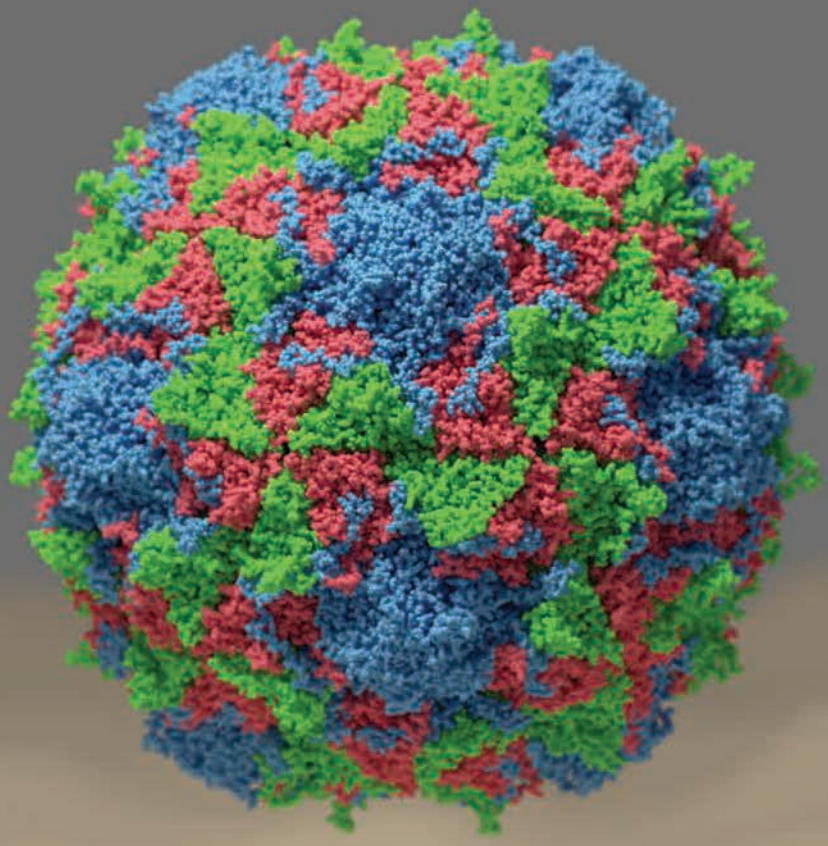
VOLUME I *Molecular Biology*

PRINCIPLES OF Virology

4TH EDITION



JANE FLINT, VINCENT R. RACANIELLO,
GLENN F. RALL, AND ANNA MARIE SKALKA

WITH LYNN W. ENQUIST

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PRINCIPLES OF Virology

4TH EDITION

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VOLUME I *Molecular Biology*

PRINCIPLES OF Virology

4TH EDITION

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We dedicate this book to the students, current and future scientists, physicians, and all those with an interest in the field of virology, for whom it was written.
We kept them ever in mind.

We also dedicate it to our families:
Jonn, Gethyn, and Amy Leedham
Doris, Aidan, Devin, and Nadia
Eileen, Kelsey, and Abigail
Rudy, Jeanne, and Chris
And
Kathy and Brian

Oh, be wiser thou!
Instructed that true knowledge leads to love.

William Wordsworth
Lines left upon a Seat in a Yew-tree
1888

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Preface

The enduring goal of scientific endeavor, as of all human enterprise, I imagine, is to achieve an intelligible view of the universe. One of the great discoveries of modern science is that its goal cannot be achieved piecemeal, certainly not by the accumulation of facts. To understand a phenomenon is to understand a category of phenomena or it is nothing. Understanding is reached through creative acts.

A. D. HERSHEY
Carnegie Institution Yearbook 65

All four editions of this textbook have been written according to the authors' philosophy that the best approach to teaching introductory virology is by emphasizing shared principles. Studying the phases of the viral reproductive cycle, illustrated with a set of representative viruses, provides an overview of the steps required to maintain these infectious agents in nature. Such knowledge cannot be acquired by learning a collection of facts about individual viruses. Consequently, the major goal of this book is to define and illustrate the basic principles of animal virus biology.

In this information-rich age, the quantity of data describing any given virus can be overwhelming, if not indigestible, for student and expert alike. The urge to write more and more about less and less is the curse of reductionist science and the bane of those who write textbooks meant to be used by students. In the fourth edition, we continue to distill information with the intent of extracting essential principles, while providing descriptions of how the information was acquired. Boxes are used to emphasize major principles and to provide supplementary material of relevance, from explanations of terminology to descriptions of trail-blazing experiments. Our goal is to illuminate process and strategy as opposed to listing facts and figures. In an effort to make the book readable, rather than comprehensive, we are selective in our choice of viruses and examples. The encyclopedic *Fields Virology* (2013) is recommended as a resource for detailed reviews of specific virus families.

What's New

This edition is marked by a change in the author team. Our new member, Glenn Rall, has brought expertise in viral immunology and pathogenesis, pedagogical clarity, and down-to-earth humor to our work. Although no longer a coauthor, our colleague Lynn Enquist has continued to provide insight, advice, and comments on the chapters.

Each of the two volumes of the fourth edition has a unique appendix and a general glossary. Links to Internet resources such as websites, podcasts, blog posts, and movies are provided; the digital edition provides one-click access to these materials.

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A major new feature of the fourth edition is the incorporation of in-depth video interviews with scientists who have made a major contribution to the subject of each chapter. Students will be interested in these conversations, which also explore the factors that motivated the scientists' interest in the field and the personal stories associated with their contributions.

Volume I covers the molecular biology of viral reproduction, and Volume II focuses on viral pathogenesis, control of virus infections, and virus evolution. The organization into two volumes follows a natural break in pedagogy and provides considerable flexibility and utility for students and teachers alike. The volumes can be used for two courses, or as two parts of a one-semester course. The two volumes differ in content but are integrated in style and presentation. In addition to updating the chapters and Appendices for both volumes, we have organized the material more efficiently and new chapters have been added.

As in our previous editions, we have tested ideas for inclusion in the text in our own classes. We have also received constructive comments and suggestions from other virology instructors and their students. Feedback from students was particularly useful in finding typographical errors, clarifying confusing or complicated illustrations, and pointing out inconsistencies in content.

For purposes of readability, references are generally omitted from the text, but each chapter ends with an updated list of relevant books, review articles, and selected research papers for readers who wish to pursue specific topics. In general, if an experiment is featured in a chapter, one or more references are listed to provide more detailed information.

Principles Taught in Two Distinct, but Integrated Volumes

These two volumes outline and illustrate the strategies by which all viruses reproduce, how infections spread within a host, and how they are maintained in populations. The principles of viral reproduction established in Volume I are essential for understanding the topics of viral disease, its control, and the evolution of viruses that are covered in Volume II.

Volume I The Science of Virology and the Molecular Biology of Viruses

This volume examines the molecular processes that take place in an infected host cell. It begins with a general introduction and historical perspectives, and includes descriptions of the unique properties of viruses (Chapter 1). The unifying principles that are the foundations of virology, including the concept of a common strategy for viral propagation, are then described. An introduction to cell biology, the principles of the infectious cycle, descriptions of the basic techniques for cultivating and assaying viruses, and the concept of the single-step growth cycle are presented in Chapter 2.

The fundamentals of viral genomes and genetics, and an overview of the surprisingly limited repertoire of viral strategies for genome replication and mRNA synthesis, are topics of Chapter 3. The architecture of extracellular virus particles in the context of providing both protection and delivery of the viral genome in a single vehicle are considered in Chapter 4. Chapters 5 through 13 address the broad spectrum of molecular processes that characterize the common steps of the reproductive cycle of viruses in a single cell, from decoding genetic information to genome replication and production of progeny virions. We describe how these common steps are accomplished in cells infected by diverse but representative viruses, while emphasizing common principles. Volume I concludes with a new chapter, "The Infected Cell," which presents an integrated description of cellular responses to illustrate the marked, and generally, irreversible, impact of virus infection on the host cell.

The appendix in Volume I provides concise illustrations of viral life cycles for members of the main virus families discussed in the text; five new families have been added in the fourth edition. It is intended to be a reference resource when reading individual chapters and a convenient visual means by which specific topics may be related to the overall infectious cycles of the selected viruses.

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Volume II Pathogenesis, Control, and Evolution

This volume addresses the interplay between viruses and their host organisms. The first five chapters have been reorganized and rewritten to reflect our growing appreciation of the host immune response and how viruses cause disease. In Chapter 1 we introduce the discipline of epidemiology, provide historical examples of epidemics in history, and consider basic aspects that govern how the susceptibility of a population is controlled and measured. With an understanding of how viruses affect human populations, subsequent chapters focus on the impact of viral infections on hosts, tissues and individual cells. Physiological barriers to virus infections, and how viruses spread in a host, invade organs, and spread to other hosts are the topics of Chapter 2. The early host response to infection, comprising cell autonomous (intrinsic) and innate immune responses, are the topics of Chapter 3, while the next chapter considers adaptive immune defenses, that are tailored to the pathogen, and immune memory. Chapter 5 focuses on the classic patterns of virus infection within cells and hosts, the myriad ways that viruses cause illness, and the value of animal models in uncovering new principles of viral pathogenesis. In Chapter 6, we discuss virus infections that transform cells in culture and promote oncogenesis (the formation of tumors) in animals. Chapter 7 is devoted entirely to the AIDS virus, not only because it is the causative agent of the most serious current worldwide epidemic, but also because of its unique and informative interactions with the human immune defenses.

Next, we consider the principles involved in treatment and control of infection. Chapter 8 focuses on vaccines, and Chapter 9 discusses the approaches and challenges of antiviral drug discovery. The topics of viral evolution and emergence have now been divided into two chapters. The origin of viruses, the drivers of viral evolution, and host-virus conflicts are the subjects of Chapter 10. The principles of emerging virus infections, and humankind's experiences with epidemic and pandemic viral infections, are considered in Chapter 11. Volume II ends with a new chapter on unusual infectious agents, viroids, satellites, and prions.

The Appendix of Volume II provides snapshots of the pathogenesis of common human viruses. This information is presented in four illustrated panels that summarize the viruses and diseases, epidemiology, disease mechanisms, and human infections.

Reference

Knipe DM, Howley PM (ed). 2013. *Fields Virology*, 6th ed. Lippincott Williams & Wilkins, Philadelphia, PA.

For some behind-the-scenes information about how the authors created the fourth edition of *Principles of Virology*, see: http://bit.ly/Virology_MakingOf

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Acknowledgments

These two volumes of *Principles* could not have been composed and revised without help and contributions from many individuals. We are most grateful for the continuing encouragement from our colleagues in virology and the students who use the text. Our sincere thanks also go to colleagues (listed in the Acknowledgments for the third edition) who have taken considerable time and effort to review the text in its evolving manifestations. Their expert knowledge and advice on issues ranging from teaching virology to organization of individual chapters and style were invaluable, and are inextricably woven into the final form of the book.

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Since the inception of this work, our belief has been that the illustrations must complement and enrich the text. Execution of this plan would not have been possible without the support of Christine Charlip (Director, ASM Press), and the technical expertise and craft of our illustrator. The illustrations are an integral part of the text, and credit for their execution goes to the knowledge, insight, and artistic talent of Patrick Lane of ScEYEnce Studios. We also are indebted to Jason Roberts (Victorian Infectious Diseases Reference Laboratory, Doherty Institute, Melbourne, Australia) for the computational expertise and time he devoted to producing the beautiful renditions of poliovirus particles on our new covers. As noted in the figure legends, many could not have been completed without the help and generosity of numerous colleagues who provided original images. Special thanks go to those who crafted figures or videos tailored specifically to our needs, or provided multiple pieces: Chantal Abergel (CNRS, Aix-Marseille Université, France), Mark Andrake (Fox Chase Cancer Center), Timothy Baker (University of California), Bruce Banfield (The University of Colorado), Christopher Basler and Peter Palese (Mount Sinai School of Medicine), Ralf Bartenschlager (University of Heidelberg, Germany), Eileen Bridge (Miami University, Ohio), Richard Compans (Emory University), Kartik Chandran (Albert Einstein College of Medicine), Paul Duprex (Boston University School of Medicine), Ramón González (Universidad Autónoma del Estado

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There is little doubt in undertaking such a massive effort that inaccuracies still remain, despite our best efforts to resolve or prevent them. We hope that the readership of this edition will draw our attention to them, so that these errors can be eliminated from future editions of this text.

This often-consuming enterprise was made possible by the emotional, intellectual, and logistical support of our families, to whom the two volumes are dedicated.

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About the Authors



Jane Flint is a Professor of Molecular Biology at Princeton University. Dr. Flint's research focuses on investigation of the molecular mechanisms by which viral gene products modulate host cell pathways and antiviral defenses to allow efficient reproduction in normal human cells of adenoviruses, viruses that are widely used in such therapeutic applications as gene transfer and cancer treatment. Her service to the scientific community includes membership of various editorial boards and several NIH study sections and other review panels. Dr. Flint is currently a member of the Biosafety Working Group of the NIH Recombinant DNA Advisory Committee.

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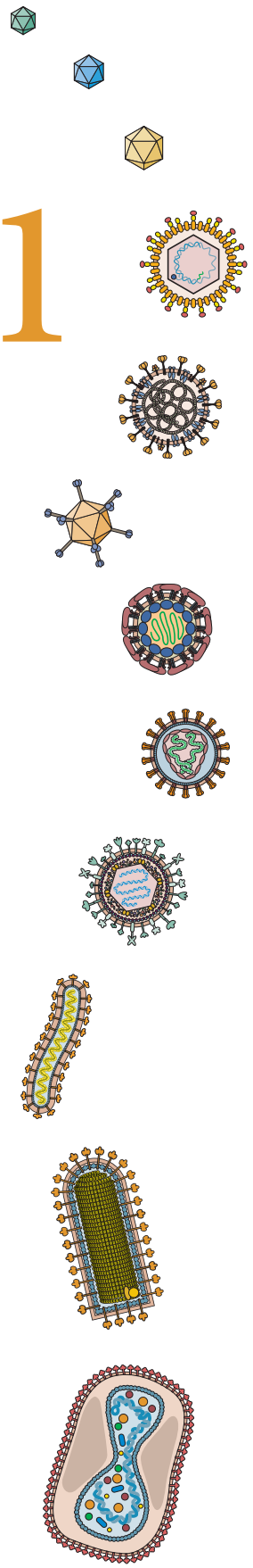
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PART I

The Science of Virology

1 Foundations
2 The Infectious Cycle

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1 Foundations

Luria's Credo

Why We Study Viruses
- Viruses Are Everywhere
- Viruses Can Cause Human Disease
- Viruses Infect All Living Things
- Viruses Can Be Beneficial
- Viruses Can Cross Species Boundaries
- Viruses "R" Us
- Viruses Are Unique Tools To Study Biology

Virus Prehistory
- Viral Infections in Antiquity
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- Microorganisms as Pathogenic Agents

Discovery of Viruses

The Definitive Properties of Viruses
- The Structural Simplicity of Virus Particles
- The Intracellular Parasitism of Viruses

Viruses Defined

Cataloging Animal Viruses
- The Classical System
- Classification by Genome Type: the Baltimore System

A Common Strategy for Viral Propagation

Perspectives

References

LINKS FOR CHAPTER 1

- ⏭ *Video: Interview with Dr. Donald Henderson*
  http://bit.ly/Virology_Henderson
- ⏭ *This Week in Virology (TWIV): A weekly podcast about viruses featuring informal yet informative discussions and interviews with guests about the latest topics in the field.*
  http://www.twiv.tv
- ⏭ *Marine viruses and insect defense*
  http://bit.ly/Virology_Twiv301
- ⏭ *Giants among viruses*
  http://bit.ly/Virology_Twiv261
- ⏭ *Latest update of virus classification from the ICTV.*
  http://www.ictvonline.org/virusTaxonomy.asp?bhcp=1
- ⏭ *The abundant and diverse viruses of the seas.*
  http://bit.ly/Virology_3-20-09
- ⏭ *How many viruses on Earth?*
  http://bit.ly/Virology_9-6-13

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Thus, we cannot reject the assumption that the effect of the filtered lymph is not due to toxicity, but rather to the ability of the agent to replicate.

F. Loeffler *1898*

Luria's Credo

"There is an intrinsic simplicity of nature and the ultimate contribution of science resides in the discovery of unifying and simplifying generalizations, rather than in the description of isolated situations—in the visualization of simple, overall patterns rather than in the analysis of patchworks." More than half a century has passed since Salvador Luria wrote this credo in the introduction to the classic textbook *General Virology*.

Despite an explosion of information in biology since Luria wrote these words, his vision of unity in diversity is as relevant now as it was then. That such unifying principles exist may not be obvious considering the bewildering array of viruses, genes, and proteins recognized in modern virology. Indeed, new viruses are being described regularly, and viral diseases such as acquired immunodeficiency syndrome (AIDS), hepatitis, and influenza continue to challenge our efforts to control them. Yet Luria's credo still stands: even as our knowledge continues to increase, it is clear that all viruses follow the same simple strategy to ensure their survival. This insight has been hard-won over many years of observation, research, and debate; the history of virology is rich and instructive.

Why We Study Viruses

Viruses Are Everywhere

Viruses are all around us, comprising an enormous proportion of our environment, in both number and total mass (Box 1.1). All living things encounter billions of virus particles every day. For example, they enter our lungs in the 6 liters of air each of us inhales every minute; they enter our digestive systems with the food we eat; and they are transferred to our eyes, mouths, and other points of entry from the surfaces we touch and the people with whom we interact. Our bodies are reservoirs for viruses that reside in our respiratory, gastrointestinal, and urogenital tracts. In addition to viruses that can infect us, our intestinal tracts are loaded with myriad plant and insect viruses, as well as hundreds of bacterial species that harbor their own constellations of viruses.

Viruses Can Cause Human Disease

With such constant exposure, it is nothing short of amazing that the vast majority of viruses that infect us have little or no impact on our health or well-being. As described in Volume II, we owe such relative safety to our elaborate immune defense systems, which have evolved to fight microbial infection. When these defenses are compromised, even the most common infection can be lethal. Despite such defenses, some of the most devastating human diseases have been or still are caused by viruses; these diseases include smallpox, yellow fever, poliomyelitis, influenza, measles, and AIDS. Viral infections can lead to life-threatening diseases that impact virtually all organs, including the lungs, liver, central nervous system, and intestines. Viruses are responsible for approximately 20% of the human cancer burden, and viral infections of the respiratory and gastrointestinal tracts kill millions of children in the developing world each year. As summarized in Volume II, Appendix, there is no question about the biomedical importance of these agents.

Viruses Infect All Living Things

While most of this textbook focuses on viral infections of humans, it is important to bear in mind that viruses also infect pets, food animals, plants, insects, and wildlife throughout

PRINCIPLES ***Foundations***

- The field of virology encompasses viral discovery, the study of virus structure and reproduction, and the importance of viruses in biology and disease.
- While this text focuses primarily on viruses that infect vertebrates, especially humans, it is important to keep in mind that viruses infect **all** living things including insects, plants, bacteria, and even other viruses.
- Viruses are not solely pathogenic nuisances; they can be beneficial. Viruses contribute to ecological homeostasis, keep our immune responses activated and alert, and can be used as molecular flashlights to illuminate cellular processes.
- Viruses have been part of all of human history: they were present long before *Homo sapiens* evolved, and the majority of human infections were likely acquired from other animals (zoonoses). As viruses continue to be discovered, our understanding of how human health and well-being are affected by these agents remains incomplete.
- Viruses are obligate intracellular parasites and depend on their host cell for all aspects of the viral life cycle.
- While Koch's postulates were essential for defining many agents of disease, not all pathogenic viruses fulfilled these criteria.
- Viruses can be cataloged based on their appearance, the hosts they infect, or the nature of their nucleic acid genome.
- The Baltimore classification allows relationships among various viral genomes and the pathway to mRNA to be determined.
- A common strategy underlies the propagation of all viruses. This textbook describes that strategy and the similarities and differences in the manner in which it is accomplished by different viruses.

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BOX 1.1

BACKGROUND

Some astounding numbers

- Viruses are the most abundant entities in the biosphere. The biomass on our planet of bacterial viruses *alone* exceeds that of all of Earth's elephants by more than 1,000-fold. There are more than 10^{30} bacteriophage particles in the world's oceans, enough to extend out into space for 200 million light-years if arranged head to tail (http://www.virology.ws/2009/03/20/the-abundant-and-diverse-viruses-of-the-seas/).
- Whales are commonly infected with a member of the virus family *Caliciviridae* that causes rashes, blisters, intestinal problems, and diarrhea and can also infect humans. Infected whales excrete more than 10^{13} calicivirus particles daily.
- The average human body contains approximately 10^{13} cells, but these are outnumbered 10-fold by bacteria and as much as 100-fold by virus particles.
- With about 10^{16} human immunodeficiency virus (HIV) genomes on the planet today, it is highly probable that somewhere there exist HIV genomes that are resistant to every one of the antiviral drugs that we have now or are likely to have in the future.

Earth and its oceans. Courtesy: NASA/Goddard Space Flight Center.



the world. They infect microbes such as algae, fungi, and bacteria, and some even interfere with the reproduction of other viruses. Viral infection of agricultural plants and animals can have enormous economic and societal impact. Outbreaks of infection by foot-and-mouth disease and avian influenza viruses have led to the destruction (**culling**) of millions of cattle, sheep, and poultry to prevent further spread. Losses in the United Kingdom during the 2001 outbreak of foot-and-mouth disease ran into billions of dollars and caused havoc for both farmers and the government (Box 1.2). More recent outbreaks of the avian influenza virus H5N1 in Asia have resulted in similar disruption and economic loss. Viruses that infect crops such as potatoes and fruit trees are common and can lead to serious food shortages as well as financial devastation.

Viruses Can Be Beneficial

Despite the appalling statistics from human and agricultural epidemics, it is important to realize that viruses can also be beneficial. Such benefit can be seen most clearly in marine ecology, where virus particles are the most abundant biological entities (Box 1.1). Indeed, they comprise 94% of all nucleic acid-containing particles in the oceans and are 15 times more abundant than the *Bacteria* and *Archaea*. Viral infections in the ocean kill 20 to 40% of marine microbes daily, converting these living organisms into particulate matter, and in so doing release essential nutrients that supply phytoplankton at the bottom of the ocean's food chain, as well as carbon dioxide and other gases that affect the climate of the earth. Pathogens can also influence one another: infection by one virus can have an ameliorating effect on the pathogenesis of a second virus or even bacteria. For example, human immunodeficiency virus-infected AIDS patients show a substantial decrease in their disease progression if they are persistently infected with hepatitis G virus, and mice latently infected with some murine herpesviruses are resistant to infection with the bacterial pathogens *Listeria monocytogenes* and *Yersinia pestis*. The idea that viruses are solely agents of disease is giving way to the notion that they can exert positive, even necessary, effects.

Viruses Can Cross Species Boundaries

Although viruses generally have a limited host range, they can and do spread across species barriers. As the world's human population continues to expand and impinge on the wilderness, cross-species (**zoonotic**) infections of humans are occurring with increasing frequency. In addition to the AIDS pandemic, the highly fatal Ebola hemorrhagic fever and the severe acute respiratory syndrome (SARS) are recent examples of viral diseases to emerge from zoonotic infections. The current pandemic of influenza virus H5N1 in avian species has much of the world riveted by the frightening possibility that a new, highly pathogenic strain might emerge following transmission from birds to human hosts. Indeed, given the eons over which viruses have had the opportunity to interact with various species, today's "natural" host may simply be a way station in viral evolution.

Viruses "R" Us

Every cell in our body contains viral DNA. Human endogenous retroviruses, and elements thereof, make up about 5 to 8% of our DNA. Most are inactive, fossil remnants from infections of germ cells that have occurred over millions of years during our evolution. Some of them are suspected to be associated with specific diseases, but the protein products of other endogenous retroviruses are essential for placental development.

Recent genomic studies have revealed that our viral "heritage" is not limited to retroviruses. Human and other

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BOX 1.2

DISCUSSION

The first animal virus discovered remains a scourge today

Foot-and-mouth disease virus infects domestic cattle, pigs, and sheep, as well as many species of wild animals. Although mortality is low, morbidity is high and infected farm animals lose their commercial value. The virus is highly contagious, and the most common and effective method of control is by the slaughter of entire herds in affected areas.

Outbreaks of foot-and-mouth disease were widely reported in Europe, Asia, Africa, and South and North America in the 1800s. The largest epidemic ever recorded in the United States occurred in 1914. After gaining entry into the Chicago stockyards, the virus spread to more than 3,500 herds in 22 states. This calamity accelerated epidemiological and disease control programs, eventually leading to the field- and laboratory-based systems maintained by the U.S. Department of Agriculture to protect domestic livestock from foreign animal and plant diseases. Similar control systems have been established in other Western countries, but this virus still presents a formidable challenge throughout the world. A 1997 outbreak of foot-and-mouth disease among pigs in Taiwan resulted in economic losses of greater than $10 billion.

In 2001, an epidemic outbreak in the United Kingdom spread to other countries in Europe and led to the slaughter of more than 3 million infected and uninfected farm animals. The associated economic, societal, and political costs threatened to bring down the British government. Images of mass graves and horrific pyres consuming the corpses of dead animals (see figure) sensitized the public as never before. Recent outbreaks and societal unrest in Turkey and regions of North Africa, including Libya and Egypt, make the threat of further spread a serious concern for other countries.

Hunt J. 3 January 2013. Foot-and-mouth is knocking on Europe's door. *Farmers Weekly*. http://www.fwi.co.uk/articles/03/01/2013/136943/foot-and-mouth-is-knocking-on-europe39s-door.htm.

Murphy FA, Gibbs EPJ, Horzinek MC, Studdert MJ. 1999. *Veterinary Virology*, 3rd ed. Academic Press, Inc, San Diego, CA.

Mass burning of cattle carcasses during the 2001 foot-and-mouth disease outbreak in the United Kingdom.



vertebrate genomes harbor sequences derived from several DNA and RNA viruses that, in contrast to the retroviruses, lack mechanisms to invade host DNA. As many of these insertions are estimated to have occurred some 40 million to 90 million years ago, this knowledge has provided unique insight into the ages and evolution of some currently circulating viruses. Furthermore, the conservation of some of the viral sequences in vertebrate genomes suggests that they may have been selected for beneficial properties over evolutionary time.

Viruses Are Unique Tools To Study Biology

Because viruses are dependent on their hosts for propagation, studies that focus on viral reprogramming of cellular mechanisms have provided unique insights into cellular biology and functioning of host defenses. Groundbreaking studies of viruses that infect bacteria, the bacteriophages, laid the foundations of modern molecular biology (Table 1.1), and crystallization of the plant virus tobacco mosaic virus was a landmark in structural biology. Studies of animal viruses established many fundamental principles of cellular function, including the presence of intervening sequences in eukaryotic genes. The study of cancer (transforming) viruses revealed the genetic basis of this disease. It seems clear that studies of viruses will continue to open up such paths of discovery in the future.

With the development of recombinant DNA technology and our increased understanding of some viral systems, it has become possible to use viral genomes as vehicles for the delivery of genes to cells and organisms for both scientific and therapeutic purposes. The use of viral vectors to introduce genes into various cells and organisms to study their function has become a standard method in biology. Viral vectors are

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Table 1.1 Bacteriophages: landmarks in molecular biology[a]

Year	Discovery (discoverer[s])
1939	One-step growth of viruses (Ellis and Delbrück)
1946	Mixed phage infection leads to genetic recombination (Delbrück)
1947	Mutation and DNA repair (multiplicity reactivation) (Luria)
1952	Transduction of genetic information (Zinder and Lederberg)
1952	DNA, not protein, found to be the genetic material (Hershey and Chase)
1952	Restriction and modification of DNA (Luria)
1955	Definition of a gene (*cis-trans* test) (Benzer)
1958	Mechanisms of control of gene expression by repressors and activators established (Pardee, Jacob, and Monod)
1958	Definition of the episome (Jacob and Wollman)
1961	Discovery of mRNA (Brenner, Jacob, and Meselson)
1961	Elucidation of the triplet code by genetic analysis (Crick, Barnett, Brenner, and Watts-Tobin)
1961	Genetic definition of nonsense codons as stop signals for translation (Campbell, Epstein, and Bernstein)
1964	Colinearity of the gene with the polypeptide chain (Sarabhai, Stretton, and Brenner)
1966	Pathways of macromolecular assembly (Edgar and Wood)
1974	Vectors for recombinant DNA technology (Murray and Murray, Thomas, Cameron, and Davis)

[a]Sources: T. D. Brock, *The Emergence of Bacterial Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1990); K. Denniston and L. Enquist, *Recombinant DNA. Benchmark Papers in Microbiology*, vol. 15 (Dowden, Hutchinson and Ross, Inc., Stroudsburg, PA, 1981); and C. K. Mathews, E. Kutter, G. Mosig, and P. Berget, *Bacteriophage T4* (American Society for Microbiology, Washington, DC, 1983).

also being used to treat human disease via "gene therapy," in which functional genes delivered by viral vectors compensate for faulty genes in the host cells.

Virus Prehistory

Although viruses have been known as distinct biological entities for little more than 100 years, evidence of viral infection can be found among the earliest recordings of human activity, and methods for combating viral disease were practiced long before the first virus was recognized. Consequently, efforts to understand and control these important agents of disease are phenomena of the 20th century.

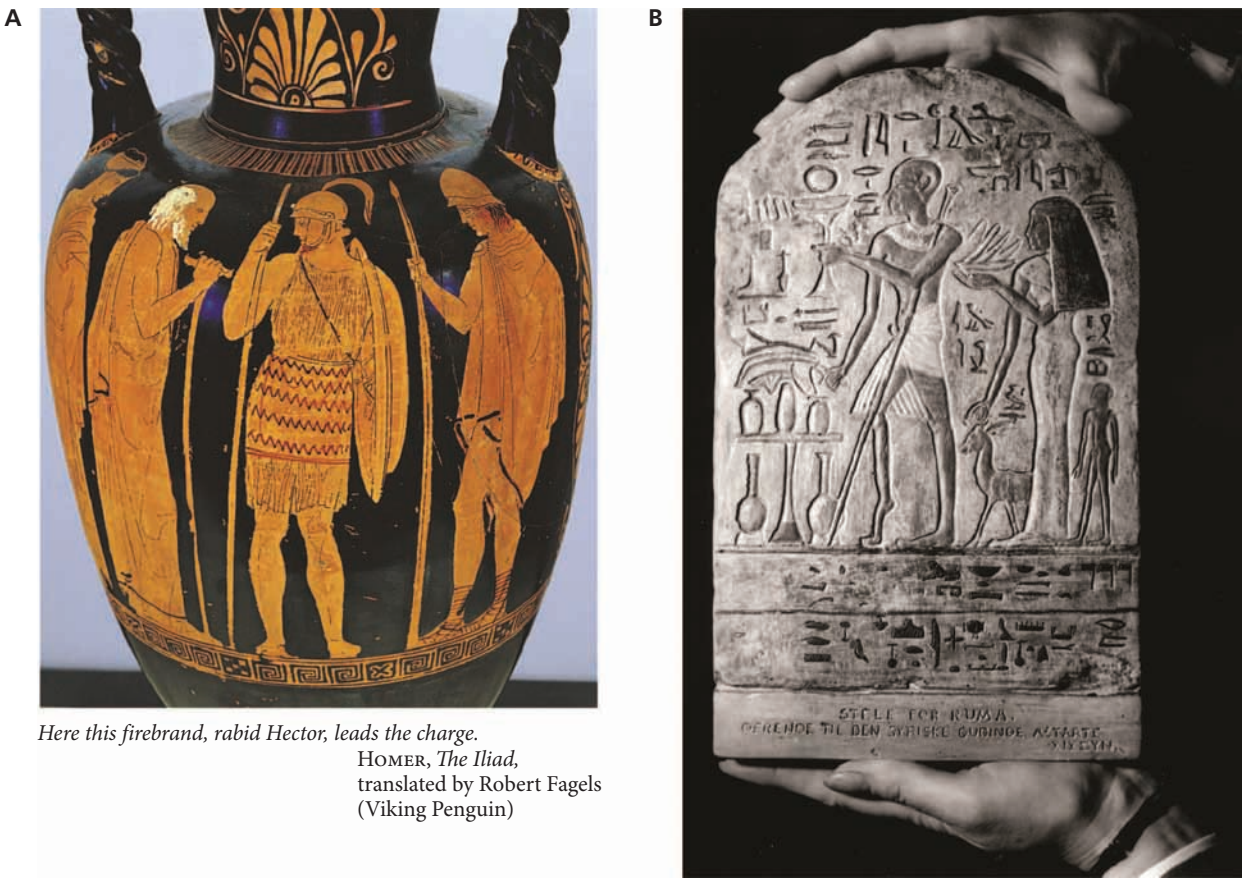
Viral Infections in Antiquity

Reconstruction of the prehistoric past to provide a plausible account of when or how viruses established themselves in human populations is a challenging task. However, extrapolating from current knowledge, we can deduce that some modern viruses were undoubtedly associated with the earliest precursors of mammals and coevolved with humans. Other viruses entered human populations only recently. The last 10,000 years of history was a time of radical change for humans and our viruses: animals were domesticated, the human population increased dramatically, large population centers appeared, and commerce drove worldwide travel and interactions among unprecedented numbers of people.

Viruses that established themselves in human populations were undoubtedly transmitted from animals, much as still happens today. Early human groups that domesticated and lived with their animals were almost certainly exposed to different viruses than were nomadic hunter societies. Similarly, as many different viruses are **endemic** in the tropics, human societies in that environment must have been exposed to a greater variety of viruses than societies established in temperate climates. When nomadic groups met others with domesticated animals, human-to-human contact could have provided new avenues for virus spread. Even so, it seems unlikely that viruses such as those that cause measles or smallpox could have entered a permanent relationship with small groups of early humans. Such highly virulent viruses, as we now know them to be, either kill their hosts or induce lifelong immunity. Consequently, they can survive only when large, interacting host populations offer a sufficient number of naive and permissive hosts for their continued propagation. Such viruses could not have been established in human populations until large, settled communities appeared. Less virulent viruses that enter into a long-term relationship with their hosts were therefore more likely to be the first to become adapted to reproduction in the earliest human populations. These viruses include the modern retroviruses, herpesviruses, and papillomaviruses.

Evidence of several viral diseases can be found in ancient records. The Greek poet Homer characterizes Hector as

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Here this firebrand, rabid Hector, leads the charge.
HOMER, *The Iliad,*
translated by Robert Fagels
(Viking Penguin)

Figure 1.1 References to viral diseases abound in the ancient literature. (A) An image of Hector from an ancient Greek vase. Courtesy of the University of Pennsylvania Museum (object 30-44-4). **(B)** An Egyptian stele, or stone tablet, from the 18th dynasty (1580–1350 B.C.) depicting a man with a withered leg and the "drop foot" syndrome characteristic of polio. Panel B is reprinted from W. Biddle, *A Field Guide to Germs* (Henry Holt and Co., LLC, New York, NY, 1995; © 1995 by Wayne Biddle), with permission from the publisher.

"rabid" in *The Iliad* (Fig. 1.1A), and Mesopotamian laws that outline the responsibilities of the owners of rabid dogs date from before 1000 B.C. Their existence indicates that the communicable nature of this viral disease was already well-known by that time. Egyptian hieroglyphs that illustrate what appear to be the consequences of poliovirus infection (a withered leg typical of poliomyelitis [Fig. 1.1B]) or pustular lesions characteristic of smallpox also date from that period. The smallpox virus, which was probably endemic in the Ganges River basin by the fifth century B.C. and subsequently spread to other parts of Asia and Europe, has played an important part in human history. Its introduction into the previously unexposed native populations of Central and South America by colonists in the 16th century led to lethal epidemics, which are considered an important factor in the conquests achieved by a small number of European soldiers. Other viral diseases known in ancient times include mumps and, perhaps, influenza. Yellow fever has been described since the discovery of Africa by Europeans, and it has been suggested that this scourge of the tropical trade was the basis for legends about ghost ships, such as the *Flying Dutchman*, in which an entire ship's crew perished mysteriously.

Humans have not only been subject to viral disease throughout much of their history but have also manipulated these agents, albeit unknowingly, for much longer than might be imagined. One classic example is the cultivation of marvelously patterned tulips, which were of enormous value in 17th-century Holland. Such efforts included deliberate spread of a virus (tulip breaking virus or tulip mosaic virus) that we now know causes the striping of tulip petals so highly prized at that time (Fig. 1.2). Attempts to control viral disease have an even more venerable history.

The First Vaccines

Measures to control one viral disease have been used with some success for the last millennium. The disease is smallpox

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Figure 1.2 *Three Broken Tulips*. A painting by Nicolas Robert (1624–1685), now in the collection of the Fitzwilliam Museum, Cambridge, United Kingdom. Striping patterns (color breaking) in tulips were described in 1576 in western Europe and were caused by a viral infection. This beautiful image depicts the remarkable consequences of infection with the tulip mosaic virus. Courtesy of the Fitzwilliam Museum, University of Cambridge.

(Fig. 1.3), and the practice is called **variolation**, inoculation of healthy individuals with material from a smallpox pustule into a scratch made on the arm. Variolation, widespread in China and India by the 11th century, was based on the recognition that smallpox survivors were protected against subsequent bouts of the disease. Variolation later spread to Asia Minor, where its value was recognized by Lady Mary Wortley Montagu, wife of the British ambassador to the Ottoman Empire. She introduced this practice into England in 1721, where it became quite widespread following the successful inoculation of children of the royal family. George Washington is said to have introduced variolation among Continental Army soldiers in 1776. However, the consequences of variolation were unpredictable and never pleasant: serious skin lesions invariably developed at the site of inoculation and were often accompanied by more generalized rash and disease, with a fatality rate of 1 to 2%. From the comfortable viewpoint of an affluent country in the 21st century, such a death rate seems unacceptably high. However, in the 18th century, variolation

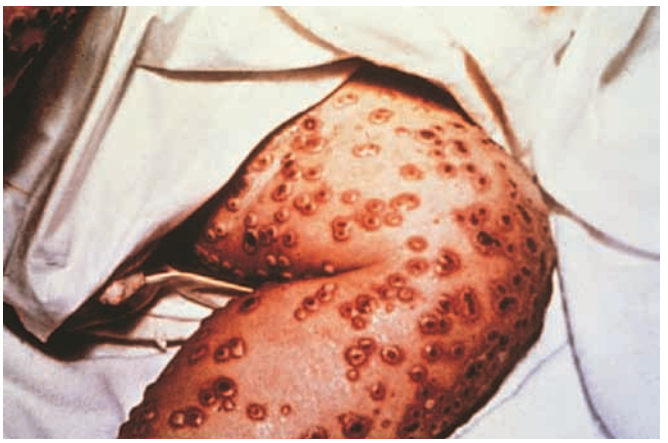


Figure 1.3 Characteristic smallpox lesions in a young victim. Illustrations like these were used as examples to track down individuals infected with the smallpox virus (variola virus) during the World Health Organization campaign to eradicate the disease. Photo courtesy of the Immunization Action Coalition (original source: Centers for Disease Control and Prevention). (See also the interview with Dr. Donald Henderson: http://bit.ly/Virology_Henderson)

was perceived as a much better alternative than contracting natural smallpox, a disease with a fatality rate of 25% in the whole population and 40% in babies and young children.

In the 1790s, Edward Jenner, an English country physician, recognized the principle on which modern methods of viral immunization are based, even though viruses themselves were not to be identified for another 100 years. Jenner himself was variolated as a boy and also practiced this procedure. He was undoubtedly familiar with its effects and risks. Perhaps this experience spurred his great insight upon observing that milkmaids were protected against smallpox if they previously contracted cowpox (a mild disease in humans). Jenner followed up this astute observation with direct experiments. In 1794 to 1796, he demonstrated that inoculation with extracts from cowpox lesions induced only mild symptoms but protected against the far more dangerous disease. It is from these experiments with cowpox that we derive the term **vaccination** (*vacca* = "cow" in Latin); Louis Pasteur coined this term in 1881 to honor Jenner's accomplishments.

Initially, the only way to propagate and maintain the cowpox vaccine was by serial infection of human subjects. This method was eventually banned, as it was often associated with transmission of other diseases such as syphilis and hepatitis. By 1860, the vaccine had been passaged in cows; later, sheep and water buffaloes were also used. While Jenner's original vaccine was based on the virus that causes cowpox, sometime during the human-to-human or cow-to-cow transfers, the poxvirus now called vaccinia virus replaced the cowpox virus. Vaccinia virus is the basis for the modern smallpox vaccine, but its origins remain a mystery: it exhibits limited genetic similarity to the viruses that cause cowpox or smallpox, or to many of the

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BOX 1.3

DISCUSSION

Origin of vaccinia virus

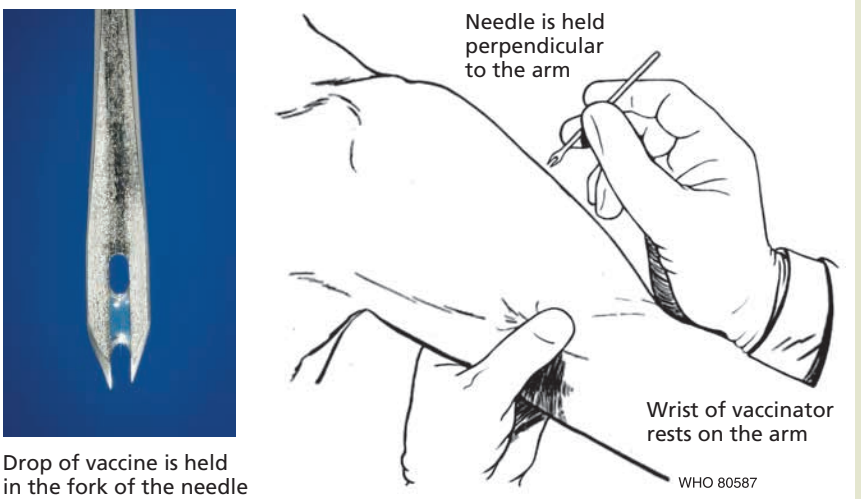
Over the years, at least three hypotheses have been advanced to explain the curious substitution of cowpox virus by vaccinia virus:

1. Recombination of cowpox virus with smallpox virus after variolation of humans
2. Recombination between cowpox virus and animal poxviruses during passage in various animals
3. Genetic drift of cowpox virus after repeated passage in humans and animals

None of these hypotheses has been proven conclusively, and all fail to account fully for the origins of the sequences in the vaccinia virus genome.

Evans DH. 2 June 2013. Episode 235, *This Week in Virology*. http://www.twiv.tv/2013/06/02/twiv-235-live-in-edmonton-eh/

Qin L, Upton C, Hazes B, Evans DH. 2011. Genomic analysis of the vaccinia virus strain variants found in Dryvax vaccine. *J Virol* **24:**13049–13060.



Smallpox vaccine is delivered via multiple punctures with a special two-pronged needle (inset) that has been dipped in the vaccine (Adapted from WHO, with permission).

other known members of the poxvirus family. Scientists have recovered the smallpox vaccine used in New York in 1876 and have verified that it contains vaccinia virus and not cowpox virus. Speculation about when and how the switch occurred has produced some possible scenarios (Box 1.3).

The first deliberately attenuated viral vaccine was made by Louis Pasteur, although he had no idea at the time that the relevant agent was a virus. In 1885, he inoculated rabbits with material from the brain of a cow suffering from rabies and then used aqueous suspensions of dried spinal cords from these animals to infect other rabbits. After several such passages, the resulting preparations caused mild disease (i.e., were **attenuated**) yet produced effective immunity against rabies. Safer and more efficient methods for the production of larger quantities of these first vaccines awaited the recognition of viruses as distinctive biological entities and parasites of cells in their hosts. Indeed, it took almost 50 years to discover the next antiviral vaccines: a vaccine for yellow fever virus was developed in 1935, and an influenza vaccine was available in 1936. These advances became possible only with radical changes in our knowledge of living organisms and of the causes of disease.

Microorganisms as Pathogenic Agents

The 19th century was a period of revolution in scientific thought, particularly in ideas about the origins of living things. The publication of Charles Darwin's *The Origin of Species* in 1859 crystallized startling (and, to many people, shocking) new ideas about the origin of diversity in plants and animals, until then generally attributed directly to the hand of God. These insights permanently undermined the perception that humans were somehow set apart from all other members of the animal kingdom. From the point of view of the science of virology, the most important changes were in ideas about the causes of disease.

The diversity of macroscopic organisms has been appreciated and cataloged since the dawn of recorded human history. A vast new world of organisms too small to be visible to the naked eye was revealed through the microscopes of Antony van Leeuwenhoek (1632–1723). Van Leeuwenhoek's vivid and exciting descriptions of living microorganisms, the "wee animalcules" present in such ordinary materials as rain or seawater, included examples of protozoa, algae, and bacteria. By the early 19th century, the scientific community had accepted the existence of microorganisms and turned to the question of their origin, a topic of fierce debate. Some believed that microorganisms arose spontaneously, for example in decomposing matter, where they were especially abundant. Others held the view that all were generated by the reproduction of like microorganisms, as were macroscopic organisms. The death knell of the spontaneous-generation hypothesis was sounded with the famous experiments of Pasteur. He demonstrated

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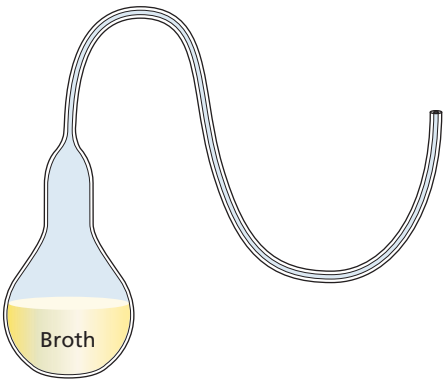


Figure 1.4 Pasteur's famous swan-neck flasks provided passive exclusion of microbes from the sterilized broth. Although the flask was freely open to the air at the end of the long curved stem, the broth remained sterile as long as the microbe-bearing dust that collected in the neck of the stem did not reach the liquid.

that boiled (i.e., sterilized) medium remained free of microorganisms as long as it was maintained in special flasks with curved, narrow necks designed to prevent entry of airborne microbes (Fig. 1.4). Pasteur also established that particular microorganisms were associated with specific processes, such as fermentation, an idea that was crucial in the development of modern explanations for the causes of disease.

From the earliest times, poisonous air (miasma) was generally invoked to account for **epidemics** of contagious diseases, and there was little recognition of the differences among causative agents. The association of particular microorganisms, initially bacteria, with specific diseases can be attributed to the ideas of the German physician Robert Koch. He developed and applied a set of criteria for identification of the agent responsible for a specific disease (a **pathogen**), articulated in an 1890 presentation in Berlin. These criteria, **Koch's postulates**, can be summarized as follows.

- The organism must be regularly associated with the disease and its characteristic lesions.
- The organism must be isolated from the diseased host and grown in culture.
- The disease must be reproduced when a pure culture of the organism is introduced into a healthy, susceptible host.
- The same organism must be reisolated from the experimentally infected host (Box 1.4).

By applying his criteria, Koch demonstrated that anthrax, a common disease of cattle, was caused by a specific bacterium (designated *Bacillus anthracis*) and that a second, distinct bacterial species caused tuberculosis in humans. Guided by these postulates and the methods for the sterile culture and isolation of pure preparations of bacteria developed by Pasteur, Joseph Lister, and Koch, many pathogenic bacteria (as well as yeasts and fungi) were identified and classified during the last part of the 19th century (Fig. 1.5). From these beginnings, investigation into the causes of infectious disease was placed on a secure scientific foundation, the first step toward rational treatment and ultimately control. Furthermore, during the last decade of the 19th century, failures of the paradigm that bacterial or fungal agents are responsible for **all** diseases led to the identification of a new class of infectious agents—submicroscopic pathogens that came to be called **viruses**.

Discovery of Viruses

The first report of a pathogenic agent smaller than any known bacterium appeared in 1892. The Russian scientist Dimitrii Ivanovsky observed that the causative agent of tobacco mosaic disease was not retained by the unglazed filters used at

BOX 1.4

DISCUSSION

New methods extend Koch's principles

While it is clear that a microbe that fulfills Koch's postulates is almost certainly the cause of the disease in question, we now know that microbes that do not fulfill such criteria may still represent the etiological agents of disease. In the latter part of the 20th century, new methods were developed to associate particular viruses with disease based on immunological evidence of infection, for example, the presence of antibodies in blood. The availability of these methods led to the proposal of modified "molecular Koch's postulates" based on the application of molecular techniques to monitor the role played by virulence genes in bacteria.

The most revolutionary advances in our ability to link particular viruses with disease (or benefit) come from the more recent development of high-throughput nucleic acid sequencing methods and bioinformatics tools that allow detection of viral genetic material directly in environmental or biological samples, an approach called viral metagenomics. Based on these developments, alternative "metagenomic Koch's postulates" have been proposed in which (i) the definitive traits are molecular markers such as genes or full genomes that can uniquely distinguish samples obtained from diseased subjects from those obtained from matched, healthy control subjects and (ii) inoculating a healthy individual with a sample from a diseased subject results in transmission of the disease as well as the molecular markers.

Falkow S. 1988. Molecular Koch's postulates applied to microbial pathogenicity. *Rev Infect Dis* **10**(Suppl 2)**:** S274–S276.

Fredericks DN, Relman DA. 1996. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clin Microbiol Rev* **9:**18–33.

Mokili JL, Rohwer F, Dutilh BE. 2012. Metagenomics and future perspectives in virus discovery. *Curr Opin Virol* **2:**63–77.

Racaniello V. 22 January 2010. Koch's postulates in the 21st century. Virology Blog. http://www.virology.ws/2010/01/22/kochs-postulates-in-the-21st-century/

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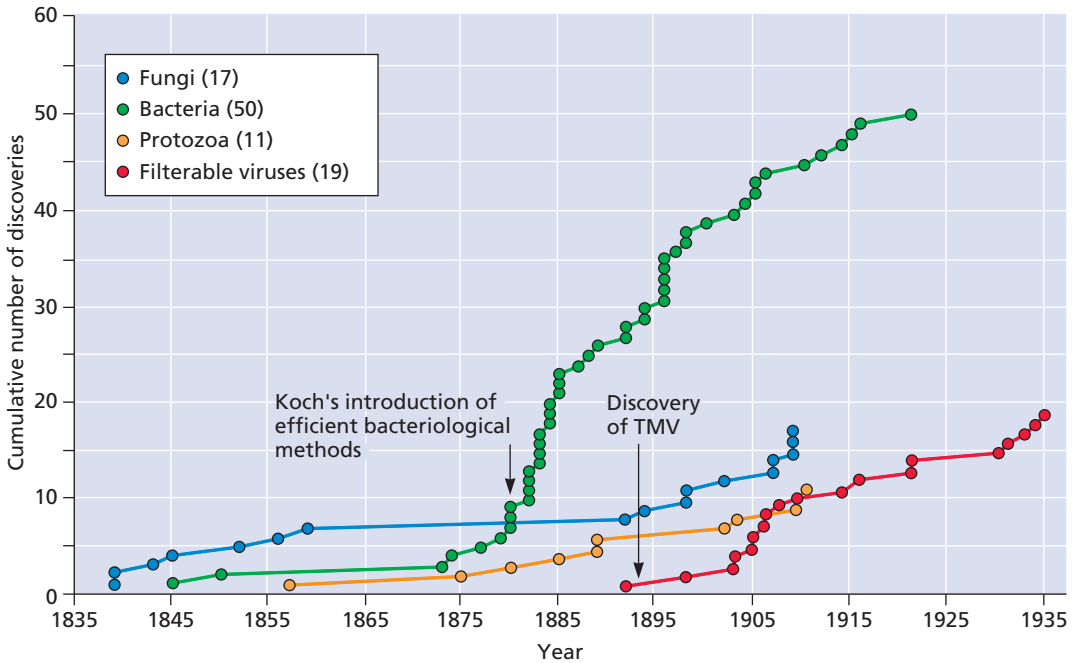


Figure 1.5 The pace of discovery of new infectious agents in the 19th and 20th centuries. Koch's introduction of efficient bacteriological techniques spawned an explosion of new discoveries of bacterial agents in the early 1880s. Similarly, the discovery of filterable agents launched the field of virology in the early 1900s. Despite an early surge of virus discovery, only 19 distinct human viruses had been reported by 1935. TMV, tobacco mosaic virus. Adapted from K. L. Burdon, *Medical Microbiology* (Macmillan Co., New York, NY, 1939), with permission.

that time to remove bacteria from extracts and culture media (Fig. 1.6A). Six years later in Holland, Martinus Beijerinck independently made the same observation. More importantly, Beijerinck made the conceptual leap that this must be a distinctive agent, because it was so small that it could pass through filters that trapped all known bacteria. However, Beijerinck thought that the agent was an infectious liquid. It was two former students and assistants of Koch, Friedrich Loeffler and Paul Frosch, who in the same year (1898) deduced that such infectious filterable agents comprised small particles: they observed that while the causative agent of foot-and-mouth disease (Box 1.2) passed through filters that held back bacteria, it could be retained by a finer filter.

Not only were the tobacco mosaic and foot-and-mouth disease pathogens much smaller than any previously recognized microorganism, but also they were replicated **only** in their host organisms. For example, extracts of an infected tobacco plant diluted into sterile solution produced no additional infectious agents until introduced into leaves of healthy plants, which subsequently developed tobacco mosaic disease. The serial transmission of infection by diluted extracts established that these diseases were not caused by a bacterial toxin present in the original preparations derived from infected tobacco plants or cattle. The failure of both pathogens to multiply in solutions that readily supported the growth of bacteria, as well as their dependence on host organisms for reproduction, further distinguished these new agents from pathogenic bacteria. Beijerinck termed the submicroscopic agent responsible for tobacco mosaic disease *contagium vivum fluidum* to emphasize its infectious nature and distinctive reproductive and physical properties. Agents passing through filters that retain bacteria came to be called ultrafilterable viruses, appropriating the term "*virus*" from the Latin for "poison." This term eventually was simplified to "virus."

The discovery of the first virus, tobacco mosaic virus, is often attributed to the work of Ivanovsky in 1892. However, he did not identify the tobacco mosaic disease pathogen as a distinctive agent, nor was he convinced that its passage through bacterial filters was not the result of some technical failure. It may be more appropriate to attribute the founding of the field of virology to the astute insights of Beijerinck, Loeffler, and Frosch, who recognized the distinctive nature of the plant and animal pathogens they were studying more than 100 years ago.

The pioneering work on tobacco mosaic and foot-and-mouth disease viruses was followed by the identification of viruses associated with specific diseases in many other organisms. Important landmarks from this early period include the identification of viruses that cause leukemias or solid tumors in chickens by Vilhelm Ellerman and Olaf Bang in 1908 and Peyton Rous in 1911, respectively. The study of viruses associated with cancers in chickens, particularly Rous

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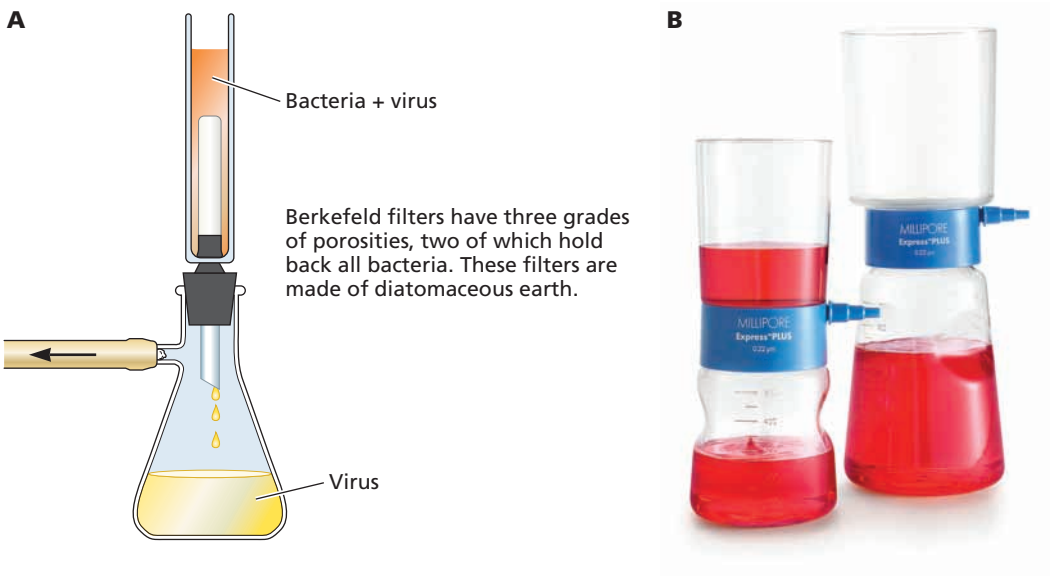


Figure 1.6 Filter systems used to characterize/purify virus particles. (A) The earliest, the Berkefeld filter, was invented in Germany in 1891. It was a "candle"-style filter comprising diatomaceous earth, or Kieselguhr, pressed into the shape of a hollow candle. The white candle is in the upper chamber of the apparatus, which is open at the top to receive the liquid to be filtered into the suction flask. The smallest pore size retained bacteria and allowed virus particles to pass through. Such filters were probably used by Ivanovsky, Loeffler, and Frosch to isolate the first plant and animal viruses. **(B)** A typical Millipore membrane filter apparatus. Such modern-day filter systems are disposable plastic laboratory items in which the upper and lower chambers are separated by a biologically inert membrane, available in a variety of pore sizes. Such filtration approaches may have limited our detection of giant viruses. Image provided courtesy of EMD Millipore Corporation.

sarcoma virus, eventually led to an understanding of the molecular basis of cancer (Volume II, Chapter 7).

The fact that bacteria could also be hosts to viruses was first recognized by Frederick Twort in 1915 and Félix d'Hérelle in 1917. d'Hérelle named such viruses **bacteriophages** because of their ability to lyse bacteria on the surface of agar plates ("phage" is derived from the Greek for "eating"). In an interesting twist of serendipity, Twort made his discovery of bacterial viruses while testing the smallpox vaccine virus to see if it would grow on simple media. He found bacterial contaminants, some of them appearing more transparent, which proved to be the result of lysis by a bacteriophage. Investigation of bacteriophages established the foundations for the field of molecular biology, as well as fundamental insights into how viruses interact with their host cells.

The Definitive Properties of Viruses

Throughout the early period of virology when many viruses of plants, animals, and bacteria were cataloged, ideas about the origin and nature of these distinctive infectious agents were quite controversial. Arguments centered on whether viruses originated from parts of a cell or were built from unique components. Little progress was made toward resolving these issues and establishing the definitive properties of viruses until the development of new techniques that allowed their visualization or propagation in cultured cells.

The Structural Simplicity of Virus Particles

Dramatic confirmation of the structural simplicity of virus particles came in 1935, when Wendell Stanley obtained crystals of tobacco mosaic virus. At that time, nothing was known of the structural organization of any biologically important macromolecules, such as proteins and DNA. Indeed, the crucial role of DNA as genetic material had not even been recognized. The ability to obtain an infectious agent in crystalline form, a state that is more generally associated with inorganic material, created much wonder and speculation about whether a virus is truly a life form. In retrospect, it is obvious that the relative ease with which tobacco mosaic virus could be crystallized was a direct result of both its structural simplicity and the ability of many particles to associate in regular arrays.

The 1930s saw the introduction of the instrument that rapidly revolutionized virology: the electron microscope. The great magnifying power of this instrument (eventually more than 100,000-fold) allowed direct visualization of virus particles for the first time. It has always been an exciting experience for investigators to obtain images of viruses, especially as they appear to be remarkably elegant (Fig. 1.7). Images of many different virus particles confirmed that these agents are very small (Fig. 1.8) and that most are far simpler in structure than any cellular organism. Many appeared

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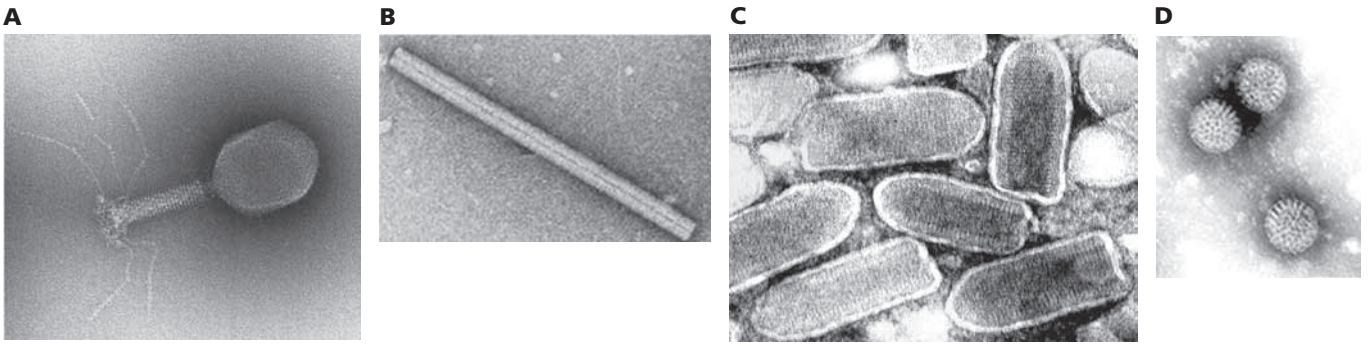
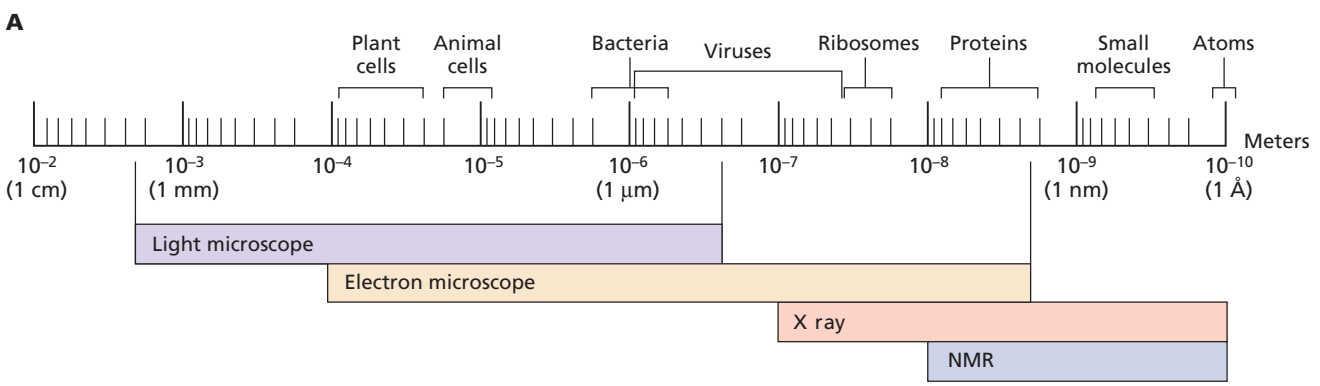
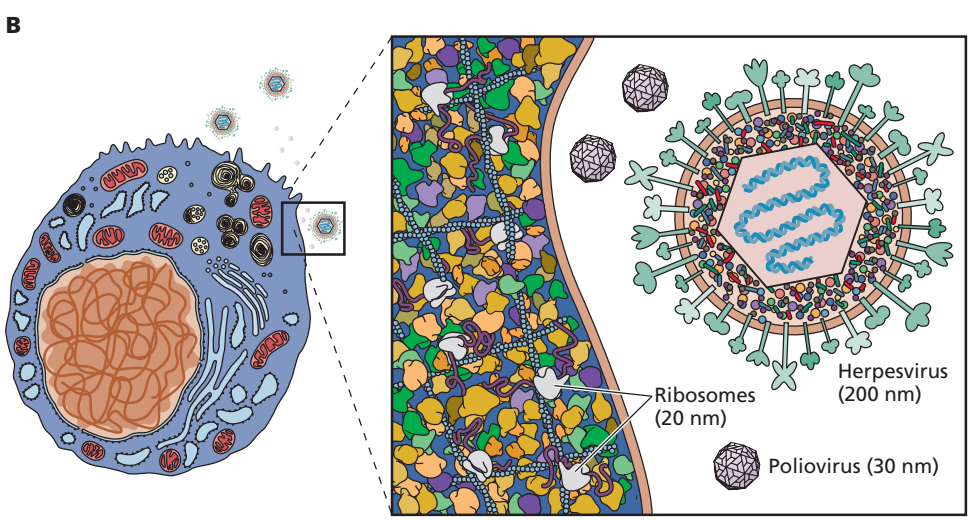


Figure 1.7 Electron micrographs of virus particles following negative staining. **(A)** The complex, nonenveloped virus bacteriophage T4. Note the intricate tail and tail fibers. Courtesy of R. L. Duda, University of Pittsburgh, Pittsburgh, PA. **(B)** The helical, nonenveloped particle of tobacco mosaic virus. Reprinted from the Universal Virus Database of the International Committee on Taxonomy of Viruses (http://ictvonline.org/), with permission. **(C)** Enveloped particles of the rhabdovirus vesicular stomatitis virus. Courtesy of F. P. Williams, University of California, Davis. **(D)** Nonenveloped, icosahedral human rotavirus particles. Courtesy of F. P. Williams, U.S. Environmental Protection Agency, Washington, DC.

Figure 1.8 Size matters. **(A)** Sizes of animal and plant cells, bacteria, viruses, proteins, molecules, and atoms are indicated. The resolving powers of various techniques used in virology, including light microscopy, electron microscopy, X-ray crystallography, and nuclear magnetic resonance (NMR) spectroscopy, are indicated. Viruses span a broad range from that equal to some small bacteria to just under ribosome size. The units commonly used in descriptions of virus particles or their components are the nanometer (nm [10^{-9} m]) and the angstrom (Å [10^{-10} m]). Adapted from A. J. Levine, *Viruses* (Scientific American Library, New York, NY, 1991); used with permission of Henry Holt and Company, LLC. **(B)** Illustration of the size differences among two viruses and a typical host cell.





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as regular helical or spherical particles. The description of the morphology of virus particles made possible by electron microscopy also opened the way for the first rational classification of viruses.

The Intracellular Parasitism of Viruses

Organisms as Hosts

The defining characteristic of viruses is their absolute dependence on a living host for reproduction: they are **obligate parasites**. Transmission of plant viruses such as tobacco mosaic virus can be achieved readily, for example, by applying extracts of an infected plant to a scratch made on the leaf of a healthy plant. Furthermore, as a single infectious particle of many plant viruses is sufficient to induce the characteristic lesion (Fig. 1.9), the concentration of the infectious agent could be measured. Plant viruses were therefore the first to be studied in detail. Some viruses of humans and other species could also be propagated in laboratory animals, and methods were developed to quantify them by determining the lethal dose. The transmission of yellow fever virus to mice by Max Theiler in 1930 was an achievement that led to the isolation of an attenuated strain, still considered one of the safest and most effective ever produced for the vaccination of humans.

After specific viruses and host organisms were identified, it became possible to produce sufficient quantities of virus particles for study of their physical and chemical properties and the consequences of infection for the host. Features such as the incubation period, symptoms of infection, and effects on specific tissues and organs were investigated. Laboratory animals remain an essential tool in investigations of the pathogenesis of viruses that cause disease. However, real progress toward understanding the mechanisms of virus reproduction was made only with the development of cell culture systems. Among the simplest, but crucial to both virology and molecular biology, were cultures of bacterial cells.

Figure 1.9 Lesions induced by tobacco mosaic virus on an infected tobacco leaf. In 1886, Adolph Mayer first described the characteristic patterns of light and dark green areas on the leaves of tobacco plants infected with tobacco mosaic virus. He demonstrated that the mosaic lesions could be transmitted from an infected plant to a healthy plant by aqueous extracts derived from infected plants. The number of local necrotic lesions that result is directly proportional to the number of infectious particles in the preparation. Courtesy J. P. Krausz; Reproduced, by permission of APS, from Scholthof, K.-B. G. 2000. Tobacco mosaic virus. The Plant Health Instructor. doi:10.1094/PHI-I-2000-1010-01.



Lessons from Bacteriophages

In the late 1930s and early 1940s, bacteriophages, or "phages," received increased attention as a result of controversy centering on how they were formed. John Northrup, a biochemist at the Rockefeller Institute in Princeton, NJ, championed the theory that a phage was a metabolic product of a bacterium. On the other hand, Max Delbrück, in his work with Emory Ellis and later with Luria, regarded phages as autonomous, stable, self-replicating entities characterized by heritable traits. According to this paradigm, phages were seen as ideal tools with which to investigate the nature of genes and heredity. Probably the most critical early contribution of Delbrück and Ellis was the perfection of the one-step growth method for synchronization of the reproduction of phages, an achievement that allowed analysis of a single cycle of phage reproduction in a population of bacteria. This approach introduced highly quantitative methods to virology, as well as an unprecedented rigor of analysis. The first experiments showed that phages indeed multiplied in the bacterial host and were liberated in a "burst" by lysis of the cell.

Delbrück was a zealot for phage research and recruited talented scientists to pursue the fundamental issues of what is now known as the field of molecular biology. This group of scientists, working together in what came to be called the "phage school," focused their attention on specific phages of the bacterium *Escherichia coli*. Progress was rapid, primarily because of the simplicity of the phage infectious cycle. Phages reproduce in bacterial hosts, which can be obtained in large numbers by overnight culture. By the mid-1950s, it was evident that viruses from bacteria, animals, and plants share many fundamental properties. However, the phages provided a far more tractable experimental system. Consequently, their study had a profound impact on the field of virology.

One critical lesson came from a definitive experiment that established that viral nucleic acid carries genetic information. It was known from studies of the "transforming principle" of pneumococcus by Oswald Avery, Colin MacLeod, and Maclyn McCarty (1944) that nucleic acid was both necessary and sufficient for the transfer of genetic traits of bacteria. However, in the early 1950s, protein was still suspected to be an important component of viral heredity. In a brilliantly simple experiment that included the use of a common kitchen food blender, Alfred Hershey and Martha Chase showed that this hypothesis was incorrect (Box 1.5).

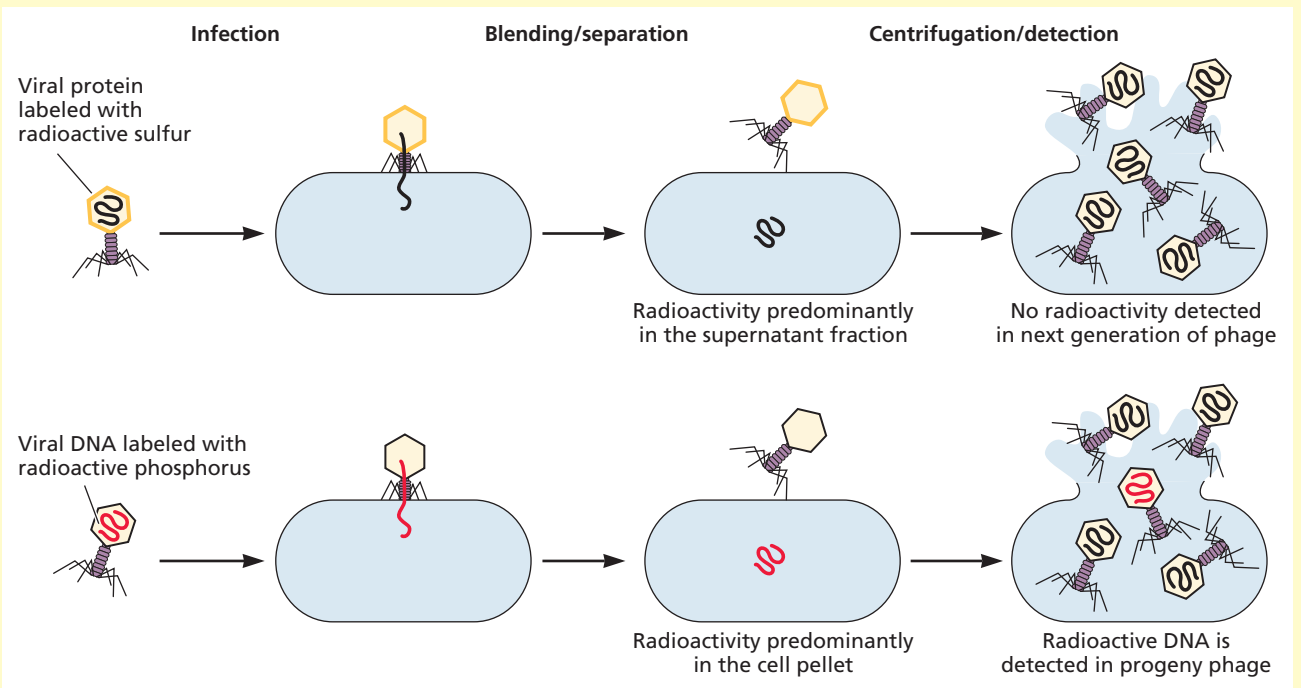
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BOX 1.5

EXPERIMENTS

The Hershey-Chase experiment

By differentially labeling the nucleic acid and protein components of virus particles with radioactive phosphorus (^{32}P) and radioactive sulfur (^{35}S), respectively, Alfred Hershey and Martha Chase showed that the protein coat of the infecting virus could be removed soon after infection by agitating the bacteria for a few minutes in a blender. In contrast, ^{32}P-labeled phage DNA entered and remained associated with the bacterial cells under these conditions. Because such blended cells produced a normal burst of new virus particles, it was clear that the DNA contained all of the information necessary to produce progeny phages.



Bacteriophages were originally thought to be lethal agents, killing their host cells after infection. In the early 1920s, a previously unknown interaction was discovered, in which the host cell not only survived the infection but also stably inherited the genetic information of the virus. It was also observed that certain bacterial strains could lyse spontaneously and produce bacteriophages after a period of growth in culture. Such strains were called **lysogenic**, and the phenomenon, **lysogeny**. Studies of lysogeny uncovered many previously unrecognized features of virus-host cell interactions (Box 1.6). Recognition of this phenomenon came from the work of many scientists, but it began with the elegant experiments of André Lwoff and colleagues at the Institut Pasteur in Paris. Lwoff showed that a viral genome exists in lysogenic cells in the form of a silent genetic element called the **prophage**. This element determined the ability of lysogenic bacteria to produce infectious bacteriophages. Subsequent studies of the *E. coli* phage lambda established a paradigm for one mechanism of lysogeny, the integration of a phage genome into a specific site on the bacterial chromosome.

Bacteriophages became inextricably associated with the new field of molecular biology (Table 1.1). Their study established many fundamental principles: for example, control of the decision to enter a lysogenic or a lytic pathway is encoded in the genome of the virus. The first mechanisms discovered for the control of gene expression, exemplified by the elegant operon theory of Nobel laureates François Jacob and Jacques Monod, were deduced in part from studies of lysogeny by phage lambda. The biology of phage lambda provided a fertile ground for work on gene regulation, but study of virulent T phages (T1 to T7, where T stands for "type") of *E. coli* paved the way for many other important advances (Table 1.1). As we shall see, these systems also

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BOX 1.6

BACKGROUND

Properties of lysogeny shared with animal viruses

Lytic versus Lysogenic Response to Infection

Some bacterial viruses can enter into either destructive (lytic) or relatively benign (lysogenic) relationships with their host cells. Such bacteriophages were called temperate. In a lysogenic bacterial cell, viral genetic information persists but viral gene expression is repressed. Such cells are called lysogens, and the quiescent viral genome, a prophage. By analogy with the prophage, an integrated DNA copy of a retroviral genome in an animal genome is termed a provirus.

Propagation as a Prophage

For some bacteriophages like lambda and Mu (Mu stands for "mutator"), prophage DNA is integrated into the host genome of lysogens and passively replicated by the host. Virally encoded enzymes, known as integrase (lambda) and transposase (Mu), mediate the covalent insertion of viral DNA into the chromosome of the host bacterium, establishing it as a prophage. The prophage DNA of other bacteriophages, such as P1, exists as a plasmid, a self-replicating, autonomous chromosome in a lysogen. Both forms of propagation have been identified in certain animal viruses.

Insertional Mutagenesis

Bacteriophage Mu inserts its genome into many random locations on the host chromosome, causing numerous mutations. This process is called insertional mutagenesis and is a phenomenon observed with retroviruses.

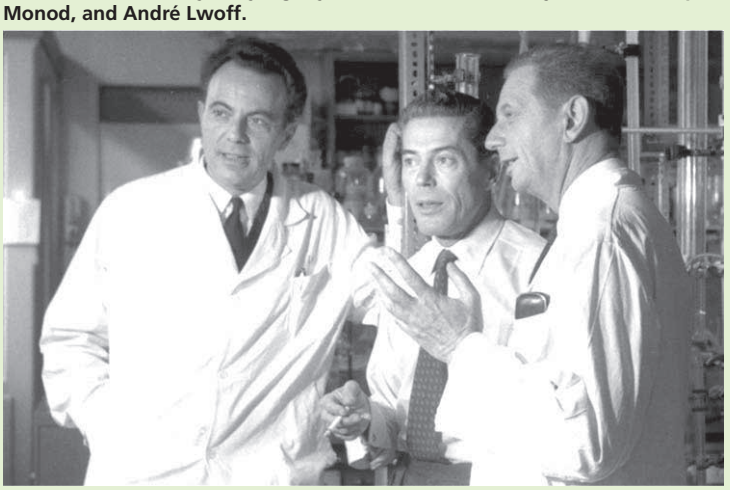
Gene Repression and Induction

Prophage gene expression in lysogens is turned off by the action of viral proteins called repressors. Expression can be turned on when repressors are inactivated (a process called induction). Elucidation of the mechanisms of these processes set the stage for later investigation of the control of gene expression in experiments with other viruses and their host cells.

Transduction of Host Genes

Bacteriophage genomes can pick up cellular genes and deliver them to new cells (a process known as transduction). The process can be generalized, with the acquisition by the virus of any segment from the host chromosome, or specialized, as is the case for viruses that integrate into specific sites in the host chromosome. For example, occasional mistakes in excision of the lambda prophage after induction result in production of unusual progeny phage that have lost some of their own DNA but have acquired the bacterial DNA adjacent to the prophage. As described in Volume II, Chapter 7, the acute transforming retroviruses also arise via capture of genes in the vicinity of their integration as proviruses. These cancer-inducing cellular genes are then transduced along with viral genes during subsequent infection.

Pioneers in the study of lysogeny: Nobel laureates François Jacob, Jacques Monod, and André Lwoff.



provided an extensive preview of mechanisms of animal virus reproduction (Box 1.7).

Animal Cells as Hosts

The culture of animal cells in the laboratory was initially more of an art than a science, restricted to cells that grew out of organs or tissues maintained in nutrient solutions under sterile conditions. The finite life span of such **primary cells**; their dependence for growth on natural components in their media such as lymph, plasma, or chicken embryo extracts; and the technical demands of sterile culture prior to the discovery of antibiotics made reproducible experimentation very difficult. However, by 1955, the work of many investigators had led to a series of important methodological advances. These included the development of defined media optimal for growth of mammalian cells, incorporation of antibiotics into cell culture media, and development of immortal cell lines such as the mouse L and human HeLa cells that are still in widespread use. These advances allowed growth of animal cells in culture to become a routine, reproducible exercise.

The availability of well-characterized cell cultures had several important consequences for virology. It allowed the discovery of new human viruses, such as adenovirus, measles virus, and rubella virus, for which animal hosts were not available. In 1949, John Enders and colleagues used cell cultures to propagate poliovirus, a feat that led to the development of polio vaccines a few years later. Cell culture technology revolutionized the ability to investigate the reproduction of viruses. Viral infectious cycles could be studied

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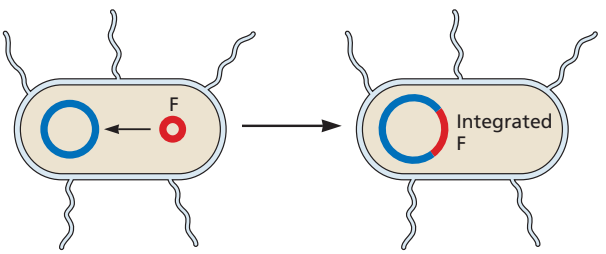
BOX 1.7

TERMINOLOGY

The episome

In 1958, François Jacob and Elie Wollman realized that lambda prophage and the *E. coli* F sex factor had many common properties. This remarkable insight led to the definition of the episome.

An episome is an exogenous genetic element that is not necessary for cell survival. Its defining characteristic is the ability to reproduce in two alternative states: while integrated in the host chromosome or autonomously. However, this term is often applied to genomes that can be maintained in cells by autonomous replication and never integrate, for example, the DNA genomes of certain animal viruses.



under precisely controlled conditions by employing the analog of the one-step growth cycle of bacteriophages and simple methods for quantification of infectious particles described in Chapter 2. Our current understanding of the molecular basis of viral parasitism, the focus of this volume, is based almost entirely on analyses of one-step growth cycles in cultured cells. Such studies established that viruses are **molecular** parasites: for example, their reproduction depends absolutely on their host cell's biosynthetic machinery for synthesis of the components from which they are built. In contrast to cells, viruses are not reproduced by growth and division. Rather, the infecting genome contains the information necessary to redirect cellular systems to the production of many copies of all the components needed for the *de novo* assembly of new virus particles.

Viruses Defined

Advances in knowledge of the structure of virus particles and the mechanisms by which they are produced in their host cells have been accompanied by increasingly accurate definitions of these unique agents. The earliest pathogenic agents, distinguished by their small size and dependence on a host organism for reproduction, emphasized the importance of viruses as agents of disease. We can now provide a much more precise definition, elaborating their relationship with the host cell and the important features of virus particles.

The definitive properties of viruses are summarized as follows:

- A virus is an infectious, obligate intracellular parasite.
- The viral genome comprises DNA or RNA.
- The viral genome directs the synthesis of viral components by cellular systems within an appropriate host cell.
- Infectious progeny virus particles, called **virions**, are formed by *de novo* self-assembly from newly synthesized components.
- A progeny virion assembled during the infectious cycle is the vehicle for transmission of the viral genome to the next host cell or organism, where its disassembly initiates the next infectious cycle.

While viruses lack the complex energy-generating and biosynthetic systems necessary for independent existence (Box 1.8), they are **not** the simplest biologically active agents: **viroids**, which are infectious agents of a variety of economically important plants, comprise a single small molecule of noncoding RNA, whereas other agents, termed **prions**, are thought to be single protein molecules (Volume II, Chapter 12).

Cataloging Animal Viruses

Virus classification was at one time a subject of colorful and quite heated controversy (Box 1.9). As new viruses were being discovered and studied by electron microscopy, the virus world was seen to be a veritable zoo of particles with different sizes, shapes, and compositions (see, for example, Fig. 1.10). Very strong opinions were advanced concerning classification and nomenclature. One camp pointed to the inability to infer, from the known properties of viruses, anything about their evolutionary origin or their relationships to one another—the major goal of classical taxonomy. The other camp maintained that despite such limitations, there were significant practical advantages in grouping isolates with similar properties. A major sticking point, however, was finding agreement on **which** properties should be considered most important in constructing a scheme for virus classification.

The Classical System

Lwoff, Robert Horne, and Paul Tournier, in 1962, advanced a comprehensive scheme for the classification of all viruses (bacterial, plant, and animal) under the classical Linnaean hierarchical system consisting of phylum, class, order, family, genus, and species. Although a subsequently formed international committee on the nomenclature of viruses did not adopt this system *in toto*, its designation of families, genera, and species was used for the classification of animal viruses.

One of the most important principles embodied in the system advanced by Lwoff and his colleagues was that viruses

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BOX 1.8

DISCUSSION

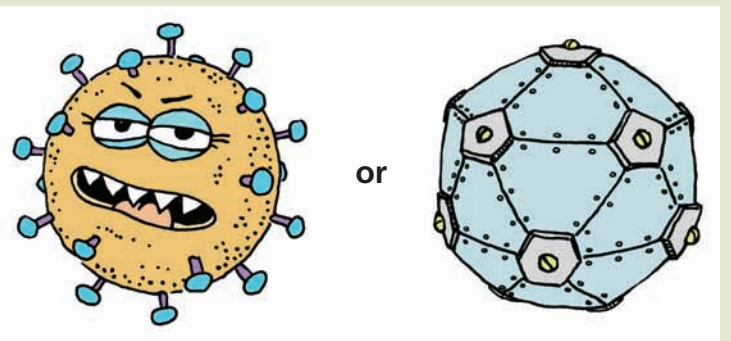
Are viruses living entities? What can/can't they do?

Viruses can be viewed as microbes that exist in two phases: an inanimate phase, the virion; and a multiplying phase in an infected cell. Some researchers have promoted the idea that viruses are organisms and that the inanimate virions may be viewed as "spores" that come "alive" in cells, or in factories within cells. This has long been a topic of intense discussion, stimulated most recently by the discovery of giant viruses such as the mimiviruses and pandoraviruses. Check out what the contemporary general public feels about this topic (http://www.virology.ws/are-viruses-alive/).

Apart from attributing "life" to viruses, many scientists have succumbed to the temptation of ascribing various **actions** and **motives** when discussing them. While remarkably effective in enlivening a lecture or an article, anthropomorphic characterizations are inaccurate and also quite misleading. Infected cells and hosts respond in many ways after infection, but viruses are **passive** agents, totally at the mercy of their environments. Therefore viruses cannot employ, ensure, synthesize, exhibit, display, destroy, deploy, depend, reprogram, avoid, retain, evade, exploit, generate, etc.

As virologists can be very passionate about their subject, it is exceedingly difficult to purge such anthropomorphic terms from virology communications. Indeed, hours were spent doing so in the preparation of this textbook, though undoubtedly there remain examples in which actions are attributed to viruses. Should you find them, let us know!

Bândea CI. 1983. A new theory on the origin and the nature of viruses. *J Theor Biol* **105:**591–602.
Claverie JM, Abergel C. 2013. Open questions about giant viruses. *Adv Virus Res* **85:**25-56.



should be grouped according to **their** shared properties rather than the properties of the cells or organisms they infect. A second principle was a focus on the nucleic acid genome as the primary criterion for classification. The importance of the genome had become clear when it was inferred from the Hershey-Chase experiment that viral nucleic acid alone can be infectious (Box 1.5). Four characteristics were to be used in the classification of all viruses:

1. Nature of the nucleic acid in the virion (**DNA** or **RNA**)
2. Symmetry of the protein shell (**capsid**)
3. Presence or absence of a lipid membrane (**envelope**)
4. Dimensions of the virion and capsid

The elucidation of evolutionary relationships by analyses of nucleic acid and protein sequence similarities is now a standard method for assigning viruses to a particular family and to order members within a family. For example, hepatitis C virus was classified as a member of the family *Flaviviridae* and the Middle East respiratory SARS-like virus (MERS) was assigned to the *Coronaviridae* based on their genome sequences. However, as our knowledge of molecular

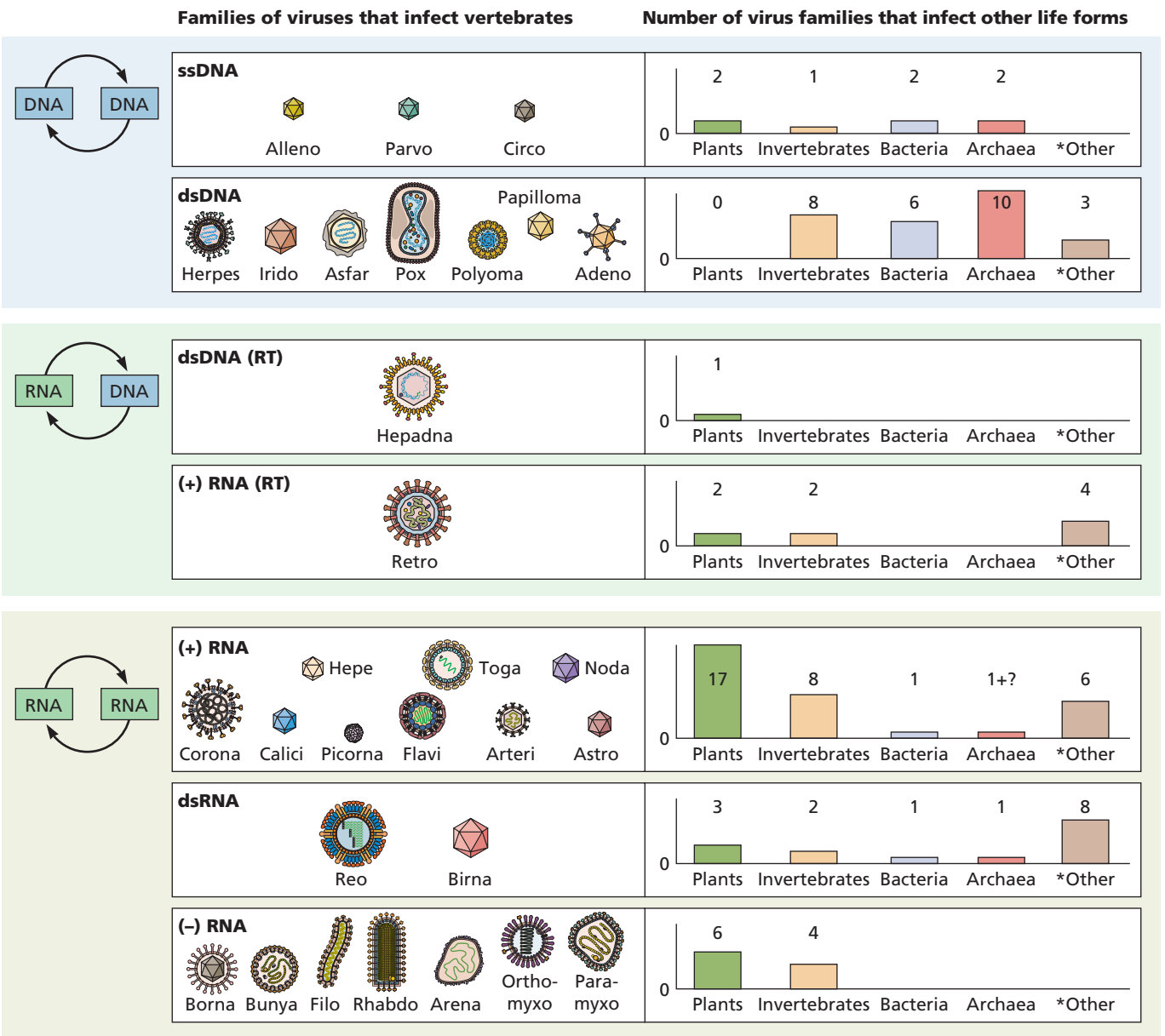
BOX 1.9

TERMINOLOGY

Complexities of viral nomenclature

No consistent system for naming viral isolates has been established by their discoverers. For example, among the vertebrate viruses, some are named for the associated diseases (e.g., poliovirus, rabies virus), for the specific type of disease they cause (e.g., murine leukemia virus), or for the sites in the body that are affected or from which they were first isolated (e.g., rhinovirus and adenovirus). Others are named for the geographic locations in which they were first isolated (e.g., Sendai virus [Sendai, Japan] and Coxsackievirus [Coxsackie, NY]) or for the scientists who first discovered them (e.g., Epstein-Barr virus). In these cases the virus names are often capitalized. Some viruses are even named for the way in which people imagined they were contracted (e.g., influenza, for the "influence" of bad air), how they were first perceived (e.g., the giant mimiviruses [Box 1.10], for the fact that they "mimic" bacteria), or totally by whimsy (e.g., pandoravirus). Finally, combinations of the above designations are also used (e.g., Rous sarcoma virus).

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*Algae, fungi, yeasts, and protozoa

Figure 1.10 Viral families sorted according to the nature of the viral genomes. A wide variety of sizes and shapes are illustrated for the families of viruses that infect vertebrates. Similar diversity exists for the families of viruses that infect other life forms, but the chart illustrates only the number found to date in each category. As noted, in some categories there are as yet no examples. The notation "1+?" for RNA archaeal viruses indicates that additional viral genomes have been predicted from metagenomic analyses but are not yet confirmed. Abbreviations: ds, double-stranded; ss, single-stranded. Adapted from A. M. Q. King et al. (ed.), *Virus Taxonomy: Classification and Nomenclature of Viruses*, Ninth Report of the International Committee on Taxonomy of Viruses (Academic Press, Inc., San Diego, CA, 2012).

properties of viruses and their reproduction has increased, other relationships have become apparent. *Hepadnaviridae*, *Retroviridae*, and some plant viruses are classified as different families on the basis of the nature of their genomes. Nevertheless, they are all related by the fact that reverse transcription is an essential step in their reproductive cycles, and the viral polymerases that perform this task exhibit important similarities in amino acid sequence. Another example is the classification of the giant protozoan *Mimiviridae* and pandoraviruses as members of a related group called nucleocytoplasmic large

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BOX 1.10

DISCUSSION

Viral giants and a new satellite

The mimivirus virion, the prototype member of the *Mimiviridae*, is large enough to be visible in a light microscope, and it was the first of the giant viruses to be discovered. The mimivirus was isolated from water in a cooling tower in England in 1992 and was initially thought to be an intracellular bacterium in its amoeba host. Not until a brief note in 2003 was it made apparent that this giant is a member of a group of nucleocytoplasmic large DNA viruses (NCLDVs) that include the poxviruses and several aquatic viruses. The mimiviruses' fiber-coated icosahedral capsid (0.75 μm in diameter) is just the right size for phagocytosis by its amoeba host. The mimivirus genome of 1.2 kbp encodes more than 900 proteins and is larger than that of some bacteria. Many of these proteins are components of the protein translational apparatus, a function for which other viruses rely entirely on the host. These unusual properties have prompted speculation that the giant viruses might represent a separate branch in the tree of life, or that they arose by reductive evolution from the nucleus of a primitive cellular life form.

The discovery of a second isolate in the family *Mimiviridae*, called mamavirus, produced yet another surprise. Mamavirus was associated with a 50-nm icosahedral satellite virus particle, called Sputnik, which differs from known satellites in that it contains a double-stranded DNA genome (<18 kbp). Nevertheless, like other satellites, Sputnik depends on proteins of its helper, mamavirus, for propagation. Because it replicates in the mamavirus "factories" within the coinfected host cell and reduces virus titer, Sputnik's discoverers consider it to be a parasite of mamavirus. For this and other reasons, they argue that Sputnik represents a new, and as yet uncharacterized, family of viruses and placed it in a new a classification, which they called virophages in analogy to the functional relationship between bacteria and bacteriophages. Other investigators have argued that the general biological behavior and genetic properties of Sputnik do not differ substantially from other known satellites, and that the virophage classification can be misleading. A metagenomic analysis of Sputnik and Sputnik-like genomes has shown that they are abundant in almost all geographical zones. The overall low sequence similarity between the shared homologous genes in the three distinguished lineages, and their distant phylogenetic relationships, suggest that the genetic diversity of these satellite viruses is much beyond what we know thus far.

Another giant virus that infects amoebae was discovered in 2013. Called pandoravirus, the largest isolate has a genome that is twice the size of that of the *Mimiviridae* and encodes 2,556 putative protein-coding sequences, most of them never seen before. All of this serves to remind us that life is a continuum, and the delineation of distinct categories can sometimes be quite difficult.

La Scola B, Audic S, Robert C, Jungang L, de Lamballerie X, Drancourt M, Birtles R, Claverie JM, Raoult D. 2003. A giant virus in amoebae. *Science* **299:**2033.

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Philippe N, Legendre M, Doutre G, Couté Y, Poirot O, Lescot M, Arslan D, Seltzer V, Bertaux L, Bruley C, Garin J, Claverie JM, Abergel C. 2013. Pandoraviruses: amoeba viruses with genomes up to 2.5 Mb reaching that of parasitic eukaryotes. *Science* **341:**281–286.

For illustrations of sputnik and mimivirus structure see: http://viralzone.expasy.org/all_by_species/670.html

See also TWiV 261: Giants among viruses. Interview with Drs. Chantal Abergel and Jean-Michel Claverie at http://www.twiv.tv/2013/12/01/twiv-261-giants-among-viruses/

DNA viruses (NCLDVs), which includes the *Poxviridae* that infect vertebrates (Box 1.10).

The 2012 report of the International Committee on Taxonomy of Viruses (ICTV) lists 2,618 virus and viroid species distributed amongst 420 genera, 22 subfamilies, 96 families, and 7 orders, as well as numerous viruses that have not yet been classified and are probably representatives of new genera and/or families. The ICTV report also includes descriptions of subviral agents (**satellites**, viroids, and prions) and a list of viruses for which information is still insufficient to make assignments. Satellites are composed of nucleic acid molecules that depend for their multiplication on coinfection of a host cell with a helper virus. However, they are not related to this helper. When a satellite encodes the coat protein in which its nucleic acid is encapsidated, it is referred to as a **satellite virus** (e.g., hepatitis delta virus is a satellite virus). The pace of discovery of new viruses has been accelerated greatly with the application of **metagenomic analyses**, direct sequencing of genomes from environmental samples, suggesting that we have barely begun to chart the viral universe.

The ICTV nomenclature has been applied widely in both the scientific and medical literature, and therefore we adopt it in this text. In this nomenclature, the Latinized virus family names are recognized as starting with capital letters and ending with *-viridae*, as, for example, in the family name *Parvoviridae*. These names are used interchangeably with their common derivatives, as, for example, parvoviruses.

Classification by Genome Type: the Baltimore System

Because the viral genome carries the entire blueprint for virus propagation, molecular virologists have long considered it the most important characteristic for classification purposes. Therefore, although individual virus families are known by their classical designations, they are more commonly placed in groups according to their genome types, as illustrated

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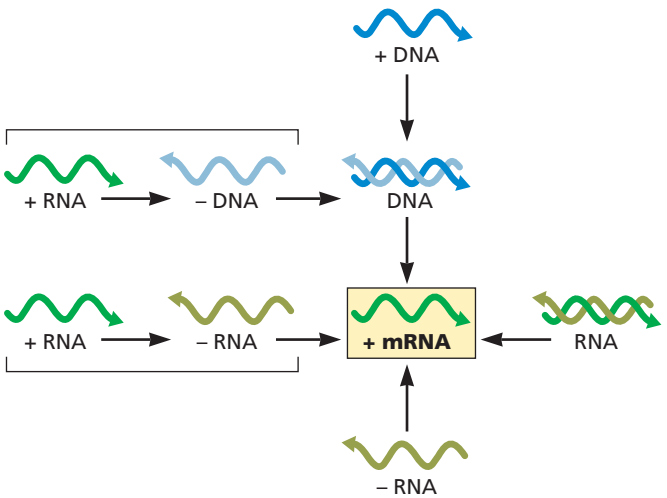


Figure 1.11 The Baltimore classification. All viruses must produce mRNA that can be translated by cellular ribosomes. In this classification system, the unique pathways from various viral genomes to mRNA define specific virus classes on the basis of the nature and polarity of their genomes.

in Fig. 1.10. There are seven genome types for all families of viruses, and all seven are represented in viruses that infect vertebrates.

Francis Crick conceptualized the central dogma for flow of genetic information:

DNA → RNA → protein

All viruses must direct the synthesis of mRNA that is decoded by the host's translational machinery. Appreciation of the essential role of the translational machinery inspired David Baltimore to devise a complementary classification scheme (Fig. 1.11). This scheme describes the pathways for formation of mRNA for viruses with either RNA or DNA genomes.

By convention, mRNA is defined as a **positive [(+)] strand** because it contains immediately translatable information. In the Baltimore classification, a strand of DNA that is of equivalent sequence is also designated a (+) strand. The RNA and DNA complements of (+) strands are designated **negative [(−)] strands**. The information embodied in this classification provides virologists with immediate insight into the steps that must take place to initiate replication and expression of the viral genome.

A Common Strategy for Viral Propagation

The basic thesis of this textbook is that **all** viral propagation can be described in the context of three fundamental properties.

- All viral genomes are packaged inside particles that mediate their transmission from host to host.
- The viral genome contains the information for initiating and completing an infectious cycle within a susceptible, permissive cell. An infectious cycle includes attachment and entry, decoding of genome information, genome replication, and assembly and release of particles containing the genome.
- All successful viruses are able to establish themselves in a host population so that virus propagation is ensured.

Perspectives

The study of viruses has increased our understanding of the importance and ubiquitous existence of these diverse agents and, in many cases, yielded new and unexpected insight into the molecular biology of host cells and organisms. Indeed, as viruses are obligate molecular parasites, every tactical solution encountered in their reproduction and propagation must of necessity tell us something about the host as well as the virus. Some of the important landmarks and achievements in the field of animal virology are summarized in Fig. 1.12. It is apparent that much has been discovered about the biology of viruses and about host defenses against them. Yet the more we learn, the more we realize that much is still unknown.

In the first edition of this textbook (published in 2000), we noted that the most recent (1995) report of the ICTV listed 71 different virus families, which covered most new isolates. We speculated therefore that: "As few new virus families had been identified in recent years, it seems likely that a significant fraction of all existing virus families are now known." In the intervening years, this prediction has been shattered, not only by the discovery of new families of viruses, including giant viruses with genome sizes that surpass those of some bacteria, but also by results from metagenomic analyses. For example, the fact that a high percentage (93%) of protein-coding sequences in the genomes of the giant pandoraviruses have **no** homologs in the current databases and the unusual morphological features and atypical reproduction process of these viruses were totally unexpected. It is also mind-boggling to contemplate that of almost 900,000 viral sequences identified in samples of only one type of ecosystem (raw sewage), more than 66% bore **no** relationship to any viral family in the current database. From these analyses and similar studies of other ecosystems (i.e., oceans and soil), it has been estimated that less than 1% of the extant viral diversity has been explored to date. Clearly, the viral universe is far more vast and diverse than suspected only a decade ago, and there is much fertile ground for gaining a deeper understanding of the biology of viruses, and their host cells and organisms, in the future.

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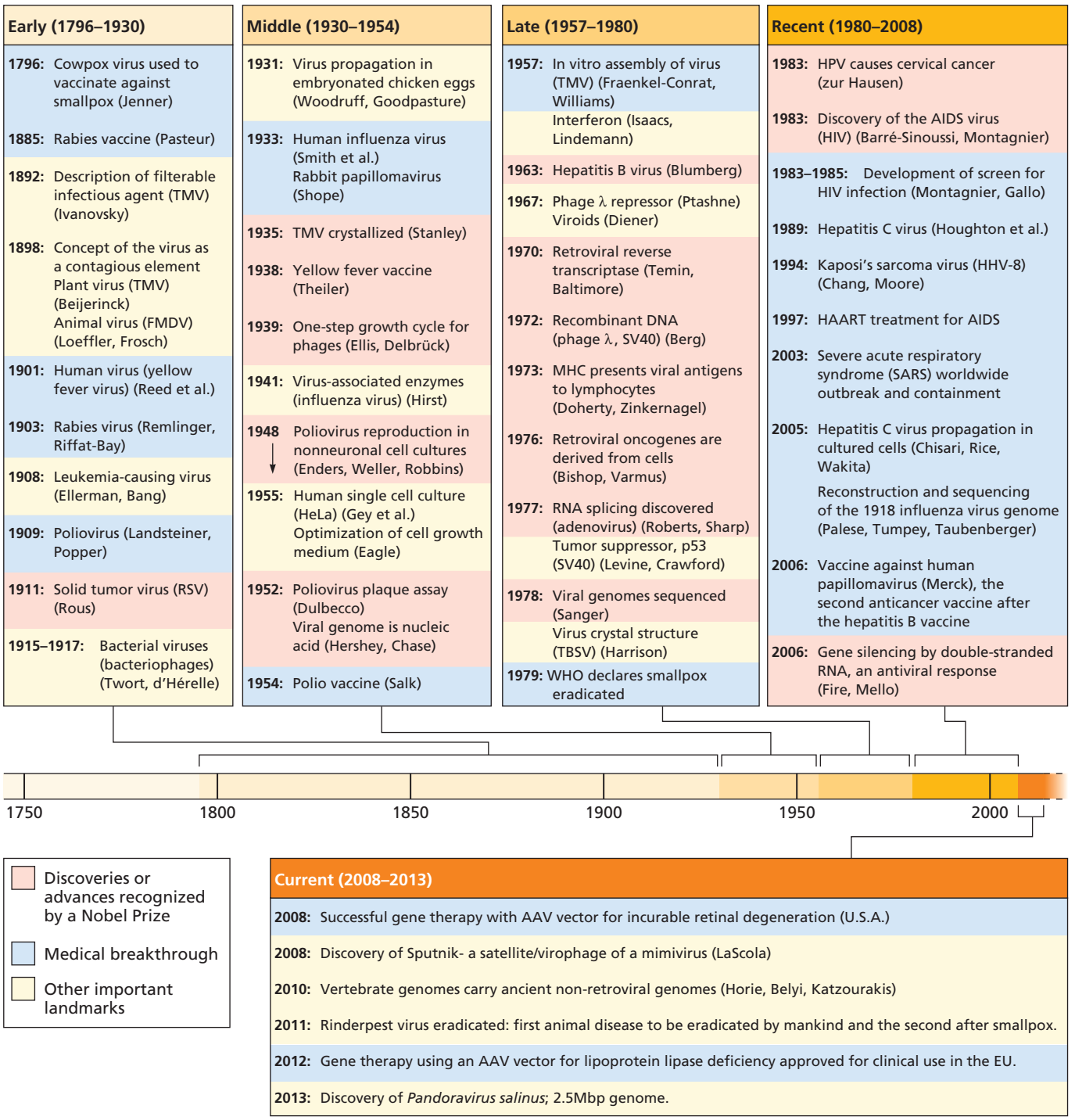


Figure 1.12 Landmarks in the study of animal viruses. Key discoveries and technical advances are listed for each time interval. The pace of discovery has increased exponentially over time. Abbreviations: AAV, adenovirus associated virus; EU, European Union; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; HPV, human papillomavirus; TBSV, tomato bushy stunt virus; TMV, tobacco mosaic virus; SV40, simian virus 40; FMDV, foot-and-mouth disease virus; WHO, World Health Organization; MHC, major histocompatibility complex; HHV-8, human herpesvirus 8; RSV, Rous sarcoma virus.

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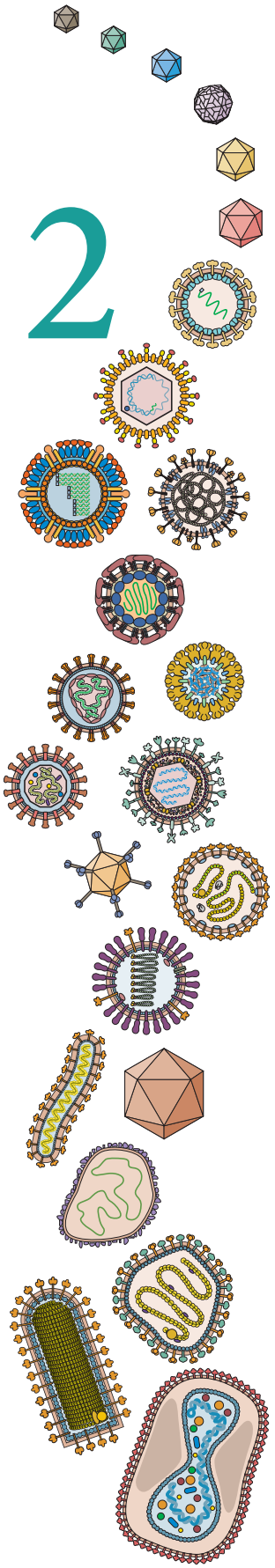
Websites

http://www.ictvonline.org/virusTaxonomy.asp?bhcp=1 *Latest update of virus classification from the ICTV.*

http://ictvonline.org/ *ICTV-approved virus names and other information as well as links to virus databases.*

http://www.twiv.tv *A weekly netcast about viruses featuring informal yet informative interviews with guest virologists who discuss their recent findings and other topics of general interest.*

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2 The Infectious Cycle

Introduction

The Infectious Cycle

The Cell

The Architecture of Cell Surfaces
- The Extracellular Matrix: Components and Biological Importance
- Properties of the Plasma Membrane
- Cell Membrane Proteins

Entering Cells

Making Viral RNA

Making Viral Proteins

Making Viral Genomes

Forming Progeny Virus Particles

Viral Pathogenesis

Overcoming Host Defenses

Cultivation of Viruses
- Cell Culture
- Embryonated Eggs
- Laboratory Animals

Assay of Viruses
- Measurement of Infectious Units
- Efficiency of Plating
- Measurement of Virus Particles and Their Components

Viral Reproduction: the Burst Concept

The One-Step Growth Cycle
- Initial Concept
- One-Step Growth Analysis: a Valuable Tool for Studying Animal Viruses

Systems Biology

Perspectives

References

LINKS FOR CHAPTER 2

- *Video: Interview with Dr. Thomas Hope*
  http://bit.ly/Virology_Hope
- *Cloning HeLa cells with Philip I. Marcus*
  http://bit.ly/Virology_Twiv197
- *Ode to a plaque*
  http://bit.ly/Virology_Twiv68
- *Movie of vaccinia virus plaque formation*
  http://www.sciencemag.org/content/suppl/2010/01/19/science.1183173.DC1/1183173s1.mov
- *Think globally, act locally*
  http://bit.ly/Virology_Twim90

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You know my methods, Watson.
SIR ARTHUR CONAN DOYLE

Introduction

Viruses are unique: they are exceedingly small, often made up of nothing more than a nucleic acid molecule within a protein shell, yet when they enter cells, they parasitize the cellular machinery to produce thousands of progeny. This simplicity is misleading: viruses can infect all known life forms, and they comprise a variety of structures and genomes. Despite such variety, viruses are amenable to study because all viral propagation can be described in the context of three fundamental properties, as described in Chapter 1: all viral genomes are packaged inside particles that mediate their transmission from cell to cell; the viral genome contains the information for initiating and completing an infectious cycle; and all viruses can establish themselves in a host population to ensure virus survival.

The objective of research in virology is to understand how viruses enter individual cells, replicate, and assemble new infectious particles. These studies are usually carried out with cell cultures, because they are a much simpler and more homogeneous experimental system than animals. Cells can be infected in such a way as to ensure that a single replication cycle occurs synchronously in every infected cell, the **one-step growth curve**. Because all viral infections take place within a cell, a full understanding of viral life cycles also requires knowledge of cell biology and cellular architecture. These are the topics of this chapter: the cell surface (the site at which viruses enter and exit cells), methods for detecting viruses and viral growth, and one-step growth analysis.

The Infectious Cycle

The production of new infectious viruses can take place only within a cell (Fig. 2.1). Virologists divide the viral infectious cycle into discrete steps to facilitate their study, although in virus-infected cells no such artificial boundaries occur. The infectious cycle comprises attachment and entry of the particle, production of viral mRNA and its translation by host ribosomes, genome replication, and assembly and release of particles containing the genome. New virus particles produced during the infectious cycle may then infect other cells. The term **virus reproduction** is another name for the sum total of all events that occur during the infectious cycle.

There are events common to virus replication in animals and in cells in culture, but there are also many important differences. While viruses readily attach to cells in culture, in nature a virus particle must encounter a host, no mean feat for nanoparticles without any means of locomotion. After encountering a host, the virus particle must pass through physical host defenses, such as dead skin, mucous layers, and the extracellular matrix. Host defenses such as antibodies and immune cells, which exist to combat virus infections, are not found in cell cultures. Virus infection of cells in culture has been a valuable tool for understanding viral infectious cycles, but the differences compared with infection of a living animal must always be considered.

The Cell

Viruses require many different functions of the host cell (Fig. 2.2) for propagation. Examples include the machinery for translation of viral mRNAs, sources of energy, and enzymes for genome replication. The cellular transport apparatus brings

PRINCIPLES *The infectious cycle*

- Many distinct functions of the host cell are required to complete a viral life cycle.
- A productive infection requires target cells that are both susceptible (i.e., allow virus entry) and permissive (i.e., support virus reproduction).
- Viral nucleic acids must be shielded from harsh environmental conditions as extracellular particles, but be readily accessible for replication once inside the cell.
- To advance their study, viruses may be propagated in cells within a laboratory animal or in cell cultures, which include immortalized cells or primary cultures derived from the natural host or other animals.
- Plaque assays are the major way to determine the concentration of infectious virus particles in a sample, though alternative strategies exist for viruses that do not form plaques.
- While the goals of quantifying and characterizing virus particles remain fundamental for research in virology, the specific techniques used evolve rapidly, based on developments in detection, ease, cost, safety, utility in the field, and amenability to large-scale implementation.
- Viral nucleic acids can be detected and characterized by multiple methods, including direct sequencing of genomes and mRNAs, PCR, and microarrays.
- Relationships among viruses can be deduced from phylogenetic trees generated from protein or nucleic acid sequences.
- Viral reproduction is distinct from cellular or bacterial replication: rather than doubling with each cycle, each single cell cycle of viral reproduction is typically characterized by the release of many (often thousands) of progeny virions.
- The multiplicity of infection (MOI) is the number of infectious units added per cell in an experimental setting; the probability that any one target cell will become infected based on the MOI can be calculated from the Poisson distribution.
- Application of systems biology approaches to virology can implicate particular cellular pathways in viral reproduction and can reveal signatures of virus-induced lethality or immune protection.

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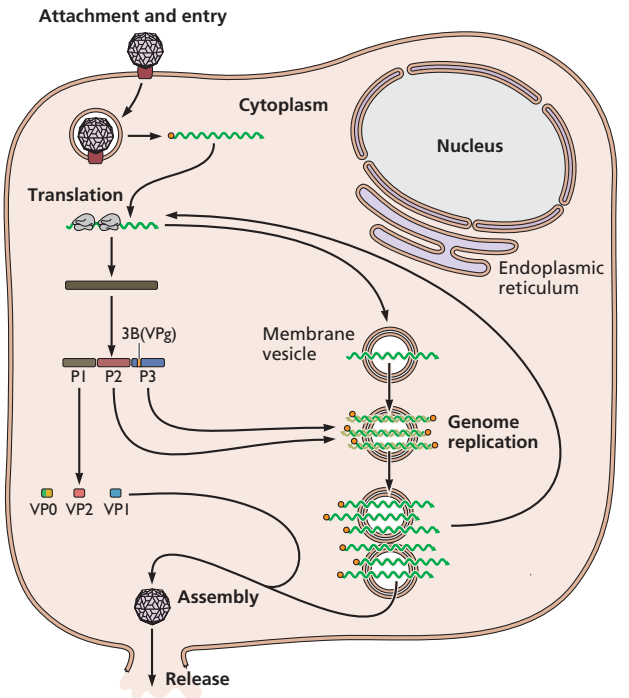
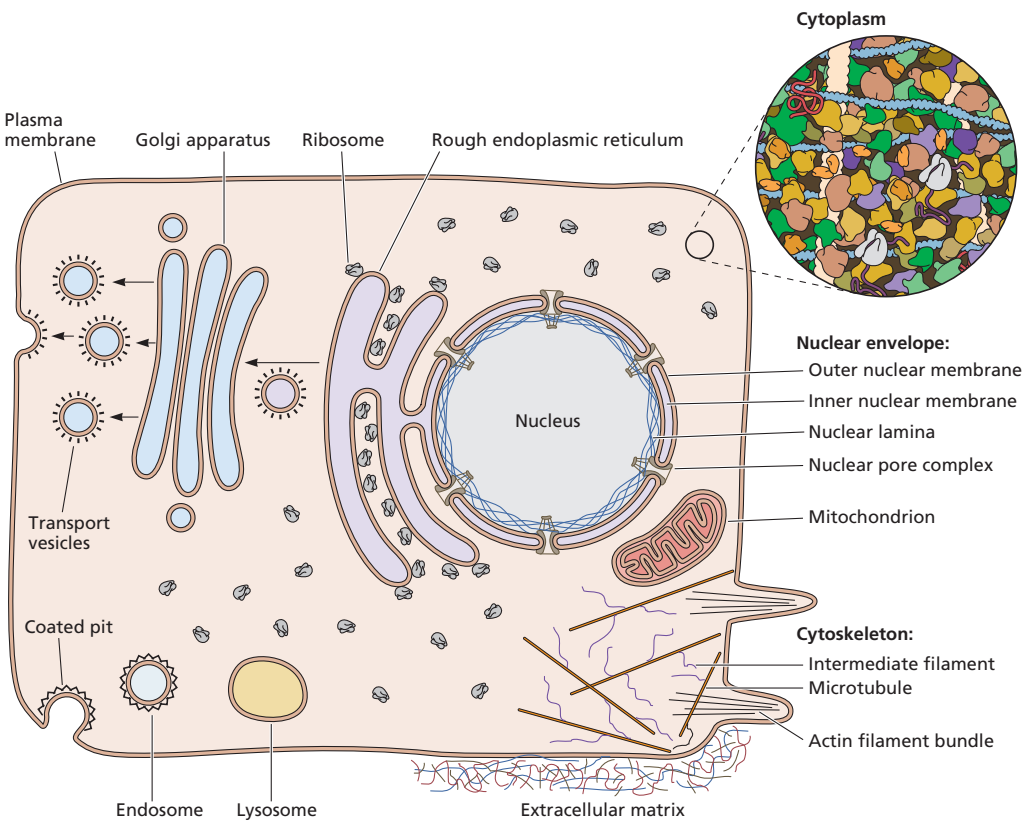


Figure 2.1 The viral infectious cycle. The infectious cycle of poliovirus is shown as an example, illustrating the steps common to all viral life cycles: attachment and entry, translation, genome replication, assembly, and release. See Appendix, Figures 21 and 22 for explanation of abbreviations.

Figure 2.2 The mammalian cell. Illustrated schematically are the nucleus, the major membrane-bound compartments of the cytoplasm, and components of the cytoskeleton that play important roles in virus reproduction. A small part of the cytoplasm is magnified, showing the crowded contents. The figure is not drawn to scale.



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viral genomes to the correct cellular compartment and ensures that viral subunits reach locations where they may be assembled into virus particles. Subsequent chapters include a discussion of cellular functions that are important for individual steps in the viral replication cycle. In the following section, we consider the architecture of cell surfaces. The cell membrane merits this special focus because it is not only the portal of entry for all animal viruses, but also the site from which many viruses leave the cell.

The Architecture of Cell Surfaces

In animals, viral infections usually begin at the surfaces of the body that are exposed to the environment (Fig. 2.3). Epithelial cells cover these surfaces, and the region of these cells exposed to the environment is called the **apical surface**. Conversely, the **basolateral surfaces** of such cells are in contact with adjacent or underlying cells or tissues. These cells exhibit a differential (polar) distribution of proteins and lipids in the plasma membranes that creates the two distinct surface domains. As illustrated in Fig. 2.3, these cell layers differ in thickness and organization. Movement of macromolecules between the cells in the epithelium is prevented by **tight junctions**, which circumscribe the cells at the apical edges of their lateral membranes. Many viral infections are initiated upon entry into epithelial or endothelial cells (which line the interior surface of blood and lymphatic vessels) at their exposed apical surfaces, often by attaching to cell surface molecules specific for these domains. Viruses that both enter and are released at apical membranes can be transmitted laterally from cell to cell without ever traversing the epithelial or endothelial layers; they generally cause localized infections. In other cases, progeny virus particles are transported to the basolateral surface and released into the underlying cells and tissues, a process that facilitates viral spread to other sites of replication.

There are also more-specific pathways by which viruses reach susceptible cells. For example, some epithelial tissues contain M cells, specialized cells that overlie the collections of lymphoid cells in the gut known as Peyer's patches. M cells transport intestinal contents to Peyer's patches by a mechanism called **transcytosis**. Certain viruses, such as poliovirus and human immunodeficiency virus type 1, can be transported through them to gain access to underlying tissues. Such specialized pathways of invasion are considered in Volume II, Chapter 2. Below, we describe briefly the structures that surround cells and tissues, as well as the membrane components that are relevant to virus replication.

The Extracellular Matrix: Components and Biological Importance

Extracellular matrices, which hold the cells and tissues of the body together, are made up of two main classes of macromolecules (Fig. 2.4). The first comprises glycosaminoglycans (such as heparan sulfate and chondroitin sulfate), which are unbranched polysaccharides made of repeating disaccharides. Glycosaminoglycans are usually linked to proteins to

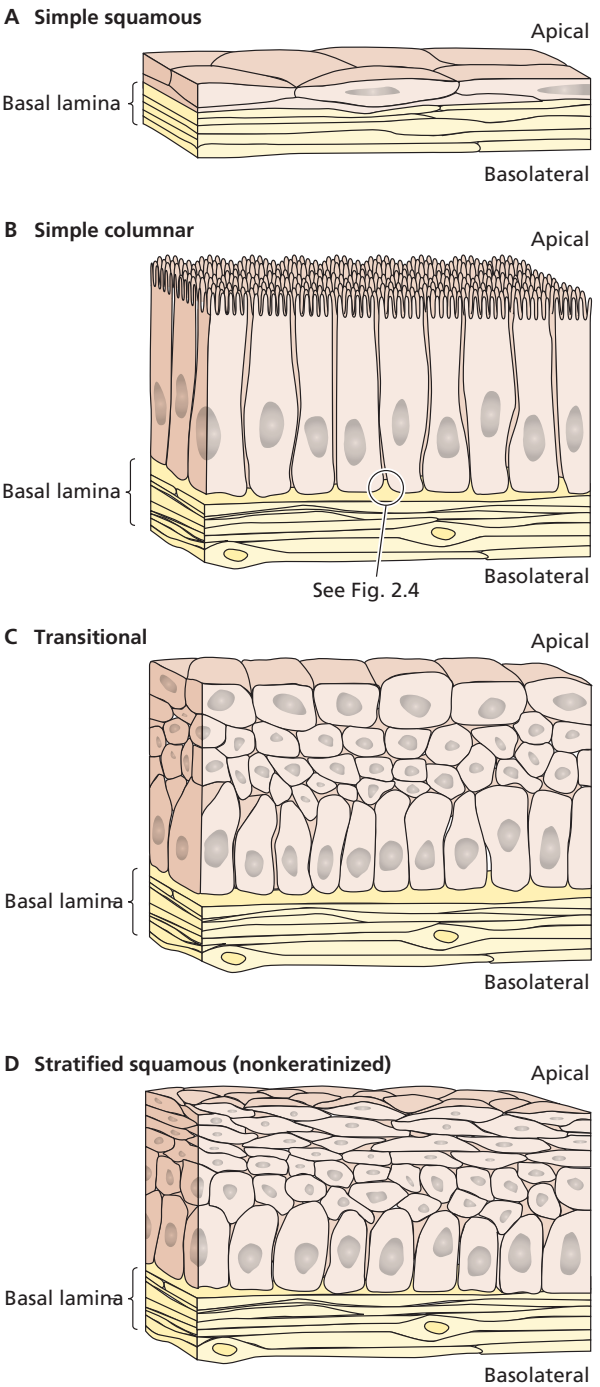


Figure 2.3 Major types of epithelia. **(A)** Simple squamous epithelium made up of thin cells such as those lining blood vessels and many body cavities. **(B)** Simple columnar epithelium found in the stomach, cervical tract, and small intestine. **(C)** Transitional epithelium, which lines cavities, such as the urinary bladder, that are subject to expansion and contraction. **(D)** Stratified, nonkeratinized epithelium lining surfaces such as the mouth and vagina. Adapted from H. Lodish et al., *Molecular Cell Biology*, 3rd ed. (W. H. Freeman & Co., New York, NY, 1995), with permission.

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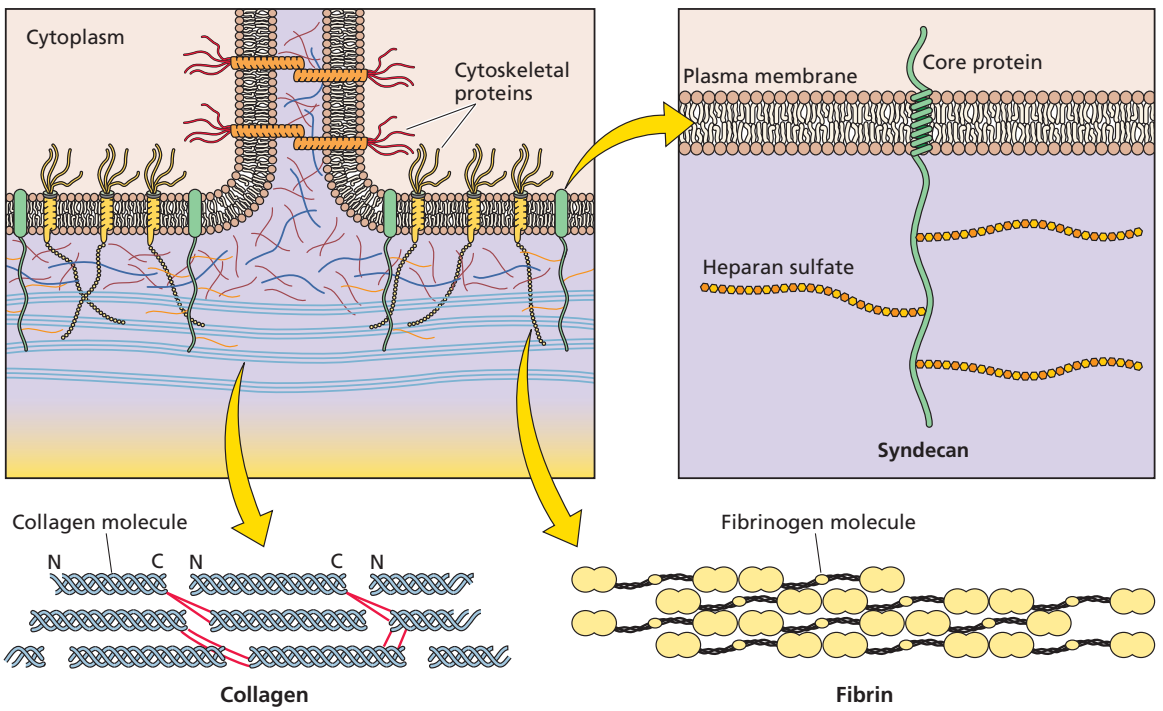
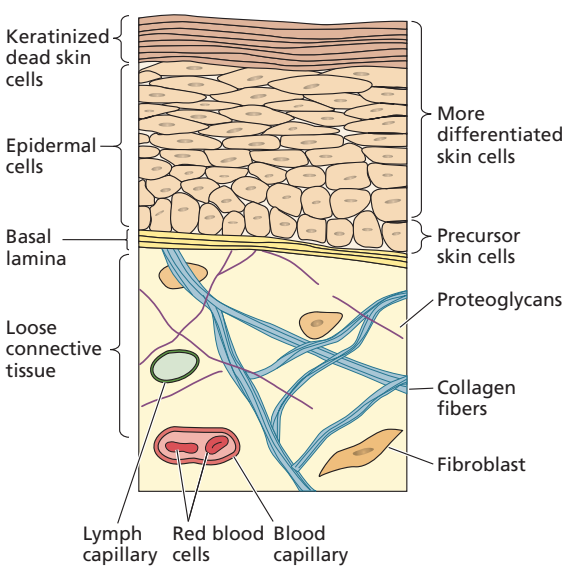


Figure 2.4 Cell adhesion molecules and components of the extracellular matrix. The diagram (expanded from Fig. 2.3B) illustrates some of the many cell surface components that contribute to cell-cell adhesion and attachment to the extracellular matrix. Adapted from G. M. Cooper, *The Cell: a Molecular Approach* (ASM Press, Washington, DC, and Sinauer Associates, Sunderland, MA, 1997).

form **proteoglycans**. The second class of macromolecules in the extracellular matrix consists of fibrous proteins with structural (**collagen** and **elastin**) or adhesive (**fibronectin** and **laminin**) functions. The proteoglycan molecules in the matrix form hydrated gels in which the fibrous proteins are embedded, providing strength and resilience. The gel provides resistance to compression and allows the diffusion of nutrients between blood and tissue cells. The extracellular matrix of each cell type is specialized for the particular function required, varying in thickness, strength, elasticity, and degree of adhesion.

Most organized groups of cells, like epithelial cells of the skin (Fig. 2.3 and 2.5), are bound tightly on their basal surface to a thin layer of extracellular matrix called the **basal lamina**. This matrix is linked to the basolateral membrane by specific receptor proteins called **integrins** (which are discussed in "Cell Membrane Proteins" below). Integrins are anchored to the intracellular structural network (the **cytoskeleton**) at the inner surface of the cell membrane. The basal lamina is attached to collagen and other material in the underlying loose connective tissue found in many organs of the body (Fig. 2.5). Capillaries, glands, and specialized cells are embedded in the connective tissue. Some viruses gain access to susceptible cells by attaching specifically to components of the extracellular matrix, including cell adhesion proteins or proteoglycans.

Figure 2.5 Cross section through skin. In this diagram of skin from a pig, the precursor epidermal cells rest on a thin layer of extracellular matrix called the basal lamina. Underneath is loose connective tissue consisting mostly of extracellular matrix. Fibroblasts in the connective tissue synthesize the connective tissue proteins, hyaluronan, and proteoglycans. Blood and lymph capillaries are also located in the loose connective tissue layer. Adapted from H. Lodish et al., *Molecular Cell Biology*, 3rd ed. (W. H. Freeman & Co., New York, NY, 1995), with permission.



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Properties of the Plasma Membrane

The plasma membrane of every mammalian cell type is composed of a similar phospholipid/glycolipid bilayer, but different sets of membrane proteins and lipids allow the cells of different tissues to carry out their specialized functions. The lipid bilayer is constructed from molecules that possess both hydrophilic and hydrophobic portions; they are known as **amphipathic** molecules, from the Greek word *amphi* (meaning "on both sides") (Fig. 2.6). They form a sheet-like structure in which polar head groups face the aqueous environment of the cell's cytoplasm (inner surface) or the surrounding environment (outer surface). The polar head groups of the inner and outer leaflets bear side chains with different lipid compositions. The fatty acyl side chains form a continuous hydrophobic interior about 3 nm thick. Hydrophobic interactions are the driving force for formation of the bilayer. However, hydrogen bonding and electrostatic interactions among the polar groups and water molecules or membrane proteins also stabilize the structure.

Thermal energy permits the phospholipid and glycolipid molecules comprising natural cell membranes to rotate freely around their long axes and diffuse laterally. If unencumbered, a lipid molecule can diffuse the length of an animal cell in only 20 s at 37°C. In most cases, phospholipids and glycolipids do not flip-flop from one side of a bilayer to the other, and the outer and inner leaflets of the bilayer remain separate. Similarly, membrane proteins not anchored to the extracellular matrix and/or the underlying structural network of the cell can diffuse rapidly, moving laterally like icebergs in this fluid bilayer. In this way, certain membrane proteins can form functional aggregates. Intracellular organelles such as the nucleus, endoplasmic reticulum, and lysosomes are also

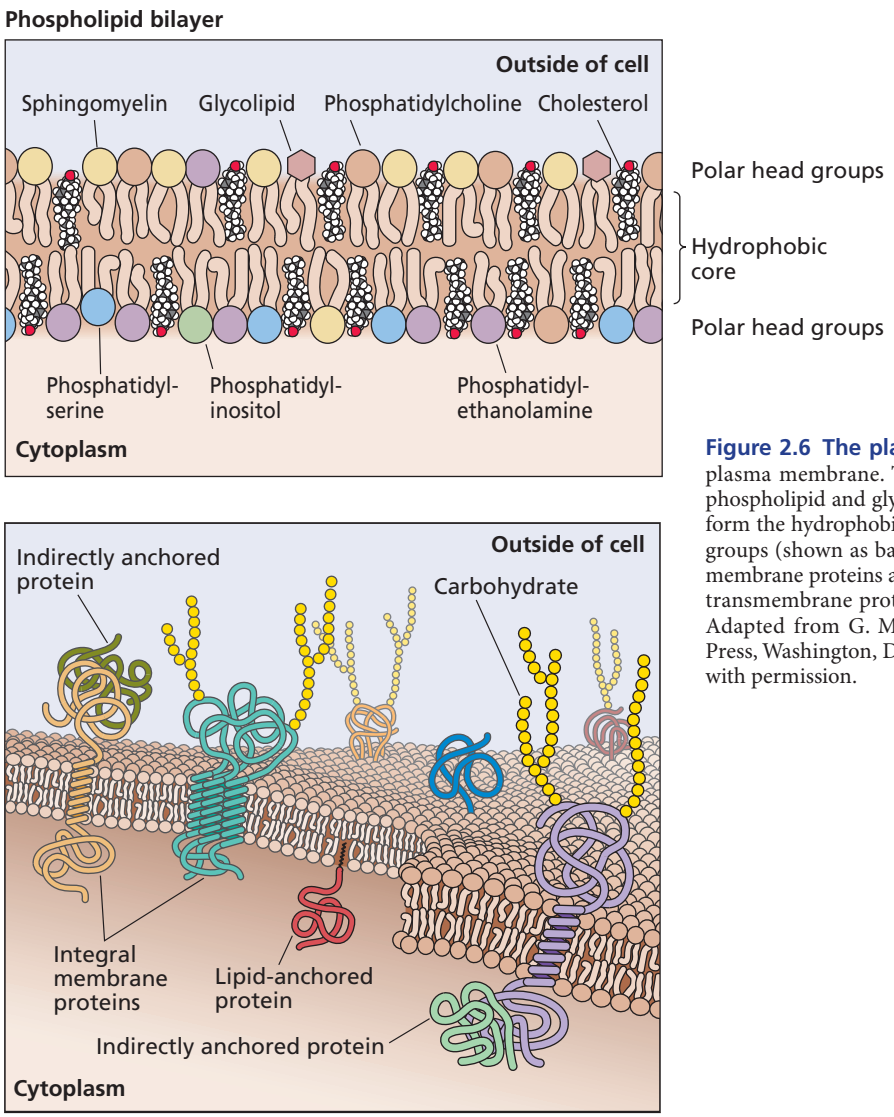


Figure 2.6 The plasma membrane. (Top) Lipid components of the plasma membrane. The membrane consists of two layers (leaflets) of phospholipid and glycolipid molecules. Their fatty acid tails converge to form the hydrophobic interior of the bilayer; the polar hydrophilic head groups (shown as balls) line both surfaces. (Bottom) Different types of membrane proteins are illustrated. Some integral membrane proteins are transmembrane proteins and are exposed on both sides of the bilayer. Adapted from G. M. Cooper, *The Cell: a Molecular Approach* (ASM Press, Washington, DC, and Sinauer Associates, Sunderland, MA, 1997), with permission.

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enclosed in lipid bilayers, although their composition and physical properties differ.

The plasma membrane was once viewed as a uniform and fluid sea, in which lipid and protein components diffused randomly in the plane of the membrane. This simplistic model has been dispelled by experimental findings, which demonstrate that plasma membranes comprise **microdomains**, regions with distinct lipid and protein composition (Box 2.1). The **lipid raft** is one type of microdomain that is important for virus replication. Lipid rafts are enriched in cholesterol and saturated fatty acids, and are more densely packed and less fluid than other regions of the membrane. The assembly of a variety of viruses takes place at lipid rafts (see Chapter 13). Furthermore, the entry of some viruses requires lipid rafts. For example, particles of human immunodeficiency virus type 1 and Ebola virus enter cells at lipid rafts. Treatment of cells with compounds that disrupt these microdomains blocks entry. One explanation for this requirement might be that cell membrane proteins required for entry are concentrated in lipid rafts: receptors and coreceptors for human immunodeficiency virus are preferentially located in these domains.

Cell Membrane Proteins

Membrane proteins are classified into two broad categories, **integral membrane proteins** and **indirectly anchored proteins**, names that describe the nature of their interactions with the plasma membrane (Fig. 2.6).

Integral membrane proteins are embedded in the lipid bilayer, via one or more **membrane-spanning domains**, and contain portions that protrude out into the exterior and interior of the cell (Fig. 2.6). Many membrane-spanning domains consist of an α-helix typically 3.7 nm long. Such a domain includes 20 to 25 generally hydrophobic or uncharged residues embedded in the membrane, with the hydrophobic side chains protruding outward to interact with the fatty acyl chains of the lipid bilayer. The first and last residues are often positively charged amino acids (lysine or arginine) that can interact with the negatively charged polar head groups of the phospho- or glycolipids to stabilize the membrane-spanning domain. Some proteins with membrane-spanning domains enable the cell to respond to external signals. Such membrane proteins are designed to bind external ligands (e.g., hormones, cytokines, or membrane proteins on the same cell or on other cells) and to signal the occurrence of such interactions to molecules in the interior of the cell. Other proteins with multiple membrane-spanning domains (Fig. 2.6) form critical components of molecular pores or pumps, which mediate the internalization of required nutrients or the expulsion of undesirable material from the cell, or maintain homeostasis with respect to cell volume, pH, and ion concentration.

In many cases, the external portions of membrane proteins are decorated by complex or branched **carbohydrate chains** linked to the peptide backbone. Linkage can be to either nitrogen (**N linked**) in the side chain of asparagine residues or oxygen (**O linked**) in the side chains of serine or threonine residues. Such membrane **glycoproteins**, as they are called, quite frequently serve as viral receptors.

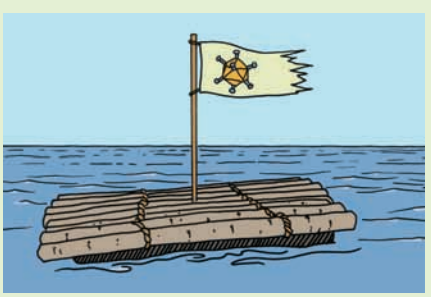
Some membrane proteins do not span the lipid bilayer, but are anchored in the inner or outer leaflet by covalently attached hydrocarbon chains (see Chapter 12). Indirectly

BOX 2.1

BACKGROUND

Plasma membrane microdomains

According to the Singer-Nicholson fluid mosaic model of membrane structure proposed in 1972, membranes are two-dimensional fluids with proteins inserted into the lipid bilayers (Fig. 2.6). Although the model accurately predicts the general organization of membranes, one of its conclusions has proven incorrect: that proteins and lipids are randomly distributed because they can freely rotate and laterally diffuse within the plane of the membrane. Beginning in the 1990s, the results of a series of experiments indicated that the movement of most proteins in the plasma membrane is partially restricted. In particular, these studies provided evidence for the existence of plasma membrane microdomains that are enriched in glycosphingolipids, cholesterol, glycosylphosphatidylinositol-anchored proteins, and certain intracellular signaling proteins. These microdomains, called lipid rafts, are defined experimentally as being resistant to extraction in cold 1% Triton X-100 and floating in the top half of a 5 to 30% sucrose density gradient. A major component of lipid rafts was found to be caveolin-1, a major coat protein of caveolae. These flask-shaped invaginations of the plasma membrane take up sphingolipids and integrins, as well as viruses, bacteria, and toxins. There are many noncaveolar lipid raft domains in the plasma membrane: detergent-insoluble microdomains are also present in cells that lack caveolin-1.



Sonnino S, Prinetti A. 2013. Membrane domains and the "lipid raft" concept. *Curr Med Chem* **20:**4–21.

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anchored proteins are bound to the plasma membrane lipid bilayer by interacting either with integral membrane proteins or with the charged sugars of the glycolipids. Fibronectin, a protein in the extracellular matrix that binds to integrins (Fig. 2.4), is an example.

Entering Cells

Viral infection is initiated by a collision between the virus particle and the cell, a process that is governed by chance. Consequently, a higher concentration of virus particles increases the probability of infection. However, a virion may not infect every cell it encounters; it must first come in contact with the cells and tissues to which it can bind. Such cells are normally recognized by means of a specific interaction of a virus particle with a cell surface receptor. These molecules do not exist for the benefit of viruses: they all have cellular functions, and viruses have evolved to bind them for cell entry. Virus-receptor interactions can be either promiscuous or highly selective, depending on the virus and the distribution of the cell receptor. The presence of such receptors determines whether the cell will be **susceptible** to the virus. However, whether a cell is **permissive** for the reproduction of a particular virus depends on other, intracellular components found only in certain cell types. Cells must be both susceptible **and** permissive if an infection is to be successful.

Viruses have no intrinsic means of locomotion, but their small size facilitates diffusion driven by Brownian motion. Propagation of viruses is dependent on essentially random encounters with potential hosts and host cells. Features that increase the probability of favorable encounters are very important. In particular, viral propagation is critically dependent on the production of large numbers of progeny virus particles with surfaces composed of many copies of structures that enable the attachment of virus particles to susceptible cells.

Successful entry of a virus into a host cell requires traversal of the plasma membrane and in some cases the nuclear membrane. The virus particle must be partially or completely disassembled, and the nucleic acid must be targeted to the correct cellular compartment. These are not simple processes. Furthermore, virus particles or critical subassemblies are brought across such barriers by specific transport pathways. To survive in the extracellular environment, the viral genome must be encapsidated in a protective coat that shields viral nucleic acid from the variety of potentially harsh conditions that may be met during transit from one host cell or organism to another. For example, UV irradiation (from sunlight), extremes of pH (in the gastrointestinal tract), dehydration (in the air), and enzymatic attack (in body fluids) are all capable of damaging viral nucleic acids. However, once in the host cell, the protective structures must become sufficiently unstable to release the genome. Virus particles cannot be viewed only as passive vehicles: they must be able to undergo structural transformations that are important for attachment and entry into a new host cell and for the subsequent disassembly required for viral replication.

Making Viral RNA

Although the genomes of viruses come in a number of configurations, they share a common requirement: they must be efficiently copied into mRNAs for the synthesis of viral proteins and progeny genomes for assembly. The synthesis of RNA molecules in cells infected with RNA viruses is a unique process that has no counterpart in the cell. With the exception of retroviruses, all RNA viruses encode an RNA-dependent RNA polymerase to catalyze the synthesis of mRNAs and genomes. For the majority of DNA viruses and retroviruses, synthesis of viral mRNA is accomplished by RNA polymerase II, the enzyme that produces cellular mRNA. Much of our current understanding of the mechanisms of cellular transcription comes from study of the transcription of viral templates.

Making Viral Proteins

Because viruses are parasites of translation, all viral mRNAs must be translated by the host's cytoplasmic protein-synthesizing machinery (see Chapter 11). However, viral infection often results in modification of the host's translational apparatus so that viral mRNAs are translated selectively. The study of such modifications has revealed a great deal about mechanisms of protein synthesis. Analysis of viral translation has also revealed new strategies, such as internal ribosome binding and leaky scanning, that have been subsequently found to occur in uninfected cells.

Making Viral Genomes

Many viral genomes are copied by the cell's synthetic machinery in cooperation with viral proteins (see Chapters 6 through 9). The cell provides nucleotide substrates, energy, enzymes, and other proteins. Transport systems are required because the cell is compartmentalized: essential components might be found only in the nucleus, the cytoplasm, or cellular membranes. Study of the mechanisms of viral genome replication has established fundamental principles of cell biology and nucleic acid synthesis.

Forming Progeny Virus Particles

The various components of a virus particle—the nucleic acid genome, capsid protein(s), and in some cases envelope proteins—are often synthesized in different cellular compartments. Their trafficking through and among the

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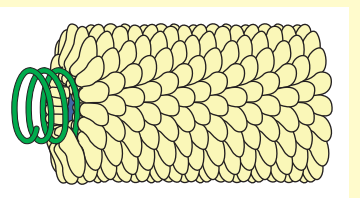
BOX 2.2

EXPERIMENTS

In vitro *assembly of tobacco mosaic virus*

The ability of the primary sequence of viral structural proteins to specify assembly is exemplified by the coat protein of tobacco mosaic virus. Heinz Fraenkel-Conrat and Robley Williams showed in 1955 that purified tobacco mosaic virus RNA and capsid protein assemble into infectious particles when mixed and incubated for 24 h. When examined by electron microscopy, the particles produced *in vitro* were identical to the rod-shaped virions produced from infected tobacco plants (Fig. 1.7B). Neither the purified viral RNA nor the capsid protein was infectious. These results indicate that the viral coat protein contains all the information needed for assembly of a virion. The spontaneous formation of tobacco mosaic virions *in vitro* from protein and RNA components is the paradigm for self-assembly in biology.

Fraenkel-Conrat H, Williams RC. 1955. Reconstitution of active tobacco mosaic virus from its inactive protein and nucleic acid components. *Proc Natl Acad Sci U S A* **40:**690–698.



cell's compartments and organelles requires that they be equipped with the proper homing signals (see Chapter 12). Components of virus particles must be assembled at some central location, and the information for assembly must be preprogrammed in the component molecules (see Chapter 13). The primary sequences of viral structural proteins contain sufficient information to specify assembly; this property is exemplified by the remarkable *in vitro* assembly of tobacco mosaic virus from coat protein and RNA (Box 2.2). Successful virus reproduction depends on redirection of the host cell's metabolic and biosynthetic capabilities, signal transduction pathways, and trafficking systems (see Chapter 14).

Viral Pathogenesis

Viruses command our attention because of their association with animal and plant diseases. The process by which viruses cause disease is called **viral pathogenesis**. To study this process, we must investigate not only the relationships of viruses with the specific cells that they infect but also the consequences of infection for the host organism. The nature of viral disease depends on the effects of viral reproduction on host cells, the responses of the host's defense systems, and the ability of the virus to spread in and among hosts (Volume II, Chapters 1 to 5).

Overcoming Host Defenses

Organisms have many physical barriers to protect themselves from dangers in their environment such as invading parasites. In addition, vertebrates possess an effective immune system to defend against anything recognized as nonself or dangerous. Studies of the interactions between viruses and the immune system are particularly instructive, because of the many viral countermeasures that can frustrate this system. Elucidation of these measures continues to teach us much about the basis of immunity (Volume II, Chapters 2 to 4).

Cultivation of Viruses

Cell Culture

Types of Cell Culture

Although human and other animal cells were first cultured in the early 1900s, contamination with bacteria, mycoplasmas, and fungi initially made routine work with such cultures extremely difficult. For this reason, most viruses were grown in laboratory animals. In 1949, John Enders, Thomas Weller, and Frederick Robbins made the discovery that poliovirus could multiply in cultured cells. As noted in Chapter 1, this revolutionary finding, for which these three investigators were awarded the Nobel Prize in Physiology or Medicine in 1954, led the way to the propagation of many other viruses in cells in culture, the discovery of new viruses, and the development of viral vaccines such as those against poliomyelitis, measles, and rubella. The ability to infect cultured cells synchronously permitted studies of the biochemistry and molecular biology of viral replication. Large-scale growth and purification allowed studies of the composition of virus particles, leading to the solution of high-resolution, three-dimensional structures, as discussed in Chapter 4.

Cells in culture are still the most commonly used hosts for the propagation of animal viruses. To prepare a cell culture, tissues are dissociated into a single-cell suspension by mechanical disruption followed by treatment with proteolytic enzymes. The cells are then suspended in culture medium and placed in plastic flasks or covered plates. As the cells divide, they cover the plastic surface. Epithelial and fibroblastic cells attach to the plastic and form a **monolayer**, whereas blood cells such as lymphocytes settle, but do not adhere. The cells are grown in a chemically defined and buffered medium optimal for their growth. Commonly used cell lines double in number in 24 to 48 h in such media. Most cells retain viability after being frozen at low temperatures (−70 to −196°C).

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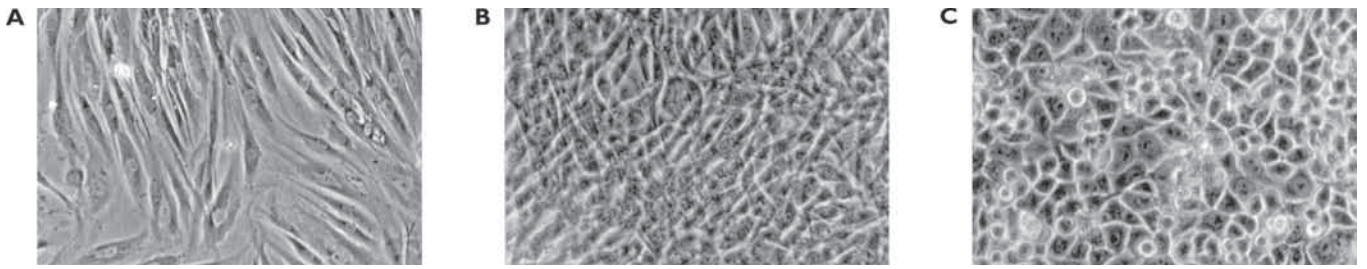


Figure 2.7 Different types of cell culture used in virology. Confluent cell monolayers photographed by low-power light microscopy. **(A)** Primary human foreskin fibroblasts; **(B)** established line of mouse fibroblasts (3T3); **(C)** continuous line of human epithelial cells (HeLa [Box 2.3]). The ability of transformed HeLa cells to overgrow one another is the result of a loss of contact inhibition. Courtesy of R. Gonzalez, Princeton University.

There are three main kinds of cell cultures (Fig. 2.7), each with advantages and disadvantages. **Primary cell cultures** are prepared from animal tissues as described above. They have a limited life span, usually no more than 5 to 20 cell divisions. Commonly used primary cell cultures are derived from monkey kidneys, human embryonic amnion and kidneys, human foreskins and respiratory epithelium, and chicken or mouse embryos. Such cells are used for experimental virology when the state of cell differentiation is important or when appropriate cell lines are not available. They are also used in vaccine production: for example, live attenuated poliovirus vaccine strains may be propagated in primary monkey kidney cells. Primary cell cultures were mandated for the growth of viruses to be used as human vaccines to avoid contamination of the product with potentially oncogenic DNA from continuous cell lines (see below). Some viral vaccines are now prepared in **diploid cell strains**, which consist of a homogeneous population of a single type and can divide up to 100 times before dying. Despite the numerous divisions, these cell strains retain the diploid chromosome number. The most widely used diploid cells are those established from human embryos, such as the WI-38 strain derived from human embryonic lung.

Continuous cell lines consist of a single cell type that can be propagated indefinitely in culture. These immortal lines are usually derived from tumor tissue or by treating a primary cell culture or a diploid strain with a mutagenic chemical or a tumor virus. Such cell lines often do not resemble the cell of origin; they are less differentiated (having lost the morphology and biochemical features that they possessed in the organ), are often abnormal in chromosome morphology and number (**aneuploid**), and can be tumorigenic (i.e., they produce tumors when inoculated into immunodeficient mice). Examples of commonly used continuous cell lines include those derived from human carcinomas (e.g., HeLa [Henrietta Lacks] cells; Box 2.3) and from mice (e.g., L and 3T3 cells). Continuous cell lines provide a uniform population of cells that can be infected synchronously for growth curve analysis (see "The One-Step Growth Cycle" below) or biochemical studies of virus replication.

In contrast to cells that grow in monolayers on plastic dishes, others can be maintained in **suspension cultures**, in which a spinning magnet continuously stirs the cells. The advantage of suspension culture is that a large number of cells can be grown in a relatively small volume. This culture method is well suited for applications that require large quantities of virus particles, such as X-ray crystallography or production of vectors.

Because viruses are obligatory intracellular parasites, they cannot reproduce outside a living cell. An exception comes from the demonstration in 1991 that infectious poliovirus could be produced in an extract of human cells incubated with viral RNA. Similar extracellular replication of the complete viral infectious cycle has not been achieved for any other virus. Consequently, most analysis of viral replication is done using cultured cells, embryonated eggs, or laboratory animals (Box 2.4).

Evidence of Viral Growth in Cultured Cells

Some viruses kill the cells in which they reproduce, and the infected cells may eventually detach from the cell culture plate. As more cells are infected, the changes become visible and are called **cytopathic effects** (Table 2.1). Many types of cytopathic effect can be seen with a simple light or phase-contrast microscope at low power, without fixing or staining the cells. These changes include the rounding up and detachment of cells from the culture dish, cell lysis, swelling of nuclei, and sometimes the formation of a group of fused cells called a syncytium (Fig. 2.8). Observation of other cytopathic effects requires high-power microscopy. These cytopathic effects include the development of intracellular masses of virus particles or unassembled viral components in the nucleus and/or cytoplasm (inclusion bodies), formation of crystalline arrays of viral proteins, membrane blebbing, duplication of membranes, and fragmentation of organelles. The time required for the development of cytopathology varies

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BOX 2.3

BACKGROUND

The cells of Henrietta Lacks

The most widely used continuous cell line in virology, the HeLa cell line, was derived from Henrietta Lacks. In 1951, the 31-year-old mother of five visited a physician at Johns Hopkins Hospital in Baltimore and found that she had a malignant tumor of the cervix. A sample of the tumor was taken and given to George Gey, head of tissue culture research at Hopkins. Gey had been attempting for years, without success, to produce a line of human cells that would live indefinitely. When placed in culture, Henrietta Lacks' cells propagated as no other cells had before.

On the day in October that Henrietta Lacks died, Gey appeared on national television with a vial of her cells, which he called HeLa cells. He said, "It is possible that, from a fundamental study such as this, we will be able to learn a way by which cancer can be completely wiped out." Soon after, HeLa cells were used to propagate poliovirus, which was causing poliomyelitis throughout the world, and they played an important role in the development of poliovirus vaccines. Henrietta Lacks' HeLa cells started a medical revolution: not only was it possible to propagate many different viruses in these cells, but the work set a precedent for producing continuous cell lines from many human tissues. Sadly, the family of Henrietta Lacks did not learn about HeLa cells, or the revolution they started, until 24 years after her death. Her family members were shocked that cells from Henrietta lived in so many laboratories, and hurt that they had not been told that any cells had been taken from her.

The story of HeLa cells is an indictment of the lack of informed consent that pervaded medical research in the 1950s. Since then, biomedical ethics have changed greatly, and now there are strict regulations about clinical research: physicians may not take samples from patients without permission. Nevertheless, in early 2013, HeLa cells generated more controversy when a research group published the cells' genome sequence. The Lacks family objected to the publication, claiming that the information could reveal private medical information about surviving family members. As a result, the sequence was withdrawn from public databases. Months later, a second HeLa cell genome sequence was published, but this time the authors were bound by an agreement brokered by the National Institutes of Health, which required an application process for any individual wishing to view the sequence.

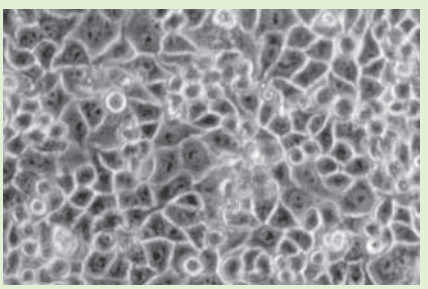
Adey A, Burton JN, Kitzman JO, Hiatt JB, Lewis AP, Martin BK, Qiu R, Lee C, Shendure J. 2013. The haplotype-resolved genome and epigenome of the aneuploid HeLa cancer cell line. *Nature* **500:**207–211.

Callaway E. 2013. Deal done over HeLa cell line. *Nature* **500:**132–133.

Callaway E. 2013. HeLa publication brews bioethical storm. *Nature* doi:10.1038/nature.2013.12689.

Skloot R. April 2000. Henrietta's dance. *Johns Hopkins Magazine*. http://pages.jh.edu/~jhumag/0400web/01.html.

Skloot R. 2011. *The Immortal Life of Henrietta Lacks*. Broadway Books, New York, NY.

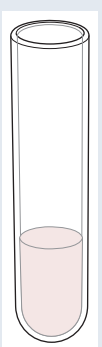


BOX 2.4

TERMINOLOGY

In vitro *and* in vivo

The terms "*in vitro*" and "*in vivo*" are common in the virology literature. *In vitro* means "in glass" and refers to experiments carried out in an artificial environment, such as a glass test tube. Unfortunately, the phrase "experiments performed *in vitro*" is used to designate not only work done in the cell-free environment of a test tube but also work done within cultured cells. The use of the phrase *in vitro* to describe living cultured cells leads to confusion and is inappropriate.



In this textbook, descriptions of experiments being carried out *in vitro* signify the absence of cells, e.g., *in vitro* translation. Work *ex vivo* is done in cells in culture, while research done in animals is carried out *in vivo*.

Table 2.1 Some examples of cytopathic effects of viral infection of animal cells

Cytopathic effect(s)	Virus(es)
Morphological alterations	
Nuclear shrinking (pyknosis), proliferation of membrane	Picornaviruses
Proliferation of nuclear membrane	Alphaviruses, herpesviruses
Vacuoles in cytoplasm	Polyomaviruses, papillomaviruses
Syncytium formation (cell fusion)	Paramyxoviruses, coronaviruses
Margination and breaking of chromosomes	Herpesviruses
Rounding up and detachment of cultured cells	Herpesviruses, rhabdoviruses, adenoviruses, picornaviruses
Inclusion bodies	
Virions in nucleus	Adenoviruses
Virions in cytoplasm (Negri bodies)	Rabies virus
"Factories" in cytoplasm (Guarnieri bodies)	Poxviruses
Clumps of ribosomes in virions	Arenaviruses
Clumps of chromatin in nucleus	Herpesviruses

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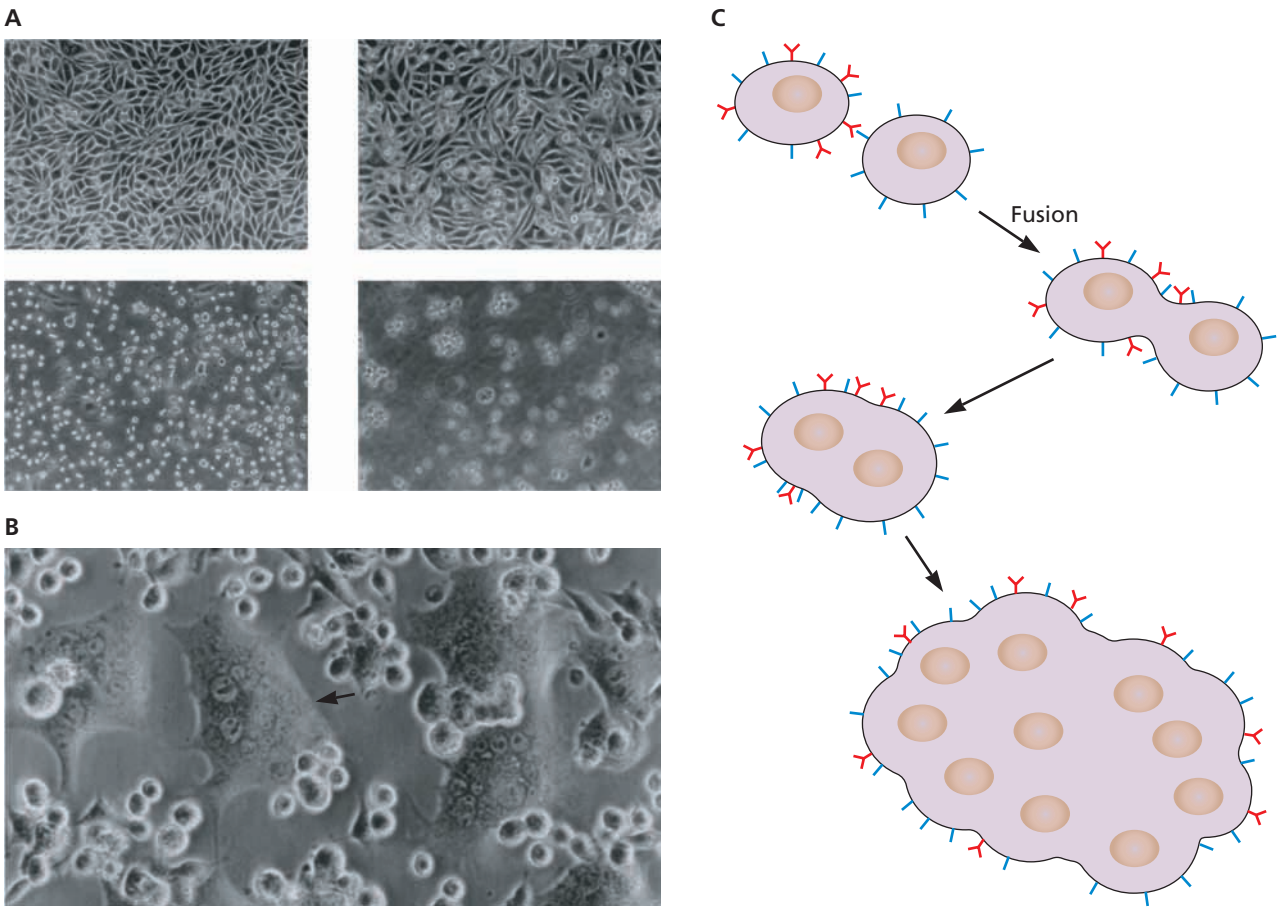


Figure 2.8 Development of cytopathic effect. **(A)** Cell rounding and lysis during poliovirus infection. (Upper left) Uninfected cells; (upper right) 5.5 h after infection; (lower left) 8 h after infection; (lower right) 24 h after infection. **(B)** Syncytium formation induced by murine leukemia virus. The field shows a mixture of individual small cells and syncytia, indicated by the arrow, which are large, multinucleate cells. Courtesy of R. Compans, Emory University School of Medicine. **(C)** Schematic illustration of syncytium formation. Viral glycoproteins on the surface of an infected cell bind receptors on a neighboring cell, causing fusion.

greatly among animal viruses. For example, depending on the size of the inoculum, enteroviruses and herpes simplex virus can cause cytopathic effects in 1 to 2 days and destroy the cell monolayer in 3 days. In contrast, cytomegalovirus, rubella virus, and some adenoviruses may not produce such effects for several weeks.

The development of characteristic cytopathic effects in infected cell cultures is frequently monitored in diagnostic virology during isolation of viruses from specimens obtained from infected patients or animals. However, cytopathic effect is also of value in the research laboratory: it can be used to monitor the progress of an infection, and is often one of the phenotypic traits by which mutant viruses are characterized.

Some viruses multiply in cells without causing obvious cytopathic effects. For example, many members of the families *Arenaviridae*, *Paramyxoviridae*, and *Retroviridae* do not cause obvious damage to cultured cells. Infection by such viruses must therefore be assayed using alternative methods, as described in "Assay of Viruses" below.

Embryonated Eggs

Before the advent of cell culture, many viruses were propagated in embryonated chicken eggs (Fig. 2.9). At 5 to 14 days after fertilization, a hole is drilled in the shell and virus is injected into the site appropriate for its replication. This method of virus propagation is now routine only for influenza virus. The robust yield of this virus from chicken eggs has led to their widespread use in research laboratories and for vaccine production.

Laboratory Animals

In the early 1900s, when viruses were first isolated, freezers and cell cultures were not available and it was necessary to maintain virus stocks by continuous passage from animal to

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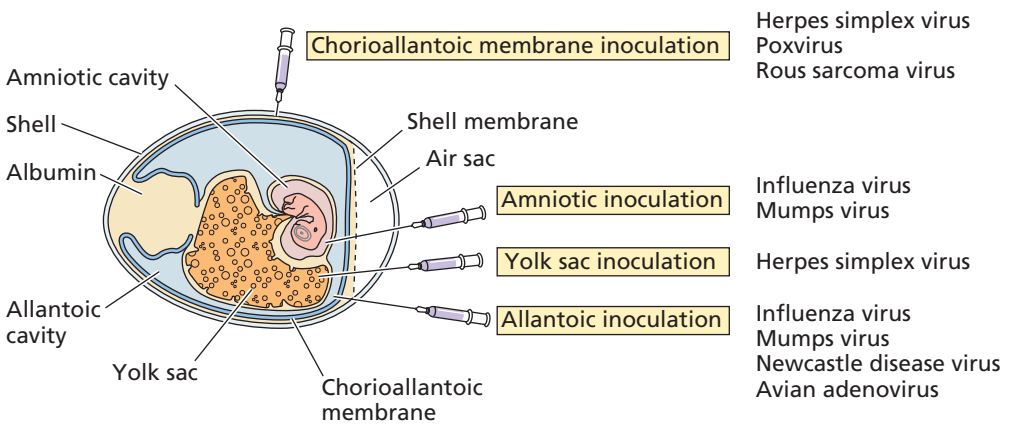


Figure 2.9 Growth of viruses in embryonated eggs. The cutaway view of an embryonated chicken egg shows the different routes by which viruses are inoculated into eggs and the different compartments in which viruses may grow. Adapted from F. Fenner et al., *The Biology of Animal Viruses* (Academic Press, New York, NY, 1974), with permission.

animal. This practice not only was inconvenient but also, as we shall see in Volume II, Chapter 8, led to the selection of viral mutants. For example, monkey-to-monkey intracerebral passage of poliovirus selected a mutant that could no longer infect chimpanzees by the oral route, the natural means of infection. Cell culture has largely supplanted the use of animals for propagating viruses, but some viruses cannot yet be grown in this way.

Experimental infection of laboratory animals has always been, and will continue to be, obligatory for studying the processes by which viruses cause disease. The use of monkeys in the study of poliomyelitis, the paralytic disease caused by poliovirus, led to an understanding of the basis of this disease and was instrumental in the development of a successful vaccine. Similarly, the development of vaccines against hepatitis B virus would not have been possible without experimental studies with chimpanzees. Understanding how the immune system or any complex organ reacts to a virus cannot be achieved without research on living animals. The development of viral vaccines, antiviral drugs, and diagnostic tests for veterinary medicine has also benefited from research on diseases in laboratory animals.

Assay of Viruses

There are two main types of assay for detecting viruses: biological and physical. Because viruses were first recognized by their infectivity, the earliest assays focused on this most sensitive and informative property. However, biological assays such as the plaque assay and end-point titration methods do not measure noninfectious particles. All such particles are included when measured by physical assays such as electron microscopy or by immunological methods. Knowledge of the number of noninfectious particles is useful for assessing the quality of a virus preparation.

Measurement of Infectious Units

One of the most important procedures in virology is measuring the **virus titer**, the concentration of a virus in a sample. This parameter is determined by inoculating serial dilutions of virus into host cell cultures, chicken embryos, or laboratory animals and monitoring for evidence of virus multiplication. The response may be quantitative (as in assays for plaques, fluorescent foci, infectious centers, or transformation) or all-or-none, in which the presence or absence of infection is measured (as in an end-point dilution assay).

Plaque Assay

In 1952, Renato Dulbecco modified the plaque assay developed to determine the titers of bacteriophage stocks for use in animal virology. The plaque assay was adopted rapidly for reliable determination of the titers of a wide variety of viruses. In this procedure, monolayers of cultured cells are incubated with a preparation of virus to allow adsorption to cells. After removal of the inoculum, the cells are covered with nutrient medium containing a supplement, most commonly agar, which forms a gel. When the original infected cells release new progeny particles, the gel restricts the spread of viruses to neighboring uninfected cells. As a result, each infectious particle produces a circular zone of infected cells, a **plaque**. If the infected cells are damaged, the plaque can be distinguished from the surrounding monolayer. In time, the plaque becomes large enough to be seen with the naked eye (Fig. 2.10). Only viruses that cause visible damage of cultured cells can be assayed in this way.

For the majority of animal viruses, there is a linear relationship between the number of infectious particles and the plaque count (Fig. 2.11). One infectious particle is therefore sufficient to initiate infection, and the virus is said to infect cells with **one-hit kinetics**. Some examples of **two-hit kinetics**,

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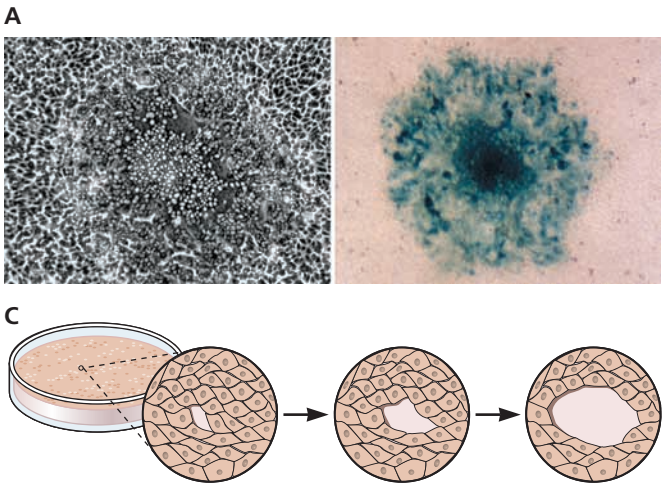
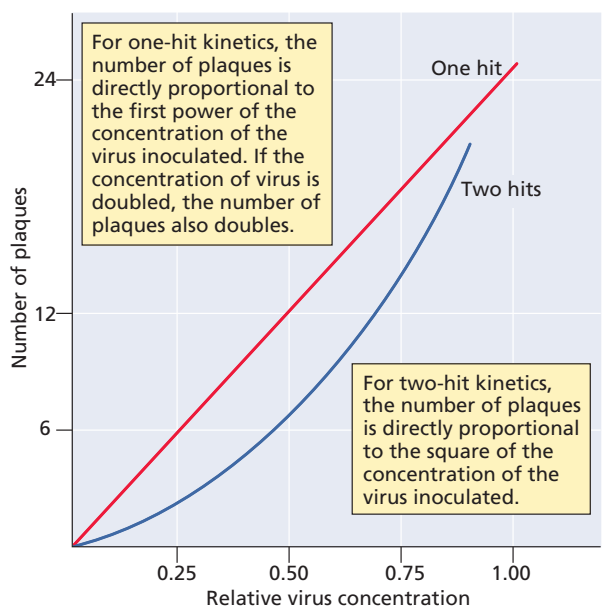


Figure 2.10 Plaques formed by different animal viruses. Plaque sizes reflect the reproductive cycle of a virus in a particular cell type. **(A)** Photomicrograph of a single plaque formed by pseudorabies virus in Georgia bovine kidney cells. (Left) Unstained cells. (Right) Cells stained with the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), which is converted to a blue compound by the product of the *lacZ* gene carried by the virus. Courtesy of B. Banfield, Princeton University. **(B)** Plaques formed by poliovirus on human HeLa cells stained with crystal violet. **(C)** Illustration of the spread of virus from an initial infected cell to neighboring cells, resulting in a plaque.

in which two different types of virus particle must infect a cell to ensure replication, have been recognized. For example, the genomes of some (+) strand RNA viruses of plants consist of two RNA molecules that are encapsidated separately. Both RNAs are required for infectivity. The dose-response curve in plaque assays for these viruses is parabolic rather than linear (Fig. 2.11).

Figure 2.11 The dose-response curve of the plaque assay. The number of plaques produced by a virus with one-hit kinetics (red) or two-hit kinetics (blue) is plotted against the relative concentration of the virus. In two-hit kinetics, there are two classes of uninfected cells, those receiving one particle and those receiving none. The Poisson distribution can be used to determine the proportion of cells in each class: they are e^{-m} and me^{-m} (Box 2.10). Because one particle is not sufficient for infection, $P(0) = e^{-m}(1 + m)$. At a very low multiplicity of infection, this equation becomes $P(i) = (1/2)m^2$ (where i = infection), which gives a parabolic curve. Adapted from B. D. Davis et al., *Microbiology* (J. B. Lippincott Co., Philadelphia, PA, 1980), with permission.



The titer of a virus stock can be calculated in **plaque-forming units (PFU) per milliliter** (Box 2.5). When one infectious virus particle initiates a plaque, the viral progeny within the plaque are clones, and virus stocks prepared from a single plaque are known as **plaque purified**. Plaque purification is employed widely in virology to establish clonal virus stocks. The tip of a small pipette is plunged into the overlay above the plaque, and the plug of agar containing the virus is recovered. The virus within the agar plug is eluted into buffer and used to prepare virus stocks. To ensure purity, this process is usually repeated at least one more time.

Fluorescent-Focus Assay

The fluorescent-focus assay, a modification of the plaque assay, can be done more rapidly and is useful in determining the titers of viruses that do not form plaques. The initial procedure is the same as in the plaque assay. However, after a period sufficient for adsorption and gene expression, cells are made permeable and incubated with an antibody raised against a viral protein. A second antibody, which recognizes the first, is then added. This second antibody is usually conjugated to a fluorescent molecule. The cells are then examined under a microscope at an appropriate wavelength. The titer of the virus stock is expressed in fluorescent-focus-forming units per milliliter. When the gene encoding a fluorescent protein is incorporated into the viral genome, foci may be detected without the use of antiviral antibodies.

Infectious-Centers Assay

Another modification of the plaque assay, the infectious-centers assay, is used to determine the fraction of cells in a culture that are infected with a virus. Monolayers of infected cells are suspended before progeny viruses are produced.

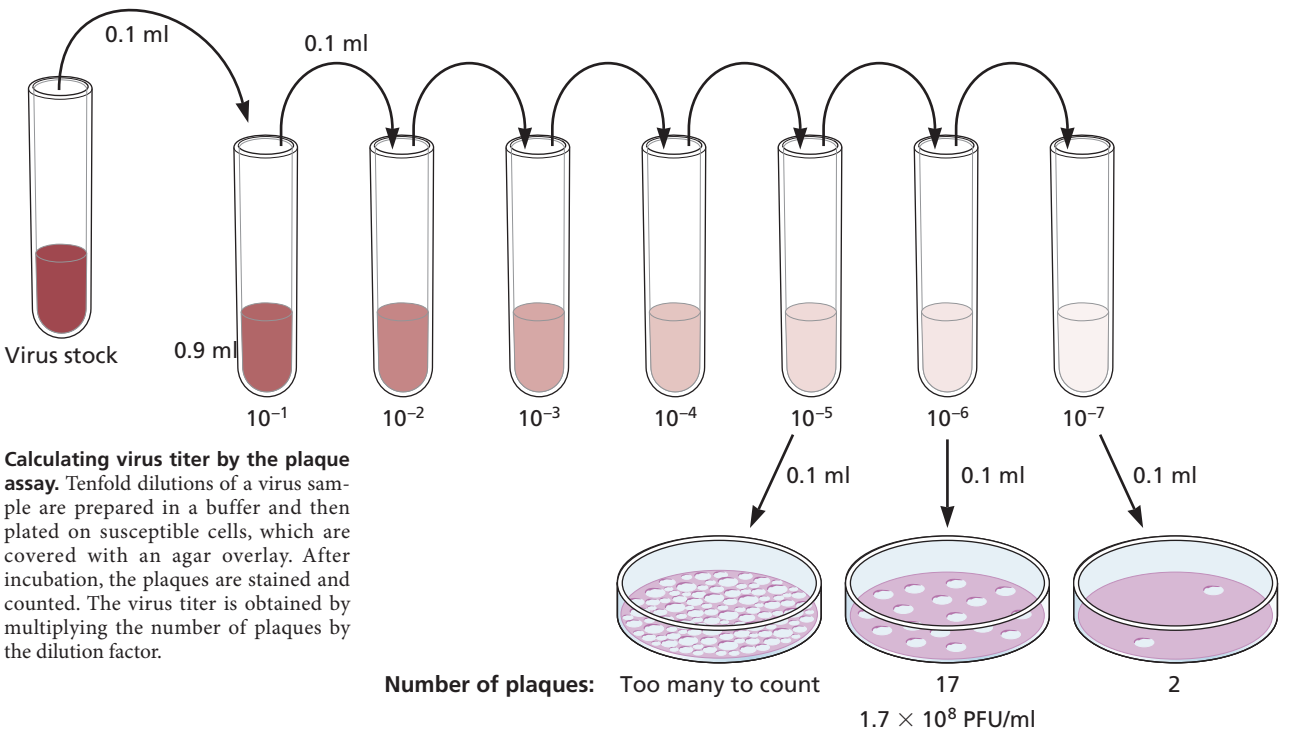
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BOX 2.5

METHODS

Calculating virus titer from the plaque assay

To calculate the titer of a virus in plaque-forming units (PFU) per milliliter, 10-fold serial dilutions of a virus stock are prepared, and 0.1-ml aliquots are inoculated onto susceptible cell monolayers (see figure). After a suitable incubation period, the monolayers are stained and the plaques are counted. To minimize error in calculating the virus titer, only plates containing between 10 and 100 plaques are counted, depending on the area of the cell culture vessel. Plates with >100 plaques are generally not counted because the plaques may overlap, causing inaccuracies. According to statistical principles, when 100 plaques are counted, the sample titer varies by ±10%. For accuracy, each dilution is plated in duplicate or triplicate (not shown in the figure). In the example shown in the figure, 17 plaques are observed on the plate produced from the 10^{-6} dilution. Therefore, the 10^{-6} dilution tube contains 17 PFU per 0.1 ml, or 170 PFU per ml, and the titer of the virus stock is 170×10^6 or 1.7×10^8 PFU/ml.



Calculating virus titer by the plaque assay. Tenfold dilutions of a virus sample are prepared in a buffer and then plated on susceptible cells, which are covered with an agar overlay. After incubation, the plaques are stained and counted. The virus titer is obtained by multiplying the number of plaques by the dilution factor.

Dilutions of a known number of infected cells are then plated on monolayers of susceptible cells, which are covered with an agar overlay. The number of plaques that form on the indicator cells is a measure of the number of cells infected in the original population. The fraction of infected cells can therefore be determined. A typical use of the infectious-centers assay is to measure the proportion of infected cells in persistently infected cultures.

Transformation Assay

The transformation assay is useful for determining the titers of some retroviruses that do not form plaques. For example, when Rous sarcoma virus transforms chicken embryo cells, the cells lose their contact inhibition (the property that governs whether cultured cells grow as a single monolayer [see Volume II, Chapter 6]) and become heaped up on one another. The transformed cells form small piles, or **foci**, that can be distinguished easily from the rest of the monolayer (Fig. 2.12). Infectivity is expressed in focus-forming units per milliliter.

End-Point Dilution Assay

The end-point dilution assay provided a measure of virus titer before the development of the plaque assay. It is still used for measuring the titers of certain viruses that do not form plaques or for determining the virulence of a virus in animals.

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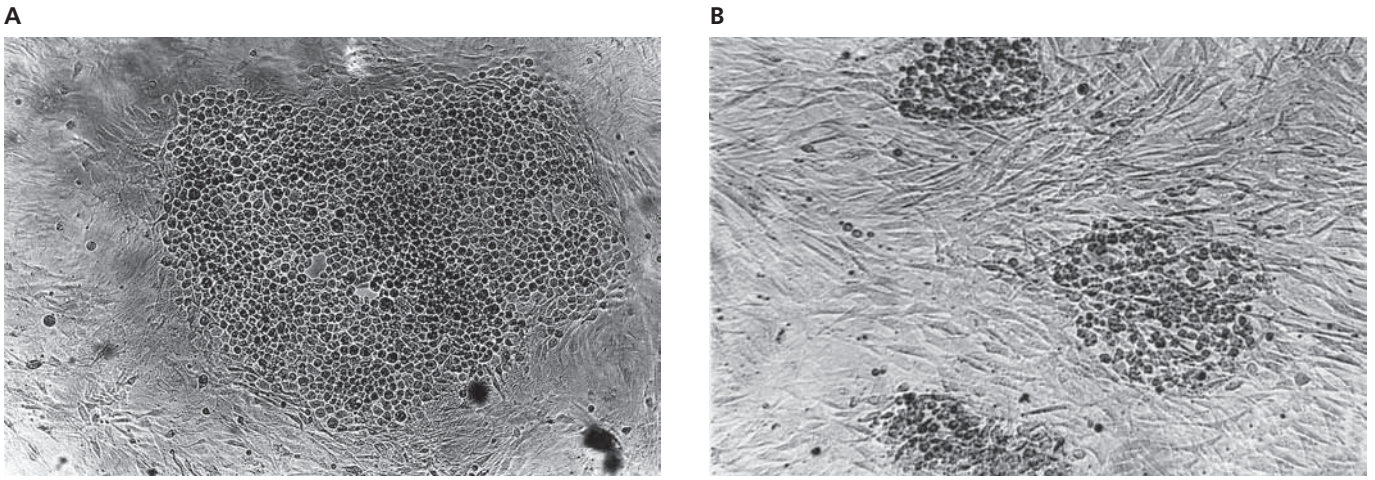


Figure 2.12 Transformation assay. Chicken cells transformed by two different strains of Rous sarcoma virus are shown. Loss of contact inhibition causes cells to pile up rather than grow as a monolayer. One focus is seen in panel **A**, and three foci are seen in panel **B** at the same magnification. Courtesy of H. Hanafusa, Osaka Bioscience Institute.

Serial dilutions of a virus stock are inoculated into replicate test units (typically 8 to 10), which can be cell cultures, eggs, or animals. The number of test units that have become infected is then determined for each virus dilution. When cell culture is used, infection may be determined by the development of cytopathic effect; in eggs or animals, infection is gauged by death or disease. An example of an end-point dilution assay using cell cultures is shown in Box 2.6. At high dilutions, none of the cell cultures are infected because no infectious particles are delivered to the cells; at low dilutions, every culture is infected. The **end point** is the dilution of virus that affects 50% of the test units. This number can be calculated from the data and expressed as 50% infectious dose (ID_{50}) per milliliter. The first preparation illustrated in Box 2.6 contains 10^5 ID_{50} per ml. This type of assay is suitable for high-throughput applications.

When the end-point dilution assay is used to assess the virulence of a virus or its capacity to cause disease (Volume II, Chapter 1), the result of the assay can be expressed in terms of 50% lethal dose (LD_{50}) per milliliter or 50% paralytic dose (PD_{50}) per milliliter, end points of death and paralysis, respectively. If the virus titer can be determined separately by plaque assay, the 50% end point determined in an animal host can be related to this parameter. In this way, the effects of the route of inoculation or specific mutations on viral virulence can be quantified.

Efficiency of Plating

Efficiency of plating is defined as the virus titer (in PFU/ml) divided by the number of virus particles in the sample. The **particle–to–plaque-forming-unit (PFU) ratio**, a term more commonly used today, is the inverse value (Table 2.2). For many bacteriophages, the particle-to-PFU ratio approaches 1, the lowest value that can be obtained. However, for animal viruses, this value can be much higher, ranging from 1 to 10,000. These high values have complicated the study of animal viruses. For example, when the particle-to-PFU ratio is high, it is never certain whether properties measured biochemically are in fact those of the infectious particle or those of the noninfectious component.

Although the linear nature of the dose-response curve indicates that a single particle is capable of initiating an infection (one-hit kinetics) (Fig. 2.11), the high particle-to-PFU ratio for many viruses demonstrates that not all virus particles are successful. High values are sometimes caused by the presence of noninfectious particles with genomes that harbor lethal mutations or that have been damaged during growth or purification. An alternative explanation is that although all viruses in a preparation are in fact capable of initiating infection, not all of them succeed because of the complexity of the infectious cycle. Failure at any one step in the cycle prevents completion. A high particle-to-PFU ratio indicates not that most particles are defective but, rather, that they failed to complete the infection.

Measurement of Virus Particles and Their Components

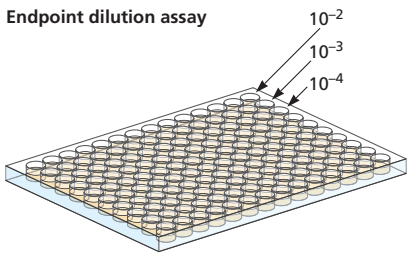
Although the numbers of virus particles and infectious units are often not equal, assays for particle number are frequently used to approximate the number of infectious particles present in a sample. For example, the concentration of viral DNA or protein can be used to estimate the particle number, assuming that the ratio of infectious units to physical particles is constant. Biochemical or physical assays are usually more

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BOX 2.6

METHODS

End-point dilution assays



Virus dilution	Cytopathic effect									
10^{-2}	+	+	+	+	+	+	+	+	+	+
10^{-3}	+	+	+	+	+	+	+	+	+	+
10^{-4}	+	+	−	+	+	+	+	+	+	+
10^{-5}	−	+	+	−	+	−	−	+	−	+
10^{-6}	−	−	−	−	−	−	+	−	−	−
10^{-7}	−	−	−	−	−	−	−	−	−	−

End-point dilution assays are usually carried out in multiwell plastic plates (see the figure). In the example shown in the first table, 10 monolayer cell cultures were infected with each virus dilution. After the incubation period, plates that displayed cytopathic effect were scored +. Fifty percent of the cell cultures displayed cytopathic effect at the 10^{-5} dilution, and therefore the virus stock contains 10^5 $TCID_{50}$ units.

In most cases, the 50% end point does not fall on a dilution tested as shown in the example; for this reason, various statistical procedures have been developed to calculate the end point of the titration. In one popular method, the dilution containing the ID_{50} is identified by interpolation between the dilutions on either side of this value. The assumption is made that the location of the 50% end point varies linearly with the log of the dilution. Because the number of test units used at each dilution is usually small, the accuracy of this method is relatively low. For example, if six test units are used at each 10-fold dilution, differences in virus titer of only 50-fold or more can be detected reliably. The method is illustrated in the second example, in which the lethality of poliovirus in mice is the end point. Eight mice were inoculated per dilution. In the method of Reed and Muench, the results are pooled, as shown in the table, which equalizes chance variations (another way to achieve the same result would be to utilize greater numbers of animals at each dilution). The interpolated value of the 50% end point, which in this case falls between the 5th and 6th dilutions, is calculated to be $10^{-6.5}$. The virus sample therefore contains $10^{6.5}$ LD_{50}s. The LD_{50} may also be calculated as the concentration of the stock virus in PFU per milliliter (1×10^9) times the 50% end-point titer. In the example shown, the LD_{50} is 3×10^2 PFU.

Reed LJ, Muench H. 1938. A simple method of estimating fifty per cent endpoints. *Am J Hyg* **27:**493–497.

Dilution	Alive	Dead	Total alive	Total dead	Mortality ratio	Mortality (%)
10^{-2}	0	8	0	40	0/40	100
10^{-3}	0	8	0	32	0/32	100
10^{-4}	1	7	1	24	1/25	96
10^{-5}	0	8	1	17	1/18	94
10^{-6}	2	6	3	9	3/12	75
10^{-7}	5	3	8	3	8/11	27

Table 2.2 Particle-to-PFU ratios of some animal viruses

Virus	Particle/PFU ratio
Papillomaviridae	
Papillomavirus	10,000
Picornaviridae	
Poliovirus	30–1,000
Herpesviridae	
Herpes simplex virus	50–200
Polyomaviridae	
Polyomavirus	38–50
Simian virus 40	100–200
Adenoviridae	20–100
Poxviridae	1–100
Orthomyxoviridae	
Influenza virus	20–50
Reoviridae	
Reovirus	10
Alphaviridae	
Semliki Forest virus	1–2

rapid and easier to carry out than assays for infectivity, which may be slow, cumbersome, or not possible. Assays for subviral components also provide information on particle number if the stoichiometry of these components in the virus particle is known.

Imaging Particles

Electron microscopy. With few exceptions, virus particles are too small to be observed directly by light microscopy. However, they can be seen readily in the electron microscope. If a sample contains only one type of virus, the particle count can be determined. First, a virus preparation is mixed with a known concentration of latex beads. The numbers of virus particles and beads are then counted, allowing the concentration of the virus particles in the sample to be determined by comparison.

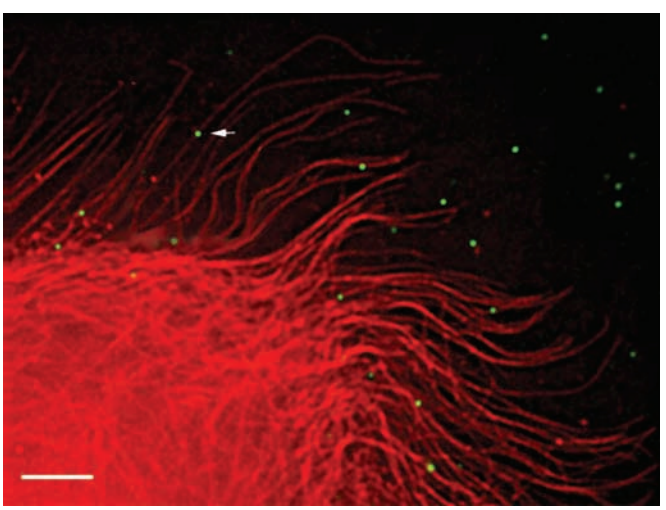
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Live-cell imaging of single fluorescent virions. The discovery of green fluorescent protein revolutionized the study of the cell biology of virus infection. This protein, isolated from the jellyfish *Aequorea victoria*, is a convenient reporter for monitoring gene expression, because it is directly visible in living cells without the need for fixation, substrates, or coenzymes. Similar proteins isolated from different organisms, which emit light of different wavelengths, are also widely used in virology. The use of fluorescent proteins has allowed visualization of single virus particles in living cells. The coding sequence for the fluorescent protein is inserted into the viral genome, often fused to the coding region of a virion protein. The fusion protein is incorporated into the viral particle, which is visible in cells by fluorescence microscopy (Fig. 2.13). Using this approach, entry, uncoating, replication, assembly, and egress of single particles can all theoretically be observed in living cells.

Hemagglutination

Members of the *Adenoviridae*, *Orthomyxoviridae*, and *Paramyxoviridae*, among others, contain proteins that can bind to erythrocytes (red blood cells); these viruses can link multiple cells, resulting in formation of a lattice. This property is called **hemagglutination**. For example, influenza viruses contain an envelope glycoprotein called hemagglutinin, which binds to *N*-acetylneuraminic acid-containing glycoproteins on erythrocytes. In practice, 2-fold serial dilutions of the virus stock are prepared, mixed with a known quantity of red blood cells, and added to small wells in a plastic tray (Fig. 2.14). Unadsorbed red blood cells tumble to the bottom of the well and form a sharp dot or button. In contrast, agglutinated red blood cells form a diffuse lattice that coats the well. Because the assay is rapid (30 min), it is often used as a quick indicator of the relative quantities of virus particles. However, it is not sufficiently sensitive to detect small numbers of particles.

Figure 2.13 Live-cell imaging of single virus particles by fluorescence. Single-virus-particle imaging with green fluorescent protein illustrates microtubule-dependent movement of human immunodeficiency virus type 1 particles in cells. Rhodamine-tubulin was injected into cells to label microtubules (red). The cells were infected with virus particles that contain a fusion of green fluorescent protein with Vpr. Virus particles can be seen as green dots. Bar, 5 μm. Courtesy of David McDonald, University of Illinois.



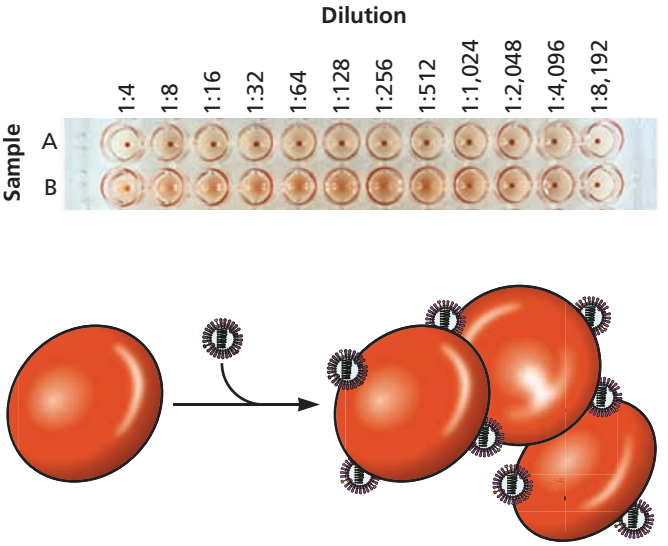


Figure 2.14 Hemagglutination assay. (Top) Samples of different influenza viruses were diluted, and a portion of each dilution was mixed with a suspension of chicken red blood cells and added to the wells. After 30 min at 4°C, the wells were photographed. Sample A does not contain virus. Sample B causes hemagglutination until a dilution of 1:512 and therefore has a hemagglutination titer of 512. Elution of the virus from red blood cells at the 1:4 dilution is caused by neuraminidase in the virus particle. This enzyme cleaves *N*-acetylneuraminic acid from glycoprotein receptors and elutes bound viruses from red blood cells. (Bottom) Schematic illustration of hemagglutination of red blood cells by influenza virus. Top, Courtesy of C. Basler and P. Palese, Mount Sinai School of Medicine of the City University of New York.

Measurement of Viral Enzyme Activity

Some animal virus particles contain nucleic acid polymerases, which can be assayed by mixing permeabilized particles with radioactively labeled precursors and measuring the incorporation of radioactivity into nucleic acid. This type of assay is used most frequently for retroviruses, many of which neither transform cells nor form plaques. The reverse transcriptase incorporated into the virus particle is assayed by mixing cell culture supernatants with a mild detergent (to permeabilize the viral envelope), an RNA template and primer, and a radioactive nucleoside triphosphate. If reverse transcriptase is present, a radioactive product will be produced by priming on the template. This product can be detected by precipitation or bound to a filter and quantified.

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Because enzymatic activity is proportional to particle number, this assay allows rapid tracking of virus production in the course of an infection. Many of these assays have been modified to permit the use of safer, nonradioactive substrates. For example, when nucleoside triphosphates conjugated to biotin are used, the product can be detected with streptavidin (which binds biotin) conjugated to a fluorochrome. Alternatively, the reaction products may be quantified by quantitative real-time PCR (see "Detection of Viral Nucleic Acids" below).

Serological Methods

The specificity of the antibody-antigen reaction has been used to design a variety of assays for viral proteins and antiviral antibodies. These techniques, such as immunostaining, immunoprecipitation, immunoblotting, and the enzyme-linked immunosorbent assay, are by no means limited to virology: all these approaches have been used extensively to study the structures and functions of cellular proteins.

Virus neutralization. When a virus preparation is inoculated into an animal, an array of antibodies is produced. These antibodies can bind to virus particles, but not all of them can block infectivity (**neutralize**), as discussed in Volume II, Chapter 4. Virus neutralization assays are usually conducted by mixing dilutions of antibodies with virus; incubating them; and assaying for remaining infectivity in cultured cells, eggs, or animals. The end point is defined as the highest dilution of antibody that inhibits the development of cytopathic effect in cells or virus replication in eggs or animals.

Some neutralizing antibodies define **type-specific antigens** on the virus particle. For example, the three **serotypes** of poliovirus are distinguished on the basis of neutralization tests: type 1 poliovirus is neutralized by antibodies to type 1 virus but not by antibodies to type 2 or type 3 poliovirus, and so forth. The results of neutralization tests were once used for virus classification, a process now accomplished largely by comparing viral genome sequences. Nevertheless, the detection of antiviral antibodies in animal sera is still extremely important for identifying infected hosts. These antibodies may also be used to map the three-dimensional structure of neutralization antigenic sites on the virus particle (Box 2.7).

Hemagglutination inhibition. Antibodies against viral proteins with hemagglutination activity can block the ability of virus to bind red blood cells. In this assay, dilutions of antibodies are incubated with virus, and erythrocytes are added as outlined above. After incubation, the hemagglutination inhibition titer is read as the highest dilution of antibody that inhibits hemagglutination. This test is sensitive, simple, inexpensive, and rapid: it is the method of choice for assaying antibodies to any virus that causes hemagglutination. It can be used to detect antibodies to viral hemagglutinin in animal and human sera or to identify the origin of the hemagglutinin of influenza viruses produced in cells coinfected with two parent

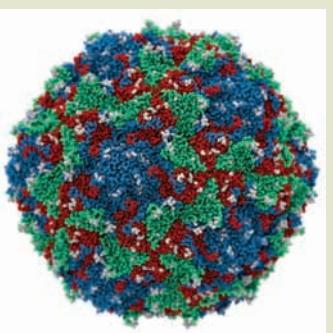
BOX 2.7

DISCUSSION

Neutralization antigenic sites

Knowledge of the antigenic structure of a virus is useful in understanding the immune response to these agents and in designing new vaccination strategies. The use of **monoclonal antibodies** (antibodies of a single specificity made by a clone of antibody-producing cells) in neutralization assays permits mapping of antigenic sites on a virus particle, or of the amino acid sequences that are recognized by neutralizing antibodies. Each monoclonal antibody binds specifically to a short amino acid sequence (8 to 12 residues) that fits into the antibody-combining site. This amino acid sequence, which may be linear or nonlinear, is known as an **epitope**. In contrast, **polyclonal antibodies** comprise the repertoire produced in an animal against the many epitopes of an antigen. Antigenic sites may be identified by cross-linking the monoclonal antibody to the virus and determining which protein is the target of the antibody.

Epitope mapping may also be performed by assessing the abilities of monoclonal antibodies to bind synthetic peptides representing viral protein sequences. When the monoclonal antibody recognizes a linear epitope, it may react with the protein in Western blot analysis, facilitating direct identification of the viral protein harboring the antigenic site. The most elegant understanding of antigenic structures has come from the isolation and study of variant viruses that are resistant to neutralization with specific monoclonal antibodies (called **monoclonal antibody-resistant variants**). By identifying the amino acid change responsible for this phenotype, the antibody-binding site can be located and, together with three-dimensional structural information, can provide detailed information on the nature of antigenic sites that are recognized by neutralizing antibodies (see the figure).



Locations of neutralization antigenic sites on the capsid of poliovirus type 1. Amino acids that change in viral mutants selected for resistance to neutralization by monoclonal antibodies are shown in white on a model of the viral capsid. These amino acids are in VP1 (blue), VP2 (green), and VP3 (red) on the surface of the virus particle. Figure courtesy of Jason Roberts, Victorian Infectious Diseases Reference Laboratory, Doherty Institute, Melbourne, Australia.

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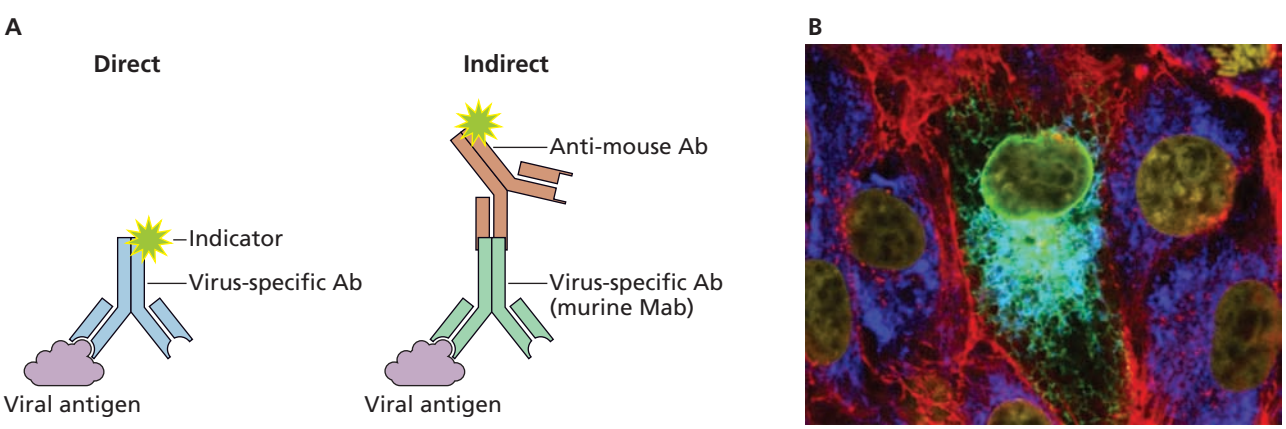
viruses. For example, hemagglutination inhibition assays were used to identify individuals who had been infected with the newly discovered avian influenza A (H7N9) virus in China during the 2013 outbreak.

Immunostaining. Antibodies can be used to visualize viral proteins in infected cells or tissues. In direct immunostaining, an antibody that recognizes a viral protein is coupled directly to an indicator such as a fluorescent dye or an enzyme (Fig. 2.15). A more sensitive approach is indirect immunostaining, in which a second antibody is coupled to the indicator. The second antibody recognizes a common region on the virus-specific antibody. Multiple second-antibody molecules bind to the first antibody, resulting in an increased signal from the indicator compared with that obtained with direct immunostaining. Furthermore, a single indicator-coupled second antibody can be used in many assays, avoiding the need to purify and couple an indicator to multiple first antibodies.

In practice, virus-infected cells (unfixed or fixed with acetone, methanol, or paraformaldehyde) are incubated with **polyclonal** or **monoclonal antibodies** directed against viral antigen. Excess antibody is washed away, and in direct immunostaining, cells are examined by microscopy. For indirect immunostaining, the second antibody is added before examination of the cells by microscopy. Commonly used indicators include fluorescein and rhodamine, which fluoresce on exposure to UV light. Filters are placed between the specimen and the eyepiece to remove blue and UV light so that the field is dark, except for cells to which the antibody has bound, which emit green (fluorescein) or red (rhodamine) light (Fig. 2.15). Even though these colors are at the opposite ends of the visible light spectrum, bleeding of red into green and vice versa still occur. Today's optics are much better at keeping the wavelengths separate, and many more colors in between red and green are now available. Antibodies can also be coupled to molecules other than fluorescent indicators, including enzymes such as alkaline phosphatase, horseradish peroxidase, and β-galactosidase, a bacterial enzyme that in a test system converts the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) to a blue product. After excess antibody is washed away, a suitable chromogenic substrate is added, and the presence of the indicator antibody is revealed by the development of a color that can be visualized.

Immunostaining has been applied widely in the research laboratory for determining the subcellular localization of proteins in cells (Fig. 2.15), monitoring the synthesis of viral proteins, determining the effects of mutation on protein production, and localizing the sites of virus replication in animal hosts. It is the basis of the fluorescent-focus assay.

Figure 2.15 Direct and indirect methods for antigen detection. (A) The sample (tissue section, smear, or bound to a solid phase) is incubated with a virus-specific antibody (Ab). In direct immunostaining, the antibody is linked to an indicator such as fluorescein. In indirect immunostaining, a second antibody, which recognizes a general epitope on the virus-specific antibody, is coupled to the indicator. Mab, monoclonal antibody. **(B)** Use of immunofluorescence to determine the intracellular location of mumps virus by direct and indirect immunofluorescence using confocal laser scanning microscopy. A mumps virus small hydrophobic protein-enhanced green fluorescent protein fusion protein was produced following transfection of Vero cells with a plasmid encoding the fusion protein. The small hydrophobic protein-enhanced green fluorescent protein was visualized by virtue of its autofluorescence by excitation at 488 nm (green). Protein disulfide isomerase, an enzyme in the endoplasmic reticulum, was detected using a monoclonal antibody and visualized indirectly by excitation at 647 nm of a fluorescent molecule, cyanine 5 (Cy5) conjugated to a secondary antibody (blue). Filamentous actin, a major component of the cytoskeleton, was detected using phalloidin conjugated to tetramethylrhodamine and visualized directly by excitation at 586 nm (red). Nuclei were counterstained using 4′,6-diamidino-2-phenylindole and visualized directly by excitation at 405 nm (yellow). Colocalization of the mumps virus small hydrophobic protein-enhanced green fluorescent protein and protein disulfide isomerase (cyan) demonstrated that the protein was present in the endoplasmic reticulum. Image provided by Paul Duprex, Boston University Medical School, with permission of the Wellcome Trust.



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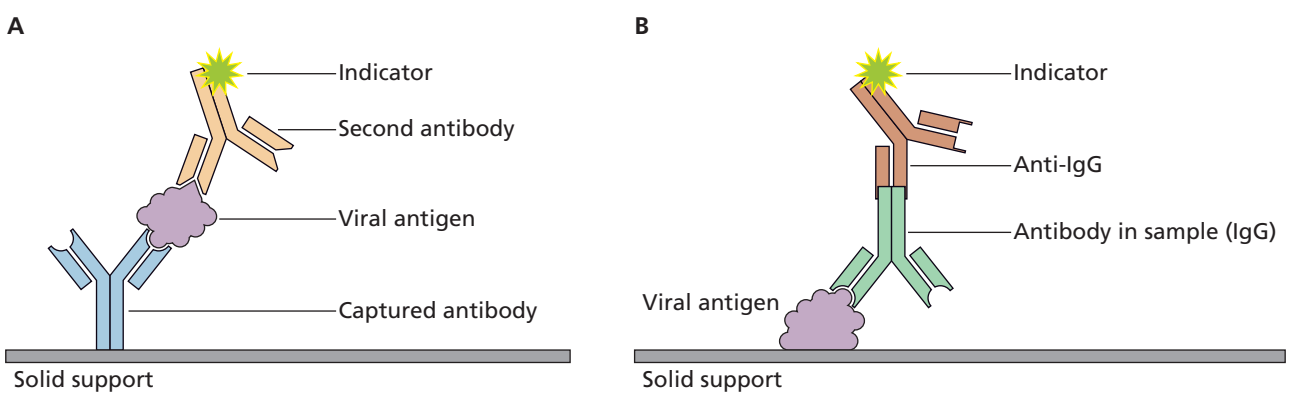
Immunostaining of viral antigens in smears of clinical specimens may be used to diagnose viral infections. For example, direct and indirect immunofluorescence assays with nasal swabs or washes are used to diagnose a variety of viruses, including influenza virus and measles virus. Viral proteins or nucleic acids may also be detected in infected animals by immunohistochemistry. In this procedure, tissues are embedded in a solid medium such as paraffin, and thin slices are produced using a microtome. Viral antigens can be detected within the cells of the sections by direct and indirect immunofluorescence assays.

Recent improvements in microscopy technology and computational image manipulation have led to unprecedented levels of resolution and contrast and the ability to reconstruct three-dimensional structures from captured images. An example is **confocal microscopy**, which utilizes a scanning point of light instead of full-sample illumination, providing improvements in optical sectioning. **Super-resolution microscopy** combines the advantages of fluorescent imaging (multicolor labeling and live-cell imaging) with the high resolution of electron microscopy. While conventional fluorescent microscopy has a resolution of 200 to 500 nm, single-molecule localization microscopy can achieve resolution below 1 nm. This resolution is achieved by combining sequential acquisition of images with random switching of fluorophores on and off. From several hundred to thousands of images are collected and processed to generate a super-resolution dataset that can resolve cellular ultrastructure.

Enzyme immunoassay. Detection of viral antigens or antiviral antibodies can be accomplished by solid-phase methods, in which antiviral antibody or protein is adsorbed to a plastic surface. To detect viral antigens in serum or clinical samples, a "capture" antibody, directed against the virus, is linked to a solid support, a plastic dish or bead (Fig. 2.16A). The specimen is added to the plastic support, and if viral antigens are present, they will be captured by the bound antibody. Bound viral antigen is detected by using a second antibody linked to an enzyme. A chromogenic molecule that is converted by the enzyme to an easily detectable product is then added. The enzyme amplifies the signal because a single catalytic enzyme molecule can generate many product molecules. To detect IgG antibodies to viruses, viral protein is first linked to the plastic support, and then the specimen is added (Fig. 2.16B). If antibodies against the virus are present in the specimen, they will bind to the immobilized antibody. The bound antibodies are then detected by using a second antibody directed against a common region on the first antibody. Like other detection methods, enzyme immunoassays are used in both experimental and diagnostic virology. In the clinical laboratory, enzyme immunoassays are used to detect a variety of viruses including rotavirus, herpes simplex virus, and human immunodeficiency virus.

A modification of the enzyme immunoassay is the lateral flow immunochromatographic assay, which has been used in rapid antigen detection test kits. In this assay, a sample is applied to a membrane and is drawn across it by capillary action. Antigens in the sample react with a specific

Figure 2.16 Detection of viral antigen or antibodies against viruses by enzyme-linked immunosorbent assay. (A) To detect viral proteins in a sample, antibodies specific for the virus are immobilized on a solid support such as a plastic well. The sample is placed in the well, and viral proteins are "captured" by the immobilized antibody. After washing to remove unbound proteins, a second antibody against the virus is added, which is linked to an indicator. Another wash is done to remove unbound second antibody. If viral antigen has been captured by the first antibody, the second antibody will bind and the complex will be detected by the indicator. **(B)** To detect antibodies to a virus in a sample, viral antigen is immobilized on a solid support. The sample is placed in the well, and viral antibodies bind the immobilized antigen. After washing to remove unbound antibodies, a second antibody, directed against a general epitope on the first antibody, is added. Another wash removes unbound second antibody. If viral antibodies are bound by the immobilized antigen, the second antibody will bind and the complex will be detected by the indicator.



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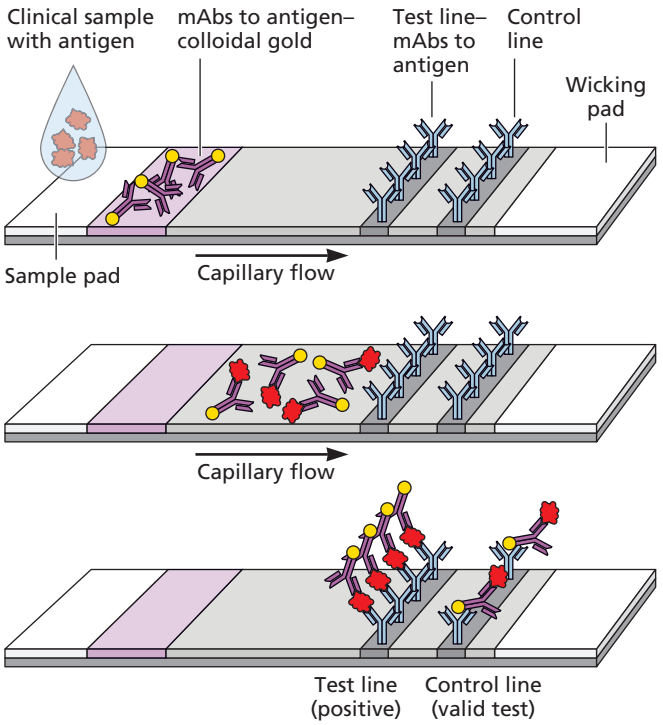


Figure 2.17 Lateral flow immunochromatographic assay. A slide or "dipstick" covered with a membrane is used to assay for the presence of viral antigens. The clinical specimen is placed on an absorbent pad at one end and is drawn across the slide by capillary action. Antigens in the sample react with a specific antibody, which is conjugated to a detector. The antigen-antibody complexes move across the membrane until they are captured by a second antibody. At this point a line becomes visible, indicating that viral antigen is present in the sample.

antibody, which is conjugated to a detector. The antigen-antibody complexes move across the membrane until they are captured by a second antibody. At this point a line becomes visible, indicating that viral antigen is present in the sample (Fig. 2.17). The lateral flow immunochromatographic assay does not require instrumentation and can be read in 5 to 20 min in a physician's office or in the field. Commercial rapid antigen detection assays are currently available for influenza virus, respiratory syncytial virus, and rotavirus.

Detection of Viral Nucleic Acids

The use of cell culture to detect viruses is being increasingly supplanted by molecular methods such as the polymerase chain reaction, DNA microarrays, and high-throughput sequencing, especially for discovery of new viruses associated with human diseases. These methods can be used to identify viruses that cannot be propagated in cell culture, necessitating new ways to fulfill Koch's postulates (Box 1.4).

Polymerase chain reaction. In this technique, specific oligonucleotides are used to amplify viral DNA sequences from infected cells or clinical specimens. Amplification is done in cycles, using a thermostable DNA polymerase. Each cycle consists of primer annealing, extension, and thermal denaturation carried out by automated cycler machines. The result is exponential amplification (a $2n$-fold increase after n cycles of amplification) of the target sequence that is located between the two DNA primers. Clinical laboratories use PCR assays to detect evidence for infection by a single type of virus (singleplex PCR), while screening for the presence of up to 30 different viruses can be done using multiplex PCR. In contrast to conventional PCR, real-time PCR can be used to quantitate the amount of DNA or RNA in a sample. In this procedure, also called quantitative PCR, the amplified DNA is detected as the reaction progresses, for example, by the use of fluorescent dyes that intercalate nonspecifically into DNA. The number of cycles needed to detect fluorescence above background can then be compared between standard and experimental samples.

DNA microarrays. This approach provides a method for studying the gene expression profile of a cell in response to virus infection (Chapter 8), and can also be used to discover new viruses. In this method, millions of unique viral DNA sequences fixed to glass or silicon wafers are incubated with complementary sequences amplified from clinical and environmental samples. Binding is usually detected by using fluorescent molecules incorporated into amplified nucleic acids.

High-throughput sequencing. Sequencing of thousands to millions of DNA molecules at the same time is a feature of this method, also known as next-generation sequencing to distinguish it from older methods. The newer approaches have not only made sequencing of DNA cheaper and faster but also helped create innovative experimental approaches to study genome organization, function, and evolution. Current high-throughput sequencing methods include 454, Illumina, and SOLiD (sequencing by oligonucleotide ligation and detection). Each has advantages and limitations in terms of read length, cost, and speed. Illumina technology provides extremely high throughput, enabling sequencing of 1 human genome per 24-h period. SOLiD is comparable in performance to Illumina technology but at a higher price.

The use of high-throughput sequencing has led to the discovery of new viruses and has given birth to the field of metagenomics, the analysis of sequences from clinical or environmental samples. These sequencing technologies can be used to study the **virome,** the collection of all viruses in a specific environment, such as sewage, the human body, or the intestinal tract.

The generation of nucleotide sequences at an unprecedented rate has spawned a new branch of bioinformatics to develop algorithms for assembling sequence reads into continuous

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strings and to determine whether they are from a virus, and if so, whether it is novel or previously discovered. Storing, analyzing, and sharing massive quantities of data, an estimated 15 quadrillion nucleotides per year, is an immense challenge. While these virus detection technologies are extremely powerful, the results obtained must be interpreted with caution. It is very easy to detect traces of a viral contaminant when searching for new agents of human disease (Box 2.8).

Genome sequences can provide considerable insight into the evolutionary relationships among viruses. Such information can be used to understand the origin of viruses and how selection pressures change viral genomes, and to assist in epidemiological investigations of viral outbreaks. When few viral genome sequences were available, pairwise homologies were often displayed in simple tables. As sequence databases increased in size, tables of multiple alignments were created, but these were still based only on pairwise comparisons. Today, phylogenetic trees are used to illustrate the relationships among numerous viruses or viral proteins (Box 2.9). Not only are such trees important tools for understanding evolutionary relationships, but they may allow conclusions to be drawn about biological functions: examination of a phylogenetic tree may allow determination of how closely or distantly a sequence relates to one of known function.

Viral Reproduction: the Burst Concept

A fundamental and important principle is that viruses replicate by the assembly of preformed components into particles. The parts are first made in cells and then assembled into the final product. The reproduction of viruses is very different from that of cells, which multiply by binary fission. This simple build-and-assemble strategy is unique to viruses, but the details for members of different virus families are astoundingly different. There are many ways to build a virus particle, and each one tells us something new about virus structure and assembly.

Modern studies of virus replication strategies have their origins in the work of Max Delbrück and colleagues, who studied the T-even bacteriophages starting in 1937. Delbrück believed that these bacteriophages were perfect models for understanding virus replication. He also thought that phages were excellent models for studying the gene: they were self-replicating (a hallmark of a gene); their mutations were inherited; and they were small, easily manipulated entities with short reproductive cycles.

Delbrück focused his attention on the fact that one bacterial cell usually makes hundreds of progeny virus particles. The yield from one cell is one viral generation; it was called the **burst** because viruses literally burst from the infected cell. Under carefully controlled laboratory conditions, most cells make, on average, about the same number of bacteriophages per cell. For example, in one of Delbrück's experiments, the average number of bacteriophage T4 particles produced from individual single-cell bursts from *Escherichia coli* cells was 150 particles per cell. If this experiment were done today, using comparable experimental conditions, the average burst would be similar.

Another important implication of the burst is that a cell has a finite capacity to produce virus. A number of parameters limit the number of particles produced per cell, such as metabolic resources, the number of sites for replication in the cell, the regulation of release of virus particles, and host defenses. In general, larger cells (e.g., eukaryotic cells) produce more virus particles per cell: yields of 1,000 to 10,000 virions per eukaryotic cell are not uncommon.

A burst occurs for viruses that kill the cell after infection, namely, the cytopathic viruses. However, some viruses do **not** kill their host cells, and virus particles are produced as long as the cell is alive. Examples include filamentous bacteriophages, some retroviruses, and hepatitis viruses.

The One-Step Growth Cycle

Initial Concept

The idea that one-step growth analysis can be used to study the single-cell life cycle of viruses originated from the work on bacteriophages by Emory Ellis and Delbrück. In their classic experiment, they added virus particles to a culture of rapidly growing *E. coli* cells. These particles adsorbed quickly to the cells. The infected culture was then diluted, preventing further adsorption of unbound particles. This simple dilution step is the key to the experiment: it reduces further binding of virus to cells and effectively synchronizes the infection. Samples of the diluted culture were then taken every few minutes and analyzed for the number of infectious bacteriophages. When the results were plotted, several key observations emerged. The results were surprising in that they did not resemble the growth curves of bacteria or cultured cells. After a short lag, bacterial cell growth becomes exponential (i.e., each progeny cell is capable of dividing) and follows a straight line (Fig. 2.18A). Exponential growth continues until the nutrients in the medium are exhausted. In contrast, numbers of new viruses do not increase in a linear fashion from the start of the infection (Fig. 2.18B, left). There is an initial lag, followed by a rapid increase in virus production, which then plateaus. This single cycle of virus reproduction produces the "burst" of virus progeny. If the experiment is repeated, so that only a few cells are initially infected, the graph looks different (Fig. 2.18B, right). Instead of a single cycle, there is a stepwise increase in numbers of new viruses with time. Each step represents one cycle of virus infection.

Once the nature of the viral growth cycle was explored using the one-step growth curve, questions emerged about what was happening in the cell before the burst. What was the fate of the incoming virus? Did it disappear? How were more

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BOX 2.8

EXPERIMENTS

Pathogen de-discovery

Deep sequencing of nucleic acids has accelerated the pace of virus discovery, but at a cost: contaminants are much easier to detect.

During a search for the causative agent of seronegative hepatitis (disease not caused by hepatitis A, B, C, D, or E virus) in Chinese patients, a new virus was discovered in sera by next-generation sequencing. This virus, provisionally called NIH-Chongquing (NIH-CQV) has a single-stranded DNA genome that is a hybrid between that of parvoviruses and circoviruses. When human sera were screened by PCR, 63 of 90 patient samples (70%) were positive for the virus, while sera from 45 healthy controls were negative. Furthermore, 84% of patients were positive for IgG antibodies against the virus and 31% were positive for IgM antibodies (suggesting a recent infection). Among healthy controls, 78% were positive for IgG and all were negative for IgM. The authors concluded that this virus was highly prevalent in some patients with seronegative hepatitis.

A second independent laboratory also identified the same virus (which they called parvovirus-like hybrid virus, PHV-1) in sera from patients in the United States with non-A-to-E hepatitis, while a third group identified the virus in diarrheal stool samples from Nigeria.

The first clue that something was amiss was the observation that the new virus identified in all three laboratories shared 99% nucleotide and amino acid identity: this similarity would not be expected in virus samples from such geographically, temporally, and clinically diverse samples. Another problem was that in the U.S. non-A-to-E hepatitis study, all patient sample pools were positive for viral sequences. These observations suggested the possibility of viral contamination.

When nucleic acids were repurified from the U.S. non-A-to-E hepatitis samples using a different method, **none** of the samples were positive for the new virus. The presence of the virus was ultimately traced to the use of column-based purification kits manufactured by Qiagen, Inc. Nearly the entire viral genome could be detected by deep sequencing of water that was passed through these columns.

The nucleic acid purification columns contaminated with the new virus were used to purify nucleic acid from patient samples. These columns, produced by a number of manufacturers, are typically an inch in length and contain a silica gel membrane that binds nucleic acids. The clinical samples are added to the column, which is then centrifuged briefly to remove liquids (hence the name "spin" columns). The nucleic acid adheres to the silica gel membrane. Contaminants are washed away, and then the nucleic acids are released from the silica by the addition of a buffer.

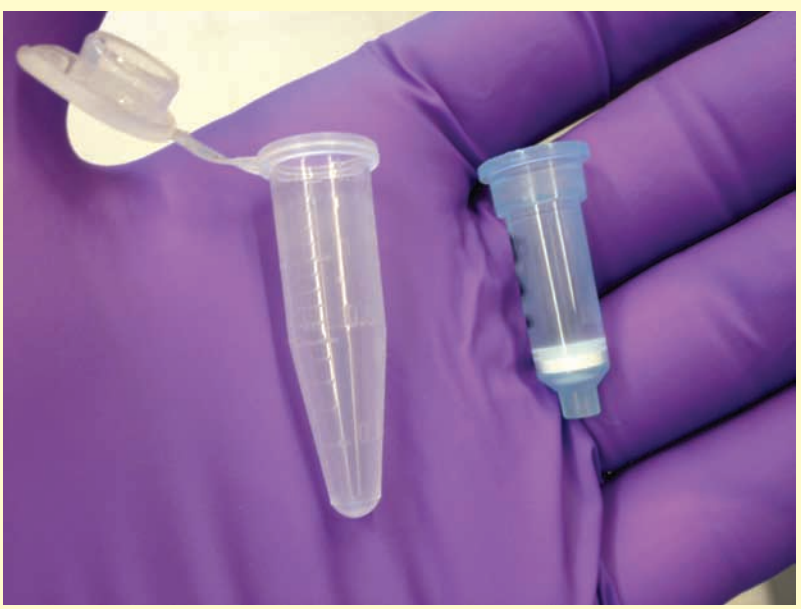
Why were the Qiagen spin columns contaminated with the parvovirus-circovirus hybrid? A search of the publicly available environmental metagenomic datasets revealed the presence of sequences highly related to PHV-1 (87 to 99% nucleotide identity). The datasets containing PHV-1 sequences were obtained from sampled seawater off the Pacific coast of North America and coastal regions of Oregon and Chile. Silica, a component of spin columns, may be produced from diatoms. The source of contamination could be explained if the silica in the Qiagen spin columns was produced from diatoms and if PHV-1 is a virus of ocean-dwelling diatoms.

In retrospect, it was easy to be fooled into believing that NIH-CQV might be a human pathogen because it was detected only in sick and not healthy patients. Why antibodies to the virus were detected in samples from both sick and healthy patients remains to be explained. However, NIH-CQV/PHV-1 is likely not associated with any human illness: when non-Qiagen spin columns were used, PHV-1 was not found in any patient sample.

The lesson to be learned from this story is clear: deep sequencing is a very powerful and sensitive method, but must be applied with great care. Every step of the virus discovery process must be carefully controlled, from the water used to the plastic reagents. Most importantly, laboratories involved in pathogen discovery must share their sequence data, something that took place during this study.

Naccache SN, Greninger AL, Lee D, Coffey LL, Phan T, Rein-Weston A, Aronsohn A, Hackett J, Jr, Delwart EL, Chiu CY. 2013. The perils of pathogen discovery: origin of a novel parvovirus-like hybrid genome traced to nucleic acid extraction spin columns. *J Virol* **87:**11966–11977.

Xu B Zhi N, Hu G, Wan Z, Zheng X, Liu X, Wong S, Kajigaya S, Zhao K, Mao Q, Young NS. 2013. Hybrid DNA virus in Chinese patients with seronegative hepatitis discovered by deep sequencing. *Proc Natl Acad Sci U S A* **110:**10264–10269.



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BOX 2.9

METHODS

How to read a phylogenetic tree

Phylogenetic dendrograms, or trees, provide information about the inferred evolutionary relationships between viruses. The example shown in the figure is a phylogenetic tree for 10 viral isolates from different individuals whose genome sequences have been determined. The horizontal dimension of the tree represents the amount of genetic change, and the scale (0.07) is the number of changes divided by the length of the sequence (in some trees this may be expressed as % change). The blue circles, called nodes, represent putative ancestors of the sampled viruses. Therefore, the branches represent chains of infections that have led to sampled viruses. The vertical distances have no significance.

The tree in the figure is *rooted*, which means that we know the common ancestor of all the sampled viruses. A rooted tree gives the order of branching from left to right: virus A existed before B, although the unit of time might not be known. The numbers next to each node represent the measure of support; these are computed by a variety of statistical approaches including "bootstrapping" and "Bayesian posterior probabilities." A value close to 1 indicates strong evidence that sequences to the right of the node cluster together better than any other sequences. Often there is no known isolate corresponding to the root of the tree; in this case, an arbitrary root may be estimated, or the tree will be unrooted. In these cases, it can no longer be assumed that the order of ancestors proceeds from left to right.

Phylogenetic trees can also be constructed by grouping sampled viruses by host of isolation. Such an arrangement sometimes makes it possible to identify the animal source of a human virus. Circular forms, such as a radial format tree, are often displayed when the root is unknown.

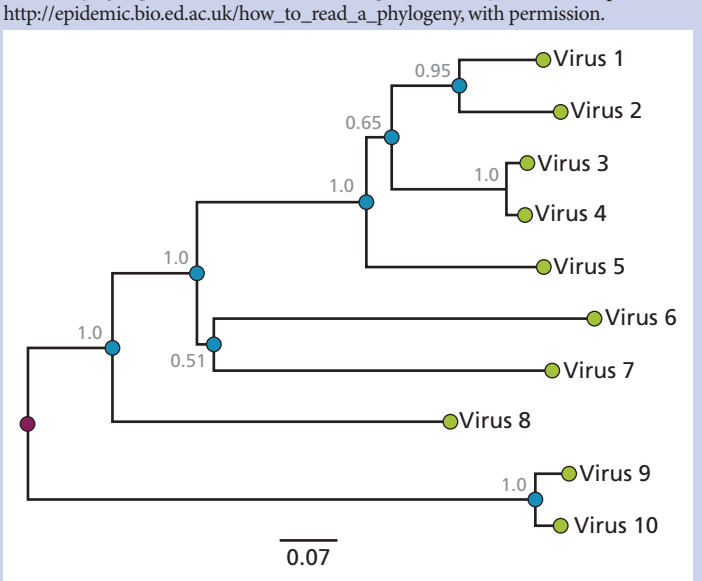
Trees relating nucleic acid sequences depict the relationships as if sampled and intermediary sequences were on a trajectory to the present sequences. This deduction is an oversimplification, because any intermediate that was lost during evolution will not be represented in the tree. In addition, any recombination or gene exchange by coinfection with similar viral genomes will scramble ordered lineages.

A fair question is whether we can predict the future trajectory or branches of the tree. We can never answer this question for two reasons: any given sample may not represent the diversity of any given virus population in an ecosystem, and we cannot predict the selective pressures that will be imposed.

Hall BG. 2011. *Phylogenetic Trees Made Easy: a How-to Manual*, 4th ed. Sinauer Associates, Sunderland, MA.

ViralZone. Phylogenetics of animal pathogens: basic principles and applications (a tutorial). http://viralzone.expasy.org/e_learning/phylogenetics/content.html

Rooted phylogenetic tree of 10 viral genome sequences. Adapted from http://epidemic.bio.ed.ac.uk/how_to_read_a_phylogeny, with permission.



virus particles produced? These questions were answered by looking inside the infected cell. Instead of sampling the diluted culture for virus after various periods of infection, researchers prematurely lysed the infected cells as the infection proceeded and then assayed for infectious virus. The results were extremely informative. Immediately after dilution, there was a complete loss, or eclipse, of infectious virus for 10 to 15 min (Fig. 2.18B). In other words, input virions disappeared and no new phage particles were produced during this period. The loss of infectivity is a consequence of the release of the genome from the virion, to allow for subsequent transcription of viral genes. Particle infectivity is lost during this phase because the released genome is not infectious under the conditions of the plaque assay. Next, new infectious particles were detected inside the cell, before they were released into the medium. These were newly assembled virus particles that had not yet been released by cell lysis. The results of these experiments defined two new terms in virology: the **eclipse period**, the phase in which infectivity is lost when virions are disassembled after penetrating cells; and the **latent period**, the time it takes to replicate, assemble, and release new virus particles before lysis, ~20 to 25 min for *E. coli* bacteriophages.

Synchronous infection, the key to the one-step growth cycle, is usually accomplished by infecting cells with a sufficient number of virus particles to ensure that most of the cells are infected rapidly.

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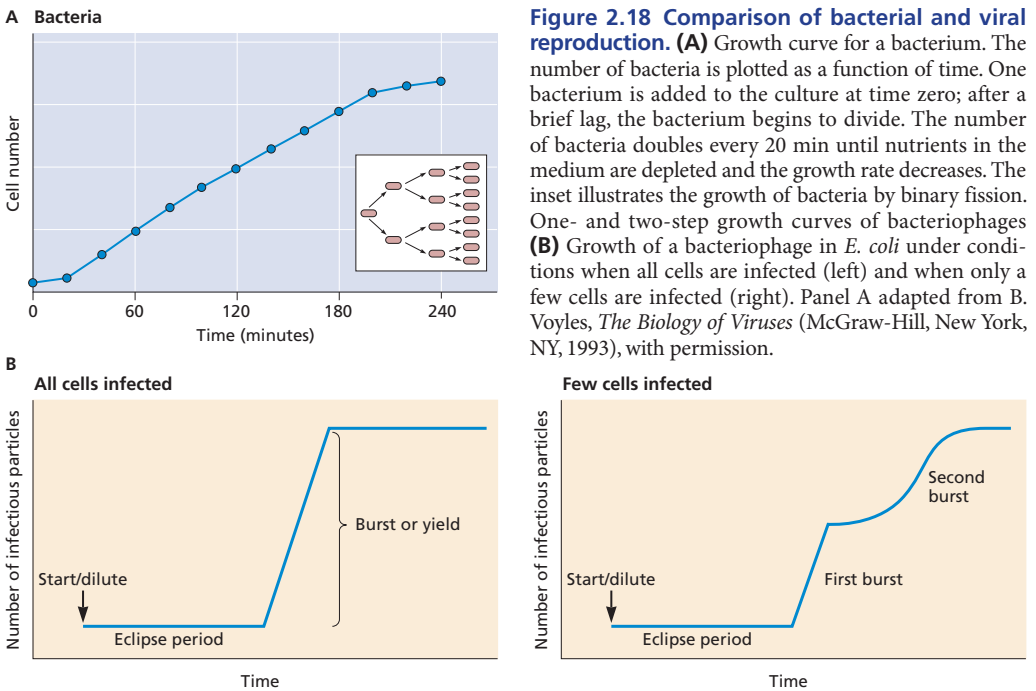


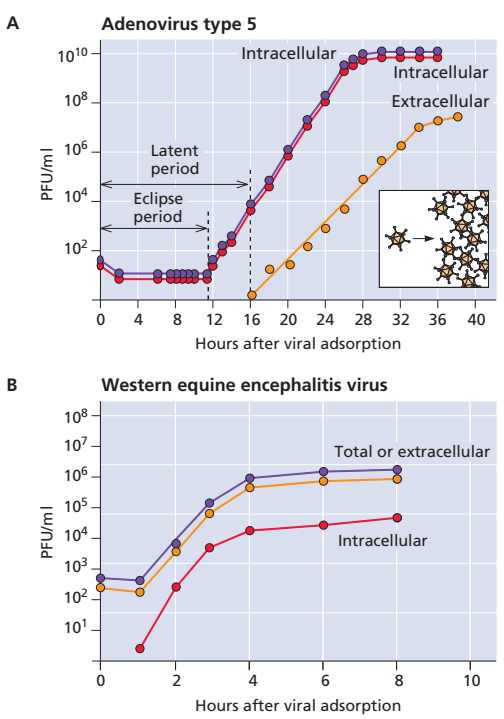
Figure 2.18 Comparison of bacterial and viral reproduction. (A) Growth curve for a bacterium. The number of bacteria is plotted as a function of time. One bacterium is added to the culture at time zero; after a brief lag, the bacterium begins to divide. The number of bacteria doubles every 20 min until nutrients in the medium are depleted and the growth rate decreases. The inset illustrates the growth of bacteria by binary fission. One- and two-step growth curves of bacteriophages **(B)** Growth of a bacteriophage in *E. coli* under conditions when all cells are infected (left) and when only a few cells are infected (right). Panel A adapted from B. Voyles, *The Biology of Viruses* (McGraw-Hill, New York, NY, 1993), with permission.

One-Step Growth Analysis: a Valuable Tool for Studying Animal Viruses

One-step growth analysis soon became adapted for studying the replication of animal viruses. The experiment begins with removal of the medium from the cell monolayer and addition of virus in a small volume to promote rapid adsorption. After ~1 h, unadsorbed inoculum containing virus particles is removed, the cells are washed, and fresh medium is added. At different times after infection, samples of the cell culture supernatant are collected and the virus titer is determined. The kinetics of intracellular virus production can be monitored by removing the medium containing extracellular particles, scraping the cells into fresh medium, and lysing them. A cell extract is prepared after removal of cellular debris by centrifugation, and the virus titer in the extract is measured.

The results of a one-step growth experiment establish a number of important features about viral replication. In the example shown in Fig. 2.19A, the first 11 h after infection constitutes the eclipse period, during which the viral nucleic acid is uncoated from its protective shell and no infectious virus can be detected inside cells. The small number of infectious particles detected during this period probably results from adsorbed virus that was not uncoated. Beginning at 12 h after adsorption, the quantity of intracellular infectious virus begins to increase, marking the onset of the synthetic phase, during which new virus particles are assembled. During the latent period, no extracellular virus can be detected. At 18 h after adsorption, virions are released from cells into the extracellular medium. Ultimately, virus production plateaus as the

Figure 2.19 One-step growth curves of animal viruses. (A) Growth of a nonenveloped virus, adenovirus type 5. The inset illustrates the concept that viruses multiply by assembly of preformed components into particles. **(B)** Growth of an enveloped virus, Western equine encephalitis virus, a member of the *Togaviridae*. This virus acquires infectivity after maturation at the plasma membrane, and therefore little intracellular virus can be detected. The small quantities observed at each time point probably represent released virus contaminating the cell extract. Adapted from B. D. Davis et al., *Microbiology* (J. B. Lippincott Co., Philadelphia, PA, 1980), with permission.



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BOX 2.10

DISCUSSION

Multiplicity of infection (MOI)

Infection depends on the random collision of cells and virus particles. When susceptible cells are mixed with a suspension of virus particles, some cells are uninfected and other cells receive one, two, three, etc., particles. The distribution of virus particles per cell is best described by the Poisson distribution:

$$P(k) = e^{-m}m^k/k!$$

In this equation, $P(k)$ is the fraction of cells infected by k virus particles. The multiplicity of infection, m, is calculated from the proportion of uninfected cells, $P(0)$, which can be determined experimentally. If k is made 0 in the above equation, then

$$P(0) = e^{-m} \text{ and } m = -1n\ P(0)$$

The fraction of cells receiving 0, 1, and >1 virus particle in a culture of 10^6 cells infected with an MOI of 10 can be determined as follows.

The fraction of cells that receive 0 particles is

$$P(0) = e^{-10} = 4.5 \times 10^{-5}$$

and in a culture of 10^6 cells, this equals 45 uninfected cells.

The fraction of cells that receive 1 particle is

$$P(1) = 10 \times 4.5 \times 10^{-5} = 4.5 \times 10^{-4}$$

and in a culture of 10^6 cells, 450 cells receive 1 particle.

The fraction of cells that receive >1 particle is

$$P(>1) = 1 - e^{-m}(m + 1) = 0.9995$$

and in a culture of 10^6 cells, 999,500 cells receive >1 particle. [The value in this equation is obtained by subtracting from 1 (the sum of all probabilities for any value of k) the probabilities $P(0)$ and $P(1)$.]

The fraction of cells receiving 0, 1, and >1 virus particle in a culture of 10^6 cells infected with an MOI of 0.001 is

$P(0) = 99.99\%$
$P(1) = 0.0999\%$ (for 10^6 cells, 10^4 are infected)
$P(>1) = 10^{-6}$

The MOI required to infect 99% of the cells in a cell culture dish is

$P(0) = 1\% = 0.01$
$m = -1n\ (0.01) = 4.6$ PFU per cell



cells become metabolically and structurally incapable of supporting additional replication.

The yield of infectious virus per cell can be calculated from the data collected during a one-step growth experiment (Fig. 2.19). This value varies widely among different viruses and with different virus-host cell combinations. For many viruses, increasing the **multiplicity of infection** (Box 2.10) above a certain point does not increase the yield: cells have a finite capacity to produce new virus particles. In fact, infecting at a very high multiplicity of infection can cause premature cell lysis and decrease virus yields.

The kinetics of the one-step growth curve can vary dramatically among different viruses. For example, enveloped viruses that mature by budding from the plasma membrane, as discussed in Chapter 13, generally become infectious only as they leave the cell, and therefore little intracellular infectious virus can be detected (Fig. 2.19B). The curve shown in Fig. 2.19A illustrates the pattern observed for a DNA virus with the long latent and synthetic phases typical of many DNA viruses, some retroviruses, and reovirus. For small RNA viruses, the entire growth curve is complete within 6 to 8 h, and the latent and synthetic phases are correspondingly shorter.

One-step growth curve analysis can provide quantitative information about different virus-host systems. It is frequently employed to study mutant viruses to determine what parts of the infectious cycle are affected by a particular genetic lesion. It is also valuable for studying the multiplication of a new virus or viral replication in a new virus-host cell combination.

When cells are infected at a low multiplicity of infection, several cycles of viral replication may occur (Fig. 2.18). Growth curves established under these conditions can also provide useful information. When infection is done at a high multiplicity of infection, a mutation may fail to have an obvious effect on viral replication. The defect may become obvious following a low-multiplicity infection. Because the effect of a mutation in each cycle is multiplied over several cycles, a small effect can be amplified. Defects in the ability of viruses to spread from cell to cell may also be revealed when multiple cycles of replication occur.

Systems Biology

The use of one-step growth analysis to study the replication cycles of many viruses has allowed a reductionist approach to understanding and defining the steps of virus attachment, entry, replication, and assembly. New experimental and computational tools that permit global analysis of viral, cellular, and host responses to infection have been developed. Systems biology analysis uses high-throughput technologies (such as next-generation sequencing of DNA and RNA, and mass spectrometry) to measure system-wide changes in DNA, RNA, proteins, and metabolites during virus infection of cells, tissues, or entire organisms. Data obtained from high-throughput measurements are integrated and analyzed using mathematical algorithms to generate models that are predictive of the system. For example, virus infections of different animals are characterized by the induction of distinct

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sets of cytokine genes, which can be correlated with different pathogenic outcomes. When a model has been developed, it can be further refined by the use of viral mutants or targeted inhibition of host genes or pathways. Systems virology is therefore a holistic, host-directed approach that complements traditional approaches to studying viruses.

Examples of systems virology approaches include the use of genome-wide transcriptional profiling to study the host response to infection. Infection of mice with the 1918 strain of influenza virus leads to a rapidly fatal infection characterized by sustained induction of proinflammatory cytokine and chemokine genes. Understanding the gene expression signature that correlates with lethality is one goal of these studies. Systems virology approaches can also be used to predict signatures of vaccine efficacy. In one study, transcriptional profiling of peripheral blood mononuclear cells from vaccinated subjects revealed that the yellow fever virus vaccine induces the expression of genes encoding members of the complement system and stress response proteins. This pattern accurately predicts subsequent $CD8^+$ T cell activation ($CD8^+$ T cell and antibody responses that are thought to mediate protection from infection with yellow fever virus). A separate signature was also identified that accurately predicts neutralizing antibody synthesis during infection. Systems virology approaches also can be used to identify and analyze all interactions between cellular and viral proteins and the roles of such interactions in replication (Box 2.11).

Perspectives

The one-step growth analysis is used nearly universally to study virus replication. When cells are infected at a high multiplicity of infection, sufficient viral nucleic acid or protein can be isolated to allow a study of events during the replication cycle. Synchronous infection is the key to this approach, because under this condition, the same steps of the reproduction cycle typically occur in all cells at the same time. Many of the experimental results discussed in subsequent chapters of this book were obtained using one-step growth analysis. The power of this approach is such that it reports on all stages of the reproduction cycle in a simple and quantitative fashion. With modest expenditure of time and reagents, virologists can deduce a great deal about viral translation, replication, or assembly.

From the humble beginnings of the one-step growth curve, many new methods have been developed that have propelled

BOX 2.11

WARNING

Determining a role for cellular proteins in viral replication can be quite difficult

Understanding the roles of both viral and cellular proteins at various stages of viral reproduction is essential for elucidating molecular mechanisms and for developing strategies for blocking pathogenic infections. As viral genomes have a limited set of genes, the viral proteins or genetic elements that are essential at each step can be deduced by introducing mutations and observing phenotypes. Identifying critical cellular genes and proteins is much more difficult. A general approach to select likely candidates has been to identify cellular proteins that are included in virus particles and/or bind to viral proteins (*in vitro* or in cells).

Once candidates are identified, the contribution of the cellular protein to viral reproduction may be evaluated by observing the effects of

- specific small-molecule inhibitors of the protein's function (inhibitory drugs)
- synthesis of an altered protein, known to have a dominant-negative effect on its normal function
- treatment with small RNAs that induce mRNA degradation (see Chapter 10) and reduce the concentration of the cellular protein
- reproduction in cells in which the candidate gene has been mutated or deleted

Even after applying the multiple approaches and methods described above, identifying relevant cellular proteins and evaluating their roles in viral reproduction is seldom easy. The problems encountered include the following.

- More than one protein may provide the required function (redundancy).
- The function of the protein might be essential to the cell, and mutation of the gene that encodes it (or inhibition of protein production) could be lethal.
- Only small quantities of the protein might be required, and reducing its activity with an inhibitor, or its concentration may be insufficient to observe a defect in viral reproduction.
- The cellular protein might provide a slight enhancement to viral reproduction that could be difficult to detect, but may be physiologically significant.
- Synthesis of an altered cellular gene or overexpression of a normal cellular gene may produce changes that affect virus reproduction for reasons that are irrelevant to the natural infection (artifacts).

Given these difficulties, it is not surprising that the literature in this area is sometimes contradictory and the results can be controversial.



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our understanding of viruses and infected cells to greater depths and at unprecedented speed. These abilities are illustrated by the new human coronavirus, Middle East respiratory syndrome coronavirus, isolated in early 2013. Within 6 months, not only had the virus been isolated and studied by one-step growth analysis, but the genome was molecularly cloned and its sequence was determined; an infectious DNA clone was made and used to produce viral mutants; the cellular receptor was identified; immunofluorescence was used to study infection of various cell types; and serological assays, including neutralization and enzyme immunoassays, were used to screen animal sera to determine the origin of the virus. We are truly in a remarkable era, when few experimental questions are beyond the reach of the techniques that are currently available.

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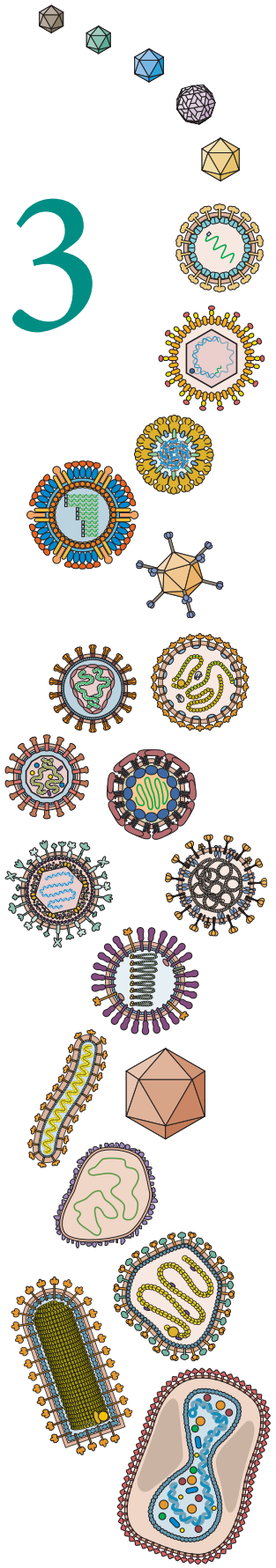
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PART II

Molecular Biology

3 Genomes and Genetics
4 Structure
5 Attachment and Entry
6 Synthesis of RNA from RNA Templates
7 Reverse Transcription and Integration
8 Synthesis of RNA from DNA Templates
9 Replication of DNA Genomes
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11 Protein Synthesis
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3 Genomes and Genetics

Introduction

Genome Principles and the Baltimore System

Structure and Complexity of Viral Genomes
- DNA Genomes
- RNA Genomes

What Do Viral Genomes Look Like?

Coding Strategies

What Can Viral Sequences Tell Us?

The Origin of Viral Genomes

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Genetic Analysis of Viruses
- Classical Genetic Methods
- Engineering Mutations into Viral Genomes
- Engineering Viral Genomes: Viral Vectors

Perspectives

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LINKS FOR CHAPTER 3

⏭ *Video: Interview with Dr. Katherine High*
http://bit.ly/Virology_High

⏭ *Virocentricity with Eugene Koonin*
http://bit.ly/Virology_Twiv275

⏭ *What if influenza virus did not reassort?*
http://bit.ly/Virology_9-15-09

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... everywhere an interplay between nucleic acids and proteins; a spinning wheel in which the thread makes the spindle and the spindle the thread.

ERWIN CHARGAFF
as quoted in A. N. H. Creager, *The Life of a Virus*, *University of Chicago Press*, 2002

Introduction

Earth abounds with uncountable numbers of viruses of great diversity. However, because taxonomists have devised methods of classifying viruses, the number of identifiable groups is manageable (Chapter 1). One of the contributions of molecular biology has been a detailed analysis of the genetic material of representatives of these major virus families. From these studies emerged the principle that the **viral genome** is the nucleic acid-based repository of the information needed to build, reproduce, and transmit a virus (Box 3.1). These analyses also revealed that the thousands of distinct viruses defined by classical taxonomic methods can be organized into seven groups, based on the structures of their genomes.

Genome Principles and the Baltimore System

A universal function of viral genomes is to specify proteins. However, these genomes do not encode the complete machinery needed to carry out protein synthesis. Consequently, one important principle is that all viral genomes must be copied to produce messenger RNAs (mRNAs) that can be read by host ribosomes. Literally, all viruses are parasites of their host cells' translation system.

A second principle is that there is unity in diversity: evolution has led to the formation of only seven major types of viral genome. The Baltimore classification system integrates these two principles to construct an elegant molecular algorithm for virologists (see Fig. 1.11). When the bewildering array of viruses is classified by this system, we find fewer than 10 pathways to mRNA. The value of the Baltimore system is that by knowing only the nature of the viral genome, one can deduce the basic steps that must take place to produce mRNA. Perhaps more pragmatically, the system simplifies comprehension of the extraordinary life cycles of viruses.

The Baltimore system omits the second universal function of viral genomes, to serve as a template for synthesis of progeny genomes. Nevertheless, there is also a finite number of nucleic acid-copying strategies, each with unique primer, template, and termination requirements. We shall combine this principle with that embodied in the Baltimore system to define seven strategies based on mRNA synthesis **and** genome replication.

Replication and mRNA synthesis present no obvious challenges for most viruses with DNA genomes, as all cells use DNA-based mechanisms. In contrast, animal cells possess no known mechanisms to copy viral RNA templates and to produce mRNA from them. For RNA viruses to survive, their RNA genomes must, by definition, encode a nucleic acid polymerase.

Structure and Complexity of Viral Genomes

Despite the simplicity of expression strategies, the composition and structures of viral genomes are more varied than those seen in the entire archaeal, bacterial, or eukaryotic kingdoms. Nearly every possible method for encoding information in nucleic acid can be found in viruses. Viral genomes can be

- DNA or RNA
- DNA with short segments of RNA
- DNA or RNA with covalently attached protein
- single stranded (+) strand, (−) strand, or ambisense (Box 3.2)
- double stranded
- linear
- circular
- segmented
- gapped

PRINCIPLES *Genomes and genetics*

- Viral genomes specify some, but never all, of the proteins needed to complete the viral life cycle.
- That only seven viral genome replication strategies exist for thousands of known viruses implies unity in viral diversity.
- Some genomes can enter the reproduction cycle upon entry into a target cell, whereas others require prior modification or other viral nucleic acids before replication can proceed.
- Although the details of replication differ, all viruses with RNA genomes must encode either an RNA-dependent RNA polymerase to synthesize RNA from an RNA template, or a reverse transcriptase to convert viral RNA to DNA.
- The information encoded in viral genomes is optimized by a variety of mechanisms; the smaller the genome, the greater the compression of genetic information.
- The genome sequence of a virus is at best a biological "parts list" and tells us little about how the virus interacts with its host.
- The genomes of viruses range from those that are extraordinarily small (<2 kb) to those that are extraordinarily large (>2,500 kbp); the diversity in size likely provides advantages in the niche in which particular viruses exist.
- Technical advances allowing the introduction of mutations into any viral gene or genome sequence were responsible for much of what we know about viruses and their lifestyles.

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BOX 3.1

BACKGROUND

What information is encoded in a viral genome?

Gene products and regulatory signals required for

- replication of the genome
- efficient expression of the genome
- assembly and packaging of the genome
- regulation and timing of the reproduction cycle
- modulation of host defenses
- spread to other cells and hosts

Information **not** contained in viral genomes:

- genes encoding a complete protein synthesis machinery (e.g., no ribosomal RNA and no ribosomal or translation proteins); note: the genomes of some large DNA viruses contain genes for transfer RNAs (tRNAs), aminoacyl-tRNA synthetases, and enzymes that participate in sugar and lipid metabolism
- genes encoding proteins of energy metabolism or membrane biosynthesis
- telomeres (to maintain genomes) or centromeres (to ensure segregation of genomes)

BOX 3.2

TERMINOLOGY

Important conventions: plus (+) and minus (−) strands

mRNA is defined as the positive (+) strand, because it can be translated. A strand of DNA of the equivalent polarity is also designated as a (+) strand; i.e., if it were mRNA, it would be translated into protein.

The RNA or DNA complement of the (+) strand is called the (−) strand. The (−) strand cannot be translated; it must first be copied to make the (+) strand. Ambisense RNA contains both (+) and (−) sequences.



The seven strategies for expression and replication of viral genomes are illustrated in Fig. 3.1 through 3.7. In some cases, genomes can enter the replication cycle directly, but in others, genomes must first be modified and additional viral nucleic acids participate in the replication cycle. Examples of specific viruses in each class are provided.

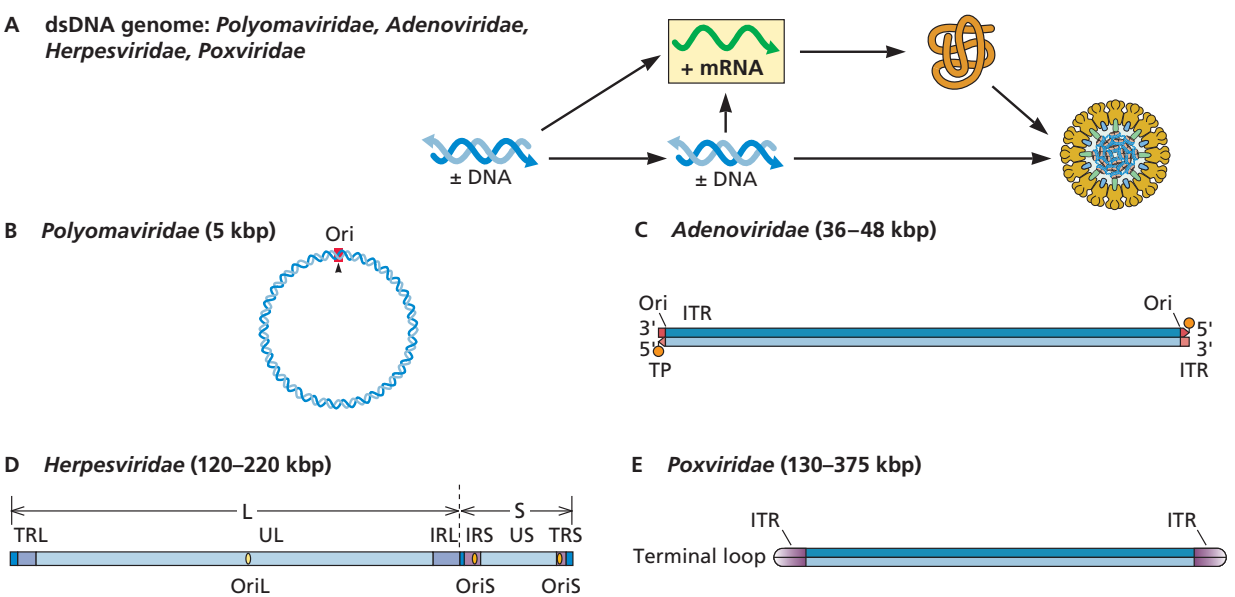
DNA Genomes

The strategy of having DNA as a viral genome appears at first glance to be simplicity itself: the host genetic system is based on DNA, so viral genome replication and expression could simply emulate the host system. Many surprises await those who believe that this is all such a strategy entails.

Double-Stranded DNA (dsDNA) (Fig. 3.1)

There are 32 families of viruses with dsDNA genomes. Those that include vertebrate viruses are the *Adenoviridae*, *Asfarviridae*, *Herpesviridae*, *Papillomaviridae*, *Polyomaviridae*,

Figure 3.1 Structure and expression of viral double-stranded DNA genomes. (A) Synthesis of genomes, mRNA, and protein. The icon represents a polyomavirus particle. **(B to E)** Genome configurations. Ori, origin of replication; ITR, inverted terminal repeat; TP, terminal protein; L, long region; S, short region; UL, US, long and short unique regions; IRL, internal repeat sequence, long region; IRS, internal repeat sequence, short region; TRL, terminal repeat sequence, long region; TRS, terminal repeat sequence, short region; OriL, origin of replication of the long region; OriS, origin of replication of the short region.



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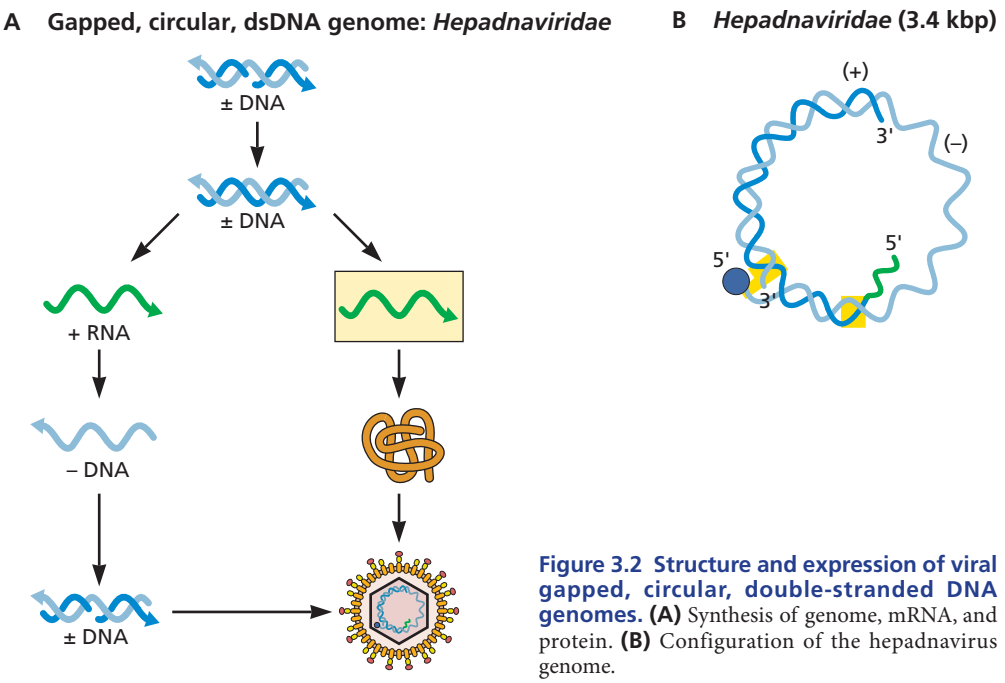


Figure 3.2 Structure and expression of viral gapped, circular, double-stranded DNA genomes. (A) Synthesis of genome, mRNA, and protein. **(B)** Configuration of the hepadnavirus genome.

Iridoviridae, and *Poxviridae* (Fig. 1.10). These genomes may be linear or circular. Genome replication and mRNA synthesis are accomplished by host or viral DNA-dependent DNA and RNA polymerases.

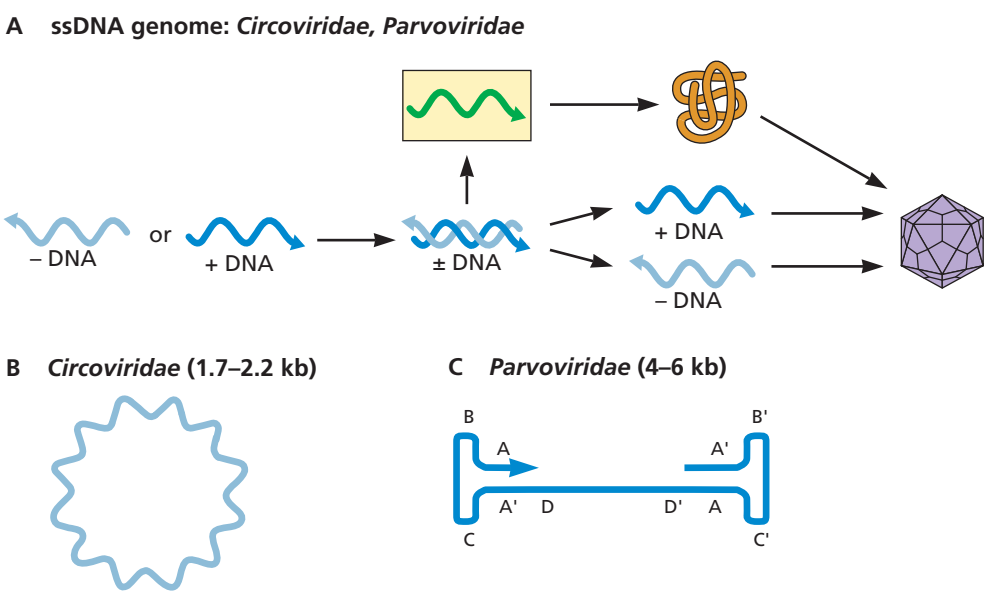
Gapped DNA (Fig. 3.2)

As the gapped DNA genome is partially double stranded, the gaps must be filled to produce perfect duplexes. This repair process must precede mRNA synthesis because the host RNA polymerase can transcribe only fully dsDNA. The unusual gapped DNA genome is produced from an RNA template by a virus-encoded enzyme, reverse transcriptase. Members of one virus family that infect vertebrates, the *Hepadnaviridae*, have a gapped DNA genome.

Single-Stranded DNA (ssDNA) (Fig. 3.3)

Seven families of viruses containing ssDNA genomes have been recognized; the families *Anelloviridae*, *Circoviridae*, and *Parvoviridae* include viruses that infect vertebrates. ssDNA must be copied into mRNA before proteins can be produced.

Figure 3.3 Structure and expression of viral single-stranded DNA genomes. (A) Synthesis of genomes, mRNA, and protein. **(B, C)** Genome configurations.



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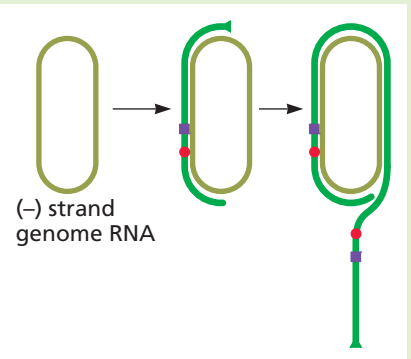
BOX 3.3

BACKGROUND

RNA synthesis in cells

There are no known host cell enzymes that can copy the genomes of RNA viruses. However, at least one enzyme, RNA polymerase II, can copy an RNA template. The 1.7-kb circular, ssRNA genome of hepatitis delta satellite virus is copied by RNA polymerase II to form multimeric RNAs (see the figure). How RNA polymerase II, an enzyme that produces pre-mRNAs from DNA templates, is reprogrammed to copy a circular RNA template is not known.

Hepatitis delta satellite (−) strand genome RNA is copied by RNA polymerase II at the indicated position. The polymerase passes the poly(A) signal (purple box) and the self-cleavage domain (red circle). For more information, see Fig. 6.14. Redrawn from J. M. Taylor, *Curr Top Microbiol Immunol* **239:**107–122, 1999, with permission.



However, RNA can be made only from a dsDNA template, whatever the sense of the ssDNA. Consequently, some DNA synthesis **must** precede mRNA production in the replication cycles of these viruses. The single-stranded viral genome is produced by cellular DNA polymerases.

RNA Genomes

Cells have no RNA-dependent RNA polymerases that can replicate the genomes of RNA viruses or make mRNA from RNA templates (Box 3.3). One solution to this problem is that RNA virus genomes encode RNA-dependent RNA polymerases that produce RNA from RNA templates. The other solution, exemplified by retrovirus genomes, is reverse transcription of the genome to dsDNA, which can be transcribed by host RNA polymerase.

dsRNA (Fig. 3.4)

There are eight families of viruses with dsRNA genomes. The number of dsRNA segments ranges from 1 (*Totiviridae* and *Endornaviridae*, viruses of fungi, protozoa, and plants) to 9 to 12 (*Reoviridae*, viruses of fungi, invertebrates, plants, protozoa, and vertebrates). While dsRNA contains a (+) strand, it cannot be translated as part of a duplex to synthesize viral proteins. The (−) strand of the genomic dsRNA is first copied into mRNAs by a viral RNA-dependent RNA polymerase. Newly synthesized mRNAs are encapsidated and then copied to produce dsRNAs.

(+) Strand RNA (Fig. 3.5)

The (+) strand RNA viruses are the most plentiful on this planet; 29 families have been recognized [not counting (+) strand RNA viruses with DNA intermediates]. The families *Arteriviridae*, *Astroviridae*, *Caliciviridae*, *Coronaviridae*, *Flaviviridae*, *Hepeviridae*, *Nodaviridae*, *Picornaviridae*, and *Togaviridae* include viruses that infect vertebrates. (+) strand RNA genomes usually can be translated directly into protein by host ribosomes. The genome is replicated in two steps. The (+) strand genome is first copied into a full-length (−) strand, and the (−) strand is then copied into full-length (+) strand genomes. In some cases, a subgenomic mRNA is produced.

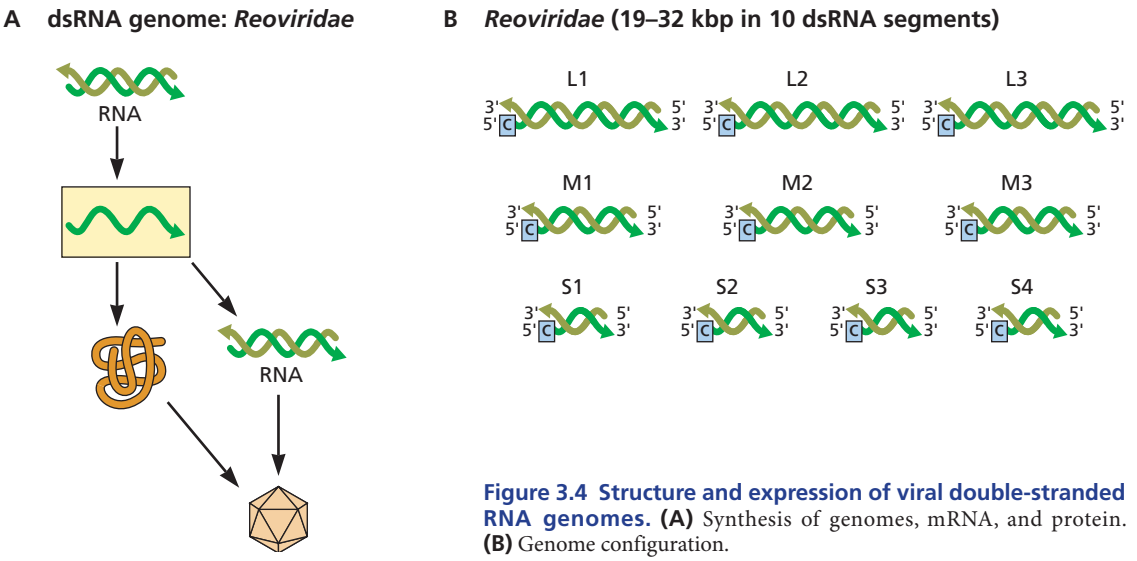


Figure 3.4 Structure and expression of viral double-stranded RNA genomes. (A) Synthesis of genomes, mRNA, and protein. **(B)** Genome configuration.

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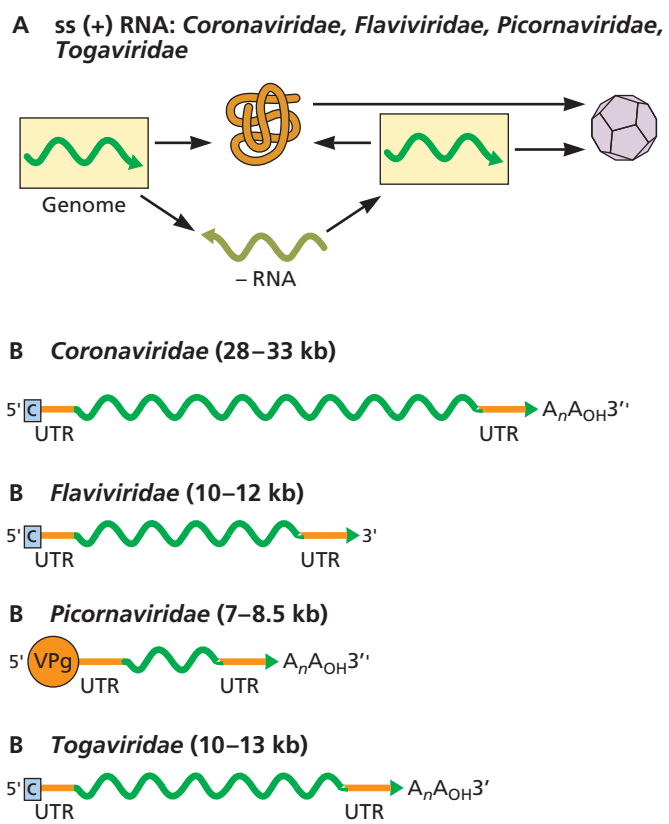


Figure 3.5 Structure and expression of viral single-stranded (+) RNA genomes. (A) Synthesis of genomes, mRNA, and protein. **(B)** Genome configurations. UTR, untranslated region.

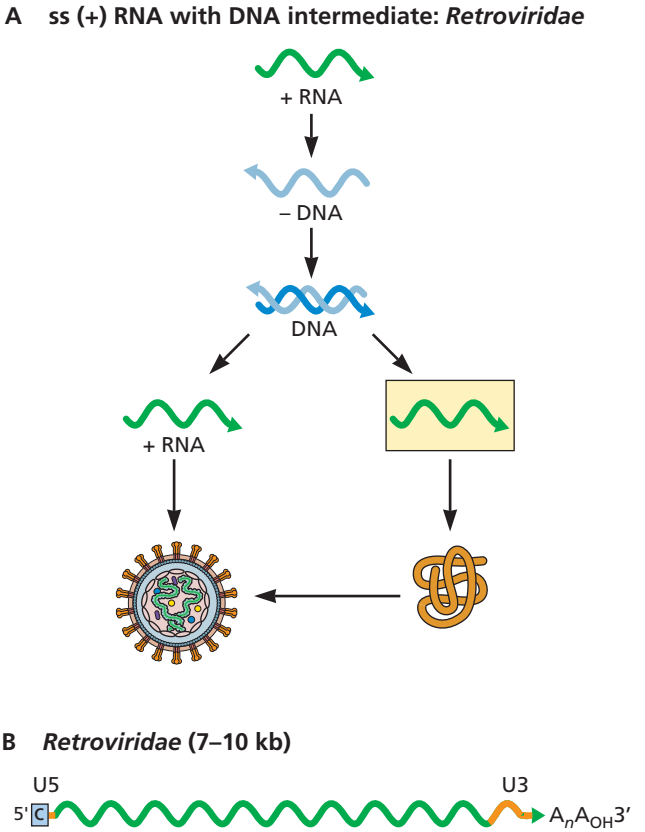


Figure 3.6 Structure and expression of viral single-stranded (+) RNA genomes with a DNA intermediate. (A) Synthesis of genomes, mRNA, and protein. **(B)** Genome configuration.

(+) Strand RNA with DNA Intermediate (Fig. 3.6)

In contrast to other (+) strand RNA viruses, the (+) strand RNA genome of retroviruses is converted to a dsDNA intermediate by viral RNA-dependent DNA polymerase (reverse transcriptase). This DNA then serves as the template for viral mRNA and genome RNA synthesis by cellular enzymes. There are three families of (+) strand RNA viruses with a DNA intermediate; members of the *Retroviridae* infect vertebrates.

(−) Strand RNA (Fig. 3.7)

Viruses with (−) strand RNA genomes are found in seven families. Viruses of this type that can infect vertebrates include members of the *Bornaviridae*, *Filoviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, and *Rhabdoviridae* families. Unlike (+) strand RNA, (−) strand RNA genomes cannot be translated directly into protein, but must be first copied to make (+) strand mRNA. There are no enzymes in the cell that can make mRNAs from the RNA genomes of (−) strand RNA viruses. These virus particles therefore contain virus-encoded RNA-dependent RNA polymerases. The genome is also the template for the synthesis of full-length (+) strands, which in turn are copied to produce (−) strand genomes. Such RNA viral genomes can be either single molecules (nonsegmented; some viruses with this configuration have been classified in the order *Mononegavirales*) or segmented.

The genomes of certain (−) strand RNA viruses (e.g., members of the *Arenaviridae* and *Bunyaviridae*) are ambisense: they contain both (+) and (−) strand information on a single strand of RNA (Fig. 3.7C). The (+) sense information in the genome is translated upon entry of the viral RNA into cells. Replication of the RNA genome yields additional (+) sense information, which is then translated.

What Do Viral Genomes Look Like?

Some small RNA and DNA genomes enter cells from virus particles as naked molecules of nucleic acid, whereas others are always associated with specialized nucleic acid-binding proteins. A fundamental difference between the genomes of viruses and those of hosts is that although viral genomes are often covered with proteins, they are usually not bound by histones (polyomaviral and papillomaviral genomes are an exception).

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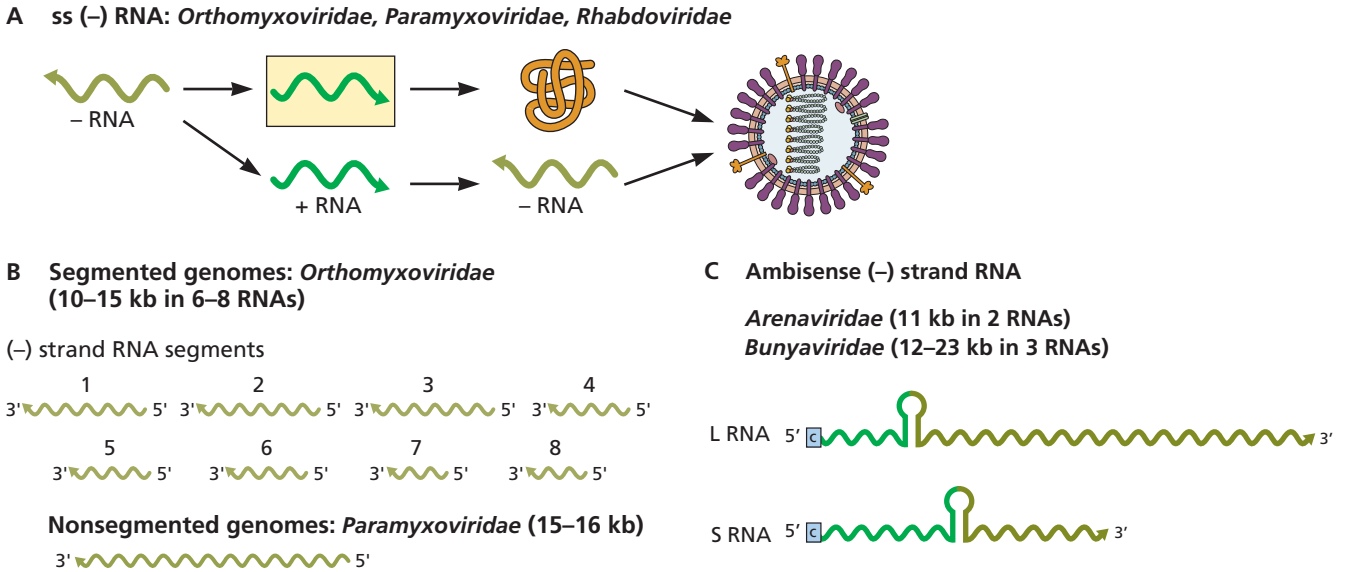


Figure 3.7 Structure and expression of viral single-stranded (–) RNA genomes. **(A)** Synthesis of genomes, mRNA, and protein. The icon represents an orthomyxovirus particle. **(B, C)** Genome configurations.

While viral genomes are all nucleic acids, they should **not** be thought of as one-dimensional structures. Virology textbooks (this one included) often draw genomes as straight, one-dimensional lines, but this notation is for illustrative purposes only; physical reality is certain to be dramatically different. Genomes have the potential to adopt amazing secondary and tertiary structures in which nucleotides may engage in long-distance interactions (Fig. 3.8).

The sequences and structures near the ends of viral genomes are often indispensable for viral replication (Fig. 3.9). For example, the DNA sequences at the ends of parvovirus genomes form T-shaped structures that are required for priming during DNA synthesis. Proteins covalently attached to 5′ ends, inverted and tandem repeats, and tRNAs may also participate in the replication of RNA and DNA genomes. Secondary RNA structures may facilitate translation (the internal ribosome entry site [IRES] of picornavirus genomes) and genome packaging (the structured packaging signal of retroviral genomes).

Coding Strategies

The compact genome of most viruses renders the "one gene, one mRNA" dogma inaccurate. Extraordinary tactics for information retrieval, such as the production of multiple subgenomic mRNAs, mRNA splicing, RNA editing, and nested transcription units (Fig. 3.10), allow the production of multiple proteins from a single viral genome. Further expansion of the coding capacity of the viral genome is achieved by posttranscriptional mechanisms, such as polyprotein synthesis, leaky scanning, suppression of termination, and ribosomal frameshifting. In general, the smaller the genome, the greater the compression of genetic information.

What Can Viral Sequences Tell Us?

Knowledge about the physical nature of genomes and coding strategies was first obtained by study of the nucleic acids of viruses. Indeed, DNA sequencing technology was perfected on viral genomes. The first genome of any kind to be sequenced was that of the *Escherichia coli* bacteriophage MS2, a linear ssRNA of 3,569 nucleotides. dsDNA genomes of larger viruses, such as herpesviruses and poxviruses (vaccinia virus), were sequenced completely by the 1990s. Since then, the complete sequences of >3,600 different viral genomes have been determined. Published viral genome sequences can be found at http://www.ncbi.nlm.nih.gov/genome/viruses/.

Viral genome sequences have many uses, including classification of viruses. Furthermore, sequence analysis has identified many relationships among diverse viral genomes, providing considerable insight into the origin of viruses. A great deal can also be learned from the lack of such relationships: >93% of the >2,500 genes of Pandoravirus salinus resemble nothing known. Consequently, their origin cannot be traced to any known cellular lineage, leading to the controversial suggestion that these giant viruses are derived from a now-extinct fourth domain of life. In outbreaks or epidemics of viral disease, even partial genome sequences can provide information about the identity of the infecting virus and its spread in different populations. New viral nucleic acid sequences can be associated with disease and characterized even in the absence of standard virological techniques (Volume II, Chapter 10). For example, human herpesvirus 8 was identified by comparing sequences present in diseased and nondiseased tissues.

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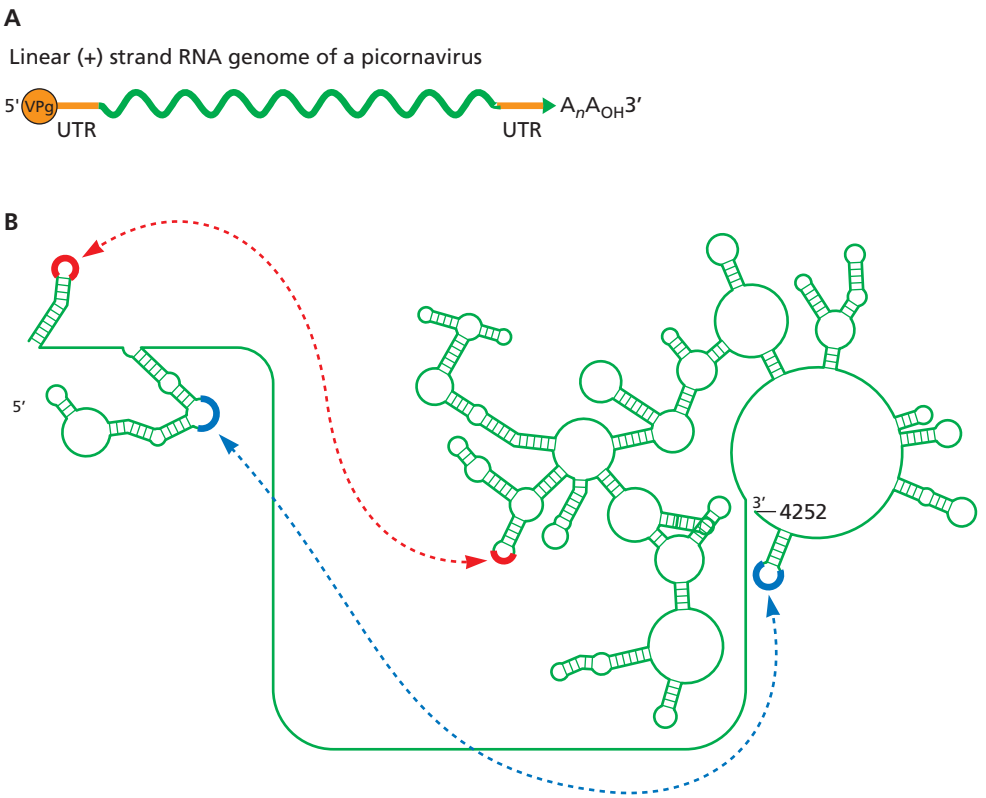


Figure 3.8 Genome structures in cartoons and in real life. (A) Linear representation of a picornavirus RNA genome. UTR, untranslated region. **(B)** Long-distance RNA-RNA interactions in a plant virus RNA genome. The 4,252-nucleotide viral genome is shown with secondary RNA structures at the 5′ and 3′ ends. Sequences that base pair are shown in blue (required for RNA frameshifting) and red (required to bring ribosomes from the 3′ end to the 5′ end). Courtesy of Anne Simon, University of Maryland.

Despite its utility, a complete understanding of how viruses reproduce cannot be obtained solely from the genome sequence or structure. The genome sequence of a virus is at best a biological "parts list": it provides some information about the intrinsic properties of a virus (e.g., predicted sequences of viral proteins and particle composition), but says little or nothing about how the virus interacts with cells, hosts, and populations. This limitation is best illustrated by the results of environmental metagenomic analyses, which reveal that the number of viruses around us (especially in the sea) is astronomical. Most are uncharacterized and, because their hosts are also unknown, cannot be studied. A reductionist study of individual components in isolation provides few answers. Although the reductionist approach is often experimentally the simplest, it is also important to understand how the genome behaves among others (population biology) and how the genome changes with time (evolution). Nevertheless, reductionism has provided much-needed detailed information for tractable virus-host systems. These systems allow genetic and biochemical analyses and provide models of infection *in vivo* and *in vitro*. Unfortunately, viruses and hosts that are difficult or impossible to manipulate in the laboratory remain understudied or ignored.

The Origin of Viral Genomes

The absence of bona fide viral fossils, i.e. ancient material from which viral nucleic acids can be recovered, might appear to make the origin of viral genomes an impenetrable mystery. However, the discovery of fragments of viral nucleic acids integrated into host genomes, coupled with an explosion in the determination of viral genome sequences, has allowed speculation on the evolutionary history of viruses. The origin of viruses is discussed in depth in Volume II, Chapter 10.

How viruses with DNA or RNA genomes arose is a compelling question. A predominant hypothesis is that RNA viruses are relics of the "RNA world," a period during which RNA was both genome and catalyst (no proteins yet existed). During this time, billions of years ago, life could have evolved from RNA, and the earliest organisms might have had RNA genomes. Viruses with RNA genomes might have evolved during this time. Later, DNA

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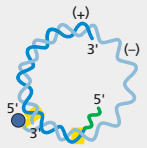
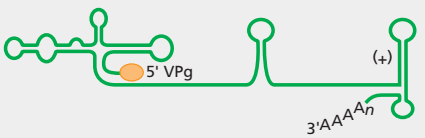
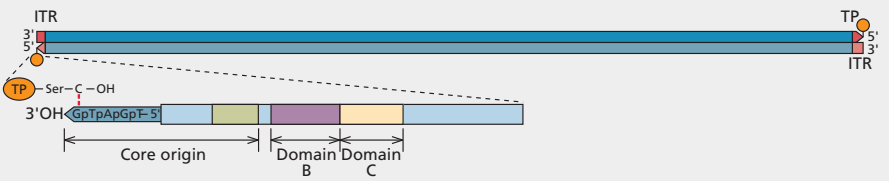
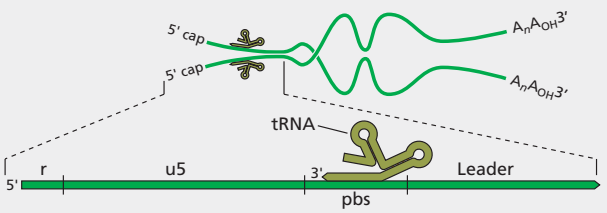
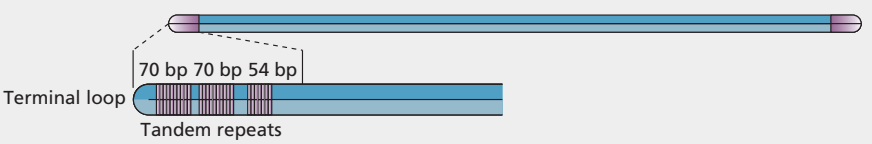
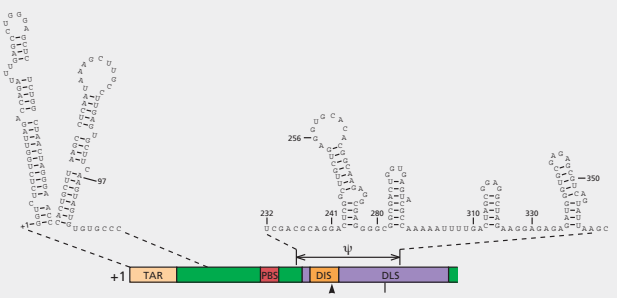
Function	Genome structure
Genome replication	
Parvovirus • T structure for priming DNA synthesis	
Hepatitis B virus • Primers for reverse transcription: 5'-linked P protein 5'-linked capped RNA	
Poliovirus • 5'-linked VPg primer • 5' cloverleaf • 3' pseudoknot • *cis*-acting replication element in coding region	
Adenovirus • Terminal protein • Inverted terminal repeat • Packaging sequences	
Retrovirus • (+) strand RNA dimer • tRNA primer • Inverted repeat sequences	
Poxvirus • Covalently joined 5′ and 3′ termini • Tandem repeats	
Translation	
Poliovirus • IRES	
Assembly	
Retrovirus • Packaging signal	

Figure 3.9 Genome structures critical for function. Abbreviations: ITR, inverted terminal repeat; TP, terminal protein; pbs and PBS, primer-binding site; IRES, internal ribosome entry site; TAR, trans-activating response element; DIS, dimerization initiation site; DLS, dimer linkage structure.

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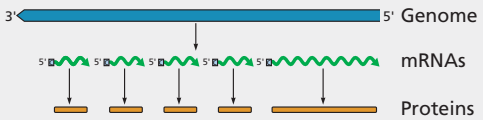
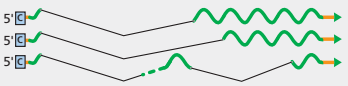
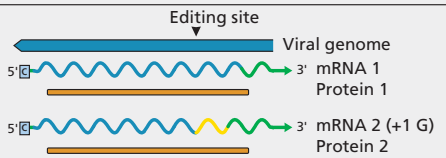
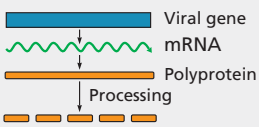
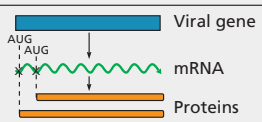
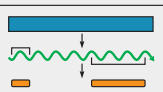
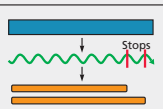
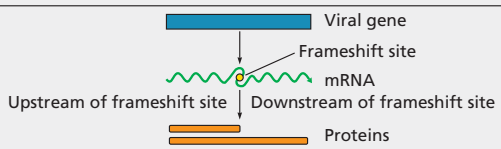
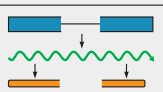
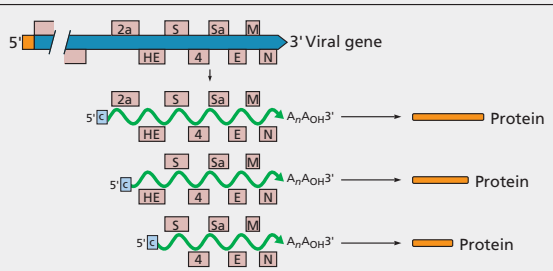
Mechanism	Diagram	Virus	Chapter(s)	Figures in appendix
Multiple subgenomic mRNAs		*Adenoviridae* *Hepadnaviridae* *Herpesviridae* *Paramyxoviridae* *Poxviridae* *Rhabdoviridae*	8, 10 7, 8 8 6 8 6	1, 2 11, 12 17, 18 25, 26 31, 32
Alternative mRNA splicing		*Adenoviridae* *Orthomyxoviridae* *Papillomaviridae* *Polyomaviridae* *Retroviridae*	8, 10 10 8, 10 8, 10 7, 10	1, 2 15, 16 23, 24 29, 30
RNA editing		*Paramyxoviridae* *Filoviridae* Hepatitis delta satellite	6, 10 10 10	
Information on both strands		*Adenoviridae* *Polyomaviridae* *Retroviridae*	8–10 8–10 7	1, 2 23, 24 29, 30
Polyprotein synthesis		*Alphaviruses* *Flaviviridae* *Picornaviridae* *Retroviridae*	6, 11 6, 11 6, 11 6, 11	33, 34 9, 10 14, 15 29, 30
Leaky scanning		*Orthomyxoviridae* *Paramyxoviridae* *Polyomaviridae* *Retroviridae*	11 11 11 11	15, 16 29, 30
Reinitiation		*Orthomyxoviridae* *Herpesviridae*	11 11	15, 16
Suppression of termination		*Alphaviruses* *Retroviridae*	11 11	33, 34 29, 30
Ribosomal frameshifting		*Astroviridae* *Coronaviridae* *Retroviridae*	11 11 11	 5, 6 29, 30
IRES		*Flaviviridae* *Picornaviridae*	11 11	 14, 15
Nested mRNAs		*Coronaviridae* *Arteriviridae*	6 6	5, 6 5, 6

Figure 3.10 Information retrieval from viral genomes. Different strategies for decoding the information in viral genomes are depicted. Cbf, CCAAT-binding factor; Usf, upstream stimulatory factor; IRES, internal ribosome entry site.

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replaced RNA as the genetic material, perhaps through the action of reverse transcriptases. With the emergence of DNA genomes came the evolution of DNA viruses. However, those with RNA genomes were and remain evolutionarily competitive, and hence they continue to survive to this day.

There is no evidence that viruses are monophyletic, i.e., descended from a common ancestor: there is no single gene shared by all viruses. Nevertheless, viruses with different genomes and replication strategies do share a small set of viral hallmark genes that encode icosahedral capsid proteins, nucleic acid polymerases, helicases, integrases, and other enzymes. There are only distant homologs of these hallmark genes in cellular genomes. It seems likely that the widespread presence of viral hallmark genes implies their ancient, possibly precellular origin.

Viral genomes display a greater diversity of genome composition, structure, and reproduction than any other organism. Understanding the function of such diversity is an intriguing problem. As viral genomes are survivors of constant selective pressure, all configurations must provide advantages. One possibility is that different genome configurations allow unique mechanisms for control over gene expression. These mechanisms include synthesis of a polyprotein from (+) strand RNA genomes or production of subgenomic mRNAs from (−) strand RNA genomes. There is some evidence that segmented RNA genomes might have arisen from monopartite genomes, perhaps to allow regulation of the production of individual proteins (Box 3.4). Segmentation probably did not emerge to increase genome size, as the largest RNA genomes are monopartite.

BOX 3.4

EXPERIMENTS

Origin of segmented RNA virus genomes

Segmented genomes are plentiful in the RNA virus world. They are found in virus particles from different families, and can be double stranded (*Reoviridae*) or single stranded, with (+) (*Closteroviridae*) or (−) (*Orthomyxoviridae*) polarity. Some experimental findings suggest that monopartite viral genomes emerged first, then later fragmented to form segmented genomes.

Insight into how a monopartite RNA genome might have fragmented to form a segmented genome comes from studies with the picornavirus foot-and-mouth disease virus (FMDV). The genome of this virus is a single molecule of (+) strand RNA. Serial passage of the virus in baby hamster kidney cells led to the emergence of genomes with two different large deletions (417 and 999 nucleotides) in the coding region. Neither mutant genome is infectious, but when they are introduced together into cells, an infectious virus population is produced. This population comprises a mixture of each of the two mutant genomes packaged separately into viral particles. Infection is successful because of complementation: when a host cell is infected with both particles, each genome provides the proteins missing in the other.

Further study of the deleted FMDV genomes revealed the presence of point mutations in other regions of the genome. These mutations had accumulated before the deletions appeared, and increased the fitness of the deleted genome compared with the wild-type genome.

These results show how monopartite viral RNAs may be divided, possibly a pathway to a segmented genome. It is interesting that the point mutations that gave the RNAs a fitness advantage over the standard RNA arose before fragmentation occurred—implying that the changes needed to occur in a specific sequence. The authors of the study conclude: "Thus, exploration of sequence space by a viral genome (in this case an unsegmented RNA) can reach a point of the space in which a totally different genome structure (in this case, a segmented RNA) is favored over the form that performed the exploration." While the fragmentation of the FMDV genome may represent a step on the path to segmentation, its relevance to what occurs in nature is unclear, because the results were obtained in cell culture.

A compelling picture of the genesis of a segmented RNA genome comes from the discovery of a new tick-borne virus in China, Jingmen tick virus (JMTV). The genome of this virus comprises four segments of (+) strand RNA. Two of the RNA segments have no known sequence homologs, while the other two are related to sequences of flaviviruses. The RNA genome of flaviviruses is not segmented: it is a single strand of (+) sense RNA. The proteins encoded by RNA segments 1 and 3 of JMTV are nonstructural proteins that are clearly related to the flavivirus NS5 and NS3 proteins (see the figure).

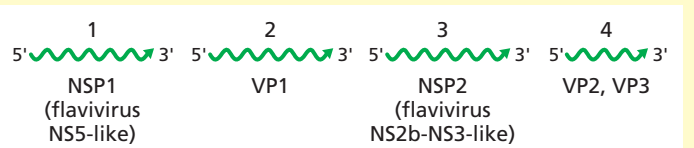
The genome structure of JMTV suggests that at some point in the past a flavivirus genome fragmented to produce the RNA segments encoding the NS3- and NS5-like proteins. This fragmentation might have initially taken place as shown for FMDV in cell culture, by fixing of deletion mutations that complemented one another. Next, coinfection of this segmented flavivirus with another unidentified virus took place to produce the precursor of JMTV.

The results provide new clues about the origins of segmented RNA viruses.

Moreno E, Ojosnegros S, García-Arriaza J, Escarmís C, Domingo E, Perales C. 2014. Exploration of sequence space as the basis of viral RNA genome segmentation. *Proc Natl Acad Sci U S A* **111:**6678–6683.

Qin XC, Shi M, Tian JH, Lin XD, Gao DY, He JR, Wang JB, Li CX, Kang YJ, Yu B, Zhou DJ, Xu J, Plyusnin A, Holmes EC, Zhang YZ. 2014. A tick-borne segmented RNA virus contains genome segments derived from unsegmented viral ancestors. *Proc Natl Acad Sci U S A* **111:**6744–6749.

RNA genome of JMTV virus. The viral genome comprises four segments of single-stranded, (+) sense RNA. Proteins encoded by each RNA are indicated. RNA segments 1 and 3 encode flavivirus-like proteins.



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The "Big and Small" of Viral Genomes: Does Size Matter?

Currently, the prize for the smallest nondefective animal virus genome goes to members of the *Circoviridae* and *Anelloviridae*, which possess circular, ssDNA genomes of 1.7 to 2.2 kb and 2 to 4 kb, respectively (Fig. 3.3B). Members of the *Circoviridae* include agriculturally important pathogens of chickens and pigs; anelloviruses such as torque teno (TT) virus infect >90% of humans with no known consequence. The consolation prize goes to the *Hepadnaviridae*, such as hepatitis B virus, which causes hepatitis and liver cancer in millions of people. Its genome comprises 3.2 kb of gapped DNA (Fig. 3.2).

The largest known virus genome, a DNA molecule of 2,500 kbp, is that of Pandoravirus salinus, which infects amoebae. The largest RNA virus genome, 31 kb, is characteristic of some coronaviruses (Fig. 3.5). Despite detailed analyses, there is no evidence that one size is more advantageous than another. All viral genomes have evolved under relentless selection, so extremes of size must provide particular advantages. One feature distinguishing large genomes from smaller ones is the presence of many genes that encode proteins for viral genome replication, nucleic acid metabolism, and countering of host defense systems. In other words, these large viruses have sufficient coding capacity to escape some restrictions imposed by host cell biochemistry. The smallest genome of a free-living cell is predicted to comprise <300 genes (based on bacterial genome sequences). Remarkably, this number is smaller than the genetic content of large viral DNA genomes. Nevertheless, the big viruses are **not** cells: their replication absolutely requires the cellular translation machinery, as well as host cell systems to make membranes and generate energy.

The parameters that limit the size of viral genomes are largely unknown. There are cellular DNA and RNA molecules that are much longer than those found in virus particles. Consequently, the rate of nucleic acid synthesis is not likely to be limiting. In some cases, the capsid volume might limit genome size. There is a penalty inherent in having a large genome: a huge particle must be provided, and this is not a simple matter. In the case of the 150-kb herpes simplex virus genome, 50 to 60 gene products are needed to build the icosahedral nucleocapsid that houses the genome. This large number of protein products is encoded by 75% of the viral genome. Two of the largest known viral DNA genomes, those of Megavirus chilensis (1,259 kbp) and mamavirus (1,192 kbp), are housed in the biggest known capsids constructed with icosahedral symmetry. Although the principles of icosahedral symmetry are quite flexible in allowing a wide range of capsid sizes, it is possible that building a very large and stable capsid that can also come apart to release the viral genome is beyond the intrinsic properties of macromolecules.

One solution to the capsid size problem is to abandon icosahedral symmetry. Particles built with helical symmetry can in principle accommodate very large genomes, for example, baculoviruses with DNA genomes up to 180 kbp. The pandoraviruses, with the largest known DNA viral genomes (2,500 kbp), are housed in decidedly nonisometric ovoid particles 1 μm in length and 0.5 μm in diameter.

There is no reason to believe that the upper limit in viral particle and genome size has been reached. The core compartment of a mimivirus particle is larger than needed to accommodate the 1,200-kbp DNA genome. A particle of this size could, in theory, house a genome of 6 million bp if the DNA were packed at the same density as in polyomaviruses. Indeed, if the genome were packed into the particle at the same density reached in bacteriophages, it could be >12 million bp, the size of that of the smallest free-living unicellular eukaryote.

In cells, DNAs are much longer than RNA molecules. RNA is less stable than DNA, but in the cell, much of the RNA is used for the synthesis of proteins and therefore need not exceed the size needed to specify the largest polypeptide. However, this constraint does not apply to viral genomes. Yet the largest viral single-molecule RNA genomes, the 27- to 31-kb (+) strand RNAs of the coronaviruses, are dwarfed by the largest (2,500-kbp) DNA virus genomes. Susceptibility of RNA to nuclease attack might limit the size of viral RNA genomes, but there is little direct support for this hypothesis. The most likely explanation is that there are few known enzymes that can correct errors introduced during RNA synthesis. An exonuclease encoded in the coronavirus genome is one exception: its presence could explain the large size of these RNAs. DNA polymerases can eliminate errors during polymerization, a process known as proofreading, and remaining errors can also be corrected after synthesis is complete. The average error frequencies for RNA genomes are about 1 misincorporation in 10^4 or 10^5 nucleotides polymerized. In an RNA viral genome of 10 kb, a mutation frequency of 1 in 10^4 would produce about 1 mutation in every replicated genome. Hence, very long viral RNA genomes, perhaps longer than 32 kb, would sustain too many lethal mutations. Even the 7.5-kb genome of poliovirus exists at the edge of viability: treatment of the virus with the RNA mutagen ribavirin causes a >99% loss in infectivity after a single round of replication.

Genetic Analysis of Viruses

The application of genetic methods to study the structure and function of animal viral genes and proteins began with development of the plaque assay by Renato Dulbecco in 1952. This assay permitted the preparation of clonal stocks of virus, the measurement of virus titers, and a convenient system for studying viruses with conditional lethal mutations. Although a limited repertoire of classical genetic methods was available, the mutants that were isolated (Box 3.5) were invaluable in elucidating many aspects of infectious cycles and cell transformation. Contemporary methods of genetic analysis based

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BOX 3.5

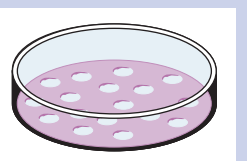
METHODS

Spontaneous and induced mutations

In the early days of experimental virology, mutant viruses could be isolated only by screening stocks for interesting phenotypes, for none of the tools that we now take for granted, such as restriction endonucleases, efficient DNA sequencing methods, and molecular cloning procedures, were developed until the mid- to late 1970s. RNA virus stocks usually contain a high proportion of mutants, and it is only a matter of devising the appropriate selection conditions (e.g., high or low temperature or exposure to drugs that inhibit viral reproduction) to select mutants with the desired phenotype from the total population. For example, the live attenuated poliovirus vaccine strains developed by Albert Sabin are mutants that were selected from a virulent virus stock (Volume II, Fig. 8.7).

The low spontaneous mutation rate of DNA viruses necessitated random mutagenesis by exposure to a chemical mutagen. Mutagens such as nitrous acid, hydroxylamine, and alkylating agents chemically modify the nucleic acid in preparations of virus particles, resulting in changes in base pairing during subsequent genome replication. Mutagens such as base analogs, intercalating agents, or UV light are applied to the infected cell to cause changes in the viral genome during replication. Such agents introduce mutations more or less at random. Some mutations are lethal under all conditions, while others have no effect and are said to be silent.

To facilitate identification of mutants, the population must be screened for a phenotype that can be identified easily in a plaque assay. One such phenotype is temperature-sensitive viability of the virus. Virus mutants with this phenotype reproduce well at low temperatures, but poorly or not at all at high temperatures. The permissive and nonpermissive temperatures are typically 33 and 39°C, respectively, for viruses that replicate in mammalian cells. Other commonly sought phenotypes are changes in plaque size or morphology, drug resistance, antibody resistance, and host range (that is, loss of the ability to reproduce in certain hosts or host cells).



on recombinant DNA technology confer an essentially unlimited scope for genetic manipulation; in principle, any viral gene of interest can be mutated, and the precise nature of the mutation can be predetermined by the investigator. Much of the large body of information about viruses and their lifestyles that we now possess can be attributed to the power of these methods.

Classical Genetic Methods

Mapping Mutations

Before the advent of recombinant DNA technology, it was extremely difficult for investigators to determine the locations of mutations in viral genomes. The **marker rescue** technique (described in "Introducing Mutations into the Viral Genome" below) was a solution to this problem, but before it was developed, other, less satisfactory approaches were exploited.

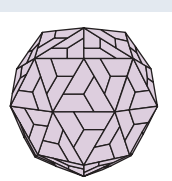
Recombination mapping can be applied to both DNA and RNA viruses. Recombination results in genetic exchange between genomes within the infected cell. The frequency of recombination between two mutations on a linear genome increases with the physical distance separating them. In practice, cells are coinfected with two mutants, and the frequency of recombination is calculated by dividing the titer of phenotypically wild-type virus (Box 3.6) obtained under restrictive conditions (e.g., high temperature) by the titer measured under permissive conditions (e.g., low temperature).

BOX 3.6

TERMINOLOGY

What is wild type?

Terminology can be confusing. Virologists often use terms such as "strains," "variants," and "mutants" to designate a virus that differs in some heritable way from a parental or wild-type virus. In conventional usage, the **wild type** is defined as the original (often laboratory-adapted) virus from which mutants are selected and which is used as the basis for comparison. A wild-type virus may **not** be identical to a virus isolated from nature. In fact, the genome of a wild-type virus may include numerous mutations accumulated during propagation in the laboratory. For example, the genome of the first isolate of poliovirus obtained in 1909 undoubtedly is very different from that of the virus we call wild type today. We distinguish carefully between laboratory wild types and new virus isolates from the natural host. The latter are called **field isolates** or **clinical isolates**.



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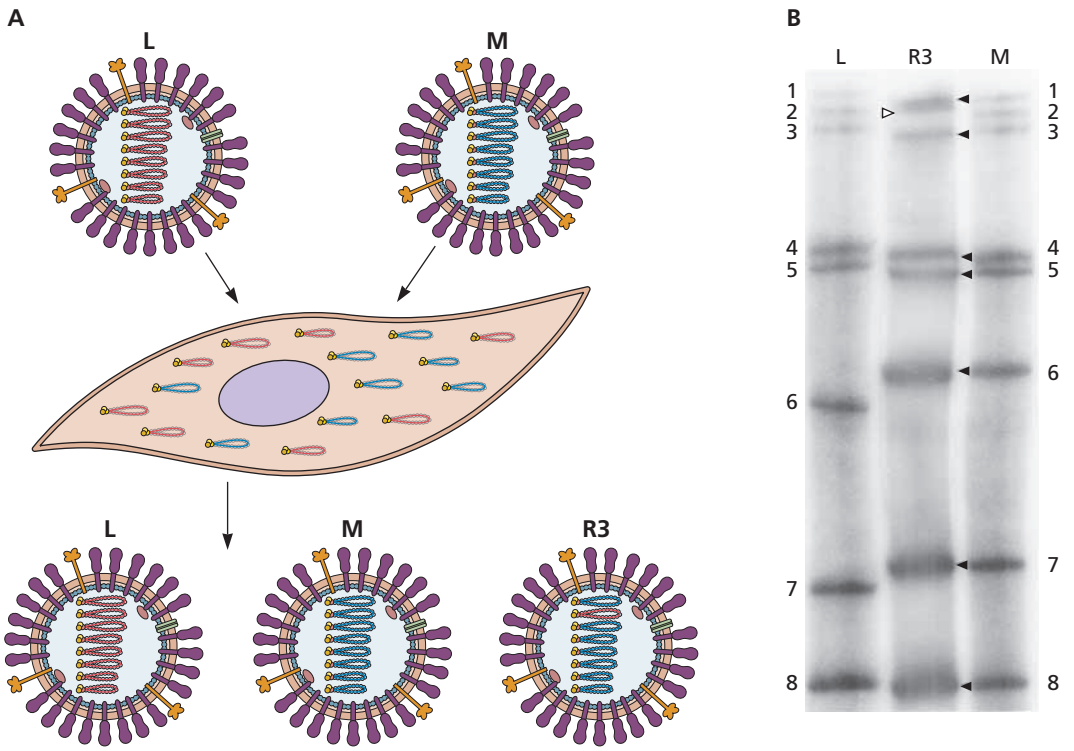


Figure 3.11 Reassortment of influenza virus RNA segments. (A) Progeny viruses of cells that are coinfected with two influenza virus strains, L and M, include both parents and viruses that derive RNA segments from them. Recombinant R3 has inherited segment 2 from the L strain and the remaining seven segments from the M strain. **(B)** ^{32}P-labeled influenza virus RNAs were fractionated in a polyacrylamide gel and detected by autoradiography. Migration differences of parental viral RNAs (M and L) permitted identification of the origin of RNA segments in the progeny virus R3. Panel B reprinted from V. R. Racaniello and P. Palese, *J Virol* **29:**361–373, 1979.

The recombination frequency between pairs of mutants is determined, allowing the mutations to be placed on a contiguous map. Although a location can be assigned for each mutation relative to others, this approach does not result in a physical map of the actual location of the base change in the genome.

In the case of RNA viruses with segmented genomes, the technique of **reassortment** allows the assignment of mutations to specific genome segments. When cells are coinfected with both mutant and wild-type viruses, the progeny includes **reassortants** that inherit RNA segments from either parent. The origins of the RNA segments can be deduced from their migration patterns during gel electrophoresis (Fig. 3.11) or by nucleic acid hybridization. By analyzing a panel of such reassortants, the segment responsible for the phenotype can be identified.

Functional Analysis

Complementation describes the ability of gene products from two different mutant viruses to interact functionally in the same cell, permitting viral reproduction. It can be distinguished from recombination or reassortment by examining the progeny produced by coinfected cells. True complementation yields only the two parental mutants, while wild-type genomes result from recombination or reassortment. If the mutations being tested are in separate genes, each virus is able to supply a functional gene product, allowing both viruses to be reproduced. If the two viruses carry mutations in the same gene, no reproduction will occur. In this way, the members of collections of mutants obtained by chemical mutagenesis were initially organized into complementation groups defining separate viral functions. In principle, there can be as many complementation groups as genes.

Engineering Mutations into Viral Genomes

Infectious DNA Clones

Recombinant DNA techniques have made it possible to introduce any kind of mutation anywhere in the genome of most animal viruses, whether that genome comprises DNA or RNA. The quintessential tool in virology today is the **infectious DNA clone**, a dsDNA copy of the viral genome that is carried on a bacterial vector such as a plasmid. Infectious DNA clones, or *in vitro* transcripts derived from them, can be introduced into cultured cells by **transfection** (Box 3.7) to

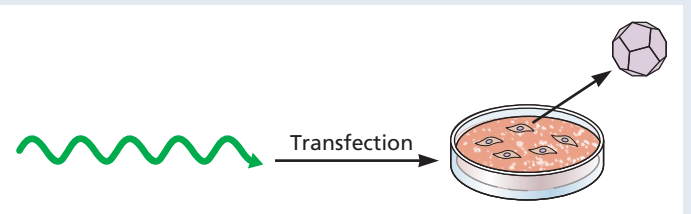
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BOX 3.7

TERMINOLOGY

DNA-mediated transformation and transfection

The introduction of foreign DNA into cells is called DNA-mediated transformation to distinguish it from the oncogenic transformation of cells caused by tumor viruses and other insults. The term "transfection" (transformation-infection) was coined to describe the production of infectious virus after transformation of cells by viral DNA, first demonstrated with bacteriophage lambda. Unfortunately, the term "transfection" is now routinely used to describe the introduction of any DNA or RNA into cells. In this textbook, we use the correct nomenclature: the term "transfection" is restricted to the introduction of viral DNA or RNA into cells with the goal of obtaining virus reproduction.



recover infectious virus. This approach is a modern validation of the Hershey-Chase experiment described in Chapter 1. The availability of site-specific bacterial restriction endonucleases, DNA ligases, and an array of methods for mutagenesis has made it possible to manipulate these infectious clones at will. Infectious DNA clones also provide a stable repository of the viral genome, a particularly important advantage for vaccine strains.

DNA viruses. Current genetic methods for the study of most viruses with DNA genomes are based on the infectivity of viral DNA. When deproteinized viral DNA molecules are introduced into permissive cells by transfection, they generally initiate a complete infectious cycle, although the infectivity (number of plaques per microgram of DNA) may be low. For example, the infectivity of deproteinized human adenoviral DNA is between 10 and 100 PFU per μg. When the genome is isolated by procedures that do not degrade the covalently attached terminal protein, infectivity is increased by 2 orders of magnitude, probably because this protein facilitates the assembly of initiation complexes on the viral origins of replication.

The complete genomes of polyomaviruses, papillomaviruses, and adenoviruses can be cloned in plasmid vectors, and such DNA is infectious under appropriate conditions. The DNA genomes of herpesviruses and poxviruses are too large to insert into conventional bacterial plasmid vectors, but they can be cloned in vectors that accept larger insertions (e.g., cosmids and bacterial artificial chromosomes). The plasmids containing such cloned herpesvirus genomes are infectious. In contrast, poxvirus DNA is not infectious, because the viral promoters cannot be recognized by cellular DNA-dependent RNA polymerase. Poxvirus DNA is infectious when early functions (viral DNA-dependent RNA polymerase and transcription proteins) are provided by a helper virus.

RNA viruses. *(+) strand RNA viruses.* The genomic RNA of retroviruses is copied into dsDNA by reverse transcriptase early during infection, a process described in Chapter 7. Such DNA is infectious when introduced into cells, as are molecularly cloned forms inserted into bacterial plasmids.

Introduction of a plasmid containing cloned poliovirus DNA into cultured mammalian cells results in the production of progeny virus (Fig. 3.12A). The mechanism by which cloned poliovirus DNA initiates infection is not known, but it has been suggested that the DNA enters the nucleus, where it is transcribed by cellular DNA-dependent RNA polymerase from cryptic, promoter-like sequences on the plasmid. The resulting (+) strand RNA transcripts initiate an infectious cycle. During genome replication, the extra terminal nucleotide sequences transcribed from the vector must be removed or ignored, because the virus particles that are produced contain RNA with the authentic 5′ and 3′ termini.

By incorporating promoters for bacteriophage T7 DNA-dependent RNA polymerase in plasmids containing poliovirus DNA, full-length (+) strand RNA transcripts can be synthesized *in vitro*. The specific infectivity of such RNA transcripts resembles that of genomic RNA (10^6 PFU per μg), which is higher than that of cloned DNA (10^3 PFU per μg). Infectious DNA clones have been constructed for many (+) strand RNA viruses.

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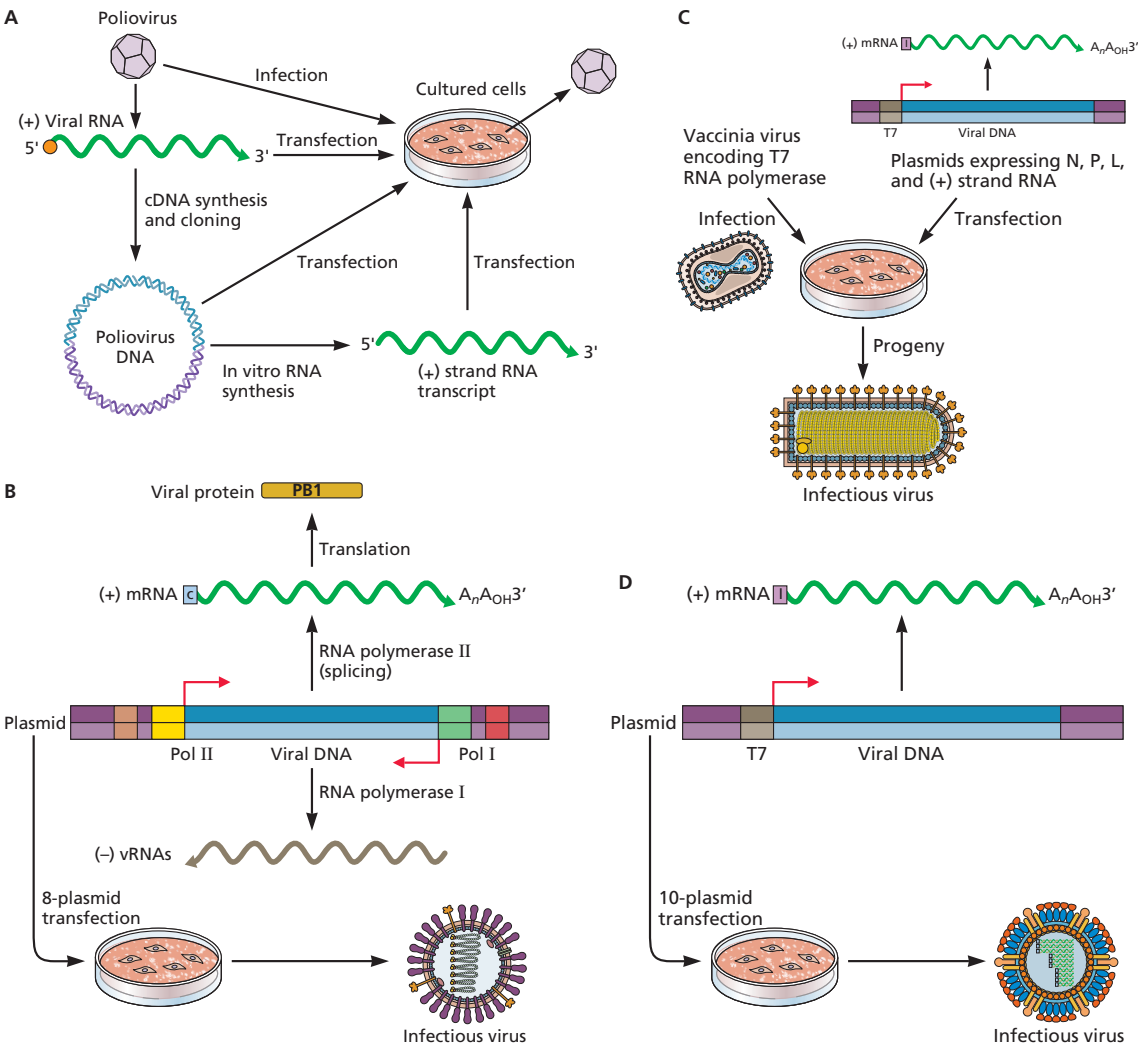


Figure 3.12 Genetic manipulation of RNA viruses. (A) Recovery of infectivity from cloned DNA of (+) strand RNA genomes as exemplified by genomic RNA of poliovirus, which is infectious when introduced into cultured cells by transfection. A complete DNA clone of the viral RNA (blue strands), carried in a plasmid, is also infectious, as are RNAs derived by *in vitro* transcription of the full-length DNA. **(B)** Recovery of influenza viruses by transfection of cells with eight plasmids. Cloned DNA of each of the eight influenza virus RNA segments is inserted between an RNA polymerase I promoter (Pol I, green) and terminator (brown), and an RNA polymerase II promoter (Pol II, yellow) and a polyadenylation signal (red). When the plasmids are introduced into mammalian cells, (−) strand viral RNA (vRNA) molecules are synthesized from the RNA polymerase I promoter, and mRNAs are produced by transcription from the RNA polymerase II promoter. The mRNAs are translated into viral proteins, and infectious virus is produced from the transfected cells. For clarity, only one cloned viral RNA segment is shown. Adapted from E. G. Hoffmann et al., *Proc Natl Acad Sci U S A* **97:**6108–6113, 2000, with permission. **(C)** Recovery of infectious virus from cloned DNA of viruses with a (−) strand RNA genome. Cells are infected with a vaccinia virus recombinant that synthesizes T7 RNA polymerase and transformed with plasmids that encode a full-length (+) strand copy of the viral genome RNA and proteins required for viral RNA synthesis (N, P, and L proteins). Production of RNA from these plasmids is under the control of the bacteriophage T7 RNA polymerase promoter (brown). Because bacteriophage T7 RNA transcripts are uncapped, an internal ribosome entry site (I) is included so the mRNAs will be translated. After the plasmids are transfected into cells, the (+) strand RNA is copied into (−) strands, which in turn are used as templates for mRNA synthesis and genome replication. The example shown is for viruses with a single (−) strand RNA genome (e.g., rhabdoviruses and paramyxoviruses). A similar approach has been demonstrated for Bunyamwera virus, with a genome comprising three (−) strand RNAs. **(D)** Recovery of infectious virus from cloned DNA of dsRNA viruses. Cloned DNA of each of the 10 reovirus dsRNA segments is inserted under the control of a bacteriophage T7 RNA polymerase promoter (brown). Because bacteriophage T7 RNA transcripts are uncapped, an internal ribosome entry site (I) is included so the mRNAs will be translated. Cells are infected with a vaccinia virus recombinant that synthesizes T7 RNA polymerase and transformed with all 10 plasmids. For clarity, only one cloned viral RNA segment is shown.

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(−) strand RNA viruses. Genomic RNA of (−) strand RNA viruses is not infectious, because it can be neither translated nor copied into (+) strand RNA by host cell RNA polymerases, as discussed in Chapter 6. Two different experimental approaches have been used to develop infectious DNA clones of these viral genomes (Fig. 3.12B and C).

The recovery of influenza virus from cloned DNA is achieved by an expression system in which cloned DNA copies of the eight RNA segments of the viral genome are inserted between two promoters, so that complementary RNA strands can be synthesized (Fig. 3.12B). When the eight plasmids carrying DNA for each viral RNA segment are introduced into cells, infectious influenza virus is produced.

The full-length (−) strand RNA of viruses with a nonsegmented genome, such as vesicular stomatitis virus (a rhabdovirus), is not infectious, because it cannot be translated into protein or copied into mRNA by the host cell. When the full-length (−) strand is introduced into cells containing plasmids that produce viral proteins required for production of mRNA, no infectious virus is recovered. Unexpectedly, when a full-length (+) strand RNA is transfected into cells that synthesize the vesicular stomatitis virus nucleocapsid protein, phosphoprotein, and polymerase, the (+) strand RNA is copied into (−) strand RNAs. These RNAs initiate an infectious cycle, leading to the production of new virus particles.

dsRNA viruses. Genomic RNA of dsRNA viruses is not infectious because the (+) strand cannot be translated. The recovery of reovirus from cloned DNA is achieved by an expression system in which cloned DNA copies of the 10 RNA segments of the viral genome are inserted under the control of an RNA polymerase promoter (Fig. 3.12D). When 10 plasmids carrying DNA for each viral dsRNA segment are introduced into cells, infectious reovirus is produced.

Types of Mutation

Recombinant DNA techniques allow the introduction of many kinds of mutation at any desired site in cloned DNA (Box 3.8). Indeed, provided that the sequence of the segment of the viral genome to be mutated is known, there is little restriction on the type of mutation that can be introduced. **Deletion mutations** can be used to remove an entire gene to assess its role in reproduction, to produce truncated gene products, or to assess the functions of specific segments of a coding sequence. Noncoding regions can be deleted to identify and characterize regulatory sequences such as promoters. **Insertion mutations** can be made by the addition of any desired sequences. **Substitution mutations**, which can correspond to one or more nucleotides, are often made in coding or noncoding regions. Included in the former class are **nonsense mutations**, in which a termination codon is introduced, and **missense mutations**, in which a single nucleotide or a codon is changed, resulting in the synthesis of a protein with a single amino acid substitution. The introduction of a termination codon is frequently exploited to cause truncation of a membrane protein so that it is secreted or to eliminate the synthesis of a protein without changing the size of the viral genome or mRNA. Substitutions are used to assess the roles of specific nucleotides in regulatory sequences or of amino acids in protein function, such as polymerase activity or binding of a viral protein to a cell receptor.

Introducing Mutations into the Viral Genome

Mutations can be introduced rapidly into a viral genome when it is cloned in its entirety. Mutagenesis is usually carried out on cloned subfragments, which are then substituted into full-length cloned DNA. The final step is introduction of the mutagenized DNA into cultured cells by transfection. This approach has been applied to cloned DNA copies of RNA and DNA viral genomes.

BOX 3.8

TERMINOLOGY

Operations on nucleic acids and on protein

A mutation is a change in DNA or RNA comprising base changes and nucleotide additions, deletions, and rearrangements. When mutations occur in open reading frames, they can be manifested as changes in the synthesized proteins. For example, one or more base changes in a specific codon may produce a single amino acid substitution, a truncated protein, or no protein. The terms "mutation" and "deletion" are often used incorrectly, or ambiguously to describe alterations in proteins. In this textbook, these terms are used to describe genetic changes, and the terms "amino acid substitution" and "truncation" are used to describe protein alterations.



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Introduction of mutagenized viral nucleic acid into cultured cells by transfection may produce one of several possible results. The mutation may have no effect on virus production, it may have a subtle effect, or it may impart a readily detectable phenotype.

Reversion Analysis

The phenotypes caused by mutation can **revert** in one of two ways: by change of the mutation to the wild-type sequence or by acquisition of a mutation at a second site, either in the same gene or a different gene. Phenotypic reversion caused by second-site mutation is known as **suppression**, or **pseudoreversion**, to distinguish it from reversion at the original site of mutation. Reversion has been studied since the beginnings of classical genetic analysis (Box 3.9). In the modern era of genetics, cloning and sequencing techniques can be used to demonstrate suppression and to identify the nature of the suppressor mutation (see below). The identification of suppressor mutations is a powerful tool for studying protein-protein and protein-nucleic acid interactions. Some suppressor mutations complement changes made at several sites, whereas **allele-specific** suppressors complement only a specific change. The allele specificity of second-site mutations provides evidence for physical interactions among proteins and nucleic acids.

Phenotypic revertants can be isolated either by propagating the mutant virus under restrictive conditions or, in the case of mutants exhibiting phenotypes (e.g., small plaques), by searching for wild-type properties. Chemical mutagenesis may be required to produce revertants of DNA viruses, but is not necessary for RNA viruses, which spawn mutants at a higher frequency. Nucleotide sequence analysis is then used to determine if the original mutation is still present in the genome of the revertant. The presence of the original mutation indicates that reversion has occurred by second-site mutation. Nucleotide sequence analysis is done to identify the suppressor mutation. The final step is introduction of the suspected suppressor mutation into the genome of the original mutant virus to confirm its effect. Several specific examples of suppressor analysis are provided below.

BOX 3.9

DISCUSSION

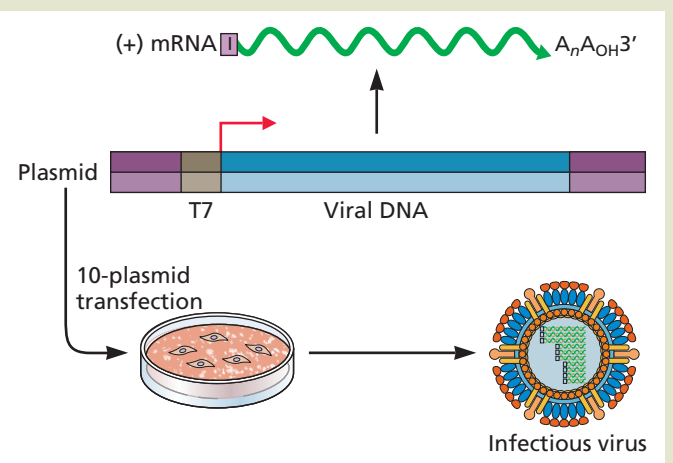
Is the observed phenotype due to the mutation?

In genetic analysis of viruses, mutations are made *in vitro* by a variety of techniques, all of which can introduce unexpected changes. Errors can be introduced during cloning, from PCR, during sequencing, and when the viral DNA or plasmid DNA is introduced into the eukaryotic cell.

With these potential problems in mind, how can it be concluded that a phenotype arises from the planned mutation? Here are some possible solutions.

- Repeat the construction. It is unlikely that an unlinked mutation with the same phenotype would occur twice.
- Look for marker rescue. Replace the mutation and all adjacent DNA with parental DNA. If the mutation indeed causes the phenotype, the wild-type phenotype should be restored in the rescued virus.
- Allow synthesis of the wild-type protein in the mutant background. If the wild-type phenotype is restored (complemented), then the probability is high that the phenotype arises from the mutation. The merit of this method over marker rescue is that the latter shows only that unlinked mutations are probably not the cause of the phenotype.

Each of these approaches has limitations, and it is therefore prudent to use more than one.



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Some mutations within the origin of replication (Ori) of simian virus 40 reduce viral DNA replication and induce the formation of small plaques. Pseudorevertants of Ori mutants were isolated by random mutagenesis of mutant viral DNA followed by their introduction into cultured cells and screening for viruses that form large plaques. The second-site mutations that suppressed the replication defects were localized to a specific region within the gene for large T antigen. These results indicated that a specific domain of large T antigen interacts with the Ori sequence during viral genome replication.

The 5′ untranslated region of the poliovirus genome contains elaborate RNA secondary-structural features, which are important for RNA replication and translation, as discussed in Chapters 6 and 11, respectively. Disruption of such features by substitution of an 8-nucleotide sequence produces a virus that replicates poorly and readily gives rise to pseudorevertants that reproduce more efficiently (Fig. 3.13). Nucleotide sequence analysis of the genomes of two pseudorevertants demonstrated that they contain base changes that restore the disrupted secondary structure. These results confirm that the RNA secondary structure is important for the biological activity of this untranslated region.

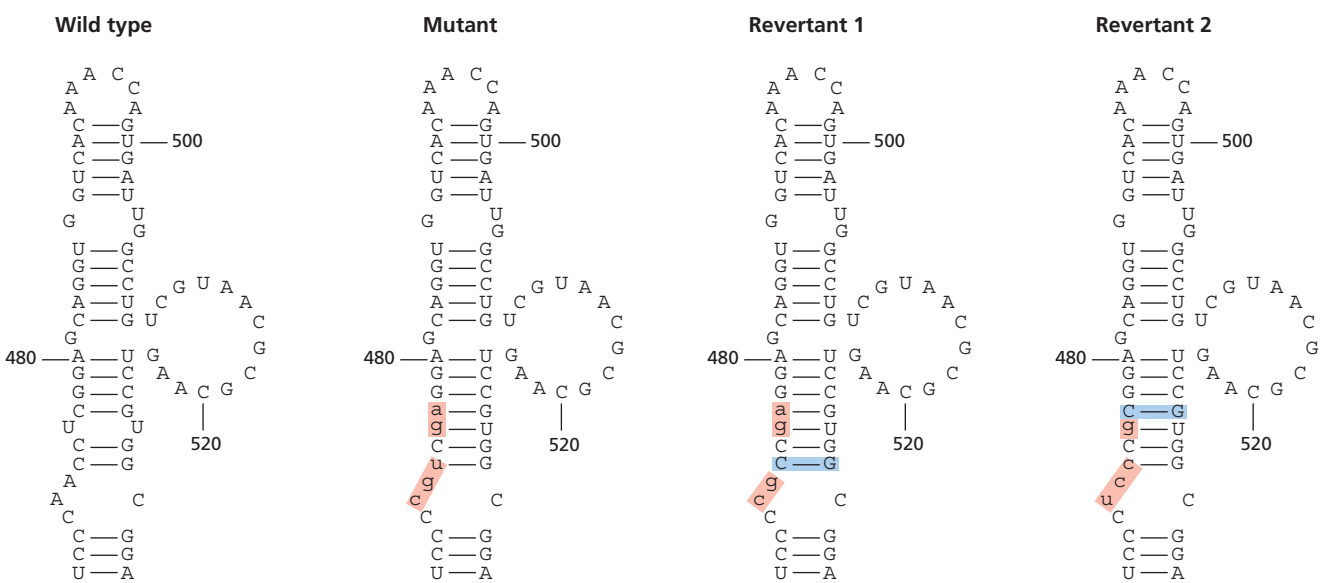
RNA interference (RNAi)

RNA interference (Chapter 10) has become a powerful and widely used tool for analyzing gene function. In such analyses, duplexes of 21-nucleotide RNA molecules, called **small interfering RNAs (siRNAs)**, which are complementary to small regions of the mRNA, are synthesized chemically or by transcription reactions. siRNAs or plasmids that encode them are then introduced into cultured cells by transformation, and these small molecules block the production of specific proteins by inducing sequence-specific mRNA degradation or inhibition of translation. The functions of specific viral or cellular proteins during infection can therefore be studied by using this procedure (Fig. 3.14). In another application of this technology, thousands of siRNAs directed at all cellular mRNAs can be introduced into cells to identify genes that stimulate or block viral reproduction.

Targeted Gene Editing with CRISPR-Cas9

While the experimental use of RNAi can lead to reduced protein production, genomic manipulation by CRISPR-Cas9 (clustered regularly interspersed short palindromic repeat-CRISPR-associated nuclease 9) has advantages of complete depletion of the protein, and fewer effects on unintended targets. Though endogenous to bacteria and archaea (Box 10.11), the CRISPR-Cas9 system can be effectively and efficiently utilized to generate targeted gene disruptions in any genome. The specificity depends on the ability of the single-stranded guide RNAs (sgRNAs) to hybridize to the correct DNA sequence within the chromosome.

Figure 3.13 Effect of second-site suppressor mutations on predicted secondary structure in the 5′ untranslated region of poliovirus (+) strand RNA. Diagrams of the region between nucleotides 468 and 534, which corresponds to stem-loop V (Chapter 11), are shown. These include, from left to right, sequences of wild-type poliovirus type 1, a mutant containing the nucleotide changes highlighted in orange, and two phenotypic revertants. Two CG base pairs present in the wild-type parent and destroyed by the mutation are restored by second-site reversion (blue shading). Adapted from A. A. Haller et al., *J Virol* **70:**1467–1474, 1996, with permission.



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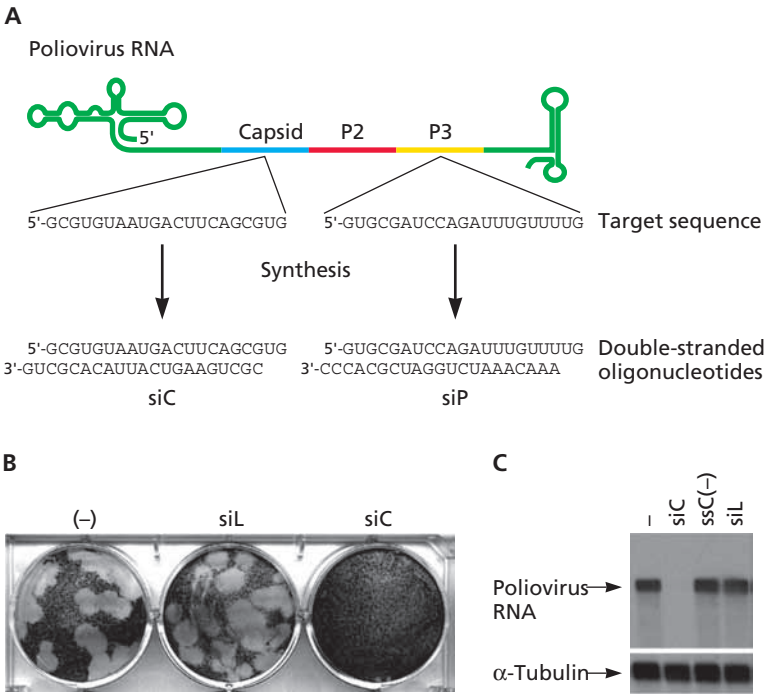


Figure 3.14 Inhibition of poliovirus replication by siRNA. siRNAs were introduced into cells by transformation, and the cells were then subjected to poliovirus infection. **(A)** Location of siRNAs siC and siP on a map of the poliovirus RNA genome. **(B)** Inhibition of plaque formation by siRNA siC. The number of plaques is not reduced in untreated cells (−) or when siRNA from *Renilla* luciferase is used (siL). Plaque formation was also inhibited with siP (not shown). **(C)** Northern blot analysis of RNA from poliovirus-infected cells 6 h after infection. Poliovirus RNA replication is blocked by siC but not by the (−) strand of siC RNA, ssC(−), or siL. The blot was rehybridized with a DNA probe directed against α-tubulin to ensure that all lanes contained equal amounts of RNA. Adapted from L. Gitlin et al., *Nature* **418:**430–434, 2002, with permission.

Once annealed, the endonuclease Cas9 catalyzes formation of a double-strand break, which is then repaired, creating frame-shifting insertion/deletion mutations within the gene. One advantage of using CRISPR-Cas9 methodology to genetically modify cell genomes is that the method can be applied to any cell type. Like siRNAs, CRISPR-Cas9 can be used to affect individual mRNAs or to identify cell genes that stimulate or block viral reproduction.

Engineering Viral Genomes: Viral Vectors

Naked DNA can be introduced into cultured animal cells as complexes with calcium phosphate or lipid-based reagents or directly by electroporation. Such DNA can direct synthesis of its gene products transiently or stably from integrated or episomal copies, respectively. Introduction of DNA into cells is a routine method in virological research and is also employed for certain clinical applications, such as the production of a therapeutic protein or a vaccine or the engineering of primary cells, progenitor cells, and stem cells for subsequent introduction into patients. However, this approach is not suitable for certain applications. For example, one goal of gene therapy is to deliver a gene to patients who either lack the gene or carry defective versions of it (Tables 3.1 and 3.2). The >7,000 monogenic human disorders, characterized by mutations in one gene, are especially amenable to viral gene therapy. In one application, DNA including the gene is introduced and expressed in cells recovered from the patient. After infusion into patients, the cells can become permanently established. If the primary cells to be used are limiting in a culture (e.g., stem cells), it is not practical to select and amplify the rare cells that receive naked DNA. Recombinant viruses carrying foreign genes can infect a greater percentage of cells and thus facilitate generation of the desired population. These viral vectors have also found widespread use in the research laboratory. A complete understanding of the structure and function of viral vectors requires knowledge of viral genome replication, a topic discussed in subsequent chapters for selected viruses and summarized in the Appendix.

Genetically engineered viruses are also being used to treat a wide variety of cancers, a field called viral oncotherapy (Box 3.10). Many tumor cells have defective innate immune signaling pathways and hence are susceptible to viral lysis. Viruses used for this purpose can be made more selective for tumor cells in a variety of ways. Another approach to viral oncotherapy is to utilize nonhuman viruses, such as the poxvirus myxoma virus and the picornavirus Seneca Valley virus, which can infect cells in human tumors but not normal tissues.

Design requirements for viral vectors include the use of an appropriate promoter; maintenance of genome size within the packaging limit of the particle; and elimination of viral virulence, the capacity of the virus to cause disease. Expression of foreign genes from viral vectors may be controlled by

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Table 3.1 Clinical uses for viral vectors: some diseases being targeted in clinical trials of gene therapy with viral vectors

Disease	Defect	Incidence	Viral vector
Severe combined immunodeficiency	Adenosine deaminase (25% of patients)	Rare, <1 in 10^5 live births	Gammaretrovirus
	Common cytokine receptor γ chain (X-linked)	1 in 50,000–100,000 live births	Self-inactivating gammaretrovirus
Lipoprotein lipase deficiency	Lipoprotein lipase	Rare, 1–2 in 10^6 live births	AAV[a,b]
Hemophilia B	Factor IX deficiency	1 in 30,000 males	AAV
Hemoglobinopathies and thalassemias	Defects in α- or β-globin gene	1 in 600 in specific ethnic groups	Self-inactivating lentivirus
α_1-Antitrypsin deficiency (inherited emphysema, liver disease)	α_1-Antitrypsin not produced	1 in 3,500	AAV
Retinal degenerative disease, Leber's congenital amaurosis (LCA)	Retinal pigment epithelium-specific 65-kDa protein	<10% of LCA cases (LCA, ~1 in 80,000 live births)	AAV
X-linked adrenoleukodystrophy	ABCD1 transporter	1 in 20,000–50,000 live births	Self-inactivating lentivirus
Wiskott-Aldrich syndrome (eczema-thrombocytopenia-immunodeficiency syndrome)	Was protein	1–10 in 10^6 males	Self-inactivating lentivirus

[a]AAV, adenovirus-associated virus.

[b]Lipoprotein lipase gene therapy is approved for clinical use in Europe.

homologous or heterologous promoters and enhancers chosen to support efficient (e.g., the human cytomegalovirus immediate-early transcriptional control region) or cell-type-specific transcription, depending on the goals of the experiment. Such genes can be built directly into the viral genome or introduced by recombination in cells, as described above (see "Introducing Mutations into the Viral Genome"). The viral vector genome generally carries deletions and sometimes additional mutations. Deletion of some viral sequences is often required to overcome the limitations on the size of viral genomes that can be packaged in virus particles. For example, adenoviral DNA molecules more than 105% of the normal length are packaged very poorly. As this limitation would allow only 1.8 kbp of exogenous DNA to be inserted, adenovirus vectors often include deletions of the E3 gene (which is not essential for reproduction in cells in culture) and of the E1A and E1B transcription units, which encode proteins that can be provided by complementing cell lines.

Table 3.2 Clinical uses for viral vectors: some oncolytic viruses tested in clinical trials

Virus	Modification(s)	Delivery	Outcomes
Human adenovirus type 5 (e.g., ONYX-015, H101)	Deletion of E1B gene (increases virus reproduction in, and lysis of, tumor cells)	Intratumoral inoculation of tumors of head and neck	Decreased tumor volume in some patients when combined with chemotherapy; H101 in clinical use in China
Herpes simplex virus 1 (e.g., talimogene laherparepvec, aka OncoVEX)	Deletions in viral genes to confer tumor selectivity (ICP34.5, US11) or allow antigen presentation (ICP47); addition of cellular GM-CSF gene to stimulate tumor-specific immune responses	Intratumoral inoculation of malignant gliomas	Complete remission in 8 of 50 patients; improved overall survival
Vaccinia virus (JX-594)	Disruption of viral gene for ribonucleotide reductase (tumor selectivity); addition of human GM-CSF gene to stimulate tumor-specific immune responses	Intratumoral inoculation into primary and metastatic liver tumors	Decreased tumor volume in ~30% of patients; dose-dependent increase in survival time
Parvovirus (ParvOryx)	None	Myeloma	Phase 1 recruitment
Measles virus	Edmonton vaccine strain of measles virus; cannot block Stat1 and Mda5; addition of human gene for sodium-iodide symporter	Myeloma	2 of 2 patients resolved bone marrow plasmacytosis; 1 in complete remission
Poliovirus	Sabin vaccine strain with IRES from rhinovirus	Glioma	Phase 1 recruiting
Vesicular stomatitis virus	Addition of human interferon β gene	Hepatocellular carcinoma	Phase 1 recruiting
Murine leukemia virus	Amphotropic env gene added; addition of cytosine deaminase	Glioma	Phase 1/2

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BOX 3.10

BACKGROUND

Viral oncotherapy

The use of viruses to treat cancer depends upon the ability of these agents to specifically infect and lyse cancer cells while not harming normal cells. These properties are made possible by a variety of tumor-specific abnormalities, including preferential production of certain proteins on the tumor cell surface that can serve as viral entry receptors; the enhanced activity of specific promoters and enhancers to drive expression of viral genes governing reproduction; the use of tumor-specific micro-RNAs to make viral gene expression cell specific; and the increased immunogenicity of tumor-specific antigens caused by the immune response to virus infection and the expression of immunostimulatory genes delivered by the vector.

Viruses from nine different families (*Adenoviridae*, *Picornaviridae*, *Herpesviridae*, *Paramyxoviridae*, *Parvoviridae*, *Reoviridae*, *Poxviridae*, *Retroviridae*, and *Rhabdoviridae*) are currently in clinical trials to test their safety and anticancer properties. The genomes of many viruses have been modified to confer greater efficacy and specificity for tumor cells. Oncolytic virotherapy has not been free of serious toxicities (see Volume II, Box 5.15), but in general, the treatments have been well tolerated after local or systemic injection.

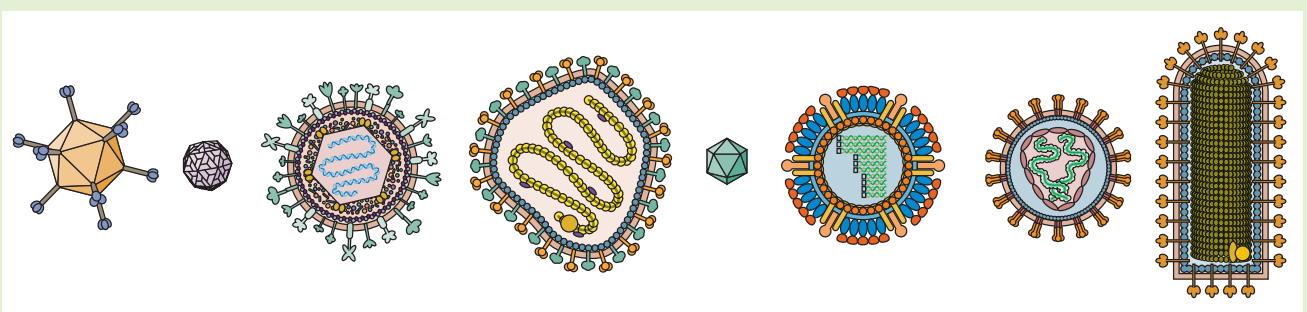
A challenge to the development of oncolytic viruses is the host antiviral immune response, which can blunt therapeutic efficacy. Several approaches have been used to address this problem, including the substitution of structural proteins from different human or animal serotypes and the production of novel serotypes by chemical modification of virus particles. Different serotypes can be used when the patient is immune to the original vector, due to either previous infection or treatment.

Viral structural proteins may also be modified to bind proteins that are specific to the target cells, conferring greater specificity for tumor lysis. Such targeting may also involve postentry steps. For example, many tumor genes are expressed at aberrantly high levels; the promoters and enhancers responsible for such high expression have been identified and used to drive synthesis of viral genes encoding proteins that mediate in cell killing. Another approach to conferring specificity for tumor cells is to insert in the viral genome targets of micro-RNAs that are produced in nontumor cells.

Enhanced killing of tumor cells has also been achieved by inserting a gene in the viral vector that makes the cell more susceptible to destruction by drugs or immune therapies. An example is the insertion of the herpes simplex virus thymidine kinase gene, which converts prodrugs such as ganciclovir to a nucleoside analog that halts DNA synthesis. Insertion of the human sodium-iodide symporter gene into measles virus allows tumor cells to concentrate lethal beta-emitting isotopes. Oncolytic viruses have also been produced that carry the gene encoding granulocyte-macrophage colony-stimulating factor (GM-CSF). The synthesis of this protein stimulates proliferation of the eponymous cells that turn the adaptive immune system against the tumor cells.

From the first use of a vaccine strain of rabies virus to treat melanomatosis in the 1950s, our progress in understanding the biology of cancer, combined with the ability to genetically modify viruses by manipulation of infectious DNA clones, has led to the development of many rationally designed oncolytic viruses with greater clinical safety and efficacy.

Miest TS, Cattaneo R. 2014. New viruses for cancer therapy: meeting clinical needs. *Nat Rev Microbiol* **12:**23–34.



When viral vectors are designed for therapeutic purposes, it is essential to prevent their reproduction as well as destruction of target host cells. The deletions necessary to accommodate a foreign gene may contribute to such disabling of the vector. For example, the E1A protein-coding sequences that are invariably deleted from adenovirus vectors are necessary for efficient transcription of viral early genes; in their absence, viral yields from cells in culture are reduced by about 3 to 6 orders of magnitude (depending on the cell type). Removal of E1A coding sequences from adenovirus vectors is therefore doubly beneficial, although it is not sufficient to ensure that the vector cannot reproduce or induce damage in a host animal. Adenovirus-associated virus vectors are not lytic, obviating the need for such manipulations. As discussed in detail in Volume II, Chapter 8, production of virus vectors that do not cause disease can be more difficult to achieve.

As of this writing, >1,800 approved gene therapy clinical

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Table 3.3 Some viral vectors

Virus	Insert size	Integration	Duration of expression	Advantages	Potential disadvantages
Adeno-associated virus	~5 kb	No	Long	Nonpathogenic, episomal, infects nondividing and dividing cells, broad tropism, low immunogenicity	Small packaging limit, helper virus needed for vector production
Adenovirus	~8–38 kb	No	Short	Efficient gene delivery, infects nondividing and dividing cells	Transient, immunogenic, high levels of preexisting immunity
Gammaretrovirus	8 kb	Yes	Short	Stable integration, broad tropism, low immunogenicity, low preexisting immunity	Risk of insertional mutagenesis, requires cell division
Herpes simplex virus	~50 kb	No	Long in central nervous system, short elsewhere	Infects nondividing cells, neurotropic, large capacity, broad tropism	Virulence, persistence in neurons, high levels of preexisting immunity, may recombine with genomes in latently infected cells
Lentivirus	9 kb	Yes	Long	Stable integration, transduces nondividing and dividing cells	Potential insertional mutagenesis; none detected in clinical trials
Rhabdovirus	~4.5 kb	No	Short	High-level expression, rapid cell killing, broad tropism, lack of preexisting immunity	Virulence, highly cytopathic, neurotropism, immunogenic
Vaccinia virus	~30 kb	No	Short	Wide host range, ease of isolation, large capacity, high-level expression, low preexisting immunity	Transient, immunogenic

trials have either been conducted or are in progress. These most often utilize adenovirus and retrovirus vectors, although poxvirus, adenovirus-associated virus, and herpes simplex virus vectors are also used. Cancer is the most common disease treated, followed by monogenetic and cardiovascular diseases.

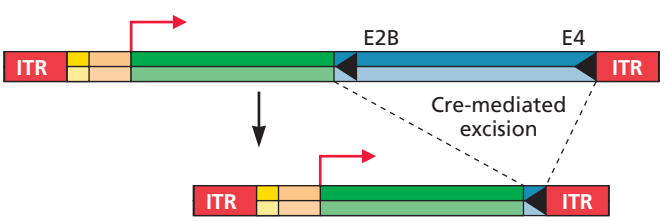
A summary of viral vectors is presented in Table 3.3, and examples are discussed below.

DNA Virus Vectors

One goal of gene therapy is to introduce genes into terminally differentiated cells. Such cells normally do not divide, and they cannot be propagated in culture. Moreover, the organs they comprise cannot be populated with virus-infected cells. DNA virus vectors have been developed to overcome some of these problems.

Adenovirus vectors were originally developed for the treatment of cystic fibrosis because of the tropism of the virus for the respiratory epithelium. Adenovirus can infect terminally differentiated cells, but only transient gene expression is achieved, as this viral DNA is not integrated into host cell DNA. Adenoviruses carrying the cystic fibrosis transmembrane conductance regulator gene, which is defective in patients with this disease, have been used in clinical trials. Many other gene products with therapeutic potential have been produced from adenovirus vectors in a wide variety of cell types. In the earliest vectors that were designed, foreign genes were inserted into the E1 and/or E3 regions. As these vectors had limited capacity, genomes with minimal adenovirus sequences have been designed (Fig. 3.15). This strategy allows up to 38 kb of foreign sequence to be introduced into the vector. In addition, elimination of most viral genes reduces the host immune response to viral proteins, simplifying multiple immunizations. Considerable efforts have been made to modify the adenovirus capsid to target the vectors to different cell types. The fiber protein, which mediates adenovirus binding to cells, has been altered by insertion of ligands that bind particular cell surface receptors. Such alter-

Figure 3.15 Adenovirus vectors. High-capacity adenovirus vectors are produced by inserting a foreign gene and promoter into the viral E1 region, which has been deleted. The E3 region also has been deleted. Two *loxP* sites for cleavage by the Cre recombinase have been introduced into the adenoviral genome (black arrowheads). Infection of cells that produce Cre leads to excision of sequences flanked by the *loxP* sites. The result is a "gutless" vector that contains only the origin-of-replication-containing inverted terminal repeats (ITR), the packaging signal (yellow), the viral E4 transcription unit (orange), and the transgene with its promoter (green). Additional DNA flanking the foreign gene must be inserted to allow packaging of the viral genome (not shown). Adapted from A. Pfeifer and I. M. Verma, *in* D. M. Knipe et al. (ed.), *Fields Virology*, 4th ed. (Lippincott Williams & Wilkins, Philadelphia, PA, 2001), with permission.



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BOX 3.11

EXPERIMENTS

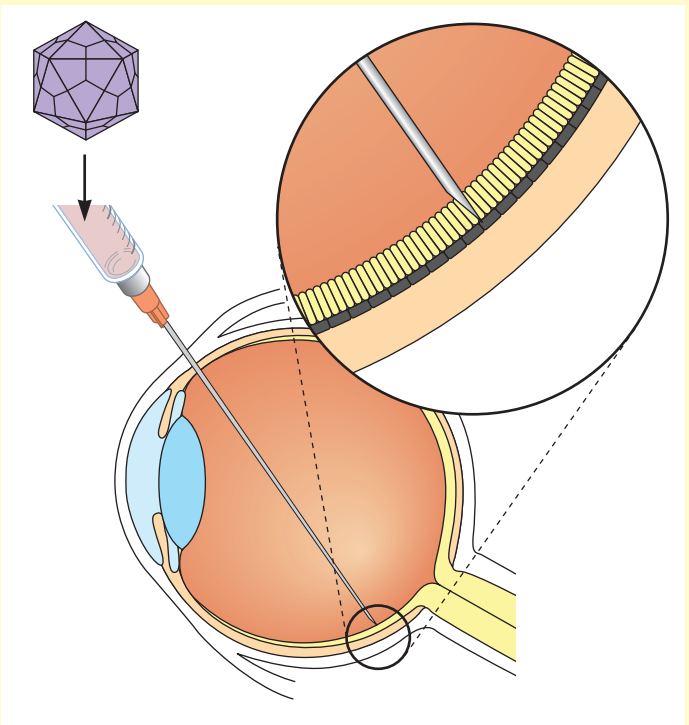
Restoring vision with viral gene therapy

Leber's congenital amaurosis is an autosomal recessive disease characterized by blindness as a consequence of retinal degeneration. Subretinal injection of adenovirus-associated virus vectors carrying the *RPE65* gene (figure) has restored patient vision is several clinical trials.

Mutations in the *RPE65* gene, which encodes a protein required for photoreceptor function in the retinal epithelium, account for ~10% of Leber's congenital amaurosis cases. Consequently, gene replacement has been studied as a therapeutic strategy for treatment of this disease. Human *RPE65* cDNA was packaged into an adenovirus-associated virus vector under the control of a chicken β-actin promoter. Infection of cells in culture with this virus, AAV2.hRPE65, leads to production of RPE65 protein. Introduction of this vector behind the retina of affected dogs led to sustained reversal of the visual deficit.

The safety and efficacy of AAV2.hRPE65 was assessed in three independent clinical trials. The results indicate that the vector is safe and in many cases leads to visual improvement for up to 1.5 years. These successful trials are likely to lead to licensure of this therapy to treat Leber's congenital amaurosis.

(See also the interview with Dr. Katherine High: http://bit.ly/Virology_High)



Pierce EA, Bennett J. 2015. The status of *RPE65* gene therapy trials: safety and efficacy. *Cold Spring Harb Perspect Med* doi:10.1101/cshperspect.a017285.

ations could increase the cell specificity of adenovirus attachment and the efficiency of gene transfer and thereby decrease the dose of virus that need be administered.

Adenovirus-associated virus has attracted much attention as a vector for gene therapy (Box 3.11). Genomes packaged into recombinant viruses replicate as an episome and persist, in some cases with high levels of expression, in many different tissues. There has been increasing interest in these vectors to target therapeutic genes to smooth muscle and other differentiated tissues, which are highly susceptible and support sustained high-level expression of foreign genes. Although the first-generation adenovirus-associated virus vectors were limited in the size of inserts that could be transferred, other systems have been developed to overcome the limited genetic capacity (Fig. 3.16). The cell specificity of adenovirus-associated virus vectors has been altered by inserting receptor-specific ligands into the capsid. In addition, many new viral serotypes that vary in their tropism and ability to trigger immune responses have been identified.

Vaccinia virus and other animal poxvirus vectors offer the advantages of a wide host range, a genome that accepts very large fragments, high expression of foreign genes, and relative ease of preparation. Foreign DNA is usually inserted into the viral genome by homologous recombination, using an approach similar to that described for marker transfer. Because of the relatively low pathogenicity of the virus, vaccinia virus recombinants have been considered candidates for human and animal vaccines.

RNA Virus Vectors

A number of RNA viruses have also been developed as vectors for foreign gene expression (Table 3.3). Vesicular stomatitis virus, a (−) strand RNA virus, has emerged as a

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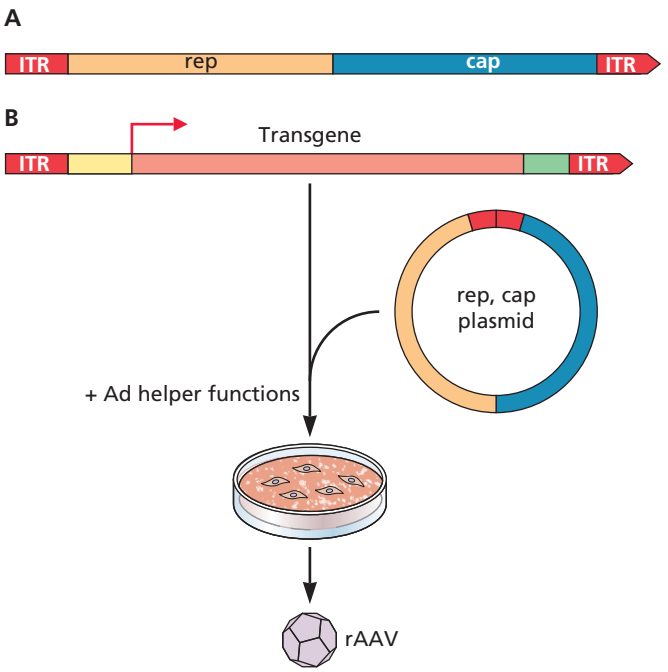


Figure 3.16 Adeno-associated virus vectors. (A) Map of the genome of wild-type adeno-associated virus. The viral DNA is single stranded and flanked by two inverted terminal repeats (ITR); it encodes capsid (blue) and nonstructural (orange) proteins. **(B)** In one type of vector, the viral genes are replaced with the transgene (pink) and its promoter (yellow) and a poly(A) addition signal (green). These DNAs are introduced into cells that have been engineered to produce capsid proteins, and the vector genome is encapsidated into virus particles. A limitation of this vector structure is that only 4.1 to 4.9 kb of foreign DNA can be packaged efficiently. Ad, adenovirus; rAAV, recombinant adenovirus-associated virus. Adapted from A. Pfeifer and I. M. Verma, *in* D. M. Knipe et al. (ed.), *Fields Virology*, 4th ed. (Lippincott Williams & Wilkins, Philadelphia, PA, 2001) with permission.

candidate for vaccine delivery (e.g., *Ebolavirus* vaccines) and for viral oncotherapy. The virus is well suited for the latter application because it reproduces preferentially in tumor cells, and recombinant vesicular stomatitis viruses have been engineered to improve tumor selectivity.

Retroviruses have enjoyed great popularity as vectors (Fig. 3.17) because their infectious cycles include the integration of a dsDNA copy of viral RNA into the cell genome, a topic of Chapter 7. The integrated provirus remains permanently in the cell's genome and is passed on to progeny during cell division. This feature of retroviral vectors results in permanent modification of the genome of the infected cell. The choice of the envelope glycoprotein carried by retroviral vectors has a significant impact on their tropism. The vesicular stomatitis virus G glycoprotein is often used because it confers a wide tissue tropism. Retrovirus vectors can be targeted to specific cell types by using other viral envelope proteins.

An initial problem encountered with the use of retroviruses in correcting genetic deficiencies is that only a few cell types can be infected by the commonly used murine retroviral vectors, and the DNA of these viruses can be integrated efficiently only in actively dividing cells. Often the cells that are targets of gene therapy, such as hepatocytes and muscle cells, do not divide. This problem can be circumvented if ways can be found to induce such cells to divide before being infected with the retrovirus. Another important limitation of the murine retrovirus vectors is the phenomenon of gene silencing, which represses foreign gene expression in many cells. An alternative approach is to use viral vectors that contain sequences from human immunodeficiency virus type 1 or other lentiviruses, which can infect nondividing cells and are less severely affected by gene silencing.

Perspectives

The information presented in this chapter can be used as a "road map" for navigating this book and for planning a virology course. Figures 3.1 to 3.7 serve as the points of departure for detailed analyses of the principles of virology. They illustrate seven strategies based on viral mRNA synthesis and genome replication. The material in this chapter can be used to structure individual reading or to design a virology course based on specific viruses or groups of viruses while adhering to the overall organization of this textbook by function. Refer to this chapter and the figures to find answers to questions about specific viruses. For example, Fig. 3.5 provides information about (+) strand RNA viruses and Fig. 3.10 indicates specific chapters in which these viruses are discussed.

Since the earliest days of experimental virology, genetic analysis has proven invaluable for studying the viral genome. Initially, methods were developed to produce viral mutants by chemical or UV mutagenesis, followed by screening for readily identifiable phenotypes. Because it was not possible to identify the genetic changes in such mutants, it was difficult to associate proteins with virus-specific processes. This limitation vanished with the development of infectious DNA clones of viral genomes, an achievement that enabled the introduction of defined mutations into any region of the viral genome. This complete genetic toolbox provides countless possibilities for studying the viral genome, limited only by the creativity and enthusiasm of the investigator. The ability to manipulate cloned DNA copies of viral genomes has also ushered in a new era of virus-based therapies. It may soon be possible to use viruses to treat genetic diseases and cancer and to deliver vaccines to prevent infectious diseases.

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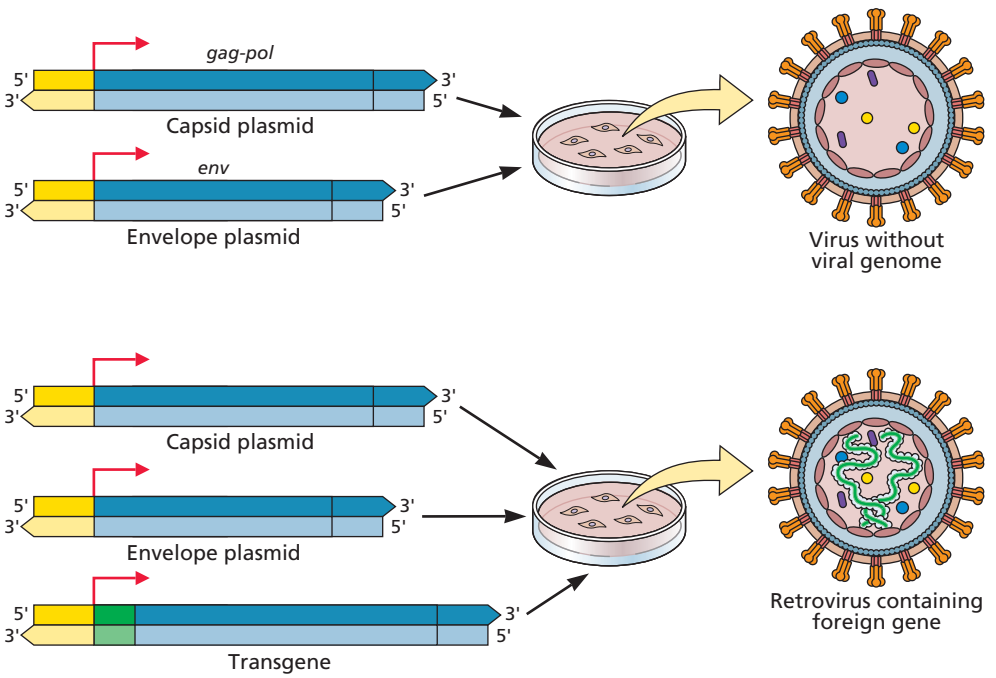


Figure 3.17 Retroviral vectors. The minimal viral sequences required for retroviral vectors are 5′- and 3′-terminal sequences (yellow and blue, respectively) that control gene expression and packaging of the RNA genome. The foreign gene (blue) and promoter (green) are inserted between the viral sequences. To package this DNA into viral particles, it is introduced into cultured cells with plasmids that encode viral proteins required for encapsidation, under the control of a heterologous promoter and containing no viral regulatory sequences. No wild-type viral RNA is present in these cells. If these plasmids alone are introduced into cells, virus particles that do not contain viral genomes are produced. When all three plasmids are introduced into cells, retrovirus particles that contain only the recombinant vector genome are formed. The host range of the recombinant vector can be controlled by the type of envelope protein. Envelope protein from amphotropic retroviruses allows the recombinant virus to infect human and mouse cells. The vesicular stomatitis virus glycoprotein G allows infection of a broad range of cell types in many species and also permits concentration with simple methods. Adapted from A. Pfeifer and I. M. Verma, *in* D. M. Knipe et al. (ed.), *Fields Virology*, 4th ed. (Lippincott Williams & Wilkins, Philadelphia, PA, 2001), with permission.

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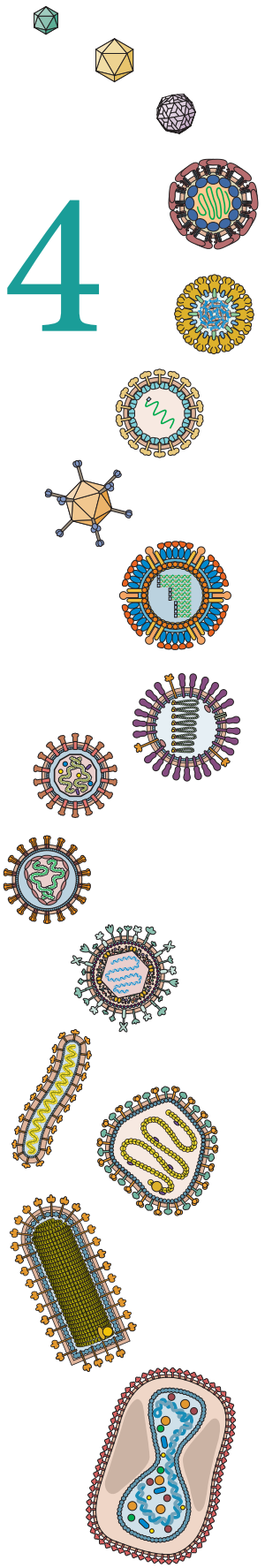
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4 Structure

Introduction
- Functions of the Virion
- Nomenclature
- Methods for Studying Virus Structure

Building a Protective Coat
- Helical Structures
- Capsids with Icosahedral Symmetry
- Other Capsid Architectures

Packaging the Nucleic Acid Genome
- Direct Contact of the Genome with a Protein Shell
- Packaging by Specialized Viral Proteins
- Packaging by Cellular Proteins

Viruses with Envelopes
- Viral Envelope Components
- Simple Enveloped Viruses: Direct Contact of External Proteins with the Capsid or Nucleocapsid
- Enveloped Viruses with an Additional Protein Layer

Large Viruses with Multiple Structural Elements
- Bacteriophage T4
- Herpesviruses
- Poxviruses
- Giant Viruses

Other Components of Virions
- Enzymes
- Other Viral Proteins
- Nongenomic Viral Nucleic Acid
- Cellular Macromolecules

Perspectives

References

LINKS FOR CHAPTER 4

- ⏭ *Video: Interview with Dr. Michael Rossmann*
  http://bit.ly/Virology_Rossmann
- ⏭ *Movie 4.1: Virus-based Piezoelectric Generator*
  http://bit.ly/Virology_V1_Movie4-1
- ⏭ *Movie 4.2: Cryo-EM reconstruction of the adenovirus type 5 capsid*
  http://bit.ly/Virology_V1_Movie4-2
- ⏭ *Sizing up adenovirus*
  http://bit.ly/Virology_Twiv101
- ⏭ *The Big Picture Book of Viruses*
  http://www.virology.net/Big_Virology/BVHomePage.html
- ⏭ *ViralZone*
  http://viralzone.expasy.org/
- ⏭ *Viruses in the extreme*
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- ⏭ *Virus particle explorer*
  http://viperdb.scripps.edu/

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In order to create something that functions properly—a container, a chair, a house—its essence has to be explored, for it should serve its purpose to perfection; i.e., it should fulfill its function practically and should be durable, inexpensive and beautiful.

WALTER GROPIUS
Neue Arbeiten der Bauhauswerkstätten, Bauhaus Book no. 7 1925

Introduction

Virus particles are elegant assemblies of viral, and occasionally cellular, macromolecules. They are marvelous examples of architecture on the molecular scale, with forms perfectly adapted to their functions. Virus particles come in many sizes and shapes (Fig. 4.1; also see Fig. 1.7) and vary enormously in the number and nature of the molecules from which they are built. Nevertheless, they fulfill common functions and are constructed according to general principles that apply to them all. These properties are described in subsequent sections, in which we also discuss some examples of the architectural detail characteristic of members of different virus families.

Functions of the Virion

Virus particles are designed for effective transmission of the nucleic acid genome from one host cell to another within a single organism or among host organisms (Table 4.1). A primary function of the **virion**, an infectious virus particle, is protection of the genome, which can be damaged irreversibly by a break in the nucleic acid or by mutation during passage through hostile environments. During its travels, a virus particle may encounter a variety of potentially lethal chemical and physical agents, including proteolytic and nucleolytic enzymes, extremes of pH or temperature, and various forms of natural radiation. In all virus particles, the nucleic acid is sequestered within a sturdy barrier formed by extensive interactions among the viral proteins that comprise the protein coat. Such protein-protein interactions maintain surprisingly stable capsids: many virus particles composed of only protein and nucleic acid survive exposure to large variations in the temperature, pH, or chemical composition of their environment. For example, when dried onto a solid surface, human rotavirus (a major cause of gastroenteritis) loses <20% of its infectivity in 30 days at room temperature, whereas the infectivity of poliovirus (a picornavirus) is reduced by some 5 orders of magnitude within 2 days. This same reduction in infectivity requires >250 days when poliovirus particles suspended in spring water are incubated at room temperature at neutral pH. Certain picornaviruses are even resistant to very strong detergents. The highly folded nature of coat proteins and their dense packing to form shells render them largely inaccessible to proteolytic enzymes. Some viruses also possess an **envelope**, typically derived from cellular membranes, into which viral glycoproteins have been inserted. The envelope adds not only a protective lipid membrane but also an external layer of protein and sugars formed by the glycoproteins. Like the cellular membranes from which they are derived, viral envelopes are impermeable to many molecules and block entry of chemicals or enzymes in aqueous solution.

To protect the nucleic acid genome, virus particles must be stable structures. However, they must also attach to an appropriate host cell and deliver the genome to the interior of that cell, where the particle is at least partially disassembled. The protective function of virus particles depends on stable intermolecular interactions among their components during assembly, egress from the virus-producing cell, and transmission. On the other hand, these interactions must be reversed readily during entry and uncoating in a new host cell. In only a few cases do we understand the molecular mechanisms by which these apparently paradoxical requirements are met. Nevertheless, it is clear that contact of a virion with the appropriate cell surface receptor or exposure to a specific intracellular environment can trigger substantial conformational changes. Virus particles are therefore **metastable structures** that have not yet attained the minimum free energy conformation. The latter state can be attained only once an unfavorable energy barrier has been surmounted, following induction of the irreversible conformational transitions that are associated

PRINCIPLES *Structure*

- Virus particles are designed for protection and delivery of the genome.
- Virus structure can be studied at an atomic level of resolution.
- Genetic economy dictates construction of capsids from a small number of subunits.
- Rod-like viruses are built with helical symmetry and spherical viruses are built with icosahedral symmetry.
- The primary determinant of capsid size is the number of subunits: the more subunits, the larger the capsid.
- There are multiple ways to achieve icosahedral symmetry, even among small viruses.
- While ordered RNA can be observed, how genomes are condensed and organized within virus particles is largely obscure.
- The elaborate capsids of larger viruses contain viral proteins dedicated to stabilizing the capsid shell. In some cases, viruses may have multiple shells.
- Some large viruses are built with structural elements recognizable from simpler viruses.
- Virus particles contain nonstructural components, including enzymes, small RNAs, and cellular macromolecules.

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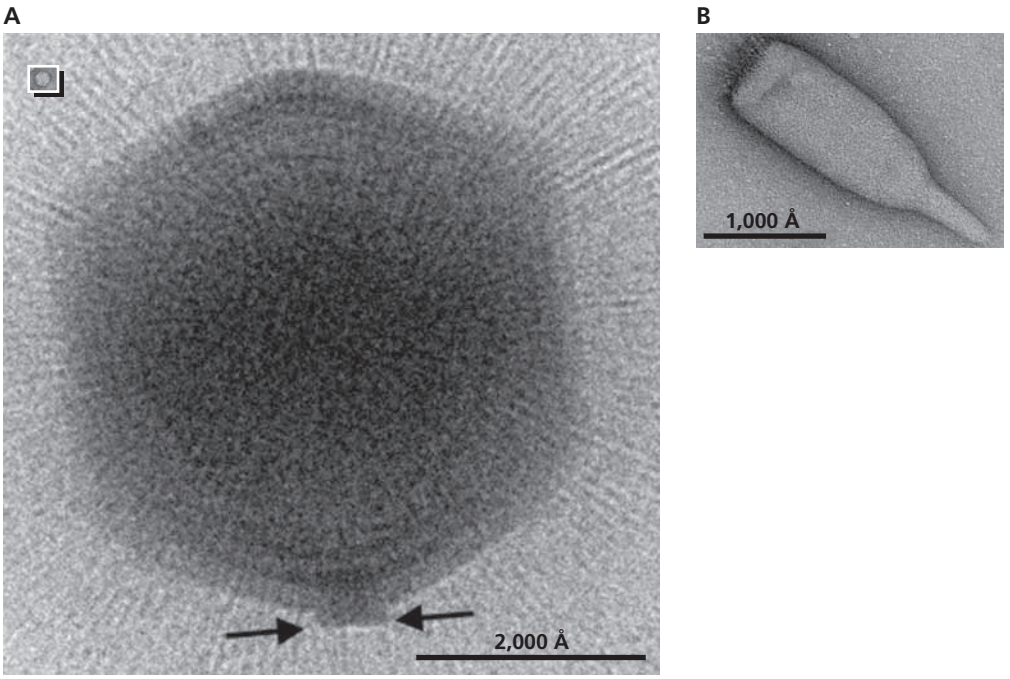


Figure 4.1 Variation in the size and shape of virus particles. (A) Cryo-electron micrographs of mimivirus and, in the inset (upper left), the parvovirus adeno-associated virus type 4, shown to scale relative to one another to illustrate the ~50-fold range in diameter among viruses that appear roughly spherical. Rod-shaped viruses also exhibit considerable variation in size, ranging in length from <200 nm to >2,000 nm. Adapted from C. Xiao et al., *J Mol Biol* **353:**493–496, 2005, and E. Pardon et al., *J Virol* **79:**5047–5058, 2005, respectively, with permission. Courtesy of M. G. Rossmann, Purdue University, and M. Agbandje-McKenna, University of Florida, Gainesville. **(B)** Non-symmetric shape of acidianus bottle virus isolated from a hot spring in Italy. The mimivirus particle (A) is also structurally complex: a large number of long, closely packed filaments project from its surface; and one vertex of the capsid carries a unique structure called the stargate, which opens in infected cells to release the viral genome. Adapted from M. Häring et al., *J Virol* **79:**9904–9911, 2005, with permission. Courtesy of D. Prangishvili, Institut Pasteur.

with attachment and entry. Virions are **not** simply inert structures. Rather, they are molecular machines (nanomachines) that play an active role in delivery of the nucleic acid genome to the appropriate host cell and initiation of the reproductive cycle.

Table 4.1 Functions of virion proteins

Protection of the genome
Assembly of a stable protective protein shell
Specific recognition and packaging of the nucleic acid genome
Interaction with host cell membranes to form the envelope
Delivery of the genome
Binding to external receptors of the host cell
Transmission of signals that induce uncoating of the genome
Induction of fusion with host cell membranes
Interaction with internal components of the infected cell to direct transport of the genome to the appropriate site
Other functions
Interactions with cellular components for transport to intracellular sites of assembly
Interactions with cellular components to ensure an efficient infectious cycle

As might be anticipated, elucidation of the structures of virus particles and individual structural proteins has illuminated the mechanisms of both assembly of viral nanomachines in the final stages of an infectious cycle and their entry into a new host cell. High-resolution structural information can also facilitate identification of targets for antiviral drugs, as well as the design of such drugs (Volume II, Chapter 9), and provide insights into the dynamic interplay between important viral pathogens and host adaptive immune responses (Volume II, Chapter 4). As we shall see, cataloguing of virus architecture has also revealed completely unanticipated relationships among viruses of different families that infect evolutionarily divergent hosts and has suggested new principles of virus classification.

Nomenclature

Virus architecture is described in terms of **structural units** of increasing complexity, from the smallest biochemical unit (the polypeptide chain) to the infectious particle (or virion). These terms, which are used throughout this text, are defined in Table 4.2. Although virus particles are complex assemblies of macromolecules exquisitely suited for protection and

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Table 4.2 Nomenclature used in description of virus structure

Term	Synonym	Definition
Subunit (protein subunit)		Single, folded polypeptide chain
Structural unit	Asymmetric unit	Unit from which capsids or nucleocapsids are built; may comprise one protein subunit or multiple, different protein subunits
Capsid	Coat	The protein shell surrounding the nucleic acid genome
Nucleocapsid	Core	The nucleic acid-protein assembly packaged within the virion; used when this assembly is a discrete substructure of a particle
Envelope	Viral membrane	The host cell-derived lipid bilayer carrying viral glycoproteins
Virion		The infectious virus particle

delivery of viral genomes, they are constructed according to the general principles of biochemistry and protein structure.

Methods for Studying Virus Structure

Electron microscopy is the most widely used method for the examination of structure and morphology of virus particles. This technique, which has been applied to viruses since the 1940s, traditionally relied on staining of purified virus particles (or of sections of infected cells) with an electron-dense material. It can yield quite detailed and often beautiful images (Fig. 1.7; see the Appendix) and provided the first rational basis for the classification of viruses.

The greatest contrast between virus particle and stain (negative contrast) occurs where portions of the folded protein chain protrude from the surface. Consequently, surface knobs or projections, termed morphological units, are the main features identified by this method of electron microscopy. However, these structures are often formed by multiple proteins and so their organization does not necessarily correspond to that of the individual proteins that make up the capsid shell. Even when structure is well preserved and a high degree of contrast can be achieved, the minimal size of an object that can be distinguished by classical electron microscopy, its **resolution**, is limited to 50 to 75 Å. This resolution is far too poor to permit molecular interpretation: for example, the diameter of an α-helix in a protein is on the order of 10 Å. Cryo-electron microscopy (cryo-EM), in which samples are rapidly frozen and examined at very low temperatures in a hydrated, vitrified (noncrystalline, glass-like) state, preserves native structure. Because samples are not stained, this technique allows direct visualization of the contrast inherent in the virus particle. When combined with computerized mathematical methods of image analysis of single particles and three-dimensional reconstruction (Fig. 4.2), cryo-EM can increase resolution to the atomic level. As described in subsequent sections, the continual improvements in this method have provided unprecedented views of virus particles not amenable to other methods of structural analysis. Indeed, structures of even quite large and structurally sophisticated viruses like human adenovirus can now be determined at a resolution directly comparable to that achieved by X-ray crystallography (Box 4.1).

The inherent symmetry of most virus particles facilitates analysis of images obtained by cryo-EM for reconstruction of three-dimensional structure. This approach can be complemented by cryo-electron tomography, in which two-dimensional images are recorded as the vitrified sample is tilted at different angles to the electron beam and subsequently combined into a three-dimensional density map (Fig. 4.2). Within the past decade, cryo-electron microscopy and tomography have become standard tools of structural biology. Their application to virus particles has provided a wealth of previously inaccessible information about the external and internal structures of multiple members of at least 20 virus families.

The first descriptions of the molecular interactions that dictate the structure of virus particles were obtained by X-ray crystallography (Fig. 4.3) (see the interview with Dr. Michael Rossmann: http://bit.ly/Virology_Rossmann). A plant virus (tobacco mosaic virus) was the first to be crystallized, and the first high-resolution virus structure determined was that of tomato bushy stunt virus. Since this feat was accomplished in 1978, high-resolution structures of increasingly larger animal viruses have been determined, placing our understanding of the principles of capsid architecture on a firm foundation.

Not all viruses can be examined directly by X-ray crystallography: some do not form suitable crystals, and the larger viruses lie beyond the power of the current procedures by which X-ray diffraction spots are converted into a structural model. However, their architectures can be determined by using a combination of structural methods. Individual viral proteins can be examined by X-ray crystallography and by multidimensional nuclear magnetic resonance techniques. The latter methods, which allow structural models to be constructed from knowledge of the distances between specific atoms in a polypeptide chain, can be applied to proteins in solution, a significant advantage.

High-resolution structures of individual proteins have been important in deciphering mechanisms of attachment

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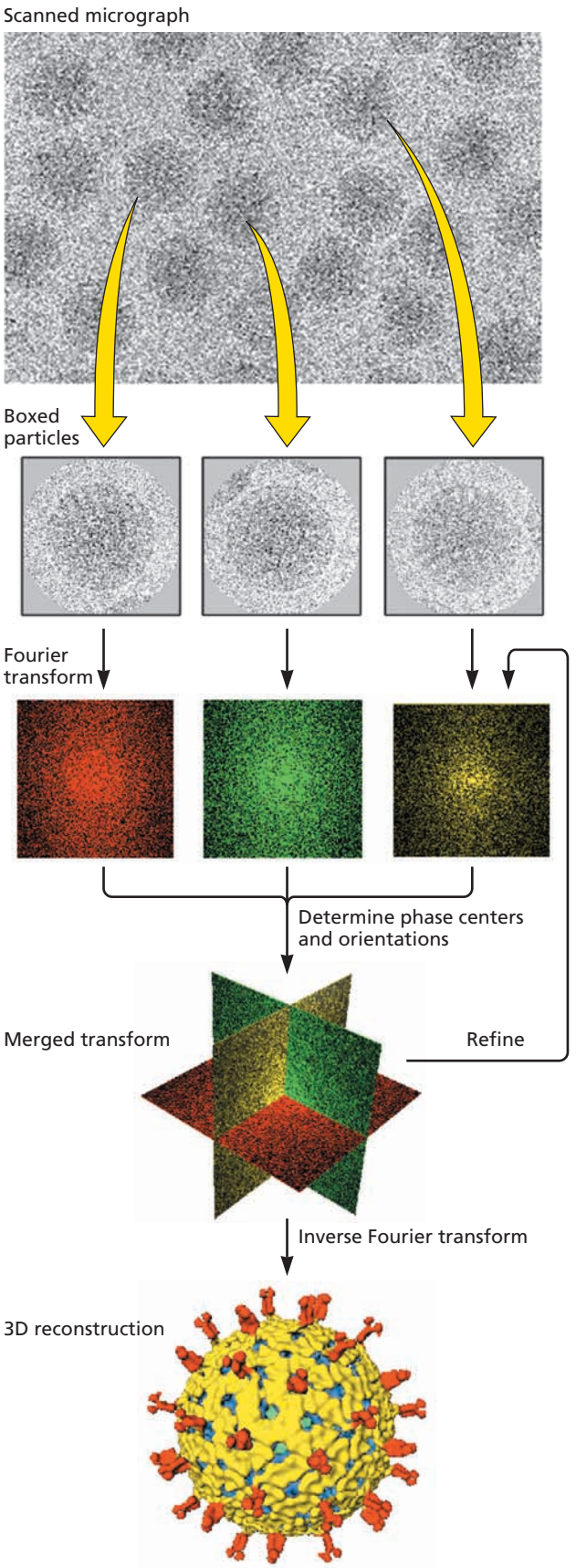


Figure 4.2 Cryo-EM and image reconstruction illustrated with images of rotavirus.

Concentrated preparations of purified virus particles are prepared for cryo-electron microscopy by rapid freezing on an electron microscope grid so that a glasslike, noncrystalline water layer is produced. This procedure avoids sample damage that can be caused by crystallization of the water or by chemical modification or dehydration during conventional negative-contrast electron microscopy. The sample is maintained at or below -160°C during all subsequent operations. Fields containing sufficient numbers of vitrified virus particles are identified by transmission electron microscopy at low magnification (to minimize sample damage from the electron beam) and photographed at high resolution (top).

These electron micrographs can be treated as two-dimensional projections (Fourier transforms) of the particles. Three-dimensional structures can be reconstructed from such two-dimensional projections by mathematically combining the information given by different views of the particles. For the purpose of reconstruction, the images of different particles are treated as different views of the same structure.

For reconstruction, micrographs are digitized for computer processing. Each particle to be analyzed is then centered inside a box, and its orientation is determined by application of programs that orient the particle on the basis of its icosahedral symmetry. In cryo-electron tomography, a series of images is collected with the sample at different angles to the electron beam and combined computationally to reconstruct a three-dimensional structure. The advantage of this approach is that no assumptions about the symmetry of the structure are required. The parameters that define the orientation of the particle must be determined with a high degree of accuracy, for example, to within 1° for even a low-resolution reconstruction (~40 Å). These parameters are improved in accuracy (**refined**) by comparison of different views (particles) to identify common data.

Once the orientations of a number of particles sufficient to represent all parts of the asymmetric unit have been determined, a low-resolution three-dimensional reconstruction is calculated from the initial set of two-dimensional projections by using computerized algorithms.

This reconstruction is refined by including data from additional views (particles). The number of views required depends on the size of the particle and the resolution sought. The reconstruction is initially interpreted in terms of the external features of the virus particle. Various computational and computer graphics procedures have been developed to facilitate interpretation of internal features. Courtesy of B. V. V. Prasad, Baylor College of Medicine.

And is it not true that even the small step of a glimpse through the microscope reveals to us images that we should deem fantastic and over-imaginative if we were to see them somewhere accidentally, and lacked the sense to understand them.

Paul Klee, *On Modern Art*, translated by Paul Findlay (London, United Kingdom, 1948)

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BOX 4.1

METHODS

Structures of human adenovirus: technical tours de force

The nonenveloped adenovirus particle is quite large, ~900 Å in diameter excluding the fibers that project from each vertex, and built from multiple proteins. Structural models of this particle were obtained initially by combining the high-resolution structures of individual viral proteins with lower-resolution images obtained by cryo-EM (see Fig. 4.4). In 2010, two papers published in the same issue of *Science* described atomic-level-resolution structures of adenovirus.

A 3.5-Å-resolution structure was obtained by X-ray crystallography of a derivative of human adenovirus type 5, one of the largest and most complicated to be determined by this method. Cryo-EM and single-particle analysis of unmodified adenovirus type 5 generated a structure of comparable resolution, 3.6 Å. This reconstruction represented the most complex structure in which polypeptide chains could be traced directly (see figure). Both models revealed protein-protein interactions important for stabilizing the capsid. However, the cryo-EM-derived structure also included segments of the major capsid protein not evident in X-ray crystal structures. Furthermore, the cryo-EM density map was judged to be clearer than that obtained by X-ray crystallography.

By allowing a direct comparison, these studies showed that current methods of cryo-EM and single-particle analysis can be as powerful as X-ray crystallography, at least when applied to highly symmetric structures like the particles of icosahedral viruses.

Harrison S. 2010. Looking inside adenovirus. *Science* **329:**1026–1027.

Liu H, Jin L, Koh SB, Atanasov I, Schein S, Wu L, Zhou ZH. 2010. Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. *Science* **329:**1038–1043.

Reddy VS, Natchiar SK, Stewart PL, Nemerow GR. 2010. Crystal structure of human adenovirus at 3.5 Å resolution. *Science* **329:**1071–1075.

The atomic model (sticks) of an α-helix present in the adenovirus type 5 major capsid protein (hexon), with amino acid side chains in red and the polypeptide backbone in blue, is shown superimposed on its cryo-EM electron density map (gray mesh). The identities of some of the side chains are labeled. Adapted from H. Liu et al., *Science* **329:**1038–1043, 2010, with permission. Courtesy of Z. H. Zhou, University of California, Los Angeles.

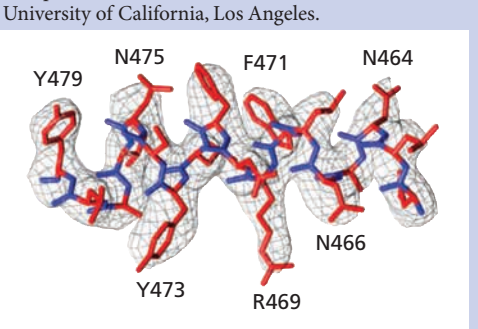
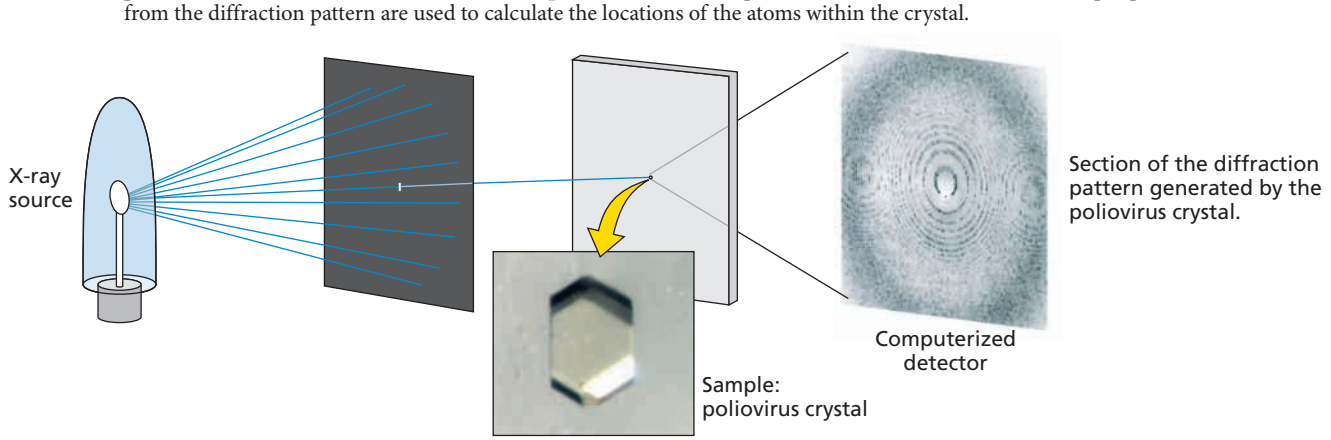


Figure 4.3 Determination of virus structure by X-ray diffraction. A virus crystal is composed of virus particles arranged in a well-ordered three-dimensional lattice. When the crystal is bombarded with a monochromatic X-ray beam traveling through the pinhole, each atom within the virus particle scatters the radiation. Interactions of the scattered rays with one another form a diffraction pattern that is recorded. Each spot contains information about the position and the identity of the atoms in the crystal. The locations and intensities of the spots are stored electronically. Determination of the three-dimensional structure of the virus from the diffraction pattern requires information that is lost in the X-ray diffraction experiment. This missing information (the phases of the diffracted rays) can be retrieved by collecting the diffraction information from otherwise identical (isomorphous) crystals in which the phases have been systematically perturbed by the introduction of heavy metal atoms at known positions. Comparison of the two diffraction patterns yields the phases. This process is called **multiple isomorphous replacement**. Alternatively, if the structure of a related molecule is known, the diffraction pattern collected from the crystal can be interpreted by using the phases from the known structure as a starting point and subsequently using computer algorithms to calculate the actual values of the phases. This method is known as **molecular replacement**. Once the phases are known, the intensities and spot positions from the diffraction pattern are used to calculate the locations of the atoms within the crystal.



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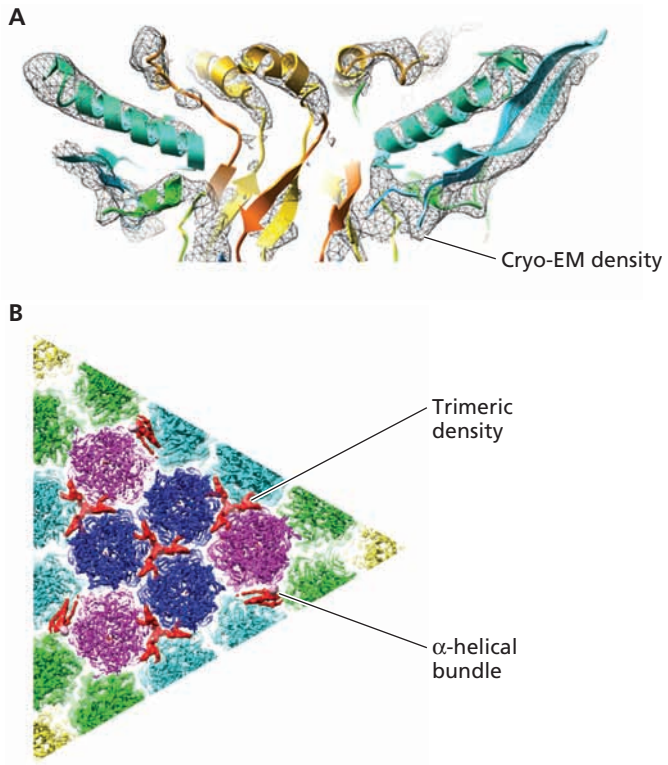


Figure 4.4 Difference mapping illustrated by a 6-Å-resolution reconstruction of adenovirus. (A) Comparison of α-helices of the penton base in the cryo-electron microscopic (cryo-EM) density and the crystal structure of this protein bound to a fiber peptide (ribbon). The excellent agreement established that α-helices could be reliably discerned in the 6-Å cryo-EM reconstruction. **(B)** Portion of the cryo-EM difference map corresponding to the surface of one icosahedral face of the capsid. The crystal structures of the penton base (yellow) and the hexons (green, cyan, blue, and magenta at different positions) at appropriate resolution were docked within the cryo-EM density at 6-Å resolution. The cryo-EM density that does not correspond to these structural units (the difference map) is shown in red. At this resolution, the difference map revealed four trimeric structures located between neighboring hexons and three bundles of coiled-coiled α-helices. The former were previously assigned to protein IX. Adapted from S. D. Saban et al., *J Virol* **80:**12049–12059, 2006, with permission. Courtesy of Phoebe Stewart, Vanderbilt University Medical Center.

and entry of enveloped viruses. Even more valuable are methods in which high-resolution structures of individual viral proteins are combined with cryo-EM reconstructions of intact virus particles. For example, in difference imaging, the structures of individual proteins are in essence subtracted from the reconstruction of the particle to yield new structural insight (Fig. 4.4). This powerful approach has provided fascinating views of interactions of viral envelope proteins embedded in lipid bilayers and even of internal surfaces and components of virus particles.

Atomic-resolution structures of individual proteins or domains can also be modeled into lower-resolution views (currently ~15 Å) obtained by small-angle X-ray scattering. This technique, which is applied to proteins in solution, provides information about the overall size and shape of flexible, asymmetric proteins and has provided valuable information about viral proteins with multiple functional domains (see, e.g., Chapter 7).

Building a Protective Coat

Regardless of their structural complexity, all virions contain at least one protein coat, the **capsid** or nucleocapsid, which encases and protects the nucleic acid genome (Table 4.2). As first pointed out by Francis Crick and James Watson in 1956, most virus particles appear to be rod shaped or spherical under the electron microscope. Because the coding capacities of viral genomes are limited, these authors proposed that construction of capsids from a small number of subunits would minimize the genetic cost of encoding structural proteins. Such genetic economy dictates that capsids be built from identical copies of a small number of viral proteins with structural properties that permit regular and repetitive interactions among them. These protein molecules are arranged to provide maximal contact and noncovalent bonding among subunits and structural units. The repetition of such interactions among a limited number of proteins results in a regular structure, with symmetry that is determined by the spatial patterns of the interactions. In fact, the protein coats of many viruses **do** display **helical** or **icosahedral symmetry**. Such well-defined symmetry has considerable practical value (Box 4.2).

Helical Structures

The **nucleocapsids** of some enveloped animal viruses, as well as certain plant viruses and bacteriophages, are rod-like or filamentous structures with helical symmetry. Helical symmetry is described by the number of structural units per turn of the helix, μ, the axial rise per unit, ρ, and the pitch of the helix, *P* (Fig. 4.5A). A characteristic feature of a helical structure is that any volume can be enclosed simply by varying the length of the helix. Such a structure is said to be **open**. In contrast, capsids with icosahedral symmetry (described below) are **closed** structures with fixed internal volume.

From a structural point of view, the best-understood helical nucleocapsid is that of tobacco mosaic virus, the very first virus to be identified. The virus particle comprises a single molecule of (+) strand RNA, about 6.4 kb in length, enclosed within a helical protein coat (Fig. 4.5B; see also Fig. 1.7). The coat is built from a single protein that folds into an extended structure shaped like a Dutch clog. Repetitive interactions among coat protein subunits form disks that have been

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BOX 4.2

METHODS

Nanoconstruction with virus particles

Nanochemistry is the synthesis and study of well-defined structures with dimensions of 1 to 100 nm. Nano-building blocks span the size range between molecules and materials such as nylon. Molecular biologists study nanochemistry, nanostructures, and molecular machines including the ribosome and membrane-bound signaling complexes. Icosahedral viruses are proving to be precision building blocks for nanochemistry. The icosahedral cowpea mosaic virus particle is 30 nm in diameter, and its atomic structure is known in detail. Grams of particles can be prepared easily from kilograms of infected leaves, insertional mutagenesis is straightforward, and precise amino acid changes can be introduced. As illustrated in panel A of the figure, cysteine residues inserted in the capsid protein provide functional groups for chemical attachment of 60 precisely placed molecules, in this case, gold particles.

High local concentrations of attached chemical agents, coupled with precise placement, and the propensity of virus-like particles for self-organization into two- and three-dimensional lattices of well-ordered arrays of particles enable rather remarkable nanoconstruction. For example, the surface of the filamentous bacteriophage M13 can be patterned to carry separate binding sites for gold and cobalt oxide and assembled into nanowires to form the anodes of small lithium ion batteries. Remarkably, this bacteriophage also displays intrinsic piezoelectric properties, that is, the ability to generate an electric charge in response to mechanical deformation, and vice versa. The basis of this property is not fully understood, but modification of the sequence of the major protein to increase its dipole moment (figure, panel B) augmented the piezoelectric strength of the bacteriophage. Assembly of the modified M13 into thin films was exploited to build a piezoelectric generator that produced up to 6 mA of current and 400 mV of potential, sufficient to operate a liquid crystal display (see Movie 4.1: http://bit.ly/Virology_V1_Movie4-1, Box 4.2).

Virus particles also have considerable potential for the delivery of drugs and other medically relevant molecules.

Viruses are not just for infections anymore! They will provide a rich source of building blocks for applications spanning the worlds of molecular biology, materials science, and medicine.

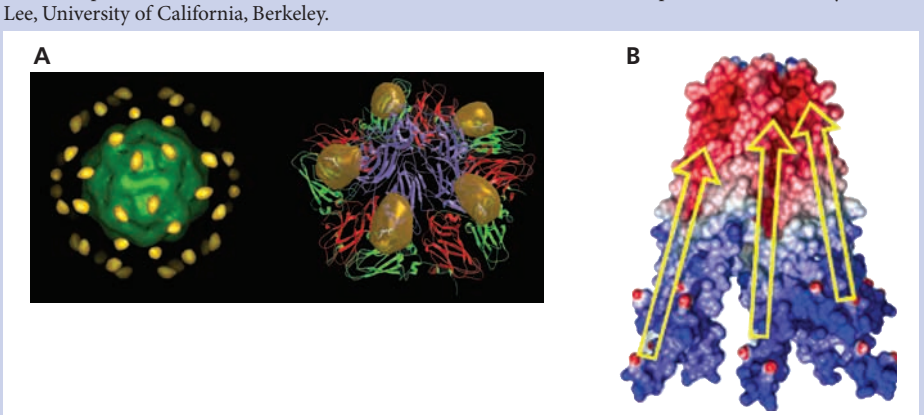
Lee BY, Zhang J, Zueger C, Chung WJ, Yoo SY, Wang E, Meyer J, Ramesh R, Lee SW. 2012. Virus-based piezoelectric energy generation. *Nat Nanotechnol* **7:**351–356.

Nam KT, Kim DW, Yoo PJ, Chiang CY, Meethong N, Hammond PT, Chiang YM, Belcher AM. 2006. Virus-enabled synthesis and assembly of nanowires for lithium ion battery electrodes. *Science* **312:** 885–888.

Tarascon JM. 2009. Nanomaterials: viruses electrify battery research. *Nat Nanotechnol* **4:**341–342.

Wang Q, Lin T, Tang L, Johnson JE, Finn MG. 2002. Icosahedral virus particles as addressable nanoscale building blocks. *Angew Chem Int Ed Engl* **41:**459–462.

Gold particles attached to cowpea mosaic virus. (A) Cryo-EM was performed on derivatized cowpea mosaic virus with a cysteine residue inserted on the surface of each of the 60 subunits and to which nanogold particles with a diameter of 1.4 nm were chemically linked. (Left) Difference electron density map obtained by subtracting the density of unaltered cowpea mosaic virus at 29 Å from the density map of the derivatized virus. This procedure reveals both the genome (green) and the gold nanoparticles. (Right) A section of the difference map imposed on the atomic model of cowpea mosaic virus. The positions of the gold indicate that it is attached at the sites of the introduced cysteine residues. Courtesy of M. G. Finn and J. Johnson, The Scripps Research Institute. **(B)** Increasing the piezoelectric strength of phage M13. Side view of a segment of M13 containing 10 copies of the helical major coat protein modified to contain four glutamine residues at its N terminus. The dipole moments (yellow arrows) are directed from the N terminus (blue) to the C terminus (red). Adapted from B. Y. Lee et al., *Nat Nanotechnol* **7:**351–356, 2012, with permission. Courtesy of S.-W. Lee, University of California, Berkeley.



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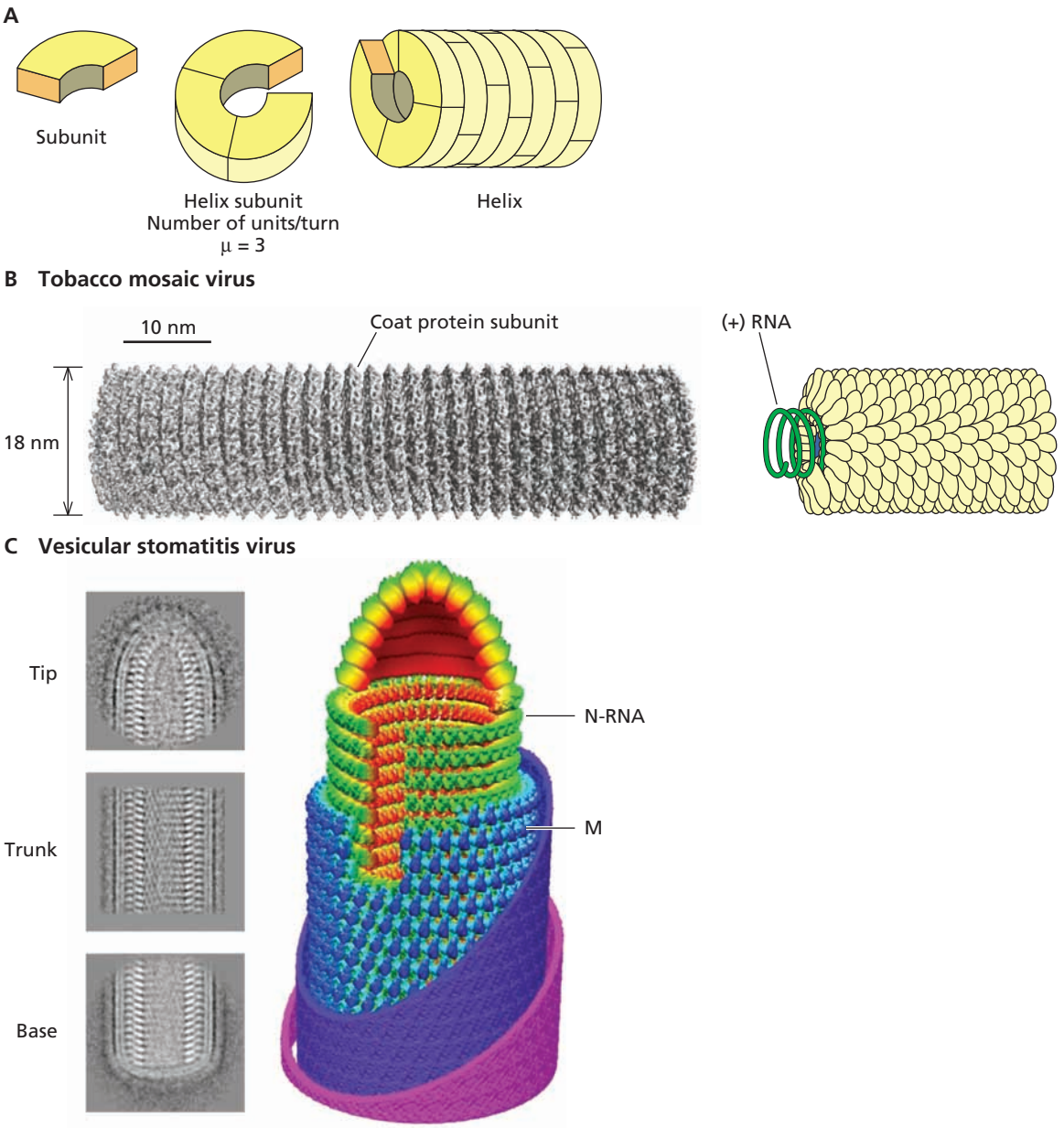


Figure 4.5 Virus structures with helical symmetry. (A) Schematic illustration of a helical particle, indicating the individual subunits, their interaction to form a helical turn, the helix, and the helical parameters ρ (axial rise per subunit) and μ (the number of subunits per turn). The pitch of the helix, *P*, is given by the formula $P = \rho \times \mu$. **(B)** Tobacco mosaic virus. (Left) A cryo-EM reconstruction at <5-Å resolution of a 70-nm segment of this particle. Each helical turn contains 16.3 protein molecules. Adapted from J. Sachse et al., *J Mol Biol* **371:**812–835, 2007, with permission. Courtesy of N. Grigorieff, Leibniz-Institut für Alterforschung, Jena, Germany. (Right) The regular interaction of the (+) strand RNA genome with coat protein subunits is illustrated in the model based on a 2.9-Å X-ray fiber diffraction structure. Adapted from K. Namba et al., *J Mol Biol* **208:**307–325, 1989, with permission. **(C)** Vesicular stomatitis virus. Representative averages of cryo-EM images of the central trunk, conical tip, and flat base of this bullet-shaped virus particle are shown at the left. The trunk and tip were analyzed and reconstructed separately to form the montage model shown on the right, with N and M proteins in green and blue, respectively, and the membrane in purple and pink. The N protein packages the (−) strand RNA genome in a left-handed helix. The crystal structure of N determined in an N-RNA complex (Fig. 4.6) fits unambiguously with the cryo-EM density of trunk N subunits. The turns of the N protein helix are not closely associated with one another, a property that accounts for the unwinding of the nucleoprotein in the absence of M (see text), which forms an outer, left-handed helix. In the trunk, the N helix contains 37.5 subunits per turn. Comparison of N-N interactions in such a turn and in rings of 10 N molecules determined by X-ray crystallography, as well as the results of mutational analysis, are consistent with formation of rings containing increasing numbers of N molecules from the apex of the tip via different modes of N-N interaction induced by association with long genomic RNA. Once a second turn of the N-RNA is stacked on the first, the M protein can bind to add rigidity. Adapted from P. Ge et al., *Science* **327:**689–693, 2010, with permission. Courtesy of Z. H. Zhou, University of California, Los Angeles.

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likened to lock washers, which in turn assemble as a long, rod-like, right-handed helix. In the interior of the helix, each coat protein molecule binds three nucleotides of the RNA genome. The coat protein subunits therefore engage in **identical** interactions with one another and with the genome, allowing the construction of a large, stable structure from multiple copies of a single protein.

The particles of several families of animal viruses with (−) strand RNA genomes, including filoviruses, paramyxoviruses, rhabdoviruses, and orthomyxoviruses, contain internal structures with helical symmetry that are encased within an envelope. In all cases, these structures contain an RNA molecule, many copies of an RNA-packaging protein (designated NP or N), and the viral RNA polymerase and associated enzymes responsible for synthesis of mRNA. Despite common helical symmetry and similar composition, the internal components of these (−) strand RNA viruses exhibit considerable diversity in morphology and organization. For example, the nucleocapsids of the filovirus ebolavirus and the paramyxovirus Sendai virus are long, filamentous structures in which the NP proteins make regular interactions with the single molecule of the RNA genome. In contrast, those of rhabdoviruses such as vesicular stomatitis virus are bullet-shaped structures (Fig. 4.5C). Furthermore, an additional viral protein is essential to maintain their organization: vesicular stomatitis virus nucleocapsids released from within the envelope retain the dimensions and morphology observed in intact particles, but become highly extended and filamentous once the matrix (M) protein is also removed (Fig. 12.23). X-ray crystallography of a ring-like N protein-RNA complex containing 10 molecules of the N protein bound to RNA has revealed that each N protein molecule binds to 9 nucleotides of RNA, which is largely sequestered within cavities within the N proteins (Fig. 4.6). Furthermore, each N subunit makes extensive and regular contacts with neighboring N molecules, exactly as predicted from first principles by Crick and Watson.

The internal components of influenza A virus particles differ more radically. In the first place, they comprise not a single nucleocapsid but, rather, multiple ribonucleoproteins, one for each molecule of the segmented RNA genome present in the virus particle (Appendix, Fig. 15). Furthermore, with the exception of terminal sequences, the RNA in these ribonucleoproteins is fully accessible to solvent. This property suggests that the RNA is not sequestered in the interior of the ribonucleoprotein. The structure of ribonucleoproteins released from influenza A virus particles determined by cryo-EM is consistent with such a model: the ribonucleoprotein comprises a double helix of NP molecules connected at one end by an NP loop (Fig. 4.7A) (currently an unusual architecture for helical viral ribonucleoproteins) with the RNA bound along the exposed surface of each NP strand (Fig. 4.7B).

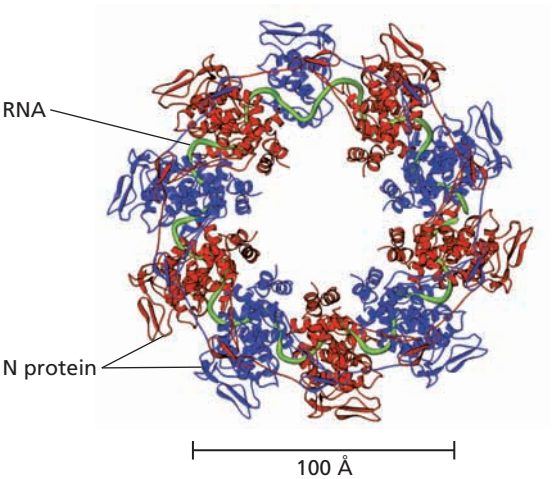


Figure 4.6 Structure of a ribonucleoprotein-like complex of vesicular stomatitis virus. Shown is the structure of the decamer of the N protein bound to RNA, determined by X-ray crystallography, with alternating monomers in the ring colored red and blue and the RNA ribose-phosphate backbone depicted as a green tube. To allow visualization of the RNA, the C-terminal domain of the monomer at the top center is not shown. The decamer was isolated by dissociation of the viral P protein from RNA-bound oligomers formed when the N and P proteins were synthesized in *Escherichia coli*. The N-terminal extension and the extended loop in the C-terminal lobe contribute to the extensive interactions among neighboring N monomers. Adapted from T. J. Green et al., *Science* **313:**357–360, 2006, with permission. Courtesy of M. Luo, University of Alabama at Birmingham.

Capsids with Icosahedral Symmetry

General Principles

Icosahedral symmetry. An icosahedron is a solid with 20 triangular faces and 12 vertices related by two-, three-, and fivefold axes of rotational symmetry (Fig. 4.8A). In a few cases, virus particles can be readily seen to be icosahedral (e.g., see Fig. 4.15 and 4.27. However, most closed capsids **look** spherical, and they often possess prominent surface structures or viral glycoproteins in the envelope that do not conform to the underlying icosahedral symmetry of the capsid shell. Nevertheless, the symmetry with which the structural units interact is that of an icosahedron.

In solid geometry, each of the 20 faces of an icosahedron is an equilateral triangle, and five such triangles interact at each of the 12 vertices (Fig. 4.8A). In the simplest protein shells, a trimer of a single viral protein (the **subunit**) corresponds to each triangular face of the icosahedron: as shown in Fig. 4.8B, such trimers interact with one another at the five-, three-, and twofold axes of rotational symmetry that define an icosahedron. As an icosahedron has 20 faces, 60 identical subunits (3 per face × 20 faces) is the minimal number needed to build a capsid with icosahedral symmetry.

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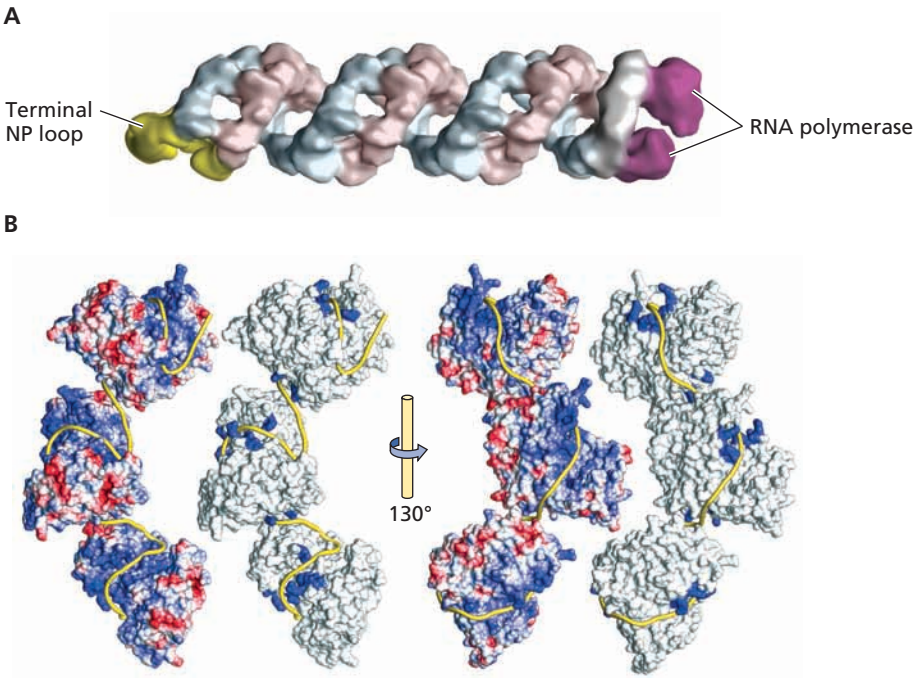


Figure 4.7 Structure of an influenza A virus ribonucleoprotein. (A) Ribonucleoproteins were isolated from purified influenza A virus particles and the central and terminal regions analyzed separately following cryo-EM. This procedure was adopted to overcome the heterogeneity in length of individual ribonucleoproteins and their flexibility. Class averaging of images of straight segments of central regions and three-dimensional reconstruction revealed that the RNA-binding NP protein forms a double helix closed by a loop at one end. In the model, the NP strands of opposite polarity are shown in blue and pink, with the NP loop in yellow and the RNA polymerase subunits at the other end in gray, green, and tan. **(B)** Four views of a single NP strand, indicating the likely localization of the (−) strand genome RNA (yellow ribbon). This localization was deduced from the surface electrostatic potential (models on the left, with positive and negative charge shown blue and red, respectively) and the positions of substitutions that impair binding of NP to RNA (blue in the models on the right). Adapted from R. Arranz et al., *Science* **338:**1634–1637, 2012, with permission. Courtesy of J. Martin-Benito, Centro Nacional de Biotecnologia, Madrid, Spain.

Large capsids and quasiequivalent bonding. In the simple icosahedral packing arrangement, each of the 60 subunits (**structural** or **asymmetric units**) consists of a single molecule in a structurally identical environment (Fig. 4.8B). Consequently, all subunits interact with their neighbors in an identical (or **equivalent**) manner, just like the subunits of helical particles such as that of tobacco mosaic virus. As the viral proteins that form such closed shells are generally $<\sim$100 kDa in molecular mass, the size of the viral genome that can be accommodated in this simplest type of particle is restricted severely. To make larger capsids, additional subunits must be included. Indeed, the capsids of the majority of animal viruses are built from many more than 60 subunits and can house quite large genomes. In 1962, Donald Caspar and Aaron Klug developed a theoretical framework accounting for the structural properties of larger particles with icosahedral symmetry. This theory has had enormous influence on the way virus architecture is described and interpreted.

The triangulation number, *T*. A crucial idea introduced by Caspar and Klug was that of **triangulation,** the description of the triangular face of a large icosahedral structure in terms of its subdivision into smaller triangles, termed **facets** (Fig. 4.9). This process is described by the triangulation number, *T*, which gives the number of structural units (small "triangles") per face (Box 4.3). Because the minimum number of subunits required is 60, the total number of subunits in the structure is 60*T*.

Quasiequivalence. A second cornerstone of the theory developed by Caspar and Klug was the proposition that when a capsid contains >60 subunits, each occupies a quasiequivalent position; that is, the noncovalent bonding

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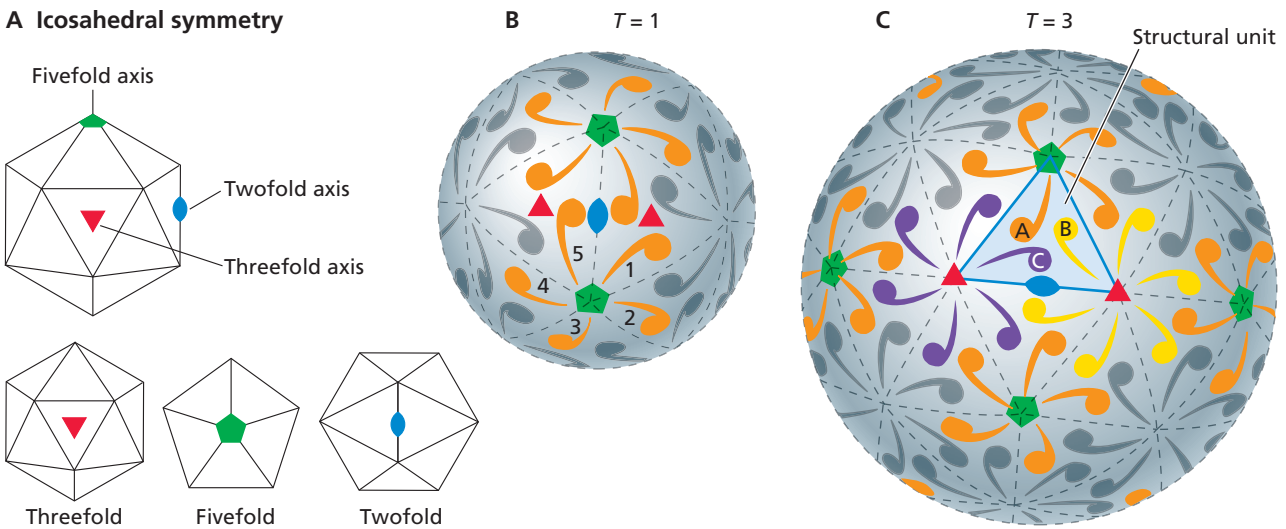


Figure 4.8 Icosahedral packing in simple structures. (A) An icosahedron, which comprises 20 equilateral triangular faces characterized by positions of five-, three-, and twofold rotational symmetry. The three views at the bottom illustrate these positions. **(B and C)** A comma represents a single protein molecule, and axes of rotational symmetry are indicated as in panel A. In the simplest case, $T = 1$ **(B)**, the protein molecule forms the structural unit, and each of the 60 molecules is related to its neighbors by the two-, three-, and fivefold rotational axes that define a structure with icosahedral symmetry. In such a simple icosahedral structure, the interactions of all molecules with their neighbors are identical. In the $T = 3$ structure **(C)** with 180 identical protein subunits, there are three modes of packing of a subunit (shown in orange, yellow, and purple): the structural unit (outlined in blue) is now the asymmetric unit, which, when replicated according to 60-fold icosahedral symmetry, generates the complete structure. The orange subunits are present in pentamers, formed by tail-to-tail interactions, and interact in rings of three (head to head) with purple and yellow subunits, and in pairs (head to head) with a purple or a yellow subunit. The purple and yellow subunits are arranged in rings of six molecules (by tail-to-tail interactions) that alternate in the particle. Despite these packing differences, the bonding interactions in which each subunit engages are similar, that is, quasiequivalent: for example, all engage in tail-to-tail and head-to-head interactions. Adapted from S. C. Harrison et al., *in* B. N. Fields et al. (ed.), *Fundamental Virology* (Lippincott-Raven, New York, NY, 1995), with permission.

properties of subunits in different structural environments are **similar**, but not identical, as is the case for the simplest, 60-subunit capsids. This property is illustrated in Fig. 4.8C for a particle with 180 identical subunits. In the small, 60-subunit structure, 5 subunits make fivefold symmetric contact at each of the 12 vertices (Fig. 4.8B). In the larger assembly with 180 subunits, this arrangement is retained at the 12 vertices, but the additional subunits are interposed to form clusters with sixfold symmetry. In such a capsid, each subunit can be present in one of three **different** structural environments (designated A, B, or C in Fig. 4.8C). Nevertheless, all subunits bond to their neighbors in similar **(quasi-equivalent)** ways, for example, via head-to-head and tail-to-tail interactions.

Capsid architectures corresponding to various values of T, some very large, have been described. The triangulation number and quasiequivalent bonding among subunits describe the structural properties of many simple viruses with icosahedral symmetry. However, it is now clear that the structures adopted by specific segments of capsid proteins can govern the packing interactions of identical subunits. Such large conformational differences between small regions of chemically identical subunits were not anticipated in early considerations of virus structure. This omission is not surprising, for these principles were formulated when little was known about the conformational flexibility of proteins. As we discuss in the next sections, the architectures of both small and more-complex viruses can, in fact, depart radically from the constraints imposed by quasiequivalent bonding. For example, the capsid of the small polyomavirus simian virus 40 is built from 360 subunits, corresponding to the $T = 6$ triangulation number excluded by the rules formulated by Caspar and Klug (Box 4.3). Moreover, a capsid stabilized by **covalent** joining of subunits to form viral "chain mail" has been described (Box 4.4). Our current view of icosahedrally symmetric virus structures is therefore one that includes greater diversity in the mechanisms by which stable capsids can be formed than was anticipated by the pioneers in this field.

Structurally Simple Capsids

Several nonenveloped animal viruses are small enough to be amenable to high-resolution analysis by X-ray

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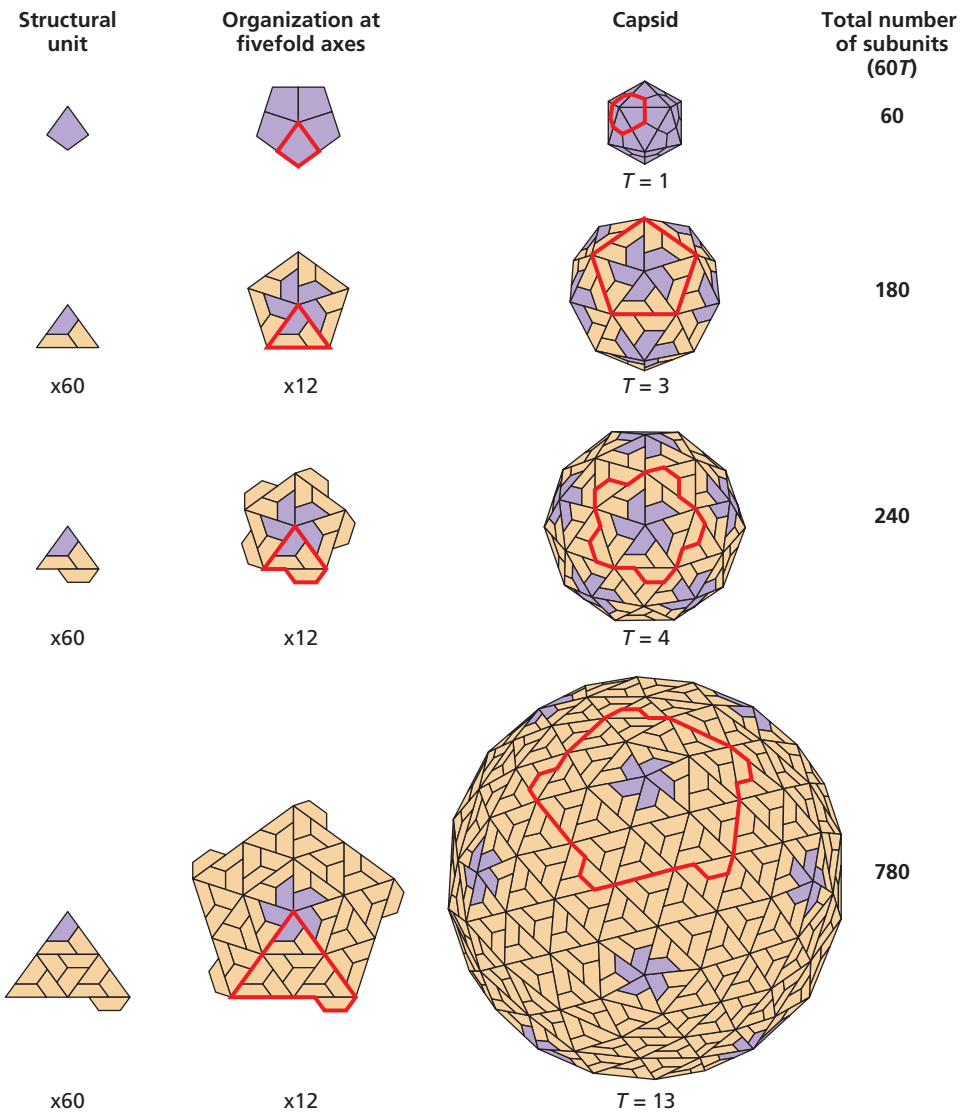


Figure 4.9 The principle of triangulation: formation of large capsids with icosahedral symmetry. The formation of faces of icosahedral particles by triangulation is illustrated by comparison of structural units, organization of structural units at fivefold axes of icosahedral symmetry, and capsids with the *T* number indicated. In each case, the protein subunits are represented by trapezoids, with those that interact at the vertices colored purple and all others tan. It is important to appreciate that protein subunits are **not**, in fact, flat, as shown here for simplicity, but highly structured (see, for examples, Fig. 4.10 and 4.12). Both the interaction of subunits around the fivefold axes of symmetry and the capsid, with an individual face outlined in red, are shown for each value of *T*, to illustrate the increase in face and particle size with increasing *T*.

crystallography. We have chosen three examples, the parvovirus adenovirus-associated virus 2, the picornavirus poliovirus, and the polyomavirus simian virus 40, to illustrate the molecular foundations of icosahedral architecture.

Structure of adeno-associated virus 2: classic $T = 1$ icosahedral design. The parvoviruses are very small animal viruses, with particles of ~25 nm in diameter that encase single-stranded DNA genomes of <5 kb. These small, naked capsids are built from 60 copies of a single subunit organized according to $T = 1$ icosahedral symmetry. The subunits of adenovirus-associated virus type 2, a member of the dependovirus subgroup of parvovirus (Appendix, Fig. 19), contain a core domain commonly found in viral

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BOX 4.3

BACKGROUND

The triangulation number, T*, and how it is determined*

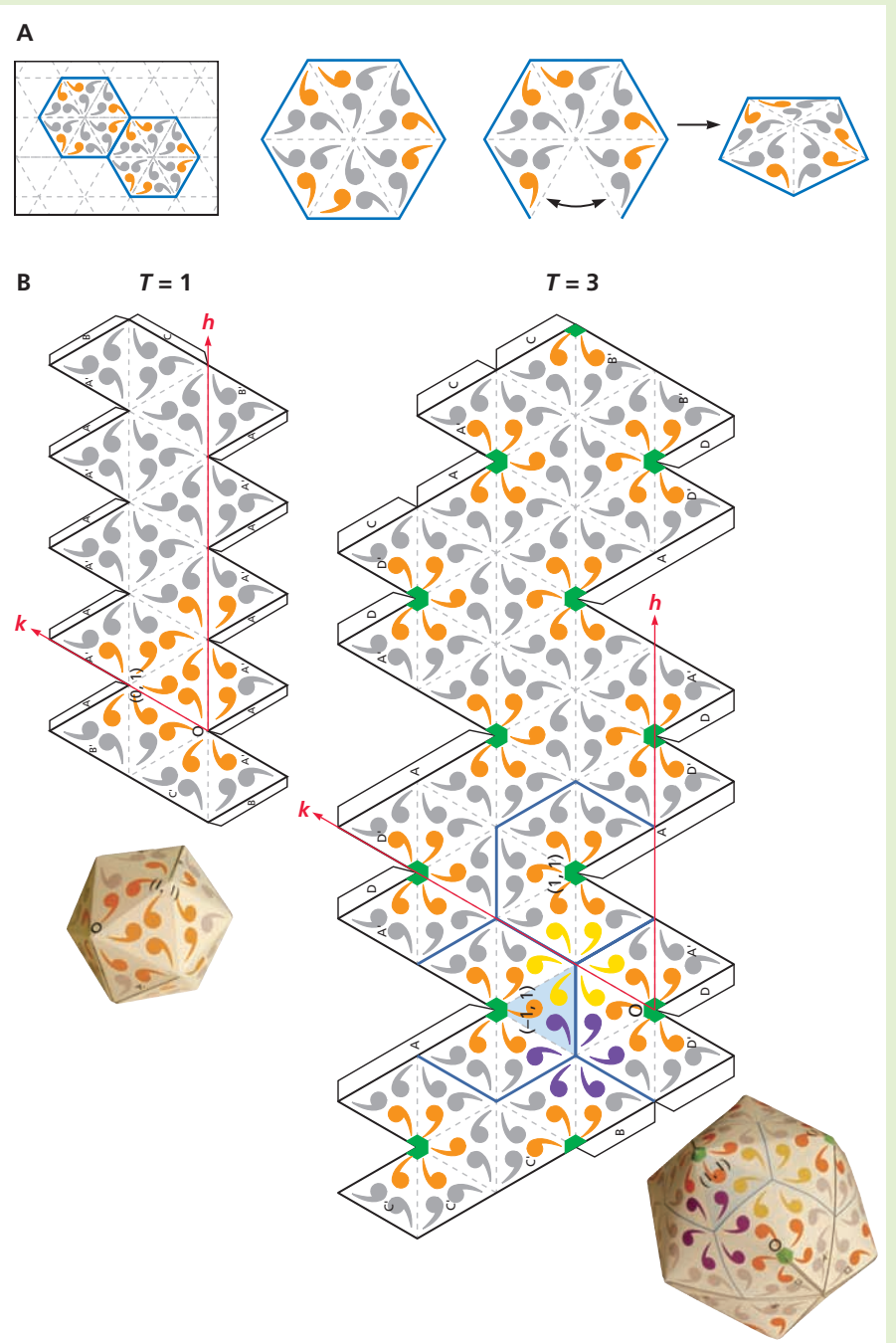
In developing their theories about virus structure, Caspar and Klug used graphic illustrations of capsid subunits, such as the net of flat hexagons shown at the top left of panel A in the figure. Each hexagon represents a hexamer, with identical subunits shown as equilateral triangles. When all subunits assemble into such hexamers, the result is a flat sheet, or lattice, which can never form a closed structure. To introduce curvature, and hence form three-dimensional structures, one triangle is removed from a hexamer to form a pentamer in which the vertex and faces project above the plane of the original lattice (A, far right). As an icosahedron has 12 axes of fivefold symmetry, 12 pentamers must be introduced to form a closed structure with icosahedral symmetry. If 12 adjacent hexamers are converted to pentamers, an icosahedron of the minimal size possible for the net is formed. This structure is built from 60 equilateral-triangle asymmetric units and corresponds to a $T = 1$ icosahedron (panel B, left). Larger structures with icosahedral symmetry are built by including a larger number of equilateral triangles (subunits) per face (B, right). In the hexagonal lattice, this is equivalent to converting 12 **nonadjacent** hexamers to pentamers at precisely spaced and regular intervals.

To illustrate this operation, we use nets in which an origin (O) is fixed and the positions of all other hexamers are defined by the coordinates along the axes labeled h and k, where h and k are any positive integer (B, left). The hexamer (h, k) is therefore defined as that reached from the origin (O) by h steps in the direction of the h axis and k steps in the direction of the k axis. In the $T = 1$ structure, $h = 1$ and $k = 0$ (or $h = 0$ and $k = 1$), and adjacent hexamers are converted to pentamers (B, left. When $h = 1$ and $k = 1$, pentamers are separated by one step in the h and one step in the k direction (B, right). Similarly, when $h = 2$ and $k = 0$ (or vice versa), two steps in a single direction separate the pentamers.

The triangulation number, T, is the number of asymmetric units per face of the icosahedron constructed in this way. It can be shown, for example by geometry, that

$$T = h^2 + hk + k^2$$

Therefore, when both h and k are 1, $T = 3$,



(continued)

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BOX 4.3

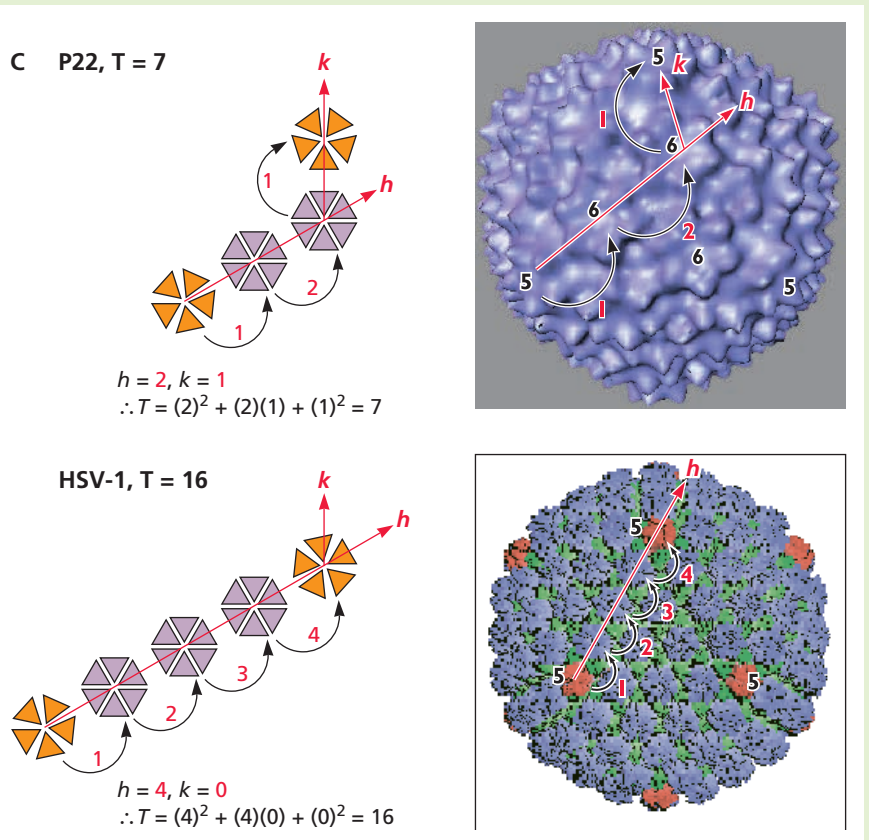
BACKGROUND

The triangulation number, T, *and how it is determined (continued)*

and each face of the icosahedron contains three asymmetric units. The total number of units, which must be 60*T*, is 180. When $T = 4$, there are four asymmetric units per face and a total of 240 units (Fig. 4.9).

As the integers *h* and *k* describe the spacing and spatial relationships of pentamers, that is, of fivefold vertices in the corresponding icosahedra, their values can be determined by inspection of electron micrographs of virus particles or their constituents (C). For example, in the bacteriophage p22 capsid (C, top), one pentamer is separated from another by two steps along the *h* axis and one step along the *k* axis, as illustrated for the bottom left pentamer shown. Hence, $h = 2$, $k = 1$, and $T = 7$. In contrast, pentamers of the herpes simplex virus type 1 (HSV-1) nucleocapsid (bottom) are separated by four and zero steps along the directions of the *h* and *k* axes, respectively. Therefore, $h = 4$, $k = 0$, and $T = 16$.

Panels A and B adapted from Fig. 2 of J. E. Johnson and A. J. Fisher, *in* R. G. Webster and A. Granoff (ed.), *Encyclopedia of Virology*, 3rd ed. (Academic Press, London, United Kingdom, 1994), with permission. Cryo-electron micrographs of bacteriophage p22 and HSV-1 courtesy of B. V. V. Prasad and W. Chiu, Baylor College of Medicine, respectively.



capsid proteins (the β-barrel jelly roll; see next section) in which β-strands are connected by loops (Fig. 4.10A). Interactions among neighboring subunits are mediated by these loops. The prominent projections near the threefold axes of rotational symmetry (Fig. 4.10B), which have been implicated in receptor binding of adenovirus-associated virus type 2, are formed by extensive interdigitation among the loops from adjacent subunits.

Structure of poliovirus: a $T = 3$ structure. As their name implies, the picornaviruses are among the smallest of animal viruses. In contrast to the $T = 1$ parvoviruses, the ~30-nm-diameter poliovirus particle is composed of 60 copies of a **multimeric** structural unit. It contains a (+) strand RNA genome of ~7.5 kb and its covalently attached 5′-terminal protein, VPg (Appendix, Fig. 21). Our understanding of the architecture of the *Picornaviridae* took a quantum leap in 1985 with the determination of high-resolution structures of human rhinovirus 14 (genus *Rhinovirus*) and poliovirus (genus *Enterovirus*).

The heteromeric structural unit of the poliovirus capsid contains one copy each of VP1, VP2, VP3, and VP4. The VP4 protein is synthesized as an N-terminal extension of VP2 and restricted to the inner surface of the particle. The poliovirus capsid is built from asymmetric units that contain one copy of each of three different proteins (VP1, VP2, and VP3), and is therefore described as a pseudo $T = 3$ structure (Fig. 4.11A). Although these three proteins are not related in amino acid sequence, all contain a central β-sheet structure termed a **β-barrel jelly roll**. The arrangement of β-strands in these β-barrel proteins is illustrated schematically in Fig. 4.11B, for comparison with the actual structures of VP1, VP2, and VP3. As can be seen in the schematic, two antiparallel β-sheets form a wedge-shaped structure. One of the β-sheets comprises one wall of the wedge, while the second, sharply twisted β-sheet forms both the second wall and the floor.

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BOX 4.4

EXPERIMENTS

Viral chain mail: not the electronic kind

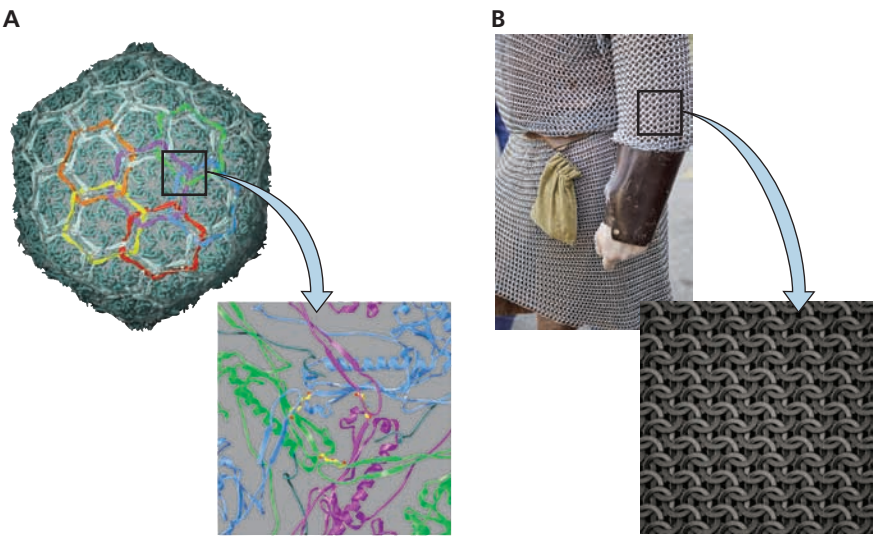
The mature capsid of the tailed, double-stranded DNA bacteriophage HK97 is a $T = 7$ structure built from hexamers and pentamers of a single viral protein, Gp5. The first hints of the remarkable and unprecedented mechanism of stabilization of this particle came from biochemical experiments, which showed the following:

- A previously unknown covalent protein-protein linkage forms in the final reaction in the assembly of the capsid: the side chain of a lysine (K) in every Gp5 subunit forms a covalent isopeptide bond with an asparagine (N) in an adjacent subunit. Consequently, **all** subunits are joined covalently to each other.
- This reaction is **autocatalytic**, depending only on Gp5 subunits organized in a particular conformational state: the capsid is enzyme, substrate, and product.
- HK97 mature particles are extraordinarily stable and cannot be disassembled into individual subunits by boiling in strong ionic detergent: it was therefore proposed that the cross-linking also interlinks the subunits from adjacent structural units to catenate rings of hexamers and pentamers.

The determination of the structure of the HK97 capsid to 3.6-Å resolution by X-ray crystallography has confirmed the formation of such capsid "chain mail" (figure, panel A), akin to that widely used in armor (B) until the development of the crossbow. The HK97 capsid is the first example of a protein catenane (an interlocked ring). This unique structure has been shown to increase the stability of the virus particle, and it may be of particular advantage as the capsid shell is very thin. The delivery of the DNA genome to host cells via the tail of the particle obviates the need for capsid disassembly.

Duda RL. 1998. Protein chainmail: catenated protein in viral capsids. *Cell* **94:**55–60.

Wikoff WR, Liljas L, Duda RL, Tsuruta H, Hendrix RW, Johnson JE. 2000. Topologically linked protein rings in the bacteriophage HK97 capsid. *Science* **289:**2129–2133.



Chain mail in the bacteriophage HK97 capsid. (A) The exterior of the HK97 capsid is shown at the top, with structural units of the Gp5 protein in cyan. The segments of subunits that are cross-linked into rings are colored the same, to illustrate the formation of catenated rings of subunits. The cross-linking is shown in the more detailed view below, down a quasithreefold axis with three pairs of cross-linked subunits. The K-N isopeptide bonds are shown in yellow. The cross-linked monomers (shown in blue) loop over a second pair of covalently joined subunits (green), which in turn cross over a third pair (magenta). Adapted from W. R. Wikoff et al., *Science* **289:**2129–2133, 2000, with permission. Courtesy of J. Johnson, The Scripps Research Institute. **(B)** Chain mail armor and schematic illustration of the rings that form the chain mail.

The protein backbones in β-barrel domains of VP1, VP2, and VP3 are folded in the same way; that is, they possess the same **topology**, and the differences among these proteins are restricted largely to the loops that connect β-strands and to the N- and C-terminal segments that extend from the central β-barrel domains.

The β-barrel jelly roll conformation of these picornaviral proteins is also seen in the core domains of capsid proteins of a number of plant, insect, and vertebrate (+) strand RNA viruses, such as tomato bushy stunt virus and Nodamura virus. This structural conservation was entirely unanticipated. Even more remarkably, this relationship is not restricted to small RNA viruses: the major capsid proteins of the DNA-containing parvoviruses and polyomaviruses also contain such β-barrel domains. It is well established that the three-dimensional structures of cellular proteins have been highly conserved during evolution, even though there may be very little amino acid sequence identity. For example, all globins possess a common three-dimensional architecture based on a particular arrangement of eight α-helices, even though their amino acid sequences are different. One interpretation of the common occurrence of the β-barrel jelly roll domain in viral capsid proteins is therefore that seemingly unrelated modern viruses (e.g., picornaviruses and parvoviruses) share some portion of their evolutionary history. It is also possible that this domain topology represents one of a limited number commensurate

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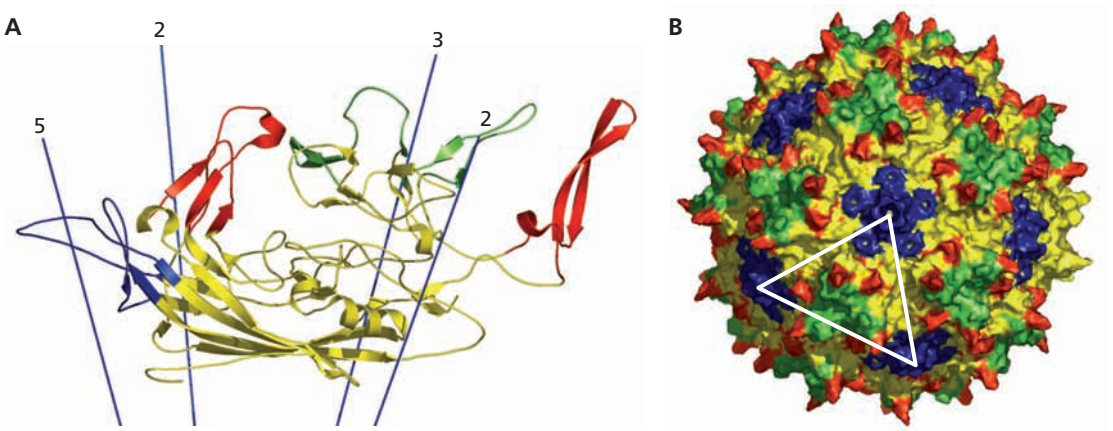
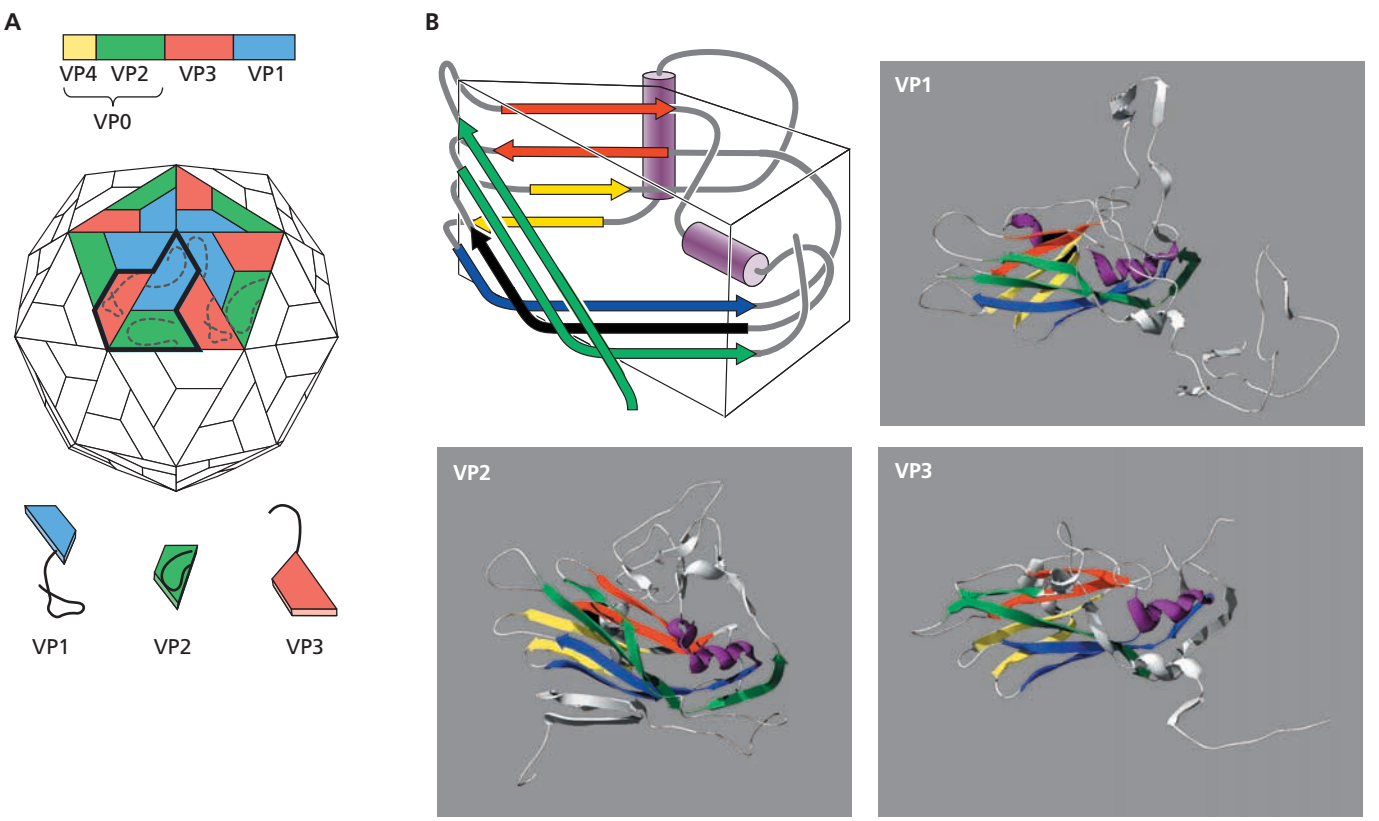


Figure 4.10 Structure of the parvovirus adeno-associated virus 2. (A) Ribbon diagram of the single coat subunit of the $T = 1$ particle. The regions of the subunit that interact around the five-, three-, and twofold axes (indicated) of icosahedral symmetry are shown in blue, green, and yellow, respectively. The red segments form peaks that cluster around the threefold axes. **(B)** Surface view of the 3-Å-resolution structure determined by X-ray crystallography of purified virions. The regions of the single subunits from which the capsid is built are colored as in panel A, and the face formed by three subunits is outlined in black. Adapted from Q. Xie et al., *Proc Natl Acad Sci U S A* **99:**10405–10410, 2002, with permission. Courtesy of Michael Chapman, Florida State University.

Figure 4.11 Packing and structures of poliovirus proteins. (A) The packing of the 60 VP1-VP2-VP3 structural units, represented by wedge-shaped blocks corresponding to their β-barrel domains. Note that the structural unit (outlined in black) contributes to two adjacent faces of an icosahedron rather than corresponding to a facet. When virus particles are assembled, VP4 is covalently joined to the N terminus of VP2. It is located on the inner surface of the capsid shell (see Fig. 4.12A). **(B)** The topology of the polypeptide chain in a β-barrel jelly roll is shown at the top left. The β-strands, indicated by arrows, form two antiparallel sheets juxtaposed in a wedge-like structure. The two α-helices (purple cylinders) that surround the open end of the wedge are also conserved in location and orientation in these proteins. As shown, the VP1, VP2, and VP3 proteins each contain a central β-barrel jelly roll domain. However, the loops that connect the β-strands in this domain of the three proteins vary considerably in length and conformation, particularly at the top of the β-barrel, which, as represented here, corresponds to the outer surface of the capsid. The N- and C-terminal segments of the protein also vary in length and structure. The very long N-terminal extension of VP3 has been truncated in this representation. Adapted from J. M. Hogle et al., *Science* **229:**1358–1365, 1985, with permission.



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BOX 4.5

DISCUSSION

Remarkable architectural relationships among viruses with double-stranded DNA genomes

Viruses with double-stranded DNA genomes are currently classified by the International Committee on the Taxonomy of Viruses into 28 families (with some unassigned) on the basis of the criteria described in Chapter 1. As might be expected, these viruses exhibit different morphologies and infect diverse organisms representing all three domains of life. They span a large size range, with genomes from a few kilobase pairs (members of the *Polyomaviridae*) to >2,500 kbp (*Pandoravirus*). Nevertheless, consideration of structural properties indicates that these very disparate virus families in fact represent a limited number of architectural types.

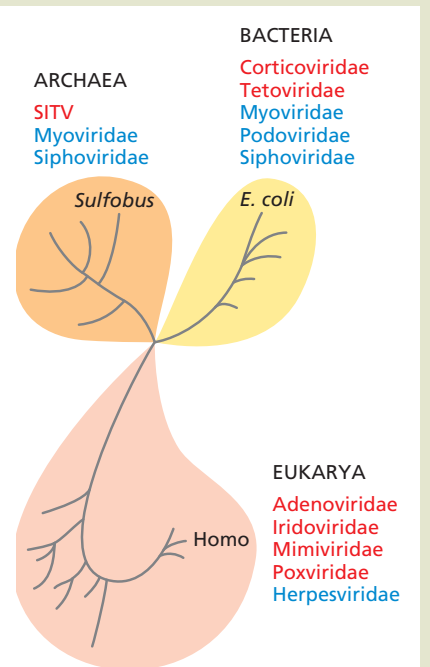
Structural information is now available for the major capsid proteins of representatives of some 20 of the 28 families of double-stranded DNA viruses. Based on the fold of the proteins, most of these families can be assigned to one of just five structural classes. It is noteworthy that the two most common major capsid protein folds, the double jelly roll and the HK97-like, are found in viruses that infect *Bacteria*, *Archaea*, and *Eukarya* (including mammals), as summarized in the figure.

The small number of building blocks seen in the major capsid proteins of these viruses might indicate convergent evolution, the compatibility of only a tiny fraction of the >1,000 distinct protein folds described to date with assembly of an infectious virus particle. However, viruses that infect hosts as divergent as bacteria and humans share more than the architectural elements of their major capsid proteins. This property is exemplified by the bacteriophage PRD1 and human species C adenoviruses, in which the major structural unit comprises a trimer of monomers each with two jelly roll domains and hence exhibits pseudohexagonal symmetry. These icosahedral capsids also share a structural unit built from different proteins at the positions of fivefold symmetry, from which project proteins that attach to the host cell receptors project; features of their linear double-stranded DNA genomes, such as the presence of inverted terminal repetitions; and mechanisms of viral DNA synthesis. Extensive similarities in morphology and the mechanisms of particle assembly and active genome packaging are also shared by tailed, double-stranded DNA viruses that infect bacteria, e.g., phage T4, and herpesviruses. It is therefore difficult to escape the conclusion that these modern viruses evolved from an ancient common ancestor (see also Volume II, Chapter 10).

Abrescia NG, Branford DH, Grimes JM, Stuart DI. 2012. Structure unifies the viral universe. *Ann Rev Biochem* **81:**795–822.

Benson SD, Bamford JK, Bamford DH, Burnett RM. 1999. Viral evolution revealed by bacteriophage PRD1 and human adenovirus coat protein structures. *Cell* **98:**825–833.

Kropovic M, Banford DH. 2011. Double-stranded DNA viruses: 20 families and only five different architectural principles for virion assembly. *Curr Opin Virol* **1:**118–124.



The simplified evolutionary tree shows just some of the branches within each domain of life, with archaeal, bacterial, and eukaryote hosts of viruses described in this chapter indicated. Viruses with major capsid proteins with the double jelly roll and HK97-like folds are listed in red and blue, respectively.

with packing of proteins to form a sphere, and is an example of convergent evolution. The structural (and other) properties of viruses with double-stranded DNA genomes provide compelling support for the first hypothesis (Box 4.5).

The overall similarity in shape of the β-barrel domains of poliovirus VP1, VP2, and VP3 facilitates both their interaction with one another to form the 60 structural units of the capsid and the packing of these structural units. How well these interactions are tailored to form a protective shell is illustrated by the model of the capsid shown in Fig. 4.12: the extensive interactions among the β-barrel domains of adjacent proteins form a dense, rigid protein shell around a central cavity in which the genome resides. The packing of the β-barrel domains is reinforced by a network of protein-protein contacts on the inside of the capsid. These interactions are particularly extensive about the fivefold axes (Fig. 4.12C). The interaction of five VP1 molecules, which is unique to the fivefold axes, results in a prominent protrusion extending to about 25 Å from the capsid shell (Fig. 4.12A). The protrusion appears as a steep-walled plateau encircled by a valley or cleft. In the capsids of many picornaviruses, these depressions, which contain the receptor-binding sites, are so deep that they have been termed **canyons**.

One of several important lessons learned from high-resolution analysis of picornavirus capsids is that their design does not conform strictly to the principle of **quasiequivalence**. For example, despite the topological identity and geometric similarity of the jelly roll domains of the proteins that form the capsid shell, the subunits do not engage in quasiequivalent bonding: interactions among VP1 molecules around the fivefold axes are neither chemically nor structurally equivalent to those in which VP2 or VP3 engage.

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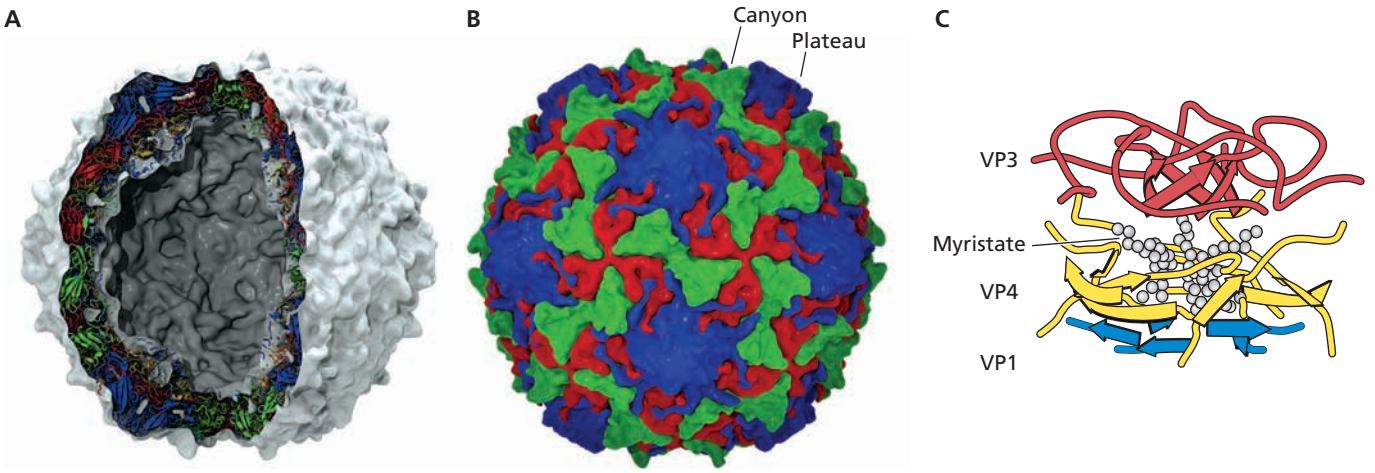


Figure 4.12 Interactions among the proteins of the poliovirus capsid. (A) Space-filling representation of the particle, with four pentamers removed from the capsid shell and VP1 in blue, VP2 in green, VP3 in red, and VP4 in yellow, as in Fig. 4.11A. Note the large central cavity in which the RNA genome resides; the dense protein shell formed by packing of the VP1, VP2, and VP3 β-barrel domains; and the interior location of VP4, which decorates the inner surface of the capsid shell. **(B)** Space-filling representation of the exterior surface showing the packing of the β-barrel domains of VP1, VP2, and VP3. Interactions among the loops connecting the upper surface of the β-barrel domains of these proteins create the surface features of the virion, such as the plateaus at the fivefold axes, which are encircled by a deep cleft or canyon. The particle is also stabilized by numerous interactions among the proteins on the inner side of the capsid. **(C)** These internal contacts are most extensive around the fivefold axes, where the N termini of five VP3 molecules are arranged in a tube-like, parallel β-sheet. The N termini of VP4 molecules carry chains of the fatty acid myristate, which are added to the protein posttranslationally. The lipids mediate interaction of the β-sheet formed by VP3 N termini with a second β-sheet structure, containing strands contributed by both VP4 and VP1 molecules. This internal structure is not completed until the final stages of, or after, assembly of virus particles, when proteolytic processing liberates VP2 and VP4 from their precursor, VP0. This reaction therefore stabilizes the capsid. Panels A and B were created by Jason Roberts, Doherty Institute, Melbourne, Australia.

An alternative icosahedral design: structure of simian virus 40. The capsids of the small DNA polyomaviruses simian virus 40 and mouse polyomavirus, ~50 nm in diameter, are organized according to a rather different design that is not based on quasiequivalent interactions. The structural unit is a pentamer of the major structural protein, VP1. The capsid is built from 72 such pentamers engaged in one of two kinds of interaction. Twelve structural units occupy the 12 positions of fivefold rotational symmetry, in which each is surrounded by five neighbors. Each of the remaining 60 pentamers is surrounded by 6 neighbors at positions of sixfold rotational symmetry in the capsid (Fig. 4.13A). Consequently, the 72 pentamers of simian virus 40 occupy a number of different local environments in the capsid, because of differences in packing around the five- and sixfold axes.

Like the three poliovirus proteins that form the capsid shell, simian virus 40 VP1 contains a large central β-barrel jelly roll domain, in this case with an N-terminal arm and a long C-terminal extension (Fig. 4.13B). However, the arrangement and packing of VP1 molecules bear little resemblance to the organization of poliovirus capsid proteins. In the first place, the VP1 β-barrels in each pentamer project outward from the surface of the capsid to a distance of about 50 Å, in sharp contrast to those of the poliovirus capsid proteins, which tilt along the surface of the capsid shell. As a result, the surface of simian virus 40 is much more "bristly" than that of poliovirus (compare Fig. 4.12A and 4.13A). Furthermore, the VP1 molecules present in adjacent pentamers in the simian virus 40 capsid do not make extensive contacts via the surfaces of their β-barrel domains. Rather, stable interactions among pentamers are mediated by their N- and C-terminal arms. The packing of VP1 pentamers in both pentameric and hexameric arrays requires different contacts among these structural units, depending on their local environment. In fact, there are just three kinds of interpentamer contact, which are the result of alternative conformations and noncovalent interactions of the long C-terminal arms of VP1 molecules. The same capsid design is also exhibited by human papillomaviruses.

Simian virus 40 and poliovirus capsids differ in their surface appearance, in the number of structural units, and in the ways in which these structural units interact. Nevertheless, they share important features, including modular organization of the proteins that form the capsid shell and a common β-barrel domain as the capsid building block. Neither poliovirus nor simian virus 40 capsids conform to strict quasiequivalent construction: all contacts made by all protein subunits are not similar, and in the case of simian virus 40, the majority of VP1 **pentamers** are packed in **hexameric** arrays. Nevertheless, close packing with icosahedral symmetry is achieved by limited variations of the contacts, either

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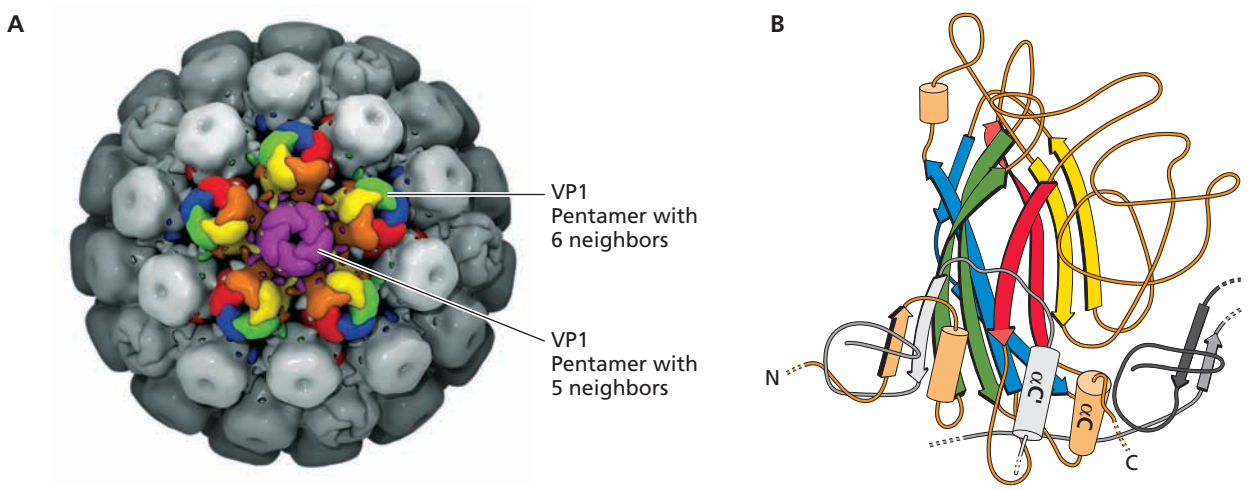


Figure 4.13 Structural features of simian virus 40. (A) View of the simian virus 40 virion showing the organization of VP1 pentamers. One of the 12 5-coordinated pentamers is shown in purple and 10 of the 60 pentamers present in hexameric arrays are in light gray. The individual VP1 molecules in the pentamers surrounding a pentamer with five neighbors are colored red, blue, green, yellow, and orange. The image was created by Jason Roberts, Doherty Institute, Melbourne, Australia. **(B)** The topology of the VP1 protein shown in a ribbon diagram, with the strands of the β-barrel jelly roll colored as in Fig. 4.11B. This β-barrel domain is perpendicular to the capsid surface. The C-terminal arm and α-helix (αC; orange) of the VP1 subunit invades a neighboring pentamer (not shown). The C-terminal arm and C α-helix shown in gray (αC′) is the invading arm from a different neighboring pentamer (not shown), which is clamped in place by extensive interactions of its β-strand with the N-terminal segment of the subunit shown. The subunit shown also interacts with the N-terminal arm from its anticlockwise neighbor in the same pentamer (dark gray). This subunit also interacts extensively with other segments from subunits in the same pentamer or in neighboring pentamers, shown in gray or black. Adapted from R. C. Liddington et al., *Nature* **354:**278–284, 1991, with permission.

among topologically similar, but chemically distinct, surfaces (poliovirus) or made by a flexible arm (simian virus 40).

Structurally simple icosahedral capsids in more complex particles. Several viruses that are structurally more sophisticated than those described in the previous sections nevertheless possess simple protein coats built from one or a few structural proteins. The complexity comes from the additional protein and lipid layers in which the capsid is enclosed (see "Viruses with Envelopes" below).

Structurally Complex Capsids

Some naked viruses are considerably larger and more elaborate than the small RNA and DNA viruses described in the previous section. The characteristic feature of such virus particles is the presence of proteins devoted to specialized structural or functional roles. Despite such complexity, detailed pictures of the organization of this type of virus particle can be constructed by using combinations of biochemical, structural, and genetic methods. Well-studied human adenoviruses and members of the *Reoviridae* exemplify these approaches.

Adenovirus. The most striking morphological features of the adenovirus particle (maximum diameter, 150 nm) are the well-defined icosahedral appearance of the capsid and the presence of long fibers at the 12 vertices (Fig. 4.14A). A fiber, which terminates in a distal knob that binds to the adenoviral receptor, is attached to each of the 12 penton bases located at positions of fivefold symmetry in the capsid. The remainder of the shell is built from 240 additional subunits, the hexons, each of which is a trimer of viral protein II (Fig. 4.14B). Formation of this capsid depends on nonequivalent interactions among subunits: the hexons that surround pentons occupy a different bonding environment than those surrounded entirely by other hexons. The X-ray crystal structures of the trimeric hexon (the major capsid protein) established that each protein II monomer contains two β-barrel domains, each with the topology of the β-barrels of the simpler RNA and DNA viruses described in the previous section (Fig. 4.14B). The very similar topologies of the two β-barrel domains of the three monomers facilitate their close packing to form the hollow base of the trimeric hexon. Interactions among the monomers are very extensive, particularly in the towers that rise above the hexon base and are formed by intertwining loops from each monomer. Consequently, the trimeric hexon is extremely stable.

The adenovirus particle contains seven additional structural proteins (Fig. 4.14A). The presence of so many proteins and the large size of the particle made elucidation of adenovirus architecture a challenging problem. One approach

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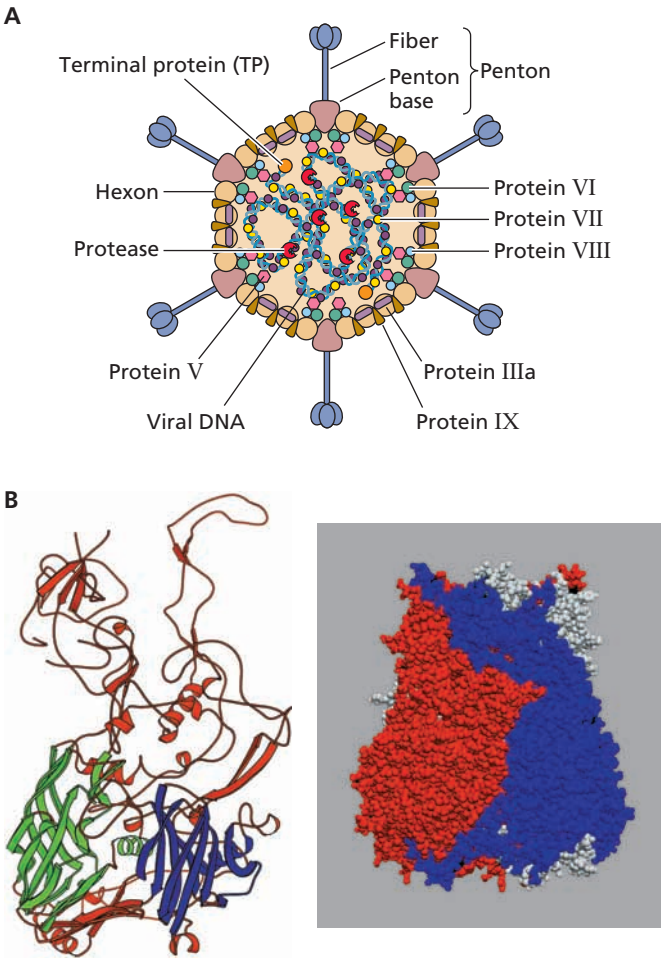


Figure 4.14 Structural features of adenovirus particles. (A) The organization of human adenovirus type 5 is shown schematically to indicate the locations of the major (hexon, penton base, and fiber) and minor (IIIa, VI, VIII, and IX) capsid proteins and of the internal core proteins, V, VII, and μ. These locations and the interactions between proteins shown were deduced from the composition of the products of controlled dissociation of viral particles and the results of cross-linking studies and confirmed in high-resolution structures of adenovirus particles. **(B)** Structure of the hexon homotrimer. The monomer (left) is shown as a ribbon diagram, with gaps indicating regions that were not defined in the X-ray crystal structure at 2.9-Å resolution, and the trimer (right) is shown as a space-filling model with each monomer in a different color. The monomer contains two β-barrel jelly roll domains colored green and blue in the left panel. The trimers are stabilized by extensive interactions within both the base and the towers. From M. M. Roberts et al., *Science* **232:**1148–1151, 1986, and F. K. Athappilly et al., *J Mol Biol* **242:**430–455, 1994, with permission. Courtesy of J. Rux, S. Benson, and R. M. Burnett, The Wistar Institute.

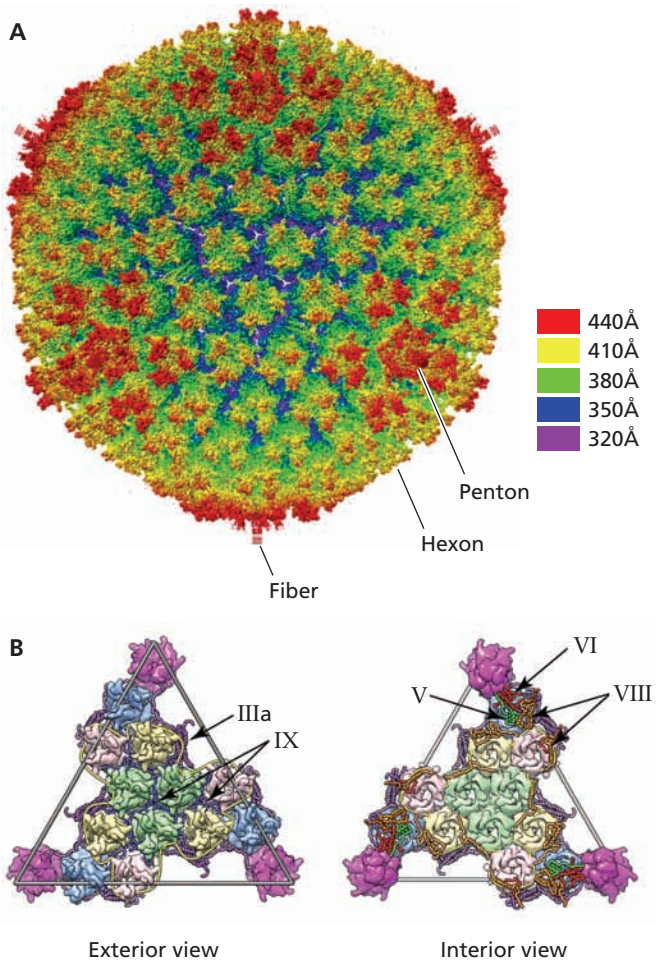


Figure 4.15 Interactions among major and minor proteins of the adenoviral capsid. (A) Cryo-EM reconstruction of the adenovirus type 5 capsid at 3.6-Å resolution radially colored by distance from the center, as indicated. This view is centered on a threefold axis of icosahedral symmetry. Only short stubs of the fibers are evident, as these structures are bent. For other views, see Movie 4.2 (http://bit.ly/Virology_V1_Movie4-2). **(B)** Views of the outer (left) and inner (right) surfaces indicating the locations of the minor capsid proteins IX (blue), IIIa (mauve), V (green), VI (red), and VIII (orange) with respect to hexons (light blue, pink, light green, and khaki) and penton base (magenta). The boundary of a group-of-nine hexon is shown by a white tube. Adapted from V. Reddy and G. Nemerow, *Proc Natl Acad Sci U S A* **111:**11715–11720, 2014, with permission. Courtesy of V. Reddy, The Scripps Research Institute.

that has proved generally useful in the study of complex viruses is the isolation and characterization of discrete subviral particles. For example, adenovirus particles can be dissociated into a core structure that contains the DNA genome, groups of nine hexons, and pentons. Analysis of the composition of such subassemblies identified two classes of proteins in addition to the major capsid proteins described above. One comprises the proteins present in the core, such as protein VII, the major DNA-binding protein. The remaining proteins are associated with either individual hexons or the groups of hexons that form an icosahedral face of the capsid, suggesting that they stabilize the structure. Protein IX has been clearly identified as capsid "cement": a mutant virus that lacks the protein IX coding sequence produces the typical yield of virions, but these particles are much less heat stable than the wild type.

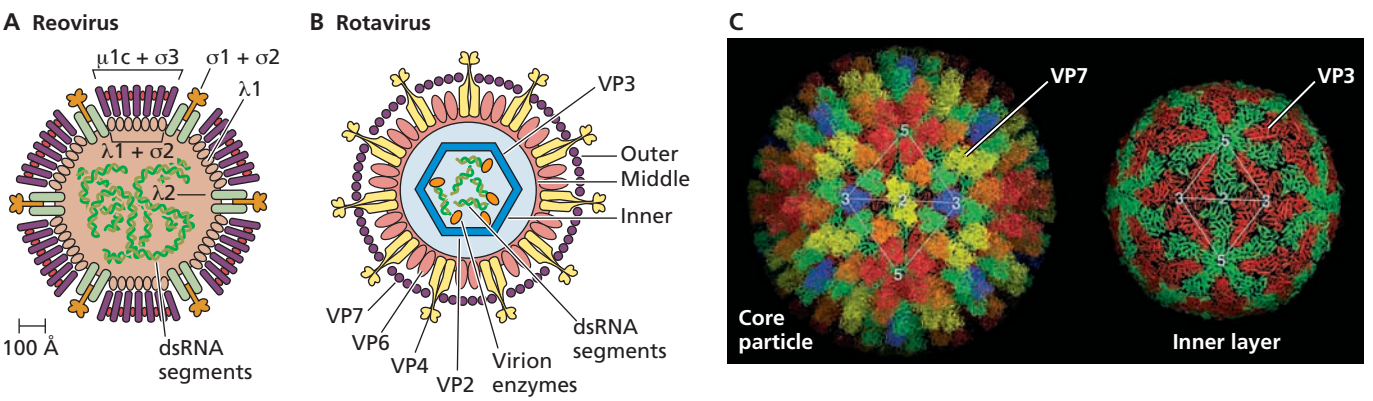
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The interactions of protein IX and other minor proteins with hexons and/or pentons were deduced initially by difference imaging (Fig. 4.4) and subsequently by X-ray crystallography or cryo-EM (Fig. 4.15A; Box 4.1). The minor capsid proteins make numerous contacts with the major structural units. For example, on the outer surface of the capsid, a network formed by extensive interactions among the extended molecules of protein IX knit together the hexons that form the groups of nine (Fig. 4.15B). Other minor capsid proteins are restricted to the inner surface, where they reinforce the groups of nine hexons, or weld the penton base to its surrounding hexons. During assembly, interactions among hexons and other major structural proteins must be relatively weak, so that incorrect associations can be reversed and corrected. However, the assembled particle must be stable enough to survive passage from one host to another. It has been proposed that the incorporation of stabilizing proteins like protein IX allows these paradoxical requirements to be met.

Reoviruses. Reovirus particles exhibit an unusual architecture: they contain multiple protein shells. They are naked particles, 70 to 90 nm in diameter with an outer $T = 13$ icosahedral protein coat, containing the 10 to 12 segments of the double-stranded genome and the enzymatic machinery to synthesize viral mRNA. The particles of human reovirus (genus Orthoreovirus) contain eight proteins organized in two concentric shells, with spikes projecting from the inner layer through and beyond the outer layer at each of the 12 vertices (Fig. 4.16A). Members of the genus Rotavirus, which includes the leading causes of severe infantile gastroenteritis in humans, contain three nested protein layers, with 60 projecting spikes (Fig. 4.16B). Although differing in architectural detail, reovirus particles have common structural features, including an unusual design of the innermost protein shell.

Removal of the outermost protein layer, a process thought to occur during entry into a host cell, yields an inner core structure, comprising one shell (orthoreoviruses) or two (rotaviruses and members of the genus *Orbivirus*, such as bluetongue virus). These subviral particles also contain the genome and virion enzymes and synthesize viral mRNAs under appropriate conditions *in vitro*. High-resolution structures have been obtained for bluetongue virus and human reovirus cores, some of the largest viral assemblies that have been examined by X-ray crystallography. The thin inner layer contains 120 copies of a single protein (termed VP3 in bluetongue virus). These proteins are not related in their primary sequences, but they nevertheless have similar topological features and the same plate-like shape. Moreover, in both cases, the dimeric proteins occupy one of two different structural environments, and to do so, they adopt one of two distinct conformational states, indicated as green and red in Fig. 4.16C (right). Because of this arrangement, the green and

Figure 4.16 Structures of members of the *Reoviridae*. The organization of mammalian reovirus **(A)** and rotavirus **(B)** particles is shown schematically to indicate the locations of proteins, deduced from the protein composition of intact particles and of subviral particles that can be readily isolated from them. **(C)** X-ray crystal structure of the core of bluetongue virus, a member of the *Orbivirus* genus of the *Reoviridae*, showing the core particle and the inner scaffold. Trimers of VP7 (VP6 in rotaviruses; panel B) project radially from the outer layer of the core particle (left). Each icosahedral asymmetric unit, two of which are indicated by the white lines, contains 13 copies of VP7 arranged as five trimers colored red, orange, green, yellow, and blue, respectively. This layer is organized with classical $T = 13$ icosahedral symmetry. As shown on the right, the inner layer is built from VP3 dimers that occupy one of two completely different structural environments, colored green and red. Green monomers span the icosahedral twofold axes and interact in rings of five around the icosahedral fivefold axes. In contrast, red monomers are organized as triangular "plugs" around the threefold axes. Differences in the interactions among monomers at different positions allow close packing to form the closed shell, the equivalent of a $T = 2$ lattice. As might be anticipated, VP7 trimers in pentameric or hexameric arrays in the outer layer make different contacts with the two classes of VP3 monomer in the inner layer. Nevertheless, each type of interaction is extensive, and in total, these contacts compensate for the symmetry mismatch between the two layers of the core. The details of these contacts suggest that the inner shell both defines the size of the virus particle and provides a template for assembly of the outer $T = 13$ structure. From J. M. Grimes et al., *Nature* **395:**470–478, 1998, with permission. Courtesy of D. I. Stuart, University of Oxford.



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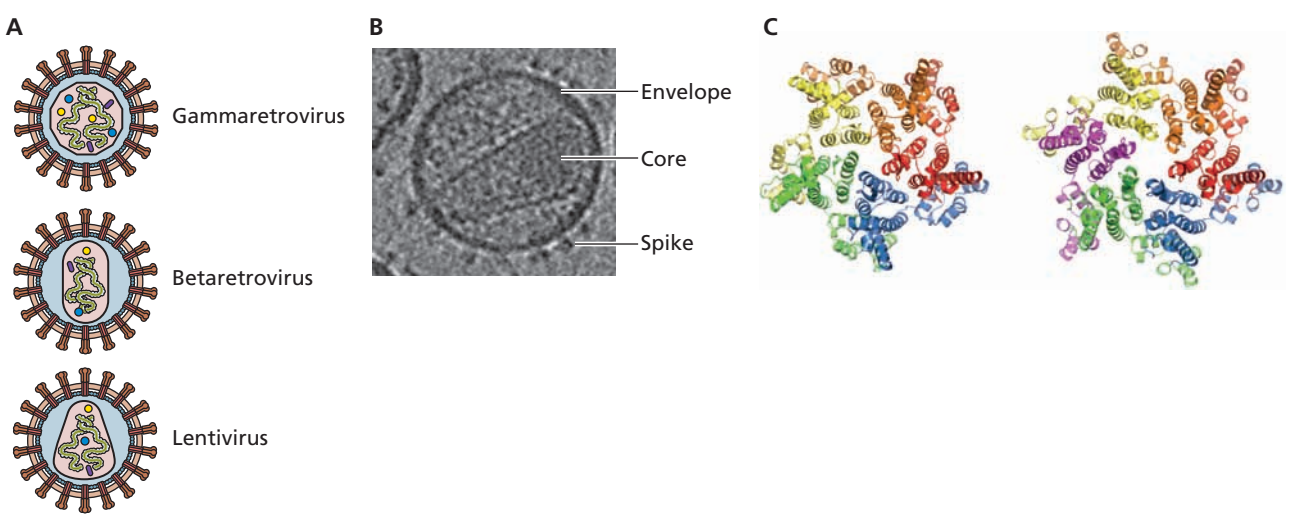
red dimers are **not** quasiequivalent, and virtually all contacts in which the two types of monomers engage are very different. However, these differences allow the formation of VP3 assemblies with either five- or threefold rotational symmetry and hence of an icosahedral shell ($T = 1$). This VP3 shell of bluetongue virus abuts directly on the inner surface of the middle layer, which comprises trimers of a single protein organized into a classical $T = 13$ lattice (Fig. 4.16C, left). A large number of different (nonequivalent) contacts between these trimers and VP3 weld the two layers together. These properties of reoviruses illustrate that a quasiequivalent structure is not the **only** solution to the problem of building large viral particles: viral proteins that interact with each other and with other proteins in multiple ways can provide an effective alternative. The architecture of the two protein shells described above appears to be conserved in all viruses with double-stranded RNA genomes. However, it is not yet known whether symmetry mismatch is also a feature of other large viruses that contain multiple protein layers.

Other Capsid Architectures

As noted previously, capsids with icosahedral or helical symmetry are characteristic of the great majority of virus particles described to date. Nevertheless, these architectures are not universal. As discussed subsequently, the composition and organization of internal structures that enclose the genome of some complex viruses, like the poxvirus vaccinia virus, are not well understood. However, the capsids of even some relatively small viruses can be constructed according to an alternative design. This property is exemplified by the capsids of retroviruses.

In the enveloped particles of all retroviruses, the capsid surrounds a nucleoprotein containing the diploid (+) strand RNA genome and is in turn encased by the viral matrix (M) protein. However, the capsid may be spherical, cylindrical, or conical, the shape exhibited by capsids of human immunodeficiency virus type 1 and other lentiviruses (Fig. 4.17A). These capsids are built from a single capsid (CA) protein, which can form both pentamers and hexamers (Fig. 4.17B). The details of the organization of the capsids of most retroviruses are not yet available. The odd appearance of the human immunodeficiency virus type 1 capsid might suggest that it represents an exception to the geometric rules that dictate the viral architectures described in previous sections. However, this is not the case: this capsid can be described by a fullerone cone model that combines principles of both icosahedral and helical symmetry. In this model, which has been confirmed by cryo-EM and modeling methods, a closed structure is formed using 12 pentamers, just as in an icosahedral capsid (Box 4.6). However, pentamers are not spaced at regular intervals throughout the

Figure 4.17 Asymmetric capsids of retroviruses. (A) Variation in the morphology of retroviruses shown schematically. Although all retrovirus particles are assembled from the same components (see the text), the cores are primarily spherical, cylindrical, or conical in the case of gammaretroviruses (e.g., Moloney murine leukemia viruses), betaretroviruses (e.g., Mason-Pfizer monkey virus), and lentiviruses (e.g., human immunodeficiency virus type 1), respectively. **(B)** Cryo-electron tomographic reconstruction of human immunodeficiency virus type 1 showing the conical core and the glycoprotein spikes projecting from the surface of the particle. Adapted from J. Liu et al., *Methods Enzymol* **483:**267–290, 2010, with permission. Courtesy of H. Winkler, Florida State University. **(C)** Structures of human immunodeficiency virus type 1 capsid (CA) pentamers and hexamers, determined by X-ray crystallography of these structures assembled *in vitro* and stabilized by disulfide bonds. Each subunit is shown in a different color with the additional subunit present in the hexamer in magenta. In each structural unit, the N-terminal domains of the CA subunits (saturated colors) interact in an inner ring surrounded by an outer belt formed by the C-terminal domains (pale colors). The interactions among N-terminal domains of individual subunits and between the N-terminal domain of one subunit and the C-terminal domain of its clockwise neighbor are very similar in pentamers and hexamers; that is, they are quasiequivalent. Reprinted from O. Pornillos et al., *Nature* **469:**424–427, 2011, with permission. Courtesy of O. Pornillos, University of Virginia.



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BOX 4.6

EXPERIMENTS

A fullerene cone model of the human immunodeficiency virus type 1 capsid

Diverse lines of evidence support a fullerone cone model of this capsid based on principles that underlie the formation of icosahedral and helical structures.

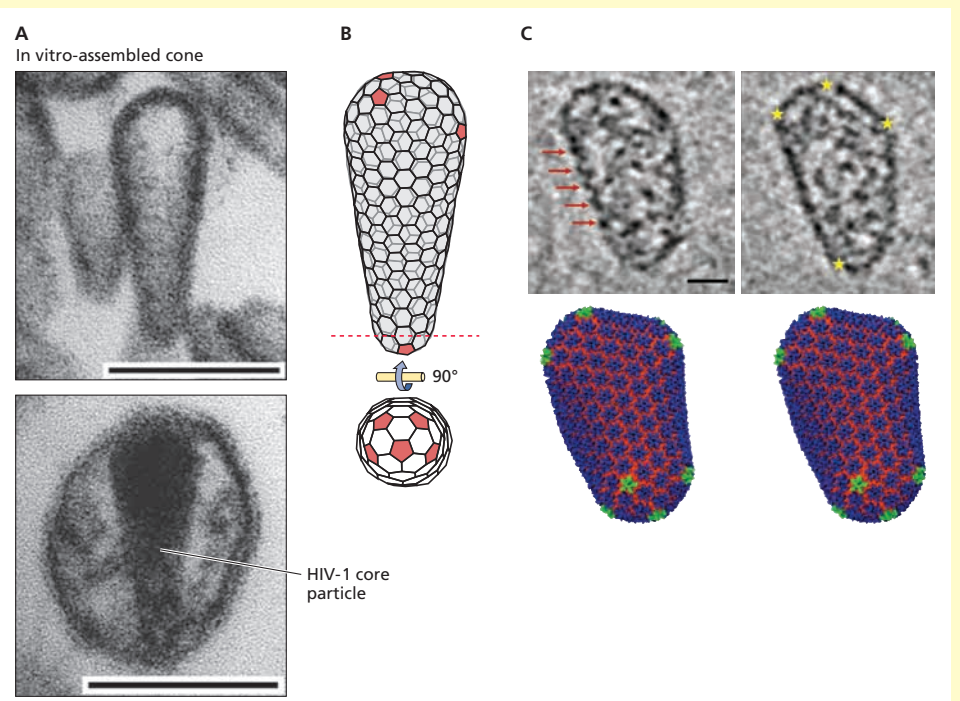
(A) A purified human immunodeficiency virus type 1 protein comprising the capsid linked to the nucleocapsid proteins, CA-NC self-assembles into cylinders and cones when incubated with a segment of the viral RNA genome *in vitro*. The cones assembled *in vitro* are capped at both ends, and many appear very similar in dimensions and morphology to cores isolated from viral particles (compare the two panels). From B. K. Ganser et al., *Science* **283:**80–83, 1999, with permission. Courtesy of W. Sundquist, University of Utah. **(B)** The very regular appearance of the synthetic CA-NC cones suggested that, despite their asymmetry, they are constructed from a regular, underlying lattice analogous to the lattices that describe structures with icosahedral symmetry discussed in Box 4.3. In fact, the human immunodeficiency virus type 1 cores can be modeled using the geometric principles that describe cones formed from carbon. Such elemental carbon cones comprise helices of hexamers closed at each end by caps of buckminsterfullerene, which are structures that contain pentamers surrounded by hexamers. As in structures with icosahedral symmetry, the positions of pentamers determine the geometry of cones. However, in cones, pentamers are present **only** in the terminal caps. The human immunodeficiency virus type 1 cones formed *in vitro* and isolated from mature virions can be modeled as a fullerene cone assembling on a curved hexagonal lattice with five pentamers (red) at the narrow end of the cone, as shown in the expanded view. The wide end would be closed by an additional 7 pentamers (because 12 pentamers are required to form a closed structure from a hexagonal lattice). In this type of structure, the cone angle at the narrow end can adopt only one of five allowed angles, determined by the number of pentamers. A narrow cap with five pentamers, as in the model shown in panel B, should exhibit a cone angle of 19.2°. Approximately 90% of all cones assembled *in vitro* examined met this prediction, consistent with the fullerene cone model. **(C)** A combination of cryo-EM of helical tubes of CA at higher resolution (8.6 Å), molecular dynamics simulation, and cryo-electron tomography of mature viral particles recently produced an atomic-level model of the human immunodeficiency virus type 1 capsid. The two top panels show representative sections through a three-dimensional reconstruction of isolated human immunodeficiency virus type 1 cores obtained by cryo-electron tomography. Red arrows indicate arrays of CA hexamers, and yellow stars indicate locations of sharp changes in curvature, i.e., the positions of pentamers. Stereoviews of the final model for a cone comprising 216 CA hexamers with N- and C-terminal domains in blue and red, respectively, and 12 pentamers (green) are shown below. Adapted from G. Zhao et al., *Nature* **497:**643–646, 2013, with permission. Courtesy of P. Zhang, University of Pittsburgh School of Medicine.

Briggs JA, Wilk T, Welker R, Kräusslich HG, Fuller SD. 2003. Structural organization of authentic, mature HIV-1 virions and cores. *EMBO J* **22:**1707–1715.

Ganser BK, Li S, Klishko VY, Finch JT, Sundquist WI. 1999. Assembly and analysis of conical models for the HIV-1 core. *Science* **283:**80–83.

Li S, Hill CP, Sundquist WI, Finch JT. 2000. Image constructions of helical assemblies of the HIV-1 CA protein. *Nature* **407:**409–413.

Zhao G, Perilla JR, Yufenyuy EL, Meng X, Chen B, Ning J, Ahn J, Gronenborn AM, Schulten K, Aiken C, Zhang P. 2013. Mature HIV-1 capsid structure by cryo-electron microscopy and all-atom molecular dynamics. *Nature* **497:**643–646.



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structure. Rather, they are restricted to the terminal caps and separated by spirals (a variant of helical symmetry) of CA hexamers that form the body of the cone.

Packaging the Nucleic Acid Genome

A definitive property of a virion is the presence of a nucleic acid genome. Incorporation of the genome requires its discrimination from a large population of cellular nucleic acid for packaging into virus particles. These processes are described in Chapter 13. The volumes of closed capsids are finite. Consequently, accommodation of viral genomes necessitates a high degree of condensation and compaction. A simple analogy illustrates vividly the scale of this problem; packing of the ~150-kbp DNA genome of herpes simplex virus type 1 into the viral capsid is equivalent to stuffing some 10 ft of 22 American gauge wire (diameter, 0.644 mm) into a tennis ball. Such confinement of the genome can result in high internal pressure, for example, some 18 and 25 atm within herpes simplex virus type 1 and phage capsids, respectively, and provides the force that powers ejection of DNA genomes. Packaging of nucleic acids is an intrinsically unfavorable process because of the highly constrained conformation imposed on the genome. In some cases, the force required to achieve packaging is provided, at least in part, by specialized viral proteins that harness the energy released by hydrolysis of ATP to drive the insertion of DNA. In many others, the binding of viral nucleic acids to capsid proteins appears to provide sufficient energy. The latter interactions also help to neutralize the negative charge of the sugar-phosphate backbone, a prerequisite for close juxtaposition of genome sequences.

We possess relatively little information about the organization of genomes within viral particles: nucleic acids or more-complex internal assemblies are not visible in the majority of high-resolution structural studies reported. This property indicates that the genomes or internal structures lack the symmetry of the capsid, do not adopt the same conformation in every viral particle, or both. Nevertheless, three mechanisms for condensing and organizing nucleic acid molecules within capsids can be distinguished and are described in the following sections.

Direct Contact of the Genome with a Protein Shell

In the simplest arrangement, the nucleic acid makes direct contact with the protein(s) that forms the protective shell of the virus particle. Proteins on the inner surfaces of the icosahedral capsids of many small RNA viruses interact with the viral genome. As we have seen, the interior surface of the poliovirus capsid can be described in detail. Nevertheless, we possess no structural information about the arrangement of the RNA genome, for the nucleic acid is not visible in the X-ray structure. However, the genome of the porcine picornavirus Seneca Valley virus has been visualized by this method (albeit at low resolution) (Fig. 4.18A). Much of the RNA genome forms an outer layer in which it makes extensive contact with the inner surface of the capsid. Highly ordered RNA genomes are also present in $T = 3$ nodaviruses, such as Flock house virus, in which an outer decahedral cage of ordered RNA surrounds additional rings.

Use of the same protein or proteins both to condense the genome and to build a capsid allows efficient utilization

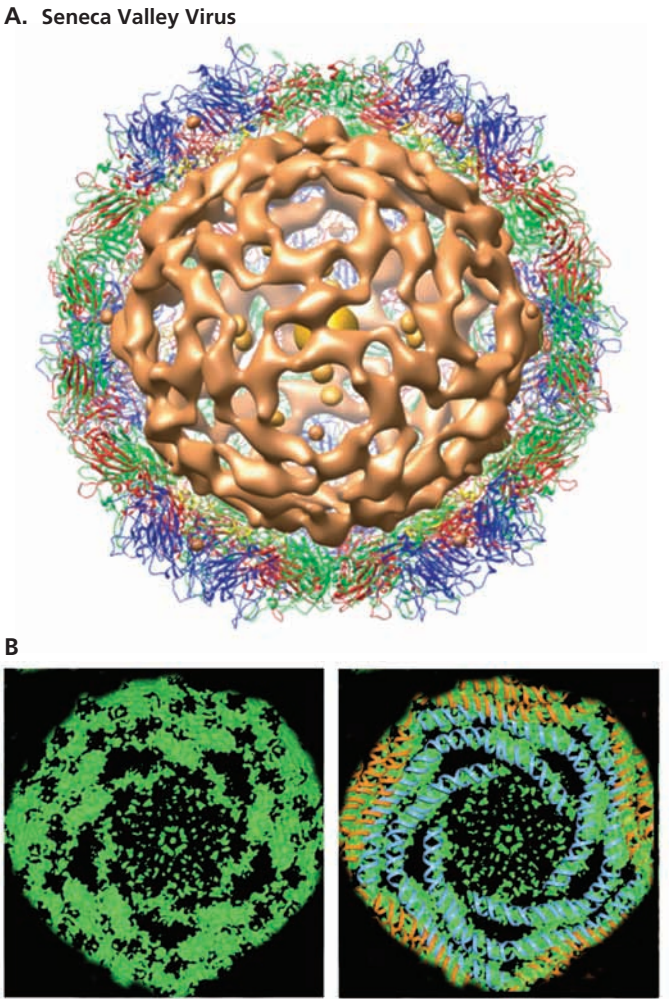


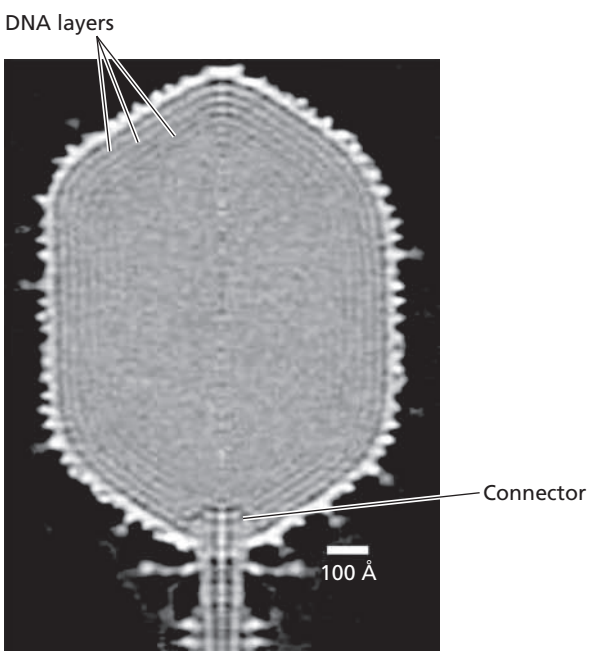
Figure 4.18: Ordered RNA genome in small and large icosahedral virus particles. (A) The 20-Å X-ray crystal structure of the picornavirus Seneca Valley virus viewed down a twofold axis of icosahedral symmetry, showing the density ascribed to the RNA genome (brown). The structural proteins are colored as in Fig. 4.11 and 4.12: VP1 (blue), VP2 (green), VP3 (red), and VP4 (yellow). **(B)** Outer layer of the double-stranded, segmented RNA genome of the rotavirus bluetongue virus observed at 6.5-Å resolution by X-ray crystallography of viral cores. The electron density of this layer of RNA from maps averaged between two closely related serotypes is shown alone (left) or with A-form duplex RNA modeled into the rods of density (right). These RNA spirals represent some 80% of the >19-kbp genome. Adapted from P. Gouet et al., *Cell* **97:**481–490, 1999, with permission. Courtesy of D. I. Stuart, University of Oxford.

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of limited genetic capacity. It is therefore an advantageous arrangement for viruses with small genomes. However, this mode of genome packing is also characteristic of some more-complex viruses, notably rotaviruses and herpesviruses. The genome of rotaviruses comprises 11 segments of double-stranded RNA located within the innermost of the three protein shells of the particle. Remarkably, as much as 80% of the RNA genome appears highly ordered within the core, with strong elements of icosahedral symmetry (Fig. 4.18B).

One of the most surprising properties of the large herpesviral capsid is the absence of internal proteins associated with viral DNA: despite intense efforts, no such core proteins have been identified, and the viral genome has not yet been visualized. In contrast, cryo-EM has allowed visualization of the large, double-stranded DNA genome of bacteriophage T4, which is organized in closely apposed, concentric layers (Fig. 4.19). This arrangement illustrates graphically the remarkably dense packing needed to accommodate such large viral DNA genomes in closed structures of fixed dimensions. This type of organization must require neutralization of the negative charges of the sugar-phosphate backbone.

Figure 4.19 Dense packing of the double-stranded DNA genome in the head of bacteriophage T4 DNA. The central section of a 22-Å cryo-EM reconstruction of the head of bacteriophage T4 viewed perpendicular to the fivefold axis is shown. The concentric layers seen underneath the capsid shell have been attributed to the viral DNA genome. The connector, which is derived from the portal structure by which the DNA genome enters the head during assembly, connects the head to the tail. Adapted from A. Fokine et al., *Proc Natl Acad Sci U S A* **101:**6003–6008, 2004, with permission. Courtesy of M. Rossmann, Purdue University.



Neutralization might be accomplished by proteins that form the inner surface of the capsid, or by the incorporation of small, basic peptides made by the host cell, such as spermine and spermidine.

Packaging by Specialized Viral Proteins

In many virus particles, the genome is associated with specialized nucleic acid-binding proteins, such as the nucleocapsid proteins of (−) strand RNA viruses and retroviruses, or the core proteins of adenoviruses. An important function of such proteins is to condense and protect viral genomes. Consequently, they do not recognize specific nucleic acid sequences but rather bind nonspecifically to RNA or DNA genomes. This mode of binding is exemplified by the structure of the vesicular stomatitis virus N protein, in which 9 nucleotides of RNA are tightly but nonspecifically bound in a cavity formed between the two domains of each N protein molecule (Fig. 4.6). These protein-RNA interactions both sequester the RNA genome and organize it into a helical structure. Formation of helical ribonucleoproteins by two-domain RNA-binding proteins is a packaging mechanism common among (−) strand RNA viruses in the order *Mononegavirales*: the N proteins of representatives of other families in the order exhibit the same two-lobed structure and mode of RNA binding (Fig. 4.20).

Electron microscopy of cores released from adenovirus particles suggested that the internal nucleoprotein is also arranged in some regular fashion. However, how the viral DNA genome is organized and condensed by the core proteins is not known: the nucleoprotein was not observed in the high-resolution structures of adenovirus particles described previously, and the structures of core proteins have not been determined. The fundamental DNA packaging unit is a multimer of protein VII, which appears as beads on a string of adenoviral DNA when other core proteins are removed. Protein VII and the other core proteins are basic, as would be expected for proteins that bind to a negatively charged DNA molecule without sequence specificity.

Packaging by Cellular Proteins

The final mechanism for condensing the viral genome, by cellular proteins, is unique to polyomaviruses, such as simian virus 40, and papillomaviruses. The circular, double-stranded DNA genomes of these viruses are organized into nucleosomes that contain the four core histones, H2A, H2B, H3, and H4. These genomes are organized within the particle (and in infected cells) like cellular DNA in chromatin to form a minichromosome. The 20 or so nucleosomes that are associated with the viral genome condense the DNA by a factor of ~7. This packaging mechanism is elegant, with two major advantages: none of the limited viral genetic information needs to be devoted to DNA-binding proteins, and the viral genome, which is transcribed by cellular RNA

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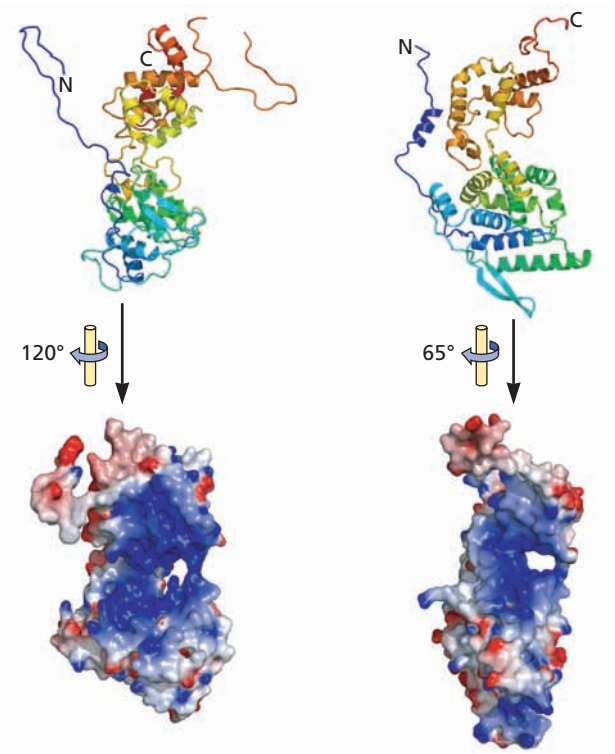


Figure 4.20 Conserved organization of the RNA-packaging proteins of nonsegmented (−) strand RNA viruses. Ribbon diagrams of the N proteins indicated are shown at the top, colored from purple at the N terminus to red at the C terminus. Their electrostatic surfaces from negative (red) to positive (blue) are shown in the space-filling models below, with the molecules rotated as indicated to show the RNA-binding cleft (blue) most clearly. Although differing in structural details, these N proteins share a two-lobed structure (top) and an RNA-binding cleft between the two lobes. Adapted from R. W. Ruigrok et al., *Curr Opin Microbiol* **14:**504–510, 2011, with permission. Courtesy of D. Kolakofsky, University of Geneva.

polymerase II, enters the infected cell nucleus as a nucleoprotein closely resembling the cellular templates for this enzyme.

Viruses with Envelopes

Many viruses contain structural elements in addition to the capsids described previously. Such virus particles possess an envelope formed by a viral protein-containing membrane that is derived from the host cell, but they vary considerably in size, morphology, and complexity. Furthermore, viral membranes differ in lipid composition, the number of proteins they contain, and their location. The envelopes form the outermost layer of enveloped animal viruses, but in bacteriophages and archaeal viruses of the PRD1 family the membrane lies **beneath** an icosahedral capsid (Box 4.7). Typical features of viral envelopes and their proteins are described in the next section, to set the stage for consideration of the structures of envelope proteins and the various ways in which they interact with internal components of the virion (Fig. 4.21).

Viral Envelope Components

The foundation of the envelopes of all animal viruses is a lipid membrane acquired from the host cell during assembly. The precise lipid composition is variable, for viral envelopes can be derived from different kinds of cellular membranes. Embedded in the membrane are viral proteins, the great majority of which are **glycoproteins** that carry covalently linked sugar chains, or **oligosaccharides**. Sugars are almost always added to the proteins posttranslationally, during transport to the cellular membrane at which progeny virus particles assemble. Intra- or interchain disulfide bonds, another common chemical feature of these proteins, are also acquired during transport to assembly sites. These covalent bonds stabilize the tertiary or quaternary structures of viral glycoproteins.

Envelope Glycoproteins

Viral glycoproteins are **integral membrane proteins** firmly embedded in the lipid bilayer by a short **membrane-spanning domain** (Fig. 4.22). The membrane-spanning domains of viral proteins are hydrophobic α-helices of sufficient length to span the lipid bilayer. They generally separate large external domains that are decorated with oligosaccharides from smaller internal segments. The former contain binding sites for cell surface virus receptors, major antigenic determinants, and sequences that mediate fusion of viral with cellular membranes during entry. Internal domains, which make contact with other components of the virion, are often essential for virus assembly.

With few if any exceptions, viral membrane glycoproteins form oligomers, which can comprise multiple copies of a single protein or may contain two or more protein chains. The subunits are held together by noncovalent interactions and disulfide bonds. On the exterior of particles, these oligomers can form surface projections, often called spikes. Because of their critical roles in initiating infection, the structures of many viral glycoproteins have been determined.

The hemagglutinin (HA) protein of human influenza A virus is a trimer that contains a globular head with a top surface that is projected ~135 Å from the viral membrane by a long stem (Fig. 4.23A). The latter is formed and stabilized by the coiling of α-helices present in each monomer. The membrane-distal globular domain contains the binding site for the host cell receptor. This important functional region is located >100 Å away from the lipid membrane of influenza virus particles. Other viral glycoproteins that mediate cell attachment and entry, such as the E protein of the flavivirus tick-borne encephalitis virus, adopt a quite different orientation (and structure); the external domain of E protein is a flat, elongated dimer that lies on the surface of the viral membrane

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BOX 4.7

DISCUSSION

A viral membrane directly surrounding the genome

The membranes present in particles of animal viruses are external structures separated from the genome by at least one protein layer. As we have seen, internal protein layers contribute to condensation and organization of the genome via interactions of the nucleic acid with specialized nucleic acid-binding proteins or the internal surfaces of capsids. Nevertheless, this arrangement is not universal: in the particles of some archaeal and bacterial viruses, a host cell-derived membrane directly abuts the genome.

This property is exemplified by *Sulfolobus* turreted icosahedral virus, which infects a hyperthermophilic archaeon. This virus has a double-stranded DNA genome, a major capsid protein containing two β-barrel jelly roll domains, and pentons built from dedicated viral proteins. The capsid of *Sulfolobus* turreted icosahedral virus encases a lipid membrane rather than an internal nucleoprotein core. As shown in panel A of the figure, a large space separates the capsid and the membrane, with contact between the capsid and the membrane limited to the fivefold axes of icosahedral symmetry, where the most internal domain of the penton base protein contacts a viral transmembrane protein. Particles purified from *Sulfolobus* turreted icosahedral virus-infected cells include forms that lack the capsid and exhibit the size and morphology of lipid cores alone. These observations suggest that the membrane, rather than the capsid, is the major determinant of particle stability.

The unusual internal membrane of the *Sulfolobus* turreted icosahedral virus is built from membrane-forming lipids synthesized specifically in thermophilic and hyperthermophilic archaea: they comprise long chains (e.g., C_{40}, compared to C_{16} to C_{18} typical of mammalian cells) of branched, isoprenoid-like units ether linked at either end to various polar head groups. Because of the latter property, these lipids can form monolayer membranes, in contrast to the lipid bilayers formed in animal cells (panel B). The ether linkages and branched acyl chains considerably increase the stability of membranes formed from these specialized archaeal lipids. This property would appear to be essential for life in the extreme conditions (e.g., pH 3 and temperature of 80°C) inhabited by the host of *Solfolobus* turreted icosahedral virus and provides an effective mechanism to protect the viral genome during transit through such harsh environments.

Khayat R, Fu CY, Ortmann AC, Young MJ, Johnson JE. 2010. The architecture and chemical stability of the archaeal *Sulfolobus* turreted icosahedral virus. *J Virol* **84:**9575–9583.

Vessler D, Ng TS, Sendamarai AK, Eilers BJ, Lawrence CM, Lok SM, Young MJ, Johnson JE, Fu CY. 2013. Atomic structure of the 75 MDa extremophile *Sulfolobus* turreted icosahedral virus determined by CryoEM and X-ray crystallography. *Proc Natl Acad Sci U S A* **110:**5504–5509.

(A) Cross section through a near-atomic-resolution reconstruction of *Sulfolobus* turreted icosahedral virus, showing the unique pentonal structures (turrets) and the separation of the capsid shell from the membrane. The internal surface of the membrane (yellow) is in direct contact with the double-stranded DNA genome (red). Adapted from D. Vessler et al., *Proc Natl Acad Sci U S A* **110:**5504–5509, 2013, with permission. Courtesy of C.-Y. Fu, The Scripps Research Institute. **(B)** Schematic comparison of archaeal monolayer membrane-forming and eukaryotic bilayer membrane-forming lipids.

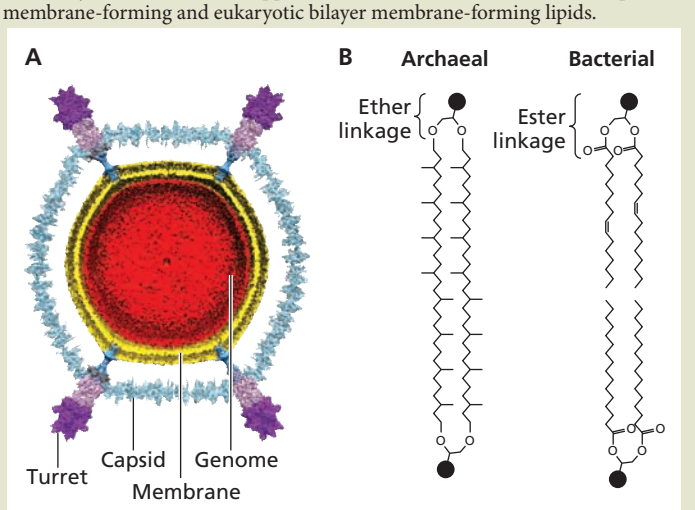
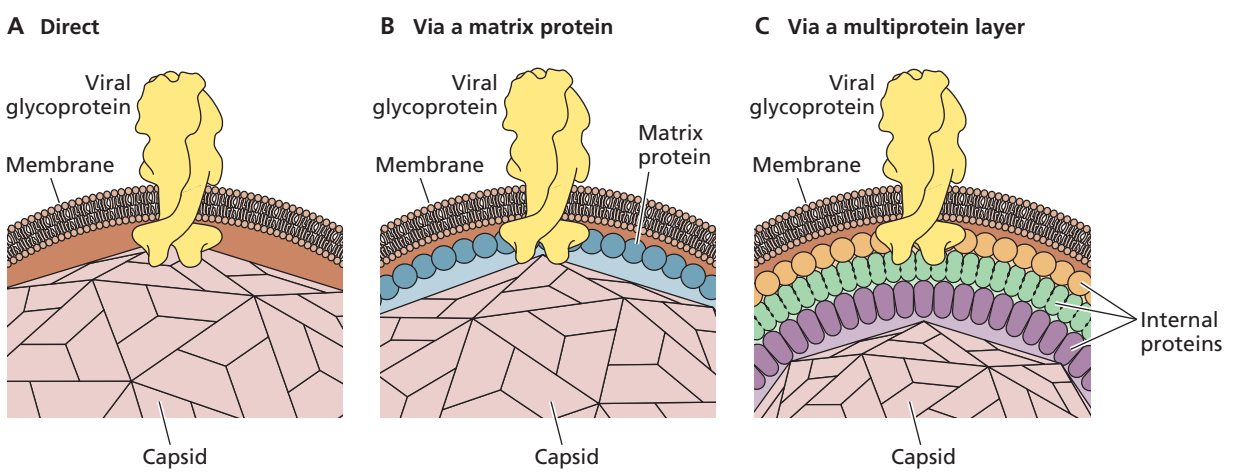


Figure 4.21 Schematic illustration of three modes of interaction of capsids or nucleocapsids with envelopes of virus particles.



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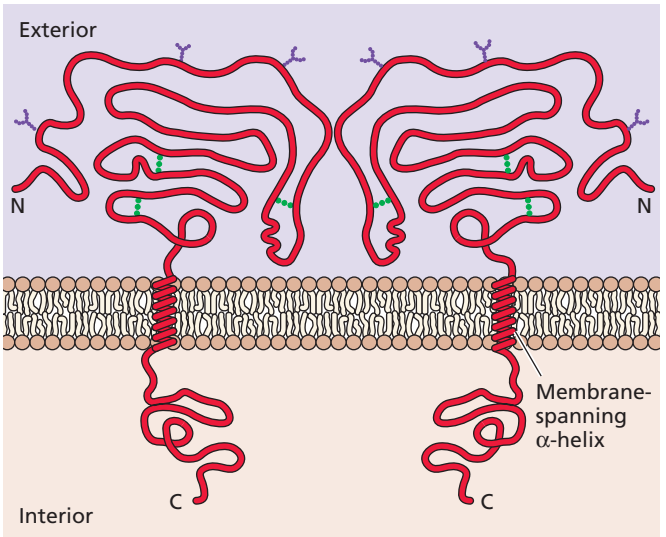


Figure 4.22 Structural and chemical features of a typical viral envelope glycoprotein shown schematically. The protein is inserted into the lipid bilayer via a single membrane-spanning domain. This segment separates a larger external domain, which is decorated with N-linked oligosaccharides (purple) and contains disulfide bonds (green), from a smaller internal domain.

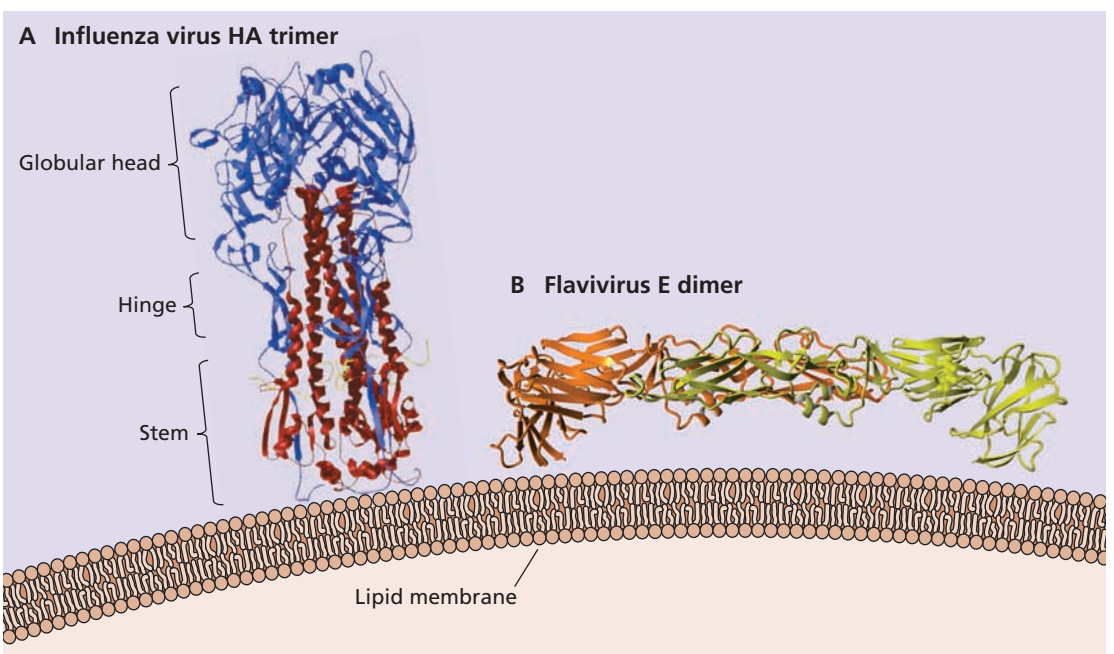
rather than projecting from it (Fig. 4.23B). Despite their lack of common structural features, both the HA protein and the E protein are primed for dramatic conformational change to allow entry of internal virion components into a host cell.

The high-resolution viral glycoprotein structures mentioned above are those of the large external domains of the proteins that had been cleaved from the viral envelope by proteases. This treatment facilitated crystallization but, of course, precluded analysis of membrane-spanning or internal segments of the proteins, both of which may operate structurally or functionally. Membrane-spanning domains can contribute to the stability of oligomeric glycoproteins, as in influenza virus hemagglutinin (HA), while internal domains can participate in anchoring the envelope to internal structures (Fig. 4.21). Improvements in resolution achieved by application of cryo-electron microscopy or tomography have allowed visualization of these segments of glycoproteins of some enveloped viruses.

Other Envelope Proteins

The envelopes of some viruses, including orthomyxoviruses, herpesviruses, and poxviruses, contain integral membrane proteins that lack large external domains or possess multiple

Figure 4.23 Structures of extracellular domains of viral glycoproteins. These extracellular domains are depicted as they are oriented with respect to the membrane of the viral envelope. **(A)** X-ray crystal structure of the influenza virus HA glycoprotein trimer. Each monomer comprises HA1 (blue) and HA2 (red) subunits covalently linked by a disulfide bond. Adapted from J. Chen et al., *Cell* **95:**409–417, 1998, with permission. **(B)** X-ray structure of the tick-borne encephalitis virus (a flavivirus) E protein dimer, with the subunits shown in orange and yellow. PDB ID: 1SVB F. A. Rey and S. C Harrison, *Nature* **375:**291–298, 1995.



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membrane-spanning segments. Among the best characterized of these is the influenza A virus M2 protein. This small (97-amino-acid) protein is a minor component of virus particles, estimated to be present at 14 to 68 copies per particle. In the viral membrane, two disulfide-linked M2 dimers associate to form a noncovalent tetramer that functions as an ion channel. The M2 ion channel is the target of the influenza virus inhibitor drug amantadine (Volume II, Fig. 9.13). The effects of this drug, as well as of mutations in the M2 coding sequence, indicate that M2 plays an important role during entry by controlling the pH of the virion interior.

Simple Enveloped Viruses: Direct Contact of External Proteins with the Capsid or Nucleocapsid

In the simplest enveloped viruses, exemplified by (+) strand RNA alphaviruses such as Semliki Forest, Sindbis, and Ross River viruses, the envelope directly abuts an inner nucleocapsid containing the (+) strand RNA genome. This inner protein layer is a $T = 4$ icosahedral shell built from 240 copies of a single capsid (C) protein arranged as hexamers and pentamers. The outer glycoprotein layer also contains 240 copies of the envelope proteins E1 and E2, which form heterodimers. They cover the surface of the particle, such that the lipid membrane is not exposed on the exterior. Strikingly, the glycoproteins are also organized into a $T = 4$ icosahedral shell (Fig. 4.24A).

The structure of Sindbis virus has been determined by cryo-EM and image reconstruction to some 9-Å resolution (Fig. 4.24A and B), while the structures of the E1 and C proteins of the related Semliki Forest virus have been solved at high resolution. The organization of the alphavirus envelope, including the transmembrane anchoring of the outer glycoprotein layer to structural units of the nucleocapsid, can therefore be described with unprecedented precision. The transmembrane segments of the E1 and E2 glycoproteins form a pair of tightly associated α-helices, with the cytoplasmic domain of E2 in close opposition to a cleft in the capsid protein (Fig. 4.24C and D). This interaction accounts for the 1:1 symmetry match between the internal capsid and exterior glycoproteins. On the outer surface of the membrane, the external portions of these glycoproteins, together with the E3 protein, form an unexpectedly elaborate structure: a thin $T = 4$ icosahedral protein layer (called the skirt) covers most of the membrane (Fig. 4.24B and C) and supports the spikes, which are hollow, three-lobed projections (Fig. 4.24D).

The structures formed by external domains of membrane proteins of the important human pathogens West Nile virus and dengue virus (family *Flaviviridae*) are quite different: they lie flat on the particle surface rather than forming protruding spikes (Fig. 4.25A; see also Box 4.8). Nevertheless, the alphavirus E1 protein and the single flavivirus envelope (E) protein exhibit the same topology (Fig. 4.25B), suggesting that the genes encoding them evolved from a common ancestor. Furthermore, the external domains of flaviviral E proteins are also icosahedrally ordered, and the envelopes of viruses of these families are described as **structured**. In contrast, as described in the next section, the arrangement of membrane proteins generally exhibits little relationship to the structure of the capsid when virus particles contain additional protein layers.

Enveloped Viruses with an Additional Protein Layer

Enveloped viruses of several families contain an additional protein layer that mediates interactions of the genome-containing structure with the viral envelope. In the simplest case, a single viral structural protein, termed the matrix protein, welds an internal ribonucleoprotein to the envelope (Fig. 4.21B). This arrangement is found in members of several groups of (−) strand RNA viruses (Fig. 4.5C; Appendix, Fig. 17 and 31). Retrovirus particles also contain an analogous, membrane-associated matrix protein (MA), which makes contact with an internal capsid in which the viral ribonucleoprotein is encased.

Because the internal capsids or nucleocapsids of these more complex enveloped viruses are not in direct contact with the envelope, the organization and symmetry of internal structures are not necessarily evident from the external appearance of the surface glycoprotein layer. Nor does the organization of these proteins reflect the symmetry of the capsid. For example, the outer surface of all retroviruses appears roughly spherical with an array of projecting knobs or spikes, regardless of whether the internal core is spherical, cylindrical, or cone shaped. Likewise, influenza virus particles, which contain helical nucleocapsids, are generally roughly spherical particles but are highly pleomorphic with long, filamentous forms common in clinical isolates (Box 4.9). Although the interior architecture of these enveloped viruses cannot be described in detail, high-resolution structures have been obtained for several matrix proteins. In conjunction with the results of *in vitro* assays for lipid binding and mutational analyses, such information allows molecular modeling of matrix protein-envelope interactions.

Internal proteins that mediate contact with the viral envelope are not embedded within the lipid bilayer but rather bind to its inner face. Such viral proteins are targeted to, and interact with, membranes by means of specific signals, which are described in more detail in Chapter 12. For example, a posttranslationally added fatty acid chain is important for membrane binding of the MA proteins of most retroviruses. The human immunodeficiency virus type 1 MA protein was the first viral peripheral membrane protein for which a high-resolution structure was determined, initially by nuclear

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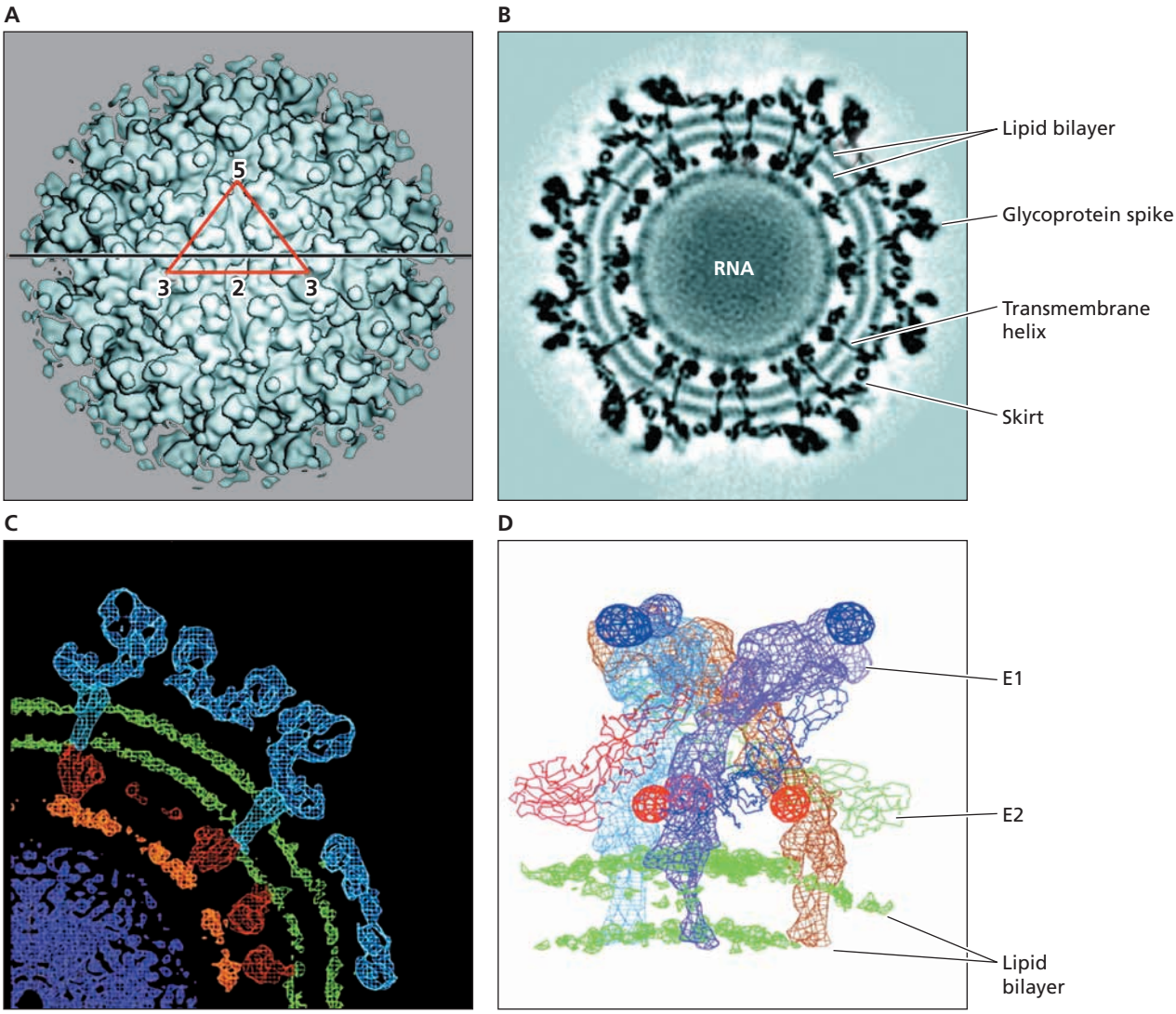


Figure 4.24 Structure of a simple enveloped virus, Sindbis virus. **(A)** The surface structure of Sindbis virus, a member of the alphavirus genus of the *Togaviridae*, at 20-Å resolution determined by cryo-EM. The boundaries of the structural (asymmetric) unit are demarcated by the red triangle, on which the icosahedral five-, three-, and twofold axes of rotational symmetry are indicated. This outer surface is organized as a $T = 4$ icosahedral shell studded with 80 spikes, each built from three copies of each of the transmembrane glycoproteins E1 and E2. These spikes are connected by a thin, external protein layer, termed the skirt. **(B)** Cross section through the density map at 11-Å resolution along the black line shown in panel A. The lipid bilayer of the viral envelope is clearly defined at this resolution, as are the transmembrane domains of the glycoproteins. **(C)** Different layers of the particle, based on the fitting of a high-resolution structure of the E1 glycoprotein into a 9-Å reconstruction of the virus particle. The nucleocapsid (red) surrounds the genomic (+) strand RNA. The RNA is the least well-ordered feature in the reconstruction, although segments (orange) lying just below the capsid protein appear to be ordered by interaction with this protein. The C protein penetrates the inner leaflet of the lipid membrane, where it interacts with the cytoplasmic domain of the E2 glycoprotein (blue). The membrane is spanned by rod-like structures that are connected to the skirt by short stems. **(D)** The structure of the E1 and E2 glycoproteins, obtained by fitting the crystal structure of the closely related Semliki Forest virus E1 glycoprotein into the 11-Å density map and assigning density unaccounted for to the E2 glycoprotein. The view shown is around a quasithree-fold symmetry axis, with the three E2 glycoprotein molecules in a trimeric spike colored light blue, dark blue, and brown and the E1 molecules shown as backbone traces colored red, green, and magenta. The portions of the proteins that cross the lipid bilayer are helical, twisting around one another in a left-handed coiled coil. Adapted from W. Zhang et al., *J Virol* **76:**11645–11658, 2002, with permission. Courtesy of Michael Rossmann, Purdue University

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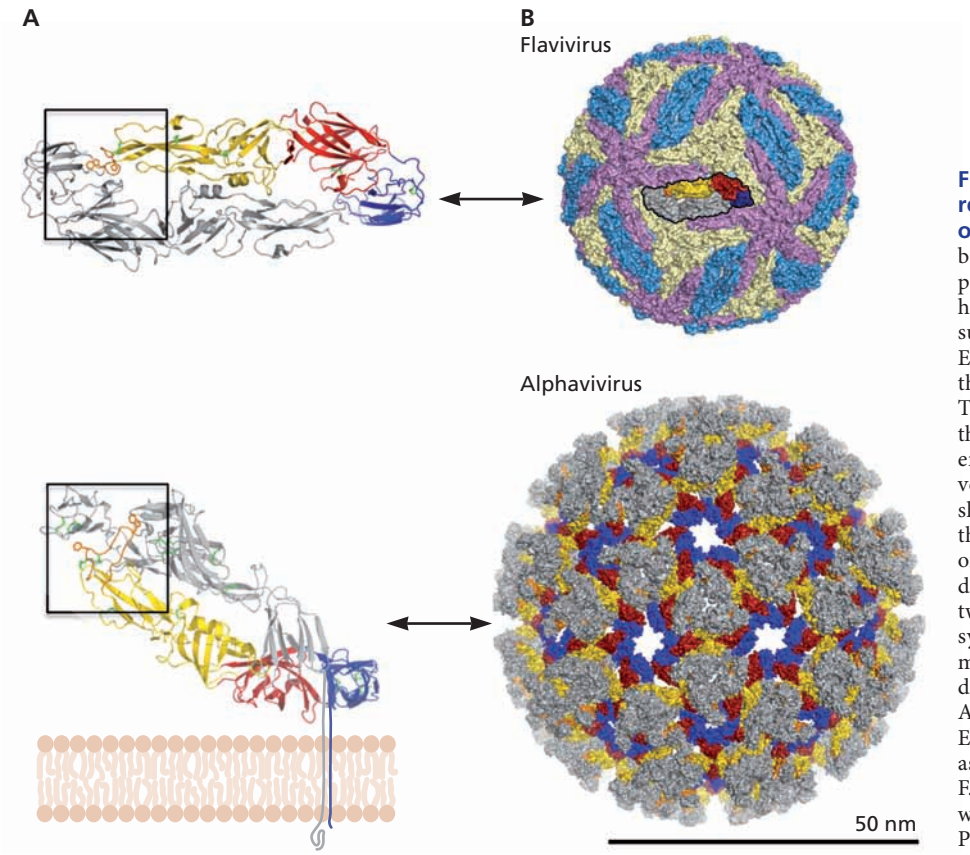


Figure 4.25 Conserved topology and regular packing of envelope proteins of small, (1) strand RNA viruses. (A) Ribbon diagrams of the flavivirus envelope (E) protein dimer (top) and the alphavirus E1/E2 heterodimer (bottom), with one E and the E2 subunit shown in gray. Conserved domains of E and E1 are colored red, yellow, and blue with the fusion loops required for entry in orange. The parallel and perpendicular orientations to the membrane of the flavivirus and alphavirus envelope proteins, respectively, result in the very different appearances of these particles shown in panel B. **(B)** Surface renderings on the same scale, showing the regular packing of flavivirus and alphavirus envelope protein dimers of flavivirus. The dimers related by two-, three-, and fivefold axes of icosahedral symmetry are colored blue, pale yellow, and mauve, respectively, except for the central dimer depicted, which is colored as in panel A. In the 80 spikes of the alphavirus envelope, E2 is shown gray and E1 colored by domain as in panel A. Adapted from M.-C. Vaney and F. A. Rey, *Cell Microbiol* **13:**1451–1459, 2011, with permission. Courtesy of F. A. Rey, Institut Pasteur.

magnetic resonance methods. Subsequent analysis by X-ray crystallography established that MA is a trimer. Each MA molecule comprises a compact, globular domain of α-helices capped by a β-sheet that contains positively charged amino acids necessary for membrane binding. As illustrated in the model of MA oriented on a membrane shown in Fig. 4.26, the basic residues form a positively charged surface, positioned for interaction with phospholipid head groups on the inner surface of the envelope. The matrix proteins of (−) strand RNA viruses such as vesicular stomatitis virus and influenza virus also contain positively charged domains required for membrane binding, despite having three-dimensional folds that are quite different from those of retroviral MA proteins (and from one another).

Large Viruses with Multiple Structural Elements

Virus particles that house large DNA genomes are structurally far more complex than any considered in previous sections. Such particles comprise obviously distinct components with different symmetries and/or multiple layers. In this section, we illustrate various ways in which multiple structural elements can be combined, using as examples bacteriophage T4, herpes simplex virus type 1, the poxvirus vaccinia virus, and giant viruses such as mimivirus. As we shall see, some of these elements are dedicated to specific functions.

Bacteriophage T4

Bacteriophage T4, which has been studied for more than 50 years, is the classic example of an architecturally elaborate virus that contains parts that exhibit both icosahedral and helical symmetry. The T4 particle, which is built from ~50 of the proteins encoded in the ~170-kbp double-stranded DNA genome, is a structurally elegant machine tailored for active delivery of the genome to host cells. The most striking feature is the presence of morphologically distinct and functionally specialized structures, notably the head containing the genome and a long tail that terminates in a baseplate from which six long tail fibers protrude (Fig. 4.27A).

The head of the mature T4 particle, an elongated icosahedron, is built from hexamers of a single viral protein (gp23*). In contrast to the other capsids considered so far, two T numbers are needed to describe the organization of gp23* in the two end structures ($T = 13$) and in the elongated midsection ($T = 20$). As in adenoviral capsids, the pentamers that occupy the vertices contain a different viral protein, and additional proteins reside on the outer or inner surfaces of the icosahedral shell (Fig. 4.27B). One of the 12 vertices is

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BOX 4.8

DISCUSSION

A virus particle with different structures in different hosts

Throughout this chapter, we describe mature virus particles in terms of a single structure: "the" structure. However, it is important to appreciate that the architectures reported are those of particles isolated and examined under a single set of specific conditions. Recent studies of the flavivirus dengue virus, an important human pathogen, illustrate the conformational plasticity of some mature virus particles.

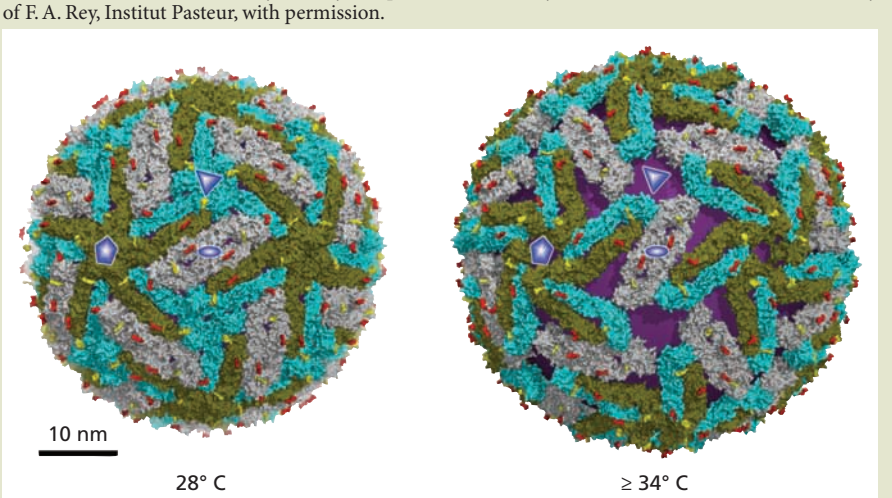
The organization of the single dengue virus envelope glycoprotein, E, described in the text (Fig. 4.25) is that observed in particles propagated in cells of the mosquito vector maintained at 28°C. As noted previously, the E protein dimers are tightly packed and icosahedrally ordered. However, the epitopes for binding of antibodies that neutralize the virus at 37°C are either partially or entirely buried, suggesting that the virus particle might undergo temperature-dependent conformational transitions. Indeed, when particles are exposed to temperatures encountered in the mammalian host (e.g., 37°C), they do expand significantly, exposing segments of the underlying membrane, and the E protein interactions are altered (compare the left and right panels in the figure). In fact, particles exposed to higher temperatures are heterogeneous, and the example shown in the figure (right) represents but one of multiple forms, identified during selection of particles for three-dimensional reconstruction. Because a heterogeneous population of particles with less well-ordered E protein dimers represent the form of dengue virus recognized by the human immune system, these observations have important implications for the design of dengue virus vaccines.

Fibriansah G, Ng TS, Kostyuchenko VA, Lee J, Lee S, Wang J, Lok SM. 2013. Structural changes in dengue virus when exposed to a temperature of 37°C. *J Virol* **87:**7585–7592.

Rey FA. 2013. Dengue virus: two hosts, two structures. *Nature* **497:**443–444.

Zhang X, Sheng J, Plevka P, Kuhn RJ, Diamond MS, Rossmann MG. 2013. Dengue structure differs at the temperatures of its human and mosquito hosts. *Proc Natl Acad Sci U S A* **110:**6795–6799.

Structures of dengue virus particles at 28°C (left) and at ≥34°C (right), with the axes of five-, three-, and twofold rotational symmetry indicated by a pentagon, triangle, and ellipse, respectively. The E protein dimers that lie at the twofold axes are shown in gray and the other dimers with one subunit in green and one in cyan. The particles exposed to higher temperatures are characterized by exposed patches of membrane (purple) and significant reduction of dimer contacts at the threefold axes of icosahedral symmetry. Adapted from F. A. Rey, *Nature* **497:**443–444, 2013. Courtesy of F. A. Rey, Institut Pasteur, with permission.



occupied by a unique structure termed the connector, which joins the head to the tail. Such structures are derived from the nanomachine that pulls DNA into immature heads termed the **portal**. Portals are a characteristic feature of the capsids of other families of DNA-containing bacteriophages, as well as of herpesviruses.

In contrast to the head, the ~100-nm-long tail, which comprises two protein layers, exhibits helical symmetry (Fig. 4.27A). The outer layer is a contractile sheath that functions in injection of the viral genome into host cells. The tail is connected to the head via a hexameric ring and at its other end to a complex, dome-shaped structure termed the baseplate, where it carries the cell-puncturing spike. Both long and short tail fibers project from the baseplate. The former, which are bent, are the primary receptor-binding structures of bacteriophage T4. As discussed in Chapter 5, remarkable conformational changes induced upon receptor binding by the tips of the long fibers are transmitted via the baseplate to initiate injection of the DNA genome.

Herpesviruses

Members of the *Herpesviridae* exhibit a number of unusual architectural features. More than half of the >80 genes of herpes simplex virus type 1 encode proteins found in the large (~200-nm-diameter) virus particles. These proteins are components of the envelope from which glycoprotein spikes project or of two distinct internal structures. The latter are the capsid surrounding the DNA genome and the protein layer encasing this structure, called the **tegument** (Fig. 4.28A).

A single protein (VP5) forms both the hexons and the pentons of the $T = 16$ icosahedral capsid of herpes simplex virus type 1 (Fig. 4.28B). Like the structural units of the smaller simian virus 40 capsid, these VP5-containing assemblies make direct contact with one another. However, the larger

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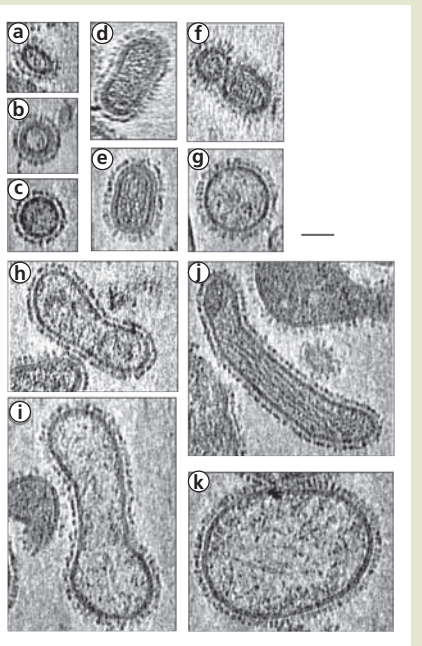
BOX 4.9

DISCUSSION

The extreme pleomorphism of influenza A virus, a genetically determined trait of unknown function

Some enveloped viruses vary considerably in size and shape. For example, the particles of paramyxoviruses, such as measles and Sendai viruses, range in size from 120 to up to 540 nm in diameter and may contain multiple copies of the (−) strand RNA genome in helical nucleocapsids of different pitch. Influenza A virus particles exhibit even more extreme pleomorphism: they appear spherical, elliptical, or filamentous, and all forms come in a wide range of sizes (see the figure). Laboratory isolates are primarily filamentous but adopt the other morphologies when adapted to propagation in the laboratory.

Several lines of evidence indicate that the filamentous phenotype is genetically determined. For example, the particles of some influenza A virus isolates are primarily filamentous, whereas those of other isolates are not. Furthermore, genetic experiments have demonstrated that the viral matrix (M1 or M2) proteins, which are encoded within the same segment of the (−) strand, segmented RNA genome, govern formation of filamentous particles. However, what determines the choice between assembly of filamentous versus spherical particles is not understood. Nor is the physiological significance of the filamentous particles, despite their predominance in clinical isolates. It has been speculated that these forms might facilitate cell-to-cell transmission of virus particles through the respiratory mucosa of infected hosts.



Cryo-electron tomogram sections of influenza A virus particles (strain PR8). Bar = 50 nm. Adapted from D. B. Nayak et al., *Virus Res* **143:**147–161, 2000. Courtesy of D. B. Nayak, University of California, Los Angeles, with permission.

herpesviral capsid is stabilized by additional proteins, VP19C and VP23, which form triplexes that link the major structural units. A second property shared with polyomaviruses (and papillomaviruses) is stabilization of the particle by disulfide bonds, which covalently link VP5, VP19C, and VP23 to one another and to specific tegument proteins. Although apparently a typical and quite simple icosahedral shell, this viral capsid is in fact an asymmetric structure: 1 of the 12 vertices is occupied not by a VP5 penton but by a unique structure termed the portal. The portal comprises 12 copies of the UL6 protein and is a squat, hollow cylinder that is wider at one end and surrounded by a two-tiered ring at the wider end (Fig. 4.28C). The asymmetry of the herpesviral capsid and the incorporation of the portal have important implications for the mechanism of assembly (see Chapter 13).

The tegument contains >20 viral proteins, viral RNAs, and cellular components. A few tegument proteins are icosahedrally ordered, as a result of direct contacts with the structural units of the capsid (Fig. 4.28D). However, some tegument proteins are **not** uniformly distributed around the capsid. Rather, they are concentrated on one side, where they form a well-defined cap-like structure (Fig. 4.28A). As this unanticipated asymmetry of herpesviral particles has been viewed only at low resolution, the molecular organization of the cap is not yet understood.

Poxviruses

Particles of poxviruses such as vaccinia virus also comprise multiple, distinct structural elements. However, none of these exhibit obvious icosahedral or helical symmetry, in contrast to components of bacteriophage T4 or herpesvirus particles. A second distinctive feature is that two forms of infectious particles are produced in vaccinia virus-infected cells (see Chapter 13), termed mature virions and enveloped extracellular virions, which differ in the number and origin of membranes. Mature virions are large, enveloped structures (~350 to 370 × 250 × 270 nm) comprising at least 75 proteins that appear in the electron microscope as brick or barrel shaped (depending on the orientation) (Fig. 4.29A). Whether one or two envelopes are present has been the subject of long-standing debate. However, there is now a growing consensus for the presence of just a single membrane. A number of internal structures have been observed by examination of thin sections through purified particles or by cryo-electron tomography (Fig. 4.29B). These features include the core wall, which surrounds the central core that contains the ~200-kbp DNA genome, and lateral bodies. Remarkably, the core contains some 20 enzymes with many different activities. Although viral proteins that contribute to these various structures have been identified, our understanding of vaccinia virus architecture remains at low resolution.

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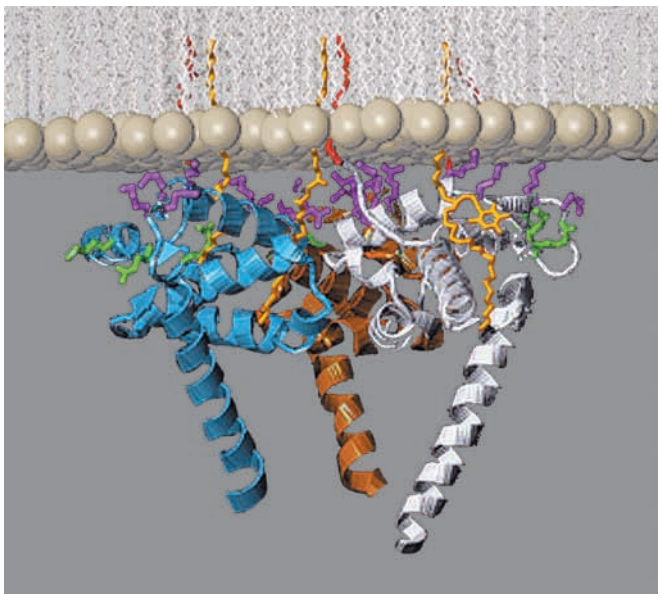


Figure 4.26 Model of the interaction of human immunodeficiency virus type 1 MA protein with the membrane. The membrane is shown with the polar head groups of membrane lipids in beige. This model is based on the X-ray crystal structure of recombinant MA protein synthesized in *E. coli* and consequently lacking the N-terminal (myristate) 14-carbon fatty acid normally added in human cells. The three monomers in the MA trimer are shown in different colors. Basic residues in the β-sheet that caps the globular α-helical domain are magenta or green. Substitution of those shown in magenta impairs reproduction of the virus in cells in culture. The positions of the N-terminal myristate (red), of MA, and of phosphatidylinositol 4,5-bisphosphate (orange) from the membrane were modeled schematically. From C. P. Hill et al., *Proc Natl Acad Sci U S A* **93:**3099–3104, 1996, with permission. Courtesy of C. P. Hill and W. I. Sundquist, University of Utah.

Giant Viruses

Since the discovery of mimivirus (first reported in 1992), a number of so-called "giant" viruses have been identified (Box 1.10). As might be anticipated, such viruses include previously unknown architectural details.

Despite their very large size (vertex-to-vertex diameter of ~5,000 Å), mimivirus particles exhibit some familiar structural features, notably icosahedral symmetry and a capsid built from a major capsid protein with the β-barrel jelly roll topology. Distinctive features include the dense coat of long fibers that cover the entire external surface with the exception of one vertex (Fig. 4.1). This vertex comprises a unique starfish-shaped structure, termed the stargate, the most distinctive structural element of this virus. The stargate opens within host *Acanthamoeba polyphaga* cells to facilitate release of the double-stranded DNA genome and also nucleates assembly of progeny virus particles.

The even larger pandoravirus and pithovirus, with double-stranded DNA genomes of 2.8 and 0.6 Mbp and particle lengths of ~1 and ~1.5 μm, respectively, bear little resemblance to any virus described previously. They share an amphora-like shape; a dense, striated outer layer surrounding an internal lipid membrane; and a rather featureless internal compartment. The apex of pithovirus is closed by a protruding "cork" with a hexagonal, grid-like appearance (Fig. 4.30). This unusual structure is expelled following uptake of virus particles into host cells by phagocytosis to allow fusion of the viral membrane with that of the cellular vacuole. Unprecedented assemblies specialized for release of the viral genome in host cells may prove to be a characteristic property of the very large viruses.

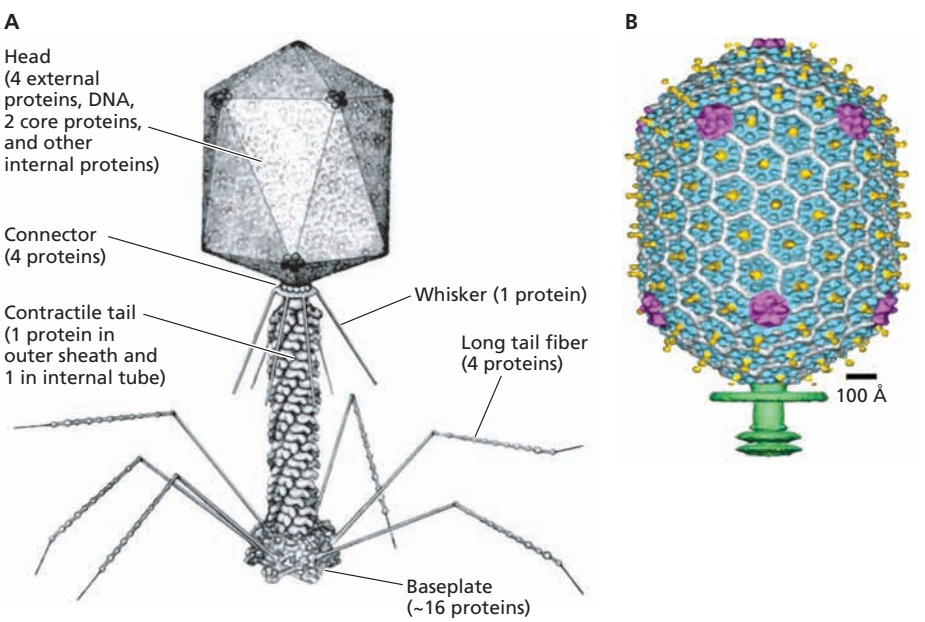


Figure 4.27 Morphological complexity of bacteriophage T4. (A) A model of the virus particle. Adapted from P. G. Leiman et al., *Cell Mol Life Sci* **60:**2356–2370, 2003, with permission. **(B)** Structure of the head (22-Å resolution) determined by cryo-EM, with the major capsid proteins shown in blue (gp23*) and magenta (gp24*), the protein that protrudes from the capsid surface in yellow, the protein that binds between gp23* subunits in white, and the beginning of the tail in green. Adapted from A. Fokine et al., *Proc Natl Acad Sci U S A* **101:**6003–6008, 2004, with permission. Courtesy of M. Rossmann, Purdue University.

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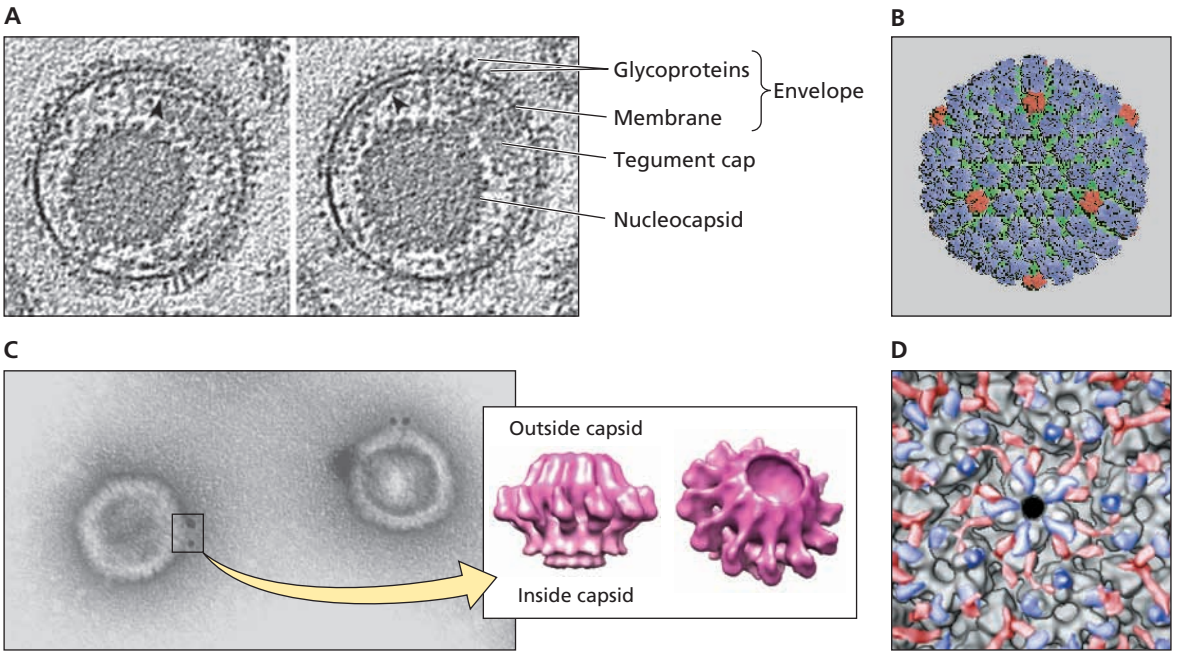


Figure 4.28 Structural features of herpesvirus particles. (A) Two slices through a cryo-electron tomogram of a single herpes simplex virus type 1 particle, showing the eccentric tegument cap. Adapted from K. Grunewald et al., *Science* **302:**1396–1398, 2003, with permission. **(B)** Reconstruction of the herpes simplex virus type 1 nucleocapsid (8.5-Å resolution), with VP5 hexamers and pentamers colored blue and red, respectively, and the triplexes that reinforce the connections among these structural units in green. VP5 hexamers, but not pentamers, are capped by a hexameric ring of VP26 protein molecules (not shown). Adapted from Z. H. Zhou et al., *Science* **288:**877–880, 2000, with permission. **(C)** The single portal of herpes simplex virus type 1 nucleocapsids visualized by staining with an antibody specific for the viral UL6 protein conjugated to gold beads is shown to the left. The gold beads are electron dense and appear as dark spots in the electron micrograph. They are present at a single vertex in each nucleocapsid, which therefore contains one portal. A 16-Å reconstruction of the UL6 protein portal based on cryo-EM is shown on the right. Adapted from B. L. Trus et al., *J Virol* **78:**12668–12671, 2004, with permission. **(D)** Interactions of two tegument proteins with the simian cytomegalovirus nucleocapsid. Tegument proteins that bind to hexons plus pentons and to triplexes are shown in blue and red, respectively. These proteins were visualized by cryo-EM, image reconstruction (to 22-Å resolution), and difference mapping of nucleocapsids purified from the nucleus and cytoplasm of virus-infected cells. The latter carry the tegument, but the former do not. Adapted from W. W. Newcomb et al., *J Virol* **75:**10923–10932, 2001, with permission. Courtesy of A. C. Steven, National Institutes of Health (A, C, and D) and W. Chiu, Baylor College of Medicine (B).

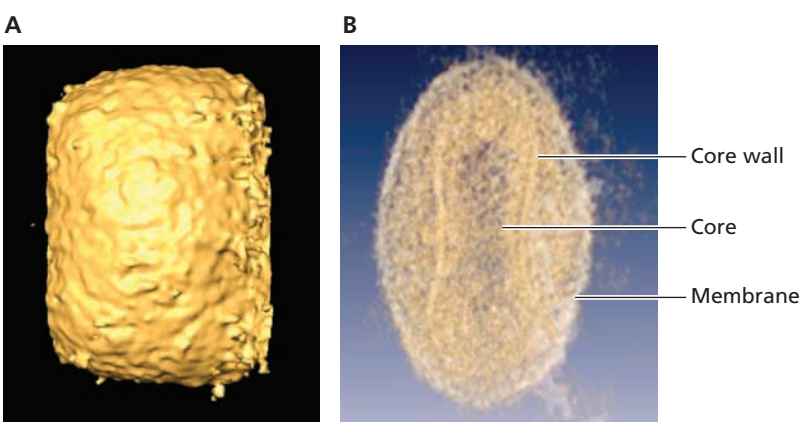


Figure 4.29 Structural features of the poxvirus vaccinia virus. (A) Surface rendering of intracellular mature particles of vaccinia virus reconstructed from cryo-electron tomograms showing the brick shape and irregular protrusions from the surface. **(B)** Translucent visualization of the reconstructed particle volume showing the dumbbell-shaped core and external membrane. Adapted from M. Cyrklaff et al., *Proc Natl Acad Sci U S A* **102:**2772–2777, 2005, with permission. Courtesy of J. L. Carrascosa, Universida Autonoma de Madrid. See also http://www.vacciniamodel.com.

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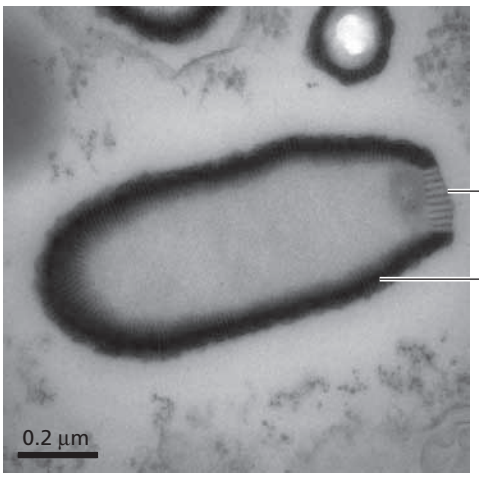


Figure 4.30 Morphology of pithovirus. The virus *Pithovirus sibericum* was isolated following culture of a suspension of soil from a sample of permafrost collected in 2000 in Siberia with the ameba *Acanthamoeba castellani*. Shown is an electron micrograph of a particle observed in infected ameba late in the infectious cycle following ultrathin sectioning of fixed cells and negative staining. Courtesy of Chantal Abergel and Jean-Michel Claverie, Aix-Marseille Université.

Other Components of Virions

Some virus particles comprise only the nucleic acid genome and structural proteins necessary for protection and delivery into a host cell. However, many contain additional viral proteins or other components, which are generally present at much lower concentrations but are essential or important for establishing an efficient infectious cycle (Table 4.3).

Enzymes

Many types of virus particles contain enzymes necessary for synthesis of viral nucleic acids. These enzymes generally catalyze reactions unique to virus-infected cells, such as synthesis of viral mRNA from an RNA template or of viral DNA from an RNA template. However, virions of vaccinia virus contain a DNA-dependent RNA polymerase, analogous to cellular RNA polymerases, as well as several enzymes that modify viral RNA transcripts (Table 4.3). This complement of enzymes is necessary because transcription of the viral double-stranded DNA genome takes place in the cytoplasm of infected cells, whereas cellular DNA-dependent RNA polymerases and the RNA-processing machinery are restricted to the nucleus. Other types of enzymes found in virus particles include integrase, cap-dependent endonuclease, and proteases. The proteases sever covalent connections within polyproteins or precursor proteins from which some virus particles assemble, a reaction that is necessary for the production of infectious particles.

Table 4.3 Some virion enzymes

Virus	Protein	Function(s)
Adenovirus		
Human adenovirus type 2	L3 23k	Protease; production of infectious particles
Herpesvirus		
Herpes simplex virus type 1	VP24	Protease; capsid maturation for genome encapsidation
	UL13	Protein kinase
	Vhs	RNase
Orthomyxovirus		
Influenza A virus	P proteins	RNA-dependent RNA polymerase; synthesis of viral mRNA and vRNA; cap-dependent endonuclease
Poxvirus		
Vaccinia virus[a]	DNA-dependent RNA polymerase (8 subunits)	Synthesis of viral mRNA
	Poly(A) polymerase (2 subunits)	Synthesis of poly(A) on viral mRNA
	Capping enzyme (2 subunits)	Addition of 5′ caps to viral pre-mRNA
	DNA topoisomerase	Sequence-specific nicking of viral DNA
	Proteases 1 and 2	Virus particle morphogenesis
Reovirus		
Reovirus type 1	λ2	Guanylyltransferase
	λ3	Double-stranded RNA-dependent RNA polymerase
Retrovirus		
Human immunodeficiency virus type 1	Pol	Reverse transcriptase; proviral DNA synthesis
	IN	Integrase; integration of proviral DNA into the cellular genome
	PR	Protease; production of infectious particles
Rhabdovirus		
Vesicular stomatitis virus	L	RNA-dependent RNA polymerase; synthesis of viral mRNA and vRNA

[a]Vaccinia virions contain some 20 enzymes, only a few of which are listed.

Other Viral Proteins

More-complex particles may also contain additional viral proteins that are not enzymes but nonetheless are important for an efficient infectious cycle. Among the best characterized are the protein primers for viral genome replication that are covalently linked to the genomes of picornaviruses such as

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poliovirus and adenoviruses. Others include several tegument proteins of herpesviruses, such as the VP16 protein, which activates transcription of viral immediate-early genes to initiate the viral program of gene expression. The cores of vaccinia virus also contain proteins that are essential for transcription of viral genes, as they allow recognition of viral early promoters. Other herpesvirus tegument proteins induce the degradation of cellular mRNA or block cellular mechanisms by which viral proteins are presented to the host's immune system. Retroviruses with complex genomes, such as human immunodeficiency virus type 1, contain additional proteins required for efficient viral reproduction in certain cell types, for example, Nef and Vpr. These proteins are discussed in Volume II, Chapter 6.

Nongenomic Viral Nucleic Acid

The presence of a viral nucleic acid genome has long been recognized as a definitive feature of virions. However, it is now clear that adenovirus, herpesvirus, and retrovirus particles also contain viral mRNAs. This property was first described for the mRNA that encodes the viral Env protein in avian sarcoma virus particles. A limited set of viral mRNAs, as well as some cellular and artificial reporter mRNAs, are packaged into particles of human cytomegalovirus, a betaherpesvirus that is an important human pathogen (Volume II, Appendix, Fig. 11), in proportion to their intracellular concentrations during the period of assembly of progeny particles. It is therefore difficult to exclude the possibility that their presence is a functionally irrelevant and secondary consequence of nonspecific nucleic acid binding by viral structural proteins. However, the viral mRNAs are translated soon after delivery to the host cell, and one has been demonstrated to encode a chemokine decoy that could modulate host immune responses.

Cellular Macromolecules

Virus particles can also contain cellular macromolecules that play important roles during the infectious cycle, such as the cellular histones that condense and organize polyomaviral and papillomaviral DNAs. Because they are formed by budding, enveloped viruses can readily incorporate cellular proteins and other macromolecules. For example, cellular glycoproteins may not be excluded from the membrane from which the viral envelope is derived. Furthermore, as a bud enlarges and pinches off during virus assembly, internal cellular components may be trapped within it. Enveloped viruses are also generally more difficult to purify than naked viruses. As a result, preparations of these viruses may be contaminated with vesicles formed from cellular membranes. Indeed, analysis by the sensitive proteomic methods provided by mass spectrometry has identified from 50 to 100 cellular proteins in purified, enveloped particles of various herpesviruses, filoviruses, and rhabdoviruses. Consequently, it can be difficult to distinguish cellular components specifically incorporated into enveloped virus particles from those trapped randomly or copurifying with the virus. Nevertheless, in some cases it is clear that cellular molecules are important components of virus particles: these molecules are reproducibly observed at a specific stoichiometry and can be shown to be essential or play important roles in the infectious cycle (Box 4.10). The cellular components captured in retrovirus particles have been particularly well characterized.

The primer for initiation of synthesis of the (−) strand DNA during reverse transcription in retroviral genomes is a specific cellular transfer RNA (tRNA). This RNA is incorporated into virus particles by virtue of its binding to a specific sequence in the RNA genome and to reverse transcriptase. A variety of cellular proteins are also present in some retroviral particles. One of the most unusual properties of human immunodeficiency virus type 1 is the presence of cellular cyclophilin A, a **chaperone** that assists or catalyzes protein folding. This protein is the major cytoplasmic member of a ubiquitous family of peptidyl-prolyl isomerases. It is incorporated within human immunodeficiency virus type 1 particles via specific interactions with the central portion of the capsid (CA) protein, and it catalyzes isomerization of a single Gly-Pro bond in this protein. Although incorporation of cyclophilin A is not a prerequisite for assembly, particles that lack this cellular chaperone have reduced infectivity. It is thought that in human cells, cyclophilin provides protection against an intrinsic antiviral defense mechanism (see Volume II, Chapter 7). Cellular membrane proteins, such as Icam-1 and Lfa1 (see Chapter 5), can also be incorporated in the viral envelope and can contribute to attachment and entry of retroviral particles. They may also influence pathogenesis (see Volume II, Chapter 6). Other cellular proteins assembled into viral particles, such as ADP-ribosylation factor 1 (Arf1) found in herpesviral particles, may facilitate intracellular transport (Box 4.10).

Cellular components present in virus particles may serve to facilitate virus reproduction, a property exemplified by the cellular tRNA primers for retroviral reverse transcription. However, incorporation of cellular components can also provide antiviral defense. As discussed in Volume II, Chapters 3 and 6, packaging of a cellular enzyme that converts cytosine to uracil (Apobec3) into retrovirus particles at the end of one infectious cycle leads to degradation and hypermutation of viral DNA synthesized early in the next cycle of infection.

It is clear from these examples that virus particles contain a surprisingly broad repertoire of biologically active molecules that are delivered to their host cells. This repertoire is undoubtedly larger than we presently appreciate, and the contributions of many components of virus particles to the infectious cycles of many viruses have yet to be established.

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BOX 4.10

EXPERIMENTS

Cellular proteins in herpes simplex virus type 1 particles: distinguishing passengers from the crew

Powerful and sensitive methods of mass spectrometry have revolutionized cataloguing of the components of virus particles, particularly the structural proteins of viruses with large genomes. This approach has also been invaluable in identifying cellular proteins that are also present, with considerable numbers detected in the particles of several families of enveloped viruses. The sizeable populations (50 to 100) of such cellular proteins emphasize the importance of distinguishing those proteins that contribute to viral reproduction from those incorporated by chance.

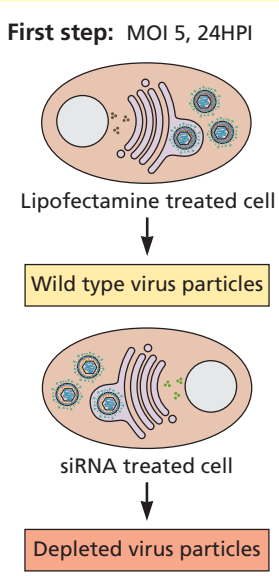
Purified extracellular herpes simplex virus type 1 particles were found by mass spectrometry to contain 49 cellular proteins. This set included proteins reported to be present in the particles of other herpesviruses, such as cyclophilin A and actin, and many not detected previously. A two-step RNA interference screen was developed to assess the contributions to viral reproduction of cellular proteins introduced into cells via virus particles. In these experiments, a phenotypically wild-type virus with a capsid protein fused to the green fluorescent protein was exploited to allow rapid and accurate measurement of yields of extracellular virus particles. This assay was validated by the demonstration that small interfering RNA (siRNA)-mediated knockdown of the viral protein VP16, which is required for efficient expression of viral immediate-early genes, significantly decreased yield, whereas a scrambled version of this siRNA did not. Inhibition of synthesis of 24 of the 49 cellular proteins by RNA interference reduced virus yield to a statistically significant degree but did not impair viability of human cells used as host.

Particles depleted of 15 of the proteins were then prepared by infection of siRNA-treated cells (see the figure) and used to infect new cells, in which production of the same cellular protein was or was not inhibited. Removal of 13 of these proteins from particles reduced virus yield significantly, even in cells that continued to synthesize the proteins. These observations established unequivocally that some cellular proteins incorporated into herpesviral particles promote the next cycle of reproduction. This result is surprising, as a viral particle seems likely to contain many fewer molecules of these cellular proteins than the host cell. Perhaps viral reproduction is facilitated by delivery of particular cellular proteins already associated with components of viral particles or delivery of the proteins to specific sites during entry. Functions of such proteins include intracellular transport (e.g., Arf1 and Rab5A) and signaling (e.g., Mif and Cd59).

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Two-step method to assess the importance of cellular proteins incorporated into herpes simplex virus type 1 particles. In the first step, particles were isolated from human cells treated with siRNA against 1 of 15 cellular proteins or, as a control, treated with transduction agent (Lipofectamine) alone. As shown, control and siRNA-treated cells were then infected by depleted and control particles, and viral yield measured. The results obtained with particles depleted for VP16 (positive control) or the cellular proteins Mif and Cd59 are summarized. MOI, multiplicity of infection; HPI, hours postinfection. Adapted from C. Stegen et al., *PLos One* **8:**e53276, 2013, with permission.



Second step: Infection of new cells MOI 0.1, 48HPI

	Wild type virus particles		Depleted virus particles	
	Lipofectamine treated cell	siRNA treated cell	Lipofectamine treated cell	siRNA treated cell
Presence of the protein in:				
Cells	+	−	+	−
Virus particles	+	+	−	−
Protein depleted:				
VP16	1.0	0.1	0.3	0.05
Mif	1.0	0.15	0.25	0.18
Cd59	1.0	0.5	0.55	0.35

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Perspectives

Virus particles are among the most elegant and visually pleasing structures found in nature, as illustrated by the images presented in this chapter. Now that many structures of particles or their components have been examined, we can appreciate the surprisingly diverse architectures they exhibit. Nevertheless, the simple principles of their construction proposed more than 50 years ago remain pertinent: with few exceptions, the capsid shells that encase and protect nucleic acid genomes are built from a small number of proteins arranged with helical or icosahedral symmetry.

The detailed views of nonenveloped virus particles provided by X-ray crystallography emphasize just how well these protein shells provide protection of the genome during passage from one host cell or organism to another. They have also identified several mechanisms by which identical or nonidentical subunits can interact to form icosahedrally symmetric structures. More-elaborate virus particles, which may contain additional protein layers, a lipid envelope carrying viral proteins, and enzymes or other proteins necessary to initiate the infectious cycle, pose greater challenges to the structural biologist. Indeed, for many years we possessed only schematic views of these structures, deduced from negative-contrast electron microscopy and biochemical or genetic methods of analysis. In the previous edition, we noted the power and promise of continuing refinements in methods of cryo-EM (or cryo-electron tomography), image reconstruction, and difference imaging. In the intervening period of just 5 years, these techniques have attained atomic-level resolution, providing remarkable views of large viruses with multiple components, viral envelopes, and, in some cases, the organization of genomes within particles. The structural descriptions of ever-increasing numbers of viruses representing diverse families have also allowed unique insights into evolutionary relationships among seemingly disparate viruses or viral proteins.

These extraordinary advances notwithstanding, important challenges remain, most obviously the visualization of structures that do not exhibit simple symmetry (or are not constructed from components that do). These structures include many genomes and the particles of some large viruses (e.g., poxviruses). The more recently described giant viruses, such as pandoravirus, with particles so large that they can be seen by light microscopy, also pose new technical challenges and suggest that unanticipated structural principles remain to be elucidated.

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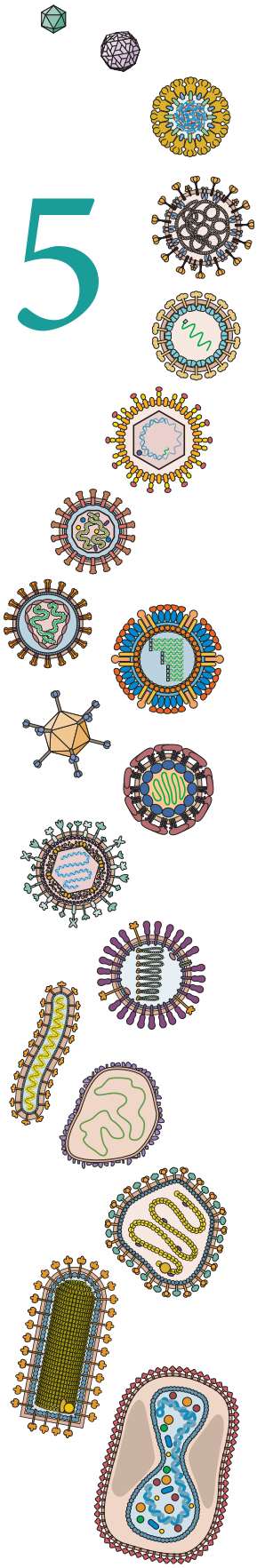
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5 Attachment and Entry

Introduction

Attachment of Virus Particles to Cells
- General Principles
- Identification of Receptors for Virus Particles
- Virus-Receptor Interactions

Entry into Cells
- Uncoating at the Plasma Membrane
- Uncoating during Endocytosis
- Membrane Fusion
- Movement of Viral and Subviral Particles within Cells
- Virus-Induced Signaling via Cell Receptors

Import of Viral Genomes into the Nucleus
- Nuclear Localization Signals
- The Nuclear Pore Complex
- The Nuclear Import Pathway
- Import of Influenza Virus Ribonucleoprotein
- Import of DNA Genomes
- Import of Retroviral Genomes

Perspectives

References

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Who hath deceived thee so often as thyself?
BENJAMIN FRANKLIN

Introduction

Because viruses are obligate intracellular parasites, the genome must enter a cell for the viral reproduction cycle to occur. The physical properties of the virion are obstacles to this seemingly simple goal. Virus particles are too large to diffuse passively across the plasma membrane. Furthermore, the viral genome is encapsidated in a stable coat that shields the nucleic acid as it travels through the harsh extracellular environment. These impediments must all be overcome during the process of viral entry into cells. Encounter of a virus particle with the surface of a susceptible host cell induces a series of events that lead to entry of the viral genome into the cytoplasm or nucleus. The first step in entry is adherence of virus particles to the plasma membrane, an interaction mediated by binding to a specific **receptor** molecule on the cell surface.

The receptor plays an important role in **uncoating**, the process by which the viral genome is exposed, so that gene expression and genome replication can begin. Interaction of the virus particle with its receptor may initiate conformational changes that prime the capsid for uncoating. Alternatively, the receptor may direct the virus particle into endocytic pathways, where uncoating may be triggered by low pH or by the action of proteases. These steps bring the genome into the cytoplasm, which is the site of replication of most RNA-containing viruses. The genomes of viruses that replicate in the nucleus are moved to that location by cellular transport pathways. Viruses that replicate in the nucleus include most DNA-containing viruses (exceptions include poxviruses and giant viruses), RNA-containing retroviruses, influenza viruses, and Borna disease virus.

Virus entry into cells is **not** a passive process but relies on viral usurpation of normal cellular processes, including endocytosis, membrane fusion, vesicular trafficking, and transport into the nucleus. Because of the limited functions encoded by viral genomes, virus entry into cells depends absolutely on cellular processes.

Attachment of Virus Particles to Cells

General Principles

Infection of cells by many, but not all, viruses requires binding to a receptor on the cell surface. Exceptions include viruses of yeasts and fungi, which have no extracellular phases, and plant viruses, which are thought to enter cells in which the cell wall has been physically damaged, for example by insects or farm machinery. A receptor is a cell surface molecule that binds the virus particle and participates in entry. It may induce conformational changes in the virus particle that lead to membrane fusion or penetration, and it may also transmit signals that cause uptake. The receptor may also bring the bound particle into endocytic pathways.

Receptors for viruses comprise a variety of cell surface proteins, carbohydrates, and lipids, all with functions in the cell unrelated to virus entry. Many virus receptors have been identified in the past 30 years and include immunoglobulin-like proteins, ligand-binding receptors, glycoproteins, ion channels, gangliosides, carbohydrates, proteoglycans, and integrins. The receptor may be the only cell surface molecule required for entry into cells, or an additional cell surface molecule, or **coreceptor**, may be needed (Box 5.1). Different receptors may serve for virus entry in diverse cell types, and unrelated viruses may bind to the same receptor (e.g., the Coxsackievirus and adenovirus receptor).

The receptor may determine the **host range** of a virus, i.e., its ability to infect a particular animal or cell culture. For example, poliovirus infects primates and primate cell cultures but not mice or mouse cell cultures. Mouse cells synthesize a protein that is homologous to the poliovirus receptor,

PRINCIPLES *Attachment and entry*

- Virus particles are too large to diffuse across the plasma membrane, and thus entry must be an active process.
- Virus particles bind to receptors on their host cells to initiate entry.
- The cell receptor may determine the host range and tissue tropism of the virus.
- Viruses may bind multiple distinct receptors, and individual cellular proteins may be receptors for multiple viruses.
- Enveloped virus particles bind via their transmembrane glycoproteins; nonenveloped virus particles bind via the capsid surface or projections from the capsid.
- Attachment proteins may not lead to internalization and viral reproduction but may still be important for dissemination in the host.
- Some viruses uncoat at the plasma membrane, while others do so from intracellular vesicles.
- Many viruses enter host cells by the same cellular pathways used to take up macromolecules.
- The entry mechanism used by a particular virus may differ depending on the nature of the target cell.
- Viral particles and subviral particles depend on the cytoskeleton to move within an infected cell.
- Binding of virions to cell receptors may activate signaling pathways that facilitate virus entry and movement, or produce cellular responses that enhance virus propagation and/or affect pathogenesis.
- For viruses that undergo replication in the nucleus, import can occur either through use of the nuclear pore complex or during cell division, when the nuclear membrane breaks down.

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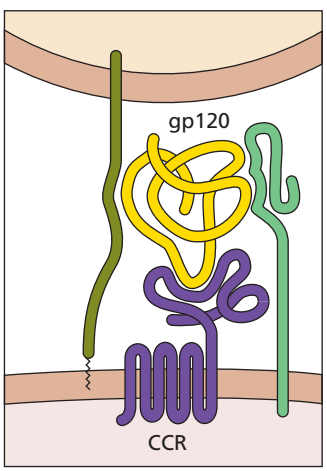
BOX 5.1

TERMINOLOGY

Receptors and coreceptors

By convention, the first cell surface molecule that is found to be essential for virus binding is called its **receptor**. Sometimes, such binding is not sufficient for entry into the cell. When binding to another cell surface molecule is needed, that protein is called a **coreceptor**. For example, human immunodeficiency virus binds to cells via a receptor, CD4, and then requires interaction with a second cell surface protein such as CXCR4, the coreceptor.

In practice, the use of receptor and coreceptor can be confusing and inaccurate. A particular cell surface molecule that is a coreceptor for one virus may be a receptor for another. Distinguishing receptors and coreceptors by the order in which they are bound is difficult to determine experimentally and is likely to be influenced by cell type and multiplicity of infection. Furthermore, as is the case for the human immunodeficiency viruses, binding only to the coreceptor may be sufficient for entry of some members. Usage of the terms "receptor" and "coreceptor" is convenient when describing virus entry, but the appellations may not be entirely precise.



but sufficiently different that poliovirus cannot attach to it. In this example, the poliovirus receptor is **the** determinant of poliovirus host range. However, production of the receptor in a particular cell type does **not** ensure that virus reproduction will occur. Some primate cell cultures produce the poliovirus receptor but cannot be infected. The restriction of viral reproduction in these cells is most probably due to a block in viral reproduction beyond the attachment step. Receptors can also be determinants of tissue **tropism**, the predilection of a virus to invade and reproduce in a particular cell type. However, there are many other determinants of tissue tropism. For example, the sialic acid residues on membrane glycoproteins or glycolipids, which are receptors for influenza virus, are found on many tissues, yet viral reproduction in the host is restricted. The basis of such restriction is discussed in Volume II, Chapter 2.

Our understanding of the earliest interactions of virus particles with cells comes almost exclusively from analysis of synchronously infected cells in culture. The initial association with cells is probably via electrostatic forces, as it is sensitive to low pH or high concentrations of salt, but higher affinity binding relies mainly on hydrophobic and other short-range forces between the viral and cellular surfaces. Although the **affinity** of a receptor for a single virus particle is low, the presence of multiple receptor-binding sites on the virion and the fluid nature of the plasma membrane allow engagement of multiple receptors. Consequently, the **avidity** (the strength conferred by multiple interactions) of virus particle binding to cells is usually very high. Binding can usually occur at 4°C (even though entry does not) as well as at body temperature (e.g., 37°C). Infection of cultured cells can therefore be synchronized by allowing binding to take place at a low temperature and then shifting the cells to a physiological temperature to allow the initiation of subsequent steps.

The first steps in virus attachment are governed largely by the probability that a virus particle and a cell will collide, and therefore by the concentrations of free particles and host cells. The rate of attachment can be described by the equation

$$dA/dt = k[V][H]$$

where A is attachment, t is time, and $[V]$ and $[H]$ are the concentrations of virus particles and host cells, respectively, and k is a constant that defines the rate of the reaction. It can be seen from this equation that if a mixture of viruses and cells is diluted after an adsorption period, subsequent binding of particles is greatly reduced. For example, a 100-fold dilution of the virus and cell mixture reduces the attachment rate 10,000-fold (i.e., $1/100 \times 1/100$). Dilution can be used to prevent subsequent virus adsorption and hence to synchronize an infection.

Many receptor molecules can move in the plasma membrane, leading to the formation of microdomains that differ in composition. Bound virus may therefore localize to specialized areas of the membrane such as lipid rafts, **caveolae**, or coated pits. Localization of virus particle-receptor complexes can also cause transmembrane signaling, changes in the cytoskeleton, and recruitment of clathrin.

Identification of Receptors for Virus Particles

The development of three crucial technologies in the past 30 years has enabled identification of many receptors for viruses. Production of monoclonal antibodies provided a powerful means of isolating and characterizing individual cell surface proteins. Hybridoma cell lines that secrete monoclonal antibodies that block virus attachment are

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obtained after immunizing mice with intact cells. Such antibodies can be used to purify the receptor protein by affinity chromatography.

A second technology that facilitated the identification of receptors was the development of DNA-mediated transformation. This method was crucial for isolating genes that encode receptors, following introduction of DNA from susceptible cells into nonsusceptible cells (Fig. 5.1). Cells that acquire DNA encoding the receptor and carry the corresponding protein on their surface are able to bind virus specifically. Clones of such cells are recognized and selected, for example, by the binding of receptor-specific monoclonal antibodies. The receptor genes can then be isolated from these selected cells by using a third technology, molecular cloning. The power of these different technologies can lead to rapid progress: the receptor for a newly identified Middle Eastern respiratory syndrome coronavirus was identified just 4 months after the first description of the virus (Box 5.2). Although these technologies have led to the identification of many cell receptors for viruses, each method has associated uncertainties (Box 5.3).

The availability of receptor genes has made it possible to investigate the details of receptor interaction with viruses by site-directed mutagenesis. Receptor proteins can be synthesized in heterologous systems and purified, and their properties can be studied *in vitro*, while animal cells producing altered receptor proteins can be used to test the effects of alterations on virus attachment. Because of their hydrophobic membrane-spanning domains, many of these cell surface proteins are relatively insoluble and difficult to work with.

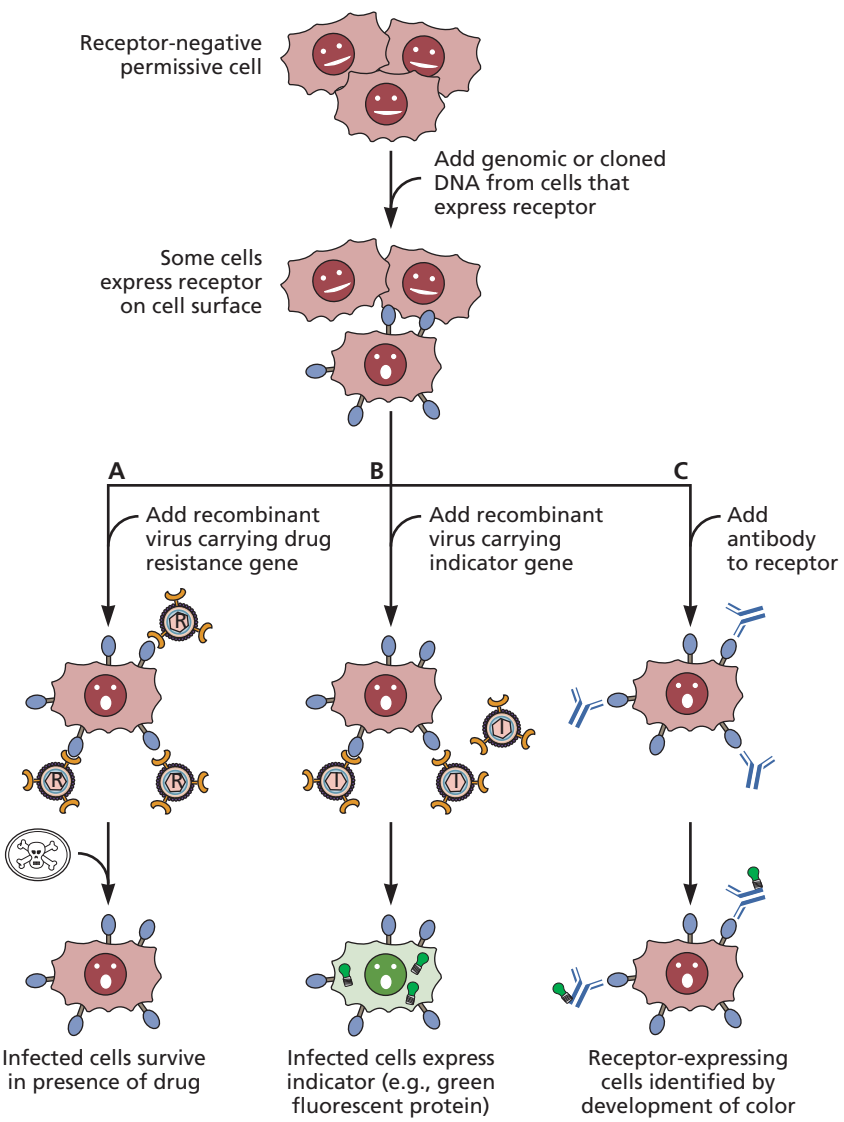


Figure 5.1 Experimental strategies for identification and isolation of genes encoding cell receptors for viruses. Genomic DNA or pools of DNA clones from cells known to synthesize the receptor are introduced into receptor-negative permissive cells. A small number of recipient cells produce the receptor. Three different strategies for identifying such rare receptor-expressing cells are outlined. **(A)** The cells are infected with a virus that has been engineered so that it carries a gene encoding drug resistance. Cells that express the receptor will become resistant to the drug. This strategy works only for viruses that persist in cells without killing them. **(B)** For lytic viruses, an alternative is to engineer the virus to express an indicator, such as green fluorescent protein or β-galactosidase. Cells that make the correct receptor and become infected with such viruses can be distinguished by a color change, such as green in the case of green fluorescent protein. **(C)** The third approach depends on the availability of an antibody directed against the receptor, which binds to cells that express the receptor gene. Bound antibodies can be detected by an indicator molecule. When complementary DNA (cDNA) cloned in a plasmid is used as the donor DNA, pools of individual clones (usually 10,000 clones per pool) are prepared and introduced individually into cells. The specific DNA pool that yields receptor-expressing cells is then subdivided, and the screening process is repeated until a single receptor-encoding DNA is identified.

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BOX 5.2

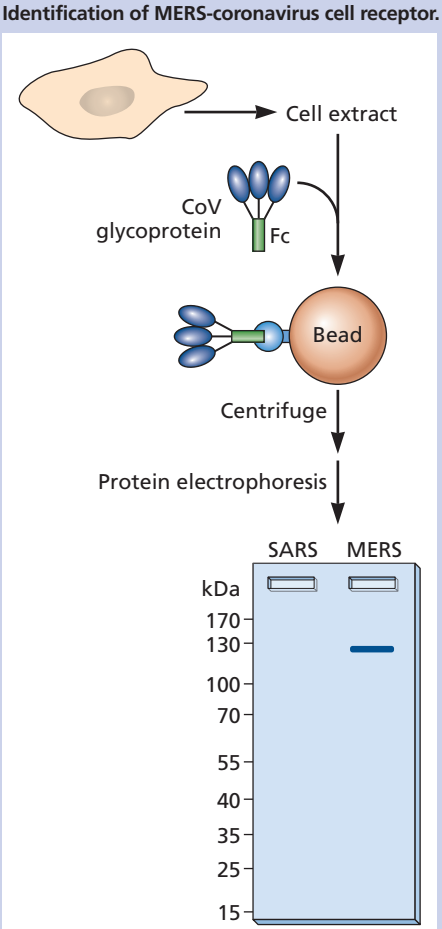
METHODS

Affinity isolation

To identify the receptor for the newly emerged Middle Eastern respiratory syndrome coronavirus, the gene encoding the viral spike glycoprotein gene was fused with sequences encoding the Fc domain of human IgG. The fusion protein was produced in cells and incubated with lysates of cells known to be susceptible to the virus, and the resulting complexes were fractionated by native polyacrylamide gel electrophoresis. A single polypeptide of ~110 kDa was obtained by this procedure. This polypeptide was excised from the polyacrylamide gel, and its amino acid sequence was determined by mass spectrometric analysis, identifying it as dipeptidyl peptidase 4. When this protein was subsequently synthesized in nonsusceptible Cos-7 cells by DNA-mediated transformation, the cells became susceptible to Middle Eastern respiratory syndrome coronavirus infection. That a single protein was identified by this procedure is remarkable: typically, this approach identifies many nonspecific binding proteins.

Raj VS, Mou H, Smits SL, Dekkers DH, Müller MA, Dijkman R, Muth D, Demmers JA, Zaki A, Fouchier RA, Thiel V, Drosten C, Rottier PJ, Osterhaus AD, Bosch BJ, Haagmans BL. 2013. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. *Nature* **495:**251–256.

Identification of MERS-coronavirus cell receptor.



Soluble extracellular protein domains (with the virus binding sites) have been essential for structural studies of receptor-virus interactions. Receptor genes have also been used to produce transgenic mice that synthesize receptor proteins. Such transgenic animals can serve as useful models in the study of human viral diseases.

Virus-Receptor Interactions

Animal viruses have multiple receptor-binding sites on their surfaces. Of necessity, one or more of the capsid proteins of nonenveloped viruses specifically interact with the cell receptor. Typically, these form projections from or indentations in the surface. Receptor-binding sites for enveloped viruses are provided by oligomeric type 1 integral membrane glycoproteins encoded by the viral genome that have been incorporated into the cell-derived membranes of virus particles. Although the details vary among viruses, most virus-receptor interactions follow one of several mechanisms illustrated by the best-studied examples described below.

Nonenveloped Viruses Bind via the Capsid Surface or Projections

Attachment via surface features: canyons and loops. Members of the enterovirus genus of the *Picornaviridae* include human polioviruses, coxsackieviruses, echoviruses, enteroviruses, and rhinoviruses. The receptor for poliovirus, CD155, was identified by using a DNA transformation and cloning strategy (Fig. 5.1). It was known that mouse cells cannot be infected with poliovirus, because they do not produce the receptor. Transfection of poliovirus RNA into mouse cells in culture leads to poliovirus reproduction, indicating that there is no intracellular block to virus multiplication. Introduction of human DNA into mouse cells confers susceptibility to poliovirus infection. The human gene recovered from receptor-positive mouse cells proved to encode CD155, a glycoprotein that is a member of the immunoglobulin (Ig) superfamily (Fig. 5.2).

Mouse cells are permissive for poliovirus reproduction, and susceptibility is limited **only** by the absence of CD155. Consequently, it was possible to develop a small-animal model for poliomyelitis by producing transgenic mice that synthesize this receptor. Inoculation of CD155 transgenic mice with poliovirus by various routes produces paralysis, as is observed in human poliomyelitis. These CD155-synthesizing mice were the first new animal model created by transgenic technology for the study of viral disease. Similar approaches have subsequently led to animal models for viral diseases caused by measles virus and echoviruses.

Rhinoviruses multiply primarily in the upper respiratory tract and are responsible for causing up to 50% of all common colds. Over 150 rhinovirus genotypes have been identified and classified on the basis of genome sequence into three species, A, B, and C. Rhinoviruses bind to at least three

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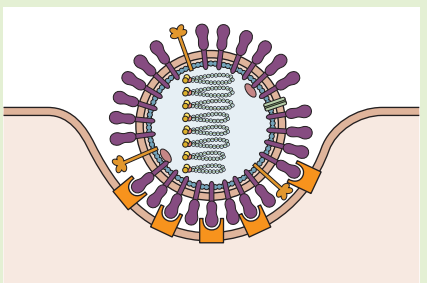
BOX 5.3

BACKGROUND

Criteria for identifying cell receptors for viruses

The use of monoclonal antibodies, molecular cloning, and DNA-mediated transformation provides a powerful approach for identifying cellular receptors for viruses, but each method has associated uncertainties. A monoclonal antibody that blocks virus attachment might recognize not the receptor but a closely associated membrane protein. To prove that the protein recognized by the monoclonal antibody **is** a receptor, DNA encoding the protein must be introduced into nonsusceptible cells to demonstrate that it can confer virus-binding activity. Any of the approaches outlined in Fig. 5.1 can result in identification of a cellular gene that encodes a putative receptor. However, the encoded protein might not be a receptor but may modify another cellular protein so that it can bind virus particles. One proof that the DNA codes for a receptor could come from the identification of a monoclonal antibody that blocks virus attachment and is directed against the encoded protein.

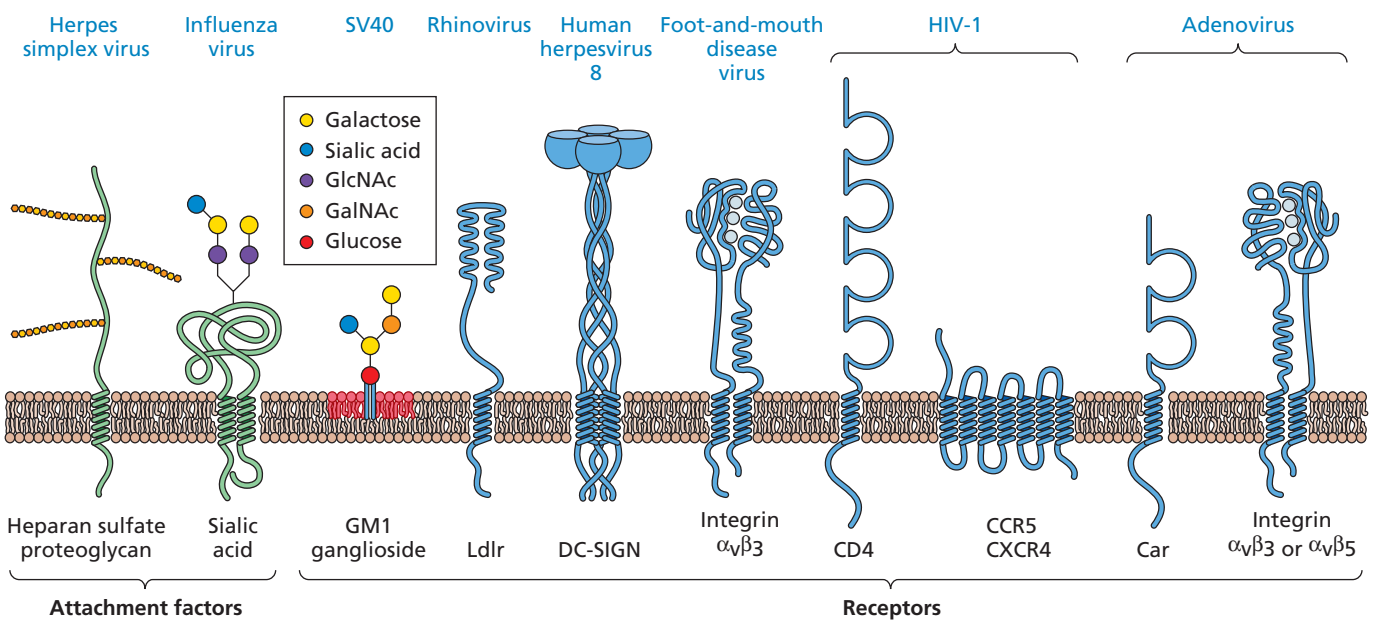
For some viruses, synthesis of the receptor on cells leads to binding but not infection. In such cases a coreceptor is required, either for internalization or for membrane fusion. The techniques of molecular cloning also can be used to identify coreceptors. For example, production of CD4 on mouse cells leads to binding of human immunodeficiency virus type 1 but not infection, because fusion of viral and cell membranes does not occur. To identify the coreceptor, a DNA clone was isolated from human cells that allowed membrane fusion catalyzed by the viral attachment protein in mouse cells synthesizing CD4.



different receptor molecules. The cell surface receptor bound by most A and B species rhinoviruses was identified by using a monoclonal antibody that blocks rhinovirus infection and that recognizes a cell surface protein. This monoclonal antibody was used to isolate a 95-kDa cell surface glycoprotein by affinity chromatography. Amino acid sequence analysis of the purified protein, which bound to rhinovirus *in vitro*, identified it as the integral membrane protein intercellular adhesion molecule 1 (Icam-1). Cell receptors for other rhinoviruses are the low-density lipoprotein receptor and cadherin-related family member 3.

The RNA genomes of picornaviruses are protected by capsids made up of four virus-encoded proteins, VP1, VP2, VP3, and VP4, arranged with icosahedral symmetry (see Fig. 4.12). The capsids of rhinoviruses and polioviruses have deep canyons surrounding the 12 5-fold axes of symmetry (Fig. 5.3),

Figure 5.2 Some cell attachment factors and receptors for viruses. Schematic diagrams of cell molecules that function during virus entry. GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; Ldlr, low-density lipoprotein receptor; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; Car, coxsackievirus-adenovirus receptor.



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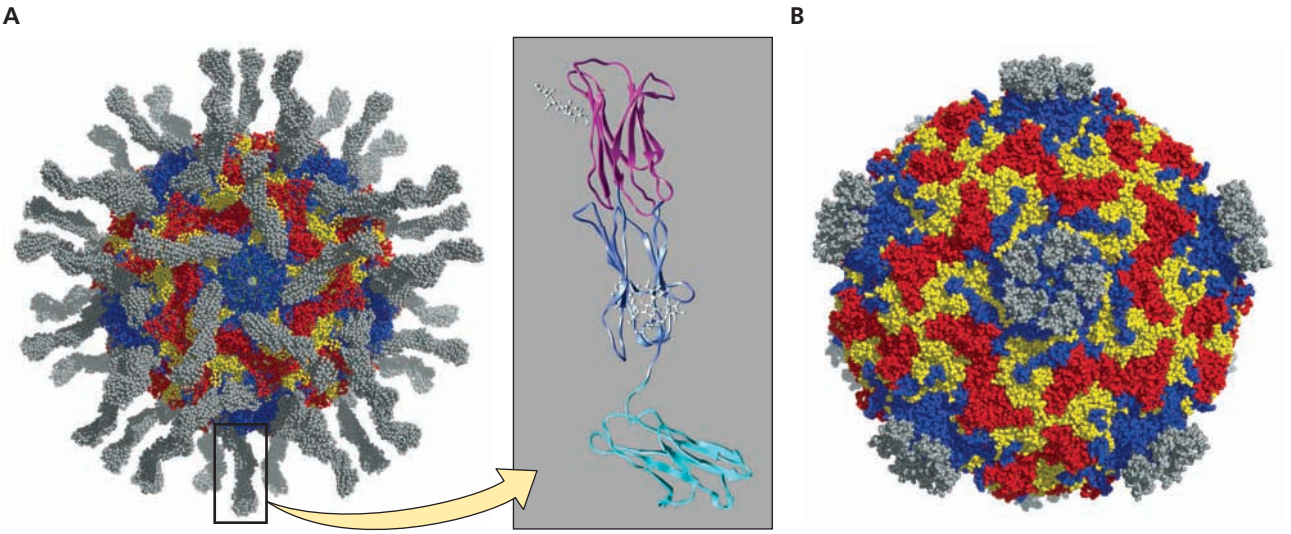


Figure 5.3 Picornavirus-receptor interactions. (A) Structure of poliovirus bound to a soluble form of CD155 (gray), derived by cryo-electron microscopy and image reconstruction. Capsid proteins are color coded (VP1, blue; VP2, yellow; VP3, red). One CD155 molecule is shown as a ribbon model in the panel to the right, with each Ig-like domain in a different color. The first Ig-like domain of CD155 (magenta) binds in the canyon of the viral capsid. **(B)** Structure of human rhinovirus type 2 bound to a soluble form of low-density lipoprotein receptor (gray). The receptor binds on the plateau at the 5-fold axis of symmetry of the capsid.

whereas cardioviruses and aphthoviruses lack this feature. The canyons in the capsids of some rhinoviruses and enteroviruses are the sites of interaction with cell surface receptors. Amino acids that line the canyons are more highly conserved than any others on the viral surface, and their substitution can alter the affinity of binding to cells. Poliovirus bound to a receptor fragment comprising CD155 domains 1 and 2 has been visualized in reconstructed images from cryo-electron microscopy. The results indicate that the first domain of CD155 binds to the central portion of the canyon in an orientation oblique to the surface of the virus particle (Fig. 5.3A).

Although canyons are present in the capsid of rhinovirus type 2, they are not the binding sites for the receptor, low-density lipoprotein receptor. Rather, this site on the capsid is located on the star-shaped plateau at the 5-fold axis of symmetry (Fig. 5.3B). Sequence and structural comparisons have revealed why different rhinovirus serotypes bind distinct receptors. A key VP1 amino acid, lysine, is conserved in all rhinoviruses that bind this receptor and interacts with a negatively charged region of low-density lipoprotein receptor. This lysine is not found in VP1 of rhinoviruses that bind Icam-1.

For picornaviruses with capsids that do not have prominent canyons, including coxsackievirus group A and foot-and-mouth disease virus, attachment is to VP1 surface loops that include amino acid sequence motifs recognized by their integrin receptors (Fig. 5.2).

Attachment via protruding fibers. The results of competition experiments indicated that members of two different virus families, group B coxsackieviruses and most human adenoviruses, share a cell receptor. This receptor is a 46-kDa member of the Ig superfamily called Car (coxsackievirus and adenovirus receptor). Binding to this receptor is not sufficient for infection by most adenoviruses. Interaction with a coreceptor, the α_v integrin $\alpha_v\beta_3$ or $\alpha_v\beta_5$, is required for uptake of the capsid into the cell by receptor-mediated endocytosis. An exception is adenovirus type 9, which can infect hematopoietic cells after binding directly to α_v integrins. Adenoviruses of subgroup B bind CD46, which is also a cell receptor for some strains of measles virus, an enveloped member of the *Paramyxoviridae*.

The nonenveloped DNA-containing adenoviruses are much larger than picornaviruses, and their icosahedral capsids are more complex, comprising at least 10 different proteins. Electron microscopy shows that fibers protrude from each adenovirus pentamer (Fig. 5.4; see the appendix in this volume, Fig. 1A). The fibers are composed of homotrimers of the adenovirus fiber protein and are anchored in the pentameric penton base; both proteins have roles to play in virus attachment and uptake.

For many adenovirus serotypes, attachment via the fibers is necessary but not sufficient for infection. A region comprising the N-terminal 40 amino acids of each subunit of the fiber protein is bound noncovalently to the penton base. The central shaft region is composed of repeating motifs of approximately 15 amino acids; the length of the shaft in different serotypes is determined by the number of these repeats. The three constituent shaft regions appear to form a rigid triple-helical structure in the trimeric fiber. The C-terminal 180 amino acids of each subunit interact to form a terminal knob. Genetic analyses and competition experiments indicate that determinants

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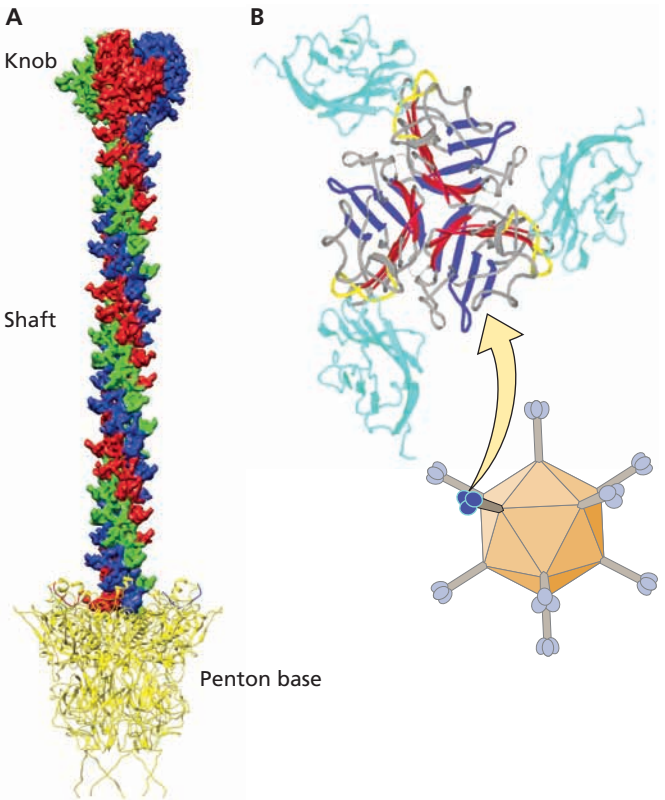


Figure 5.4 Structure of the adenovirus 12 knob bound to the Car receptor. (A) Structure of fiber protein, with knob, shaft, and tail domains labeled. Figure provided by Hong Zhou, University of California, Los Angeles, and Hongrong Liu, Hunan Normal University. **(B)** Ribbon diagram of the knob-Car complex as viewed down the axis of the viral fiber. The trimeric knob is in the center. The AB loop of the knob protein, which contacts Car, is in yellow. The first Ig-like domains of three Car molecules bound to the knob are colored blue. The binding sites of both molecules require trimer formation.

for the initial, specific attachment to host cell receptors reside in this knob. The structure of this receptor-binding domain bound to Car reveals that surface loops of the knob contact one face (Fig. 5.4).

Glycolipids, unusual cell receptors for polyomaviruses. The family *Polyomaviridae* includes simian virus 40 (SV40), mouse polyomavirus, and human BK virus. These viruses are unusual because they bind to ganglioside rather than protein receptors. Gangliosides are glycosphingolipids with one or more sialic acids linked to a sugar chain. There are over 40 known gangliosides, which differ in the position and number of sialic acid residues and are critical for virus binding. Simian virus 40, polyomavirus, and BK virus bind to three different types of ganglioside. Structural studies have revealed that sialic acid linked to galactose by an α(2,3) linkage binds to a pocket on the surface of the polyomavirus capsid (Fig. 5.5). Gangliosides are highly concentrated in lipid rafts (Chapter 2, Box 2.1) and participate in signal transduction, two properties

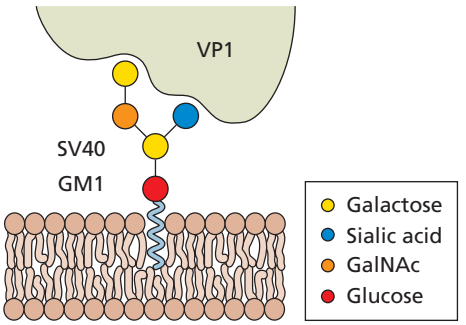


Figure 5.5 Interaction of polyomaviruses with ganglioside receptors. JC polyomavirus binds to a pentasaccharide with a terminal sialic acid linked by an α(2,6) bond to the penultimate galactose. The linear nature of this receptor differs from other branched polyomavirus ganglioside receptors. JC polyomavirus also appears to bind to a serotonin receptor for cell entry.

that are important during polyomavirus entry into cells. After binding a ganglioside, mouse polyomavirus interacts with α4β1 integrin to allow virus entry.

Enveloped Viruses Bind via Transmembrane Glycoproteins

The lipid membranes of enveloped viruses originate from those of the host cells. Membrane spanning viral proteins are inserted into these by the same mechanisms as cellular integral membrane proteins. Attachment sites on one or more of these envelope proteins bind to specific receptors. The two best-studied examples of enveloped virus attachment and its consequences are provided by the interactions of influenza A virus and the retrovirus human immunodeficiency virus type 1 with their receptors.

Influenza virus. The family *Orthomyxoviridae* comprises the three genera of influenza viruses, A, B, and C. These viruses bind to negatively charged, terminal sialic acid moieties present in oligosaccharide chains that are covalently attached to cell surface glycoproteins or glycolipids. The presence of sialic acid on most cell surfaces accounts for the ability of influenza virus particles to attach to many types of cell. The interaction of influenza virus with individual sialic acid moieties is of low affinity. However, the opportunity for multiple interactions among the numerous hemagglutinin (HA) molecules on the surface of the virus particle and multiple sialic acid residues on cellular glycoproteins and glycolipids results in a high overall avidity of the virus particle for the cell surface. The surfaces of influenza viruses were shown in the early 1940s to contain an enzyme that, paradoxically, removes the receptors for attachment from the surface of cells. Later, this enzyme was identified as the virus-encoded envelope glycoprotein neuraminidase, which cleaves the glycoside linkages of sialic acids (Fig. 5.6B). This enzyme is required for release of virus particles bound to the surfaces of

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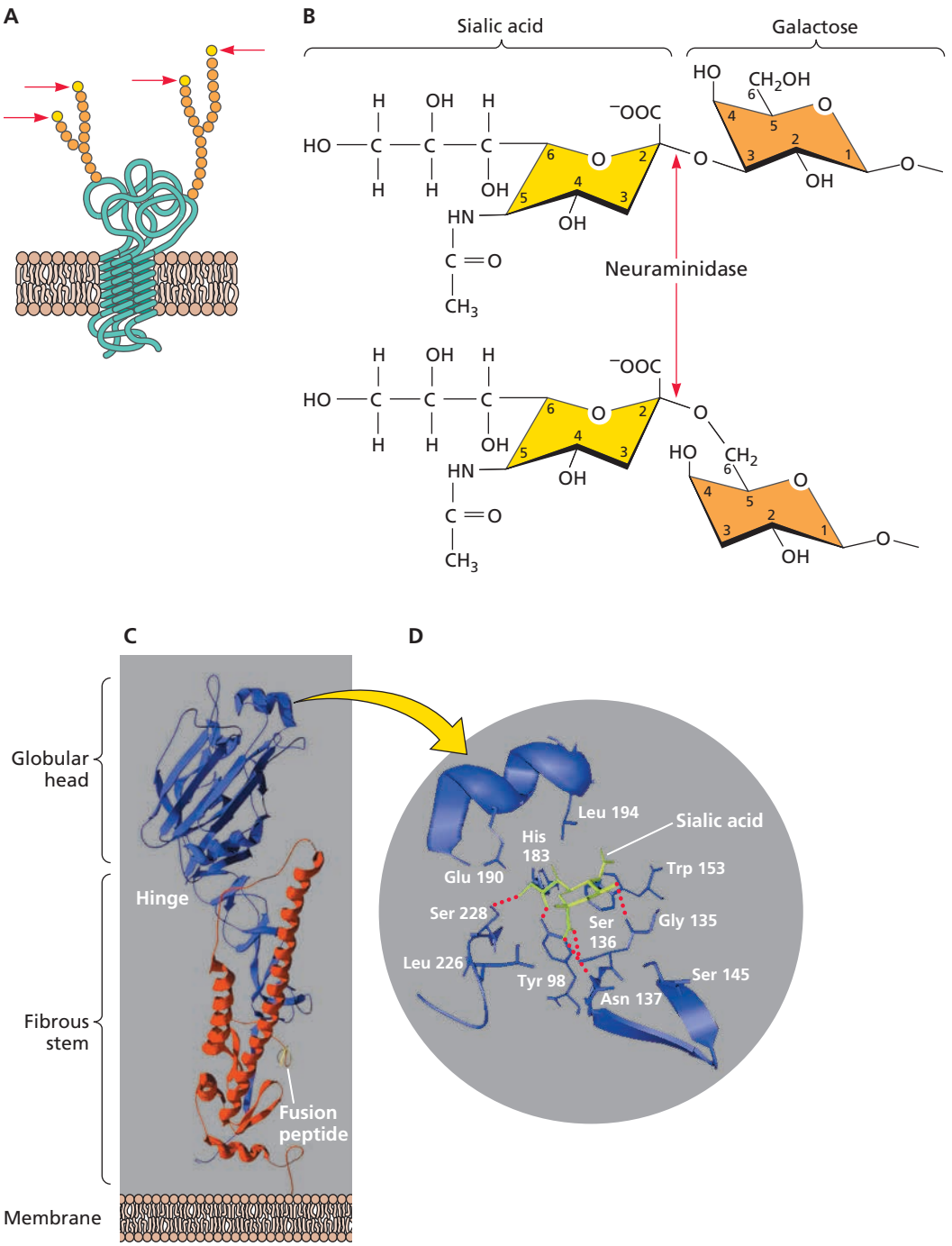


Figure 5.6 Interaction of sialic acid receptors with the hemagglutinin of influenza viruses. **(A)** An integral membrane glycoprotein; the arrows point to terminal sialic acid units that are attachment sites for influenza virus. **(B)** The structure of a terminal sialic acid moiety that is recognized by the viral envelope protein hemagglutinin. Sialic acid is attached to galactose by an α(2,3) (top) or an α(2,6) (bottom) linkage. The site of cleavage by the influenza virus envelope glycoprotein neuraminidase is indicated. The sialic acid shown is *N*-acetylneuraminic acid, which is the preferred receptor for influenza A and B viruses. These viruses do not bind to 9-*O*-acetyl-*N*-neuraminic acid, the receptor for influenza C viruses. **(C)** HA monomer modeled from the X-ray crystal structure of the natural trimer. HA1 (blue) and HA2 (red) subunits are held together by a disulfide bridge as well as by many noncovalent interactions. The fusion peptide at the N terminus of HA2 is indicated (yellow). **(D)** Close-up of the receptor-binding site with a bound sialic acid molecule. Side chains of the conserved amino acids that form the site and hydrogen-bond with the receptor are included.

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infected cells, facilitating virus spread through the respiratory tract (Volume II, Chapter 9).

Influenza virus HA is the viral glycoprotein that binds to the cell receptor sialic acid. The HA monomer is synthesized as a precursor that is glycosylated and subsequently cleaved to form HA1 and HA2 subunits. Each HA monomer consists of a long, helical stalk anchored in the membrane by HA2 and topped by a large HA1 globule, which includes the sialic acid-binding pocket (Fig. 5.6C, D). While attachment of all influenza A virus strains requires sialic acid, strains vary in their affinities for different sialyloligosaccharides. For example, human virus strains bind preferentially sialic acids attached to galactose via an α(2,6) linkage, the major sialic acid present on human respiratory epithelium (Fig. 5.6B). Avian virus strains bind preferentially to sialic acids attached to galactose via an α(2,3) linkage, the major sialic acid in the duck gut epithelium. Amino acids in the sialic acid-binding pocket of HA (Fig. 5.6D) determine which sialic acid is preferred and can therefore influence viral host range. It is thought that an amino acid change in the sialic acid-binding pocket of the 1918 influenza virus, which may have evolved from an avian virus, allowed it to recognize the α(2,6)-linked sialic acids that predominate in human cells.

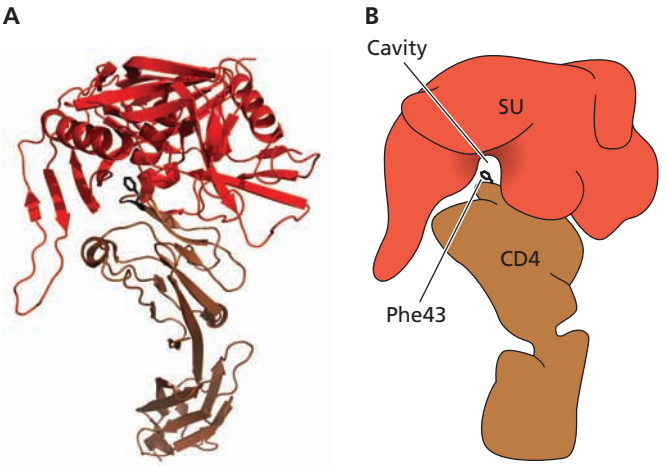


Figure 5.7 Interaction of human immunodeficiency virus type 1 SU with its cell receptor, CD4. (A) Ribbon diagram of SU (red) bound to CD4 (brown), derived from X-ray crystallographic data. The side chain of CD4 Phe43 is shown. **(B)** Cartoon of the CD4-SU complex. Mutagenesis has identified CD4 Phe43 as a residue critical for binding to SU. Phe43 is shown penetrating the hydrophobic cavity of SU. This amino acid, which makes 23% of the interatomic contacts between CD4 and SU, is at the center of the interface and appears to stabilize the entire complex.

Human immunodeficiency virus type 1. Animal retroviruses have long been of interest because of their ability to cause a variety of serious diseases, especially cancers (caused by oncogenic retroviruses) and neurological disorders (caused by lentiviruses). The acquired immunodeficiency syndrome (AIDS) pandemic has focused great attention on the lentivirus human immunodeficiency virus type 1 and its close relatives. The cell surface receptors of this virus have been among the most intensively studied and currently are the best understood.

When examined by electron microscopy, the envelopes of human immunodeficiency virus type 1 and other retroviruses appear to be studded with "spikes" (see Fig. 4.19). These structures are composed of trimers of the single viral envelope glycoprotein, which bind the cell receptor (Fig. 5.7). The monomers of the spike protein are synthesized as heavily glycosylated precursors that are cleaved by a cellular protease to form SU and TM. The latter is anchored in the envelope by a single membrane-spanning domain and remains bound to SU by numerous noncovalent bonds.

The cell receptor for human immunodeficiency virus type 1 is CD4 protein, a 55-kDa rodlike molecule that is a member of the Ig superfamily and has four Ig-like domains (Fig. 5.2). A variety of techniques have been used to identify the site of interaction with human immunodeficiency virus type 1, including site-directed mutagenesis and X-ray crystallographic studies of a complex of CD4 bound to the viral attachment protein SU (Fig. 5.7). The interaction site for SU in domain 1 of CD4 is in a region analogous to the site in CD155 that binds to poliovirus. Remarkably, two viruses with entirely different architectures bind to analogous surfaces of these Ig-like domains.

The atomic structure of a complex of human immunodeficiency virus type 1 SU, a two-domain fragment of CD4, and a neutralizing antibody against SU has been determined by X-ray crystallography (Fig. 5.7). The CD4-binding site in SU is a deep cavity, and the opening of this cavity is occupied by CD4 amino acid Phe43, which is critical for SU binding. Comparison with the structure of SU in the absence of CD4 indicates that receptor binding induces conformational changes in SU. These changes expose binding sites on SU for the chemokine receptors, which are required for fusion of viral and cell membranes (see "Uncoating at the Plasma Membrane" below).

Alphaherpesviruses. The alphaherpesvirus subfamily of the *Herpesviridae* includes herpes simplex virus types 1 and 2, pseudorabies virus, and bovine herpesvirus. Initial contact of these viruses with the cell surface is made by low-affinity binding of two viral glycoproteins, gC and gB, to glycosaminoglycans (preferentially heparan sulfate), abundant components of the extracellular matrix (Fig. 5.8). This interaction concentrates virus particles near the cell surface and facilitates subsequent attachment of the viral glycoprotein gD to an integral membrane protein, which is required for entry into the cell (Fig. 5.8). Members of at least two different protein families serve as entry receptors for alphaherpesviruses. One of these families, the nectins, comprises the poliovirus receptor CD155 and related proteins, yet another example of

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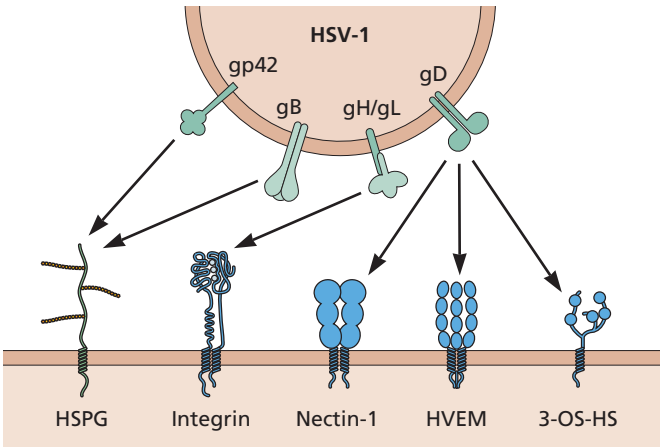


Figure 5.8 Cell receptors for herpes simplex virus type 1. Four viral glycoproteins, gp42, gB, gH/gL, and gD are shown binding with attachment molecule HSPG (heparin sulfate proteoglycan) or cell receptors (integrin, nectin-1, HVEM [herpesvirus entry mediator], 3-O-sulfate heparin sulfate). Virus entry does not require all interactions.

receptors shared by different viruses. When members of these two protein families are not present, 3-O-sulfated heparan sulfate can serve as an entry receptor for these viruses.

Multiple and Alternative Receptors

One type of receptor is not sufficient for infection by some viruses. Decay-accelerating protein (CD55), a regulator of the complement cascade, is the cell receptor for many enteroviruses, but infection also requires the presence of a coreceptor. Coxsackievirus A21 can bind to cell surface decay-accelerating protein, but this interaction does not lead to infection unless Icam-1 is also present. It is thought that Icam-1 inserts into the canyon where it triggers capsid uncoating. Some viruses bind to different cell receptors, depending on the nature of the virus isolate or the cell line. Often passage of viruses in cell culture selects variants that bind heparin sulfate. Infection of cells with foot-and-mouth disease virus type A12 requires the RGD-binding integrin $\alpha_v\beta_3$. However, the receptor for the O strain of this virus, which has been extensively passaged in cell culture, is not integrin $\alpha_v\beta_3$ but cell surface heparan sulfate. On the other hand, the type A12 strain cannot infect cells that lack integrin $\alpha_v\beta_3$, even if heparan sulfate is present. In a similar way, adaptation of Sindbis virus to cultured cells has led to the selection of variants that bind heparan sulfate. When receptors are rare, viruses that can bind to the more abundant glycosaminoglycan are readily selected.

Cell Surface Lectins and Spread of Infection

Cell surface **lectins** may bind to glycans present in viral glycoproteins, leading to dissemination within the host. An example is the lectin Dc-sign (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin), a tetrameric C-type lectin present on the surface of dendritic cells. This lectin binds high-mannose, N-linked glycans, such as those produced in insect cells. Viruses that reproduce in insects are delivered to the human skin via a bite and may bind and sometimes infect dendritic cells. These cells then carry the viruses to other parts of the body, particularly lymph nodes. However, not all viruses that bind Dc-sign replicate in insect cells. In humans, Dc-sign on the surface of dendritic cells binds human immunodeficiency virus type 1 virus particles, but cell entry does not take place. When the dendritic cells migrate to the lymph node, infectious virus is released where it can enter and reproduce in T cells. While the interaction of human immunodeficiency virus type 1 with Dc-sign is nonproductive, it leads to viral dissemination in the host.

Entry into Cells

Uncoating at the Plasma Membrane

The particles of many enveloped viruses, including members of the family *Paramyxoviridae* such as Sendai virus and measles virus, fuse directly with the plasma membrane at neutral pH. These virions bind to cell surface receptors via a viral integral membrane protein (Fig. 5.9). Once the viral and cell membranes have been closely juxtaposed by this receptor-ligand interaction, fusion is induced by a second viral glycoprotein known as fusion (F) protein, and the viral nucleocapsid is released into the cell cytoplasm (Fig. 5.10).

F protein is a type I integral membrane glycoprotein (the N terminus lies outside the viral membrane) with similarities to influenza virus HA in its synthesis and structure. It is a homotrimer that is synthesized as a precursor called F0 and cleaved during transit to the cell surface by a host cell protease to produce two subunits, F1 and F2, held together by disulfide bonds. The newly formed N-terminal 20 amino acids of the F1 subunit, which are highly hydrophobic, form a region called the **fusion peptide** because it inserts into target membranes to initiate fusion. Viruses with the uncleaved F0 precursor can be produced in cells that lack the protease responsible for its cleavage. Such virus particles are noninfectious; they bind to target cells but the viral genome does not enter. Cleavage of the F0 precursor is necessary for fusion, not only because the fusion peptide is made available for insertion into the plasma membrane, but also to generate the metastable state of the protein that can undergo the conformational rearrangements needed for fusion.

Because cleaved F-protein-mediated fusion can occur at neutral pH, it must be controlled, both to ensure that virus particles fuse with only the appropriate cell and to prevent aggregation of newly assembled virions. The fusion peptide of F1 is buried between two subunits of the trimer in the pre-fusion protein. Conformational changes in F protein lead to refolding of the protein, assembly of an α-helical coiled

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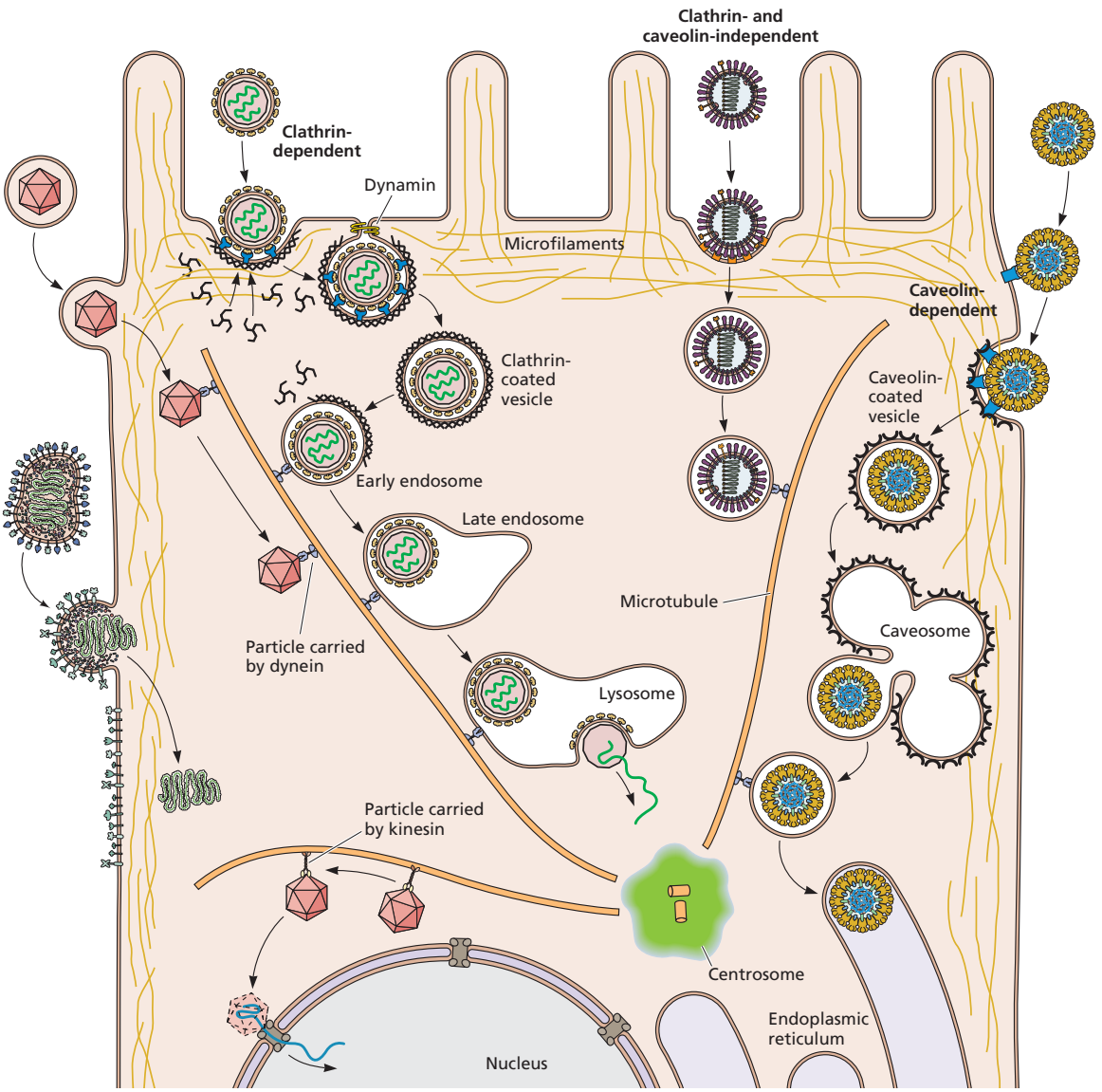


Figure 5.9 Virus entry and movement in cells. Examples of genome uncoating at the plasma membrane are shown on the left side of the cell. Fusion at the plasma membrane releases the nucleocapsid into the cytoplasm. In some cases, the subviral particle is transported on microtubules toward the nucleus, where the nucleic acid is released. Uptake of virions by clathrin-dependent endocytosis commences with binding to a specific cell surface receptor. The ligand-receptor complex diffuses into an invagination of the plasma membrane coated with the protein clathrin on the cytosolic side (clathrin-coated pits). The coated pit further invaginates and pinches off, a process that is facilitated by the GTPase dynamin. The resulting coated vesicle then fuses with an early endosome. Endosomes are acidic, as a result of the activity of vacuolar proton ATPases. Particle uncoating usually occurs from early or late endosomes. Late endosomes then fuse with lysosomes. Virus particles may enter cells by a dynamin- and caveolin-dependent endocytic pathway (right side of the cell). Three types of caveolar endocytosis have been identified. Dynamin 2-dependent endocytosis by caveolin 1-containing **caveolae** is observed in cells infected with simian virus 40 and polyomavirus. Dynamin 2-dependent, noncaveolar, lipid raft-mediated endocytosis occurs during echovirus and rotavirus infection, while dynamin-independent, noncaveolar, raft-mediated endocytosis is also observed during simian virus 40 and polyomavirus infection. This pathway brings virions to the endoplasmic reticulum via the caveosome, a pH-neutral compartment. Clathrin- and caveolin-independent endocytic pathways of viral entry have also been described (center of cell). Movement of endocytic vesicles within cells occurs on microfilaments or microtubules, components of the cytoskeleton. Microfilaments are two-stranded helical polymers of the ATPase actin. They are dispersed throughout the cell but are most highly concentrated beneath the plasma membrane, where they are connected via integrins and other proteins to the extracellular matrix. Transport along microfilaments is accomplished by myosin motors. Microtubules are 25-nm hollow cylinders made of the GTPase tubulin. They radiate from the **centrosome** to the cell periphery. Movement on microtubules is carried out by kinesin and dynein motors.

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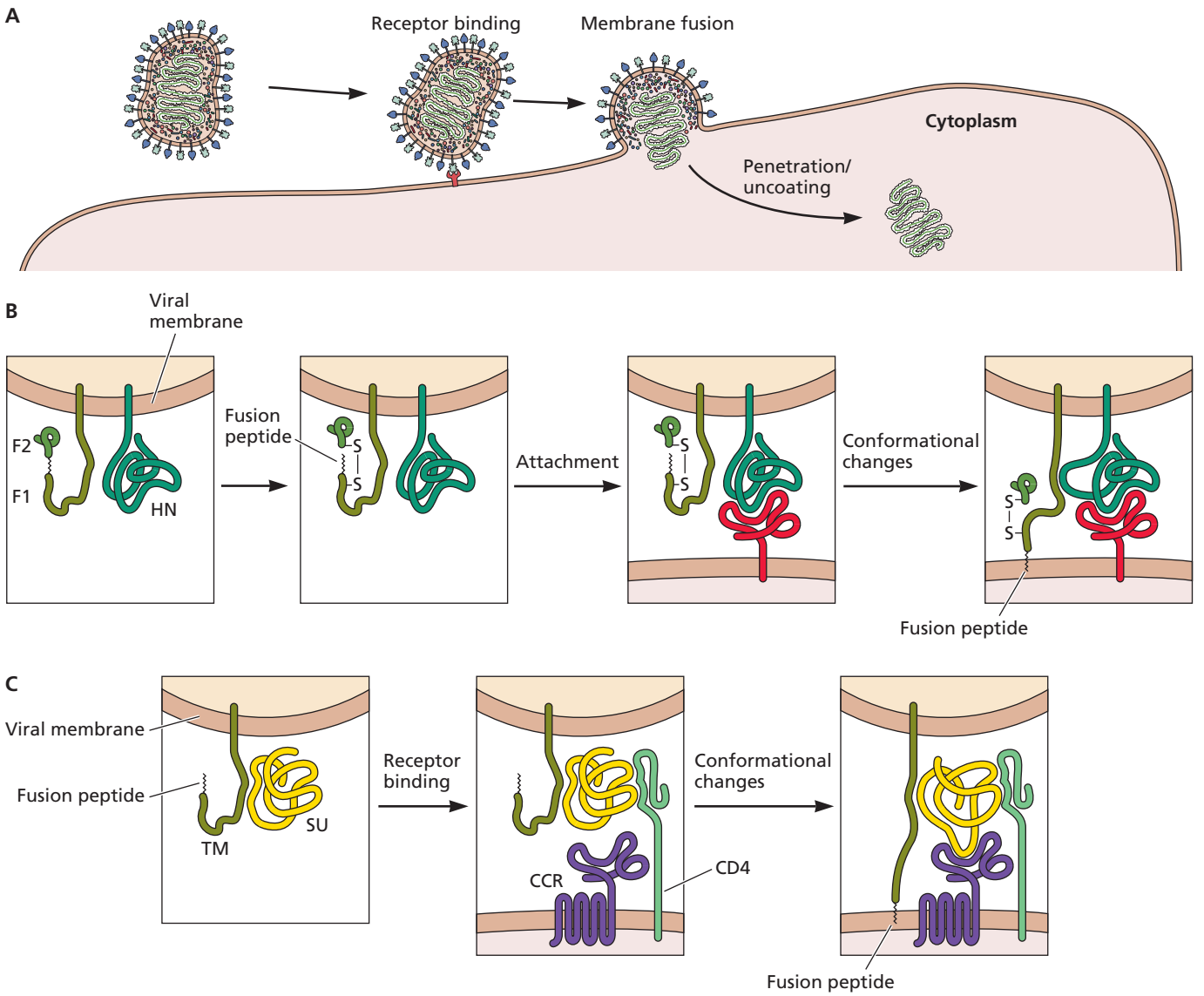


Figure 5.10 Penetration and uncoating at the plasma membrane. (A) Overview. Entry of a member of the *Paramyxoviridae*, which bind to cell surface receptors via the HN, H, or G glycoprotein. The fusion protein (F) then catalyzes membrane fusion at the cell surface at neutral pH. The viral nucleocapsid, as RNP, is released into the cytoplasm, where RNA synthesis begins. **(B)** Model for F-protein-mediated membrane fusion. Binding of HN to the cell receptor (red) induces conformational changes in HN that in turn induce conformational changes in the F protein, moving the fusion peptide from a buried position nearer to the cell membrane. **(C)** Model of the role of chemokine receptors in human immunodeficiency virus type 1 fusion at the plasma membrane. For simplicity, the envelope glycoprotein is shown as a monomer. Binding of SU to CD4 exposes a high-affinity chemokine receptor-binding site on SU. The SU-chemokine receptor interaction leads to conformational changes in TM that expose the fusion peptide and permit it to insert into the cell membrane, catalyzing fusion in a manner similar to that proposed for influenza virus (cf. Fig. 5.12 and 5.13).

coil, and movement of the fusion peptide toward the cell membrane (Fig. 5.10). Such movement of the fusion peptide has been described in atomic detail by comparing structures of the F protein before and after fusion.

The trigger that initiates conformational changes in the F protein is not known. The results of experiments in which hemagglutinin-neuraminidase (HN) and F glycoproteins are synthesized in cultured mammalian cells indicate that the fusion activity of F protein is absent or inefficient if HN is not present. It has therefore been hypothesized that an interaction between HN and F proteins is essential for fusion. It is thought that binding of HN protein to its cellular receptor induces

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conformational changes, which in turn trigger conformational change in the F protein, exposing the fusion peptide and making the protein fusion competent (Fig. 5.10). The requirement for HN protein in F fusion activity has been observed only with certain paramyxoviruses, including human parainfluenza virus type 3 and mumps virus.

As a result of fusion of the viral and plasma membranes, the viral nucleocapsid, which is a ribonucleoprotein (RNP) consisting of the (−) strand viral RNA genome and the viral proteins L, NP, and P, is released into the cytoplasm (Fig. 5.10). Once in the cytoplasm, the L, NP, and P proteins begin the synthesis of viral messenger RNAs (mRNAs), a process discussed in Chapter 6. Because members of the *Paramyxoviridae* replicate in the cytoplasm, fusion of the viral and plasma membranes achieves uncoating and delivery of the viral genome to this cellular compartment in a single step.

Fusion of human immunodeficiency virus type 1 with the plasma membrane requires participation not only of the cell receptor CD4 but also of an additional cellular protein. These proteins are cell surface receptors for small molecules produced by many cells to attract and stimulate cells of the immune defense system at sites of infection; hence, these small molecules are called **chemotactic cytokines** or **chemokines**. The chemokine receptors on such cells comprise a large family of proteins with seven membrane-spanning domains and are coupled to intracellular signal transduction pathways. There are two major coreceptors for human immunodeficiency virus type 1 infection. CXCr4 (a member of a family of chemokines characterized by having their first two cysteines separated by a single amino acid) appears to be a specific coreceptor for virus strains that infect T cells preferentially. The second is CCr5, a coreceptor for the macrophage-tropic strains of the virus. The chemokines that bind to this receptor activate both T cells and macrophages, and the receptor is found on both types of cell. Individuals who are homozygous for deletions in the CCr5 gene and produce nonfunctional coreceptors have no discernible immune function abnormality, but they appear to be resistant to infection with human immunodeficiency virus type 1. Even heterozygous individuals seem to be somewhat resistant to the virus. Other members of the CC chemokine receptor family (CCr2b and CCr3) were subsequently found to serve as coreceptors for the virus.

Attachment to CD4 appears to create a high-affinity binding site on SU for CCr5. The atomic structure of SU bound to CD4 revealed that binding of CD4 induces conformational changes that expose binding sites for chemokine receptors (Fig. 5.10). Studies of CCr5 have shown that the first N-terminal extracellular domain is crucial for coreceptor function, suggesting that this sequence might interact with SU. An antibody molecule fused to both the CD4 and CCr5 binding sites is being explored as a therapeutic compound to block infection (Box 5.4).

Human immunodeficiency virus type 1 TM mediates envelope fusion with the cell membrane. The high-affinity SU-CCr5 interaction may induce conformational changes in TM to expose the fusion peptide, placing it near the cell membrane, where it can catalyze fusion (Fig. 5.10). Such changes are similar to those that influenza virus HA undergoes upon exposure to low pH. X-ray crystallographic analysis of fusion-active human immunodeficiency virus type 1 TM revealed that its structure is strikingly similar to that of the low-pH fusogenic form of HA (see "Acid-Catalyzed Membrane Fusion" below).

Uncoating during Endocytosis

Many viruses enter cells by the same pathways by which cells take up macromolecules. The plasma membrane, the limiting membrane of the cell, permits nutrient molecules to enter and waste molecules to leave, thereby ensuring an appropriate internal environment. Water, gases, and small hydrophobic molecules such as ethanol can freely traverse the lipid bilayer, but most metabolites and ions cannot. These essential components enter the cell by specific transport processes. Integral membrane proteins are responsible for the transport of ions, sugars, and amino acids, while proteins and large particles are taken into the cell by phagocytosis or endocytosis. The former process (Fig. 5.11) is nonspecific, which means that any particle or molecule can be taken into the cell, and only occurs in specialized cell types such as dendritic cells and macrophages.

Clathrin-Mediated Endocytosis

A wide range of ligands, fluid, membrane proteins, and lipids are selectively taken into cells from the extracellular milieu by **clathrin-mediated endocytosis** (Fig. 5.9 and 5.11), also the mechanism of entry of many viruses. Ligands in the extracellular medium bind to cells via specific plasma membrane receptor proteins. The receptor-ligand assembly diffuses along the membrane until it reaches an invagination that is coated on its cytoplasmic surface by a cage-like lattice composed of the fibrous protein clathrin (Fig. 5.9). Such clathrin-coated pits can comprise as much as 2% of the surface area of a cell, and some receptors are clustered over these areas even in the absence of their ligands. Following the accumulation of receptor-ligand complexes, the clathrin-coated pit invaginates and then pinches off to form a clathrin-coated vesicle. Within a few seconds, the clathrin coat is lost and the vesicles fuse with small, smooth-walled vesicles located near the cell surface, called early **endosomes**. The lumen of early endosomes is mildly acidic (pH 6.5 to 6.0), a result of energy-dependent transport of protons into the interior of the vesicles by a membrane proton pump. The contents of the early endosome are then transported via endosomal carrier vesicles to late endosomes located close to the nucleus. The lumen of late endosomes is more acidic (pH 6.0 to 5.0). Late endosomes in turn

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BOX 5.4

EXPERIMENTS

Blocking human immunodeficiency virus infection with two soluble cell receptors

Because viruses must bind to cell surface molecules to initiate replication, the use of soluble receptors to block virus infection has long been an attractive therapeutic option. Soluble CD4 receptors that block infection with human immunodeficiency virus type 1 (HIV-1) have been developed, but these have not been licensed because of their suboptimal potency. A newly designed soluble receptor for HIV-1 overcomes this problem and provides broad and effective protection against infection of cells and of nonhuman primates.

A soluble form of CD4 fused to an antibody molecule can block infection of most HIV-1 isolates and has been shown to be safe in humans, but its affinity for gp120 is low. Furthermore, human immunodeficiency virus can also be spread from cell to cell by fusion, a process that is not blocked by circulating, soluble CD4. Similarly, peptide mimics of the CCR5 coreceptor have been shown to block infection, but their affinity for gp120 is also low.

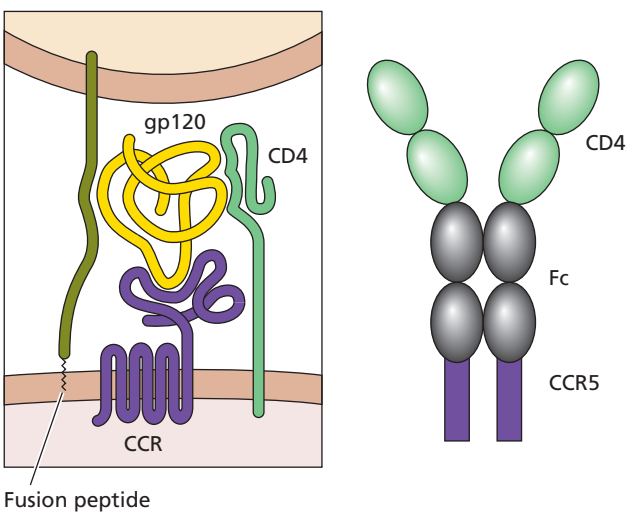
Combining the two gp120-binding molecules solved the problem of low affinity and in addition provided protection against a wide range of virus isolates. The entry inhibitor, called eCD4-Ig, is a fusion of the first two domains of CD4 to the Fc domain of an antibody molecule, with the CCR5-mimicking peptide at the carboxy terminus (illustrated). It binds strongly to gp120 and blocks infection with many different isolates of HIV-1, HIV-2, simian immunodeficiency virus (SIV), and HIV-1 resistant to broadly neutralizing monoclonal antibodies. The molecule blocks viral infection at concentrations that might be achieved in humans (1.5 to 5.2 micrograms per milliliter).

When administered to mice, eCD4-Ig protected the animals from HIV-1. Rhesus macaques inoculated with an adenovirus-associated virus (AAV) recombinant containing the gene for eCD4-Ig were protected from infection with large quantities of virus for up to 34 weeks after immunization. Concentrations of eCD4-Ig in the sera of these animals ranged from 17 to 77 micrograms per milliliter.

These results show that eCD4-Ig blocks HIV infection with a wide range of isolates more effectively than previously studied broadly neutralizing antibodies. Emergence of HIV variants resistant to neutralization with eCD4-Ig would likely produce viruses that infect cells less efficiently, reducing their transmission. eCD4-Ig is therefore an attractive candidate for therapy of HIV-1 infections. Whether sustained production of the protein in humans will cause disease remains to be determined. Because expression of the AAV genome persists for long periods, it might be advantageous to include a kill-switch in the vector: a way of turning it off if something should go wrong.

Gardner MR, Kattenhorn LM, Kondur HR, von Schaewen M, Dorfman T, Chiang JJ, Haworth KG, Decker JM, Alpert MD, Bailey CC, Neale ES, Jr, Fellinger CH, Joshi VR, Fuchs SP, Martinez-Navio JM, Quinlan BD, Yao AY, Mouquet H, Gorman J, Zhang B, Poignard P, Nussenzweig MC, Burton DR, Kwong PD, Piatak M, Jr, Lifson JD, Gao G, Desrosiers RC, Evans DT, Hahn BH, Ploss A, Cannon PM, Seaman MS, Farzan M. 2015. AAV-expressed eCD4-Ig provides durable protection from multiple SHIV challenges. *Nature* **519:**87–91.

Left, binding of HIV-1 SU (gp120) to CD4 and a chemokine receptor, CCR. Right, illustration of soluble eCD4-Ig. The Fab domains of the antibody molecule are replaced with the first two Ig-like domains of CD4, and the gp120-binding part of CCR5 is added to the C terminus of the Fc domain.



fuse with **lysosomes**, which are vesicles containing a variety of enzymes that degrade sugars, proteins, nucleic acids, and lipids. Viruses with a high pH threshold for fusion, such as vesicular stomatitis virus, enter from early endosomes; most enter the cytoplasm from late endosomes, and a few enter from lysosomes.

Clathrin-mediated endocytosis is a continuous but regulated process. For example, the uptake of vesicular stomatitis virus into cells may be influenced by over 90 different cellular protein kinases. Influenza virus, vesicular stomatitis virus, and reovirus particles are taken into cells, not into preexisting pits but mainly by clathrin-coated pits that form after virus binds to the cell surface. It is not known how virus binding to the plasma membrane induces the formation of the clathrin-coated pit.

Caveolar and Lipid Raft-Mediated Endocytosis

Although uptake of most viruses occurs by the clathrin-mediated endocytic pathway, some viruses enter by caveolin- or raft-mediated endocytosis (Fig. 5.9). The caveolar pathway

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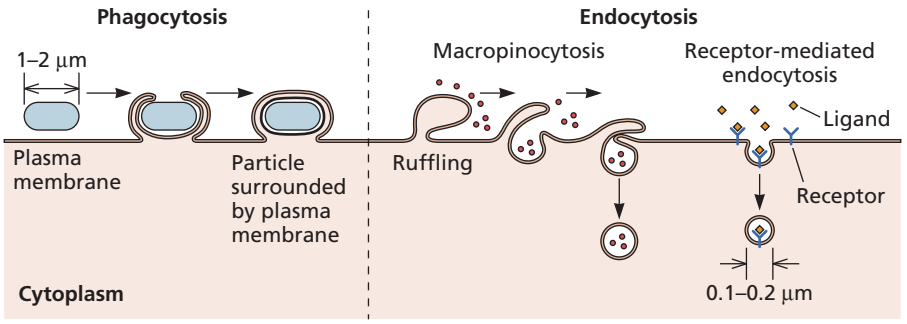


Figure 5.11 Mechanisms for the uptake of macromolecules from extracellular fluid. During phagocytosis, large particles such as bacteria or cell fragments that come in contact with the cell surface are engulfed by extensions of the plasma membrane. Phagosomes ultimately fuse with lysosomes, resulting in degradation of the material within the vesicle. Macrophages use phagocytosis to ingest bacteria and destroy them. Endocytosis comprises the invagination and pinching off of small regions of the plasma membrane, resulting in the nonspecific internalization of molecules (macropinocytosis) or the specific uptake of molecules bound to cell surface receptors (receptor-mediated endocytosis). Macropinocytosis is a mechanism for the uptake of extracellular fluid. It is triggered by ligand binding which initiates formation of plasma membrane ruffling, which traps material in large vacuoles. Adapted from J. Darnell et al., *Molecular Cell Biology* (Scientific American Books, New York, NY, 1986), with permission.

requires cholesterol (a major component of lipid rafts). Caveolae are distinguished from clathrin-coated vesicles by their flask-like shape, their smaller size, the absence of a clathrin coat, and the presence of a marker protein called caveolin. In the uninfected cell, caveolae participate in transcytosis, signal transduction, and uptake of membrane components and extracellular ligands. Binding of a virus particle to the cell surface activates signal transduction pathways required for pinching off of the vesicle, which then moves within the cytoplasm. Disassembly of filamentous actin also occurs, presumably to facilitate movement of the vesicle deeper into the cytoplasm. There it fuses with the **caveosome**, a larger membranous organelle that contains caveolin (Fig. 5.9). In contrast to endosomes, the pH of the caveosome lumen is neutral. Some viruses (e.g., echovirus type 1) penetrate the cytoplasm from the caveosome. Others (simian virus 40, polyomavirus, coxsackievirus B3) are sorted to the endoplasmic reticulum (ER) by a transport vesicle that lacks caveolin. These viruses enter the cytoplasm by a process mediated by thiol oxidases present in the lumen of the endoplasmic reticulum and by a component of the protein degradation pathway present in the membrane.

The study of virus entry by endocytosis can be confusing because some viruses may enter cells by multiple routes, depending on cell type and multiplicity of infection. For example, herpes simplex virus can enter cells by three different routes and influenza A virus may enter cells by both clathrin-dependent and clathrin-independent pathways.

Macropinocytosis

Macropinocytosis is a process by which extracellular fluid is taken into cells via large vacuoles. It is triggered by ligands and dependent on actin and a signaling pathway. It differs from phagocytosis by the signaling pathways needed and can take place in many cell types. This process serves as a pathway of entry for many viruses, including vaccinia virus, herpesviruses, and ebolaviruses. Upon receptor binding, viruses that enter cells via macropinocytosis trigger a signaling cascade that leads to changes in cortical actin and ruffling of the plasma membrane (Fig. 5.11). When these plasma membrane extensions retract, the viruses are brought into macropinosomes and eventually leave these vesicles via membrane fusion.

Membrane Fusion

The membranes of enveloped viruses fuse with those of the cell as a first step in delivery of the viral nucleic acid. Membrane fusion takes place during many other cellular processes, such as cell division, myoblast fusion, and exocytosis.

Membrane fusion must be regulated in order to maintain the integrity of the cell and its intracellular compartments. Consequently, membrane fusion does not occur spontaneously but proceeds by specialized mechanisms mediated by proteins. The two membranes must first come into close proximity. In cells, this reaction is mediated by interactions of integral membrane proteins that protrude from the lipid bilayers, a targeting protein on one membrane and a docking protein on the other. During entry of enveloped viruses, the virus and cell membranes are first brought into close contact by interaction of a viral glycoprotein with a cell receptor. The next step, fusion, requires an even closer approach of the membranes, to within 1.5 nm of each other. This step depends on the removal of water molecules from the membrane surfaces,

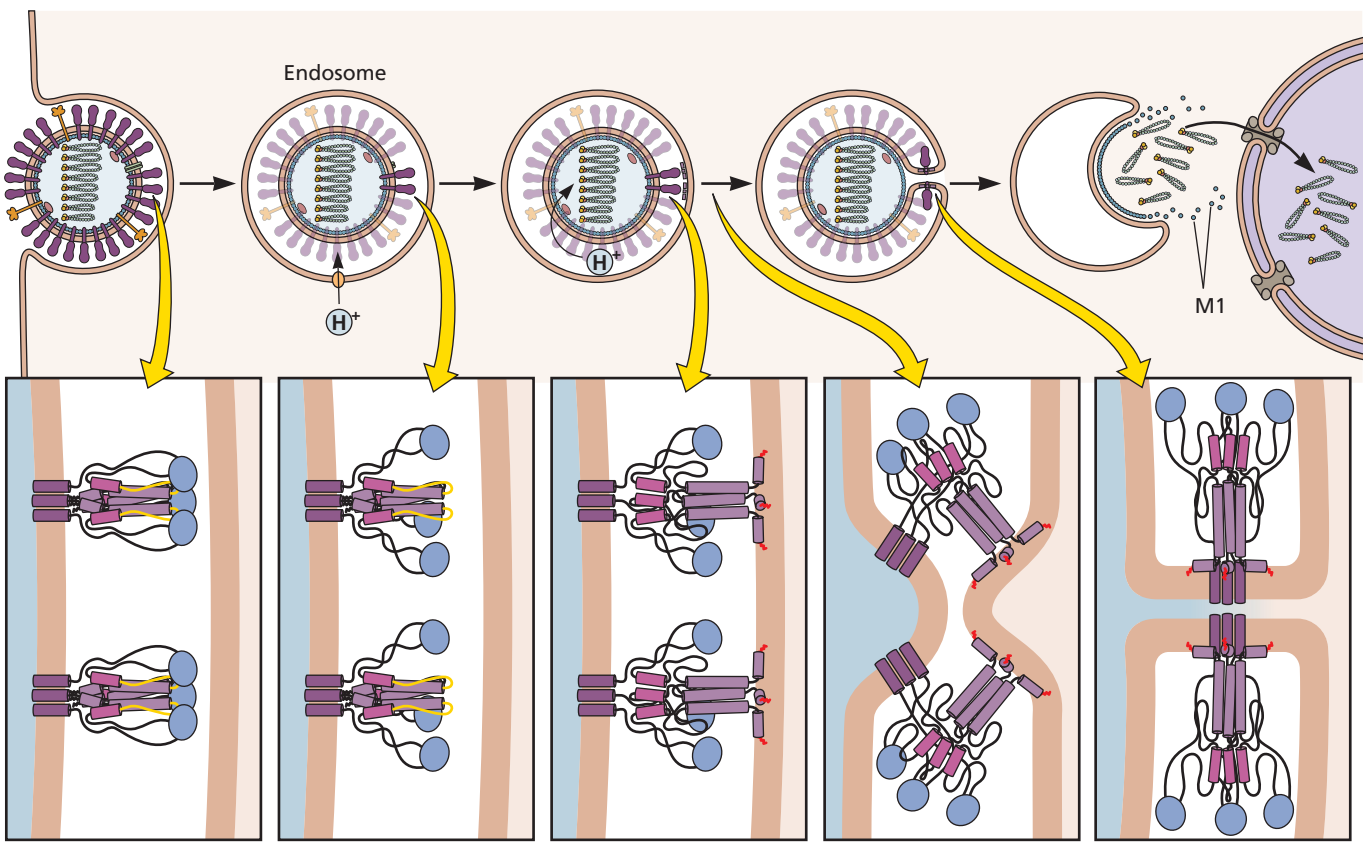
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an energetically unfavorable process. This step is hypothesized to occur when the viral glycoprotein undergoes a structural rearrangement called "hairpinning" (Fig. 5.12).

The precise mechanism by which lipid bilayers fuse is not completely understood, but the action of fusion proteins is thought to result in the formation of an opening called a **fusion pore**, allowing exchange of material across the membranes (Box 5.5). The viral glycoprotein bound to a cell receptor, or a different viral integral membrane protein, then catalyzes the fusion of the juxtaposed membranes. Viral fusion proteins are integral membrane proteins, often glycoproteins, that form homo- or hetero-oligomers.

Virus-mediated fusion must be regulated to prevent viruses from aggregating or to ensure that fusion does not occur in the incorrect cellular compartment. In some cases, fusogenic potential is masked until the fusion protein interacts with other integral membrane proteins. In others, low pH is required to expose fusion domains. The activity of fusion proteins may also be regulated by cleavage of a precursor. This requirement probably prevents premature activation of fusion potential during virus assembly. Viral fusion proteins are often primed for fusion by proteolytic cleavage as they move through the trans-Golgi network as described in Chapter 12. Proteases that catalyze such cleavage are typically furin family convertases that either cleave the fusion proteins directly (orthomyxoviruses, retroviruses, paramyxoviruses) or cleave a protein that masks the fusion protein (alphaviruses, flaviviruses).

Figure 5.12 Influenza virus entry. The globular heads of native HA mediate binding of the virus to sialic acid-containing cell receptors. The virus-receptor complex is endocytosed, and import of H^+ ions into the endosome acidifies the interior. Upon acidification, the viral HA undergoes a conformational rearrangement that produces a fusogenic protein. The loop region of native HA (yellow) becomes a coiled coil, moving the fusion peptides (red) to the top of the molecule near the cell membrane. At the viral membrane, the long α-helix (purple) packs against the trimer core, pulling the globular heads to the side. The long coiled coil bends, or hairpins, bringing the fusion peptides and the transmembrane domains together. This movement moves the cell and viral membranes close together so that fusion can occur. To allow release of vRNP into the cytoplasm, the H^+ ions in the acidic endosome are pumped into the particle interior by the M2 ion channel. As a result, vRNP is primed to dissociate from M1 after fusion of the viral and endosomal membranes. The released vRNPs are imported into the nucleus through the nuclear pore complex via a nuclear localization signal-dependent mechanism (see "Import of Influenza Virus Ribonucleoprotein" below). Adapted from C. M. Carr and P. S. Kim, *Science* **266:**234–236, 1994, with permission.



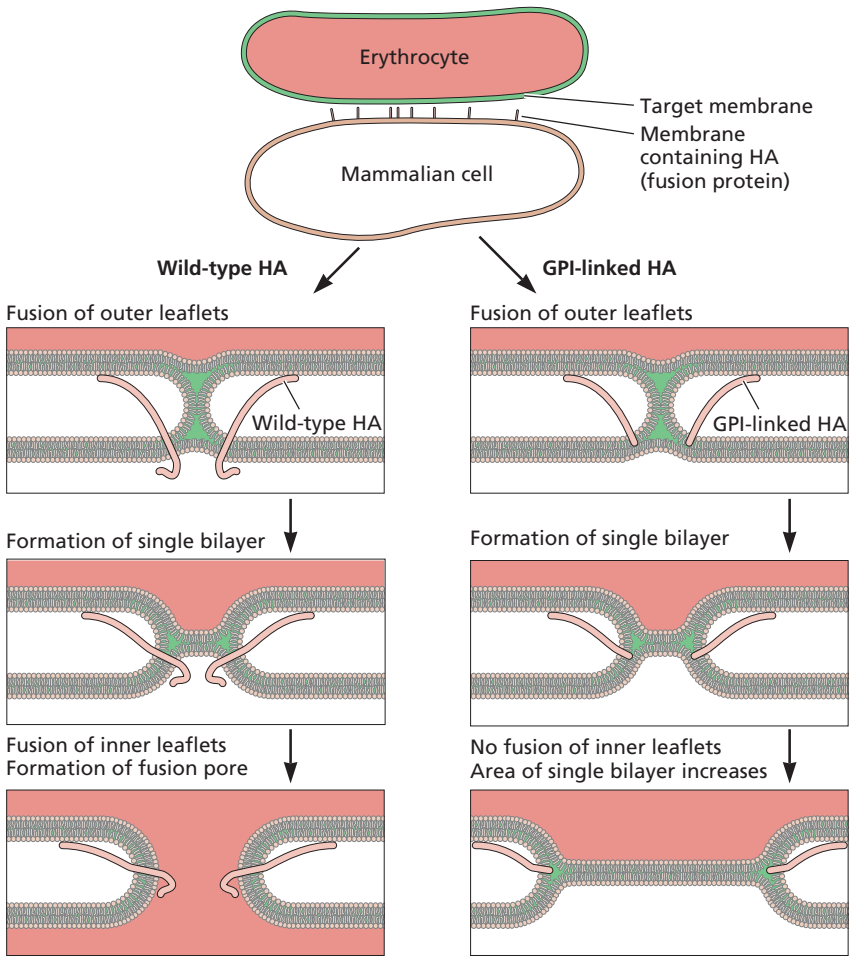
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BOX 5.5

EXPERIMENTS

Membrane fusion proceeds through a hemifusion intermediate

Fusion is thought to proceed through a hemifusion intermediate in which the outer leaflets of two opposing bilayers fuse (see figure), followed by fusion of the inner leaflets and the formation of a fusion pore. Direct evidence that fusion proceeds via a hemifusion intermediate has been obtained with influenza virus HA (see figure). **(Left)** Cultured mammalian cells expressing wild-type HA are fused with erythrocytes containing two different types of fluorescent dye, one in the cytoplasm and one in the lipid membrane. Upon exposure to low pH, HA undergoes conformational change and the fusion peptide is inserted into the erythrocyte membrane. The green dye is transferred from the lipid bilayer of the erythrocyte to the bilayer of the cultured cell. The HA trimers tilt, causing reorientation of the transmembrane domain and generating stress within the hemifusion diaphragm. Fusion pore formation relieves the stress. The red dye within the cytoplasm of the erythrocyte is then transferred to the cytoplasm of the cultured cell. **(Right)** An altered form of HA was produced, lacking the transmembrane and cytoplasmic domains and with membrane anchoring provided by linkage to a glycosylphosphatidylinositol (GPI) moiety. Upon exposure to low pH, the HA fusion peptide is inserted into the erythrocyte membrane, and green dye is transferred to the membranes of the mammalian cell. When the HA trimers tilt, no stress is transmitted to the hemifusion diaphragm because no transmembrane domain is present, and the diaphragm becomes larger. Fusion pores do not form, and there is no mixing of the contents of the cytoplasm, indicating that complete membrane fusion has not occurred. These results prove that hemifusion, or fusion of only the inner leaflet of the bilayer, can occur among whole cells. The findings also demonstrate that the transmembrane domain of the HA polypeptide plays a role in the fusion process.



Glycosylphosphatidylinositol-anchored influenza virus HA induces hemifusion. (Left) Model of the steps of fusion mediated by wild-type HA. **(Right)** Effect on fusion by an altered form of HA lacking the transmembrane and cytoplasmic domains. Adapted from G. B. Melikyan et al., *J. Cell Biol* **131:**679–691, 1995, with permission.

Proteolytic cleavage not only is a mechanism to regulate where fusion occurs, but also generates the metastable states of viral glycoproteins that can subsequently undergo the conformational rearrangements required for fusion activity without additional energy. These structural changes expose the hidden fusion peptide so that it can insert into the target membrane and likely provide the energy needed to overcome the hydration force that prevents spontaneous membrane fusion. As a consequence the fusion protein is anchored in both viral and cellular membranes.

Acid-Catalyzed Membrane Fusion

The entry of influenza virus from the endosomal pathway is one of the best-understood viral entry mechanisms. At the cell surface, the virus attaches to sialic acid-containing receptors via the viral HA glycoprotein (Fig. 5.12). The virus-receptor complex is then internalized into the clathrin-dependent receptor-mediated endocytic pathway. When the endosomal pH reaches approximately 5.0, HA undergoes an acid-catalyzed conformational rearrangement, exposing a fusion peptide. The viral and endosomal membranes

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then fuse, allowing penetration of the viral RNP (vRNP) into the cytoplasm. Because influenza virus particles have a low pH threshold for fusion, uncoating occurs in late endosomes.

The fusion reaction mediated by the influenza virus HA protein is a remarkable event when viewed at atomic resolution (Fig. 5.13). In native HA, the fusion peptide is joined to the three-stranded coiled-coil core by which the HA monomers interact via a 28-amino-acid sequence that forms an extended loop structure buried deep inside the molecule, about 100 Å from the globular head. In contrast, in the low-pH HA structure, this loop region is transformed into a three-stranded coiled coil. In addition, the long α-helices of the coiled coil bend upward and away from the viral membrane. The result is that the fusion peptide is moved a great distance toward the endosomal membrane (Fig. 5.13). Despite these dramatic changes, HA remains trimeric and the globular heads can still bind sialic acid. In this conformation, HA holds the viral and endosome membranes 100 Å apart, too distant for fusion to occur. To bring the viral and cellular membranes closer, it is thought that the HA molecule bends, bringing together the fusion peptide and the transmembrane segment (Fig. 5.12). This movement brings the two membranes close enough to fuse.

In contrast to cleaved HA, the precursor HA0 is stable at low pH and cannot undergo structural changes. Cleavage of the covalent bond between HA1 and HA2 might simply allow movement of the fusion peptide, which is restricted in the uncleaved molecule. Another possibility is suggested by the observation that cleavage of HA is accompanied by movement of the fusion peptide into a cavity in the protein (Fig. 5.13). This movement buries ionizable residues of the fusion peptide, perhaps setting the low-pH "trigger." It should be emphasized that after cleavage, the N terminus of HA2 is tucked into the hydrophobic interior of the trimer (Fig. 5.13). This rearrangement presumably buries the fusion peptide so that newly synthesized virions do not aggregate and lose infectivity.

Viral fusion proteins have been placed into three structural classes called I, II, and III. Common characteristics of all three classes include insertion of a fusion peptide into the target membrane and refolding of the fusion protein so that cell and viral membranes are brought together. In class I fusion proteins (Fig. 5.14), the fusion peptides are presented to membranes on top of a three-stranded α-coiled coil.

The envelope proteins of alphaviruses and flaviviruses exemplify class II viral fusion proteins. Defining characteristics include a three-domain subunit, a fusion loop, and tight association with a second viral protein. Proteolytic cleavage of the second protein converts the fusion protein to a metastable state that can undergo structural rearrangements at low pH to promote fusion. In contrast, the fusion peptide of the influenza virus HA is adjacent to the cleavage point and becomes the N terminus of the mature fusion protein. The envelope proteins of alphaviruses (E1) and flaviviruses (E) do not form coiled coils, as do type I fusion proteins. Rather, they contain predominantly β-barrels that tile the surface of the virus particles as dimers. At low pH they extend toward the endosome membrane, allowing insertion of the fusion peptide (Fig. 5.15).

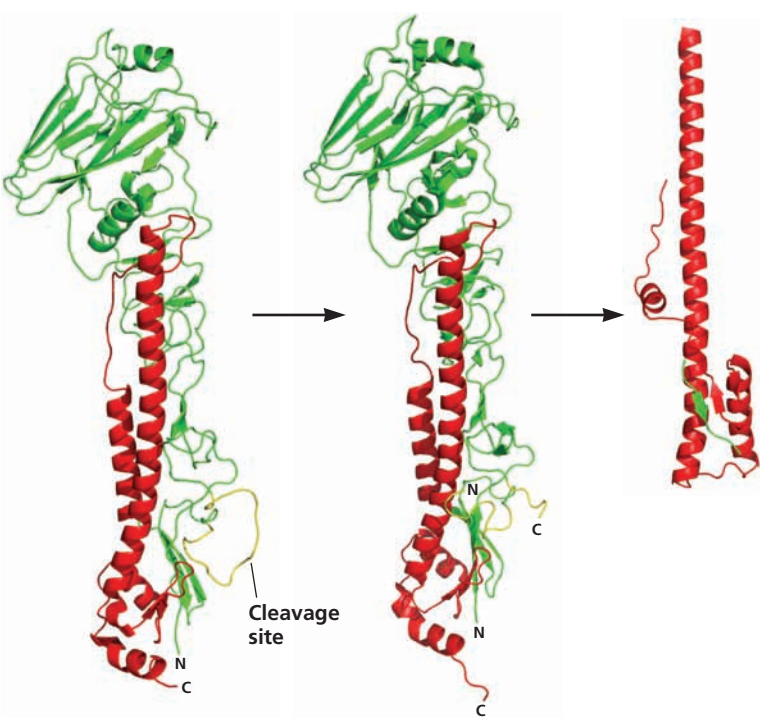


Figure 5.13 Cleavage- and low-pH-induced structural changes in the extracellular domains of influenza virus HA. **(Left)** Structure of the uncleaved HA0 precursor extracellular domain at neutral pH. HA1 subunits are green, HA2 subunits are red, residues 323 of HA1 to 12 of HA2 are yellow, and the locations of some of the N and C termini are indicated. The viral membrane is at the bottom, and the globular heads are at the top. The cleavage site between HA1 and HA2 is in a loop adjacent to a deep cavity. **(Middle)** Structure of the cleaved HA trimer at neutral pH. Cleavage of HA0 generates new N and C termini, which are separated by 20 Å. The N and C termini visible in this model are labeled. The cavity is now filled with residues 1 to 10 of HA2, part of the fusion peptide. **(Right)** Structure of the low-pH trimer. The protein used for crystallization was treated with proteases, and therefore the HA1 subunit and the fusion peptide are not present. This treatment is necessary to prevent aggregation of HA at low pH. At neutral pH the fusion peptide is close to the viral membrane, linked to a short α-helix, and at acidic pH this α-helix is reoriented toward the cell membrane, carrying with it the fusion peptide. The structures are aligned on a central α-helix that is unaffected by the conformational change. Only monomers of all three structures are shown. Adapted from J. Chen et al., *Cell* **95:**409–417, 1998, with permission.

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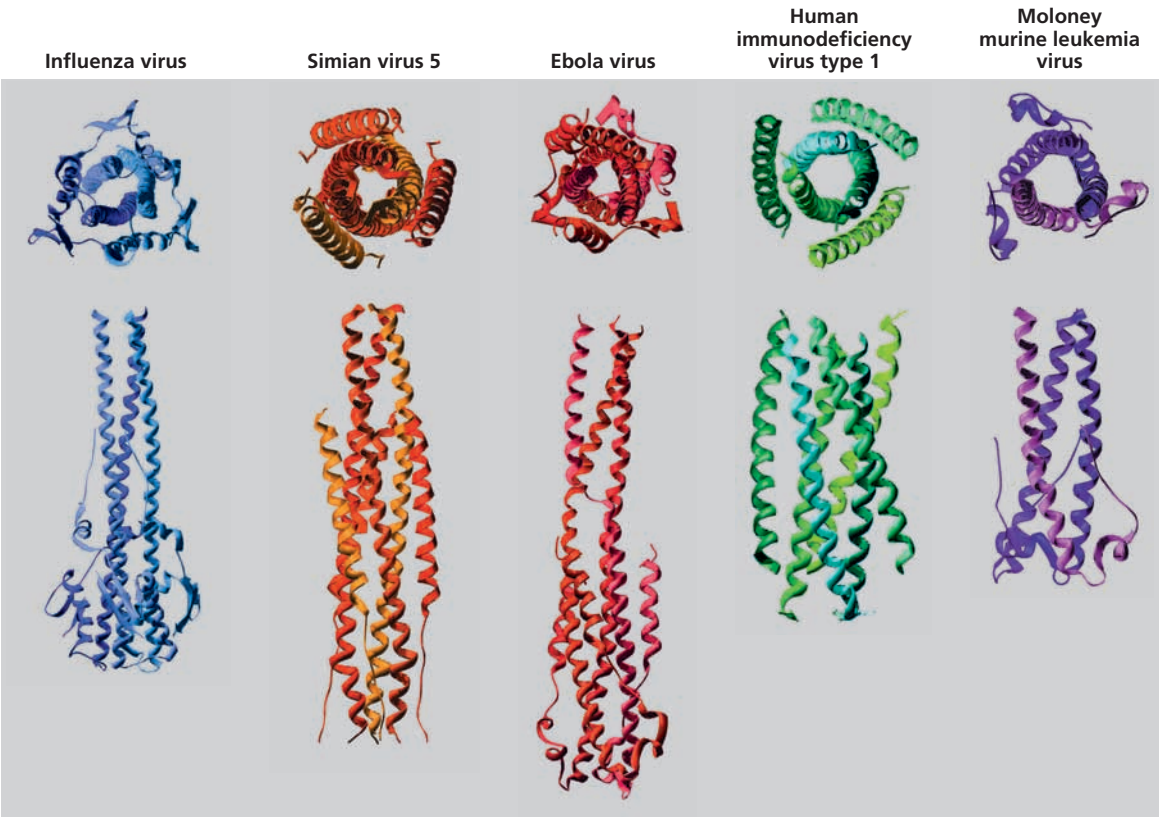


Figure 5.14 Similarities among five viral fusion proteins. **(Top)** View from the top of the structures. **(Bottom)** Side view. The structure shown for HA is the low-pH, or fusogenic, form. The structure of simian virus 5 F protein is of peptides from the N- and C-terminal heptad repeats. Structures of retroviral TM proteins are derived from interacting human immunodeficiency virus type 1 peptides and a peptide from Moloney murine leukemia virus and are presumed to represent the fusogenic forms because of structural similarity to HA. In all three molecules, fusion peptides would be located at the membrane-distal portion (the tops of the molecules in the bottom view). All present fusion peptides to cells on top of a central three-stranded coiled coil supported by C-terminal structures. Adapted from K. A. Baker et al., *Mol. Cell* **3:**309–319, 1999, with permission.

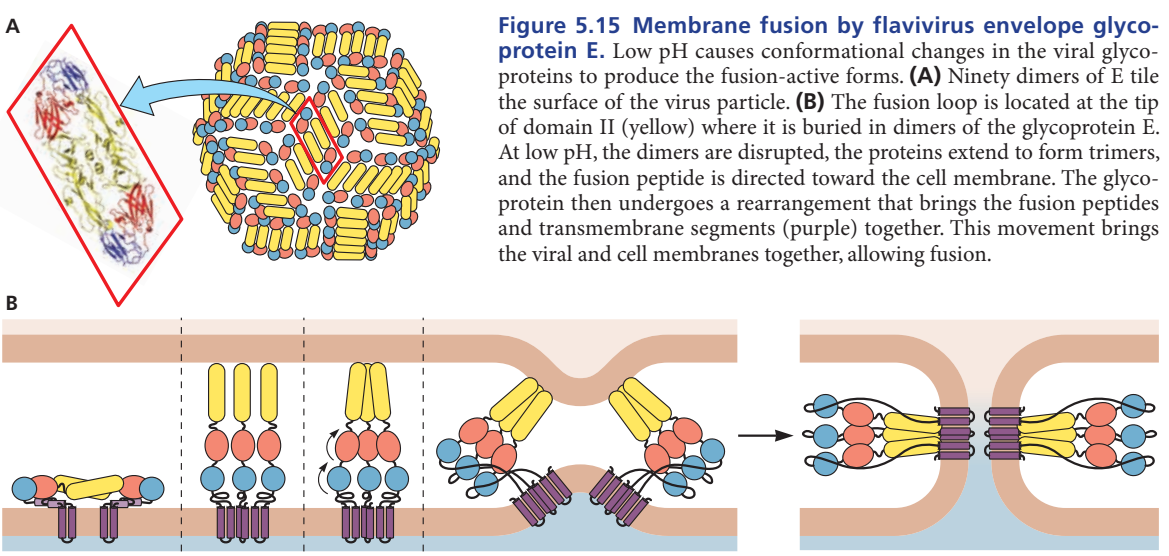


Figure 5.15 Membrane fusion by flavivirus envelope glycoprotein E. Low pH causes conformational changes in the viral glycoproteins to produce the fusion-active forms. **(A)** Ninety dimers of E tile the surface of the virus particle. **(B)** The fusion loop is located at the tip of domain II (yellow) where it is buried in dimers of the glycoprotein E. At low pH, the dimers are disrupted, the proteins extend to form trimers, and the fusion peptide is directed toward the cell membrane. The glycoprotein then undergoes a rearrangement that brings the fusion peptides and transmembrane segments (purple) together. This movement brings the viral and cell membranes together, allowing fusion.

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In the alphavirus particle, the second viral protein, E2, acts as a clamp to hold the fusion protein in place; at low pH the clamp is released. In contrast to the situation with other viruses, proteolytic cleavage of E1 is not required to produce a fusogenic protein. However, protein processing controls fusion potential in another way: in the endoplasmic reticulum, E1 protein is associated with the precursor of E2, called p62. In this heterodimeric form, p62-E1, E1 protein cannot be activated for fusion by mildly acidic conditions. Only after p62 has been cleaved to E2 can low pH induce disruption of E1-E2 heterodimers and formation of fusion-active E1 homotrimers.

Receptor Priming for Low-pH Fusion: Two Entry Mechanisms Combined

During the entry of avian leukosis virus into cells, binding of the virus particle to the cell receptor primes the viral fusion protein for low-pH-activated fusion. Avian leukosis virus, like many other retroviruses with simple genomes, was believed to enter cells at the plasma membrane in a pH-independent mechanism resembling that of members of the *Paramyxoviridae* (Fig. 5.10). It is now known that binding of the viral membrane glycoprotein (SU) to the cellular receptors of avian leukosis viruses induces conformational rearrangements that convert the viral protein from a native metastable state that is insensitive to low pH to a second metastable state. In this state, exposure to low pH within the endosomal compartment leads to membrane fusion and release of the viral capsid.

An Endosomal Fusion Receptor

The study of Ebolavirus entry into cells has revealed a new mechanism in which the viral fusion protein binds to a specific fusion receptor in the endosome membrane. Previously, all known cases of fusion catalyzed by viral glycoproteins have taken place when the fusion peptide inserts into the endosomal membrane by virtue of its hydrophobic properties.

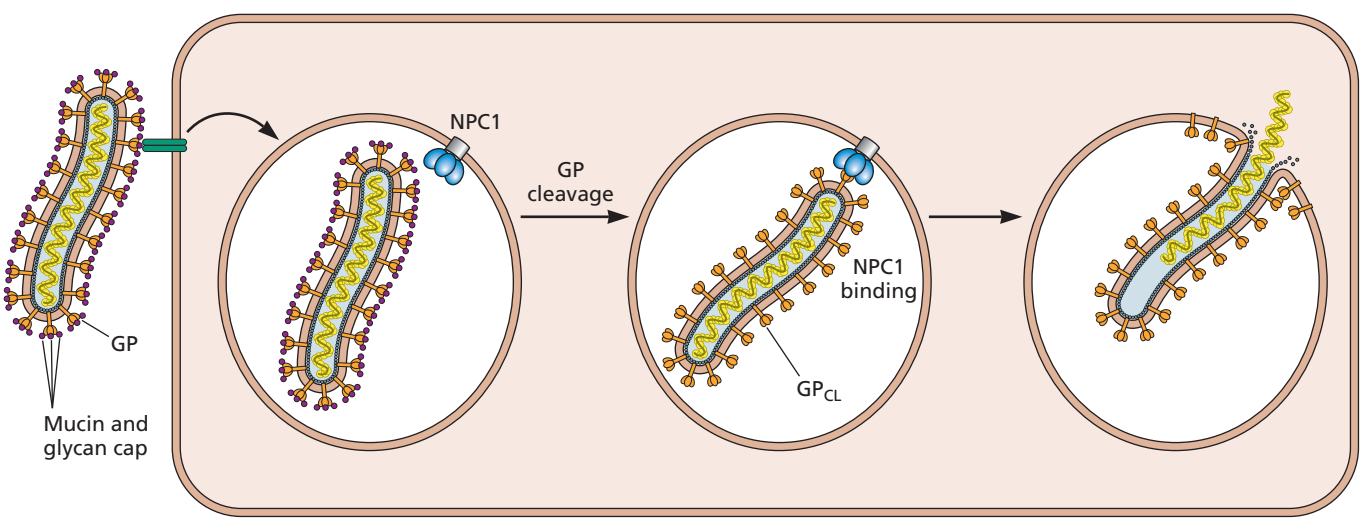
Following attachment to cells via the viral glycoprotein GP, viral particles are internalized and move to late endosomes. There, cysteine proteases cleave GP to remove heavily glycosylated sequences at the C terminus of the protein, producing GP1 and GP2 subunits. The cleaved glycoprotein then binds Niemann-Pick C1 protein, catalyzing fusion of the viral and endosomal membranes. Niemann-Pick C1 is a multiple membrane-spanning protein that resides in the late endosomes and lysosomes and participates in the transport of lysosomal cholesterol to the ER and other cellular sites. Individuals with Niemann-Pick type C1 disease lack the protein and consequently have defects in cholesterol transport; fibroblasts from these patients are resistant to Ebola virus infection (Fig. 5.16).

The binding site on the viral glycoprotein for Niemann-Pick C1 protein is located beneath the heavily glycosylated mucin and glycan cap of the protein, explaining why proteolytic removal of these sequences is needed for binding of viral GP. These observations demonstrate that Niemann-Pick C1 is an intracellular receptor for Ebola virus that promotes a late step in viral entry. It is believed that this receptor assists in dissociating GP1 and GP2, allowing conformational rearrangements needed for membrane fusion.

Release of Viral Ribonucleoprotein

The genomes of many enveloped RNA viruses are present as vRNP in the virus particle. One mechanism for release of

Figure 5.16 Entry of Ebolavirus into cells. Virus particles bind cells via an unidentified attachment receptor and enter by endocytosis. The mucin and glycan cap on the viral glycoprotein is removed by cellular cysteine proteases, exposing binding sites for NPC1. The latter is required for fusion of the viral and cell membranes, releasing the nucleocapsid into the cytoplasm. Courtesy of Kartik Chandran, Albert Einstein College of Medicine.



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vRNP during virus entry has been identified by studies of influenza virus. Each influenza virus vRNP is composed of a segment of the RNA genome bound by nucleoprotein (NP) molecules and the viral RNA polymerase. This structure interacts with viral M1 protein, an abundant protein in virus particles that underlies the envelope and provides rigidity (Fig. 5.12). The M1 protein also contacts the internal tails of HA and neuraminidase proteins in the viral envelope. This arrangement presents two problems. Unless M1-vRNP interactions are disrupted, vRNPs might not be released into the cytoplasm. Furthermore, the vRNPs must enter the nucleus, where mRNA synthesis takes place. However, vRNP cannot enter the nucleus if M1 protein remains bound, because this protein masks a nuclear localization signal (see "Import of Influenza Virus Ribonucleoprotein" below).

The influenza virus M2 protein, the first viral protein discovered to be an ion channel, provides the solution to both problems. The envelope of the virus particle contains a small number of molecules of M2 protein, which form a homotetramer. When purified M2 was reconstituted into synthetic lipid bilayers, ion channel activity was observed, indicating that this property requires only M2 protein. The M2 protein channel is structurally much simpler than other ion channels and is the smallest channel discovered to date.

The M2 ion channel is activated by the low pH of the endosome before HA-catalyzed membrane fusion occurs. As a result, protons enter the interior of the virus particle. It has been suggested that the reduced pH of the particle interior leads to conformational changes in the M1 protein, thereby disrupting M1-vRNP interactions. When fusion between the viral envelope and the endosomal membrane takes place, vRNPs are released into the cytoplasm free of M1 and can then be imported into the nucleus (Fig. 5.12). Support for this model comes from studies with the anti-influenza virus drug amantadine, which specifically inhibits M2 ion channel activity (Volume II, Fig. 9.11). In the presence of this drug, influenza virus particles can bind to cells, enter endosomes, and undergo HA-mediated membrane fusion, but vRNPs are not released from endosomes.

Uncoating in the Cytoplasm by Ribosomes

Some enveloped RNA-containing viruses, such as Semliki Forest virus, contain nucleocapsids that are disassembled in the cytoplasm by pH-independent mechanisms. The icosahedral nucleocapsid of this virus is composed of a single viral protein, C protein, which encloses the (+) strand viral RNA. This structure is surrounded by an envelope containing viral glycoproteins E1 and E2, which are arranged as heterodimers clustered into groups of three, each cluster forming a spike on the virus surface.

Fusion of the viral and endosomal membranes exposes the nucleocapsid to the cytoplasm (Fig. 5.17). To begin translation

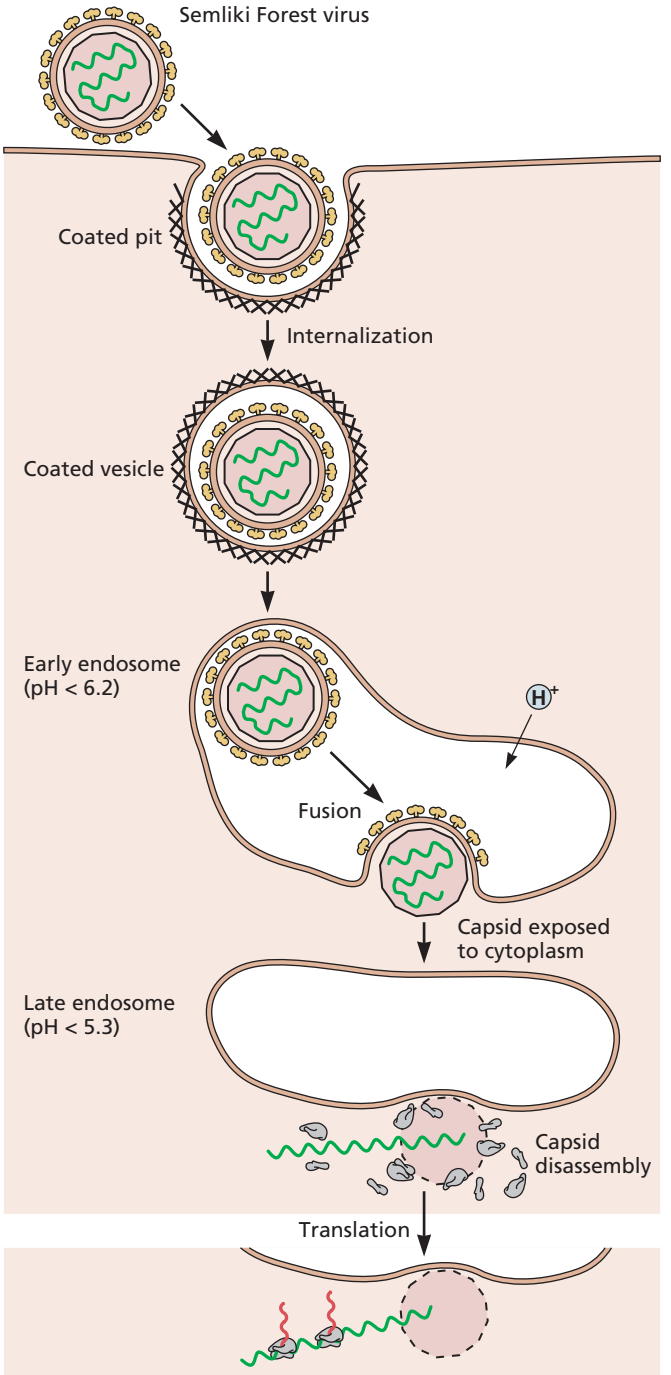


Figure 5.17 Entry of Semliki Forest virus into cells. Semliki Forest virus enters cells by clathrin-dependent receptor-mediated endocytosis, and membrane fusion is catalyzed by acidification of endosomes. Fusion results in exposure of the viral nucleocapsid to the cytoplasm, although the nucleocapsid remains attached to the cytosolic side of the endosome membrane. Cellular ribosomes then bind the capsid, disassembling it and distributing the capsid protein throughout the cytoplasm. The viral RNA is then accessible to ribosomes, which initiate translation. Adapted from M. Marsh and A. Helenius, *Adv. Virus Res.* **36:**107–151, 1989, with permission.

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of (+) strand viral RNA, the nucleocapsid must be disassembled, a process mediated by an abundant cellular component—the ribosome. Each ribosome binds three to six molecules of C protein, disrupting the nucleocapsid. This process occurs while the nucleocapsid is attached to the cytoplasmic side of the endosomal membrane (Fig. 5.17) and ultimately results in disassembly. The uncoated viral RNA remains associated with cellular membranes, where translation and replication begin.

Disrupting the Endosomal Membrane

Adenoviruses are composed of a double-stranded DNA genome packaged in an icosahedral capsid (Chapter 4). Internalization of most adenovirus serotypes by receptor-mediated endocytosis requires attachment of fiber to an integrin or Ig-like cell surface receptor and binding of the penton base to a second cell receptor, the cellular vitronectin-binding integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Attachment is mediated by amino acid sequences in each of the five subunits of the adenovirus penton base that mimic the normal ligands of cell surface integrins. As the virus particle is transported via the endosomes from the cell surface toward the nuclear membrane, it undergoes multiple uncoating steps as structural proteins are removed sequentially (Fig. 5.18). As the endosome becomes acidified, the viral capsid is destabilized, leading to release of proteins from the capsid. Among these is protein VI, which causes disruption of the endosomal membrane, thereby delivering the remainder of the particle into the cytoplasm. An N-terminal amphipathic α-helix of protein VI is probably responsible for its pH-dependent membrane disruption activity. This region of the protein appears to be masked in the native capsid by the hexon protein. The liberated subviral particle then docks onto the nuclear pore complex (see "Import of DNA Genomes" below).

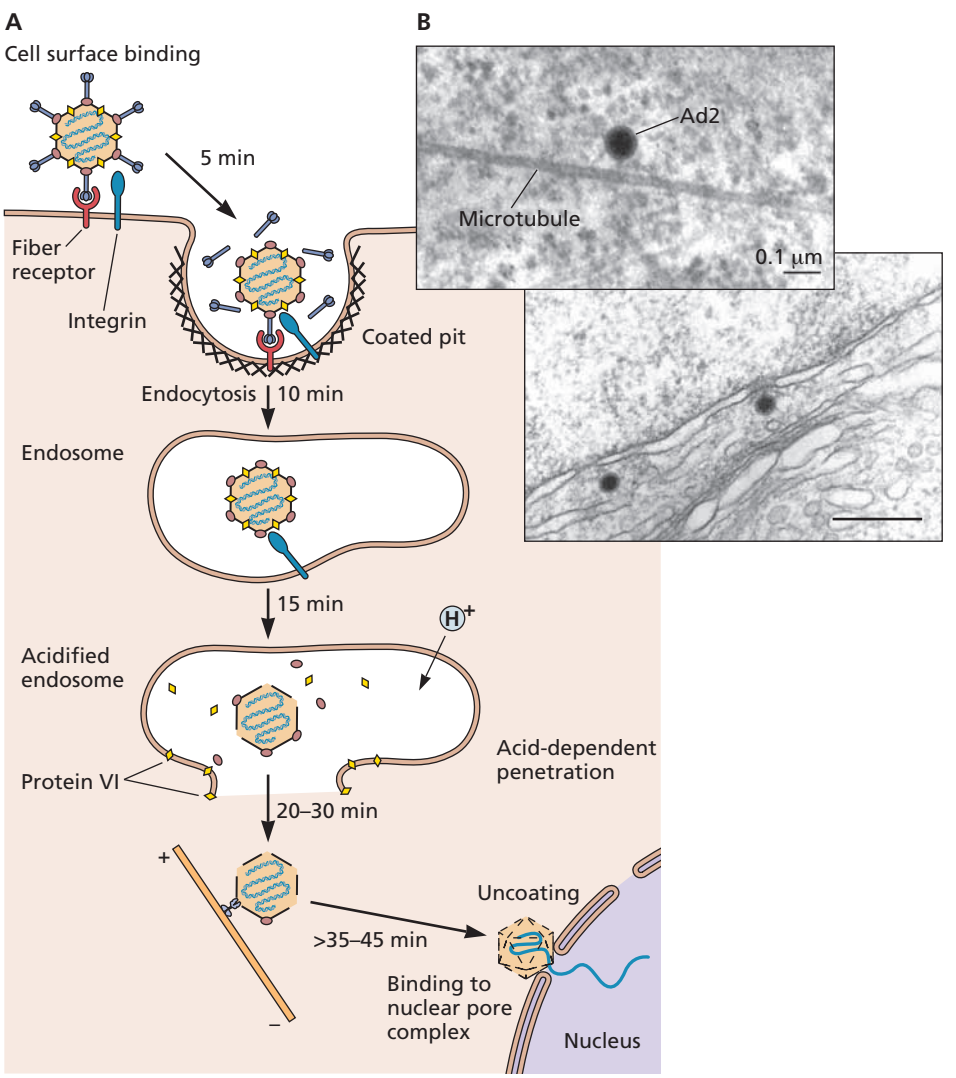


Figure 5.18 Stepwise uncoating of adenovirus. **(A)** Adenoviruses bind the cell receptor via the fiber protein. Interaction of the penton base with an integrin receptor leads to internalization by endocytosis. Fibers are released from the capsid during uptake. Low pH in the endosome causes destabilization of the capsid and release of protein VI (yellow diamonds). The hydrophobic N terminus of protein VI disrupts the endosome membrane, leading to release of a subviral particle into the cytoplasm. The capsid is transported in the cytoplasm along microtubules and docks onto the nuclear pore complex. **(B)** Electron micrograph of adenovirus type 2 particles bound to a microtubule (top) and bound to the cytoplasmic face of the nuclear pore complex (bottom). Bar in bottom panel, 200 nm. (A) Data from U. F. Greber et al., *Cell* **75:**477–486, 1993, and L. C. Trotman et al., *Nat. Cell Biol.* **3:**1092–1100, 2001. (B) Reprinted from U. F. Greber et al., *Trends Microbiol.* **2:**52–56, 1994, with permission. Courtesy of Ari Helenius, Urs Greber, and Paul Webster, University of Zurich.

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Forming a Pore in the Endosomal Membrane

The genomes of nonenveloped picornaviruses are transferred across the cell membrane by a different mechanism, as determined by structural information at the atomic level and complementary genetic and biochemical data obtained from studies of cell entry. The interaction of poliovirus with its Ig-like cell receptor, CD155, leads to major conformational rearrangements in the virus particle and the production of an expanded form called an altered (A) particle (Fig. 5.19A). Portions of two capsid proteins, VP1 and VP4, move from the inner surface of the capsid to the exterior. These polypeptides are thought to form a pore in the cell membrane that allows transport of viral RNA into the cytoplasm (Fig. 5.19B). In support of this model, ion channel activity can be detected when A particles are added to lipid bilayers.

The properties of a virus with an amino acid change in VP4 indicate that this protein is required for an early stage of cell entry. Virus particles with such amino acid alterations can bind to target cells and convert to A particles but are blocked at a subsequent, unidentified step. During poliovirus assembly, VP4 and VP2 are part of the precursor VP0, which remains uncleaved until the viral RNA has been encapsidated. The cleavage of VP0 during poliovirus assembly therefore primes the capsid for uncoating by separating VP4 from VP2.

In cells in culture, release of the poliovirus genome occurs from within early endosomes located close (within 100 to 200 nm) to the plasma membrane (Fig. 5.19A). Uncoating is dependent upon actin and tyrosine kinases, possibly for movement of the capsid through the network of actin filaments, but not on dynamin, clathrin, caveolin, or flotillin (a marker protein for clathrin- and caveolin-independent endocytosis), endosome acidification, or microtubules. The trigger for RNA release from early endosomes is not known but is clearly dependent on prior interaction with CD155. This conclusion derives from the finding that antibody-poliovirus complexes can bind to cells that produce Fc receptors but cannot infect them. As the Fc receptor is known to be endocytosed, these results suggest that interaction of poliovirus with CD155 is required to induce conformational changes in the particle that are required for uncoating.

A critical regulator of the receptor-induced structural transitions of poliovirus particles appears to be a hydrophobic tunnel located below the surface of each structural unit (Fig. 5.19C). The tunnel opens at the base of the canyon and extends toward the 5-fold axis of symmetry. In poliovirus type 1, each tunnel is occupied by a molecule of sphingosine. Similar lipids have been observed in the capsids of other picornaviruses. Because of the symmetry of the capsid, each virus particle may contain up to 60 lipid molecules.

The lipids are thought to contribute to the stability of the native virus particle by locking the capsid in a stable conformation. Consequently, removal of the lipid is probably necessary to endow the particle with sufficient flexibility to permit the RNA to leave the shell. These conclusions come from the study of antiviral drugs known as WIN compounds (named after Sterling-Winthrop, the pharmaceutical company at which they were discovered). These compounds displace the lipid and fit tightly in the hydrophobic tunnel (Fig. 5.19C). Polioviruses containing bound WIN compounds can bind to the cell receptor, but A particles are not produced. WIN compounds may therefore inhibit poliovirus infectivity by preventing the receptor-mediated conformational alterations required for uncoating. The properties of poliovirus mutants that cannot replicate in the absence of WIN compounds underscore the role of the lipids in uncoating. These drug-dependent mutants spontaneously convert to altered particles at 37°C, in the absence of the cell receptor, probably because they do not contain lipid in the hydrophobic pocket. The lipids are therefore viewed as switches, because their presence or absence determines whether the virus is stable or will be uncoated. The interaction of the virus particle with its receptor probably initiates structural changes in the virion that lead to the release of lipid. Consistent with this hypothesis is the observation that CD155 docks onto the poliovirus capsid just above the hydrophobic pocket.

It is usually assumed that the 5′-end of (+) strand RNAs is the first to leave the capsid, to allow immediate translation by ribosomes. This assumption is incorrect for rhinovirus type 2: exit of viral RNA starts from the 3′-end. This directionality is a consequence of how the viral RNA is packaged in the virion, with the 3′-end near the location of pore formation in the altered particle. Whether such directionality is a general feature of nonenveloped (+) strand RNA viruses is unknown.

Uncoating in the Lysosome

Most virus particles that enter cells by receptor-mediated endocytosis leave the pathway before the vesicles reach the lysosomal compartment. This departure is not surprising, for lysosomes contain proteases and nucleases that would degrade virus particles. However, these enzymes play an important role during the uncoating of members of the *Reoviridae*.

Orthoreoviruses are naked icosahedral viruses containing a double-stranded RNA genome of 10 segments. The viral capsid is a double-shelled structure composed of eight different proteins. These virus particles bind to cell receptors via protein σ1 and are internalized into cells by endocytosis (Fig. 5.20A). Infection of cells by reoviruses is sensitive to bafilomycin A1, an inhibitor of the endosomal proton pump, indicating that acidification is required for entry. Low pH activates lysosomal proteases, which then modify several capsid proteins, enabling the virus to cross the vesicle membrane. One viral outer capsid protein is cleaved and another is removed from the particle, producing an infectious subviral particle. These particles have the viral μ1 protein, a myristoylated protein

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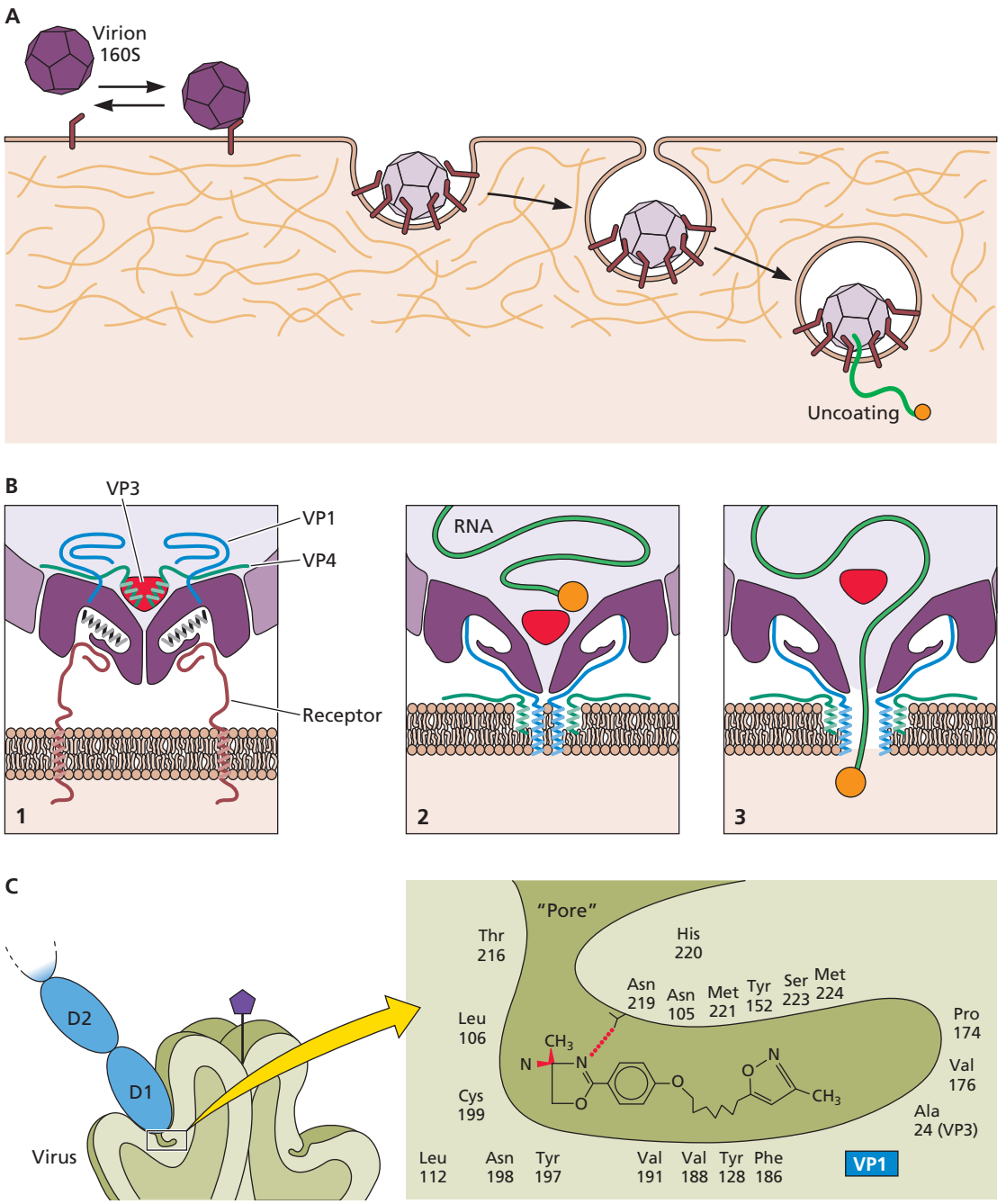


Figure 5.19 Model for poliovirus entry into cells. (A) Overview. The native virion (160S) binds to its cell receptor, CD155, and undergoes a receptor-mediated conformational transition resulting in the formation of altered (A) particles. The viral RNA, shown as a curved green line, leaves the capsid from within early endosomes close to the plasma membrane. **(B)** Model of the formation of a pore in the cell membrane after poliovirus binding. 1, poliovirus (shown in cross section, with capsid proteins purple) binds to CD155 (brown). 2, a conformational change leads to displacement of the pocket lipid (black). The pocket may be occupied by sphingosine in the capsid of poliovirus type 1. The hydrophobic N termini of VP1 (blue) are extruded and insert into the plasma membrane. 3, a pore is formed in the membrane by VP4 and the VP1 N termini, through which the RNA is released from the capsid into the cytosol. **(C)** Schematic diagram of the hydrophobic pocket below the canyon floor. Inset shows a WIN compound in the hydrophobic pocket. Adapted from J. M. Hogle and V. R. Racaniello, p. 71–83, *in* B. L. Semler and E. Wimmer (ed.), *Molecular Biology of Picornaviruses* (ASM Press, Washington, DC, 2002).

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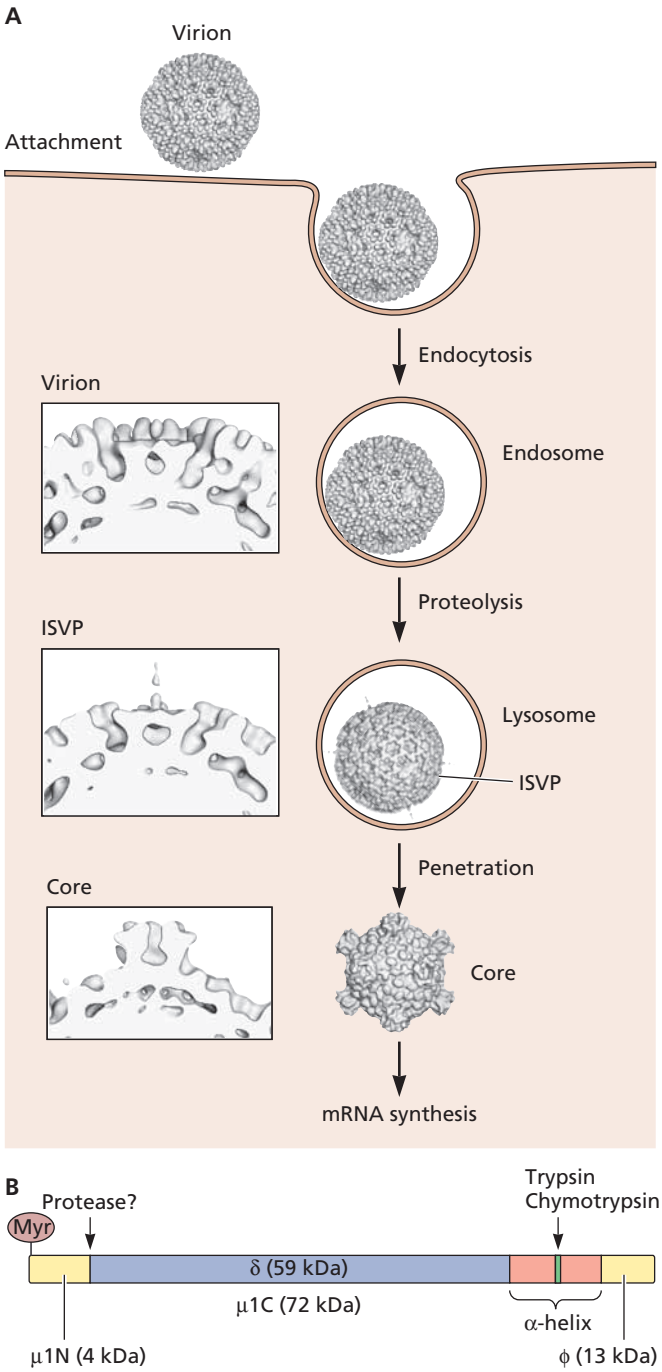


Figure 5.20 Entry of reovirus into cells. (A) The different stages in cell entry of reovirus. After the attachment of σ1 protein to the cell receptor, the virus particle enters the cell by receptor-mediated endocytosis. Proteolysis in the late endosome produces the infectious subviral particle (ISVP), which may then cross the lysosomal membrane and enter the cytoplasm as a core particle. The intact virion is composed of two concentric, icosahedrally organized protein capsids. The outer capsid is made up largely of σ3 and μ1. The dense core shell is formed mainly by λ1 and σ2. In the ISVP, 600 σ3 subunits have been released by proteolysis, and the σ1 protein changes from a compact form to an extended flexible fiber. The μ1 protein, which is thought to mediate interaction of the ISVP with membranes, is present as two cleaved fragments, μ1N and μ1C (see schematic of μ1 in panel B). The N terminus of μ1N is modified with myristate, suggesting that the protein functions in the penetration of membranes. A pair of amphipathic α-helices flank a C-terminal trypsin/chymotrypsin cleavage site at which μ1C is cleaved by lysosomal proteases. Such cleavage may release the helices to facilitate membrane penetration. The membrane-penetrating potential of μ1C in the virion may be masked by σ3; release of the σ3 in ISVPs might then allow μ1C to interact with membranes. The core is produced by the release of 12 σ1 fibers and 600 μ1 subunits. In the transition from ISVP to core, domains of λ2 rotate upward and outward to form a turretlike structure. (Insets) Close-up views of the emerging turretlike structure as the virus progresses through the ISVP and core stages. This structure may facilitate the entry of nucleotides into the core and the exit of newly synthesized viral mRNAs. **(B)** Schematic of the μ1 protein, showing locations of myristate, protease cleavage sites, and amphipathic α-helices. Virus images reprinted from K. A. Dryden et al., *J. Cell Biol.* **122:**1023–1041, 1993, with permission. Courtesy of Norm Olson and Tim Baker, Purdue University.

that interacts with membranes, on the surface. Consequently, subviral particles penetrate the lysosomal membrane and escape into the cytosol. Isolated infectious subviral particles cause cell membranes to become permeable to toxins and produce pores in artificial membranes. These particles can initiate an infection by penetrating the plasma membrane, entering the cytoplasm directly. Their infectivity is not sensitive to bafilomycin A1, further supporting the idea that these particles are primed for membrane entry and do not require further acidification for this process. The core particles generated from infectious subviral particles after penetration into the cytoplasm carry out viral mRNA synthesis.

Movement of Viral and Subviral Particles within Cells

Viral and subviral particles move within the host cell during entry and egress (Chapters 12 and 13). However, movement of molecules larger than 500 kDa does not occur by passive diffusion, because the cytoplasm is crowded with organelles, high concentrations of proteins, and the cytoskeleton. Rather, viral particles and their components are transported via the actin and microtubule cytoskeletons. Such movement can be visualized in live cells by using fluorescently labeled viral proteins (Chapter 2).

The cytoskeleton is a dynamic network of protein filaments that extends throughout the cytoplasm. It is composed of three types of filament—microtubules, intermediate filaments, and microfilaments. Microtubules are organized in a polarized manner, with minus ends situated at the microtubule-organizing center near the nucleus, and plus ends located at the cell periphery. This arrangement permits directed movement of cellular and viral components over long distances. Actin filaments (microfilaments) typically assist in virus movement close to the plasma membrane (Fig. 5.9).

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Transport along actin filaments is accomplished by myosin motors, and movement on microtubules is carried out by kinesin and dynein motors. Hydrolysis of adenosine triphosphate (ATP) provides the energy for the motors to move their cargo along cytoskeletal tracks. Dyneins and kinesins participate in movement of viral components during both entry and egress (Chapters 12 and 13). In some cases, the actin cytoskeleton is remodeled during these processes, for example, when viruses bud from the plasma membrane.

There are two basic ways for viral or subviral particles to travel within the cell—within a membrane vesicle such as an endosome, which interacts with the cytoskeletal transport machinery, or directly in the cytoplasm (Fig. 5.9). In the latter case, some form of the virus particle must bind directly to the transport machinery. After leaving endosomes, the subviral particles derived from adenoviruses and parvoviruses are transported along microtubules to the nucleus. Although adenovirus particles have an overall net movement toward the nucleus, they exhibit bidirectional plus- and minus-end-directed microtubule movement. Adenovirus binding to cells activates two different signal transduction pathways that increase the net velocity of minus-end-directed motility. The signaling pathways are therefore required for efficient delivery of the viral genome to the nucleus. Adenovirus subviral particles are loaded onto microtubules through interaction of the capsid protein, hexon, with dynein. The particles move towards the **centrosome** and are then released and dock onto the nuclear pore complex, prior to viral genome entry into the nucleus.

Some virus particles move along the surfaces of cells prior to entry until a clathrin-coated pit is encountered. If the cell receptor is rare or inaccessible, particles may first bind to more abundant or accessible receptors, such as carbohydrates, and then migrate to receptors that allow entry into the cell. For example, after binding, polyomavirus particles move laterally ("surf") on the plasma membrane for 5 to 10 s before they are internalized. They can be visualized moving along the plasma membrane toward the cell body on **filopodia**, thin extensions of the plasma membrane (Fig. 5.11). Movement along filopodia occurs by an actin-dependent mechanism. Filopodial bridges mediate cell-to-cell spread of a retrovirus in cells in culture. The filopodia originate from uninfected cells and contact infected cells with their tips. The interaction of the viral envelope glycoprotein on the surface of infected cells with the receptor on uninfected cells stabilizes the interaction. Particles move along the outside of the filipodial bridge to the uninfected cell. Such transport is a consequence of actin-based movement of the viral receptor toward the uninfected cell.

A number of different viruses enter the peripheral nervous system and spread to the central nervous system via axons. As no viral genome encodes the molecular motors or cytoskeletal structures needed for long-distance axonal transport, viral adapter proteins are required to allow movement within nerves. An example is axonal transport of alphaherpesviruses. After fusion at the plasma membrane, the viral nucleocapsid is carried by retrograde transport to the neuronal cell body. Such transport is accomplished by the interaction of a major component of the tegument, viral protein VP1/2, with minus-end-directed dynein motors. In contrast, other virus particles are carried to the nerve cell body within endocytic vesicles. After endocytosis of poliovirus, virus particles remain attached to the cellular receptor CD155. The cytoplasmic domain of the receptor engages the dynein light chain Tctex-1 to allow retrograde transport of virus-containing vesicles.

Virus-Induced Signaling via Cell Receptors

Binding of virus particles to cell receptors not only concentrates the particles on the cell surface, but also activates signaling pathways that facilitate virus entry and movement within the cell or produce cellular responses that enhance virus propagation and/or affect pathogenesis. Virus binding may lead to activation of protein kinases that trigger cascades of responses at the plasma membrane, cytoplasm, and nucleus (Chapter 14). Second messengers that participate in signaling include phosphatidylinositides, diacylglycerides, and calcium; regulators of membrane trafficking and actin dynamics also contribute to signaling. Virus-receptor interactions also stimulate antiviral responses (Volume II, Chapter 3).

Signaling triggered by binding of coxsackievirus B3 to cells makes receptors accessible for virus entry. The coxsackievirus and adenovirus receptor, Car, is not present on the apical surface of epithelial cells that line the intestinal and respiratory tracts. This membrane protein is a component of tight junctions and is inaccessible to virus particles. Binding of group B coxsackieviruses to its receptor, CD55, which is present on the apical surface, activates Abl kinase, which in turn triggers Rac-dependent actin rearrangements. These changes allow movement of virus particles to the tight junction, where they can bind Car and enter cells.

Signaling is essential for the entry of simian virus 40 into cells. Binding of this virus particle to its glycolipid cell receptor, GM1 ganglioside, causes activation of tyrosine kinases. The signaling that ensues induces reorganization of actin filaments, internalization of the virus in caveolae, and transport of the caveolar vesicles to the endoplasmic reticulum. The activities of more than 50 cellular protein kinases regulate the entry of this virus into cells.

Import of Viral Genomes into the Nucleus

The reproduction of most DNA viruses, and some RNA viruses including retroviruses and influenza viruses, begins in the cell nucleus. The genomes of these viruses must therefore

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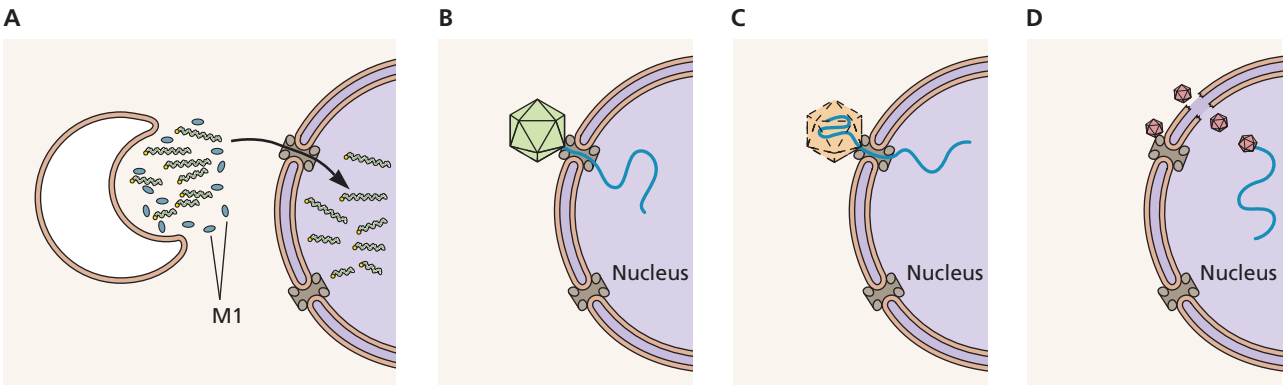


Figure 5.21 Different strategies for entering the nucleus. (A) Each segment of the influenza virus genome is small enough to be transported through the pore complex. **(B)** The herpes simplex virus type 1 capsid docks onto the nuclear pore complex and is minimally disassembled to allow transit of the viral DNA into the nucleus. **(C)** The adenovirus subviral particle is dismantled at the nuclear pore, allowing transport of the viral DNA into the nucleus. **(D)** The capsids of some viruses (parvovirus and hepadnavirus) are small enough to enter the nuclear pore complex without disassembly but do not enter by this route. These virus particles bind the nuclear pore complex, which causes disruption of the nuclear envelope followed by nuclear entry.

be imported from the cytoplasm. One way to accomplish this movement is via the cellular pathway for protein import into the nucleus. An alternative, observed in cells infected by some retroviruses, is to enter the nucleus after the nuclear envelope breaks down during cell division. When the nuclear envelope is reformed, the viral DNA is incorporated into the nucleus together with cellular chromatin. This strategy restricts infection to cells that undergo mitosis.

Many subviral particles are too large to pass through the nuclear pore complex. There are several strategies to overcome this limitation (Fig. 5.21). The influenza virus genome, which consists of eight segments that are each small enough to pass through the nuclear pore complex, is uncoated in the cytoplasm. Adenovirus subviral particles dock onto the nuclear pore complex and are disassembled by the import machinery, allowing the viral DNA to pass into the nucleus. Herpes simplex virus capsids also dock onto the nuclear pore but remain largely intact, and the nucleic acid is injected into the nucleus through a portal in the nucleocapsid. The DNA of some bacteriophages is packaged in virus particles at high pressure, which provides sufficient force to insert the viral DNA genome into the bacterial cell. A similar mechanism may allow injection of herpesviral DNA (Box 5.6). This mechanism would overcome the problem that transport through the nuclear pore complex depends upon hydrophobic interactions with nucleoporins: the charged and hydrophilic viral nucleic acids would have difficulty passing through the pore.

Nuclear Localization Signals

Proteins that reside within the nucleus are characterized by the presence of specific nuclear targeting sequences. Such **nuclear localization signals** are both necessary for nuclear entry of the proteins in which they are present and sufficient to direct heterologous, nonnuclear proteins to enter this organelle. Nuclear localization signals identified by these criteria share a number of common properties: they are generally fewer than 20 amino acids in length, and they are usually rich in basic amino acids. Despite these similarities, no consensus nuclear localization sequence can be defined.

Most nuclear localization signals belong to one of two classes, simple or bipartite sequences (Fig. 5.22). A particularly well characterized example of a simple nuclear localization signal is that of simian virus 40 large T antigen, which comprises five contiguous basic residues flanked by hydrophobic amino acids. This sequence is sufficient to relocate normally cytoplasmic proteins to the nucleus. Many other viral and cellular nuclear proteins contain short, basic nuclear localization signals, but these signals are not identical in primary sequence to that of T-antigen. The presence of a nuclear localization signal is all that is needed to target a macromolecular substrate for import into the nucleus.

The Nuclear Pore Complex

The nuclear envelope is composed of two typical lipid bilayers separated by a luminal space (Fig. 5.23). Like all other cellular membranes, it is impermeable to macromolecules such as proteins. However, the nuclear pore complexes that stud the nuclear envelopes of all eukaryotic cells provide aqueous channels that span both the inner and outer nuclear membranes for exchange of small molecules, macromolecules, and macromolecular assemblies between nuclear and cytoplasmic compartments. Numerous experimental techniques, including direct visualization of gold particles attached to proteins or RNA molecules as they are transported, have established that

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BOX 5.6

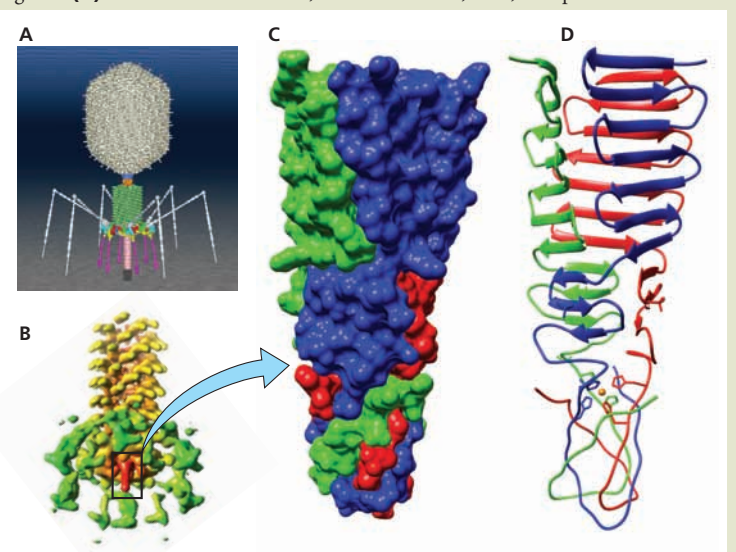
DISCUSSION

The bacteriophage DNA injection machine

The mechanisms by which the bacteriophage genome enters the bacterial host are unlike those for viruses of eukaryotic cells. One major difference is that the bacteriophage particle remains on the surface of the bacterium as the nucleic acid passes into the cell. The DNA genome of some bacteriophages is packaged under high pressure (up to 870 lb/in^2) in the capsid and is injected into the cell in a process that has no counterpart in the entry process of eukaryotic viruses. The complete structure of bacteriophage T4 illustrates this remarkable process (see figure). To initiate infection, the tail fibers attach to receptors on the surface of *Escherichia coli*. Binding causes a conformational change in the baseplate, which leads to contraction of the sheath. This movement drives the rigid tail tube through the outer membrane, using a needle at the tip. When the needle touches the peptidoglycan layer in the periplasm, the needle dissolves and three lysozyme domains in the baseplate are activated. These disrupt the peptidoglycan layer of the bacterium, allowing DNA to enter.

Browning C, Shneider MM, Bowman VD, Schwarzer D, Leiman PG. 2011. Phage pierces the host cell membrane with the iron-loaded spike. *Structure* **20:**236–339.

Structure of bacteriophage T4. (A) A model of the 2,000-Å bacteriophage as produced from electron microscopy and X-ray crystallography. Components of the virion are color coded: virion head (beige), tail tube (pink), contractile sheath around the tail tube (green), baseplate (multicolored), and tail fibers (white and magenta). In the illustration, the virion contacts the cell surface, and the tail sheath is contracted prior to DNA release into the cell. Courtesy of Michael Rossmann, Purdue University. **Structure of bacteriophage membrane-piercing spike. (B)** CryoEM reconstruction of phi92 basplate. The spike is shown in red. **(C, D)** Trimers of bacteriophage phi92 gp138, shown as surface **(C)** and ribbon diagrams **(D)**. From P.G. Leiman et al., *Cell* **118:**419-430, 2004, with permission.



nuclear proteins enter and RNA molecules exit the nucleus by transport through the nuclear pore complex. The functions of the nuclear pore complex in both protein import and RNA export are not completely understood, not least because this important cellular machine is large (molecular mass, approximately 125 × 10^6 kDa in vertebrates), built from many different proteins, and architecturally complex (Fig. 5.23).

The nuclear pore complex allows passage of cargo in and out of the nucleus by either passive diffusion or facilitated translocation. Passive diffusion does not require interaction between the cargo and components of the nuclear pore complex and becomes inefficient as molecules approach 9 nm in diameter. Objects as large as 39 nm in diameter can pass through nuclear pore complexes by facilitated translocation. This process requires specific interactions between the cargo and components of the nuclear pore complex.

The Nuclear Import Pathway

Import of a protein into the nucleus via nuclear localization signals occurs in two distinct, and experimentally separable, steps (Fig. 5.23C). A protein containing such a signal first binds to a soluble cytoplasmic receptor protein. This complex then engages with the cytoplasmic surface of the nuclear pore complex, a reaction often called docking, and is translocated through the nuclear pore complex. In the nucleus, the complex is disassembled, releasing the protein cargo.

Different groups of proteins are imported by specific receptor systems. In what is known as the "classical system" of import, cargo proteins containing basic nuclear localization signals bind to the cytoplasmic nuclear localization signal receptor protein importin-α (Fig. 5.23C). This complex then binds importin-β, which mediates docking with the nuclear pore complex by binding to members of a family of nucleoporins. Some of these nucleoporins are found in the cytoplasmic filaments of the nuclear pore complex (Fig. 5.23), which associate with import substrates. The complex is translocated to the opposite side of the nuclear envelope, where the cargo is released. Other importins can bind cargo proteins directly without the need for an adapter protein.

A single translocation through the nuclear pore complex does not require energy consumption. However, maintenance of a gradient of the guanosine nucleotide-bound forms of Ran,

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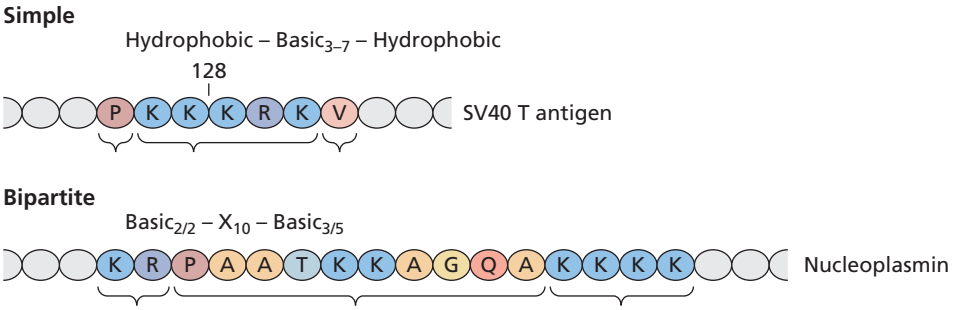


Figure 5.22 Nuclear localization signals. The general form and a specific example of simple and bipartite nuclear localization signals are shown in the one-letter amino acid code, where X is any residue. Bipartite nuclear targeting signals are defined by the presence of two clusters of positively charged amino acids separated by a spacer region of variable sequence. Both clusters of basic residues, which often resemble the simple targeting sequences of proteins like simian virus 40 T antigen, are required for efficient import of the proteins in which they are found. The subscript indicates either length (3–7) or composition (e.g., 3/5 means at least 3 residues out of 5 are basic). Gold particles with diameters as large as 26 nm are readily imported following their microinjection into the cytoplasm, as long as they are coated with proteins or peptides containing a nuclear localization signal.

with Ran-GDP and Ran-GTP concentrated in the cytoplasm and nucleus, respectively, is absolutely essential for continued transport. For example, conversion of Ran-GDP to Ran-GTP in the nucleus, catalyzed by the guanine nucleotide exchange protein Rcc-1, promotes dissociation of imported proteins from importins (Fig. 5.23).

Import of Influenza Virus Ribonucleoprotein

Influenza virus is among the few RNA-containing viruses with genomes that replicate in the cell nucleus. After vRNPs separate from M1 and are released into the cytosol, they are rapidly imported into the nucleus (Fig. 5.12). Such import depends on the presence of a nuclear localization signal in the NP protein, a component of vRNP: naked viral RNA does not dock onto the nuclear pore complex, nor is it taken up into the nucleus.

Import of DNA Genomes

The capsids of many DNA-containing viruses are larger than 39 nm and cannot be imported into the nucleus from the cytoplasm. One mechanism for crossing the nuclear membrane comprises docking of a capsid onto the nuclear pore complex, followed by delivery of the viral DNA into the nucleus. Adenoviral and herpesviral DNAs are transported into the nucleus via this mechanism. However, the strategies for DNA import are distinct: adenovirus DNA is covered with proteins and is recognized by the import system, while HSV DNA is naked and is injected.

Partially disassembled adenovirus capsids dock onto the nuclear pore complex by interaction with Nup214 (Fig. 5.24). Release of the viral genome requires capsid protein binding to kinesin-1, the motor protein that mediates transport on microtubules from the nucleus to the cell periphery. As the capsid is held on the nuclear pore, movement of kinesin-1 towards the plasma membrane is thought to pull apart the capsid. The released protein VII-associated viral DNA is then imported into the nucleus, where viral transcription begins. Herpesvirus capsids also dock onto the nuclear pore complex, and interaction with nucleoporins destabilizes a viral protein, pUL25, which locks the genome inside the capsid. This event causes the naked viral DNA, which is packaged in the nucleocapsid under very high pressure, to exit through the portal.

The 26-nm capsid of parvoviruses is small enough to fit through the nuclear pore (39 nm), and it has been assumed that these virus particles enter by this route. However, there is no experimental evidence that parvovirus capsids pass intact through the nuclear pore. Instead, virus particles bind to the nuclear pore complex, followed by disruption of the nuclear envelope and the nuclear lamina, leading to entry of virus particles (Fig. 5.21). After release from the endoplasmic reticulum, the 45-nm capsid of SV40 also docks onto the nuclear pore, initiating disruption of the nuclear envelope and lamina. Such nuclear disruption appears to involve cell proteins that also participate in the increased nuclear permeability that takes place during mitosis, raising the possibility that nuclear entry of these viral genomes is a consequence of remodeling a cell process.

Import of Retroviral Genomes

Fusion of retroviral and plasma membranes releases the viral core into the cytoplasm. The retroviral core consists of the viral RNA genome, coated with NC protein, and the enzymes reverse transcriptase (RT) and integrase (IN), enclosed by capsid (CA) protein. Retroviral DNA synthesis commences in the cytoplasm, within the nucleocapsid core, and after 4 to 8 h of DNA synthesis the preintegration complex, comprising

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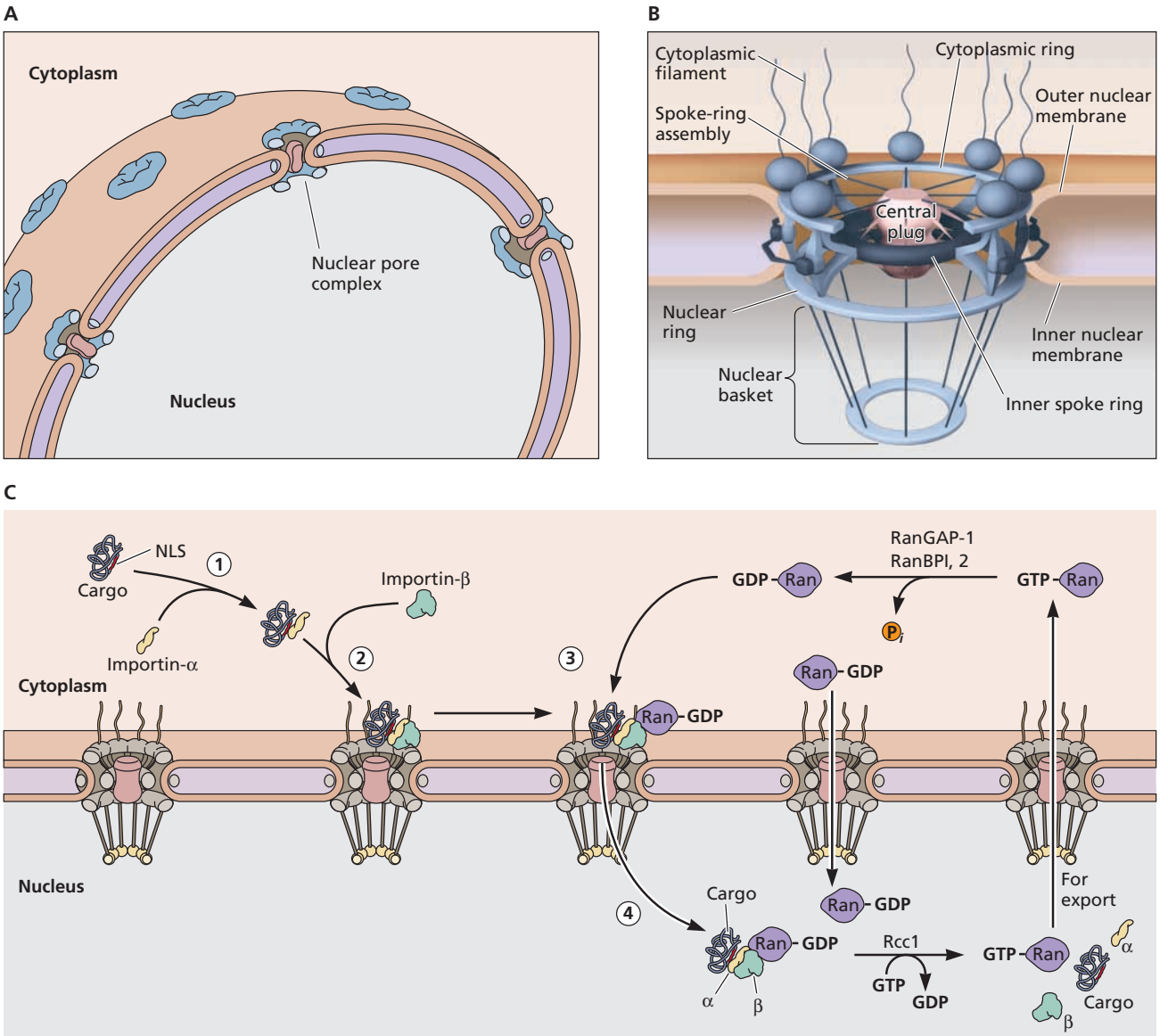


Figure 5.23 Structure and function of the nuclear pore complex. **(A)** Overview of the nuclear membrane, showing the topology of the nuclear pore complexes. **(B)** Schematic drawing of the nuclear pore complex, showing the spoke-ring assembly at its waist and its attachment to cytoplasmic filaments and the nuclear basket. The latter comprises eight filaments, extending 50 to 100 nm from the central structure and terminating in a distal annulus. The nuclear pore channel is shown containing the transporter. **(C)** An example of the classical protein import pathway for proteins with a simple nuclear localization signal (NLS). This pathway is illustrated schematically from left to right. Cytoplasmic and nuclear compartments are shown separated by the nuclear envelope studded with nuclear pore complexes. In step 1, a nuclear localization signal on the cargo (red) is recognized by importin-α. In step 2, importin-β binds the cargo–importin-α complex and docks onto the nucleus, probably by associating initially with nucleoporins present in the cytoplasmic filaments of the nuclear pore complex. Translocation of the substrate into the nucleus (step 4) requires additional soluble proteins, including the small guanine nucleotide-binding protein Ran (step 3). A Ran-specific guanine nucleotide exchange protein (Rcc1) and a Ran-GTPase-activating protein (RanGap-1) are localized in the nucleus and cytoplasm, respectively. The action of RanGAP-1, with the accessory proteins RanBp1 and RanBp2, maintains cytoplasmic Ran in the GDP-bound form. When Ran is in the GTP-bound form, nuclear import cannot occur. Following import, the complexes are dissociated when Ran-GDP is converted to Ran-GTP by Rcc1. Ran-GTP participates in export from the nucleus. The nuclear pool of Ran-GDP is replenished by the action of the transporter Ntf2/p10, which efficiently transports Ran-GDP from the cytoplasm to the nucleus. Hydrolysis of Ran-GTP in the cytoplasm and GTP-GDP exchange in the nucleus therefore maintain a gradient of Ran-GTP/Ran-GDP. The asymmetric distribution of RanGap-1 and Rcc1 allows for the formation of such a gradient. This gradient provides the driving force and directionality for nuclear transport. A monomeric receptor called transportin mediates the import of heterogeneous nuclear RNA-binding proteins that contain glycine- and arginine-rich nuclear localization signals. Transportin is related to importin-β, as are other monomeric receptors that mediate nuclear import of ribosomal proteins. (B) Adapted from Q. Yang, M. P. Rout, and C. W. Akey, *Mol. Cell* **1:**223–234, 1998, with permission.

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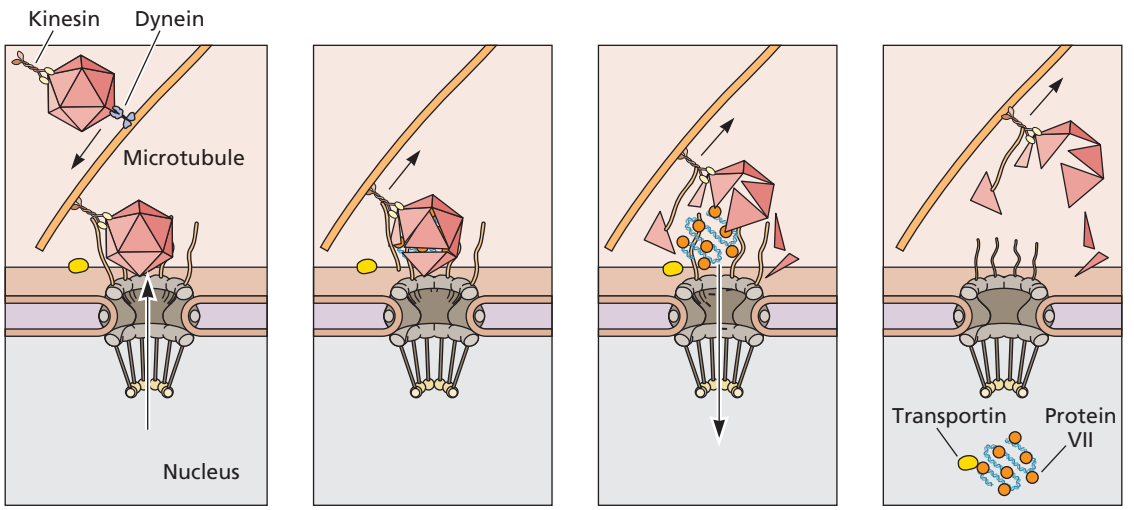


Figure 5.24 Uncoating of adenovirus at the nuclear pore complex. After release from the endosome, the partially disassembled capsid moves towards the nucleus by dynein-driven transport on microtubules. The particle docks onto the nuclear pore complex protein Nup214. The capsid also binds kinesin-1 light chains, which move away from the nucleus, pulling the capsid apart. The viral DNA, bound to protein VII, is delivered into the nucleus by the import protein transportin and other nuclear import proteins.

viral DNA, IN, and other proteins, localizes to the nucleus. There the viral DNA is integrated into the cellular genome, and viral transcription begins. The mechanism of nuclear import of the preintegration complex is poorly understood, but it is quite clear that this structure is too large to pass through the nuclear pore complex. The betaretrovirus Moloney murine leukemia virus can efficiently infect only dividing cells. The viral preintegration complex is tethered to chromatin when the nuclear membrane is broken down during mitosis and remains associated with chromatin as the nuclear membrane reforms in daughter cells, avoiding the need for active transport.

In contrast to Moloney murine leukemia virus, other retroviruses can reproduce in nondividing cells. The preintegration complex of these viruses must therefore be transported into an intact nucleus. The exact mechanism for nuclear entry is still unclear, but for the lentivirus human immunodeficiency virus type 1, there is evidence that CA-mediated attachment of the preintegration complex to the nuclear pore is required. In addition, various viral proteins that contain nuclear localization signals may facilitate the process (e.g., Vpr, MA, and IN).

Perspectives

The study of how viruses enter cells provides critical insight into the very first steps of virus reproduction. Virus entry comprises binding to receptors, transport within the cell, dismantling of the virus particle, and release of the genome. It has become clear that there are many pathways for virus entry into cells. Clathrin- and dynamin-dependent endocytosis is no longer the sole entry pathway known; other routes are caveolin-dependent endocytosis and clathrin- and caveolin-independent endocytosis. The road used seems to depend on the virus, the cell type, and the conditions of infection. As most of our current knowledge has been derived from studies with cells in culture, a crucial unanswered question is whether these same pathways of viral entry are utilized during infection of the heterogeneous tissues of living animals.

Virus binding to the cell surface leads to major alterations in cell activities, effects mediated by signal transduction. These responses include providing access to coreceptors, formation of pits, pinching off of vesicles, and rearrangement of actin filaments to facilitate vesicle movement. The precise signaling pathways required need to be elucidated. Such efforts may identify specific targets for inhibiting virus movement in cells.

The development of single-particle tracking methods has advanced considerably in the past 5 years. As a consequence, our understanding of the routes that viruses travel once they are inside the cell has improved markedly. The role of cellular transport pathways in bringing viral genomes to the site appropriate for their replication within the cell is beginning to be clarified. Yet many questions remain. How are viruses or subviral particles transported on the cytoskeletal network? What are the signals for their attachment and release from microtubules and filaments?

The genomes of many viruses are replicated in the nucleus. Some viral genomes enter this cellular compartment by transport through the nuclear pore complex. Studies of adenoviral DNA import into the nucleus have revealed an active role for components of both the nuclear pore complex and the

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microtubule network in subviral particle disassembly. Other viral DNA genomes, such as that of the herpesviruses, pass naked through the nuclear pore, raising the question of how these hydrophilic molecules move through the hydrophobic pore, against a steep gradient of nucleic acid in the nucleus. Nuclear import of retroviral DNA is barely understood. What signal allows transport of the large preintegration complex of retroviruses through the nuclear pore?

Nearly all the principles and specific features discussed in this chapter were derived from studies of viral infection in cultured cells. How viruses attach to and enter cells of a living animal remains an uncharted territory. Methods are being developed to study virus entry in whole animals, and the results will be important for understanding how viruses spread and breach host defenses to reach target cells.

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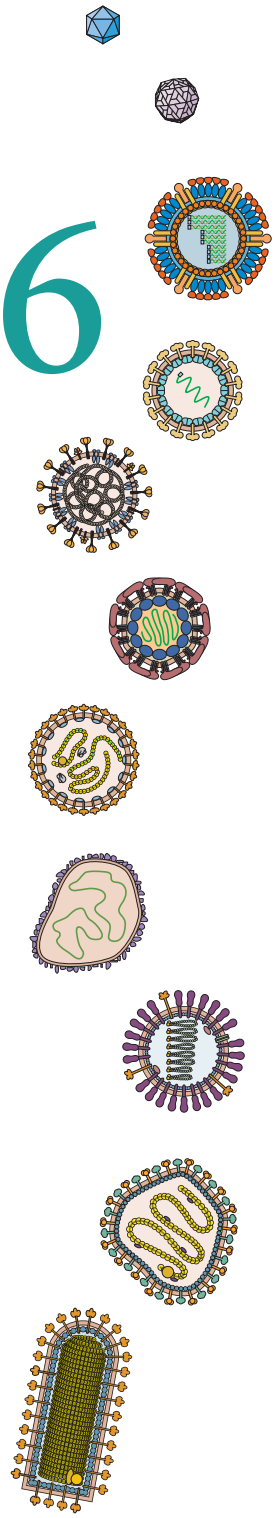
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6 Synthesis of RNA from RNA Templates

Introduction

The Nature of the RNA Template
- Secondary Structures in Viral RNA
- Naked or Nucleocapsid RNA

The RNA Synthesis Machinery
- Identification of RNA-Dependent RNA Polymerases
- Sequence Relationships among RNA Polymerases
- Three-Dimensional Structure of RNA-Dependent RNA Polymerases

Mechanisms of RNA Synthesis
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- Capping
- Elongation
- Template Specificity
- Unwinding the RNA Template
- Role of Cellular Proteins

Paradigms for Viral RNA Synthesis
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- Synthesis of Nested Subgenomic mRNAs
- (−) Strand RNA
- Ambisense RNA
- Double-Stranded RNA
- Unique Mechanisms of mRNA and Genome Synthesis of Hepatitis Delta Satellite Virus
- Why Are (−) and (+) Strands Made in Unequal Quantities?
- Do Ribosomes and RNA Polymerases Collide?

Cellular Sites of Viral RNA Synthesis

Origins of Diversity in RNA Virus Genomes
- Misincorporation of Nucleotides
- Segment Reassortment and RNA Recombination
- RNA Editing

Perspectives

References

LINKS FOR CHAPTER 6

⏭ *Video: Interview with Dr. Karla Kirkegaard*
http://bit.ly/Virology_Kirkegaard

⏭ *A swinging gate*
http://bit.ly/Virology_Twiv330

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Truth is ever to be found in the simplicity, and not in the multiplicity and confusion of things.

SIR ISAAC NEWTON

Introduction

The genomes of RNA viruses may be unimolecular or segmented, single stranded of (+), (−), or ambisense polarity, double stranded, or circular. These structurally diverse viral RNA genomes share a common requirement: they must be efficiently copied within the infected cell to provide both genomes for assembly into progeny virus particles and messenger RNAs (mRNAs) for the synthesis of viral proteins. The synthesis of these RNA molecules is a unique process that has no parallel in the cell. The genomes of all RNA viruses except retroviruses encode an **RNA-dependent RNA polymerase** (Box 6.1) to catalyze the synthesis of new genomes and mRNAs.

Virus particles that contain (−) strand or double-stranded RNA genomes must contain the RNA polymerase, because the incoming viral RNA can be neither translated nor copied by the cellular machinery. Consequently, the deproteinized genomes of (−) strand and double-stranded RNA viruses are noninfectious. In contrast, viral particles containing a (+) strand RNA genome lack a viral polymerase; the deproteinized RNAs of these viruses **are** infectious because they are translated in cells to produce, among other viral proteins, the viral RNA polymerase. An exception is the retrovirus particle, which contains a (+) stranded RNA genome that is not translated but rather copied to DNA by reverse transcriptase (Chapter 7).

The mechanisms by which viral mRNA is made and the RNA genome is replicated in cells infected by RNA viruses appear even more diverse than the structure and organization of viral RNA genomes (Fig. 6.1). For example, the genomes of both picornaviruses and alphaviruses are single molecules of (+) strand genomic RNA, but the strategies for the production of viral RNA are quite different. Nevertheless, each mechanism of viral RNA synthesis meets two essential requirements common to all infectious cycles: (i) during replication the RNA genome must be copied from one end to the other with no loss of nucleotide sequence; and (ii) viral mRNAs that can be translated efficiently by the cellular protein synthetic machinery must be made.

In this chapter we consider the mechanisms of viral RNA synthesis, the mechanism for switching from mRNA production to genome replication, and how the process of RNA-directed RNA synthesis leads to genetic diversity. Much of our understanding of viral RNA synthesis comes from experiments with purified components. Because it is possible that events proceed differently in infected cells, the results of such *in vitro* studies are used to build models for the different steps in RNA synthesis. While many models exist for each reaction, those presented in this chapter were selected because they are consistent with experimental results obtained in different laboratories or have been validated with simplified systems in cells in culture. The general principles of RNA synthesis deduced from such studies are illustrated with a few viruses as examples.

The Nature of the RNA Template

Secondary Structures in Viral RNA

RNA molecules are not simple linear chains but can form secondary structures that are important for RNA synthesis, translation, and assembly (Fig. 6.2). The first step in identifying a structural feature in RNA is to scan the nucleotide sequence with software designed to fold the nucleic acid into energetically stable structures. Comparative sequence analysis

PRINCIPLES ***Synthesis of RNA from RNA templates***

- Viral RNA genomes must be copied to provide both genomes for assembly into progeny virus particles, and mRNAs for the synthesis of viral proteins.
- Viral RNA genomes may be naked in the virus particle (typically (+) strand RNAs) or organized into nucleocapsids in which proteins are bound to the genomic RNAs.
- Some viral RNA-dependent RNA polymerases can initiate RNA synthesis without a primer, while others are primer dependent.
- Viral RNA-dependent RNA polymerases, like the other three types of nucleic acid enzymes, resemble a right hand consisting of palm, fingers, and thumb domains, with the active site located in the palm.
- Viral RNA polymerases that initiate RNA synthesis without a primer appear to have an extra protein domain in the active site that acts as a "protein priming platform" to provide support for the initiating NTP.
- Primers for RNA polymerases may be capped fragments of cellular mRNAs or protein-linked nucleotides.
- Specificity of RNA polymerases for viral RNAs is conferred by the recognition of RNA sequences or structures.
- Host cell proteins are required for the activity of viral RNA polymerases.
- The single-stranded RNA genome of hepatitis delta virus is copied by host cell DNA-dependent RNA polymerase.
- Viral RNA synthesis takes place on specific structures in the cell, either nucleocapsids, subviral particles, or membrane-bound replication complexes.
- RNA synthesis is error prone, and this process, together with reassortment and recombination, yields diversity that is required for viral evolution.

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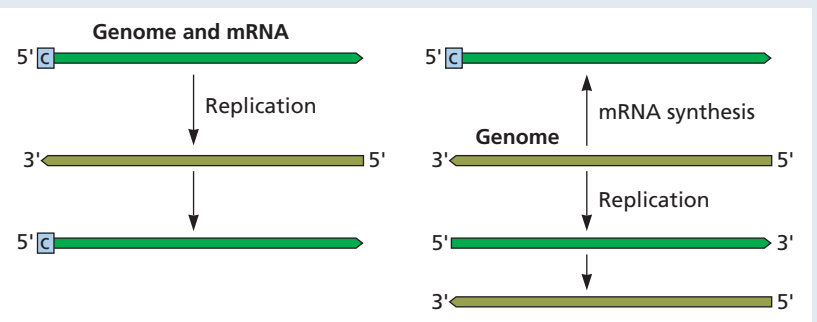
BOX 6.1

TERMINOLOGY

What should we call RNA polymerases and the processes they catalyze?

Historically, viral RNA-dependent RNA polymerases were given two different names depending on their activities during infection. The term **replicase** was used to describe the enzyme that copies the viral RNA to produce additional genomes, while the enzyme that synthesizes mRNA was called **transcriptase.** In some cases, this terminology indicates true differences in the enzymes that carry out synthesis of functionally different RNAs, but for other RNA viruses, genomic replication and mRNA synthesis are the **same** reaction (Figure). For double-stranded RNA viruses, mRNA synthesis produces templates that can also be used for genomic replication. As these terms can therefore be inaccurate and misleading, they are not used here.

The production of mRNAs from viral RNA templates is often designated **transcription.** However, this term refers to a specific process, the copying of genetic information carried in DNA into RNA. Consequently, it is not used here to describe synthesis of the mRNAs of viruses with RNA genomes. Similarly, use of the term **promoter** is reserved to designate sequences controlling transcription of DNA templates.



can predict RNA secondary structures. For example, comparison of the RNA sequences of several related viruses might establish that the structure, but not the sequence, of a stem-loop is conserved. Direct evidence for RNA structures comes from experiments in which RNAs are treated with enzymes or chemicals that attack single- or double-stranded regions specifically. The results of such analyses can confirm that predicted stem regions are base paired while loops are unpaired. Structures of RNA hairpins and pseudoknots have been determined by X-ray crystallography or nuclear magnetic resonance (Fig. 6.2C).

Naked or Nucleocapsid RNA

The genomes of (−) strand viruses are organized into nucleocapsids in which protein molecules, including the RNA-dependent RNA polymerase and accessory proteins, are bound to the genomic RNAs at regular intervals (Fig. 6.3). These tightly wound ribonucleoprotein complexes are very stable and generally resistant to RNase. The RNA polymerases of (−) strand viruses copy viral RNAs **only** when they are present in the nucleocapsid, such as that formed by the N protein of vesicular stomatitis virus bound to genomic RNA. In contrast, the genomes of (+) strand RNA viruses are not coated with proteins in the virus particle (exceptions are the (+) strand RNA genomes of members of the *Coronaviridae, Arteriviridae*, and *Retroviridae*). This difference is consistent with the fact that mRNAs are produced from the genomes of (−) strand RNA viruses upon cell entry, whereas the genomes of (+) strand RNA viruses are translated.

The viral nucleoproteins (NP) are cooperative, single-stranded RNA-binding proteins, as are the single-stranded nucleic acid-binding proteins required during DNA-directed DNA and RNA synthesis. Their function during replication is to keep the RNA single stranded and prevent base pairing between the template and product, so that additional rounds of RNA synthesis can occur. The nucleoproteins of nonsegmented (−) strand RNA viruses have a two-lobe architecture that forms a positively charged groove that binds and shields the genomic RNA (Fig. 6.3). Interactions between nucleoproteins lock monomers tightly, resulting in rigid NP-RNA complexes. The NP structures from segmented (−) strand RNA viruses are more varied and display less coordinated contacts between nucleoprotein subunits. These differences may explain why the NP-RNAs of these viruses are more susceptible to RNase digestion than those of nonsegmented (−) RNA viruses. The varied structures of the NP-RNA complexes also influence the access of the viral RNA polymerase to the template. The RNA polymerase of

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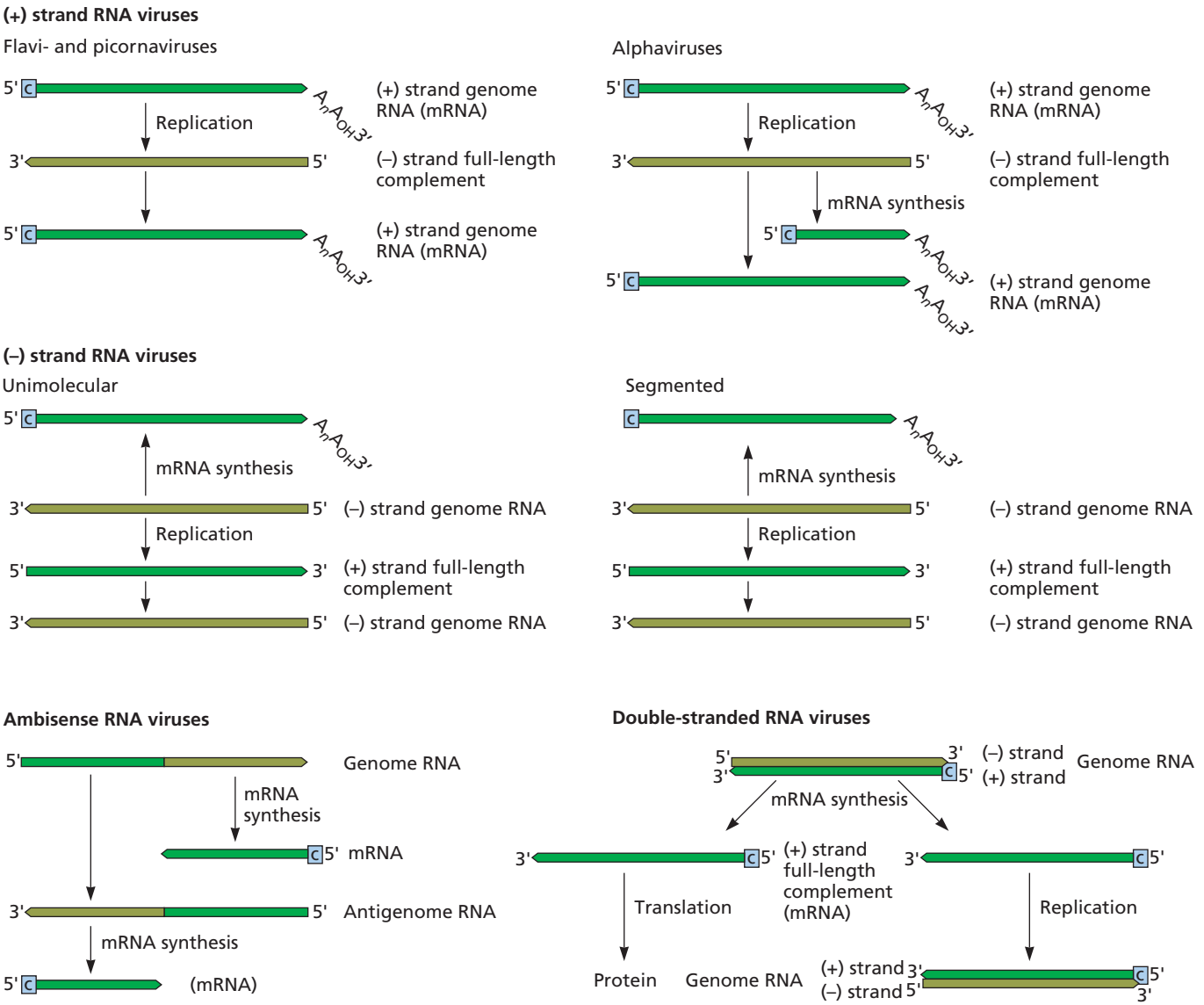


Figure 6.1 Strategies for replication and mRNA synthesis of RNA virus genomes are shown for representative virus families. Picornaviral genomic RNA is linked to VPg at the 5′ end. The (+) genomic RNA of some flaviviruses does not contain poly(A). Only one RNA segment is shown for segmented (−) strand RNA viruses.

segmented (−) strand RNA viruses can bind the NP-RNA template directly, whereas those of nonsegmented (−) strand RNA viruses cannot: a phosphoprotein (P) is required to recruit the RNA polymerase to the NP-RNA.

The genomes of many (+) strand RNA viruses encode helicases that serve a similar function as the nucleoproteins of (−) strand RNA viruses (see "Unwinding the RNA Template" below). In addition to its enzymatic activity, the poliovirus RNA polymerase ($3D^{pol}$) is a cooperative single-stranded RNA-binding protein and can unwind RNA duplexes without the hydrolysis of ATP that is characteristic of helicase-mediated unwinding. Polioviral RNA polymerase is therefore functionally similar to the RNA-binding nucleoproteins of (−) strand viruses.

The RNA Synthesis Machinery

Identification of RNA-Dependent RNA Polymerases

The first evidence for a viral RNA-dependent RNA polymerase emerged in the early 1960s from studies of mengovirus and poliovirus, both (+) strand RNA viruses. In these experiments, extracts were prepared from virus-infected cells and incubated with the four ribonucleoside

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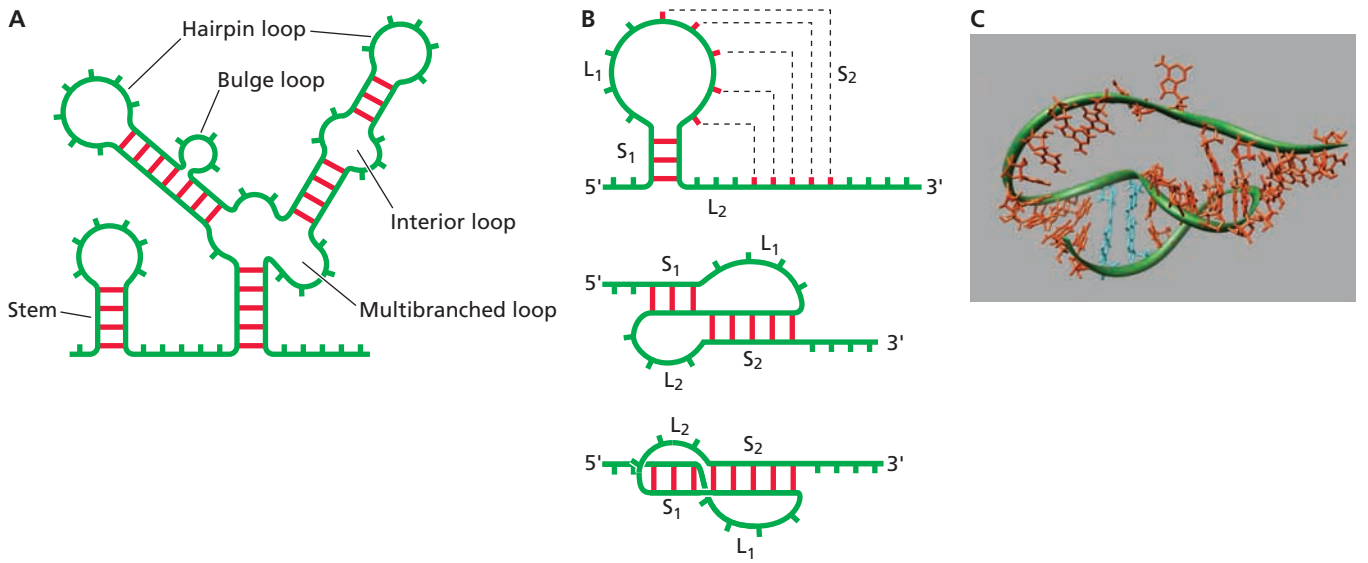
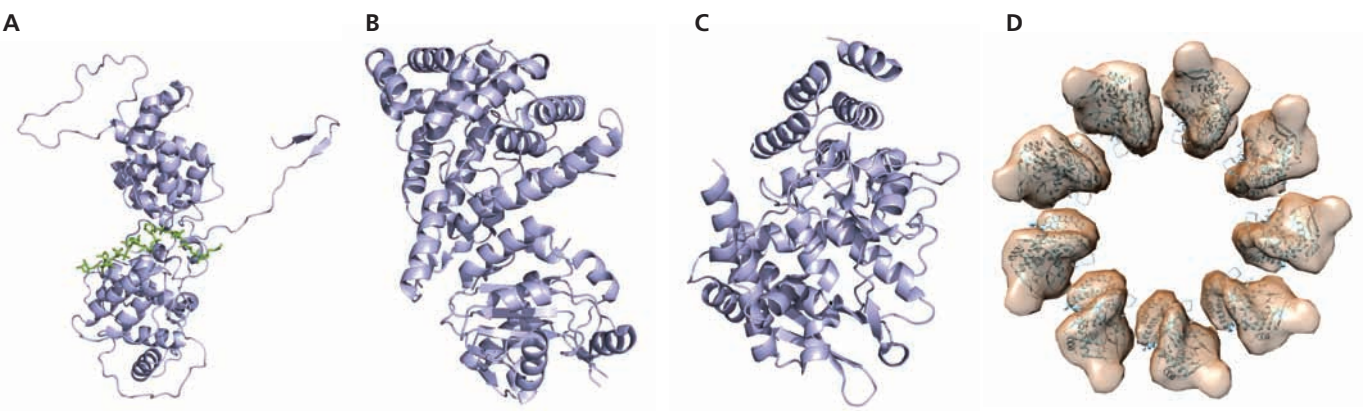


Figure 6.2 RNA secondary structure. **(A)** Schematic of different structural motifs in RNA. Red bars indicate base pairs; green bars indicate unpaired nucleotides. **(B)** Schematic of a pseudoknot. (Top) Stem 1 (S_1) is formed by base pairing in the stem-loop structure, and stem 2 (S_2) is formed by base pairing of nucleotides in the loop with nucleotides outside the loop. (Middle) A different view of the formation of stems S_1 and S_2. (Bottom) Coaxial stacking of S_1 and S_2 resulting in a quasicontinuous double helix. **(C)** Structure of a pseudoknot as determined by X-ray crystallography. The sugar backbone is highlighted with a green tube. Stacking of the bases in the areas of S_1 and S_2 can be seen. From Protein Data Bank file 1l2x. Adapted from C. W. Pleij, *Trends Biochem. Sci* **15:**143–147, 1990, with permission.

triphosphates (adenosine triphosphate [ATP], uridine triphosphate [UTP], cytosine triphosphate [CTP], and guanosine triphosphate [GTP]), one of which was radioactively labeled. The incorporation of nucleoside monophosphate into RNA was then measured. Infection with mengovirus or poliovirus led to the appearance of a cytoplasmic enzyme that could synthesize viral RNA in the presence of actinomycin D, a drug that was known to inhibit cellular DNA-directed RNA synthesis by intercalation into the double-stranded template. Lack of sensitivity to the drug suggested that the enzyme was virus specific and could copy RNA from an RNA template and not from a DNA template. This enzyme was presumed

Figure 6.3 Structure of viral ribonucleoproteins. **(A)** Ribbon diagram of vesicular stomatitis N protein molecule bound to RNA. The ribose-phosphate backbone of the RNA is shown as a green tube, and is bound in a groove located between N- and C-terminal lobes of the protein. From Protein Data Bank file 2qvj. **(B)** Ribbon diagram of Lassa virus NP bound to RNA. From Protein Data Bank file 3q7b. **(C)** Ribbon diagram of influenza virus NP bound to RNA. From Protein Data Bank file 2wfs. **(D)** Model of influenza A virus RNP. The polymerase complex is bound to a short NP-RNA template. Atomic structures of the NP are placed inside the model determined by cryo-electron microscopy. From Protein Data Bank file 2wfs and Electron Microscopy Databank entry 1603.



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to be an RNA-dependent RNA polymerase. Similar assays were later used to demonstrate that the virions of (−) strand viruses and of double-stranded RNA viruses contain an RNA-dependent RNA polymerase that synthesizes mRNAs from the (−) strand RNA present in the particles.

The initial discovery of a putative RNA polymerase in poliovirus-infected cells was followed by attempts to purify the enzyme and show that it can copy viral RNA. Because polioviral genomic RNA contains a 3′-poly(A) sequence, polymerase activity was measured with a poly(A) template and an oligo(U) **primer**. After several fractionation steps, a poly(U) polymerase that could copy polioviral genomic RNA in the presence of this primer was purified from infected cells. Poly(U) polymerase activity coincided with a single polypeptide, now known to be the polioviral RNA polymerase $3D^{pol}$ (see Appendix, Fig. 21, for a description of this nomenclature). Purified $3D^{pol}$ RNA polymerase cannot copy polioviral genomic RNA in the absence of a primer.

Assays for RNA polymerase activity have been used to demonstrate virus-specific enzymes in virus particles or in extracts of cells infected with a wide variety of RNA viruses. Amino acid sequence alignments can be used to identify viral proteins with motifs characteristic of RNA-dependent RNA polymerases (see "Sequence Relationships among RNA Polymerases" below). These approaches were used to identify the L proteins of paramyxoviruses and bunyaviruses, the PB1 protein of influenza viruses, and the nsP4 protein of alphaviruses as candidate RNA polymerases. When the genes encoding these polymerases are expressed in cells, the proteins that are produced can copy viral RNA templates.

RNA-directed RNA synthesis follows a set of universal rules that differ slightly from those followed by DNA-dependent DNA polymerases. RNA synthesis initiates and terminates at specific sites in the template and is catalyzed by virus-encoded polymerases, but viral accessory proteins and even host cell proteins may also be required. Like cellular DNA-dependent RNA polymerases, some RNA-dependent RNA polymerases can initiate RNA synthesis *de novo*. Others require a primer with a free 3′-OH end to which nucleotides complementary to the template are added. Some RNA primers are protein linked, while others bear a 5′-cap structure (the cap structure is described in Chapter 10). A comparison of the structures and sequences of polynucleotide polymerases has led to the hypothesis that all polymerases catalyze synthesis by a mechanism that requires two metals (Box 6.2). RNA is usually synthesized by template-directed, stepwise incorporation of ribodeoxynucleoside monophosphates (NMPs) into the 3′-OH end of the growing RNA chain, which undergoes **elongation** in the 5′ → 3′ direction.

Sequence Relationships among RNA Polymerases

The amino acid sequences of viral RNA polymerases have been compared to identify conserved regions and to provide information about their evolution. Although polymerases have very different amino acid sequences, four conserved sequence motifs (A to D) have been identified in all RNA-dependent RNA polymerases (Fig. 6.4). Motif A and motif C contain the Asp residues that bind metal ions in the active site (Box 6.2), and motif C includes a Gly-Asp-Asp sequence conserved in the RNA polymerases of most (+) strand RNA viruses. It was suggested that this sequence is part of the active site of the enzyme. In support of this hypothesis, alterations in this sequence in polioviral $3D^{pol}$ and many other viral polymerases inactivate the enzyme. Evidence that a viral protein is an RNA polymerase is considerably strengthened when this 3-amino-acid sequence is found (Box 6.3).

Motifs A and C are also present in the sequences of RNA-dependent DNA polymerases that copy RNA templates, while all DNA-dependent polymerases share conserved sequence motifs A, B, and C (Fig. 6.4). These sequence comparisons indicate that all four classes of nucleic acid polymerases have a similar core catalytic domain (the palm domain) and most probably evolved from a common ancestor.

Three-Dimensional Structure of RNA-Dependent RNA Polymerases

The crystal structures of RNA-dependent RNA polymerases have confirmed the hypothesis that all polynucleotide polymerases are structurally similar. The shapes of all four types of polymerases resemble a right hand consisting of a palm, fingers, and a thumb, with the active site of the enzyme located in the palm (Fig. 6.4B). This shape supports the correct arrangement of substrates and metal ions at the catalytic site optimal for catalysis, and allows the dynamic changes needed during RNA synthesis. The structures of RNA-dependent RNA polymerases differ in detail from those of other polymerases, presumably to accommodate different templates and priming mechanisms.

All known RNA-dependent RNA polymerases adopt closed structures in which the active site is completely encircled (Fig. 6.4). In contrast, structures of other polynucleotide polymerases resemble an open hand. The closed structure, which is formed by interactions between the fingers and thumb domains, creates a nucleoside triphosphate (NTP) entry tunnel on one face of the enzyme and a template-binding site on the other. Residues within motif F, a conserved region unique to RNA-dependent RNA polymerases (Fig. 6.4), form the NTP entry tunnel.

The palm domain of RNA-dependent RNA polymerases is structurally similar to that of other polymerases, and contains the four motifs (A to D) that are conserved in all these enzymes (Fig. 6.4). The motifs confer specific functions, such as nucleotide recognition and binding (A and B), phosphoryl transfer (A and C), and determine the structure of the palm domain (D). The fifth motif, E, which is present in

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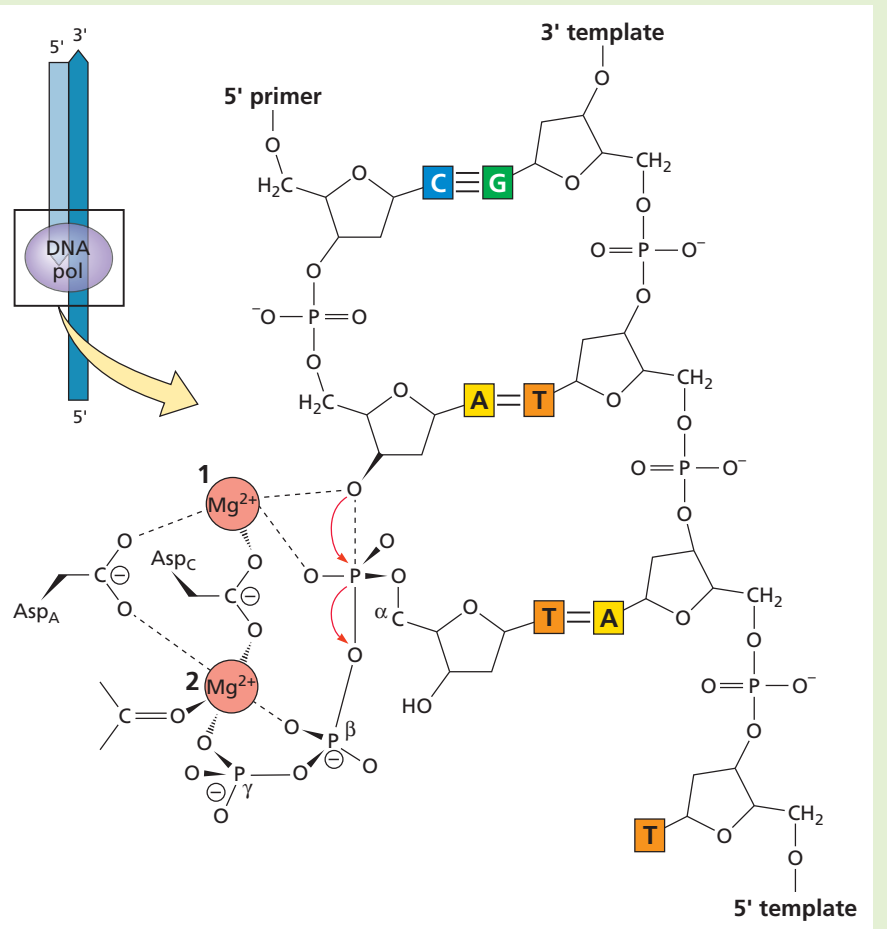
BOX 6.2

BACKGROUND

Two-metal mechanism of catalysis by polymerases

All polynucleotide polymerases are thought to catalyze synthesis by a two-metal mechanism that requires two conserved aspartic acid residues (see figure). The carboxylate groups of these amino acids coordinate two divalent metal ions, shown as Mg^{2+} in the figure. One metal ion promotes deprotonation of the 3′-OH group of the nascent strand, and the other ion stabilizes the transition state at the α-phosphate of NTP and facilitates the release of pyrophosphate (PP_i).

Two-metal mechanism of polymerase catalysis. Red arrows indicate the net movement of electrons.



RNA-dependent, but not in DNA-dependent, polymerases, lies between the palm and thumb domains and binds the primer.

RNA-dependent RNA polymerases prefer to incorporate NTPs rather than deoxyribonucleoside triphosphates (dNTPs). NTP recognition by poliovirus $3D^{pol}$ is regulated by Asp238, which forms a hydrogen bond with the ribose 2′-OH (Fig. 6.5). dNTPs are not bound because Asp238 cannot form a hydrogen bond with 2′-deoxyribose. An Asp is present at this position in all RNA-dependent RNA polymerases. A Tyr at this position in RNA-dependent DNA polymerases is responsible for discriminating against NTPs and selecting dNTPs. Motif C of $3D^{pol}$ contains the Asp-Asp sequence conserved in RNA-dependent polymerases; the first Asp is also conserved in DNA-dependent polymerases. The two Asp residues of motif C and the conserved Asp238 of motif A form a cluster that coordinates the triphosphate moiety of the NTP and the metal ions required for catalysis (Fig. 6.5).

The interaction of RNA polymerase and the RNA template has been revealed by structural studies of poliovirus $3D^{pol}$ together with elongation complexes produced after several rounds of nucleotide incorporation (Fig. 6.6). In contrast to that by other types of nucleic acid polymerase, catalysis by $3D^{pol}$ does not depend on repositioning by the fingers domain of the nascent template-NTP from a preinsertion site to the active site. Closure of the active site is accomplished by base pairing of the initial NTP to a template nucleotide, leading to structural changes in the palm domain that cause Mg^{2+} binding and catalysis.

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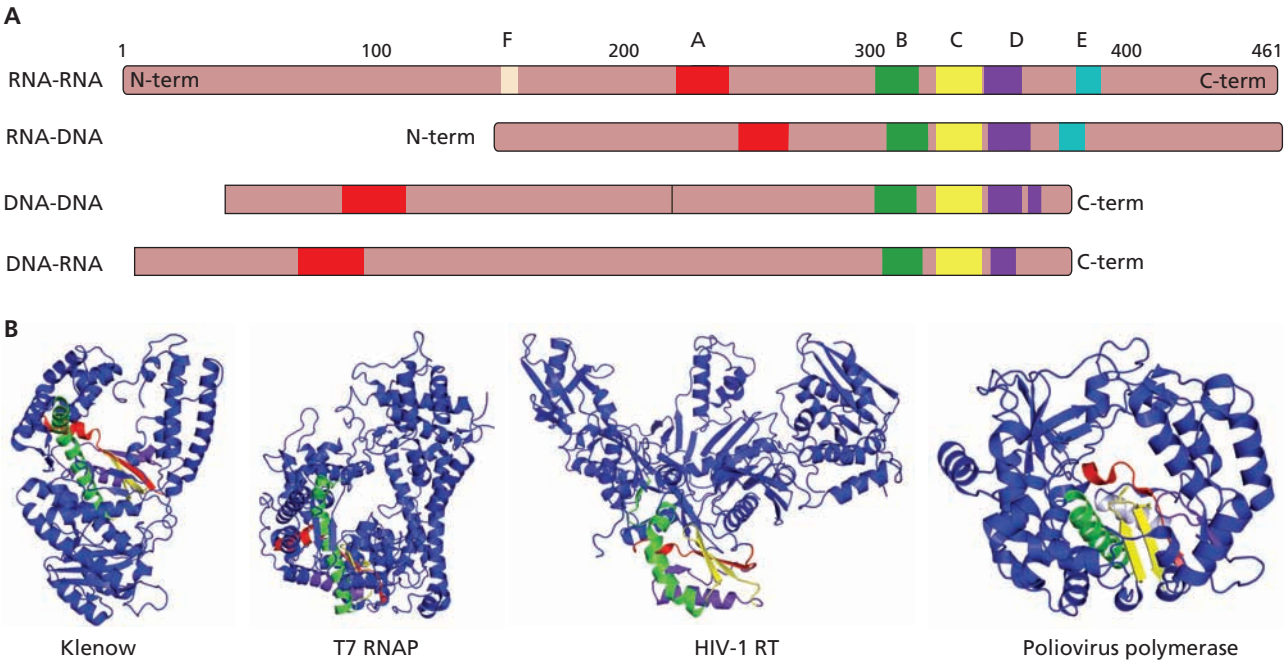


Figure 6.4 Protein domain alignments for the four categories of nucleic acid polymerases. (A) Schematic diagrams of polymerases. Numbers at the top are from the $3D^{pol}$ amino acid sequence. Sequence and structure motifs in each polymerase category are colored. Motif F is found only in RNA-dependent RNA polymerases. **(B)** Representative structures of each of the four types of nucleic acid polymerases. Ribbon diagrams of the polymerase domain of the large (Klenow) fragment of *Escherichia coli* DNA polymerase I, a DNA-dependent DNA polymerase; T7 RNA polymerase (T7 RNAP), a DNA-dependent RNA polymerase; human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT), an RNA-dependent DNA polymerase; and polioviral $3D^{pol}$, an RNA-dependent RNA polymerase. The thumb domain is at the right, and the fingers domain is at the left. The conserved structure/sequence motifs A, B, C, D, and E are red, green, yellow, cyan, and purple, respectively. From Protein Data Bank files 1qsl, 1s77, 3hvt, and 1ra6.

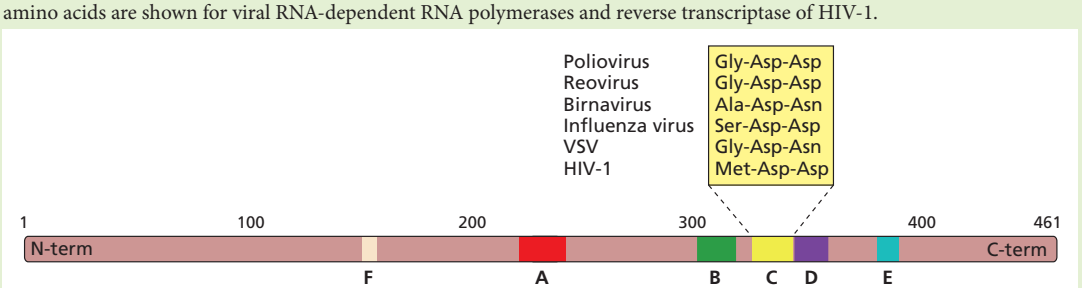
BOX 6.3

BACKGROUND

The Gly-Asp-Asp sequence of RNA polymerase motif C

The Asp-Asp sequence of motif C is also conserved in RNA-dependent DNA polymerases of retroviruses and in RNA polymerases of double-stranded RNA and segmented (−) strand viruses. The RNA polymerases of nonsegmented (−) strand viruses contain Gly-Asp-Asn instead of Gly-Asp-Asp. Mutational studies have shown that this sequence in the RNA polymerase (L protein) of vesicular stomatitis virus is essential for RNA synthesis. The RNA polymerase of birnavirus, an insect virus with a double-stranded RNA genome, has Ala-Asp-Asn instead of Gly-Asp-Asp. An RNA polymerase with Ala-Asp-Asn substituted with Gly-Asp-Asp has increased enzymatic activity. This observation has led to the suggestion that Ala-Asp-Asn may have been selected during the evolution of these birnaviruses to reduce pathogenicity and facilitate virus spread.

RNA-dependent RNA polymerase is depicted as a brown rectangle, with motifs A to F colored. Conserved motif C amino acids are shown for viral RNA-dependent RNA polymerases and reverse transcriptase of HIV-1.



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Figure 6.5 Structure of UTP bound to poliovirus 3D^{pol}. The NTP bridges the fingers (top) and palm (bottom) domains. The base is stacked with Arg174 from the fingers. Hydrogen bonds are shown as dashed lines. The Asp238 of motif A, which is conserved in all RNA-dependent RNA polymerases, hydrogen bonds with the 2′-OH of the ribose moiety; this interaction discriminates NTPs from dNTPs. Asp328 and Asp329, which coordinate Mg^{2+}, are also labeled. From Protein Data Bank file 2im2.

Mechanisms of RNA Synthesis

Initiation

As polymerases synthesize nucleic acid in a 5′ to 3′ direction, the nucleotidyl transfer reaction is initiated at the 3′ end of the template strand. The requirement for a primer for initiation of nucleic acid synthesis varies among the different classes of polymerases. All DNA polymerases are primer-dependent enzymes, while DNA-dependent RNA polymerases initiate RNA synthesis *de novo*. Some RNA-dependent RNA polymerases (e.g., those of flaviviruses and rhabdoviruses) can also initiate RNA synthesis *de novo*, while others require a primer (Fig. 6.7). Nucleic acid synthesis by these RNA polymerases is initiated by a protein-linked primer (picornaviruses) or an oligonucleotide cleaved from the 5′ end of cellular mRNA (influenza viruses).

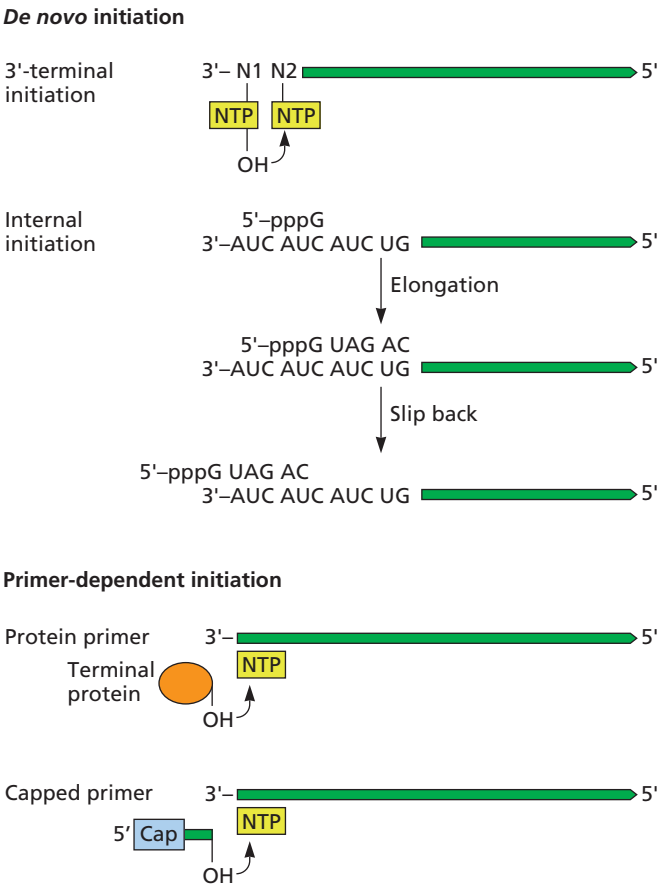
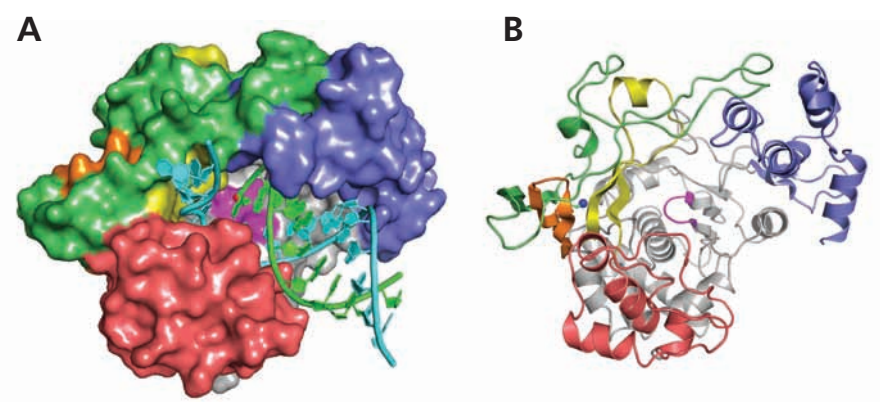


Figure 6.7 Mechanisms of initiation of RNA synthesis. *De novo* initiation may occur at the 3′ end of the viral RNA or from an internal base. When a primer is required, it may be a capped or protein-linked oligonucleotide.

De Novo Initiation

In this process the first phosphodiester bond is made between the 3′-OH of the initiating NTP and the second NTP (Fig. 6.7). The viral polymerase then copies the entire viral genome without dissociating. Initiation takes place at the exact 3′ end of the template, except during replication

Figure 6.6 Structure of RNA polymerase with template and primer. (A, B) Views from the top of poliovirus 3D^{pol} polymerase looking down into the active site. **(A)** Surface representation of the elongation complex is shown with bound template (cyan) and product (green) RNAs showing how the duplex RNA is clamped in place between the pinky finger and thumb structures. From Protein Data Bank file 1ra6.



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of the genomes of some (−) strand RNA viruses, such as bunyaviruses and arenaviruses (Fig. 6.7). Initiation begins at an internal C, and after extension of a few nucleotides, the daughter strand is shifted in the 3′ direction so that the 5′-terminal G residue is not base paired with the template strand. Because the daughter strand slips, this mechanism is called "prime and realign."

The structure of the RNA-dependent RNA polymerase of hepatitis C virus reveals how a primer-independent RNA polymerase positions the first nucleotide on the RNA template: a dinucleotide primer is synthesized by the polymerase using a beta-loop insertion in the thumb domain as a "protein platform" in the active site (Fig. 6.8). After the product reaches a certain length, the polymerase undergoes a conformational change that moves the priming platform out of the way and allows the newly synthesized complementary RNA to exit as the enzyme moves along the template strand.

A protein platform also appears to be involved in *de novo* priming by the reovirus RNA polymerase, a cubelike structure with a catalytic site in the center that is accessible by four tunnels. One tunnel allows template entry, one serves for the exit of newly synthesized double-stranded RNA, a third permits exit of mRNA, and a fourth is for substrate entry. A priming loop that is not observed in this region of other RNA polymerases is present in the palm domain. The loop supports the initiating NTP, then retracts into the palm and fits into the minor groove of the double-stranded RNA product. This movement assists in the transition between initiation and elongation, and also allows the newly synthesized RNA to exit the polymerase.

Protein platforms also appear to be involved in the *de novo* priming of RNA synthesis by other flaviviruses (dengue and West Nile viruses), influenza virus (genome RNA synthesis is primer independent), and bacteriophage Φ6.

Primer-Dependent Initiation

Protein priming. A protein-linked oligonucleotide serves as a primer for RNA synthesis by RNA polymerases of members of the *Picornaviridae* and *Caliciviridae*. Protein priming also occurs during DNA replication of adenoviruses, certain DNA-containing bacteriophages (Chapter 9), and hepatitis B virus (Chapter 7). A terminal protein provides a hydroxyl group (in a tyrosine or serine residue) to which the first oligonucleotide can be linked, by viral polymerases, via a phosphodiester bond. The protein-linked primer is then used for elongation.

Polioviral genomic RNA, as well as newly synthesized (+) and (−) strand RNAs, are covalently linked at their 5′ ends to the 22-amino-acid protein VPg (Fig. 6.9A), initially suggesting that VPg might function as a primer for RNA synthesis. This hypothesis was supported by the discovery of a uridylylated form of the protein, VPg-pUpU, in infected cells.

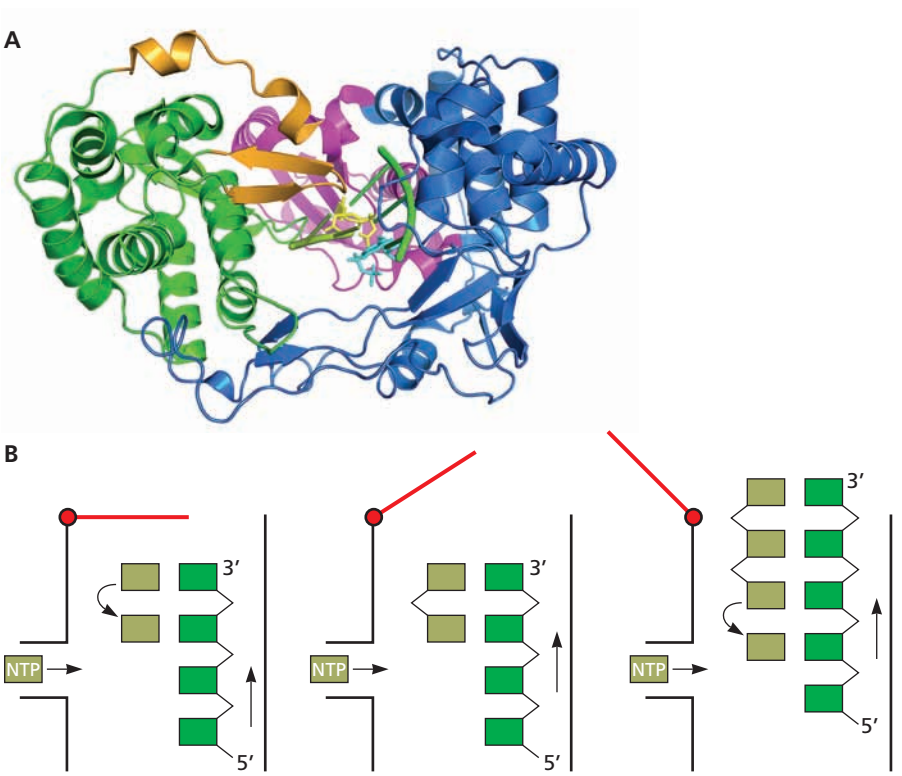


Figure 6.8 Mechanism of *de novo* initiation. (A) Ribbon diagram of RNA polymerase of hepatitis C virus. Fingers, palm, and thumb domain are colored blue, green, and magenta. The C-terminal loop that blocks the active site is shown in brown. Active site residues are yellow. Produced from Protein Data Bank file 4wtm. **(B)** Swinging-gate model of initiation. With the RNA template (green) in the active site of the enzyme, a short beta-loop (red) provides a platform on which the first complementary nucleotide (light green) is added to the template (left). The second nucleotide is then added, producing a dinucleotide primer for RNA synthesis (middle). At this point nothing further can happen because the priming platform blocks the exit of the RNA product from the enzyme. The solution to this problem is that the polymerase undergoes a conformational change that moves the priming platform out of the way and allows the newly synthesized complementary RNA (right) to exit as the enzyme moves along the template strand.

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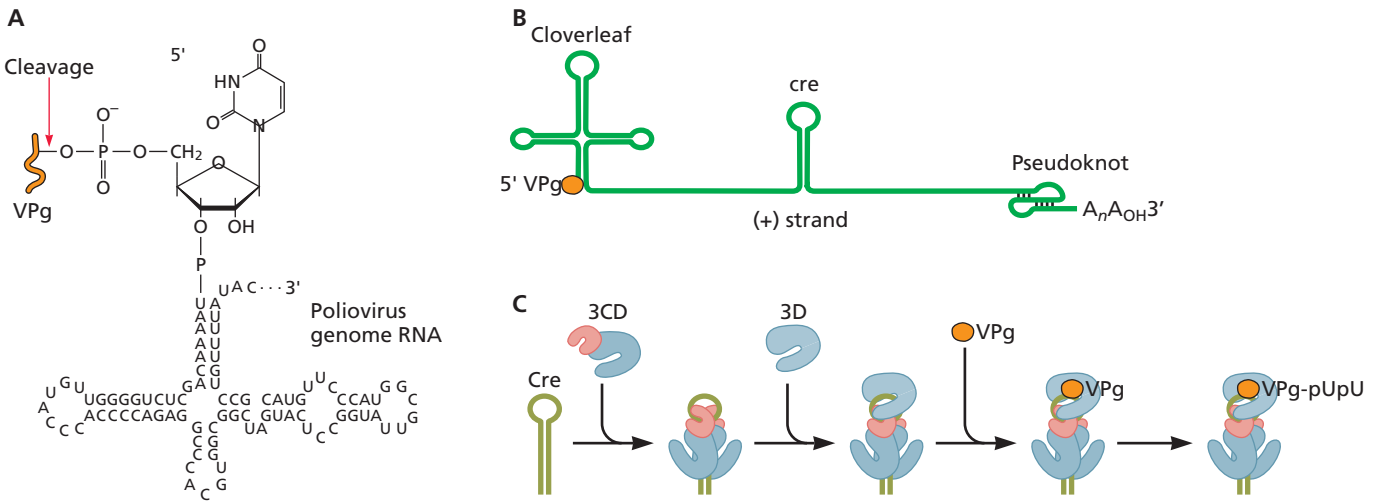


Figure 6.9 Uridylylation of VPg. (A) Linkage of VPg to polioviral genomic RNA. Polioviral RNA is linked to the 22-amino-acid VPg (orange) via an *O*4-(5′-uridylyl)-tyrosine linkage. This phosphodiester bond is cleaved at the indicated site by a cellular enzyme to produce the viral mRNA containing a 5′-terminal pU. **(B)** Structure of the poliovirus (+) strand RNA template, showing the 5′-cloverleaf structure, the internal cre (*cis*-acting replication element) sequence, and the 3′ pseudoknot. **(C)** Model for assembly of the VPg uridylylation complex. Two molecules of 3CD bind to cre. The 3C dimer melts part of the stem. $3D^{pol}$ binds to the complex by interactions between the back of the thumb domain and the surface of 3C. VPg then binds the complex and is linked to two U moieties in a reaction templated by the cre sequence.

VPg can be uridylylated *in vitro* by $3D^{pol}$ and can then prime the synthesis of VPg-linked poly(U) from a poly(A) template. The template for uridylylation of VPg is either the 3′-poly(A) on (+) strand RNA (during synthesis of (−) strand RNA, Fig. 6.10), or an RNA hairpin, the *cis*-acting replication element (cre), located in the coding region (during synthesis of (+) strand RNA (Fig. 6.9B and C).

Structures of the RNA polymerases of different picornaviruses and caliciviruses indicate that the active site is more accessible than in polymerases with a *de novo* mechanism of initiation. The small thumb domains of these polymerases leave a wide central cavity that can accommodate the template primer and the protein primer.

Uridylylation of VPg can be achieved in a reaction containing $3D^{pol}$, a template $(rA)_{10}$, UTP, and Mg^{2+} and Mn^{2+}. Crystallographic analysis of this structure reveals that VPg-pU is bound in the template-binding channel, with the N terminus of VPg in the NTP entry channel and the C terminus pointing toward the template-binding channel. The hydroxyl group of a tyrosine in VPg is covalently linked to the α-phosphate of UMP and interacts with a divalent metal ion that binds an Asp of the Gly-Asp-Asp motif in the active site. This arrangement of VPg is similar to that of the primer terminus in the nucleotidyl transfer reaction, demonstrating that $3D^{pol}$ catalyzes VPg uridylylation using the same two-metal mechanism as the nucleotidyl transfer reaction.

When VPg uridylylation begins at the 3′-poly(A) tail of the (+) strand template, the polymerase continues nucleotidyl transfer reactions and replicates the entire genome. However, when uridylylation of VPg takes place on the CRE, the protein must dissociate and transfer to the 3′ end of the RNA. How this process is accomplished is not known.

Protein priming by the birnavirus RNA polymerase VP1 is unusual because the primer **is** the polymerase, not a separate protein. Even in the absence of a template, VP1 has self-guanylylation activity that is dependent on divalent metal ions. The guanylylation site is a serine located approximately 23 Å from the catalytic site of the polymerase. The long distance between these sites suggests that guanylylation may be carried out at a second active site. The finding that some altered polymerases that are inactive in RNA synthesis retain self-guanylylation activity supports this hypothesis. After two G residues are added to VP1, it binds to a conserved CC sequence at the terminus of the viral RNA template to initiate RNA synthesis. The 5′ ends of mRNAs and genomic double-stranded RNAs produced by this reaction are therefore linked to a VP1 molecule.

Priming by capped RNA fragments. Influenza virus mRNA synthesis is blocked by treatment of cells with the fungal toxin α-amanitin at concentrations that inhibit cellular DNA-dependent RNA polymerase II. This surprising finding demonstrated that the viral RNA polymerase is dependent on a host cell RNA polymerase II. Inhibition by α-amanitin is explained by a requirement for newly synthesized cellular transcripts made by this enzyme to provide primers for viral mRNA synthesis. Presumably, these transcripts must be

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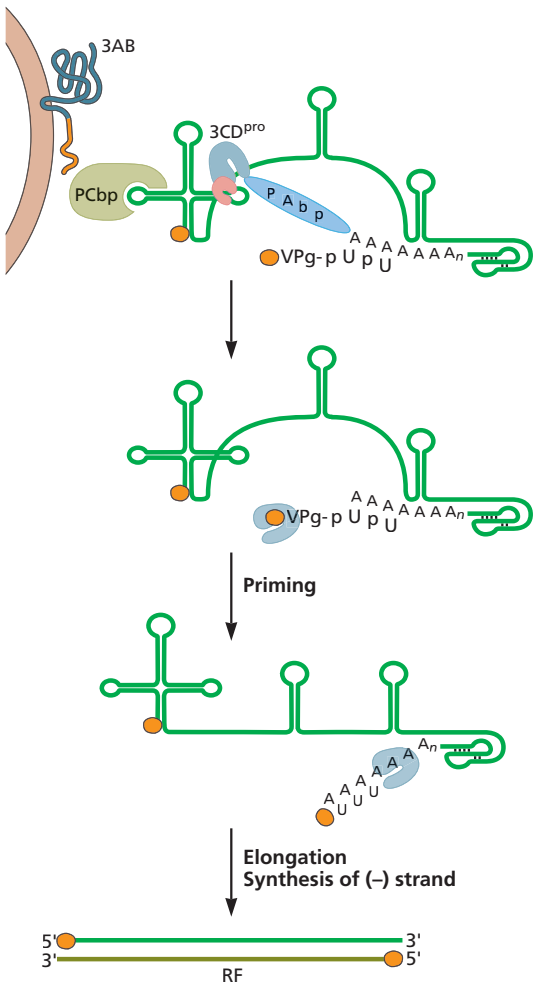


Figure 6.10 Poliovirus (−) strand RNA synthesis. The precursor of VPg, 3AB, contains a hydrophobic domain and is a membrane-bound donor of VPg. A ribonucleoprotein complex is formed when poly(rC)-binding protein 2 (PCbp2) and $3CD^{pro}$ bind the cloverleaf structure located within the first 108 nucleotides of (+) strand RNA. The ribonucleoprotein complex interacts with poly(A)-binding protein 1 (PAbp1), which is bound to the 3′-poly(A) sequence, bringing the ends of the genome into close proximity. Protease $3CD^{pro}$ cleaves membrane-bound 3AB, releasing VPg and 3A. VPg-pUpU is synthesized by $3D^{pol}$ using the 3′-poly(A) sequence as a template, and used by $3D^{pol}$ as a primer for RNA synthesis. Modified from A. V. Paul, p. 227–246, *in* B. L. Semler and E. Wimmer (ed), *Molecular Biology of Picornaviruses* (ASM Press, Washington, DC, 2002).

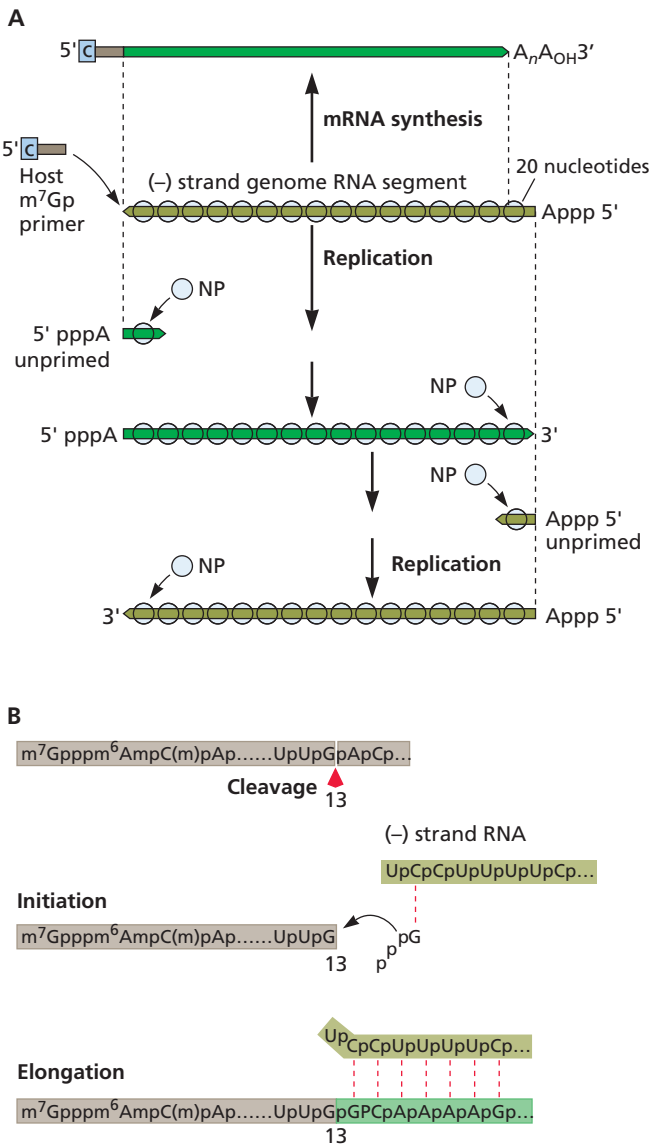


Figure 6.11 Influenza virus RNA synthesis. (A) Viral (−) strand genomes are templates for the production of either subgenomic mRNAs or full-length (+) strand RNAs. The switch from viral mRNA synthesis to genomic RNA replication is regulated by both the number of nucleocapsid (NP) protein molecules and the acquisition by the viral RNA polymerase of the ability to catalyze initiation without a primer. Binding of the NP protein to elongating (+) strands enables the polymerase to read to the 5′ end of genomic RNA. **(B)** Capped RNA-primed initiation of influenza virus mRNA synthesis. Capped RNA fragments cleaved from the 5′ ends of cellular nuclear RNAs serve as primers for viral mRNA synthesis. The 10 to 13 nucleotides in these primers do not need to hydrogen bond to the common sequence found at the 3′ ends of the influenza virus genomic RNA segments. The first nucleotide added to the primer is a G residue templated by the penultimate C residue of the genomic RNA segment; this is followed by elongation of the mRNA chains. The terminal U residue of the genomic RNA segment does not direct the incorporation of an A residue. The 5′ ends of the viral mRNAs therefore comprise 10 to 13 nucleotides plus a cap structure snatched from host nuclear pre-mRNAs. Adapted from S. J. Plotch et al., *Cell* **23:**847–858, 1981, with permission.

made continuously because they are exported rapidly from the nucleus once processed. Such transcripts are cleaved by a virus-encoded, cap-dependent endonuclease that is part of the RNA polymerase (Fig. 6.11). The resulting 10- to 13-nucleotide capped fragments serve as primers for the initiation of viral mRNA synthesis.

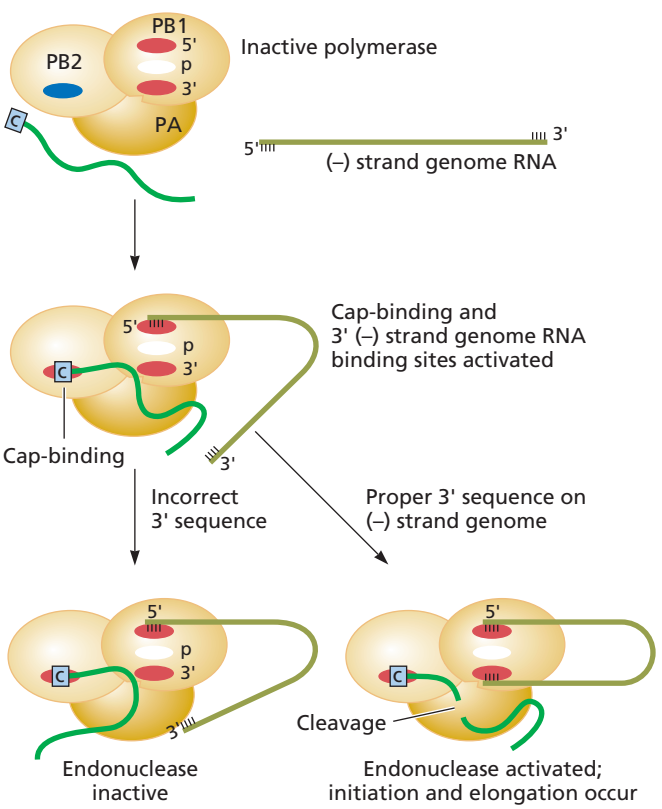
Bunyaviral mRNA synthesis is also primed with capped fragments of cellular RNAs. In contrast to that of influenza virus, bunyaviral mRNA synthesis is not inhibited by

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α-amanitin because it occurs in the cytoplasm, where capped cellular RNAs are abundant.

The influenza virus RNA polymerase is a heterotrimer composed of PA, PB1, and PB2 proteins (Fig. 6.12). The PB1 protein is the RNA polymerase, the PB2 subunit binds capped host mRNAs, and the PA protein harbors endonuclease activity. In contrast, the bunyavirus RNA polymerase is a single protein (L). The N-terminal domains of influenza PA and bunyavirus L have endonuclease activity that participates in cap snatching. The structures of endonuclease domains from these viruses reveal the presence of a common nuclease fold.

Figure 6.12 Activation of the influenza virus RNA polymerase by specific virion RNA sequences. The three P proteins form a multisubunit assembly that can neither bind to capped primers nor synthesize mRNAs. Addition of a sequence corresponding to the 5′-terminal 11 nucleotides of the viral RNA, which is highly conserved in all eight genome segments, activates the cap-binding activity of the P proteins. The PB1 protein binds this RNA sequence and activates the cap-binding PB2 subunit, probably by conformational change. Concomitantly with activation of cap binding, the P proteins acquire the ability to bind to a conserved sequence at the 3′ ends of genomic RNA segments. This second interaction activates the endonuclease that cleaves host cell RNAs 10 to 13 nucleotides from the cap, producing the primers for viral mRNA synthesis. The RNA polymerase can then carry out initiation and elongation of mRNAs. p, polymerase active site. 5′ and 3′ indicate the binding sites for the 5′ and 3′ ends, respectively, of (−) strand genomic RNA. Blue indicates an inactive site, and red indicates an active site. The polymerase is bound to both the 5′ and 3′ ends of the genomic RNA, with the capped RNA primer associated with the PB2 protein. Adapted from D. M. Knipe et al. (ed), *Fields Virology*, 4th ed. (Lippincott Williams & Wilkins, Philadelphia, PA, 2001), with permission.



Capping

Most viral mRNAs carry a 5′-terminal cap structure (exceptions include picornaviruses and the flavivirus hepatitis C virus), but the modification is made in different ways. Three mechanisms can be distinguished: acquisition of preformed 5′ cap structures from cellular pre-mRNAs or mRNAs, or during priming of mRNA synthesis as described in the previous section. Details of the latter processes can be found in Chapter 10.

Elongation

After a polymerase has associated stably with the nucleic acid template, the enzyme then adds nucleotides without dissociating from the template. Most polymerases are highly **processive**; that is, they can add thousands of nucleotides before dissociating. The poliovirus RNA polymerase $3D^{pol}$ can add 5,000 and 18,000 nucleotides in the absence or presence, respectively, of the accessory protein 3AB. The vesicular stomatitis virus P protein enhances the processivity of the RNA polymerase L, possibly as a result of conformational changes that occur upon binding of P. The increased processivity induced by P protein is enhanced in the presence of N, possibly because the template must be kept unstructured so as not to impede the progress of L. Full processivity of the influenza virus RNA polymerase also requires the presence of NP.

All nucleic acid synthesis begins with the formation of a complex of polymerase, template-primer, and initiating NTP. This complex becomes activated, a process that includes a conformational change from an open to a closed form. The nucleotidyl transfer reaction then takes place, pyrophosphate is released, and the template-primer moves by one base. RNA-dependent RNA polymerases have been visualized in template-primer, open NTP, and closed NTP forms. Interactions between the fingertips and the thumb domains of RNA-dependent RNA polymerases precede the large-scale conformational changes upon binding of template, primer, or NTP in the template-binding channel, and are characteristic of other polymerases.

The template-primer double-stranded RNA binds to the template-binding channel of picornavirus $3D^{pol}$ with the 3′ end of the template leaving the active site through one face of the enzyme. The template strand interacts mainly with the fingers domain, and the primer strand interacts with the thumb and palm domains. Seven nucleotides can fit into the template-binding channel. The 3′-hydroxyl of the primer forms a hydrogen bond with the catalytic Asp in motif C (Asp338) in the active site. This Asp also binds a metal ion, the only one observed in the active site in this state.

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Catalysis begins with the template above the active site, followed by loading of the first NTP and closure of the active site to produce a precatalytic state. The phosphate group of the incoming NTP binds a metal ion near Asp338 in motif C, and the 3′-OH of this NTP forms a hydrogen bond with Asp245 in motif A. This structure cannot carry out nucleotidyl transfer reactions until the 3′-hydroxyl of the primer terminus binds the second metal ion. After catalysis, the active site is opened, followed by translocation of the template-product. Such translocation requires conformational flexibility of a loop sequence in the B motif of the palm domain.

The closed conformation has been visualized in an elongation assembly composed of the norovirus RNA polymerase, self-complementary RNA, incoming NTP, and Mg^{2+} and Mn^{2+}. This structure is ready to carry out the nucleotidyl transfer reaction. Binding of the template-primer-NTP complex is associated with rotation of helices in the thumb domain. This movement creates a binding groove for a primer strand. The incoming NTP base pairs with the template, and the phosphates bind two Mn^{2+} ions in the active site. Motif A Asp242, and Asp343 and Asp344 of the Asp-Gly-Asp motif also coordinate the divalent metal ions. The interaction of polymerase with the 2′-hydroxyl of the rNTP also participates in addition of NTP to the nascent strand. The 2′-hydroxyl of the rNTP forms hydrogen bonds with Ser and Asn residues in motif B of the fingers domain. In the "open" conformation this interaction does not occur and the incoming NTP is not correctly assembled for catalysis. This conformation therefore represents a closed complex trapped immediately prior to catalysis.

Template Specificity

Viral RNA-dependent RNA polymerases must select viral templates from among a vast excess of cellular mRNAs and then initiate correctly to ensure accurate RNA synthesis. Different mechanisms that contribute to template specificity have been identified. Initiation specificity may be regulated by the affinity of the RNA polymerase for the initiating nucleotide. For example, the RNA polymerases of bovine viral diarrhea virus and bacteriophage ϕ6 prefers 3′-terminal C. Reovirus RNA polymerase prefers a G at the second position of the template RNA. This preference is controlled by hydrogen bonding of carbonyl and amino groups of the G with two amino acids of the enzyme. Both preferences would exclude initiation on cellular mRNAs that end in poly(A) (the great majority).

Template specificity may also be conferred by the recognition of RNA sequences or structures at the 5′ and 3′ ends of viral RNAs by viral proteins. RNA synthesis initiates specifically within a polypyrimidine tract in the 3′-untranslated region of hepatitis C virus RNA. The 3′-noncoding region of polioviral genomic RNA contains an **RNA pseudoknot** structure that is conserved among picornaviruses (Fig. 6.10). A viral protein (3AB-3CD) binds this structure and may direct the polymerase to that site for the initiation of (−) strand RNA synthesis. The precursor to the poliovirus RNA polymerase ($3CD^{pro}$) plays an important role in viral RNA synthesis by participating in the formation of a ribonucleoprotein at the 5′ end of the (+) strand RNA. This protein, together with cellular poly(rC)-binding protein 2, binds to a cloverleaf structure in the viral RNA (Fig. 6.10). Alterations within the RNA-binding domain of 3CD inhibit binding to the cloverleaf and RNA synthesis.

Internal RNA sequences may confer initiation specificity to RNA polymerases. The *cis*-acting replication elements (cre) in the coding sequence of poliovirus protein 2C and rhinovirus capsid protein VP1 contain short RNA sequences that are required for RNA synthesis. These sequences are binding sites for $3CD^{pro}$ and, as discussed previously, serve as a template for uridylylation of the VPg protein (Fig. 6.10).

During mRNA synthesis by influenza virus polymerase, sequences at the RNA termini ensure that the 5′ ends of newly synthesized viral mRNAs are not cleaved and used as primers (Fig. 6.12). If such cleavage were to occur, there would be no net synthesis of viral mRNAs. Such binding to two sites in the genomic RNA blocks access of a second P protein and protects newly synthesized viral mRNA from endonucleolytic cleavage by P proteins.

Protein-protein interactions can also direct RNA polymerases to the RNA template. The vesicular stomatitis virus RNA polymerase for mRNA synthesis consists of the P protein and the L protein, the catalytic subunit. The P protein binds both the L protein and the ribonucleoprotein containing N and the (−) strand RNA. In this way the P protein brings the L polymerase to the RNA template (See "(−) Strand RNA" below). Cellular general initiation proteins have a similar function in bringing RNA polymerase II to the correct site to initiate transcription of DNA templates.

While viral RNA polymerases copy only viral RNAs in the infected cell, purified polymerases often lack template specificity. The replication complex in the infected cell may contribute to template specificity by concentrating reaction components to create an environment that copies viral RNAs selectively. Replication of viral RNAs on membranous structures might contribute to such specificity (see "Cellular Sites of Viral RNA Synthesis").

Unwinding the RNA Template

Base-paired regions in viral RNA must be disrupted to permit copying by RNA-dependent RNA polymerase. RNA helicases, which are encoded in the genomes of many RNA viruses, are thought to unwind the genomes of double-stranded RNA viruses, as well as secondary structures in template RNAs. They also prevent extensive base pairing between template RNA

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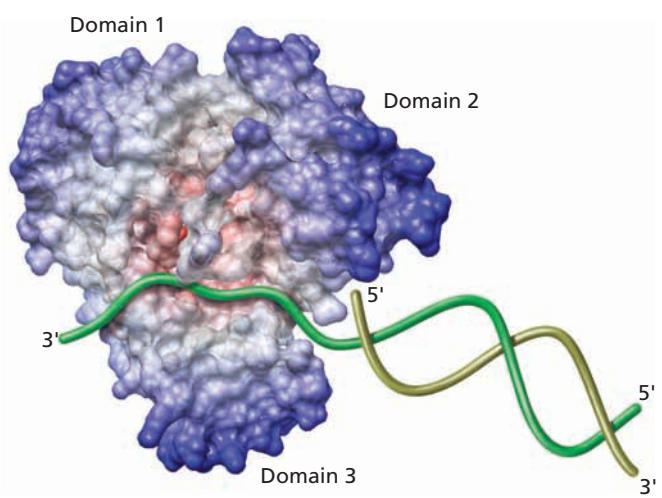
and the nascent complementary strand. The RNA helicases of several viruses that are important human pathogens, including the flaviviruses hepatitis C virus and dengue virus, have been studied extensively because these proteins are potential targets for therapeutic intervention. To facilitate the development of new agents that inhibit these helicases, their three-dimensional structures have been determined by X-ray crystallography. These molecules comprise three domains that mediate hydrolysis of NTPs and RNA binding (Fig. 6.13). Between the domains is a cleft that is large enough to accommodate single-stranded but not double-stranded RNA. Unwinding of double-stranded RNA probably occurs as one strand of RNA passes through the cleft and the other is excluded.

The bacteriophage ϕ6 RNA polymerase can separate the strands of double-stranded RNA without the activity of a helicase. Examination of the structure of the enzyme suggests how such melting might be accomplished. This RNA polymerase has a plowlike protuberance around the entrance to the template channel that is thought to separate the two strands, allowing only one to enter the channel.

Role of Cellular Proteins

Host cell components required for viral RNA synthesis were initially called "host factors," because nothing was known about their chemical composition. Evidence that cellular proteins are essential components of a viral RNA polymerase first came from studies of the bacteriophage Qβ enzyme. This viral RNA-dependent RNA polymerase is a multisubunit enzyme, consisting of a 65-kDa virus-encoded protein and four host proteins: ribosomal protein S1, translation elongation proteins (EF-Tu and EF-Ts), and an RNA-binding protein. Proteins S1 and EF-Tu contain RNA-binding sites that enable the RNA polymerase to recognize the viral RNA template. The 65-kDa viral protein exhibits no RNA polymerase activity in the absence of the host proteins, but has sequence and structural similarity to known RNA-dependent RNA polymerases.

Figure 6.13 Structure of a viral RNA helicase. The RNA helicase of yellow fever virus is shown in surface representation, colored red, white, or blue depending on the distance of the amino acid from the center of the molecule. A model for melting of double-stranded RNA is shown. From Protein Data Bank file 1yks.



Polioviral RNA synthesis also requires host cell proteins. When purified polioviral RNA is incubated with a cytoplasmic extract prepared from uninfected permissive cells, the genomic RNA is translated and the viral RNA polymerase is made. If guanidine hydrochloride is included in the reaction mixture, the polymerase assembles on the viral genome, but RNA synthesis is not initiated. The RNA polymerase-template assembly can be isolated free of guanidine, but RNA synthesis does not occur unless a new cytoplasmic extract is added, indicating that soluble cellular proteins are required for initiation. A similar conclusion comes from studies in which polioviral RNA was injected into oocytes derived from the African clawed toad *Xenopus laevis*: the viral RNA cannot replicate in *Xenopus* oocytes unless it is coinjected with a cytoplasmic extract from human cells. These observations can be explained by the requirement of the viral RNA polymerase for one or more mammalian proteins that are absent in toad oocytes.

One of these host cell proteins is poly(rC)-binding protein, which binds to a cloverleaf structure that forms in the first 108 nucleotides of (+) strand RNA (Fig. 6.10). Formation of a ribonucleoprotein composed of the 5′ cloverleaf, 3CD, and poly(rC)-binding protein is essential for the initiation of viral RNA synthesis. Interaction of poly(rC)-binding protein with the cloverleaf facilitates the binding of viral protein 3CD to the opposite side of the same cloverleaf.

Another host protein that is essential for polioviral RNA synthesis is poly(A)-binding protein 1. This protein brings together the ends of the viral genome by interacting with poly(rC)-binding protein 2, $3CD^{pro}$, and the 3′-poly(A) tail of poliovirus RNA. (Fig. 6.10). Formation of this circular ribonucleoprotein complex is required for (−) strand RNA synthesis.

Recent technical advances have facilitated identification of host cell proteins required for viral RNA synthesis. Interactions among cellular and viral proteins can be identified readily by mass spectrometry, and their function in viral replication can be determined by silencing their production by RNA interference or other methods. This approach has been used to show that the cellular RNA helicase A participates in influenza viral RNA synthesis, and heat shock protein 70 associates with the replication complex of Japanese encephalitis virus and positively regulates RNA synthesis.

Paradigms for Viral RNA Synthesis

Exact replicas of the RNA genome must be made for assembly of infectious viral particles. However, the mRNAs of most RNA viruses are **not** complete copies of the viral genome.

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The replication cycle of these viruses must therefore include a switch from mRNA synthesis to the production of full-length genomes. The majority of mechanisms for this switch regulate either the initiation or the termination of RNA synthesis.

(+) Strand RNA

For some (+) strand RNA viruses, the genome and mRNA are identical. The genome RNA of the *Picornaviridae* and *Flaviviridae* is translated upon entry into the cytoplasm to produce viral proteins, including the RNA-dependent RNA polymerase and accessory proteins. The (+) strand RNA genome is copied to a (−) strand, which in turn is used as a template for the synthesis of additional (+) strands (Fig. 6.1). Newly synthesized (+) strand RNA molecules can serve as templates for further genomic replication, as mRNAs for the synthesis of viral proteins, or as genomic RNAs to be packaged into progeny virions. Because picornaviral mRNA is identical in sequence to the viral RNA genome, all RNAs needed for the reproduction of these viruses can be made by a simple set of RNA synthesis reactions (Fig. 6.1). Such simplicity comes at a price, because synthesis of individual viral proteins cannot be regulated. However, polioviral gene expression can be controlled by the rate and extent of polyprotein processing. For example, the precursor of the viral RNA polymerase, $3D^{pol}$, cannot polymerize RNA. Rather, this protein is a protease that cleaves at certain Gln-Gly amino acid pairs in the polyprotein. Regulating the processing of the precursor $3CD^{pro}$ controls the concentration of RNA polymerase.

The mRNAs synthesized during infection by most RNA viruses contain a 3′-poly(A) sequence, as do the vast majority of cellular mRNAs (exceptions are mRNAs of arenaviruses and reoviruses). The poly(A) sequence is encoded in the genome of (+) strand viruses. For example, polioviral (+) strand RNAs contain a 3′ stretch of poly(A), approximately 62 nucleotides in length, which is required for infectivity. The (−) strand RNA contains a 5′ stretch of poly(U), which is copied to form this poly(A).

The mechanisms of mRNA synthesis of other (+) strand RNA viruses allow structural and nonstructural proteins (generally needed in greater and lesser quantities, respectively) to be made separately. The latter are synthesized from full-length (+) strand (genomic) RNA, while structural proteins are translated from subgenomic mRNA(s). This strategy is a feature of the replication cycles of coronaviruses, caliciviruses, and alphaviruses. Translation of the Sindbis virus (+) strand RNA genome yields the nonstructural proteins that synthesize a full-length (−) strand (Fig. 6.14). Such RNA molecules contain not only a 3′-terminal sequence for initiation of (+) strand RNA synthesis, but also an internal initiation site, used for production of a 26S subgenomic mRNA.

Alphaviral genome and mRNA synthesis is regulated by the sequential production of three RNA polymerases with

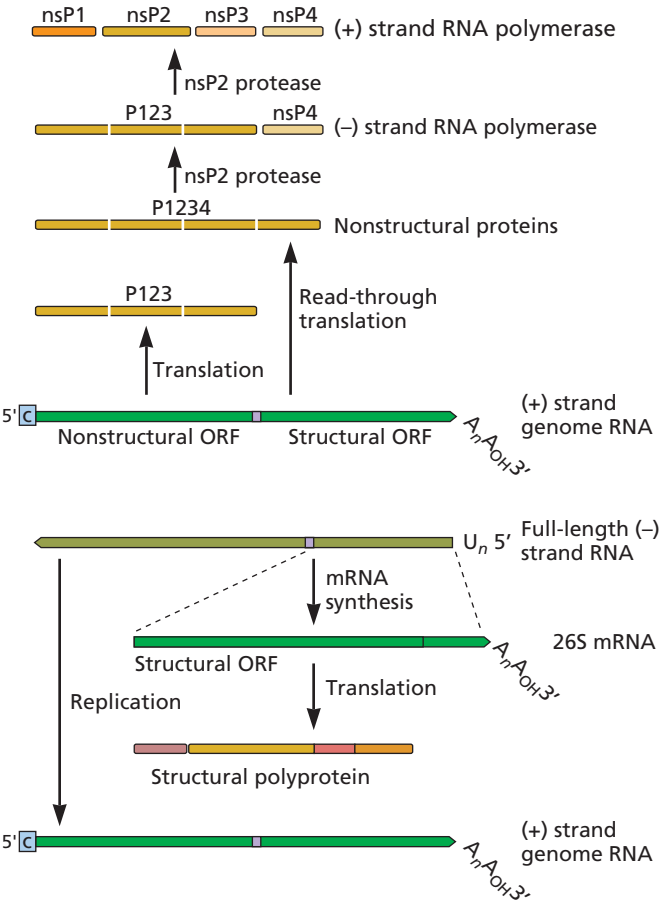


Figure 6.14 Genome structure and expression of an alphavirus, Sindbis virus. The 11,703-nucleotide Sindbis virus genome contains a 5′-terminal cap structure and a 3′-poly(A) tail. A conserved RNA secondary structure at the 3′ end of (+) strand genomic RNA is thought to control the initiation of (−) strand RNA synthesis. At early times after infection, the 5′ region of the genomic RNA (nonstructural open reading frame [ORF]) is translated to produce two nonstructural polyproteins: P123, whose synthesis is terminated at the first translational stop codon (indicated by the box), and P1234, produced by an occasional (15%) readthrough of this stop codon. The P1234 polyprotein is proteolytically cleaved to produce the enzymes that catalyze the various steps in genomic RNA replication: the synthesis of a full-length (−) strand RNA, which serves as the template for (+) strand synthesis, and either full-length genomic RNA or subgenomic 26S mRNA. The 26S mRNA, shown in expanded form, is translated into a structural polyprotein (p130) that undergoes proteolytic cleavage to produce the virion structural proteins. The 26S RNA is not copied into a (−) strand because a functional initiation site fails to form at the 3′ end.

different template specificities. All three enzymes are derived from the nonstructural polyprotein P1234 and contain the complete amino acid sequence of this precursor (Fig. 6.14). The covalent connections among the segments of the polyprotein are successively broken, with ensuing alterations in the specificity of the enzyme (Fig. 6.15). It seems likely that each proteolytic cleavage induces a conformational change in the polymerase that alters its template specificity.

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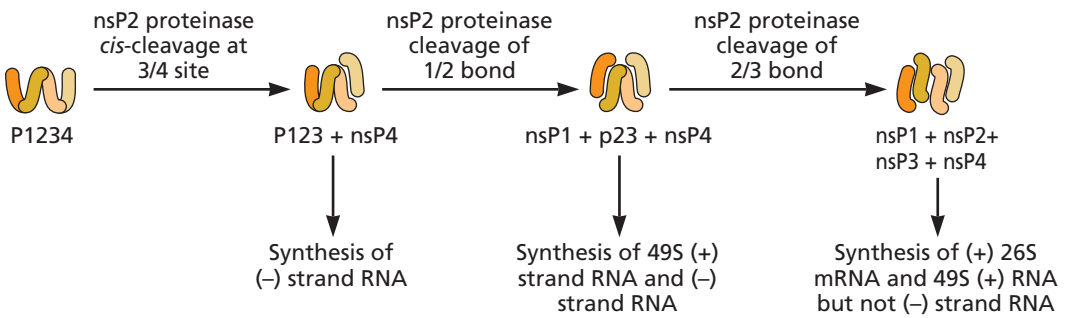


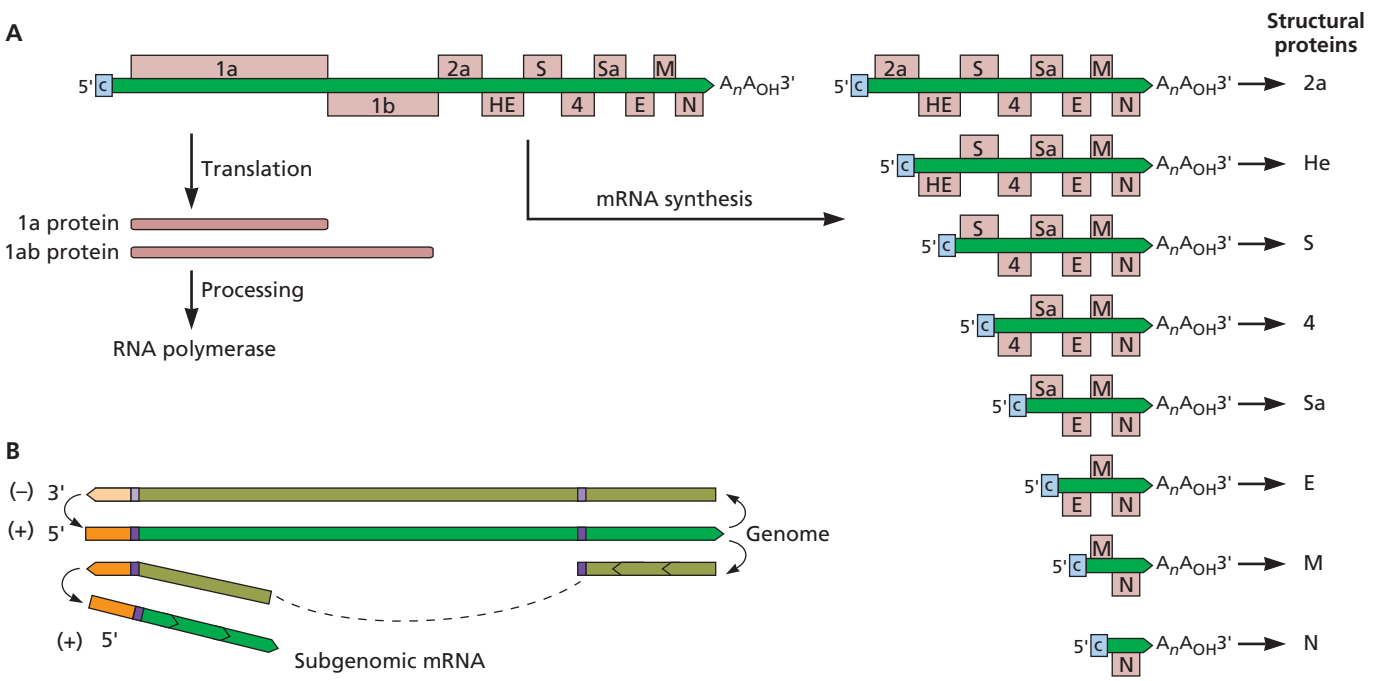
Figure 6.15 Three RNA polymerases with distinct specificities in alphavirus-infected cells. These RNA polymerases contain the entire sequence of the P1234 polyprotein and differ only in the number of proteolytic cleavages in this sequence.

Synthesis of Nested Subgenomic mRNAs

An unusual pattern of mRNA synthesis occurs in cells infected with members of the families *Coronaviridae* and *Arteriviridae*, in which subgenomic mRNAs that form a 3′-coterminal nested set with the viral genome are synthesized (Fig. 6.16). These viral families were combined into the order *Nidovirales* to denote this shared property (*nidus* is Latin for nest).

The subgenomic mRNAs of these viruses comprise a leader and a body that are synthesized from noncontiguous sequences at the 5′ and 3′ ends, respectively, of the viral (+) strand genome (Fig. 6.16A). The leader and body are separated by a conserved junction sequence encoded both at the 3′ end of the leader and at the 5′ end of the mRNA body. Subgenome-length (−) strands are produced when

Figure 6.16 Nidoviral genome organization and expression. (A) Organization of open reading frames. The (+) strand viral RNA is shown at the top, with open reading frames as boxes. The genomic RNA is translated to form polyproteins 1a and 1ab, which are processed to form the RNA polymerase. Structural proteins are encoded by nested mRNAs. **(B)** Model of the synthesis of nested mRNAs. Discontinuous transcription occurs during (−) strand RNA synthesis. Most of the (+) strand template is not copied, probably because it loops out as the polymerase completes synthesis of the leader RNA (orange). The resulting (−) strand RNAs, with leader sequences at the 3′ ends, are then copied to form mRNAs.



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the template loops out as the polymerase completes synthesis of the leader RNA (Fig. 6.16B). These (−) strand subgenome-length RNAs then serve as templates for mRNA synthesis.

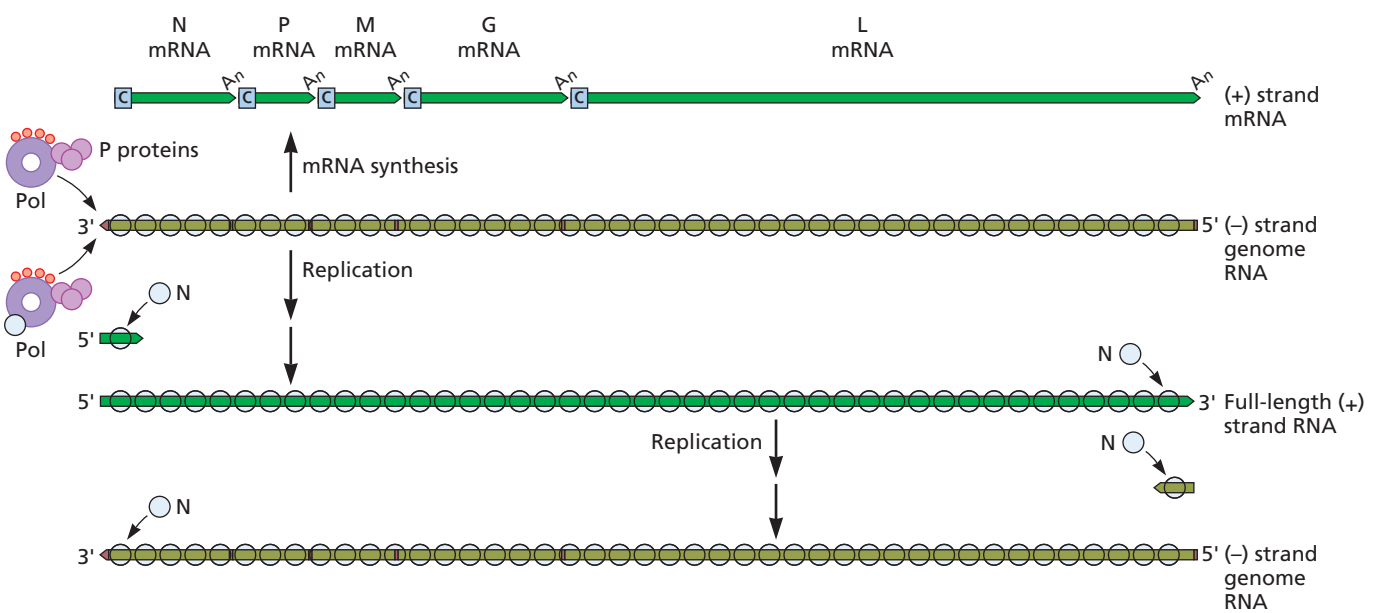
(−) Strand RNA

The genes of RNA viruses with a nonsegmented (−) strand RNA genome are expressed by the production of subgenomic mRNAs in infected cells (Fig. 6.17). An RNA polymerase composed of one molecule of L protein associated with four molecules of P protein is thought to carry out vesicular stomatitis virus mRNA synthesis. Individual mRNAs are produced by a series of initiation and termination reactions as the RNA polymerase moves down the viral genome (Fig. 6.18). This start-stop mechanism accounts for the observation that 3′-proximal genes must be copied before downstream genes (Box 6.4). The viral RNA polymerase is unable to initiate synthesis of each mRNA independently.

Vesicular stomatitis virus mRNA synthesis illustrates a second mechanism for poly(A) addition, reiterative copying of, or "stuttering" at, a short U sequence in the (−) strand template. After initiation, vesicular stomatitis virus mRNAs are elongated until the RNA polymerase reaches a conserved stop-polyadenylation signal [3′-AUACU$_7$-5′] located in each intergenic region (Fig. 6.19). Poly(A) (approximately 150 nucleotides) is added by reiterative copying of the U stretch, followed by termination.

The transition from mRNA to genome RNA synthesis in cells infected with vesicular stomatitis virus is dependent on the viral nucleocapsid (N) protein. To produce a full-length (+) strand RNA, the stop-start reactions at intergenic regions must be suppressed, a process that depends on the synthesis of the N and P proteins. The P protein maintains the N protein in a soluble form so that it can encapsidate the newly synthesized RNA. N-P assemblies bind to leader RNA and cause antitermination, signaling the polymerase to begin processive RNA synthesis. Additional N protein molecules then associate with the (+) strand RNA as it is elongated, and eventually bind to the seven A bases in the intergenic region. This interaction blocks reiterative copying of the seven U bases in the genome because the A bases cannot slip backward along the genomic RNA template. Consequently, RNA synthesis continues through the intergenic regions. The number of N-P protein complexes in infected cells therefore regulates

Figure 6.17 Vesicular stomatitis viral RNA synthesis. Viral (−) strand genomes are templates for the production of either subgenomic mRNAs or full-length (+) strand RNAs. The switch from mRNA synthesis to genomic RNA replication is mediated by two RNA polymerases and by the N protein. mRNA synthesis initiates at the beginning of the N gene, near the 3′ end of the viral genome. Poly(A) addition is a result of reiterative copying of a sequence of seven U residues present in each intergenic region. Chain termination and release occur after approximately 150 A residues have been added to the mRNA. The RNA polymerase then initiates synthesis of the next mRNA at the conserved start site 3′UUGUC . . . 5′. This process is repeated for all five viral genes. Synthesis of the full-length (+) strand begins at the exact 3′ end of the viral genome and is carried out by the RNA polymerase L-N-(P)4. The (+) strand RNA is bound by the viral nucleocapsid (N) protein, which is associated with the P protein in a 1:1 molar ratio. The N-P complexes bind to the nascent (+) strand RNA, allowing the RNA polymerase to read through the intergenic junctions at which polyadenylation and termination take place during mRNA synthesis.



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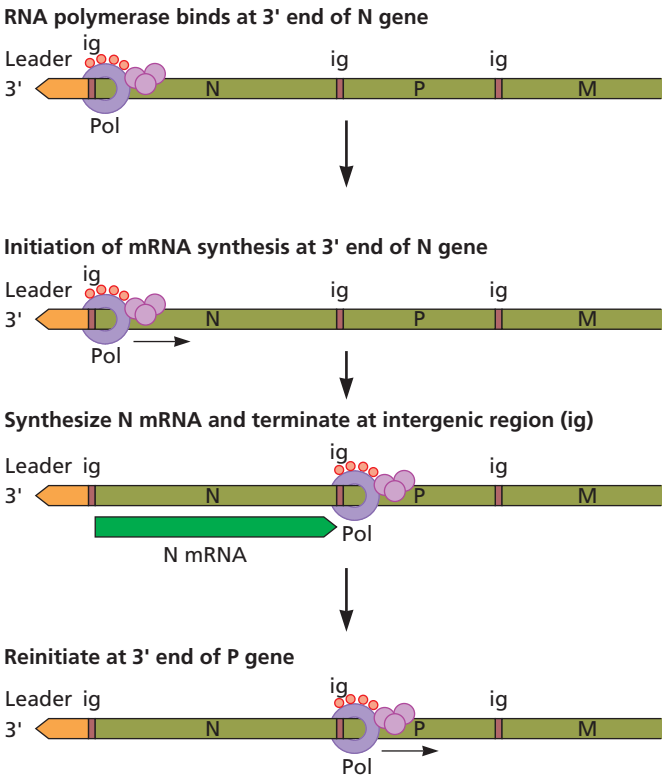


Figure 6.18 Stop-start model of vesicular stomatitis mRNA synthesis. The RNA polymerase (Pol) initiates RNA synthesis at the 3′ end of the N gene. After synthesis of the N mRNA, RNA synthesis terminates at the intergenic region, followed by reinitiation at the 3′ end of the P gene. This process continues until all five mRNAs are synthesized. Reinitiation does not occur after the last mRNA (the L mRNA) is synthesized, and, as a consequence, the 59 5′-terminal nucleotides of the vesicular stomatitis virus genomic RNA are not copied. Only a fraction of the polymerase molecules successfully make the transition from termination to reinitiation of mRNA synthesis at each intergenic region.

the relative efficiencies of mRNA synthesis and genome RNA replication. The copying of full-length (+) strand RNAs to (−) strand genomic RNAs also requires the binding of N-P protein complexes to elongating RNA molecules. Newly synthesized (−) strand RNAs are produced as nucleocapsids that can be readily packaged into progeny viral particles.

The (−) strand RNA genome of paramyxoviruses is copied efficiently only when its length in nucleotides is a multiple of 6. This requirement, called the **rule of six**, is probably a consequence of the association of each N monomer with exactly six nucleotides. Assembly of the nucleocapsid begins with the first nucleotide at the 5′ end of the RNA and continues until the 3′ end is reached. If the genome length is not a multiple of 6, then the 3′ end of the genome will not be precisely aligned with the last N monomer. Such misalignment reduces the efficiency of initiation of RNA synthesis at the 3′ end. Curiously, although the rhabdovirus N protein binds nine nucleotides of RNA, the genome length need not be a multiple of this number for efficient copying.

The segmented (−) strand RNA genome of influenza virus is expressed by the synthesis of subgenomic mRNAs in infected cells by a heterotrimeric RNA polymerase described previously (Fig. 6.12). Individual mRNAs are initiated with a capped primer derived from host cell mRNA, and terminate 20 nucleotides short of the template 3′ end. Polyadenylation of these mRNAs is achieved by a similar mechanism to that observed during vesicular stomatitis virus mRNA synthesis, reiterative copying of a short U sequence in the (−) strand template. Such copying is thought to be a consequence of the RNA polymerase specifically binding the 5′ end of (−) strand RNA and remaining at this site throughout mRNA synthesis. The genomic RNAs are threaded through the polymerase in a 3′ → 5′ direction as mRNA synthesis proceeds (Fig. 6.20). Eventually the template is unable to move, leading to reiterative copying of the U residues.

The influenza virus NP protein also regulates the switch from viral mRNA to full-length (+) strand synthesis (Fig. 6.11). The RNA polymerase for genome replication reads through the polyadenylation and termination signals for mRNA production only if NP is present. This protein is thought to bind nascent (+) strand transcripts and block poly(A) addition by a mechanism analogous to that described for vesicular stomatitis virus N protein. Copying of (+) strand RNAs into (−) strand RNAs also requires NP protein. Intracellular concentrations of NP protein are therefore an important determinant of whether mRNAs or full-length (+) strands are synthesized.

Ambisense RNA

Although arenaviruses are considered (−) strand RNA viruses, their genomic RNA is in fact **ambisense**: mRNAs are produced both from (−) strand genomic RNA and from complementary full-length (+) strands. The arenavirus genome comprises two RNA segments, S (small) and L (large) (Fig. 6.21) . Shortly after infection, RNA polymerase that enters from viral particles synthesizes mRNAs from the 3′ region of both RNA segments. Synthesis of each mRNA terminates at a stem-loop structure. These mRNAs, which are translated to produce the nucleocapsid (NP) protein and RNA polymerase (L) protein, respectively, are the only viral RNAs made during the first several hours of infection. Later in infection, the block imposed by the stem-loop structure is overcome, permitting the synthesis of full-length S and L (+) RNAs. It was initially thought that melting of the stem-loop structure by the NP protein allowed the transcription termination signal to be bypassed. It now seems more likely that two different RNA polymerases are made in infected cells, one that produces mRNAs and a second that synthesizes full-length copies of the genome. The finding that viral mRNAs are capped while genomes are not is consistent with this hypothesis.

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BOX 6.4

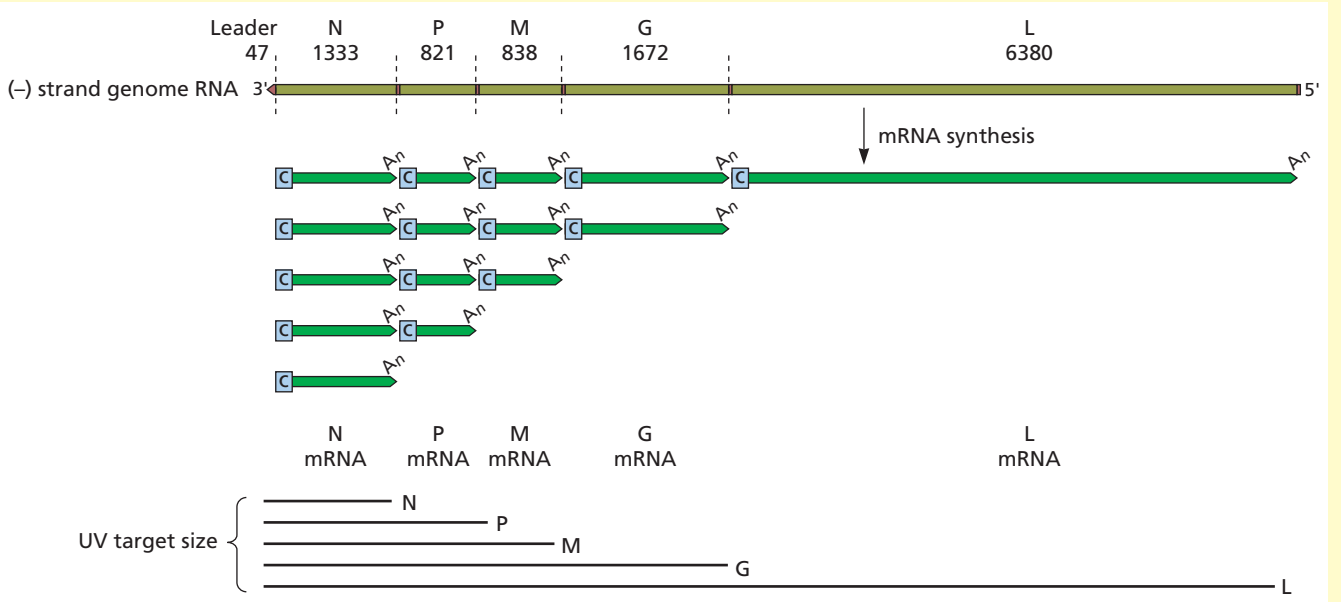
EXPERIMENTS

Mapping gene order by UV irradiation

The effects of ultraviolet (UV) irradiation provided insight into the mechanism of vesicular stomatitis virus mRNA synthesis. In these experiments, virus particles were irradiated with UV light, and the effect on the synthesis of individual mRNAs was assessed. UV light causes the formation of pyrimidine dimers that block passage of the RNA polymerase. In principle, larger genes require less UV irradiation to inactivate mRNA synthesis and have a larger **target size.** The dose of UV irradiation needed to inactivate synthesis of the N mRNA corresponded to the predicted size of the N gene, but this was not the case for the other viral mRNAs. The target size of each other mRNA was the sum of its size plus the size of other genes located 3′ to it. For example, the UV target size of the L mRNA is the size of the entire genome. These results indicate that these mRNAs are synthesized sequentially, in the 3′ → 5′ order in which their genes are arranged in the viral genome: N-P-M-G-L.

Ball LA, White CN. 1976. Order of transcription of genes of vesicular stomatitis virus. *Proc Natl Acad Sci. U S A* **73:**442–446.

Vesicular stomatitis virus mRNA map and UV map. The genome is shown as a dark green line at the top, and the N, P, M, G, and L genes and their relative sizes are indicated. The 47-nucleotide leader RNA is encoded at the 3′ end of the genomic RNA. The leader and intergenic regions are shown in orange. The RNAs encoded at the 3′ end of the genome are made in larger quantities than the RNAs encoded at the 5′ end of the genome. UV irradiation experiments determined the size of the vesicular stomatitis virus genome (UV target size) required for synthesis of each of the viral mRNAs. The UV target size of each viral mRNA corresponded to the size of the genomic RNA sequence encoding the mRNA plus all of the genomic sequence 3′ to this coding sequence. The transition from reiterative copying and termination to initiation is not perfect, and only about 70 to 80% of the polymerase molecules accomplish this transition at each intergenic region. Such inefficiency accounts for the observation that 3′-proximal mRNAs are more abundant than 5′-proximal mRNAs.



Double-Stranded RNA

A distinctive feature of the infectious cycle of double-stranded RNA viruses is the production of mRNAs and genomic RNAs from distinct templates in different viral particles. Because the viral genomes are double stranded, they cannot be translated. Therefore, the first step in infection is the production of mRNAs from each viral RNA segment by the virion-associated RNA polymerase (Fig. 6.22). Reoviral mRNAs carry 5′-cap structures but lack 3′-poly(A) sequences.

In the reovirus core, the λ3 polymerase molecules are attached to the inner shell at each fivefold axis, below an RNA exit pore. Viral mRNAs are synthesized by the polymerase inside the viral particle and then extruded into the cytoplasm through this pore. Attachment of the polymerase molecules to the pores ensures that mRNAs are actively threaded out of the particle, without depending upon diffusion, which would be very inefficient. Examination of the structure of actively transcribing rotavirus, a member of the *Reoviridae*, has allowed a three-dimensional visualization of how mRNAs are released from the particle (Box 6.5). Viral (+) strand RNAs that will serve as templates for (−) strand RNA synthesis are first packaged into newly assembled subviral particles (Fig. 6.22). Each (+) strand RNA is then copied just once within this particle to produce double-stranded RNA.

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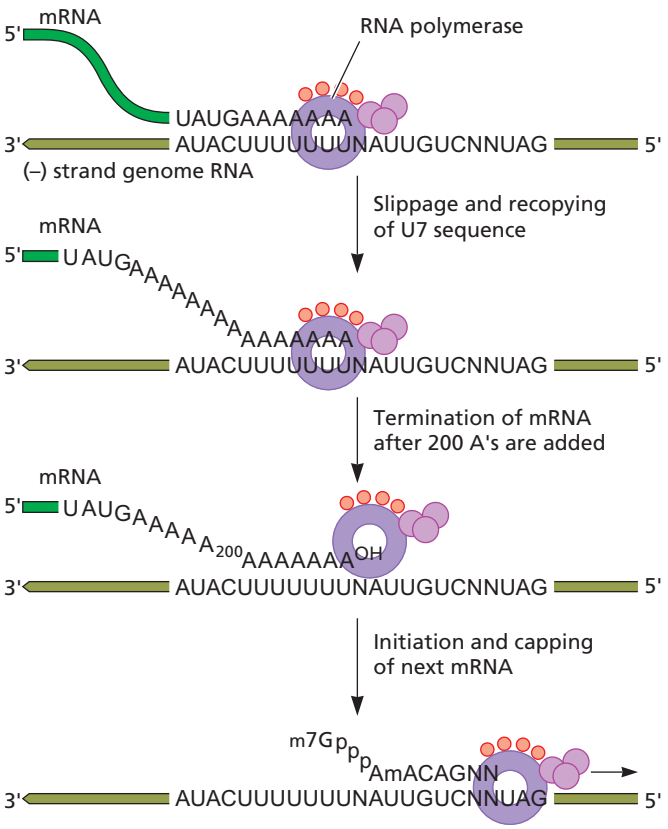


Figure 6.19 Poly(A) addition and termination at an intergenic region during vesicular stomatitis virus mRNA synthesis. Copying of the last seven U residues of an mRNA-encoding sequence is followed by slipping of the resulting seven A residues in the mRNA off the genomic sequence, which is then recopied. This process continues until approximately 200 A residues are added to the 3′ end of the mRNA. Termination then occurs, followed by initiation and capping of the next mRNA. The dinucleotide NA in the genomic RNA is not copied.

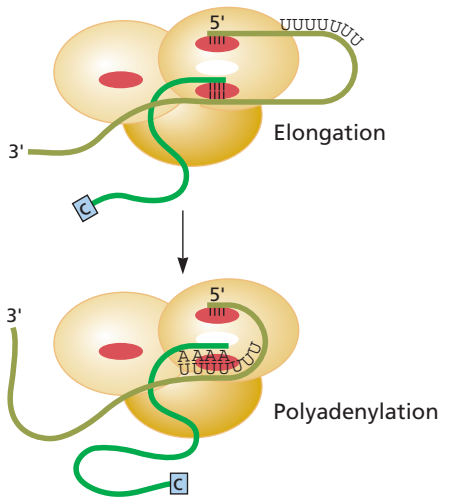


Figure 6.20 Moving-template model for influenza virus mRNA synthesis. During RNA synthesis, the polymerase remains bound to the 5′ end of the genomic RNA, and the 3′ end of the genomic RNA is threaded through (or along the surface of) the polymerase as the PB1 protein catalyzes each nucleotide addition to the growing mRNA chain. This threading process continues until the mRNA reaches a position on the genomic RNA that is close to the binding site of the polymerase. At this point the polymerase itself blocks further mRNA synthesis, and reiterative copying of the adjacent U_7 tract occurs. After about 150 A residues are added to the 3′ end of the mRNA, mRNA synthesis terminates. Adapted from D. M. Knipe et al. (ed), *Fields Virology*, 4th ed. (Lippincott Williams & Wilkins, Philadelphia, PA, 2001), with permission.

Members of different families of double-stranded RNA viruses carry out RNA synthesis in diverse ways. Replication of the genome of bacteriophage ϕ6 (3 double-stranded RNA segments) and birnaviruses (2 double-stranded RNA segments) is semiconservative, whereas that of reoviruses (10 to 12 double-stranded RNA segments) is conservative (Fig. 6.23). During conservative replication, the double-stranded RNA that exits the polymerase must be melted, so that the newly synthesized (+) strand is released and the template (−) strand reanneals with the original (+) strand. In reovirus particles, each double-stranded RNA segment is attached to a polymerase molecule, by interaction of the 5′-cap structure with a cap-binding site on the RNA polymerase. Attachment of the 5′ cap to the polymerase facilitates insertion of the 3′ end of the (−) strand into the template channel. This arrangement allows very efficient reinitiation of RNA synthesis in the crowded core of the particle. The RNA polymerase of bacteriophage ϕ6 and birnaviruses do not have such a cap-binding site, as would be expected for enzymes that copy both strands of the double-stranded RNA segments. This strategy appears less efficient, but may be sufficient when the genome consists of only two or three double-stranded RNA segments.

Unique Mechanisms of mRNA and Genome Synthesis of Hepatitis Delta Satellite Virus

The strategy for synthesis of the (+) strand RNA genome of hepatitis delta satellite virus is apparently unique among animal viruses (Fig. 6.24). The genome does not encode an RNA polymerase: viral RNAs are produced by host cell RNA polymerase II (Box 6.6), and the hepatitis delta virus RNAs are RNA catalysts, or **ribozymes** (Box 6.7). The genome of hepatitis delta virus is a 1,700-nucleotide (−) strand circular RNA, the only RNA with this structure that has been found in animal cells. As approximately 70% of the nucleotides are base paired, the viral RNA is folded into a rodlike structure.

All hepatitis delta satellite virus RNAs are synthesized in the nucleus. The switch from mRNA synthesis to the production of full-length (+) RNA is controlled by suppression of a poly(A) signal. Full-length (−) and (+) strand RNAs are copied by a rolling-circle mechanism, and ribozyme self-cleavage

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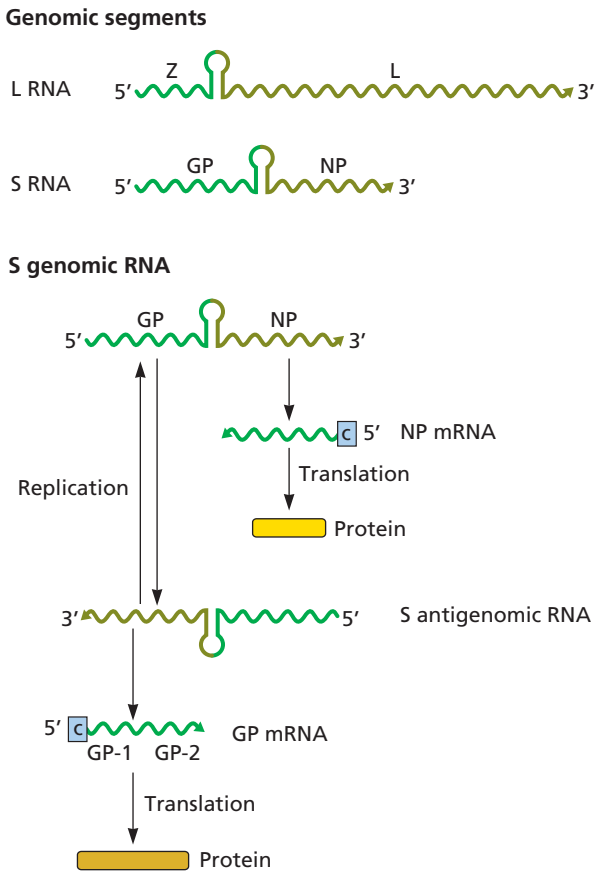


Figure 6.21 Arenavirus RNA synthesis. Arenaviruses contain two genomic RNA segments, L (large) and S (small) (top). At early times after infection, only the 3′ region of each of these segments is copied to form mRNA: the N mRNA from the S genomic RNA and the L mRNA from the L genomic RNA. Copying of the remainder of the S and L genomic RNAs may be blocked by a stem-loop structure in the genomic RNAs. After the S and L genomic RNAs are copied into full-length strands, their 3′ regions are copied to produce mRNAs: the glycoprotein precursor (GP) mRNA from S RNA and the Z mRNA (encoding an inhibitor of viral RNA synthesis) from the L RNA. Only RNA synthesis from the S RNA is shown in detail.

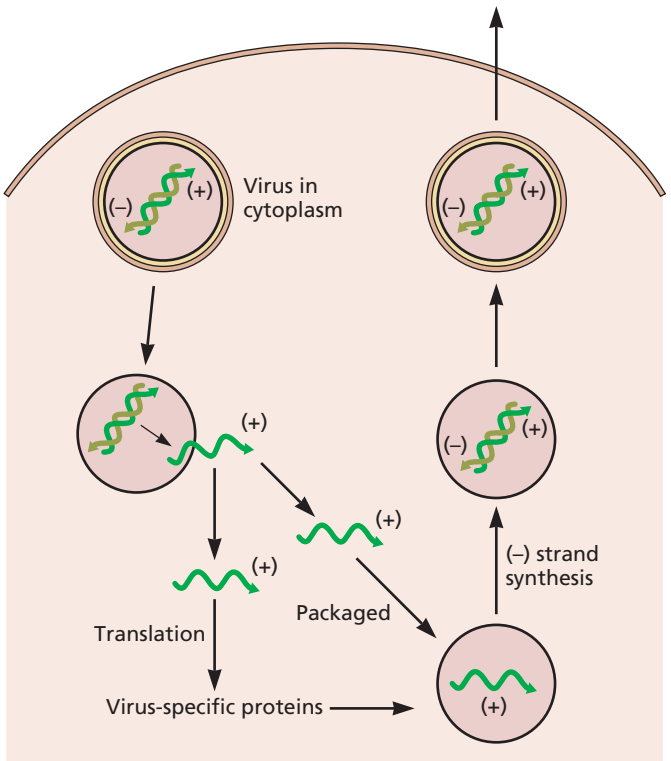


Figure 6.22 mRNA synthesis and replication of double-stranded RNA genomes. These processes occur in subviral particles containing the RNA templates and necessary enzymes. During cell entry, the virion passes through the lysosomal compartment, and proteolysis of viral capsid proteins activates the RNA synthetic machinery. Single-stranded (+) viral mRNAs, which are synthesized in parental subviral particles, are extruded into the cytoplasm, where they serve either as mRNAs or as templates for the synthesis of (−) RNA strands. In the latter case, viral mRNAs are first packaged into newly assembled subviral particles in which the synthesis of (−) RNAs to produce double-stranded RNAs occurs. These subviral particles become infectious particles. Only 1 of the 10 to 12 double-stranded RNA segments of the reoviral genome is shown.

releases linear monomers. Subsequent ligation of the two termini by the same ribozyme produces a monomeric circular RNA. The hepatitis delta virus ribozymes are therefore needed to process the intermediates of rolling-circle RNA replication. This enzyme initiates viral mRNA synthesis at a position on the genome near the beginning of the delta antigen-coding region. Once the polymerase has moved past a polyadenylation signal and the self-cleavage domain (Fig 6.24), the 3′-poly(A) of the mRNA is made by host cell enzymes. The RNA downstream of the poly(A) site is not degraded, in contrast to that of other mRNA precursors made by RNA polymerase II, but is elongated until a complete full-length (+) strand is made. The poly(A) addition site in this full-length (+) RNA is not used. The delta antigen bound to the rodlike RNA may block access of cellular enzymes to the poly(A) signal, thereby inhibiting polyadenylation.

Why Are (−) and (+) Strands Made in Unequal Quantities?

Different concentrations of (+) and (−) strands are produced in infected cells. For example, in cells infected with poliovirus, genomic RNA is produced at 100-fold higher concentrations than its complement. There are different explanations for these observations. RNA genomes and their complementary strands might have different stabilities, or the two strands might be synthesized by mechanisms with different efficiencies.

Viral (−) strand RNA is approximately 20 to 50 times more abundant than (+) strand RNA in cells infected with vesicular stomatitis virus. It was suggested that such asymmetry is

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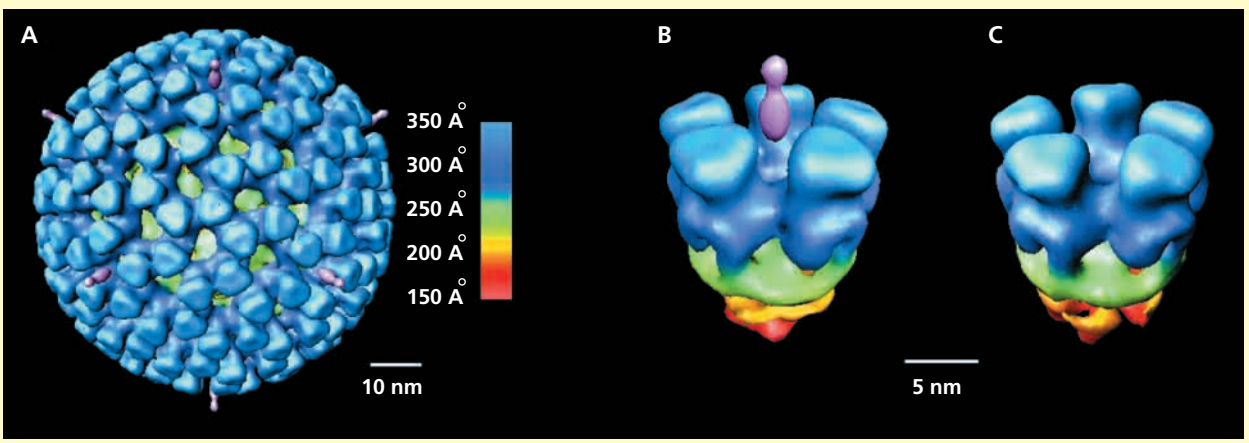
BOX 6.5

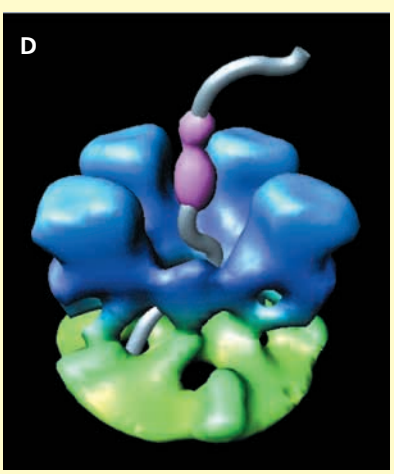
EXPERIMENTS

Release of mRNA from rotavirus particles

Rotaviruses, the most important cause of gastroenteritis in children, are large icosahedral viruses made of a three-shelled capsid containing 11 double-stranded RNA segments. The structure of this virus indicated that a large portion of the viral genome (~25%) is ordered within the particle and forms a dodecahedral structure (see Fig. 4.18). In this structure, the RNAs interact with the inner capsid layer and pack around the RNA polymerase located at the fivefold axis of symmetry. Further analysis of rotavirus particles in the process of synthesizing mRNA has shown that newly synthesized molecules are extruded from the capsid through several channels located at the fivefold axes (see figure). Multiple mRNAs are released at the same time from such particles. On the basis of these observations, it has been suggested that each double-stranded genomic RNA segment is copied by an RNA polymerase located at a fivefold axis of symmetry. This model may explain why no double-stranded RNA virus with more than 12 genomic segments, the maximum number of fivefold axes, has been found.

Lawton JA, Estes MK, Prasad BV. 1997.Three-dimensional visualization of mRNA release from actively transcribing rotavirus particles. *Nat Struct Biol* **4:**118–121.





Three-dimensional visualization of mRNA release from rotavirus particles synthesizing mRNA. (A) Structure of a rotavirus particle in the process of synthesizing mRNA. The capsid is depth cued according to the color chart. Parts of newly synthesized mRNA that are ordered, and therefore structurally visible, are shown in magenta at the fivefold axes of symmetry. **(B)** Close-up view of the channel at the fivefold axis and the visible mRNA. The mRNA is surrounded by five trimers of capsid protein VP6. **(C)** Close-up view of the channel at the fivefold axis of a particle not in the process of synthesizing mRNA. **(D)** Model of the pathway of mRNA transit through the capsid. One VP6 trimer has been omitted for clarity. The green protein is VP2, and the mRNA visible in the structure is shown in pink. The gray tube represents the possible path of an mRNA molecule passing through the VP2 and VP6 layers through the channel. Courtesy of B. V. V. Prasad, Baylor College of Medicine. Reprinted from J. A. Lawton et al., *Nat Struct Biol* **4:**118–121, 1997, with permission.

a consequence of more efficient initiation of RNA synthesis at the 3′ end of (+) strand RNA than of (−) strand RNA. An elegant proof of this hypothesis came from the construction and study of a second rhabdovirus genome, that of rabies virus, with identical initiation sites at the 3′ ends of both (−) and (+) strand RNAs. In cells infected with this virus, the ratio of (−) to (+) strands is 1:1.

In alphavirus-infected cells, the abundance of genomic RNA is explained by the fact that (−) strand RNAs are synthesized for but a short time early in infection. The RNA polymerase that catalyzes (−) strand RNA synthesis is present only during this period. The synthesis of (+) strands continues for much longer, leading to accumulation of mRNA and (+) strand genomic RNA.

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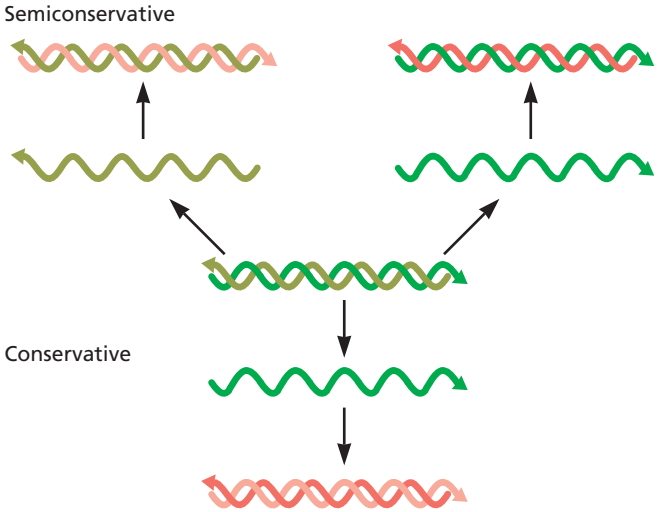


Figure 6.23 Two mechanisms for copying nucleic acids. During semiconservative replication, both strands of nucleic acid serve as templates for the synthesis of new strands (shown in red). In contrast, only one strand is copied during conservative replication.

Do Ribosomes and RNA Polymerases Collide?

The genomic RNA of (+) strand viruses can be translated in the cell, and the translation products include the viral RNA polymerase. At a certain point in infection, the RNA polymerase copies the RNA in a 3′ → 5′ direction while ribosomes traverse it in an opposite direction (Fig. 6.25), raising the question of whether the viral polymerase avoids collisions with ribosomes. When ribosomes are frozen on poliovirus RNA by using inhibitors of protein synthesis; replication is blocked. In contrast, when ribosomes are run off the template, replication of the RNA increases. These results suggest that ribosomes must be cleared from viral RNA before it can serve as a template for (−) strand RNA synthesis; in other words, replication and translation cannot occur simultaneously.

The interactions of viral and cellular proteins with the poliovirus 5′-untranslated region might determine whether the genome is translated or replicated. In this model, binding of cellular poly(rC)-binding protein 2 within the 5′-untranslated region initially stimulates translation. Once the viral protease has been synthesized, it cleaves poly(rC)-binding protein; consequently, binding of the cellular protein is reduced. However, cleaved poly(rC)-binding protein can still bind to a different segment of the 5′-untranslated region (the cloverleaf) (Fig. 6.10) and promote viral genome synthesis.

Restricting translation and RNA synthesis to distinct compartments may prevent collisions of ribosomes and polymerases. Viral mRNA synthesis takes place in the reovirus capsid, where the enzymes responsible for this process are located. The viral mRNAs are exported to the cytoplasm for translation. Retroviral RNAs are synthesized in the cell nucleus, where translation does not take place. The architecture of membranous replication complexes of (+) strand RNA viruses may favor RNA synthesis and exclude translation.

Even if mechanisms exist for controlling whether the genomes of RNA viruses are translated or replicated, some ribosome-RNA polymerase collisions are likely to occur. The isolation of a poliovirus mutant with a genome that contains an insertion of a 15-nucleotide sequence from 28S ribosomal RNA (rRNA) is consistent with this hypothesis. After colliding with a ribosome, the RNA polymerase apparently copied 15 nucleotides of rRNA.

Cellular Sites of Viral RNA Synthesis

Genomes and mRNAs of most RNA viruses are made in the cytoplasm, invariably in specific structures such as the nucleocapsids of (−) strand RNA viruses, subviral particles of double-stranded RNA viruses, and membrane-bound replication complexes in the case of (+) strand RNA viruses. Such replication complexes are morphologically diverse, and the membranes originate from various cellular compartments (Chapter 14). Alphaviral RNA synthesis occurs on the cytoplasmic surface of endosomes and lysosomes, and poliovirus RNA polymerase is located on the surfaces of small, membranous vesicles. Different viral proteins have been implicated in the formation of these viral replicative organelles.

The membrane vesicles observed early in poliovirus-infected cells are thought to originate from two sources. One appears to be the endoplasmic reticulum (ER), specifically vesicles whose production is regulated by proteins of coat protein complex II (CopII) (Chapter 12). Unlike vesicles produced from the ER in uninfected cells, those in poliovirus-infected cells do not fuse with the Golgi and therefore accumulate in the cytoplasm. The vesicles produced later during poliovirus infection bear several hallmarks of autophagosomes, including a double membrane and colocalization with protein markers of these vesicles. Synthesis of poliovirus 2BC and 3A proteins in uninfected cells leads to production of such autophagosomes. Similar double-membrane vesicles are observed during infection with a variety of (+) strand RNA viruses, indicating that they may serve as a general replication platform.

Flavivirus RNA replication takes place on perinuclear, double-membraned vesicles derived from the ER (Fig. 6.26). These vesicles are connected to the cytoplasm through a pore and are often near sites of virus assembly. Coronaviral proteins remodel cellular membranes to produce the replication complex, which consists of a network of convoluted membranes, double-membrane vesicles, and vesicle packets, all of which are continuous with the ER (Fig. 6.26).

It is thought that membrane association of viral replication assemblies ensures high local concentrations of relevant components, and hence increases the rates or efficiencies of

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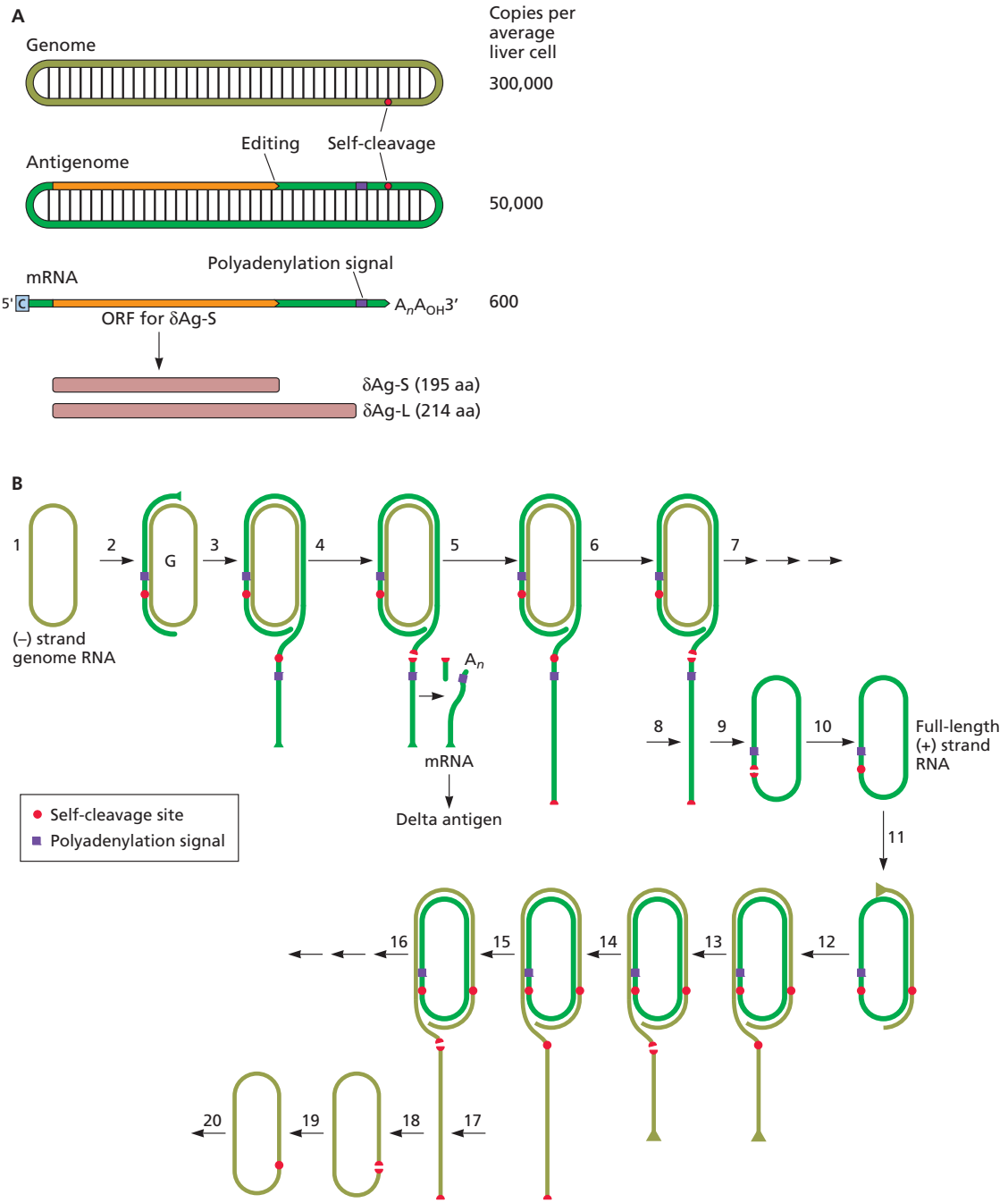


Figure 6.24 Hepatitis delta satellite virus RNA synthesis. (A) Schematic of the forms of hepatitis delta virus RNA and δ antigen found in infected cells. aa, amino acids; ORF, open reading frame. **(B)** Overview of hepatitis delta virus mRNA and genomic RNA synthesis. In steps 1 to 3, RNA synthesis is initiated, most probably by host RNA polymerase II, at the indicated position on the (−) strand genomic RNA. The polymerase passes the poly(A) signal (purple box) and the self-cleavage domain (red circle). In steps 4 and 5, the 5′ portion of this RNA is processed by cellular enzymes to produce delta mRNA with a 3′-poly(A) tail, while RNA synthesis continues beyond the cleavage site and the RNA undergoes self-cleavage (step 6). RNA synthesis continues until at least one unit of the (−) strand genomic RNA template is copied. The poly(A) signal is ignored in this full-length (+) strand. In steps 7 to 10, after self-cleavage to release a full-length (+) strand, self-ligation produces a (+) strand circular RNA. In steps 11 to 20, mRNA synthesis initiates on the full-length (+) strand to produce (−) strands by a rolling-circle mechanism. Unit-length genomes are released by the viral ribozyme (step 15) and self-ligated to form (−) strand circular genomic RNAs. Adapted from J. M. Taylor, *Curr Top Microbiol Immunol* **239:**107–122, 1999, with permission.

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BOX 6.6

DISCUSSION

RNA-dependent RNA polymerase II

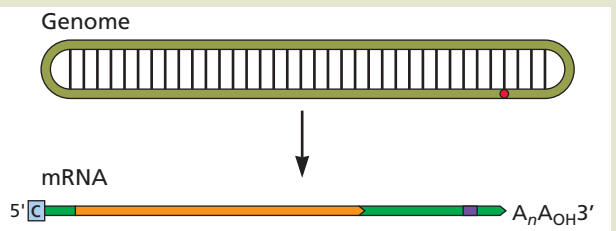
The mRNAs produced during hepatitis delta satellite virus infection of cells have typical properties of DNA-dependent RNA polymerase II products, including a 5′ cap and 3′-poly(A) tail. Production of these satellite mRNAs is also sensitive to α-amanitin, an inhibitor of DNA-dependent RNA polymerase II. Furthermore, the RNA genome of plant viroids can be copied by plant DNA-dependent RNA polymerase II. Based on these observations, it was suggested that the RNA genome of hepatitis delta satellite virus is copied by RNA polymerase II. Experimental support for this hypothesis has been obtained. When purified mammalian RNA polymerase II was incubated with NTPs and an RNA template-primer, an RNA product was produced. Similar results were obtained when the antigenome of hepatitis delta satellite virus was used in the reaction. Structural studies revealed that the RNA template-product duplex occupies the same site on the enzyme as the DNA-RNA hybrid during transcription. When transcription protein IIS was added to the reaction mixture, the satellite genome was cleaved, and the new 3′ end was used as a primer. Compared with DNA-dependent RNA synthesis, RNA-dependent RNA synthesis by RNA polymerase II was slower and less processive. These properties may explain why the enzyme can copy only short RNA templates.

The ability of DNA-dependent RNA polymerase II to copy an RNA template provides a missing link in molecular evolution. This activity supports the hypothesis that an ancestor of RNA polymerase II copied RNA genomes that are thought to have existed during the ancient RNA world. During the transition from RNA to DNA genomes, this enzyme evolved to copy DNA templates. Today these enzymes can still copy small RNAs such as the genome of hepatitis delta satellite virus.

Chang J, Nie X, Chang HE, Han Z, Taylor J. 2008. Transcription of hepatitis delta virus RNA by RNA polymerase II. *J Virol* **82:**1118–1127.

Lehmann E, Brueckner F, Cramer P. 2007. Molecular basis of RNA-dependent RNA polymerase II activity. *Nature* **450:**445–449.

Rackwitz HR, Rohde W, Sanger HL. 1981. DNA-dependent RNA polymerase II of plant origin transcribes viroid RNA into full-length copies. *Nature* **291:**297–301.



BOX 6.7

BACKGROUND

Ribozymes

A **ribozyme** is an enzyme in which RNA, not protein, carries out catalysis. The first ribozyme discovered was the group I intron of the ciliate *Tetrahymena thermophila*. Other ribozymes have since been discovered, including RNase P of bacteria, group II self-splicing introns, hammerhead RNAs of viroids and satellite RNAs, and the ribozyme of hepatitis delta virus. These catalytic RNAs are very diverse in size, sequence, and the mechanism of catalysis. For example, the hepatitis delta satellite virus ribozyme (see figure) catalyzes a transesterification reaction that yields products with 2′,3′-cyclic phosphate and 5′-OH termini. Only an 85-nucleotide sequence is required for activity of this ribozyme, and can cleave optimally with as little as a single nucleotide 5′ to the site of cleavage.

Ribozymes have been essential for producing infectious RNAs from cloned DNA copies of the genomes of (−) strand RNA viruses. Such transcripts often have extra sequences at the 3′ end. By joining the 85-nucleotide ribozyme fragment to upstream sequences, accurate 3′ ends of heterologous RNA transcripts synthesized *in vitro* can be obtained.

Kruger K, Grabowski PJ, Zaug AJ, Sands J, Gottschling DE, Cech TR. 1982. Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of Tetrahymena. *Cell* **31:**147–157.

Westhof E, Michel F. 1998. Ribozyme architectural diversity made visible. *Science* **282:**251–252.

Whelan SP, Ball LA, Barr JN, Wertz GT. 1995. Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. *Proc Natl Acad. Sci U S A* **92:**8388–8392.



Crystal structure of the hepatitis delta satellite virus ribozyme. The RNA backbone is shown as a ribbon. The two helical stacks are shown in red and blue, and unpaired nucleotides are gray. The 5′ nucleotide, which marks the active site, is green. Produced from Protein Data Bank file 1cx0.

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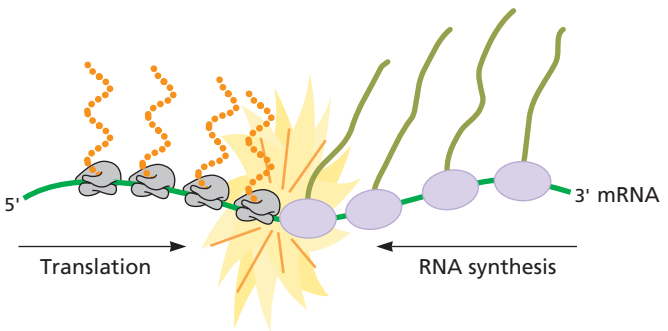


Figure 6.25 Ribosome-RNA polymerase collisions. A strand of viral RNA is shown, with ribosomes translating in the 5′ → 3′ direction and RNA polymerase copying the RNA chains in the 3′ → 5′ direction. Ribosome-polymerase collisions would occur in cells infected with (+) strand RNA viruses unless mechanisms exist to avoid simultaneous translation and replication.

these reactions. As we have seen, this property may contribute to the specificity of polioviral $3D^{pol}$ for viral RNA templates. Membrane association may also have other functions, such as allowing efficient packaging of progeny RNA into virus particles, or providing lipid components or physical support to the replication machinery. The surfaces of membranous replication complexes isolated from poliovirus-infected cells appear to be coated with two-dimensional arrays of polymerase. These arrays are formed by interaction of $3D^{pol}$ molecules in a head-to-tail fashion. Surface catalysis is known to confer several advantages, including a higher probability of collision among reactants, an increase in substrate affinity from clustering of multiple binding sites, and retention of reaction products. The last property would facilitate multiple rounds of copying (+) and (−) strand RNA templates.

In addition to providing a location for efficient viral RNA synthesis, membranous replication structures may also protect nucleic acids from nucleases and shield RNAs that activate host intrinsic defenses (Volume II, Chapter 3).

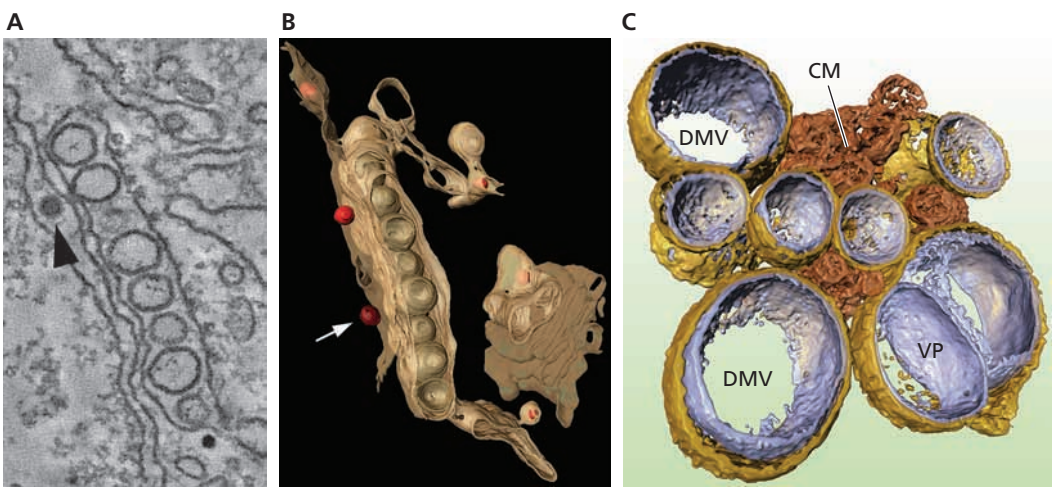
Viral RNA polymerases are recruited to membranous replication compartments in different ways. A C-terminal, transmembrane segment of the hepatitis C virus RNA polymerase, NS5b, is responsible for attachment of the enzyme to cellular membrane replication complexes. Polioviral $3D^{pol}$ cannot by itself associate with membranes but is brought to the replication complex by binding to protein 3AB. When the membrane association of this protein is disrupted by amino acid changes, viral RNA synthesis is inhibited. The hydrophobic domain of 3AB can be substituted for the C-terminal transmembrane segment of NS5b with little effect on RNA polymerase activity, indicating that membrane association is the sole function of this sequence.

Origins of Diversity in RNA Virus Genomes

Misincorporation of Nucleotides

All nucleic acid polymerases insert incorrect nucleotides during chain elongation. DNA-directed DNA polymerases have **proofreading** capabilities in the form of exonuclease activities that can correct such mistakes. Most RNA-dependent RNA polymerases do not possess

Figure 6.26 Membranous sites of viral RNA synthesis. (A) Tomogram of dengue virus-infected cell showing virus-induced vesicles as invaginations of the ER **(B)** Three-dimensional rendering of dengue virus-induced vesicles in the ER. Virus particles are colored red. **(C)** Model of SARS-coronavirus induced convoluted membranes (CM), double-membrane vesicles (DMV), and vesicle packets (VP). The cluster of large and small double-membrane vesicles (outer membrane, gold; inner membrane, silver) are connected to a vesicle packet and convoluted membrane structure (bronze). A, B from S. Welsch et al., *Cell Host Microbe* **23:**365–375, 2009; C from K. Knoops et al. *PLoS Biol* **6:**e226, 2008, with permission.



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this capability. The result is that error frequencies in RNA replication can be as high as one misincorporation per 10^3 to 10^5 nucleotides polymerized, whereas the frequency of errors in DNA replication is about 10,000-fold lower. Many of these polymerization errors cause lethal amino acid changes, while other mutations may appear in the genomes of infectious virus particles. This phenomenon has led to the realization that RNA virus populations are **quasispecies**, or mixtures of many different genome sequences. The errors introduced during RNA replication have important consequences for viral pathogenesis and evolution, as discussed in Volume II, Chapter 10. Because RNA viruses exist as mixtures of genotypically different viruses, viral mutants may be isolated readily. For example, live attenuated poliovirus vaccine strains are viral mutants that were isolated from an unmutagenized stock of wild-type virus.

Fidelity of copying by RNA-dependent RNA polymerases is determined by how the template, primer, and NTP interact at the active site. Nucleotide binding occurs in two steps: first, the NTP is bound in such a way that the ribose cannot interact properly with the Asp of motif A and the Asn of motif B (Fig. 6.5). Next, if the NTP is correctly base paired with the template, there is a conformational change in the enzyme, which reorients the triphosphate and allows phosphoryl transfer to occur. This change requires reorientation of the Asp and Asn residues, which would stabilize the position of the ribose in the binding pocket, and is thought to be a key fidelity checkpoint for the picornaviral RNA polymerase. This model is based on the structures of $3D^{pol}$ bound to a template primer and NTP (Fig. 6.6) and the study of an altered poliovirus $3D^{pol}$ with higher fidelity than the wild-type enzyme. The increased fidelity of this enzyme, which has a single amino acid substitution in the fingers domain, is the result of a change in the equilibrium constant for the conformational transition. Although this amino acid is remote from the active site, it participates in hydrogen bonding to motif A, which, as discussed above, is important in holding the NTP in a catalytically appropriate conformation. Of great interest is the observation that a similar interaction between fingers and motif A can be observed in RNA polymerases from a wide variety of viruses. This mechanism of enhancing fidelity may therefore be conserved in all RNA-dependent RNA polymerases.

These studies also provide mechanistic information on how ribavirin, an antiviral compound, causes lethal mutagenesis. The structure of foot-and-mouth disease virus RNA polymerase $3D^{pol}$ bound to ribavirin shows the compound positioned in the active site of the enzyme, adjacent to the 3′ end of the primer. The ribose of ribavirin is bound in the pocket, indicating that it has bypassed the fidelity checkpoint and induced the conformational change that holds the analog in a position ready for catalysis. Therefore, ribavirin is a mutagen because it can be substituted for any of the four NTPs in newly synthesized RNA molecules.

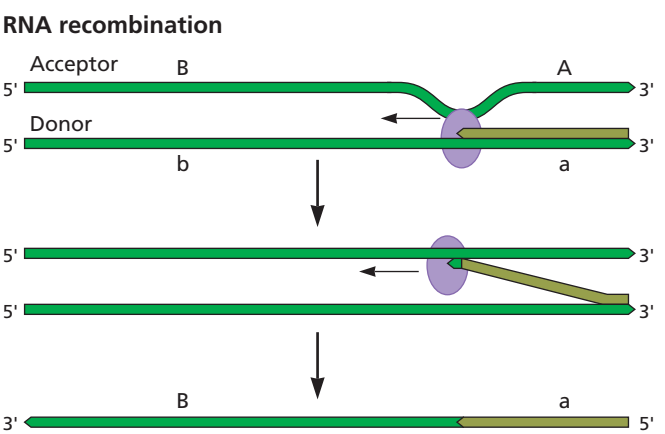
The RNA polymerase of members of the *Nidovirales* (Fig. 6.16) allows faithful replication of the large (up to 32 kb) RNA genomes. The RNA synthesis machinery includes proteins not found in other RNA viruses, such as ExoN, a 3′-5′ exonuclease. Inactivation of this enzyme does not impair viral replication, but leads to 15- to 20-fold increases in mutation rates. This observation suggests that ExoN provides a proofreading function for the viral RNA polymerase, similar to the activity associated with DNA synthesis. Viruses lacking the ExoN gene display attenuated virulence in mice, and are being considered as vaccine candidates.

Segment Reassortment and RNA Recombination

Reassortment is the exchange of entire RNA molecules between genetically related viruses with segmented genomes. In cells coinfected with two different influenza viruses, the eight genome segments of each virus replicate. When new progeny virus particles are assembled, they can package RNA segments from **either** parental virus. Because reassortment is the simple exchange of RNA segments, it can occur at high frequencies.

In contrast to reassortment, recombination is the exchange of nucleotide sequences among different genomic RNA molecules (Fig. 6.27). Recombination, a feature of many RNA viruses, is an important mechanism for producing new genomes with selective growth advantages. This process has shaped the RNA virus world by rearranging genomes, or creating new ones. RNA recombination was first discovered in

Figure 6.27 RNA recombination. Schematic representation of RNA recombination occurring during template switching by RNA polymerase, or copy choice. Two parental genomes are shown. The RNA polymerase (purple oval) has copied the 3′ end of the donor genome and is switching to the acceptor genome. The resulting recombinant molecule is shown.



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cells infected with poliovirus, and was subsequently observed for other (+) strand RNA viruses. The frequency of recombination can be relatively high: it has been estimated that 10 to 20% of polioviral genomic RNA molecules recombine in a single growth cycle. Recombinant polioviruses are readily isolated from the feces of individuals immunized with the three serotypes of Sabin vaccine. The genome of such viruses, which are recombinants of the vaccine strains with other enteroviruses found in the human intestine, may possess an improved ability to reproduce in the human alimentary tract and have a selective advantage over the parental viruses. Recombination can also lead to the production of pathogenic viruses (Box 6.8).

Polioviral recombination is predominantly **base pairing dependent**: it occurs between nucleotide sequences that have a high percentage of nucleotide identity. Other viral genomes undergo **base-pairing-independent** recombination between very different nucleotide sequences. RNA recombination is coupled with the process of genomic RNA replication: it occurs by template exchange during (−) strand synthesis, as first demonstrated in poliovirus-infected cells. The RNA polymerase first copies the 3′ end of one parental (+) strand and then exchanges one template for another at the corresponding position on a second parental (+) strand. Template exchange in poliovirus-infected cells occurs predominantly during (−) strand synthesis, presumably because the concentration of (+) strands is 100-fold higher than that of (−) strands. This template exchange mechanism of recombination is also known as **copy choice**. The exact mechanism of template exchange is not known, but it might be triggered by pausing of the polymerase during chain elongation or damage to the template.

If the RNA polymerase skips sequences during template switching, deletions will occur. Such RNAs will replicate if

BOX 6.8

DISCUSSION

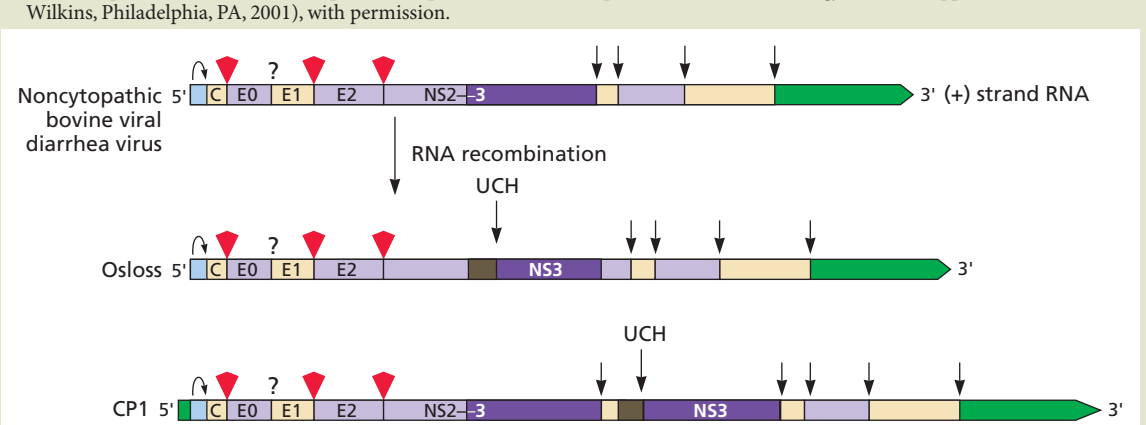
RNA recombination leading to the production of pathogenic viruses

A remarkable property of pestiviruses, members of the *Flaviviridae,* is that RNA recombination generates viruses that cause disease. Bovine viral diarrhea virus causes a usually fatal gastrointestinal disease. Infection of a fetus with this virus during the first trimester is noncytopathic, but RNA recombination produces a cytopathic virus that causes severe gastrointestinal disease after the animal is born.

Pathogenicity of bovine viral diarrhea virus is associated with the synthesis of a nonstructural protein, NS3, encoded by the recombinant cytopathic virus (see figure). The NS3 protein cannot be made in cells infected by the noncytopathic parental virus because its precursor, the NS2-3 protein, is not proteolytically processed. In contrast, NS3 is synthesized in cells infected by the cytopathic virus because RNA recombination adds an extra protease cleavage site in the viral polyprotein, precisely at the N terminus of the NS3 protein (see figure). This cleavage site can be created in several ways. One of the most frequent is insertion of a cellular RNA sequence coding for ubiquitin, which targets cellular proteins for degradation. Insertion of ubiquitin at the N terminus of NS3 permits cleavage of NS2-3 by any member of a widespread family of cellular proteases. This recombination event provides a selective advantage, because pathogenic viruses outgrow nonpathogenic ones. Why cytopathogenicity is associated with release of the NS3 protein, which is thought to be part of the machinery for genomic RNA replication, is not known.

Retroviruses acquire cellular genes by recombination, and the resulting viruses can have lethal disease potential, as described in Volume II, Chapters 6 and 11.

Pathogenicity of bovine viral diarrhea virus is associated with production of the NS3 protein. Two cytopathic viruses, Osloss and CP1, in which the ubiquitin sequence (UCH) has been inserted at different sites, are shown. In Osloss, UCH has been inserted into the NS2-3 precursor, and NS3 is produced. In CP1, a duplication has also occurred such that an additional copy of NS3 is present after the UCH sequence. Adapted from D. M. Knipe et al. (ed), *Fields Virology,* 4th ed. (Lippincott Williams & Wilkins, Philadelphia, PA, 2001), with permission.



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they contain the appropriate signals for the initiation of RNA synthesis. Because of their smaller size, subgenomic RNAs replicate more rapidly than full-length RNA, and ultimately compete for the components of the RNA synthesis machinery. Because of these properties, they are called **defective interfering RNAs**. Such RNAs can be packaged into viral particles only in the presence of a **helper virus** that provides viral proteins.

Defective interfering particles accumulate during the replication of both (+) and (−) strand RNA viruses. Production of these particles requires either a high multiplicity of infection or serial passaging, conditions that are achieved readily in the laboratory but rarely in nature. It is not known whether defective interfering viruses generally play a role in viral pathogenesis. However, some recombination reactions that lead to the appearance of cytopathic bovine viral diarrhea viruses delete viral RNA sequences rather than inserting cellular sequences (Box 6.8). Such deletions create a new protease cleavage site at the N terminus of the NS3 protein, and the defective interfering viruses also cause severe gastrointestinal disease in livestock.

RNA Editing

Diversity in RNA viral genomes is also achieved by RNA editing. Viral mRNAs can be edited either by insertion of a nontemplated nucleotide during synthesis or by alteration of the base after synthesis. Examples of RNA editing have been documented in members of the *Paramyxoviridae* and *Filoviridae* and in hepatitis delta satellite virus. This process is described in Chapter 10.

Perspectives

Structural biology has made important contributions toward understanding the mechanisms of RNA synthesis carried out by RNA-dependent RNA polymerases of (+) and double-stranded RNA viruses. These structures underscore the relationship of these enzymes to other nucleic acid polymerases, but also highlight differences that accommodate the wide diversity of RNA genome configurations. The first structure of an RNA polymerase of a (−) strand RNA virus, influenza virus, reveals how the endonuclease and cap-binding proteins are arranged with respect to the catalytic subunit. This structure should be the basis for an understanding at the atomic level of the different functions of this enzyme. We expect that forthcoming structures from other (−) strand RNA viruses will detail the conformational movements that take place during the switch between initiation and elongation, and the changes that occur as the polymerase moves from an open to a closed conformation.

RNA viral genetic diversity, and the ability to undergo rapid evolution, is made possible by errors made during nucleic acid synthesis, as well as recombination and reassortment. The importance of polymerase errors is underscored by the dramatic decrease in poliovirus fitness caused by a single amino acid change in the polymerase that decreases error rate. A different amino acid change in the RNA polymerase, which increases error frequency, has a similar effect. These observations demonstrate that the mutational diversity of RNA viruses is almost precisely where it must be, a place that is determined in large part by the error frequency of the RNA polymerase. A recent suggestion that not all mutations in the poliovirus genome are attributable to the viral RNA polymerase merits further investigation.

The function of host proteins that are required for viral RNA synthesis remain largely obscure, as does the reason why RNA synthesis is restricted to certain cellular compartments and structures induced by viral proteins. Some kinds of membranes are the sites of RNA replication for nearly all eukaryotic (+) strand RNA viruses; their architectures vary within viral families or orders. How these membranes are assembled, and their contribution to RNA synthesis, remain obscure. Prokaryotic (+) strand RNA viral replication does not occur on membranes and therefore this property cannot be a fundamental characteristic of RNA-directed RNA synthesis. Perhaps membrane-bound RNA synthesis was an adaptation required for colonization of eukaryotes. If so, it would important to determine if membranes are a requirement for RNA-directed RNA synthesis in *Archaea*.

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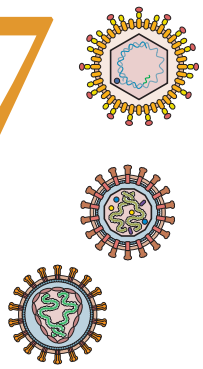
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7 Reverse Transcription and Integration



Retroviral Reverse Transcription
- Discovery
- Impact
- The Process of Reverse Transcription
- General Properties and Structure of Retroviral Reverse Transcriptases
- Other Examples of Reverse Transcription

Retroviral DNA Integration Is a Unique Process
- The Pathway of Integration: Integrase-Catalyzed Steps
- Integrase Structure and Mechanism

Hepadnaviral Reverse Transcription
- A DNA Virus with Reverse Transcriptase
- The Process of Reverse Transcription

Perspectives

References

LINKS FOR CHAPTER 7

- ⏭ *Video: Interview with Dr. David Baltimore*
  http://bit.ly/Virology_Baltimore
- ⏭ *Movie 7.1: Crystal structure of the prototype foamy virus integrase tetramer bound to viral DNA ends and a target sequence*
  http://bit.ly/Virology_V1_Movie7-1
- ⏭ *Retroviruses and cranberries*
  http://bit.ly/Virology_Twiv320
- ⏭ *Retroviral influence on human embryonic development*
  http://bit.ly/Virology_4-23-15
- ⏭ *A retrovirus makes chicken eggshells blue*
  http://bit.ly/Virology_9-11-13
- ⏭ *Museum pelts help date the Koala retrovirus*
  http://bit.ly/Virology_10-11-12
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  http://bit.ly/Virology_6-5-14

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"One can't believe impossible things," said Alice.
"I dare say you haven't had much practice," said the Queen.
"Why, sometimes I've believed as many as six impossible things before breakfast."

LEWIS CARROLL
Alice in Wonderland

Retroviral Reverse Transcription

Discovery

Back-to-back reports from the laboratories of Howard Temin and David Baltimore in 1970 provided the first concrete evidence for the existence of an RNA-directed DNA polymerase activity in retrovirus particles. The pathways that led to this unexpected finding were quite different in the two laboratories. In Temin's case, the discovery came about through attempts to understand how infection with this group of viruses, which have (+) strand RNA genomes, could alter the heredity of cells permanently, as they do in the process of oncogenic transformation. Temin believed that retroviral RNA genomes became integrated into the host cell's chromatin in a DNA form: studies of bacterial viruses such as bacteriophage lambda had established a precedent for viral DNA integration into host DNA (Box 7.1). However, it was a difficult hypothesis to test with the technology available at the time, and attempts by Temin and others to demonstrate the existence of such a phenomenon in infected cells were generally met with skepticism. Baltimore's entrée into the problem of reverse transcription came from his interest in virus-associated polymerases, in particular one that he had found to be present in particles of vesicular stomatitis virus, which contain a (−) strand RNA genome. It occurred to Baltimore and Temin independently that retrovirus particles might also contain such an enzyme, in this case the sought-after RNA-dependent DNA polymerase. Subsequent experiments confirmed this prediction; the enzyme that had earlier eluded Temin was discovered to be an integral component of these virus particles. Five years later, Temin and Baltimore were awarded the Nobel Prize in physiology or medicine for their independent discoveries of retroviral reverse transcriptase (RT).

Impact

The immediate impact of the discovery of RT was to amend the accepted central dogma of molecular biology, that the transfer of genetic information is unidirectional: DNA → RNA → protein. It was now apparent that there could also be a "retrograde" flow of information from RNA to DNA, and the name **retroviruses** eventually came to replace the earlier designation of RNA tumor viruses. In the years following this revision of dogma, many additional reverse transcription reactions were discovered. Furthermore, as Temin hypothesized, study of oncogenic retroviruses has provided a framework for current concepts of the genetic basis of cancer (Volume II, Chapter 6). Analysis of the reverse transcription and integration processes has enhanced our understanding of how retroviral infections persist and clarified aspects of the pathogenesis of acquired immunodeficiency syndrome (AIDS), caused by the human immunodeficiency virus. Finally, RT itself, first purified from virus particles and now produced in bacteria, has become an indispensable tool in molecular biology, for example, allowing experimentalists to capture cellular messenger RNAs (mRNAs) as complementary DNAs (cDNAs), which can then be converted into double-stranded DNAs, cloned, and expressed by well-established methodologies. Furthermore, the high efficiency of DNA integration mediated by the retroviral RT partner enzyme, integrase (IN), has been widely exploited for construction of viral vectors for gene transfer. For such reasons, we devote an entire chapter to these very important reactions (see interview with Dr. David Baltimore for background and personal account: http://bit.ly/Virology_Baltimore).

The Process of Reverse Transcription

Insight into the mechanism of reverse transcription can be obtained by comparing the amino acid sequences of RTs

PRINCIPLES ***Reverse transcription and integration***

- Reverse transcriptases (RTs) are enzymes that synthesize DNA from both RNA and DNA templates.
- One immediate consequence of the discovery of the first (retroviral) RT was amendment of the central dogma, DNA → RNA → protein.
- Retrotransposons are elements in cellular DNAs that are copied from an RNA intermediate by RTs and inserted at other loci. Such intracellular transposable elements are present in the genomes of most, if not all, members of the tree of life.
- Retroviral RT is an indispensable tool in molecular biology, allowing capture of cellular mRNAs as complementary DNAs (cDNAs).
- As with all DNA polymerases, DNA synthesis by RTs requires a primer, either a fragment of RNA or a protein.
- Reverse transcription is an error-prone process, as RTs lack proofreading activity.
- Reverse transcription of retroviral and hepadnaviral RNAs is facilitated by the presence of terminal repeat sequences, and the dynamic and multifunctional properties of their RTs.
- Retroviruses are the only animal viruses with genomes that encode integrase proteins.
- Integrase is a specialized recombinase that mediates the insertion of retroviral DNA into its host genome, where it is called a provirus.
- Genomic RNA templates are packaged by all viruses that replicate via reverse transcription, but the RTs of some of these viruses convert the RNA to a DNA copy before infection of a new cell.

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BOX 7.1

TRAILBLAZER

Bacteriophage lambda, a paradigm for the joining of viral and host DNAs

In 1962, Allan Campbell proposed an elegant, but at the time revolutionary, model for site-specific integration of DNA of the bacteriophage lambda into the chromosome of its host, *Escherichia coli*. The model was deduced from the fact that different linkage maps could be constructed for viral genomes at different stages in its life cycle. One linkage map, that of the integrated prophage, was obtained from the study of lysogenic bacteria. A different linkage map was obtained by measuring recombination frequencies of phage progeny (see part A of figure).

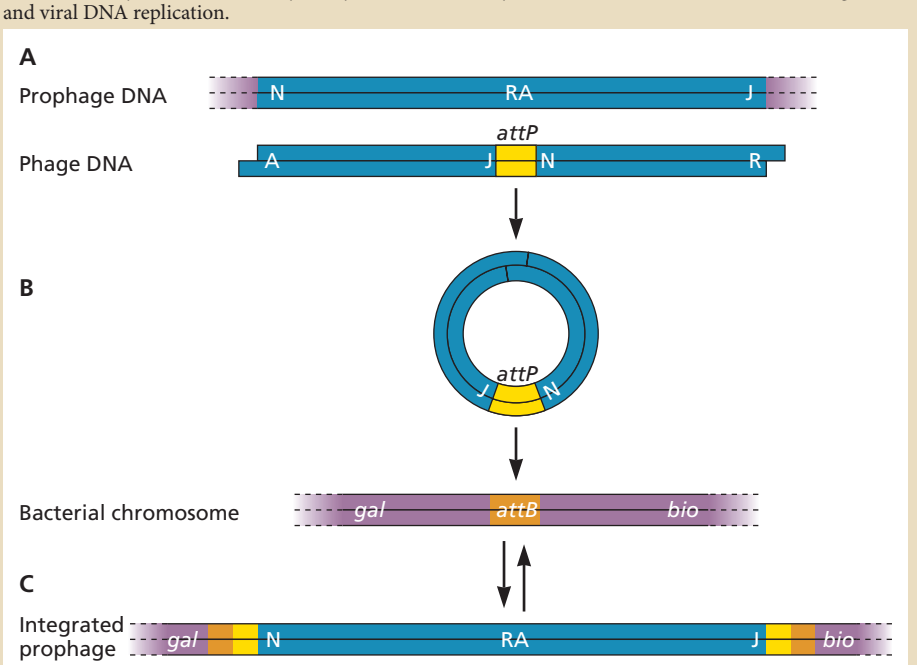
Campbell proposed that these unique features could be explained by a model for integration in which the incoming, linear, double-stranded DNA phage genome must first circularize. Subsequent recombination between a specific, internal sequence in the phage genome (called *attP*) and a specific sequence in the bacterial chromosome (called *attB*) would produce an integrated viral genome, with a linkage map that was a circular permutation of that of the linear phage genome, as had been observed (see part B of figure).

Although this model seems obvious today, it was not so in the 1960s. An alternative, in which the linear viral DNA was attached by a partial binding or "synapse" with the bacterial chromosome, was favored by a number of investigators. However, shortly after Campbell's elaboration of his model, circular molecules of lambda DNA were detected in infected cells, and the linear DNA extracted from purified phage particles was found to possess short, complementary single-strand extensions, "cohesive ends," that could promote circle formation. Other predictions of the model were also validated in several laboratories, and viral and cellular proteins that catalyzed integration were identified.

Lambda DNA integration remains an important paradigm for understanding the molecular mechanisms of DNA recombination and the parameters that influence the joining of viral and host DNAs.

Campbell AM. 1962. Episomes. *Adv Genet* **11:**101–145.

Distinct orientations of the bacteriophage lambda genetic map. (A) Comparison of the integrated prophage map, in which genes N and J are flanked by bacterial DNA and genes R and A are located centrally, with DNA extracted from virus particles, in which genes N and J are in the center of the genetic map and viral genes A and R are at the termini. Complementary single-strand ends in the viral DNA are shown in expanded scale. **(B)** Organization of viral genes in the circular form of DNA produced by annealing and ligation of the single-strand ends of viral DNA following infection of host cells. The *attP* site (yellow box) lies between genes N and J. The bacterial insertion site, *attB* (orange box), is shown below the circle, flanked by genes that encode enzymes required for galactose metabolism (*gal*) and biosynthesis of the vitamin biotin (*bio*). **(C)** Map of the integrated prophage, flanked by hybrid *att* sites. Upon induction, recombination at the hybrid *att* sites, catalyzed by another viral enzyme, leads to excision of a circular viral genome and viral DNA replication.



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with those of other enzymes that catalyze similar reactions. For example, RTs share certain sequence motifs with RNA and DNA polymerases of bacteria, archaea, and eukaryotes, in regions known to include critical active-site residues (Fig. 6.4). Consequently, it is not surprising that these enzymes employ similar catalytic mechanisms for nucleic acid polymerization reactions. Like DNA polymerases, viral RTs cannot initiate DNA synthesis *de novo*, but require a specific primer. It should be noted that even as arcane and distinct as the viral systems may appear, they do not exhaust the repertoire for reverse transcription reactions that exist in nature. Wide varieties of primers, as well as sites and modes of initiation, are used by other RTs.

Much of what has been learned about reverse transcription in retroviruses comes from the identification of intermediates in the reaction pathway that are formed in infected cells. Reverse transcription intermediates have also been detected in **endogenous reactions**, which take place within purified virus particles, using the encapsidated viral RNA template. It was amazing to discover that intermediates and products virtually identical to those made in infected cells can actually be synthesized in purified virus particles; all that is required is treatment with a mild detergent to permeabilize the envelope and addition of the metal cofactor and deoxynucleoside triphosphate (dNTP) substrates. The fidelity and robustness of the endogenous reaction suggests that the reverse transcription system is poised for action as soon as the virus particle enters the cell. Retroviral reverse transcription intermediates have also been analyzed in fully reconstituted reactions with purified enzymes and model RNA templates.

Retroviral RT is the only protein required to accomplish all the diverse steps in the pathway described below. However, as the reactions that take place inside cells are more efficient than those observed in either endogenous or reconstituted systems, it is unlikely that all of the significant molecular interactions have been reproduced *in vitro*.

Essential Components

Genomic RNA. Retrovirus particles contain two copies of the RNA genome held together by multiple regions of base pairing. (See Box 7.2 for labeling conventions.) When purified from virus particles, this RNA sediments at 70S, as expected for a dimer of 35S genomes. Partial denaturation and electron microscopic analyses of the 70S RNA indicate that the most stable pairing is via sequences located near the 5′ ends of the two genomes (Fig. 7.1A). Sequence interactions that promote dimerization have been identified in human immunodeficiency virus type 1 RNA (Fig. 7.1B). The 70S RNA also includes two molecules of a specific cellular transfer RNA (tRNA) that serves as a primer for the initiation of reverse transcription, discussed in the following section.

Despite the fact that two genomes are encapsidated, only one copy of integrated retroviral DNA is typically detected after infection with a single particle. Therefore, retroviral virus particles are said to be **pseudodiploid**. The availability of two RNA templates could help retroviruses survive extensive damage to their genomes. At least parts of both genomes can be, and typically are, used as templates during the reverse transcription process, accounting for the high rates of genetic recombination in these viruses. Presumably, being able to patch together one complete DNA copy from two randomly interrupted or mutated RNA genomes would provide survival value. Nevertheless, genetic experiments have shown that the use of two RNA templates is not an essential feature of the reverse transcription process. Therefore, all of the known steps in reverse transcription can take place on a single genome.

Like the genomes of (−) strand RNA viruses, the retroviral genome is coated along its length by a viral nucleocapsid protein (NC), with approximately one molecule for every 10 nucleotides. This small, basic protein can bind nonspecifically to both RNA and DNA and promote the annealing of nucleic acids. Biochemical experiments suggest that NC may facilitate template exchanges and function in reverse transcription like the bacterial single-stranded-DNA-binding proteins. In the synthesis of DNA catalyzed by bacterial DNA polymerases, the single-stranded-DNA-binding proteins enhance **processivity** (ability to continue synthesis without dissociating from the template). The ability of NC to organize RNA genomes within the virus particle and to facilitate reverse transcription within the infected cell may account for some of the differences in efficiency observed when comparing reactions reconstituted *in vitro* with those that take place in a natural infection.

Primer tRNA. In addition to the viral genome, retrovirus particles contain a collection of cellular RNAs. These include ~100 copies of a nonrandom sampling of tRNAs, some 5S rRNA, 7S RNA, and traces of cellular mRNAs. We do not know how most of these cellular RNAs become incorporated into virus particles, and most have no obvious function. However, one particular tRNA molecule is critical: it serves as a primer for the initiation of reverse transcription. The tRNA primer is positioned on the template genome during virus assembly, in a reaction that is facilitated by interactions with the viral polyprotein precursors (Gag and Gag-Pol; see Appendix, Fig. 29 and 30) during particle assembly. The primer tRNA

BOX 7.2

TERMINOLOGY

Conventions for designating sequences in nucleic acids

For clarity, lowercase designations are used throughout this chapter to refer to RNA sequences; uppercase designations identify the same or complementary sequences in DNA (e.g., pbs in RNA; PBS in DNA).

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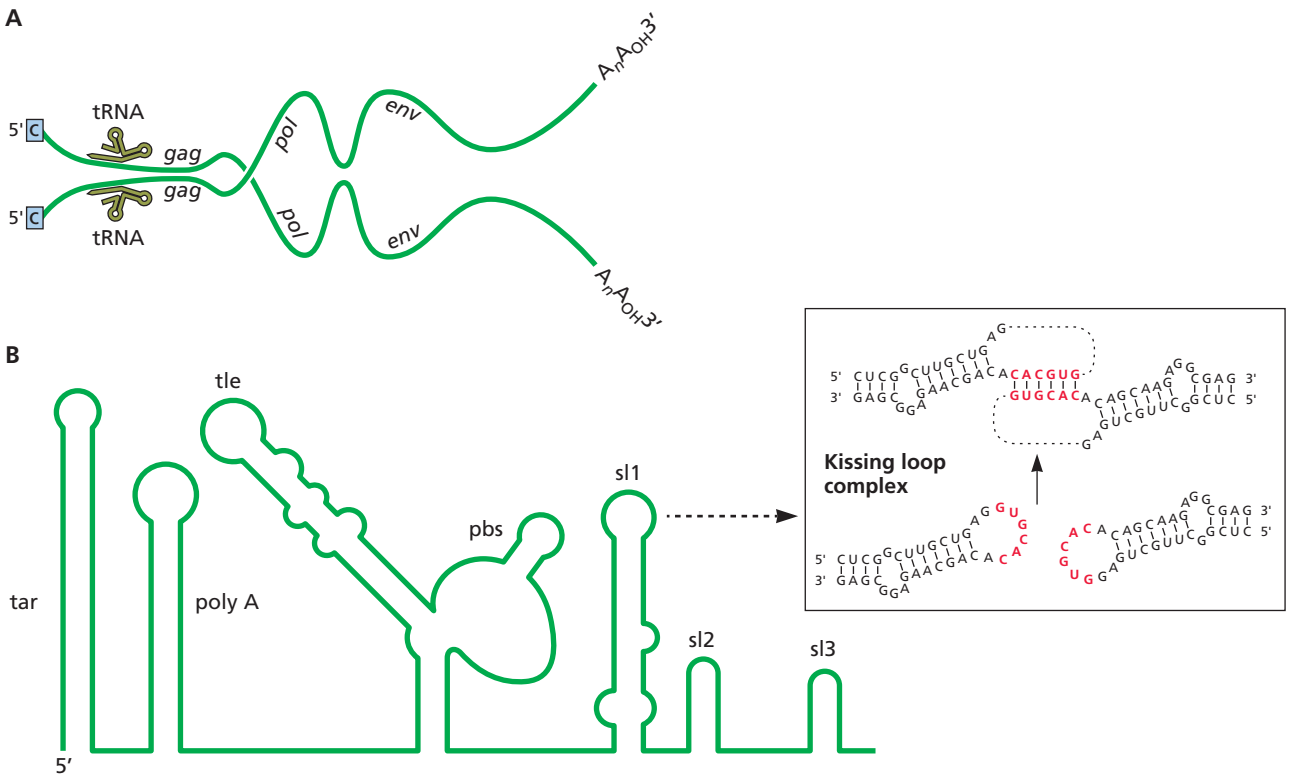


Figure 7.1 The diploid retroviral genome and a dimerization domain. (A) The diploid retroviral genome includes the following, from 5′ to 3′: the m^7Gppp cap; the coding regions for viral structural proteins and enzymes; *gag*, *pol*, and *env*; and the 3′-poly(A) sequence. The cell-derived primer tRNA is also shown. Points of contact represent multiple short regions of complementary base pairing. From J. M. Coffin, p 1767–1848, *in* B. N. Fields et al (ed), *Fields Virology*, 3rd ed (Lippincott-Raven Publishers, Philadelphia, PA, 1996), with permission. **(B)** Structural elements in the 5′ end of genomic RNA comprise distinct stem-loop structures. In the human immunodeficiency virus RNA, these elements include the Tat-binding site (tar), a poly(A) stem-loop, and a section that resembles a tRNA anticodon loop called the tle (for tRNA-like element). The adjacent primer-binding site (pbs), comprising a sequence complementary to the 3′ end of the tRNA primer, is followed by a stem-loop structure, sl1, that initiates genome dimerization by hybridizing with sl1 in a second viral RNA molecule to form a "kissing loop," as illustrated in the box. The sl1, sl2, and sl3 elements are required for efficient viral RNA packaging.

is partially unwound and hydrogen-bonded to complementary sequences near the 5′ end of each RNA genome in a region called the **primer-binding site (pbs)** (Fig. 7.2). The RTs of all retroviruses studied to date are primed by one of only a few classes of cellular tRNAs. Most mammalian retroviral RTs rely on $tRNA^{Pro}$, $tRNA^{Lys3}$, or $tRNA^{Lys1,2}$ for this function, and the relevant primer RNAs are packaged selectively into virus particles. Lysyl-tRNA synthetase is also packaged in human immunodeficiency virus type 1 particles: this cellular protein binds to viral RNA and facilitates positioning of the $tRNA^{Lys3}$ primer on the pbs (Box 7.3). It seems possible that a similar mechanism promotes primer binding on other retroviral genomes, but the generality of this process has not yet been tested.

In addition to the 3′-terminal 18 nucleotides that anneal to the pbs, other regions in the tRNA primer contact the RNA template and modulate reverse transcription. The template-primer interaction has been studied extensively in reconstituted reactions with RNA and RT of avian sarcoma/leukosis virus. In these *in vitro* analyses, the ability of the viral RNA to form stem-loop structures, and specific interactions between the primer $tRNA^{Trp}$ and one of these loops, appear to be critical for reverse transcription (Fig. 7.2). Similar interactions have been reported for human immunodeficiency virus RNA and its primer. Although the interactions are likely to be significant biologically, we do not yet know how RTs recognize structural features in these template-primer complexes.

Reverse transcriptase. Each retrovirus particle contains 50 to 100 molecules of RT. Reducing the number of enzymatically active copies of RT by more than 2- to 3-fold dramatically inhibits the process of reverse transcription in cells infected in culture. However, the number of molecules that are actually engaged in reverse transcription in each virus particle is not known. Reverse transcription can be initiated in

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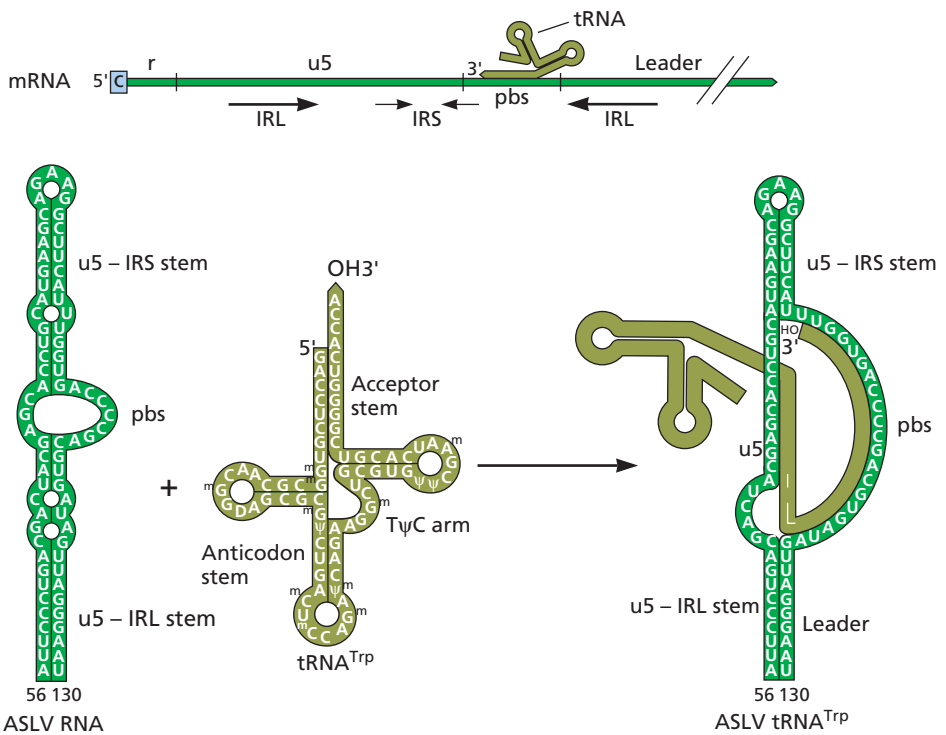


Figure 7.2 Primer tRNA binding to the retroviral RNA genome. (Top) Linear representation of the 5′ terminus of retroviral RNA, indicating locations of the r, u5, and leader regions. A tRNA primer is annealed schematically to the pbs. Two inverted-repeat (IRS and IRL) sequences that flank the pbs are represented by arrows. (Bottom) The avian sarcoma/leukosis virus (ASLV) RNA can form an extended hairpin structure around the pbs in the absence of primer tRNA (left). Primer $tRNA^{Trp}$ is shown in the cloverleaf structure (middle). Modified bases are indicated. Viral RNA annealed to $tRNA^{Trp}$, with flanking u5-leader and u5-IR stem structures (right). The TΨC arm of the primer and u5 RNA also form hydrogen bonds. Bottom diagram is from J. Leis et al, p 33–47, *in* A. M. Skalka and S. P. Goff (ed), *Reverse Transcriptase* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1993), with permission.

virus particles as soon as the viral envelope is made permeable to dNTP substrates, and it has been established that DNA synthesis takes place in the cytoplasm shortly after entry, within a subviral nucleoprotein structure that retains a partially dissociated capsid. Enzymes of the three retroviruses that have been studied most extensively, avian sarcoma/leukosis virus, murine leukemia virus, and human immunodeficiency virus type 1, are used as examples throughout this chapter.

Retroviral RTs are intricate molecular machines with moving parts and multiple activities. The distinct catalytic activities brought into play at various stages in the pathway of reverse transcription include RNA-directed and DNA-directed DNA polymerization, DNA unwinding, and the hydrolysis of RNA in RNA-DNA hybrids (RNase H). The first three activities reside within the polymerase domain, while the RNase H is in a separate domain. The RNase H of RT functions as an endonuclease, producing fragments of 2 to 15 nucleotides from the genomic RNA after it has been copied into cDNA. RNase H activity also produces the primer for (+) strand DNA synthesis from the genomic RNA, and removes this primer and the tRNA primer from the 5′ ends of the nascent viral DNA strands at specific steps in the reaction.

Distinct Steps in Reverse Transcription

Initiation of (−) strand DNA synthesis. Our understanding of DNA synthesis indicates that the simplest way of copying an RNA template to produce full-length complementary DNA would be to start at its 3′ end and finish at its 5′ end. It was therefore somewhat of a shock for early researchers to discover that retroviral reverse transcription in fact starts near the 5′ end of the viral genome—only to run out of template after little more than ~100 nucleotides (Fig. 7.3). However, this counterintuitive strategy for initiation of DNA synthesis allows the duplication and translocation of essential transcription and integration signals encoded in both the 5′ and 3′ ends of the genomic RNA, called u5 and u3, respectively.

The 5′ end of the genome RNA is degraded by the RNase H domain of RT, after (or as) it is copied to form (−) strand DNA. The short (ca. 100-nucleotide) DNA product of this first reaction, attached to the tRNA primer, accumulates in large quantities in the endogenous and reconstituted systems and is called **(−) strong-stop DNA**. For simplicity, the reactions illustrated in Fig. 7.3 to 7.6 are shown as taking place on a single RNA genome.

The first template exchange. In the next distinct step (Fig. 7.4), the 3′ end of the RNA genome is engaged as a template via hydrogen bonding between the R sequence in the (−) strong-stop DNA and the complementary r sequence upstream of the poly(A) tail. This reaction corresponds to the substitution of one end of the RNA for another to be copied by the RT "machine." As (−) strong-stop DNA is barely detectable in infected cells, this first template exchange must be efficient. Once the 3′ end of the genome RNA is engaged, the RNA-dependent DNA polymerase activity of RT can continue

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BOX 7.3

EXPERIMENTS

tRNA mimicry and the primer-binding site of human immunodeficiency virus type 1

A highly conserved region in the 5′ end of human immunodeficiency virus type 1 genomic RNA contains multiple sequences that are critical for control of viral transcription, genome replication, and genome packaging (Fig. 7.1 and 7.2). Not only are specific nucleotides important, but numerous regions of base pairing mediate formation of particular structures that are also critical for function. Recent analysis of the three-dimensional structures of functional sections of this region has shown that molecular mimicry may explain how the tRNA primer is positioned selectively on one of these sections, the primer-binding site (pbs).

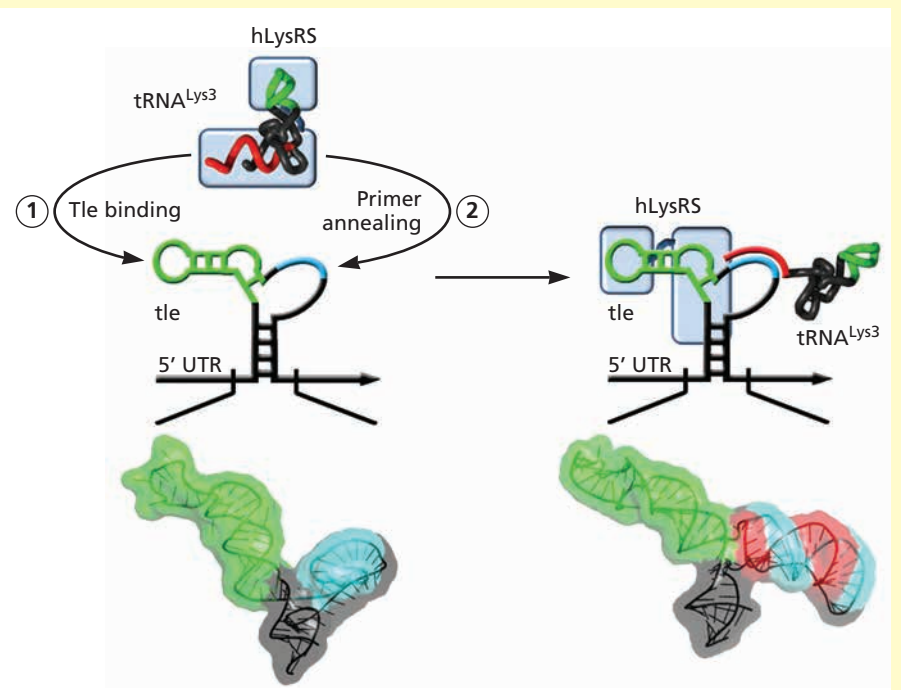
Human $tRNA^{Lys3}$ is the primer for synthesis of cDNA by the RT of human immunodeficiency virus type 1. While only one primer (or perhaps two) is needed for the two copies of genomic RNA in a virus particle, approximately 20 to 25 $tRNA^{Lys}$ molecules are encapsidated, along with equal amounts of their major binding protein, human lysyl-tRNA synthetase (hLysRS). The enrichment of $tRNA^{Lys}$ in virus particles is due, in part, to the interaction of hLysRS with the viral Gag and Gag-Pol proteins during particle assembly. It has also been established that hLysRS binds tightly to viral RNA, and that such binding depends on sequences in a tRNA-like element (tle) proximal to the pbs, which resembles the anticodon loop of $tRNA^{Lys}$. Annealing of the tRNA primer has also been proposed to promote a conformation required for genome dimerization via the downstream initiation site.

Structural analysis of a 99-nucleotide fragment corresponding to the tle-pbs region of human immunodeficiency virus type 1 RNA shows that this region, either alone or with an annealed 18-deoxynucleotide primer sequence, adopts a bent conformation in solution that resembles the shape of a tRNA (see figure, bottom). This capacity for molecular mimicry and analysis of the relative affinities of hLysRS for the tle and tRNA have suggested the model illustrated at the top of the figure. In the model, competition by the tle for binding to hLysRS leads to release of $tRNA^{Lys3}$, which can now be positioned selectively on the adjacent pbs.

Jones CP, Cantara WA, Olson ED, Musier-Forsyth K. 2014. Small-angle X-ray scattering-derived structure of the HIV-1 5′ UTR reveals 3D tRNA mimicry. *Proc Natl Acad Sci U S A* **111:**3395–3400.

Jones CP, Saadatmand J, Kleiman L, Musier-Forsyth K. 2013. Molecular mimicry of human $tRNA^{Lys}$ anti-codon domain by HIV-1 RNA genome facilitates tRNA primer annealing. *RNA* **19:** 219–229.

Seif E, Niu M, Kleiman L. 2013. Annealing to sequences within the primer binding site loop promotes an HIV-1 RNA conformation favoring RNA dimerization and packaging. *RNA* **19:**1384–1393.



Model for $tRNA^{Lys3}$ primer placement onto the pbs. The $tRNA^{Lys3}$ primer (top left) shares 18 nucleotides of complementarity (red) to human immunodeficiency virus type 1 RNA pbs (blue), to which the primer must be annealed for reverse transcription to begin from its CCA-3′-OH end. The tle is part of an hLysRS-binding domain that effectively competes with tRNA for binding to hLysRS (left, step 1). Such competition is believed to facilitate release of bound $tRNA^{Lys3}$ from the synthetase, the 3′ end of which can then be annealed to the pbs in viral RNA (left, step 2). The final annealed complex with hLysRS bound to the tle is shown at the right. The three-dimensional models at the bottom of the figure are derived from small-angle X-ray scattering in conjunction with molecular dynamics and simulated annealing of a 99-nucleotide RNA that includes the tle-pbs domain alone (left) or annealed to an 18-nucleotide complementary DNA fragment, anti-PBS, that represents the priming, 3′ end of $tRNA^{Lys3}$ (right). The color scheme is the same as at the top, with the tle shown in green, the pbs in blue, and anti-PBS in red. Figure courtesy of W. A. Cantara, E. D. Olson, C. P. Jones, and K. Musier-Forsyth, Ohio State University.

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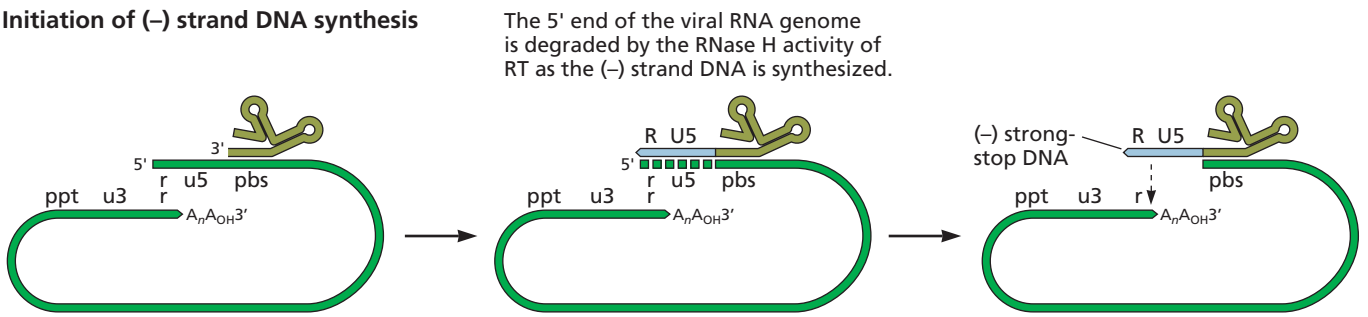


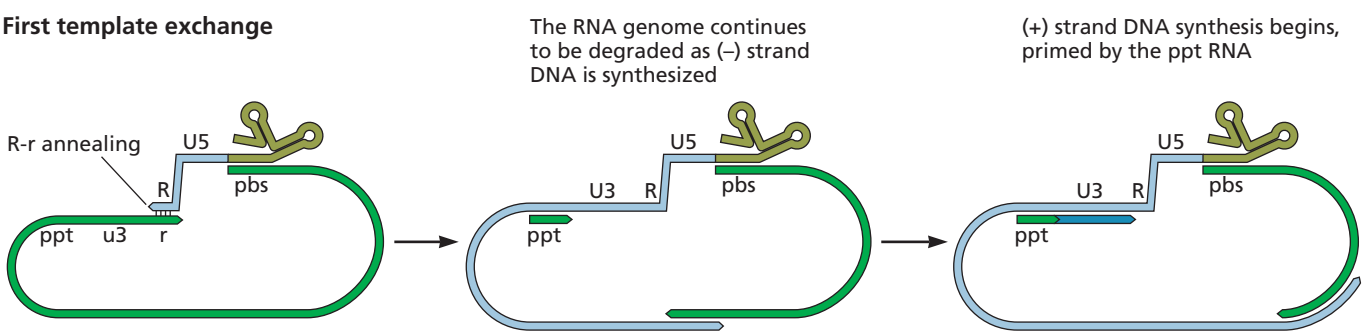
Figure 7.3 Retroviral reverse transcription: initiation of (−) strand DNA synthesis from the tRNA primer. Retroviral DNA synthesis begins with copying of the 5′ end of the viral RNA genome, using the 3′ end of a tRNA as the primer.

copying all the way to the 5′ end of the template, with the RNase H activity digesting the RNA template in its wake.

Initiation of (+) strand DNA synthesis. Among the early products of RNase H degradation of genomic RNA is a fragment comprising a **polypurine tract (ppt)** of ~13 to 15 nucleotides. This RNA fragment is especially important as it serves as the primer for (+) strand DNA synthesis, which begins even before (−) strand DNA synthesis is completed (Fig. 7.4). Following initiation from the ppt, synthesis of (+) strand DNA proceeds to the nearby end of the (−) strand DNA template and terminates after copying the first 18 nucleotides of the primer tRNA, when it encounters a modified base that cannot be copied. This product is called **(+) strong-stop DNA** (Fig. 7.5, left). The (−) strand DNA synthesis continues to the end of the viral DNA template, which includes the pbs sequence that had been annealed to the tRNA primer. The production of (+) strong-stop DNA and the converging (−) strand DNA synthesis disengages the template ends. The product is a (−) strand of viral DNA comprising the equivalent of an entire genome (but in permuted order) annealed to the (+) strong-stop DNA. The ppt and tRNA primer are removed by the RNase H, probably via recognition of structural features in these RNA-DNA junctions. The single-stranded 3′ end of the (−) strong-stop DNA then becomes available for annealing to complementary sequences in the single-stranded PBS at the 3′ end of the (+) strand DNA (Fig. 7.5, right).

The second template exchange. The next steps in the pathway of reverse transcription begin with a **second template exchange** in which annealing of the complementary PBS sequences provides a circular DNA template for polymerization by RT (Fig. 7.6, top). Synthesis of the (+) strand DNA can now continue, using (−) strand DNA as a template. The (−) strand DNA synthesis also continues to the end of U3, displacing the 5′ end of the (+) strand, a reaction that opens the DNA circle. Synthesis stops when RT reaches the terminus of each template strand (Fig. 7.6, left). The final product is a linear, DNA duplex copy of the viral genome with **long terminal repeats (LTRs)**, containing critical *cis*-acting signals at either end. This linear form of viral DNA is the major product of reverse transcription found in the nucleus of infected cells.

Figure 7.4 Retroviral reverse transcription: first template exchange, mediated by annealing of short terminal repeat sequences. Although a template exchange of the 5′ end of one RNA genome for the 3′ end of the second RNA genome can also occur, the principles illustrated and the final end products would be the same.



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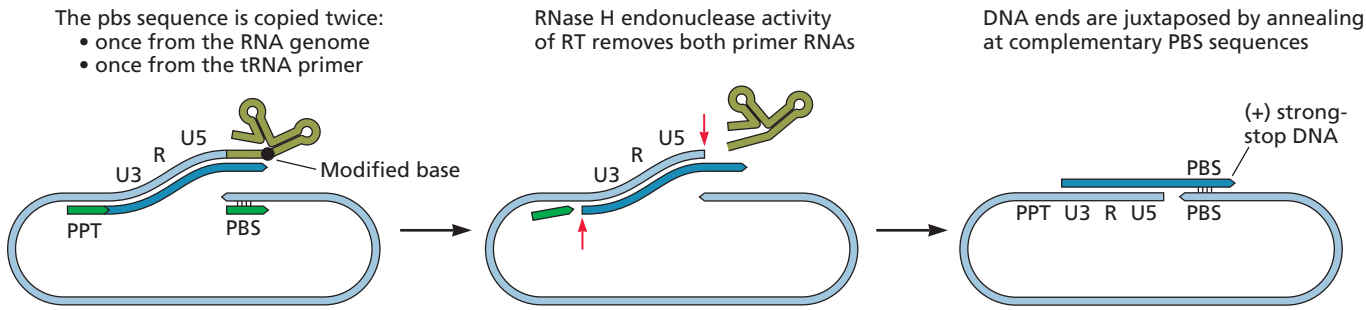
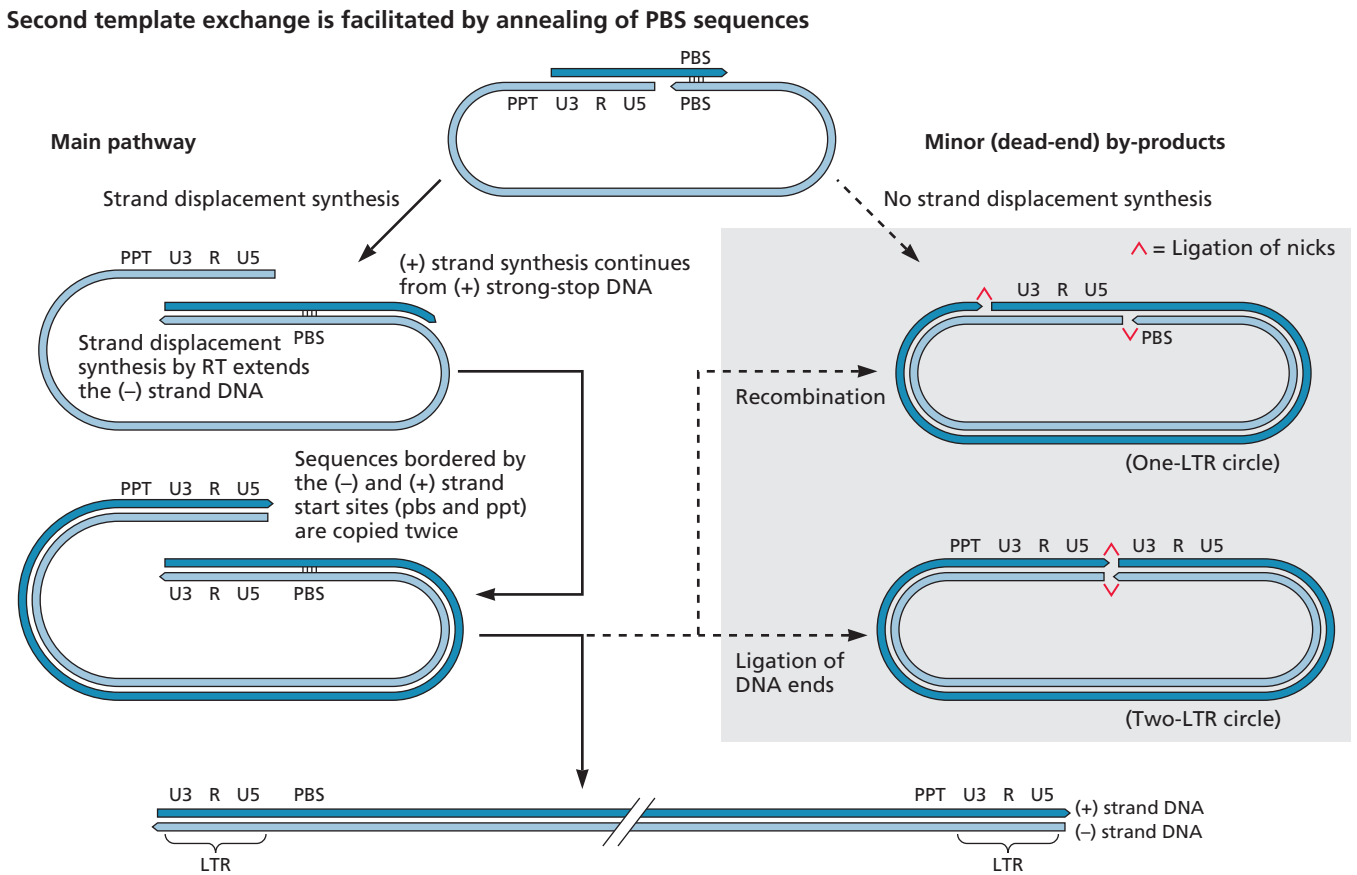


Figure 7.5 Retroviral reverse transcription: (+) strand DNA synthesis primed from ppt RNA.

Figure 7.6 Retroviral reverse transcription: the second template exchange and formation of the final linear DNA product. The second exchange is facilitated by annealing of PBS sequences in (+) and (−) strands of retroviral DNA. Formation of two minor (normally <1%), circular products is shown in the shaded box on the right. The smaller of the two circles contains only one LTR, and can arise either from a failure of strand displacement synthesis of RT or by recombination between the terminal LTR sequences in the linear molecule. The circle with two LTRs is presumed to arise by ligation of the ends of the linear viral DNA. Formation of this product requires a nuclear enzyme, DNA ligase, and it is easy to detect by PCR techniques. Consequently, the two-LTR circles are typically used as convenient markers for the transport of viral DNA into the nucleus.



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Small quantities of two circular DNA products are also invariably present in the nucleus. These are nonfunctional, dead-end products; their presumed origin is illustrated in Fig. 7.6 (right).

Retroviral reverse transcription has been called "destructive replication," as there is no net gain of genomes, but rather a substitution of one double-stranded DNA for two molecules of single, (+) strand RNA. However, by this rather intricate but elegant pathway, RT not only makes a linear DNA copy of the retroviral genome to be integrated, but also produces the LTRs that contain signals necessary for transcription of integrated DNA, which is called the **provirus**. The promoter in the upstream LTR is now in the appropriate location for synthesis of progeny RNA genomes and viral mRNAs by host cell RNA polymerase II (Chapter 8). Integration also ensures subsequent replication of the provirus via the host's DNA synthesis machinery as the cell divides.

Reverse transcription promotes recombination. The high rate of genetic recombination, a hallmark of retrovirus reproduction, is facilitated by the presence of two RNA templates within the reverse transcriptase complex. Although only one viral DNA molecule is normally produced by each infecting virion, recombination can occur during reverse transcription. The incorporation of two distinct RNA templates in a single virus particle can lead to new combinations of sequences (Box 7.4).

The above description of reverse transcription has been idealized for clarity. Analyses of reaction intermediates show that

BOX 7.4

WARNING

Retroviral recombination and the rise and fall of XMRV

Because a mutation in a human gene encoding a viral defense protein (RNase L) is a known risk factor for prostate cancer, a 2006 report of the isolation of a new retrovirus from tissue samples of individuals homozygous for this mutation attracted considerable attention. The excitement was compounded by a report in 2009 that the same virus could be isolated from the blood of patients suffering from chronic fatigue syndrome (CFS). While the association with CFS was controversial and never confirmed (indeed, it was later retracted), some desperate CFS patients were nevertheless treated with RT inhibitors.

The new virus was called XMRV (for xenotropic murine leukemia virus-related virus) because its sequence is closely related to well-known murine virus strains. Such strains are called xenotropic because they can infect foreign cells, such as human cells, in culture but are unable to reinfect mouse cells. The discovery was also noteworthy because XMRV is a gammaretrovirus, and this genus was not previously known to include human pathogens. Numerous investigators took up the study of this new virus, but the scientific literature was soon filled with contradictory reports concerning its association with prostate cancer, most based on the results from extremely sensitive PCR assays. Many investigators began to wonder if XMRV was indeed a human virus, and questioned its association with cancer.

These issues were addressed in a careful study, published in 2011, which showed that XMRV was derived from the recombination of two previously unknown defective murine endogenous retroviruses, and that the event probably occurred between 1993 and 1996 when a human tumor was implanted into nude mice, a process that is necessary to establish prostate cancer cell lines. These findings suggested that the reported PCR-based evidence of XMRV in clinical specimens could be explained by laboratory contamination. This explanation was supported by results from another team of investigators, which included some of the initial "discoverers" of XMRV, who established that the original archived prostate cancer tissue was indeed negative for XMRV, but the archival extracted RNA from the original study was positive for the viral genome. They also found that the source of XMRV contamination in the archival extracted RNA was the XMRV-infected cell line used in the laboratory. The contradictory results reported by numerous laboratories can be attributed therefore to the superb sensitivity of the PCR methods used to detect XMRV sequences and the ubiquitous presence of mouse DNA and/or sources of likely contamination.

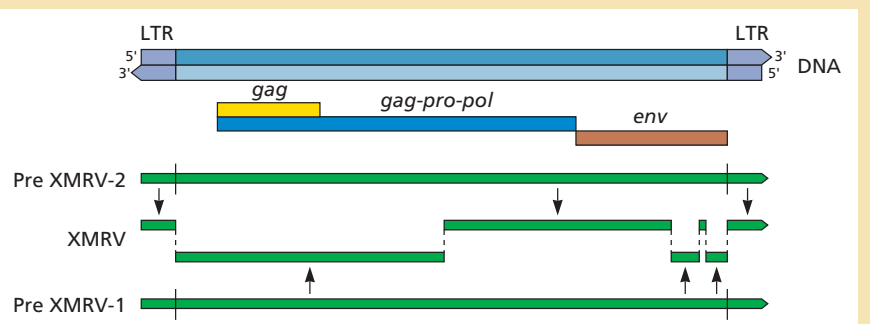
Lee D, Das Gupta J, Gaughan C, Steffan I, Tang N, Luk KC, Qiu X, Urisman A, Fischer N, Molinaro R, Broz M, Schochetman G, Klein EA, Ganem D, DeRisi JL, Simmons G, Hackett J, Jr, Silverman RH, Chiu CY. 2012. In-depth investigation of archival and prospectively collected samples reveals no evidence for XMRV infection in prostate cancer. *PLoS One* **7:**e44954. doi:10.1371/journal.pone.0044954.

Lombardi VC, Ruscetti FW, Das Gupta J, Pfost MA, Hagen KS, Peterson DL, Ruscetti SK, Bagni RK, Petrow-Sadowski C, Gold B, Dean M, Silverman RH, Mikovits JA. 2009. Detection of an infectious retrovirus, XMRV, in blood cells of patients with chronic fatigue syndrome. *Science* **326:**585–589.

Paprotka T, Delviks-Frankenberry KA, Cingoz O, Martinez A, Kung HJ, Tepper CG, Hu WS, Fivash MJ, Jr, Coffin JM, Pathak VK. 2011. Recombinant origin of the retrovirus XMRV. *Science* **333:**97–101.

Urisman A, Molinaro RJ, Fisher N, Plummer SJ, Casey G, Klein EA, Malathi K, Magi-Galluzzi C, Tubbs RR, Ganem D, Silverman RH, DeRisi JL. 2006. Identification of a novel gammaretrovirus in prostate tumors of patients homozygous for R462Q *RNASEL* variant. *PLoS Pathog* **2:**e25. doi:10.1371/journal.ppat.0020025.

Origin of XMRV. XMRV arose via recombination between two previously unknown defective murine endogenous retroviruses (arbitrarily called Pre XMRV-1 and -2), which included six separate crossover events. For discussion, see http://www.virology.ws/2011/05/31/xmrv-is-a-recombinant-virus-from-mice/.

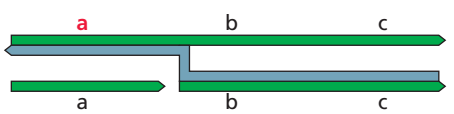


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RT pauses periodically during synthesis, presumably at some sequences, structures, or breaks that impede copying. If a break is encountered in one RNA template, synthesis can be completed by utilization of the second RNA genome. Such internal template exchanges (known to occur even in the absence of breaks) probably proceed at regions of homology via the same steps outlined for the first template exchange. Internal exchanges that take place during RNA-directed DNA synthesis are estimated to be the main source of genetic recombination, a mechanism known as **copy choice**. Exchange of single-stranded ends from one DNA template to another during (+) strand synthesis can also lead to recombination via a mechanism known as **strand displacement synthesis** (Fig. 7.7).

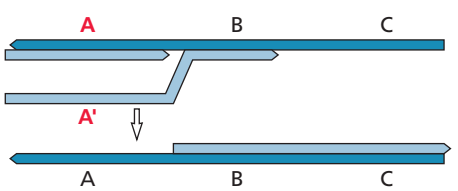
Figure 7.7 Two models for recombination during reverse transcription. Virtually all retroviral recombination occurs between coencapsidated genomes at the time of reverse transcription. The copy choice model **(A)** postulates a mechanism for genetic recombination during RNA-directed (−) strand DNA synthesis. This mechanism predicts that a homoduplex DNA product is formed as the recombined (−) strand of DNA is copied to form a (+) complementary strand. The strand displacement synthesis model **(B)** postulates a mechanism for genetic recombination that can occur when (+) strand DNA synthesis is initiated at internal sites on the (−) strand DNA template. Such internal initiations are known to arise frequently during reverse transcription by avian sarcoma/leukosis virus and human immunodeficiency virus RTs. Recombination can occur if (−) strand DNA has been synthesized from both RNA genomes in the particle. This mechanism can be distinguished from copy choice because a heteroduplex DNA product will be formed, i.e., A′/A, in which only the (+) strand (light blue in the figure) is a recombinant. The two mechanisms are not mutually exclusive, and while copy choice is most frequent, there is experimental support for both. Viral genetic markers, arbitrarily labeled *a*, *b*, and *c*, are indicated to illustrate recombination. While multiple crossovers are frequently observed, single recombination events are shown for simplicity, focusing on the hypothetical *a* allele, with the mutant form in red. For more details, see R. Katz and A. M. Skalka, *Annu Rev Genet* **24:**409–445, 1990.

A Copy Choice



(–) (first)-strand DNA synthesis starts on one genome and switches to the second at a break point, pause site, or random location

B Strand displacement synthesis



(+) (second)-strand DNA synthesis accompanied by strand displacement and assimilation of single-strand DNA tails onto DNA from the second genome (white arrow)

General Properties and Structure of Retroviral Reverse Transcriptases

Domain Structure and Variable Subunit Organization

The RTs of retroviruses are encoded in the *pol* genes. Despite the sequence homologies and similar organization of coding sequences, retroviral species-specific differences in proteolytic processing of the Gag-Pol polyprotein precursors leads to the inclusion of additional sequences or domains in the RTs. For example, the avian sarcoma/leukosis virus RT incudes a C-terminal integrase domain, and that of the prototype foamy virus includes an N-terminal protease domain (Fig. 7.8). Furthermore, although most retroviral RTs function as monomers, the enzymes of the avian sarcoma/leukosis and human immunodeficiency viruses function as heterodimers. It is difficult to gauge the significance of this structural diversity, which may simply be the result of different evolutionary histories.

Catalytic Properties

DNA polymerization is slow. The biochemical properties of retroviral RTs have been studied with enzymes purified from virus particles or synthesized in bacteria, using model templates and primers. Kinetic analyses have identified an ordered reaction pathway for DNA polymerization similar to that of other polymerases. Like cellular polymerases and nucleases, RTs require divalent cations as cofactors (most likely Mg^{2+} in the infected cell). The rate of elongation by RT on natural RNA templates *in vitro* is 1 to 1.5 nucleotides per s, approximately 1/10 the rate of other eukaryotic DNA polymerases. Assuming that DNA synthesis is initiated promptly upon viral entry, the long time period required to produce a complete copy of retroviral RNA after infection (~4 h for ~9,000 nucleotides) supports the view that reverse transcription is also a relatively slow process *in vivo*.

In reactions *in vitro*, the rate of dissociation of the enzyme from the template-primer increases considerably after addition of the first nucleotide, suggesting that initiation and elongation are distinct steps in reverse transcription, as is the case during DNA synthesis by DNA-dependent DNA polymerases. In contrast to most other DNA polymerases, retroviral RTs dissociate from their template-primers frequently *in vitro*, a property described as "poor processivity." This may not be a limitation *in vivo*, where genomic RNA is reverse transcribed within the confines of a subviral particle.

Fidelity is low. Retroviral genomes, like those of other RNA viruses, accumulate mutations at much higher rates than do cellular genomes. RTs not only are error prone, but also lack an endonuclease capable of excising mispaired nucleotides. These properties contribute to the high mutation rate of retroviruses in infected cells.

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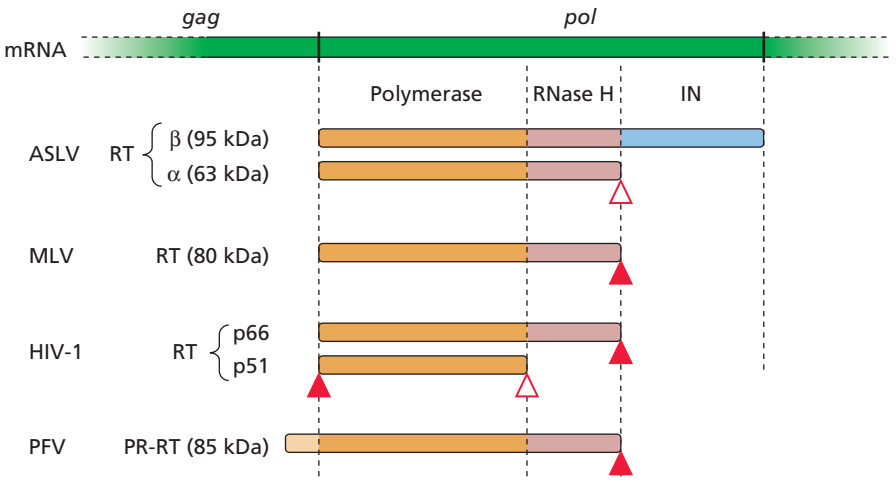
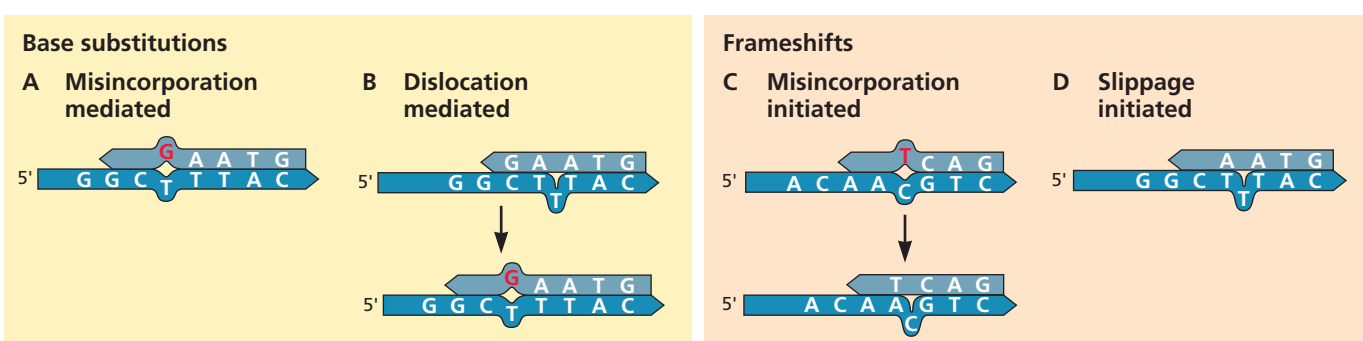


Figure 7.8 Domain and subunit relationships of the RTs of different retroviruses. The organization of open reading frames in the mRNAs of all but the spumavirus prototype foamy virus (PFV) is indicated at the top. PFV RT is expressed from a spliced *pro-pol* mRNA. Protein products (not to scale) are shown below, with arrows pointing to the sites of proteolytic processing that produce the diversity of RT subunit composition. Open red arrows indicate partial (asymmetric) processing, and solid red arrows indicate complete processing. ASLV, the alpharetrovirus avian sarcoma/leukosis virus; MLV, the gammaretrovirus murine leukemia virus; HIV-1, the lentivirus human immunodeficiency virus type 1; PFV, the spumavirus, prototype foamy virus.

Errors introduced by purified enzymes include not only misincorporations but also rearrangements such as deletions and additions (Fig. 7.9). Misincorporations by human immunodeficiency virus type 1 RT can occur as frequently as 1 per 70 copies at some template positions, and as infrequently as 1 per 10^6 copies at others. Both deletions and insertions are also known to occur during reverse transcription within an infected cell, apparently because template exchanges can take place within short sequence repeats (e.g., 4 or 5 nucleotides) that are not in homologous locations on the two RNA templates. Many types of genetic experiments have been conducted in attempts to determine the error rates of RTs in a single infectious cycle within a cell (see Volume II, Box 10.2). The general conclusion is that such rates are also quite high, with reported misincorporations in the range of 1 per 10^4 to 1 per 10^6 nucleotides polymerized, in contrast to 1 per 10^7 to 1 per 10^{11} for cellular DNA replication. As retroviral genomes are $\sim 10^4$ nucleotides in length, ~ 1 lesion per retroviral genome per replication cycle can be expected, simply by misincorporation. This high mutation rate explains, in part, the difficulties inherent in treating AIDS patients with inhibitors of RT or other viral proteins; a large population of mutant viruses preexist in every chronically infected individual, some encoding drug-resistant proteins. These mutants can propagate in the presence of a drug and quickly comprise the bulk of the population (see Volume II, Chapters 7 and 9).

Figure 7.9 Mutational intermediates for base substitution and frameshift errors. Several unique activities of RTs are likely to contribute to their high error rates. The avian sarcoma/ leukosis and human immunodeficiency virus enzymes are both proficient at extending mismatched terminal base pairs, such as those that result from nontemplated addition **(A)**. This process facilitates incorporation of mismatched (red) nucleotides into the RT product. A certain type of slippage within homopolymeric runs in which one or more bases are extruded on the template strand can also happen during reverse transcription **(B, C)**; mispairing occurs after the next deoxyribonucleotide is added and the product strands attempt to realign with the template. Deletions can also be produced by this mechanism **(D)**. Slippage and dislocations are assumed to be mediated by looping out of nucleotides in the template. Only single-nucleotide dislocations are shown here, but large dislocations leading to deletions are also possible. From K. Bebenek and T. A. Kunkel, p 85–102, *in* A. M. Skalka and S. P. Goff (ed), *Reverse Transcriptase* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1993), with permission.



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RNase H. The RNase H of RT also requires a divalent cation, most likely Mg^{2+}, which is abundant in cells. Like other RNase H enzymes, present in all bacterial, archaeal, and eukaryotic cells, the RNase H of RT digests only RNA that is annealed to DNA. Three activities of the RNase H of human immunodeficiency virus type 1 and murine leukemia virus RTs have been distinguished: endonucleases that are directed to the ends of hybrid duplexes in which the 3′ terminus of the DNA is either extended or recessed, and an internal endonuclease that is not end directed. A loose consensus site for all three activities has also been observed.

Structure of RT

Although RTs from avian and murine retroviruses have been studied extensively *in vitro*, the importance of human immunodeficiency virus type 1 RT as a target for drugs to treat AIDS has focused intense interest and resources on this enzyme. As a consequence, we know more about this RT than any other. Three aspartic acid residues in the polymerase region are included in conserved motifs in a large number of polymerases (see Fig. 6.4). These residues coordinate the required metal ions and contribute to binding deoxyribonucleoside triphosphates and subsequent catalysis.

The primary sequence of the smaller (p51) subunit of human immunodeficiency virus RT is the same as that of the larger (p66), minus the RNase H domain (Fig. 7.8). Consequently, it was somewhat surprising when the first crystallographic studies of this RT revealed **structural asymmetry** of these subunits in the heterodimer. Not only are analogous portions arranged quite differently (Fig. 7.10, bottom); they also perform different functions in the enzyme. All catalytic functions are contributed by p66. Nevertheless, p51 is required for enzyme activity and may perform a unique function in the RT heterodimer, that of binding the tRNA primer. In the heterodimer, these two subunits are nestled on top of each other, with an extensive interface (Fig. 7.10, top). The p66 polymerase domain is divided into three subdomains denoted "finger," "palm," and "thumb" by analogy to the convention used for describing the topology of the *Escherichia coli* DNA polymerase I Klenow fragment, described in Chapter 6 (Fig. 6.4). A fourth subdomain called the "connection" lies between the remainder of the polymerase and the RNase H domain. This subdomain contains the major contacts between the two subunits. The extended thumb of p51 contacts the RNase H domain of p66, an interaction that appears to be required for RNase H activity. Not only are human immunodeficiency virus type 1 RT and *E. coli* DNA polymerase similar topologically, but this retroviral RT can actually substitute for the bacterial enzyme in *E. coli* cells that lack a functional DNA polymerase I.

Highly dynamic interactions between template-primer, dNTP substrates, and RT must occur during reverse transcription. A schematic rendition (Fig. 7.11) of an RNA-DNA heteroduplex bound in the cleft region of the human immunodeficiency virus type 1 RT illustrates how the RNA strand is fed into the polymerizing site. The substrates are bound in a defined order: the template-primer first, and then the complementary dNTP to be added. The dNTP substrate interacts directly with two fingertip residues, and this contact induces closure of the binding pocket. This conformational change facilitates attack of the 3′-OH of the primer on the α-phosphate of the dNTP. Release of the diphosphate product and reopening of the fingers allows the template-primer to **translocate** by one nucleotide in preparation for the binding and addition of the next dNTP. As a new DNA chain is synthesized by addition of deoxyribonucleotides to the primer, the template RNA is moved in stepwise fashion toward the RNase H.

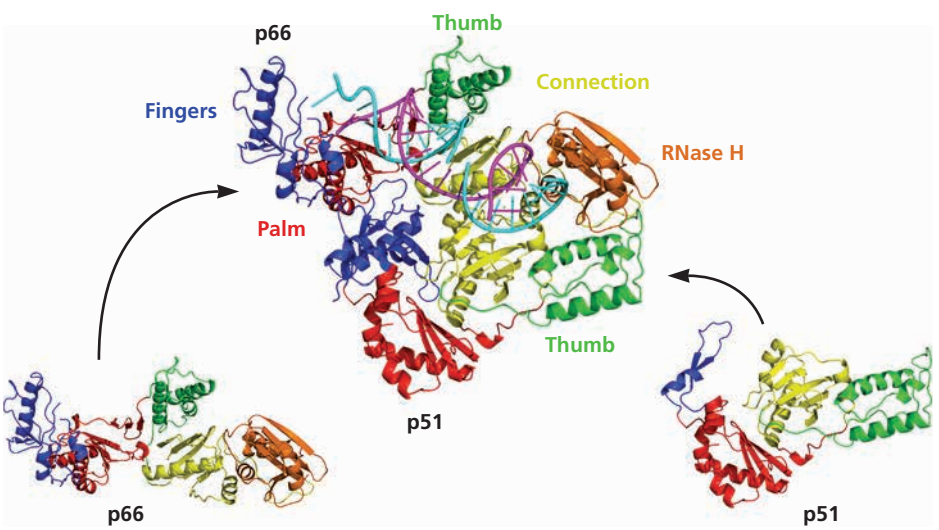


Figure 7.10 Ribbon representation of human immunodeficiency virus type 1 RT in complex with a model RNA template-DNA primer. The p61-p51 heterodimer is shown at the top, with subdomains in the catalytic subunit, p66, identified. The light blue RNA strand in the template-primer model is nicked; the DNA strand is shown in purple. The p61 and p51 subunits are shown separated at the bottom to emphasize the distinct organization of subdomains in each. For additional details, see M. Lapkouski et al., *Nat Struct Mol Biol* **20:**230–236, 2013. Courtesy of S. F. Le Grice, National Cancer Institute, Frederick, MD.

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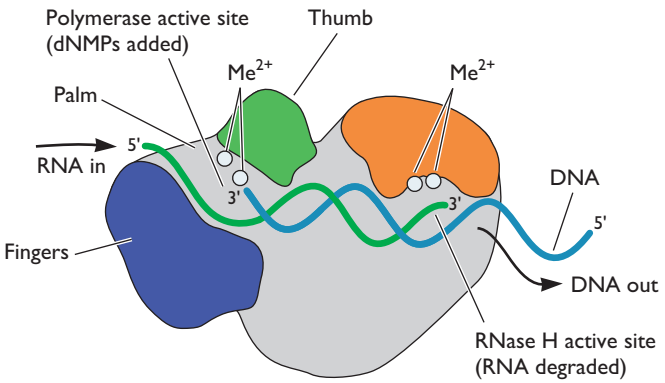


Figure 7.11 Model for a DNA-RNA hybrid bound to human immunodeficiency virus type 1 RT. The RNA template-DNA product duplex is shown lying in a cleft. The polymerase active site and the putative RNase H active site are indicated. Me^{2+} signifies a divalent metal ion. As illustrated, the RNA template enters at the polymerizing site and is degraded at the RNase H active site after being copied into DNA, which then exits from the RNase H site. Adapted from L. A. Kohlstaedt et al., p 223–250, *in* A. M. Skalka and S. P. Goff (ed), *Reverse Transcriptase* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1993), with permission.

Remarkable dynamic capabilities of human immunodeficiency virus RT have been revealed in studies of the purified enzyme. The RNase H and polymerase sites do not act simultaneously; conformational changes are required to optimize each function. Furthermore, while synthesizing DNA from an RNA template, the enzyme is able to bind the primer in a position poised for polymerization or one that is "flipped" 180°; sliding and flipping can occur without RT disengaging from the DNA (Box 7.5). Such molecular contortions may explain the observation that polymerase-coupled RNase H activity results in cleavage of the RNA template about once for every 50 to 100 dNTPs polymerized.

Production of two protein subunits that possess identical amino acid sequences, but have structures and functions that are distinct, is an excellent example of viral genetic economy. The C terminus of the p51 subunit, at the end of the connection domain, is buried within the N-terminal β-sheet of the RNase H domain of p66. This organization suggests a model for proteolytic processing in which a p66 homodimer intermediate is arranged asymmetrically and the RNase H domain of the subunit destined to become p51 is unfolded. Such an

BOX 7.5

DISCUSSION

Reverse transcriptase can reverse direction

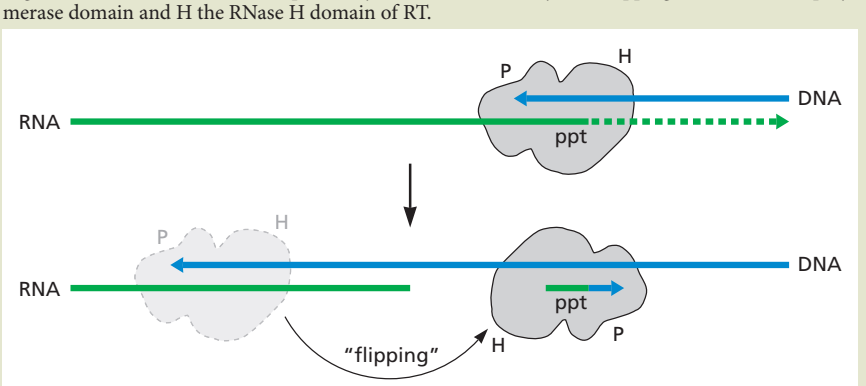
The exchange of one template for another to be copied by either DNA or RNA polymerases is sometimes referred to as enzyme "jumping." This inappropriate term comes from a too literal reading of simplified illustrations of the process, in which the templates to be exchanged may be opposite ends of the nucleic acid or different nucleic acid molecules. In actuality, such enzyme movement is quite improbable, and use of this terminology can cloud thinking about these processes. In almost all cases, these polymerases are components of large assemblies with architecture designed to bring different parts of the template, or different templates, close to each other. Consequently, it is likely that most of the "movement" is made by the flexible nucleic acid templates. Nevertheless, some dynamic changes in protein conformation must occur to accommodate template exchanges, as implied by another dynamic property ascribed to RT, called "flipping."

Application of a single-molecule assay to measure enzyme-substrate interactions has shown that RT of the human immunodeficiency virus type 1 can switch rapidly from one orientation to another on a single primer-template. The assay made use of surface-immobilized template-primer oligonucleotide substrate molecules: the protein and a nucleic acid end were labeled with donor and acceptor fluorophores. The position of the enzyme relative to the substrate was then measured by fluorescence resonance energy transfer. The results showed that a single RT heteroduplex can switch from one orientation to another without dissociating from the substrate (see figure).

Abbondanzieri EA, Bokinsky G, Rausch JW, Zhang JX, Le Grice SF, Zhuang X. 2008. Dynamic binding orientations direct activity of HIV reverse transcriptase. *Nature* **453:**184–189.

Liu S, Abbondanzieri EA, Rausch JW, Le Grice SF, Zhuang X. 2008. Slide into action: dynamic shuttling of HIV reverse transcriptase on nucleic acid substrates. *Science* **322:**1092–1097.

RT dynamics. (Top) During reverse transcription, DNA is synthesized and the RNA template is degraded by RT. (Bottom) RT can adopt an alternative orientation and switch from RNA-directed to DNA-directed synthesis without disengaging from the substrate. Polymerase-coupled RNase H degradation of the viral RNA template may also be facilitated by such flipping. P indicates the polymerase domain and H the RNase H domain of RT.



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arrangement could account for asymmetric cleavage by the viral protease.

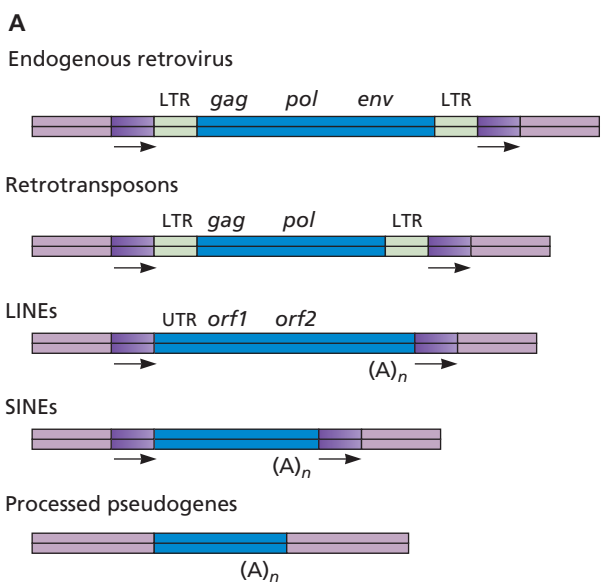
Other Examples of Reverse Transcription

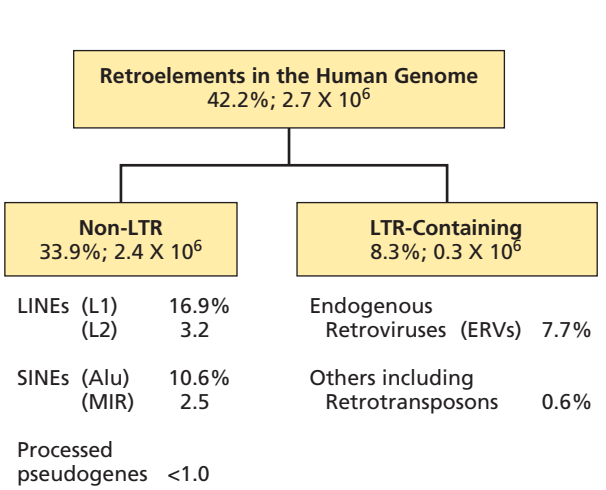
When it was first discovered, RT was thought to be a peculiarity of retroviruses. We now know that other animal viruses, hepadnaviruses, and some plant viruses, such as the caulimoviruses, synthesize genomic DNA via an RNA intermediate. All are therefore classified as **retroid viruses**. In fact, the discovery of RT activity in some strains of myxobacteria and *E. coli* places the evolutionary origin of this enzyme before the separation of bacteria and eukaryotes. It is now widely held that the biological world was initially based on RNA molecules, functioning as both catalysts and genomes. Consequently, the development of the modern (DNA) stage of evolution would have required an RT activity. If so, the retroid viruses may also be viewed as living fossils, shining the first dim light into an ancient evolutionary passageway from the primordial world (Volume II, Chapter 10).

During the retroviral life cycle (Appendix, Fig. 30), the double-stranded DNA molecule synthesized by reverse transcription is integrated into the genomes of infected animal cells by the retroviral integrase. In some cases, retroviral DNA may be integrated into the DNA of germ line cells in a host organism. These integrated DNAs are then passed on to future generations in Mendelian fashion as **endogenous proviruses**. Such proviruses are often replication defective, a property that may facilitate coexistence with their hosts. Some 8% of the human genome comprises endogenous proviruses (Fig. 7.12). While many of these proviruses were established in the primate lineage millions of years ago, a present-day example of this phenomenon can be observed in another mammalian species (Box 7.6).

Since the discovery of RT in retroviruses, additional RT-related sequences have been found in **retroelements**,

Figure 7.12 Retroelements resident in eukaryotic genomes and their representation in the human genome. (A) Gene arrangements of retroelements in eukaryotic genomes. The genetic content and organization of endogenous proviruses and LTR-containing retrotransposons are similar (Appendix, Fig. 29), but most retrotransposons lack an *env* gene. LINEs (for long interspersed nuclear elements) are a distinct class of retrotranposon; they lack LTRs but contain untranslated sequences (UTRs) that include an internal promoter for transcription by cellular RNA polymerase II. The LINE *orf1* gene encodes a protein chaperone, and *orf2* encodes a protein with endonuclease and RT activities, which catalyzes reverse transcription of mRNA intermediates and integration of the DNA product. As with the other retrotransposons, the presence of flanking duplications of cellular DNA (represented by arrows below the maps) is a hallmark of transposition by LINEs. SINEs (for short interspersed nuclear elements) are classified as retroposons. They have no known open reading frames, and RNA from these sequences is retrotransposed in *trans* by the RTs of active LINEs. Processed pseudogenes comprise a less abundant group of such non-autonomously transposed retroelements. They have no introns (hence "processed"), and their sequences are related to exons in functional genes that map elsewhere in the genome. Processed pseudogenes include long, A-rich stretches. However, they contain no promoter for transcription and no RT, and are thought to arise from reverse transcription of cellular mRNAs catalyzed by the RTs of retroviruses or nondefective LINEs. Genetic maps of the retroelements are not to scale. **(B)** Retroelements in the human genome. The percentage of the human genome that each element represents and the total number of retroelements in each major class are indicated in the boxes. The percentage of the human genome that is represented by each type of element is shown beneath the boxes. Data are excerpted from N. Bannert and R. Kurth, *Proc Natl Acad Sci U S A* **101:**14572–14579, 2004.





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BOX 7.6

BACKGROUND

Present-day establishment of an endogenous retrovirus

Endogenous retroviruses are common in the genomes of humans and other mammals; most are ancient, defective relics of germ line infections that occurred millions of years ago. The koala retrovirus recently isolated from wild and captive animals in Australia, and in koala populations in zoos in other countries, appears to be a contemporary exception.

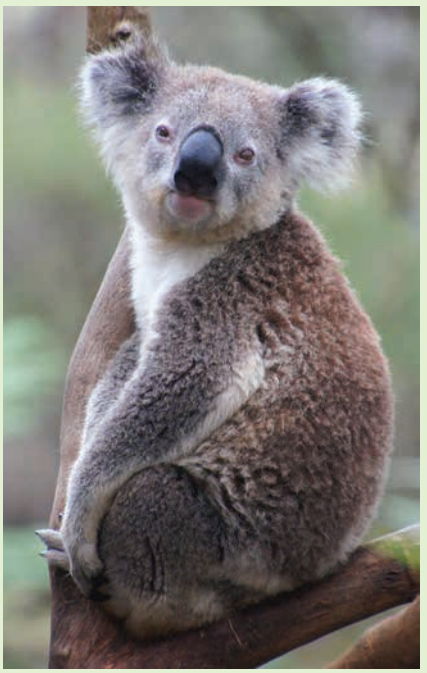
The koala virus is a gammaretrovirus, related to the gibbon ape leukemia and feline leukemia viruses. The first subtype to be isolated, KoRV-A, uses the same cell surface receptor for entry as the gibbon ape leukemia virus (the sodium-dependent phosphate transporter PiT1) and is thought to have been acquired by koalas as a result of cross-species transmission from rodents or bats. This viral subtype is widely distributed among koalas in northern Australia and, based on analysis of museum specimens, was circulating in this population more than 150 years ago. KoRV-A proviruses have been found in multiple copies in the genomes of every cell (including germ cells) in koalas in northern Australia. A smaller fraction of koalas in southern Australia were found to carry the virus, but genomic studies have identified several independent germ line insertions in animals from this region as well. In contrast, KoRV-A infection has been detected in only ~14% of the animals on an island off the southern coast of Australia to which koalas were introduced in the early 1900s. It was suggested, therefore, that both exogenous infection and the establishment of endogenous KoRV-A proviruses are probably still occurring in this host species as the virus spreads southward from a focus in northern Australia.

Denner J, Young PR. 2013. Koala retroviruses: characterization and impact on the life of koalas. *Retrovirology* **10:**108. doi:10.1186/1742-4690-10-108.

Ishida Y, Zhao K, Greenwood AD, Roca AL. 2015. Proliferation of endogenous retroviruses in the early stages of a host germ line invasion. *Mol Biol Evol* **32:**109–120.

Tarlinton RE, Meers J, Young PR. 2006. Retroviral invasion of the koala genome. *Nature* **442:**79–81.

Xu W, Stadler CK, Gorman K, Jensen N, Kim D, Zheng H, Tang S, Switzer WM, Pye GW, Eiden MV. 2013. An exogenous retrovirus isolated from koalas with malignant neoplasias in a US zoo. *Proc Natl Acad Sci U S A* **110:**11547–11552.



sequences that can be propagated from one locus to others in a cellular genome via reverse transcription (Fig. 7.12). One class of such transposable elements, the LTR-containing **retrotransposons**, is widely dispersed in nature. The gene contents and arrangements of these retrotransposons are similar to those of retroviruses. Most are distinguished from retroviruses by lack of an extracellular phase. They have no *env* gene, and hence the virus-like particles formed within the cell are not infectious. As with retroviruses, such particles contain RNA copies of the integrated sequences, and element-specific RT and integrase. DNA is synthesized and inserted into additional loci following entry into the nucleus of the same cell in which the particles are produced. However, members of one genus in the family *Metaviridae* do include open reading frames corresponding to *env*, and at least one of these elements, the *Drosophila* gypsy, produces infectious, extracellular particles. Phylogenetic comparisons of LTR-retrotransposons from invertebrates provide evidence that several have acquired *env* sequences via genetic recombination with both RNA and DNA viruses. These results support the view that LTR-containing retrotransposons were retroviral progenitors. An alternative possibility, but with less phylogenetic support, is that they are degenerate forms of retroviruses.

While there are distinct structural and biochemical differences among the RTs of retroviruses and LTR-containing retrotransposons, X-ray crystallographic comparisons have revealed striking topological similarities. For example, in the monomeric RT of a murine leukemia-related virus, the catalytic subunits of the heterodimeric RT of human immunodeficiency virus (p66), and the homodimeric RT of the yeast retrotransposon Ty3 (subunit A), the fingers-palm-thumb subdomains and the position of bound nucleic acids are almost superimposable (Fig. 7.13). Topologies of the two protein dimers are also superimposable despite the fact that the arrangements of their subdomains are quite distinct, having been modified and repositioned through evolution to provide analogous functions.

A second class of retrotransposons that is dispersed widely in the human genome is called **LINEs** (for long interspersed nuclear elements). LINEs can be up to 6 kbp in length; they lack LTRs, but contain internal promoters for transcription by cellular RNA polymerase II. All have A-rich stretches at one terminus, presumed to be derived by reverse transcription of the 3′-poly(A) tails in their RNA intermediates. Most LINEs encode RT-related sequences, but these often contain large deletions and translational stop codons; such elements cannot mediate their own retrotransposition and are considered to be "dead." However, ~80 to 100 human LINEs encode functional RTs, and a number of disease-causing genetic lesions resulting from

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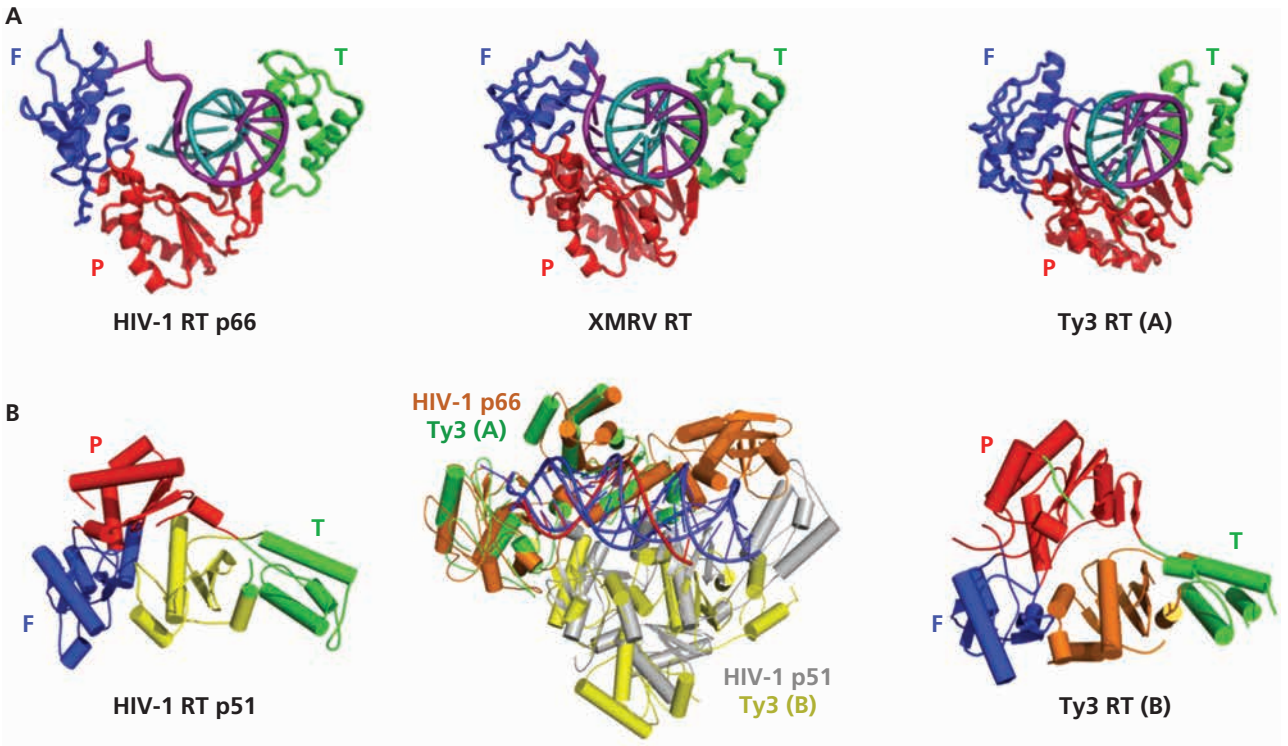


Figure 7.13 Comparison of the structures of three RTs. (A) The DNA polymerase domains of the lentivirus human immunodeficiency virus type 1 (HIV-1 p66), a gammaretrovirus related to murine leukemia virus (XMRV), and the yeast LTR-retrotransposon Ty3 (subunit A) RTs. Fingers, palm, and thumb subdomains are designated F, P, and T, respectively. RNA is shown in magenta and DNA in blue. **(B)** Architectures of the noncatalytic subunits of the dimeric RTs: HIV-1 p51 and Ty3 subunit B. Both subunits contain F, P, and T subdomains in analogous positions. Unexpectedly, the p51 connection and Ty3 (subunit B) RNase H domain, shown in yellow and brown, respectively, are also in similar positions. Superposition of the asymmetric p66-p51 HIV-1 RT heterodimer and the symmetric Ty3 (A)-(B) homodimer, together with bound nucleic acids (blue and orange strands), is shown in the center. HIV RT subunits are in orange and gray and Ty3 subunits in green and yellow. For additional details, see E. Nowak et al., *Nat Struct Mol Biol* **21:**389–396, 2014. Illustration prepared by Jason Rausch and Stuart Le Grice, National Cancer Institute, Frederick, MD, and Marcin Nowotny, International Institute of Molecular and Cell Biology, Poland.

LINE-mediated retrotransposition events have been documented. Reverse transcription by LINE RTs accounts for the wide distribution of genetic elements that lack this enzyme, including short interspersed nuclear elements (**SINEs**) and processed pseudogenes that are related to exons in functional genes but map elsewhere in the genome. Some 40% of the human genome is now known to comprise retroelements (Fig. 7.12).

Retroviral DNA Integration Is a Unique Process

The **integrase (IN)** of retroviruses and the related retrotransposons catalyzes specific and efficient insertion of the DNA product of RT into host cell DNA. This activity is unique in the eukaryotic virus world. Establishment of an integrated copy of the genome is a critical step in the life cycle of retroviruses, as this reaction ensures stable association of viral DNA with the host cell genome. The integrated **proviral DNA** is transcribed by cellular RNA polymerase II to produce the viral RNA genome and the mRNAs required to complete the infectious cycle.

IN is encoded in the 3′ region of the retroviral *pol* gene (Fig. 7.8), and the mature protein is produced by viral protease (PR)-mediated processing of the Gag-Pol polyprotein precursor. During progeny virus assembly, all three viral enzymes (PR, RT, and IN) are incorporated into the viral capsid. Virus particles contain equimolar quantities of RT and IN (some 50 to 100 molecules per particle). The viral DNA product of RT is the direct substrate for IN, and genetic and biochemical studies indicate that these enzymes function in concert within infecting particles. As already noted, IN sequences actually are present in one of the subunits of avian sarcoma/leukosis virus RT, and gentle extraction of murine leukemia virus particles yields RT-IN complexes. However, as with RT, virtually nothing is known about the molecular organization of IN within virus particles.

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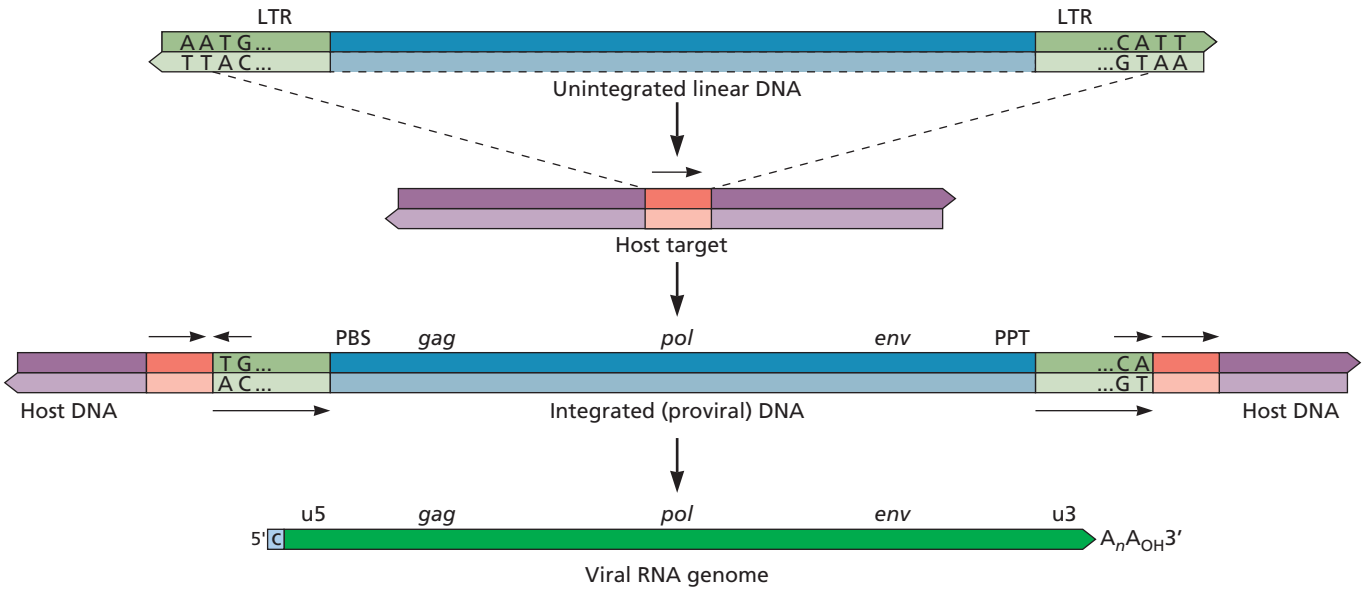


Figure 7.14 Characteristic features of retroviral integration. Unintegrated linear DNA of the avian retrovirus avian sarcoma/leukosis virus (top) after reverse transcription has produced blunt-ended LTRs (Fig. 7.6). The dashes under the bottom (+) strand indicate that this strand may include discontinuities, whereas the top (−) strand must be continuous (Fig. 7.7). Two base pairs (AA·TT) are lost from both termini upon completion of the integration process, and a 6-bp "target site" in host DNA (pink, indicated by an arrow) is duplicated on either side of the proviral DNA. The integrated proviral DNA (middle) includes short, imperfect inverted repeats at its termini, which end with the conserved 5′-TG...CA-3′ sequence; these repeats are embedded in the LTR, which is itself a direct repeat. The gene order is identical in unintegrated and proviral DNA, and is colinear with that in the viral RNA genome (bottom), for which a provirus serves as a template (described more fully in Chapter 8).

The first insights into the mechanism of the integration process came in the early 1980s, when it was established that proviral DNA is flanked by LTRs and the coding sequences are colinear in the unintegrated viral DNA and the RNA genome (Fig. 7.14). Nucleotide sequencing of cloned retroviral DNAs and host-virus DNA junctions revealed several unique features of the process. Both viral and cellular DNAs undergo characteristic changes. Viral DNA is cropped, usually by 2 bp from each end, and a short duplication of host DNA flanks the provirus on either end. Finally, the proviral ends of all retroviruses include the same dinucleotide. This dinucleotide is often embedded in an extended, imperfect inverted repeat that can be as long as 20 bp for some viral genomes. The fact that the length of the host cell DNA duplication is characteristic of the virus provided the first clue that a viral protein must play a critical role in the integration process.

The inverted terminal repeat, conserved terminal dinucleotide sequence, and flanking direct repeats of host DNA are strikingly reminiscent of features observed earlier in a number of bacterial transposons and the *E. coli* bacteriophage Mu (for "mutator"). Homologies to the predicted amino acid sequences of a portion of the retroviral IN were also found in the transposases of certain bacterial transposable elements such as Tn*5*. This observation suggested that, like RT, IN probably evolved before the divergence of bacteria and eukaryotes. These similarities predicted what is now known to be a common mechanism for retroviral DNA integration and DNA transposition.

The Pathway of Integration: Integrase-Catalyzed Steps

A generally accepted model for the IN-catalyzed reactions has been developed from the results of many different types of experiment, including studies of infected cells and reconstituted systems (Box 7.7). IN functions as a multimer, and two reactions occur in biochemically and temporally distinct steps (Fig. 7.15). In the first step, nucleotides (usually two) are removed from each 3′ end of the viral DNA. This "processing" step requires a virus-specific nucleotide sequence and duplex DNA ends. As this reaction can only occur when RT has completed synthesis of the viral DNA ends, the probability that defective molecules with imperfect ends will be integrated is limited. It has been shown that processing can take place in the cytoplasm of an infected cell before viral DNA enters the nucleus, within a subviral structure commonly referred to as the **preintegration complex** (described below). Although there is strong genetic evidence for sequence specificity for processing, only limited sequence-specific binding

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BOX 7.7

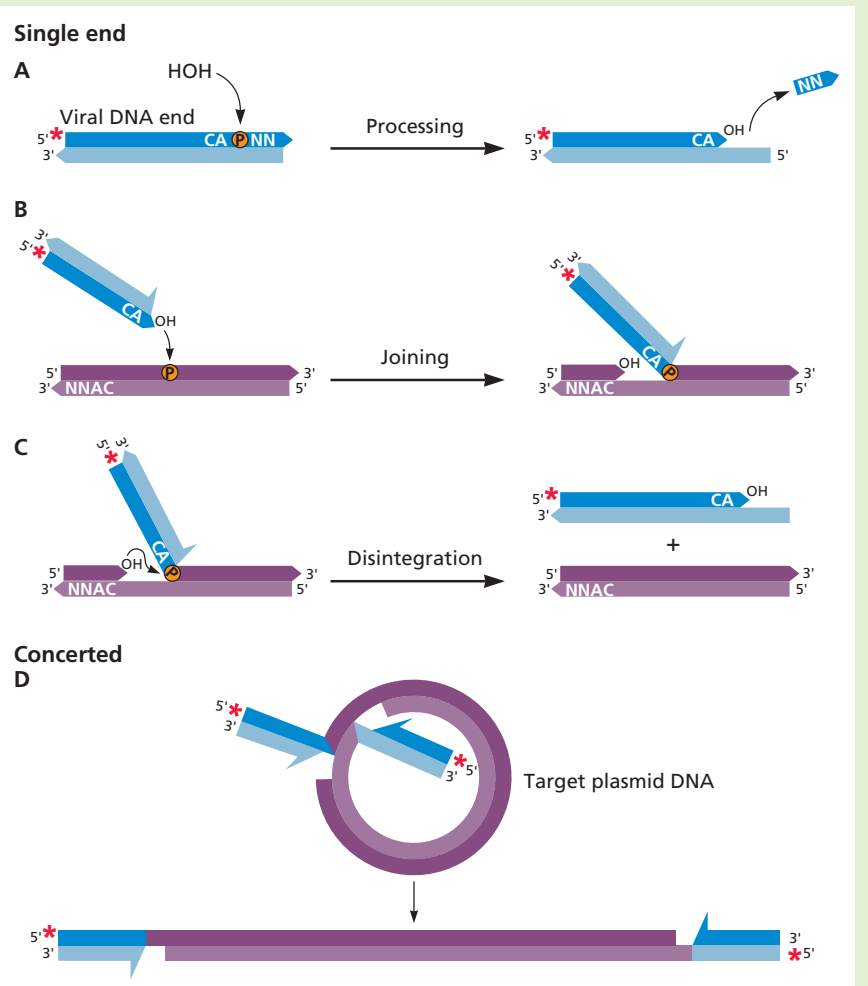
BACKGROUND

Model in vitro *reactions elucidate catalytic mechanisms*

The development of simple *in vitro* assays for the processing and joining steps catalyzed by IN marked an important turning point for investigation of the biochemistry of these reactions. With such assays, it was discovered that the retroviral IN protein is both necessary and sufficient for catalysis; that no exogenous source of energy (ATP or an ATP-generating system) is needed; and that the only required cofactor is a divalent cation, Mn^{2+} or Mg^{2+}. Use of simple substrates with purified IN protein helped to delineate the sequence and structural requirements for DNA recognition. Such *in vitro* assays also formed the basis for drug screening, enabling development of FDA-approved IN inhibitors for the treatment of AIDS.

In the simplest assays, substrates comprise short duplex DNAs (ca. 25 bp), with sequences corresponding to one retroviral DNA terminus, with labeled terminal nucleotides (red asterisk in the figure). IN can also catalyze an apparent reversal of this joining reaction *in vitro*, which has been called disintegration. While the processing, joining, and disintegration reactions produce different products, their underlying chemistry is the same: all comprise a nucleophilic attack on a phosphorus atom by the oxygen in an OH group, and result in cleavage of a phosphodiester bond in the DNA backbone. In **processing (panel A of the figure),** the -OH comes from a water molecule. In **joining (B),** the -OH is derived from the processed 3′ end of the viral DNA, and the result is a direct transesterification. In **disintegration (C),** also a direct transesterification, a 3′-OH end in the interrupted duplex attacks an adjacent phosphorus atom, forming a new phosphodiester bond and releasing the overlapping DNA. The products of all these reactions can be distinguished by gel electrophoresis.

Although assays with short, single-viral-end model substrates have been invaluable in elucidation of the catalytic mechanisms of IN, they are limited in that the major products represent "half reactions" in which only one viral end is processed and joined to a target. Subsequently, conditions for efficient, **concerted processing and joining (D)** of two viral DNA ends to a target DNA were described, with a variety of specially designed "miniviral" model DNA substrates. After preincubation, excess plasmid DNA is added as target, and the concerted joining of two donor fragments produces a linear DNA product.



of purified IN protein to retroviral DNA has been detected in reconstituted systems. It seems likely, therefore, that some structural features or interactions among components within the preintegration complex help to place IN at its site of action near the viral DNA termini. The second step catalyzed by IN is a concerted cleavage and ligation reaction in which the two newly processed 3′ viral DNA ends are joined to staggered (4- to 6-bp) phosphates at the target site in host DNA (Fig. 7.15). The product of the joining step is a **gapped intermediate** in which the 5′-PO_4 ends of the viral DNA are not linked to the 3′-OH ends of host DNA.

Host Proteins Are Recruited for Repair of the Integration Intermediate

Retroviral DNA integration creates a discontinuity in the host cell chromatin, and repair of this damage is required

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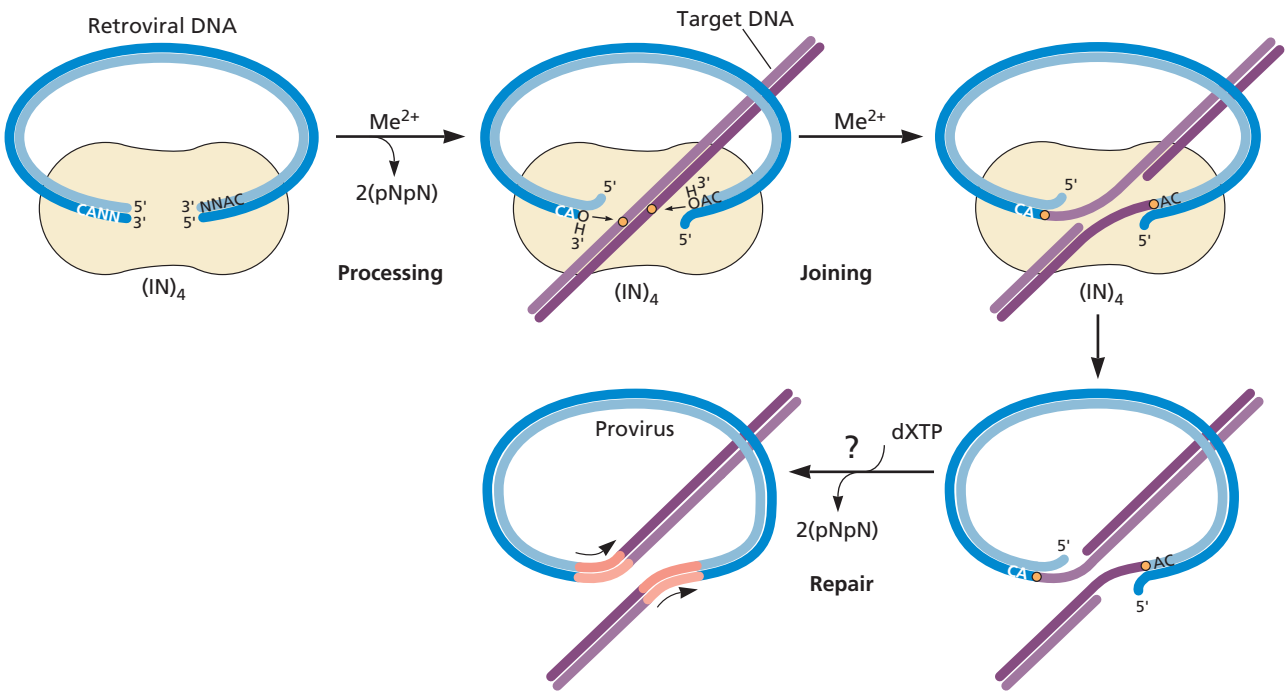


Figure 7.15 Three steps in the retroviral DNA integration process. Endonucleolytic nicking adjacent to the conserved dinucleotide near each DNA end results in the removal of a terminal dinucleotide, and formation of a new, recessed CA_{OH}-3′ end that will be joined to target DNA in the second step of the IN-catalyzed reaction. Both processing and joining reactions require a divalent metal, Mg^{2+} or Mn^{2+}. The viral DNA ends are bound by a tetramer of IN protein, $(IN)_4$, and the complex is called an intasome. Results of site-directed mutagenesis of viral DNA ends established that the conserved CA_{OH}-3′ dinucleotide is essential for correct and efficient integration. The small gold circles represent the phosphodiester bonds cleaved and re-formed in the joining reaction. The final step in the integration process is a host cell-mediated repair process.

to complete the integration process (Fig. 7.15). As with double-strand breaks produced by ionizing radiation or genotoxic drugs, retroviral DNA integration promotes recruitment of proteins of the DNA damage-sensing pathways. Components of the nonhomologous end-joining DNA repair pathway (DNA-dependent protein kinase, ligase IV, and Xrcc4) are required for postintegration repair. Retroviral DNA integration can trigger either cell cycle arrest or programmed cell death in cells that are defective in any of these proteins. It is likely that other host proteins play a role in both postintegration repair and reconstitution of chromatin structure at the site of integration, among them cellular DNA polymerases to fill any gaps, and histones and chromatin remodeling proteins to position nucleosomes on the proviral DNA.

Rapidly sedimenting preintegration complexes have been isolated from the cytoplasm of cells infected with several retroviruses. These nucleoprotein assemblies contain IN and viral DNA in a form that can be joined to exogenously provided plasmid or bacteriophage DNA. Such *ex vivo* reactions exhibit all the features expected for products of authentic integration. The mechanisms by which the preintegration complexes of different retroviruses gain access to the nucleus are likely to vary, but the details are still unclear (see Chapter 5).

Multiple Parameters Affect Selection of Host DNA Target Sites

DNA sequence features. A preference for retroviral integration into DNA sequences that are intrinsically bent, or underwound as a consequence of being wrapped around a nucleosome, was established in early analyses, but only limited sequence specificity was detected. Subsequently, advances in bioinformatics and high-throughput sequencing methodologies provided a wealth of information concerning the selection of integration sites. Weak consensus sequences for host target sites have been identified by a number of investigators, who, collectively, have mapped thousands of integration sites in human and other cell lines. As illustrated for the two cases in Fig. 7.16, the preferred sequence patterns for different retroviral genera are distinct. All target sequences studied to date form imperfect palindromes. The combinations of adjacent purine and pyrimidine nucleotides at the center of the palindromes possess different base stacking properties and hence different flexibility. The compositions of the palindromes

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Random

Base	–5	–4	–3	–2	–1	1	2	3	4	5	6	7	8	9	10
A	0.31	0.29	0.35	0.30	0.28	0.28	0.30	0.28	0.30	0.32	0.30	0.30	0.27	0.31	0.30
C	0.18	0.20	0.20	0.21	0.21	0.22	0.22	0.21	0.23	0.19	0.19	0.22	0.23	0.18	0.25
G	0.21	0.22	0.19	0.22	0.21	0.22	0.22	0.22	0.19	0.21	0.20	0.18	0.19	0.22	0.20
T	0.30	0.29	0.26	0.28	0.29	0.27	0.26	0.30	0.28	0.28	0.31	0.31	0.31	0.29	0.25

ASLV

Base	–4	–3	–2	–1	1	2	3	4	5	6	7	8	9	10
A	0.21	0.22	0.27	0.34	0.18	0.28	0.29	0.32	0.28	0.13	0.26	0.34	0.43	0.23
C	0.28	0.13	0.21	0.14	0.32	0.20	0.23	0.24	0.21	0.32	0.24	0.23	0.17	0.29
G	0.24	0.18	0.24	0.20	0.39	0.19	0.21	0.22	0.23	0.30	0.13	0.21	0.15	0.26
T	0.36	0.47	0.28	0.32	0.11	0.33	0.27	0.23	0.28	0.25	0.31	0.22	0.25	0.22
Preferred base		T			G/C					G/C			A	

HIV-1

Base	–5	–4	–3	–2	–1	1	2	3	4	5	6	7	8	9	10
A	0.34	0.25	0.22	0.29	0.34	0.22	0.24	0.32	0.46	0.10	0.27	0.35	0.46	0.34	0.31
C	0.19	0.25	0.11	0.08	0.13	0.29	0.10	0.16	0.14	0.41	0.34	0.23	0.22	0.16	0.19
G	0.19	0.19	0.24	0.26	0.31	0.40	0.12	0.16	0.10	0.25	0.13	0.10	0.13	0.19	0.21
T	0.28	0.32	0.43	0.37	0.22	0.09	0.54	0.36	0.30	0.24	0.26	0.32	0.19	0.31	0.29
Preferred base			T		G	G	T		A	C	C		A		

Figure 7.16 Palindromic consensus sequences at retroviral integration sites. The frequency of each base at each position around the integration sites was calculated, where 1 equals 100%. Integration occurs between positions −1 and 1 on the top strand, shown in the figure. Colored positions have statistically different frequencies of bases from those of randomly generated sequences shown at the top. Bases with a >10% increase of frequency at a position are blue, and bases with a >10% decrease of frequency at a position are yellow. The preferred bases are listed below. Inferred duplicated target sites are in the blue box, and joining to the 3′ ends of viral DNA occurs at positions labeled by arrows. The symmetry of the palindromic patterns is centered on the duplicated target sites. Adapted from X. Wu et al., *J Virol* **79:**5211–5214, 2005.

determine the degree of "bendability" of the target sequences that are optimal for interaction with specific integrases. For example, more bending is required by IN proteins that join viral DNA ends to their target 4 bp apart than those that join 6 bp apart. The symmetry in the patterns is consistent with the idea that IN complexes function as symmetrical multimers in the preintegration complex (discussed below).

These same large-scale, global analyses have also shown that all human chromosomes are targets for integration, but that different retroviruses display distinct preferences for particular chromosomal features (Table 7.1). For example, human immunodeficiency virus type 1 DNA is integrated preferentially inside genes, especially in those that are highly transcribed, whereas murine leukemia virus DNA is integrated preferentially in and near transcription start sites. These observations suggest that the interaction of preintegration complexes with different chromatin-bound proteins promotes integration into distinct chromosomal locations.

Cellular tethers. Cellular tethers, proteins that bind to both cellular chromatin and IN proteins, were first described for yeast retrotransposons: Pol III components for Ty3 IN, and heterochromatin proteins for Ty5 IN. The mechanism for tethering of retroviral IN protein is understood most fully for the lentivirus proteins, which bind directly to the transcriptional coactivator Ledgf (lens epithelium-derived growth factor/p75 protein) (Fig. 7.17A). The efficiency of integration of human immunodeficiency virus type 1 is greatly reduced in cells in which Ledgf has been depleted by treatment with small interfering RNA (siRNA) or in which the gene encoding Ledgf has been deleted. Furthermore, the pattern

Table 7.1 Comparison of retroviral integration site preferences

	% Integration[a]					
	Human cells[b]				Mouse cells[c]	
Site or region	Random	ASLV	MLV	HIV	$HIV^{LEDGF+/-}$	$HIV^{LEDGF-/-}$
Within genes	26	42	40	60–70	62	44
Transcription start sites	5	8	20	10	6	17

[a]HIV, human immunodeficiency virus; ASLV, avian sarcoma/leukosis virus; MLV, murine leukemia virus.

[b]Percentages are approximates for integration into human cells and are from A. Narezkina et al., *J Virol* **78:** 11656–11663, 2004.

[c]Percentages for mouse embryo fibroblasts are from M. C. Shun et al., *Genes Dev* **21:**1767–1768, 2007. Calculations performed in this study indicated HIV gene usage in mouse $LEDGF^{-/-}$ cells at ~8% above random, which was ~3% less than ASLV/MLV gene usage in human cells.

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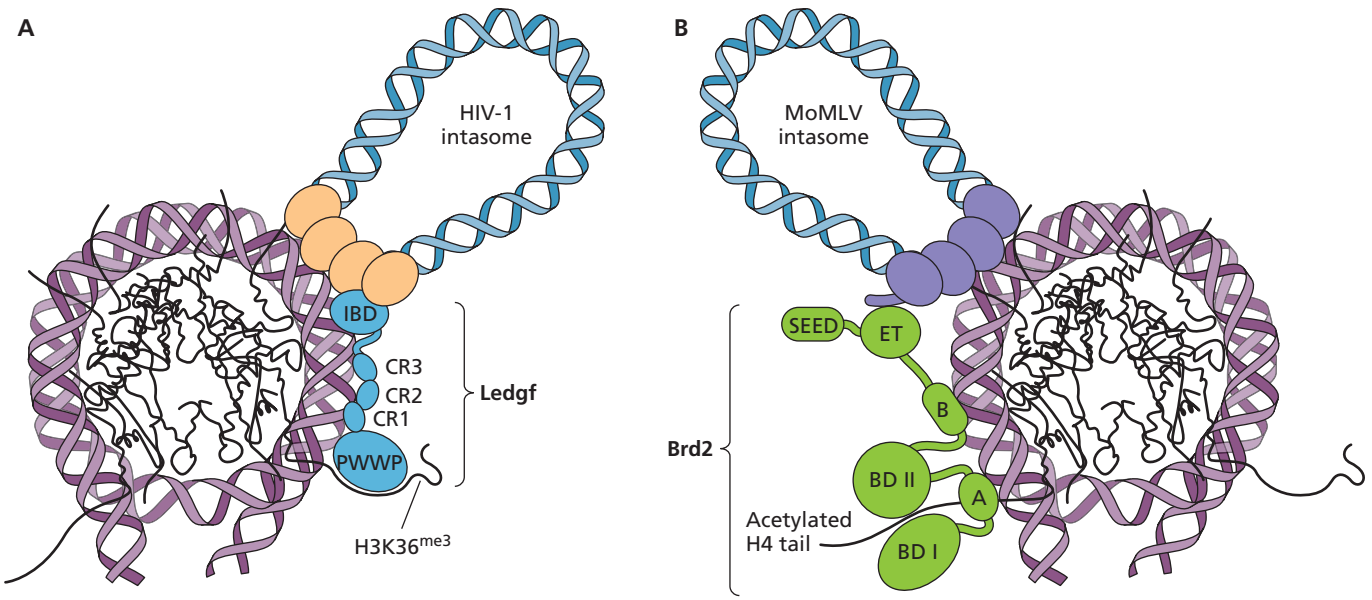


Figure 7.17 Models for chromatin tethering of intasomes by cellular proteins. (A) The intasome of human immunodeficiency type 1 virus, comprising a tetramer of IN bound to processed ends of viral DNA, is tethered to chromatin by attachment to the cellular protein Ledgf/p75. Histones are represented by black lines. The IN-binding domain (IBD) at the C-terminal end of Ledgf/p75 binds in the dimer interface(s) of the IN tetramer, and is anchored to nucleosomes by cooperative binding of the PWWP domain in Ledgf/p75 with the H3K36^{me3} modified histone tails associated with transcribed genes and by the three charge regions (CR1 to -3) that bind DNA. **(B)** The intasome of Moloney murine leukemia virus (MoMLV) is tethered to chromatin by Brd proteins. The ET domain in the C-terminal region of these proteins binds to the C-terminal tail of the IN protein(s) of this virus, and is anchored to nucleosomes by the interaction of two N-terminal bromodomains with acetylated H3 and H4 histone tails and by binding of motifs A and B to the host DNA. For more detailed information, see M. Kvaratskhelia et al., *Nucleic Acids Res* **42:**10209–10225, 2014.

of preference for various chromosomal features is altered in the small percentage of Ledgf-deficient cells in which integration does occur (Table 7.1). Target site selection is also correlated with histone modifications. Integration of human immunodeficiency virus type 1 is disfavored in regions of human chromosomes that contain modifications associated with transcriptional repression but is favored in regions with modifications that are associated with active transcription. X-ray crystallographic studies of the IN-binding domain of Ledgf bound to the catalytic core dimer interface of human immunodeficiency virus IN proteins have provided sufficient atomic detail to develop small-molecule inhibitors that reduce integration efficiency in cultured cells (see Volume II, Box 9.1).

Studies with the gammaretroviruses murine and feline leukemia viruses have identified the cellular bromodomain-containing proteins Brd2, -3, and -4 as specific tethers of these retroviral IN proteins. Conserved extraterminal (ET) domains in the Brd proteins bind to the 27-amino-acid C-terminal extension in gammaretroviral IN proteins and direct integration of the associated viral DNA to transcription start sites, which are rich in acetylated histones H3 and H4. A chromatin-tethering function for these cellular proteins has been established for papillomaviral genomes, via interaction with the DNA-binding viral E protein. In this case, viral genomes are tethered to condensed mitotic chromosomes, thereby ensuring their distribution to daughter cells following cell division. As Brd proteins promote assembly of transcriptional activators at transcription start sites, their tethering of the gammaretroviral IN provides a satisfying explanation for the integration preferences of these proviral genomes.

Although cellular tethers have not yet been identified for other retrovirus IN proteins, the examples described above support a general two-step mechanism: (i) binding to chromatin-associated proteins brings preintegration complexes in close proximity to host DNA (Fig. 7.17B); (ii) IN-catalyzed joining of viral DNA ends then occurs at viral-specific, preferred host DNA sequences nearby (Fig. 7.16).

Other Host Proteins May Affect Integration

Close to 100 cellular proteins have been identified as possible participants in the integration reactions of murine leukemia viruses or human immunodeficiency viruses, based on their association with preintegration complexes and/or their ability to bind to IN protein. Roles for candidate host proteins with DNA-binding properties, such as transcriptional regulators, chromatin components, and DNA repair enzymes, are not unexpected. The tethering functions of the Ledgf and Brd proteins are two validated examples. The possible

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contributions of other candidates are less apparent, and only a few have been characterized extensively.

One of the first of the candidate host proteins to be investigated was the 89-amino-acid cellular protein called barrier-to-autointegration factor (Baf), detected as a component of the preintegration complex of murine leukemia virus. Baf was shown to prevent integration into the newly synthesized viral DNA (autointegration), a reaction that would be suicidal for the virus. Purified Baf forms dimers in solution, binds to DNA, and can produce intermolecular bridges that compact the DNA, a reaction that prevents autointegration. The homologous human protein has been shown to block autointegration in isolated human immunodeficiency virus preintegration complexes. As purified virus particles do not contain this cellular protein, it must be acquired from the cytoplasm of a newly infected host cell (Fig. 7.18).

Another cellular protein thought initially to affect the integration process directly is the human immunodeficiency virus type 1 IN-interacting protein 1 (INi-1), which is a core component of the Swi/Snf chromatin-remodeling complex. Although INi-1 stimulates IN catalysis *in vitro*, this does not appear to be a physiologically important activity. Interestingly, IN mutants that cannot bind to INi-1 exhibit defects in virus particle morphology and reverse transcription and may provide clues to noncatalytic functions of IN.

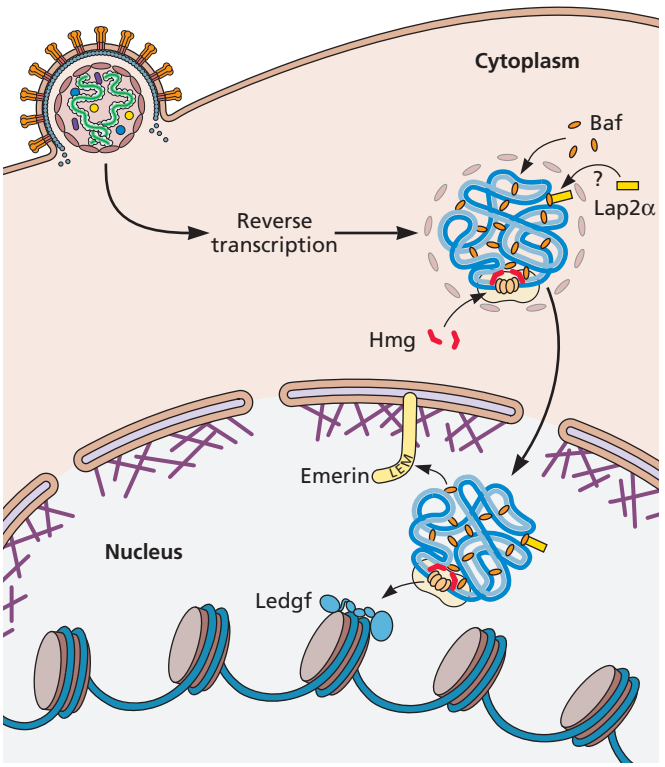


Figure 7.18 Host proteins may regulate the integration process. The barrier-to-autointegration factor protein (Baf binds to newly synthesized murine leukemia virus and human immunodeficiency virus type 1 DNAs, causing them to condense. Such DNA compaction prevents autointegration. The high mobility group 1a protein (Hmg), present in preintegration complexes of human immunodeficiency virus type 1, was originally thought to target integration to chromatin. Because cells that lack this protein have no obvious defect in virus reproduction, the protein is either redundant or not essential for integration. As this Hmg protein has been found to enhance transcription of the integrated provirus by recruitment of chromatin-remodeling complexes, it may facilitate a postintegration step. Lap2α proteins accumulate in the preintegration complexes of the human and murine viruses, respectively, but their functions, if any, are still uncertain. Once inside the nucleus, binding of Baf to emerin is proposed to facilitate access of the IN-DNA complex to chromatin, but this interaction does not appear to be essential for integration. IN binding to cellular tethers (e.g., Ledgf/p75 for lentiviruses or Brd family proteins for gammaretroviruses) anchors the intasomes to chromatin, thereby increasing integration efficiency. Fluorescence *in situ* hybridization studies with human immunodeficiency virus type 1–infected primary CD4 T cells indicate that viral DNA is integrated into actively transcribed regions of the host chromatin that are in close proximity to nuclear pores, and that the process is facilitated by particular nuclear pore proteins (nucleoporins).

Integrase Structure and Mechanism

IN Proteins Are Composed of Three Structural Domains

Retroviral IN proteins are ~300 amino acids in length and include three common domains connected by linkers of varying length (Fig. 7.19). The N-terminal domain is characterized by two pairs of invariant, Zn^{2+}-chelating histidine and cysteine residues (HHCC motif). The catalytic core domain contains a constellation of three invariant acidic amino acids, the last two separated by 35 residues [D,D(35)E motif]. These acidic amino acids chelate the two Mg^{2+} cofactors that are required for both processing and joining. Topologies of the catalytic core domains of the human and avian viral proteins established a relationship of IN to a large superfamily of nucleases and recombinases that includes the RNase H domain of RT. The amino acid sequence of the C-terminal domain is the least conserved among IN proteins from different retroviral genera, but the three-dimensional structures of this domain are quite similar in all examples analyzed to date. Some retroviral IN proteins (e.g., murine leukemia virus and prototype foamy virus) have an additional domain at their N termini, called the N-terminal extension domain (Fig. 7.19).

A Multimeric Form of IN Is Required for Catalysis

Properties of the integration reaction were first delineated by analysis of purified proteins. While a dimeric form appears to be sufficient to perform the processing reaction *in vitro*, a tetramer is required for the concerted integration of two viral DNA ends into a target DNA. The IN tetramer is stabilized by interaction with a pair of viral DNA ends, and each end is held mainly through contacts with C-terminal domain residues in one IN monomer, but acted upon by the catalytic core domain of another. The viral DNA ends do not remain double-stranded when bound at the active site of the enzyme, but the strands are partially unwound and distorted.

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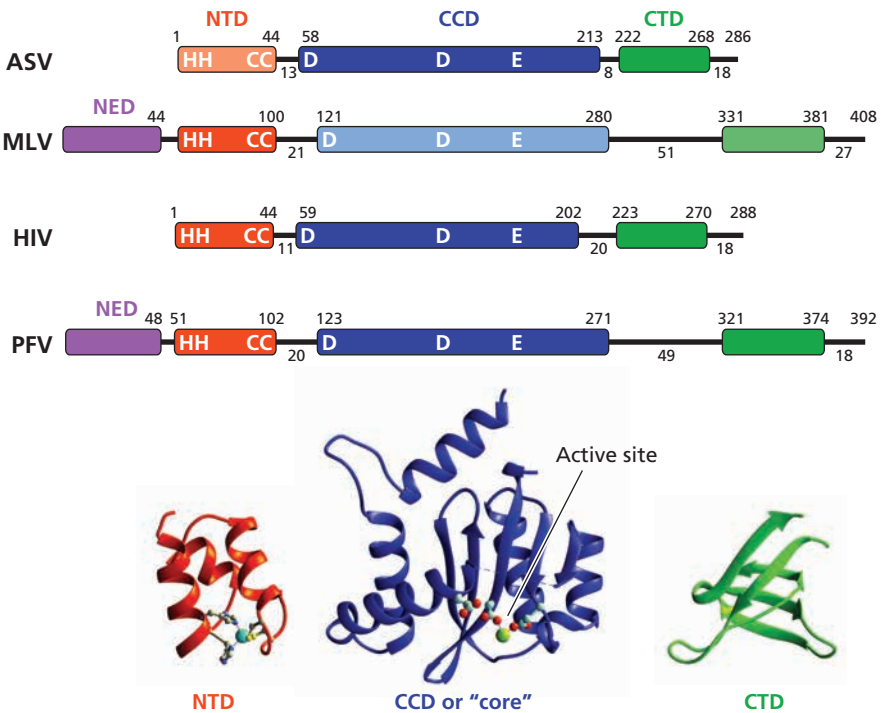


Figure 7.19 Domain maps of integrase proteins from different retroviral genera, and the structures of conserved domains in human immunodeficiency virus type 1 protein. (Top) Numbers above the maps indicate amino acid residues, starting with 1 at the N termini. Flexible linkers between the conserved domains, or C-terminal "tails," are represented by straight lines. Domain color coding is as follows: red, N-terminal domain (NTD); blue, catalytic core domain (CCD) or "core"; green, C-terminal domain (CTD); purple, N-terminal extension domain (NED). Domains for which atomic detail structures are not yet available are shown in faded colors. Evolutionarily conserved amino acids are indicated in the single-letter code within the domains. ASV, the alpharetrovirus avian sarcoma/leukosis virus; MLV, the gammaretrovirus murine leukemia virus; HIV, the lentivirus human immunodeficiency virus type 1; PFV, the spumavirus prototype foamy virus. (Bottom) The domain models are from crystal structures of the HIV-1 NTD, CCD, and CTD (PDB codes 1K6Y, 1BIU, and 1EX4, respectively). The Zn^{2+} ion in the NTD is shown as an aqua sphere, and in this structure of the HIV IN CCD with metal, only one of the two Mg^{2+} ions is bound in the active site, as indicated by the green sphere. The conserved Glu residue of the D,D(35)E motif is presumed to chelate the second metal ion together with the first conserved Asp residue.

These and other results suggested a model in which the core domains of only two of the four subunits in the IN tetramer provide catalytic function.

The retroviral INs are unusual enzymes with very low turnover rates *in vitro* (ca. 0.1 sec^{-1}). This low rate may not be a limitation *in vivo*, as only one concerted joining reaction is required to attach viral to host DNA in an infected cell.

Characterization of an Intasome

Since the last edition of this textbook was prepared, solutions of crystal structures of the prototype foamy virus IN-DNA complexes have confirmed many of the predictions from biochemical studies. In the presence of short duplex DNA fragments, with sequence corresponding to a viral DNA end, the prototype foamy virus IN protein assembles into a tetramer (a "dimer of dimers"). It was somewhat surprising to find that in this structure, called an **intasome** (Fig. 7.20A), the two "inner" subunits of the tetramer not only perform catalysis but also make **all** of the contacts with the viral DNA. The N-terminal domains in each of these inner subunits span the structure to bind the opposing viral DNA ends. The common N-terminal domains also reach over to interact with the opposing catalytic core domains. The C-terminal domains of the inner subunits are in the center, positioned to promote fraying of the unprocessed viral DNA ends and, following processing, to interact with a 30-bp target DNA fragment that contains the preferred palindromic sequence of the prototype foamy virus. Analysis of crystals with and without target DNA showed that the overall topology of the IN-DNA complex does not change upon target binding. However, as predicted from earlier biochemical studies, the target DNA must bend to fit into the active site. Crystals of IN-containing processed viral DNA ends and the DNA target were catalytically active in the presence of the Mg^{2+} cofactor, performing a concerted joining reaction 4 bp apart on the target, as expected for this viral IN protein (Fig. 7.20B).

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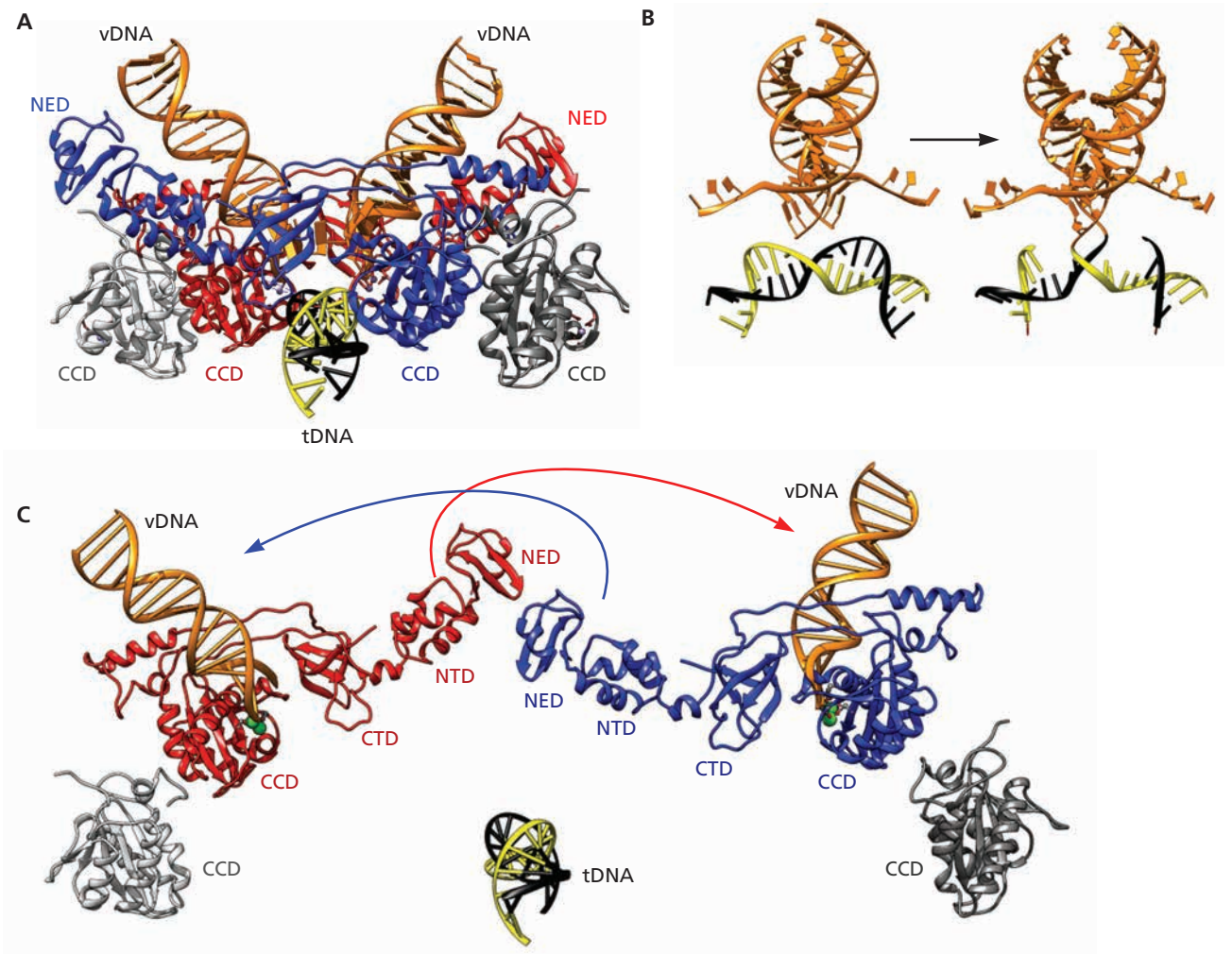


Figure 7.20 Crystal structure of the prototype foamy virus integrase tetramer bound to viral DNA ends and a target sequence. **(A)** The assembled complex (PDB code 4E7K) is shown in ribbon representation with the inner subunits in red and blue. Only the catalytic core domains (CCDs) of the outer subunits (gray) were resolved. Viral DNA (vDNA) oligonucleotides are in orange ribbon-ladder representation and the target DNA fragment (tDNA) in yellow and black. The locations of the N-terminal extension domains (NEDs) and CCDs are indicated. **(B)** DNA components of the complex portrayed before and after joining, rotated 90° about the *y* axis from panel A. The left view shows processed vDNA ends prior to joining, and the right view after joining to the target DNA. **(C)** The complex shown in panel A is pulled apart to show the positions of all domains in the inner subunits. Interactions between the distal N-terminal domain (NTD) and NED of one inner subunit and vDNA held in the CCD of the other inner subunit are indicated by the arrows. CTD, C-terminal domain. Assembly of the complex is shown in Movie 7.1 (http://bit.ly/Virology_V1_Movie7-1). For more detail on the prototype foamy virus structures, see G. N. Maertens et al., *Nature* **468:**326–329, 2010, and K. Gupta et al., *Structure* **20:**1918–1928, 2012. Image and movie courtesy of Mark Andrake, Fox Chase Cancer Center, Philadelphia, PA.

Analysis of the prototype foamy virus IN crystal structures allowed elucidation of the roles of the metal cofactors in catalyzing the reaction, and resolved details of interactions with metal-chelating, Food and Drug Administration (FDA)-approved inhibitors of human immunodeficiency virus IN. Furthermore, despite the differences in amino acid sequence, domain composition, and linker length, the prototype foamy virus IN-DNA structures have also been valuable in modeling similar assemblies for other retroviruses. While the terminal domains of the outer subunits in the prototype foamy virus IN crystals were not resolved, subsequent analysis of this complex in solution showed that these domains are extended outward from the complex and have no contact with the substrate DNAs (Fig. 7.20C).

With no obvious role for the outer subunits *in vitro*, it has been proposed that they may stabilize the tetrameric structure or promote its assembly (Box 7.8). It is also possible that the outer subunits facilitate integration *in vivo* by interacting

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BOX 7.8

DISCUSSION

Intasome assembly

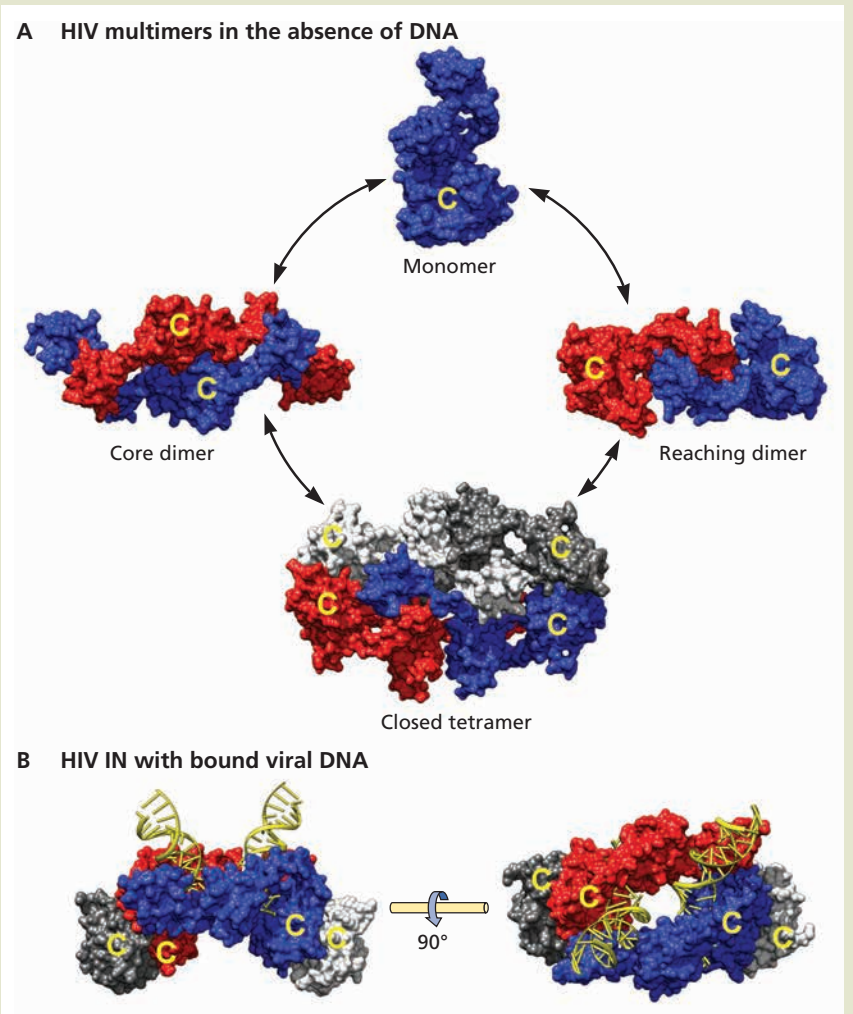
The concerted integration of two viral DNA ends into a target DNA requires assembly of an IN tetramer. The multimerization properties of purified retroviral IN proteins differ substantially. For example, at a concentration in which human immunodeficiency virus type 1 IN is mainly tetrameric, avian sarcoma/leukosis viral IN is a dimer, while prototype foamy virus IN is a monomer at even twice the concentration of the other two proteins. In solution, human immunodeficiency virus IN can form two apparently equally stable dimers: one (called a core dimer) resembles the outer dimer of the foamy virus intasome, and the other (called a reaching dimer) resembles the inner dimer of the intasome. However, the tetrameric form, a dimer of dimers, has no room for the DNA substrates without undergoing substantial conformational change (ergo, closed tetramer) (panel A in the figure).

The tetramer in the foamy virus intasome assembles from IN monomers *in vitro*, with substrate DNA imparting stability to the complex. Some evidence suggests that human immunodeficiency virus intasomes (panel B) are also assembled from monomers *in vitro*, but whether this is the case for other retroviral INs and how intasome assembly is accomplished in infected cells are currently unknown.

Bojja RS, Andrake MD, Merkel G, Weigand S, Dunbrack RL Jr, Skalka AM. 2013. Architecture and assembly of HIV integrase multimers in the absence of DNA substrates. *J Biol Chem* **288:**7373–7386.

Bojja RS, Andrake MD, Weigand S, Merkel G, Yarychkivska O, Henderson A, Kummerling M, Skalka AM. 2011. Architecture of a full-length retroviral integrase monomer and dimer, revealed by small angle X-ray scattering and chemical cross-linking. *J Biol Chem* **286:**17047–17059.

Gupta K, Curtis JE, Krueger S, Hwang Y, Cherepanov P, Bushman FD, Van Duyne GD. 2012. Solution conformations of prototype foamy virus integrase and its stable synaptic complex with U5 viral DNA. *Structure* **20:**1918–1928.



Multimers of human immunodeficiency virus type 1 (HIV-1) IN. (A) Models for the forms of HIV-1 IN that are at equilibrium in solution. Surface representations of the monomer, dimers, and a "closed" tetramer structure are based on architectures determined by small-angle X-ray scattering analysis, chemical cross-linking, and mass spectrometry with avian sarcoma/leukosis virus IN and HIV IN proteins in the absence of viral DNA substrates. In the dimers, one monomer is blue and the second is red. In the closed tetramer, additional subunits are dark and light gray. **(B)** Surface representations of HIV-1 IN with bound viral DNA end oligonucleotides, modeled from the prototype foamy virus crystal structure depicted in Fig. 7.20, in which only the catalytic core domains of the outer dimers were resolved (L. Krishnan et al., *Proc Natl Acad Sci U S A* **107:**15010–15915, 2010). The inner dimer comprises red and blue monomers as in panel A. The core domains of the outer dimers are shown in dark and light gray. Viral DNA substrate oligonucleotides are in gold ribbon-ladder representation. For aid in orientation, positions of the catalytic core domains are marked "C." Courtesy of Mark Andrake, Fox Chase Cancer Center, Philadelphia, PA.

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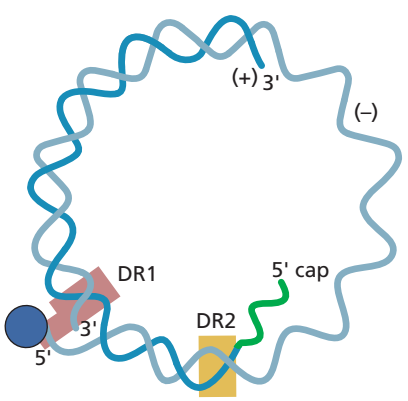
with nucleosomes when the target is chromatin. While only 4 IN molecules comprise the intasome, virus particles contain some 50 to 100 molecules of this viral protein. DNA protection experiments with preintegration complexes isolated from Moloney murine leukemia virus-infected cells suggest that several hundred base pairs of the viral DNA ends are protected by association with IN, and consequently, many monomers may be bound. IN may also provide a noncatalytic structural function, as the C-terminal domain of the human immunodeficiency virus protein has been reported to interact with the capsid protein, and virus particles that lack IN possess empty capsids and displaced genomes.

Hepadnaviral Reverse Transcription

A DNA Virus with Reverse Transcriptase

The revolutionary concept that a virus with an RNA genome can replicate by means of a DNA intermediate was followed, about a decade later, by another big surprise: RNA as an intermediate in the replication of a virus with a DNA genome. Early hints that a mechanism other than semiconservative DNA synthesis was responsible for hepadnaviral replication came from the discovery of asymmetries in the genomic DNA and in the product of an endogenous DNA polymerase reaction in isolated virus particles. The viral DNA comprises one full-length (−) strand and an incomplete, complementary (+) strand (Fig. 7.21), but the endogenous polymerase reaction could extend only the (+) strand. The replication intermediates isolated from infected cells were also unusual, comprising mainly (−) strands of less than unit length, few of which were associated with (+) strands. All this seemed suspiciously like retroviral reverse transcription, and landmark studies published in 1982 disclosed the unique features of hepadnaviral replication with duck hepatitis B virus. Unlike the endogenous reaction typical of extracellular virus particles, newly formed intracellular "core" particles were found to incorporate dNTPs into both strands. As with RNA-dependent DNA polymerization in retroviral particles, synthesis of the (−) strand was resistant to the DNA-intercalating drug actinomycin D, whereas synthesis of the (+) strand was inhibited by this compound. Furthermore, a portion of the newly synthesized (−) strand DNA sedimented with the density of RNA-DNA hybrids. These and related findings marked an important turning point in our understanding of hepadnaviruses and greatly extended our knowledge of reverse transcription. (See Box 7.9 for a subsequent surprise.)

Figure 7.21 Hepadnaviral DNA. The DNA in extracellular hepadnavirus particles is a partially duplex molecule of ~3 kb with circularity that is maintained by overlapping 5′ ends. The (−) strand is slightly longer than unit length, and the polymerase, shown as a blue ball, is attached to its 5′ end. The (+) strand has a capped RNA of 18 nucleotides at its 5′ end and is less than unit length. The 5′ ends are near or in (10- to 12-bp) direct repeats called DR1 and DR2 (colored purple and yellow, respectively). As in retroviruses, these repeat sequences play the critical role of facilitating template transfers during reverse transcription. In mammalian hepadnavirus genomes, the (+) strand is shorter than the (−) strand and has heterogeneous ends. In avian hepadnavirus genomes, the (+) strand is almost the same length as the (−) strand. Details of the genetic content are provided in the Appendix, Fig. 11.



Function of Reverse Transcription in the Hepadnaviral Life Cycle

Analyses of the single-cell reproduction cycle of hepadnaviruses have established that the gapped DNA of an entering virus particle is imported into the nucleus, where it is repaired to produce a covalently closed circular molecule (Fig. 7.22). The exact mechanism of formation of these circular molecules is not yet known. One attractive hypothesis is that, just as with retroviral DNA integration, the incoming hepadnaviral genome is seen as "damaged" by the cell. Enzymes of cellular DNA repair pathways that normally excise damaged bases or DNA adducts might then remove the bound P protein and capped RNA (Fig. 7.21) so that the viral DNA ends can then be filled in and ligated. The hepadnaviral genomes encode no integrase, and hepadnaviral DNA is not normally integrated into the host's genome. However, the covalently closed circular DNA, with acquired cellular histones, persists in the nucleus as a nonreplicating minichromosome from which cellular RNA polymerase II transcribes viral RNAs.

The 3.5-kb pregenomic mRNA is exported to the cytoplasm, where it serves as the template for reverse transcription. This process takes place in a newly formed subviral "core" particle that includes the pregenomic mRNA, plus capsid and polymerase proteins (products of the C and P genes). P protein provides all the activities required for reverse transcription. The DNA-containing, nascent core particles can then follow one of two pathways. Late in infection, when the cisternae of the endoplasmic reticulum contain an abundance of viral envelope glycoprotein, they can bud into the endoplasmic reticulum and eventually be secreted as progeny virus particles (Chapter 13). Alternatively, if they do not become enveloped, the core particles can be directed to the nucleus, where their

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BOX 7.9

BACKGROUND

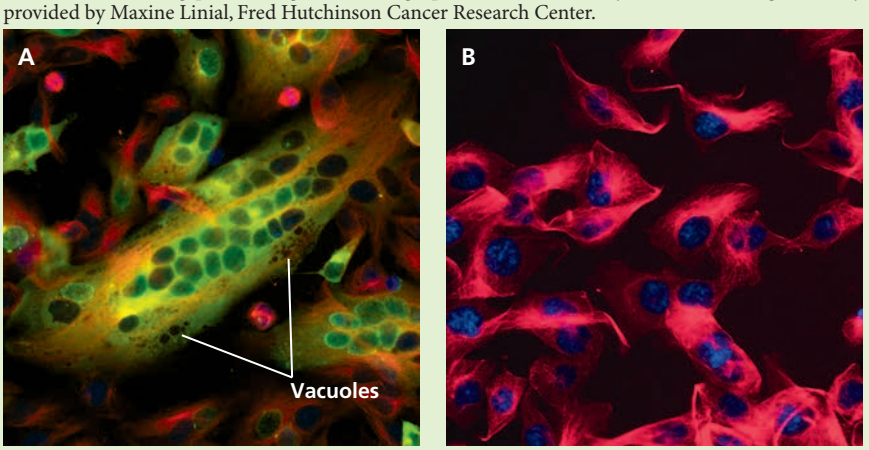
A retrovirus with a DNA genome?

The *Spumavirinae* comprise a subfamily of retroviruses isolated from primate, feline, and bovine species, among others. Spumaviruses are commonly called **foamy viruses**, because they cause vacuolization and formation of syncytia in cultured cells. These viruses exhibit no known pathogenesis and received little attention from virologists until recently. However, it is now clear that the foamy viruses are most unconventional retroviruses, with many properties that seem more similar to those of hepadnaviruses than of other retroviral family members, in the following respects.

- Reverse transcription is a late event in foamy virus production, and is largely complete before extracellular virus particles infect new host cells. Furthermore, although they contain both RNA and DNA, the genome-length DNA extracted from foamy virus particles can account entirely for viral infectivity. Like other retroviral family members, foamy virus genome replication requires an RNA intermediate, but as with hepadnaviruses, the functional nucleic acid in extracellular foamy virus particles appears to be DNA.
- Although the arrangement of genes and the mechanism of reverse transcription are the same as those in other retroviruses, the prototype foamy virus RT is not synthesized as part of a Gag-Pol precursor, but rather by translation of a separate *pol* mRNA, as is also the case for hepadnaviral RT.
- Mature foamy virus particles do not include the usual processed retroviral structural proteins (MA, CA, and NC), but instead contain two large Gag proteins that differ only by a 3-kDa extension at the C terminus. These Gag proteins contain glycine-arginine-rich domains that bind with equal affinity to RNA and DNA, much like the hepadnaviral core (C) protein.
- As with the hepadnaviruses, foamy virus budding requires both Gag and Env proteins, and most budding occurs into the endoplasmic reticulum.
- Most foamy virus particles remain within the infected cell. This property probably accounts for the large quantities of intracellular viral DNA, and might explain why persistently infected cells contain numerous integrated proviruses. It is possible that some foamy virus DNA integration occurs via an intracellular recycling pathway of progeny genomes, similar to that which occurs with hepadnaviruses.

Linial MA. 1999. Foamy viruses are unconventional retroviruses. *J Virol* **73:**1747–1755.

Cells infected with primate foamy virus (A) show large syncytia and numerous vacuoles. Uninfected cells **(B)** lack such vacuoles and have only single nuclei. Nuclei are stained blue, α-tubulin is red, and viral Gag protein is green. Micrographs were obtained by Alison Yu and generously provided by Maxine Linial, Fred Hutchinson Cancer Research Center.



DNA is converted to additional copies of the covalently closed circular molecules. This pathway predominates at early times after infection, when little envelope protein is available. Eventually, as many as 30 hepadnaviral episomes can accumulate in the nucleus.

The DNA in hepadnaviral episomes is not replicated by the host's DNA synthesis machinery; **all** hepadnaviral DNA is produced by reverse transcription. This situation contrasts with that of retroviruses, in which the integrated nuclear form, the provirus, is replicated along with the host DNA. Consequently, both retroviral and hepadnaviral DNAs are maintained in infected cells for the life of those cells, but in quite different ways.

Analysis of hepadnaviral reverse transcription has been difficult for a number of technical reasons. Suitable tissue culture systems were not available until hepatoma cell lines were identified in which virus reproduction could take place following transfection with cloned viral DNA. Furthermore, mutational studies are confounded by the compact coding organization of the DNA. The tiny genome (~3 kb) is organized very efficiently, with more than half of its nucleotides translated in more than one reading frame. This arrangement makes it more difficult to produce mutations that change only one gene product. Finally, although reverse transcription takes place in newly assembled core particles, it was not possible initially to prepare enzymatically active P protein to study the reaction. Nevertheless, currently available details reveal fascinating analogies, but also striking differences, in the reverse transcription of hepadnaviruses and retroviruses.

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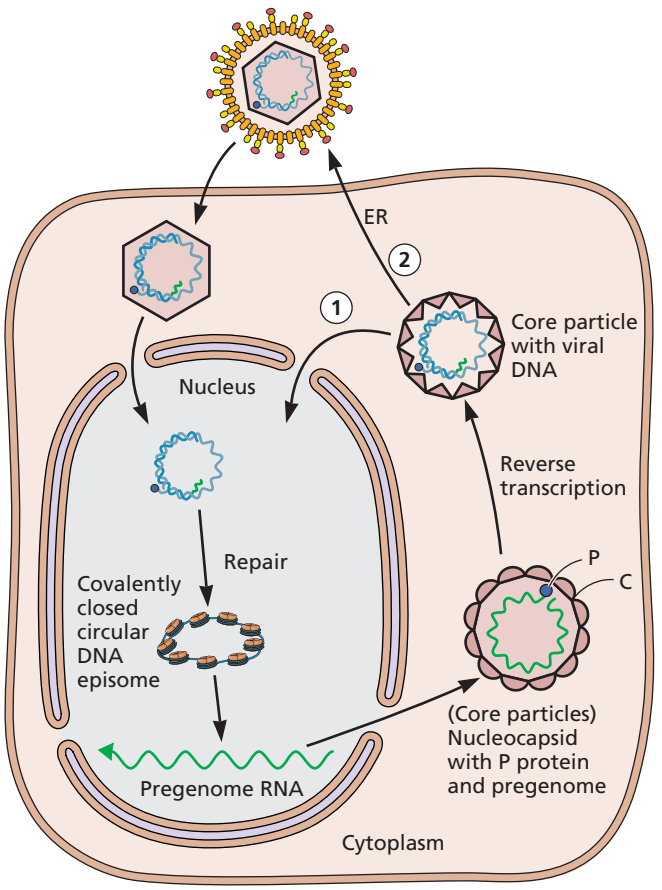


Figure 7.22 Single-cell replication cycle for hepadnaviruses. Pathway 1 provides additional copies of covalently closed circular minichromosomes. Pathway 2 represents exit of enveloped particles through the endoplasmic reticulum (ER). Additional details of the single-cell reproductive cycle are provided in the Appendix, Fig. 12.

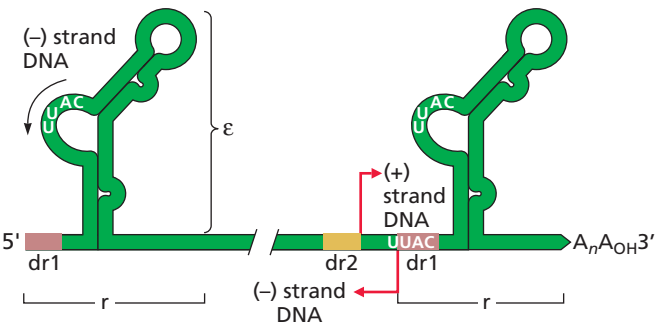


Figure 7.23 Essential *cis*-acting signals in pregenomic mRNA. The viral pregenomic mRNA bears terminal repetitions of ca. 200 nucleotides (r) that contain copies of the packaging signal (ε), but only the 5′ copy has functional activity *in vivo*. Indicated are positions for initiation of the 5′ ends of (−) and (+) strand DNAs, and the 5′-UUAC-3′ motifs in duck hepatitis B virus within ε and at dr1, that are important for (−) strand DNA synthesis. Both the structural features of ε and the specific sequence in the loop are critical for its function. Adapted from C. Seeger and W. S. Mason, p 815–832, *in* M. L. DePamphilis (ed), *DNA Replication in Eukaryotic Cells* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1996), with permission.

The Process of Reverse Transcription

Essential Components

Pregenomic mRNA. The **pregenomic mRNA** that provides the template for production of hepadnaviral genomic DNA is capped and polyadenylated, and also serves as the mRNA for both capsid (C) and reverse transcription (P) proteins. The transcription of pregenomic mRNA from covalently closed circular DNA in the nucleus is initiated at a position ~6 bp upstream of one copy of a short direct repeat called DR1 (Fig. 7.21). Transcription then proceeds along the entire DNA molecule, past the initiation site, to terminate after a polyadenylation signal just downstream of DR1. Consequently, pregenomic mRNA is longer than its template DNA. Because the region from the transcription initiation site to the polyadenylation site is copied twice, there is a long direct repeat (~200 nucleotides) (r) at either end of the RNA. This long repeat includes dr1 and a structural element of about 100 nucleotides called **epsilon (ε)** (Fig. 7.23). Deletion of ε within the 3′ copy of r has no impact on genome replication. In contrast, ε at the 5′ end is essential as it provides both the site for initiation of (−) strand synthesis and the signal for encapsidation of DNA into core particles. Although all viral transcripts have ε at their 3′ ends, only the pregenomic mRNA has this important copy of ε at its 5′ end.

There is a marked preference for reverse transcription of the pregenomic mRNA molecules from which P protein is translated. The basis of such *cis*-selectivity is unknown; C protein, which is also translated from this RNA and has nucleic acid-binding properties, appears to function perfectly well in *trans*. It is possible that the nascent P polypeptide binds to its own mRNA cotranslationally. An attendant benefit from such a mechanism would be the selection for genomes that express functional P protein. Analysis of cytoplasmic core particles suggests that there is one molecule of P protein per molecule of DNA, implying that hepadnaviruses contain one copy of the viral genome per virus particle (Table 7.2). This selectivity would be determined, in part, by the presence of the encapsidation signal(s) at the 5′ end of the pregenomic mRNA.

Primers. The primers for hepadnaviral RT remain attached to the 5′ ends of the viral DNA strands. They are, for (−) strand synthesis, the P protein itself, and, for (+) strand synthesis, a capped RNA fragment derived from the 5′ end of pregenomic RNA. A protein-priming mechanism (Chapter 9) was first described for adenovirus DNA replication and later for the bacteriophage ϕ29. Priming by a viral protein, VPg, is also a feature of poliovirus RNA synthesis. Hepadnaviral reverse transcription is distinguished by the fact that the primer and the polymerase are within a single protein.

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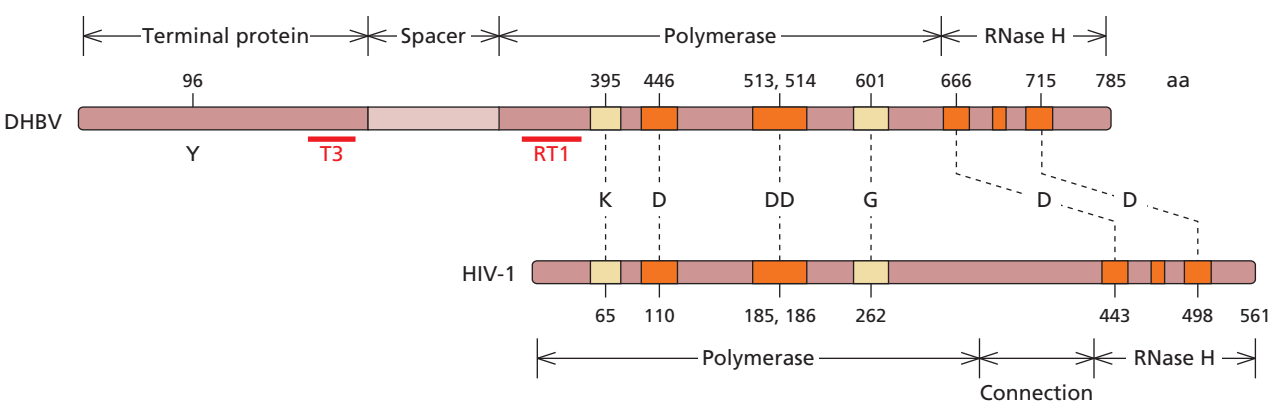
Table 7.2 Comparison of retroviral and hepadnaviral reverse transcription

Parameter	Retroviruses[a]	Hepadnaviruses[a]
Viral genome	RNA (pseudodiploid)	DNA (incomplete duplex)
Template RNA also serves as:	Genomic *RNA mRNA* (*gag*, *pol*)	Pregenomic *RNA mRNA* (C and P proteins)
DNA intermediate	*Circular DNA with 5′ overlaps*	*Circular DNA with 5′ overlaps*
Virus-encoded enzyme	RT	P protein
No. of molecules/core	50–100	1
Functions	*DNA polymerase*, *RNase H*, helicase (strand displacement)	*DNA polymerase*, *RNase H*, protein priming, template RNA encapsidation
Primer, first (−) DNA strand	tRNA (host)	Viral P protein (TP domain)
Site of initiation	*Near 5′ end of genome*	*Near 5′ end of pregenome*
First DNA product	(−) strong-stop DNA, ca. 100 nucleotides	4 nucleotides copied from bulge in 5′ ε
First template exchange	*To complementary sequence in repeated sequence, r, at 3′ end of template RNA*	*To complementary sequence in repeated sequence, R, at 3′ end of template RNA*
Primer, second (+) DNA strand	*Derived from template RNA*, internal RNase H product (ppt)	*Derived from template RNA*, 5′ cap, terminal RNase H product
Site of initiation	*Near 5′ end of (−) DNA*	*Near 5′ end of (−) DNA*
Time of initiation	Before completion of (−) strand	After completion of (−) strand
Type of priming	Priming *in situ*	Primer translocated
Second template exchange	*To the 3′ end of (−) strand DNA via complementary* pbs *sequence*	*To the 3′ end of (−) strand DNA via complementary sequence*
Reverse transcribing nucleoprotein complex	*Subviral "core"* particles, deposited in the *cytoplasm* upon viral entry	Nascent *subviral "cores"*; *cytoplasmic* intermediates in viral assembly
Final product(s)	Double-stranded linear DNA	Circular viral DNA or covalently closed episomal DNA
DNA maintained in the nucleus	Integrated into host genome, proviral DNA	Nonintegrated episome in host nucleus

[a]Italics indicate similarities.

P protein is a self-priming reverse transcriptase. P protein has C-terminal enzymatic domains that were first identified by amino acid sequence alignment with the retroviral RTs (Fig. 7.24). The highly conserved residues in the homologous domains are essential for hepadnaviral reverse transcription. Hepadnaviral P protein also contains an N-terminal domain separated from the RT region by a spacer, believed to provide a flexible hinge between these two regions of the protein. The N-terminal domain, referred to as the terminal protein region, includes a tyrosine residue utilized for priming (−) strand DNA synthesis. In addition to its other functions, P protein is required for encapsidation of viral RNA, a process

Figure 7.24 Comparison of hepadnaviral and retroviral RTs. Linear maps of the duck hepatitis B virus (DHBV) and human immunodeficiency virus type 1 (HIV-1) *pol* gene products. The maps were aligned relative to amino acids that are generally conserved among all RTs. Approximate locations of motifs (T3 and RT1) in the hepadnaviral P proteins that interact with epsilon (ε) in pregenome mRNA are indicated.



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that depends on the interaction of both the RT and terminal protein domains with the 5′ ε structure. This mechanism represents a departure from the retroviral scheme, in which the NC protein sequences in the Gag polyprotein serve this purpose (Table 7.2). Indeed, the requirement for a DNA polymerase in hepadnaviral RNA encapsidation is unique among retroelements.

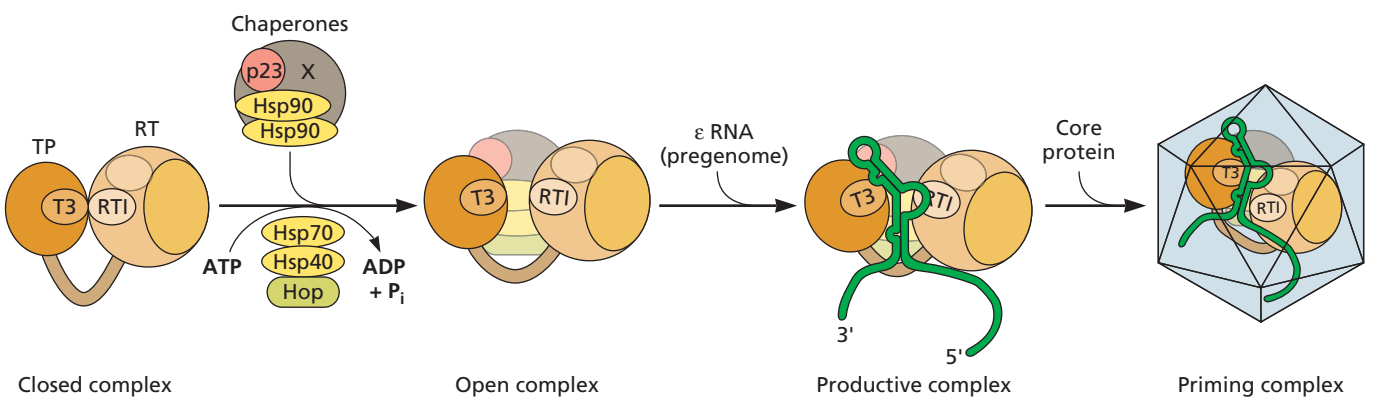
Host proteins may facilitate P-protein folding. An important breakthrough in the study of hepadnaviral reverse transcription was achieved with the demonstration that enzymatically active P protein can be produced upon translation of P mRNA from duck hepatitis B virus in a cell-free rabbit reticulocyte lysate. P protein is the only viral protein required for initiation of hepadnaviral DNA synthesis. If ε is not present during synthesis of P protein in yeast, the enzyme is inactive, even if ε is supplied later. Furthermore, P protein that is synthesized when ε is present in the mRNA is more resistant to proteolysis. Consequently, binding to ε may be required for the P protein to fold into an active conformation. Host cell proteins also appear to affect P-protein folding; synthesis of active P protein in the cell-free system requires the presence of cellular chaperone proteins and a source of energy (ATP). Furthermore, incorporation of these host cell proteins into viral capsids appears to require the polymerase activity of P protein. It has been proposed that chaperones are needed to maintain this viral protein in a conformation that is competent to bind to ε and prime DNA synthesis, and also to interact with assembling capsid subunits (Fig. 7.25).

Critical Steps in Reverse Transcription

Initiation and the first template exchange. Synthesis of the (−) strand of duck hepatitis B virus DNA is initiated by the polymerization of three or four nucleotides primed by the -OH group of a tyrosine residue located in the terminal protein domain of the single P-protein molecule present in the capsid. This single protein molecule acts both as primer and catalyst for all subsequent steps in reverse transcription (Box 7.10). This initial synthesis is followed by a template exchange in which the enzyme-bound, 4-nucleotide product anneals to a complementary sequence at the edge of dr1 at the 3′ end of the pregenomic RNA (Fig. 7.23 and 7.26, step 2). Although the sequence at this end is complementary to the short, initial product, it is not unique in the pregenomic mRNA (Fig. 7.23). In human hepadnavirus genome replication, appropriate positioning of the nascent DNA strand is promoted by a *cis*-acting sequence (ϕ), which anneals to the upper stem of the 5′ ε to which P protein is bound. It seems likely that selection of the normal site is also guided by the specific organization of pregenomic RNA in core particles. P protein remains covalently attached to the 5′ end of the (−) strand during the first template exchange and, as noted previously, through **all** subsequent steps.

Elongation and RNase H degradation of the RNA template. Following the first template exchange, (−) strand DNA synthesis continues all the way to the 5′ end of the pregenomic RNA template (Fig. 7.26, steps 3 and 4). Because synthesis is initiated in the 3′ dr1, a short repeat of 7 to

Figure 7.25 Model for the assembly of hepadnavirus nucleocapsids. P protein is synthesized in an inactive conformation (labeled "closed complex"). Interaction with a chaperone assembly (heat shock protein 90 [Hsp90], together with four cochaperones, Hsp70, Hop, Hsp40, and p23) induces a conformational change (open complex) that allows binding of P protein to ε RNA, facilitated by interaction with the T3 and RT1 motifs in the terminal protein (TP) and RT domains of P, respectively (productive complex). Such binding provides the signal for nucleocapsid assembly and initiation of viral DNA synthesis (priming complex) at the active site of the RT (indicated by a pale oval). TP provides the primer tyrosine residue for the initiation of reverse transcription. For additional details, see C. Seeger et al., p 2185–2221, *in* D. M. Knipe and P. M. Howley (ed), *Fields Virology*, 6th ed, vol 2 (Lippincott Williams & Wilkins, Philadelphia, PA, 2013).



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BOX 7.10

DISCUSSION

A single P-protein molecule does it all?

It is quite difficult to envision how a single protein can perform all of the gymnastics required for synthesis of a double-stranded circular DNA product while remaining attached to one viral DNA end. Nevertheless, it is widely believed among hepadnavirus researchers that there is only **one** P molecule in an infectious particle. Several observations support this view.

- Hepadnavirus assembly requires binding of the P protein to the ε stem-loop structure in a single pregenome mRNA molecule. In the absence of ε, there is no RNA packaging or assembly of core particles with RT activity.
- P protein in virus particles, and immature cores that contain nascent (−) DNA strands, do not use exogenous templates. Because such templates are copied by RT in permeabilized retroviral particles, one might expect that if one or more additional P proteins (not covalently attached to the genome) were present, they would bind and copy an exogenous template.
- P protein synthesized *in vitro* does not form dimers. Furthermore, even though the two functional domains are separated by a hinge, a P variant with a mutation in the terminal protein cannot complement a variant with a mutation in the polymerase domain.
- Single image particle reconstructions of RNA-filled cores reveal a structure consistent with a single P protein anchored in a unique position, touching the pregenome mRNA, which is aligned along the inner surface of the core (see the figure).

It seems likely that hepadnaviral core architecture and components help to ensure that the required interactions between P protein and nucleic acid templates can occur in such a way that exchanges are facilitated and templates can transit to the active site as product strands are synthesized.

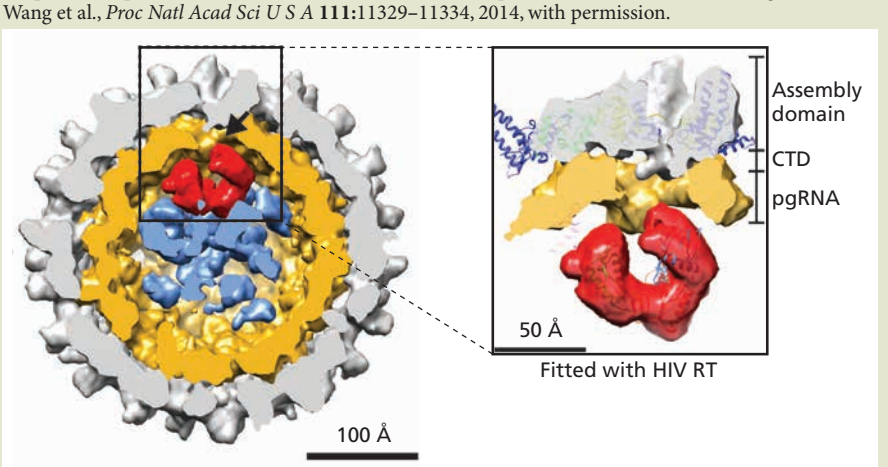
Hirsch RC, Lavine JE, Chang LJ, Varmus HE, Ganem D. 1990. Polymerase gene products of hepatitis B viruses are required for genomic RNA packaging as well as for reverse transcription. *Nature* **344:**552–555.

Junker-Niepmann M, Bartenschlager R, Schaller H. 1990. A short *cis*-acting sequence is required for hepatitis B virus pregenome encapsidation and sufficient for packaging of foreign RNA. *EMBO J* **9:**3389–3396.

Kaplan PM, Greenman RL, Gerin JL, Purcell RH, Robinson WS. 1973. DNA polymerase associated with human hepatitis B antigen. *J Virol* **12:**995–1005.

Radziwill G, Tucker W, Schaller H. 1990. Mutational analysis of the hepatitis B virus P gene product: domain structure and RNase H activity. *J Virol* **64:**613–620.

Electron microscopic reconstruction of a hepadnavirus RNA-filled core showing possible location of P protein. In a cross section, the outer portion of the core is assembled from C protein dimers (gray). Pregenome mRNA density (pgRNA, yellow) coats the inner surface of the core. A uniquely positioned density (red) is tentatively assigned to the P protein. Additional internal (blue) density represents unidentified encapsidated proteins and/or misaligned capsid. A close-up shows that the homologous polymerase domain of RT from human immunodeficiency virus type 1 in blue ribbon representation (PDB ID code 1RTD) can fit neatly into the right-hand doughnut-shaped red density of the putative P protein. The TP and RNase H domains of P protein are unresolved. From Fig. 4 in J. C.-Y. Wang et al., *Proc Natl Acad Sci U S A* **111:**11329–11334, 2014, with permission.



8 nucleotides (3′R) is produced at the end of this elongation step when the 5′ dr1 sequence is copied (Fig. 7.26, step 4). The RNA template is degraded by the RNase H activity of P protein as (−) strand synthesis proceeds. Unlike the retroviral RNase H products, none of these hepadnaviral RNA fragments are used as primers for (+) strand DNA synthesis (Table 7.2). The final product of RNase H digestion is a short RNA molecule, corresponding to the capped end of the pregenomic RNA, which includes the 5′ dr1 and serves as a primer for (+) strand DNA synthesis. It is noteworthy that (+) strand DNA synthesis can begin only after completion of (−) strand DNA synthesis, because such completion is required for formation of this primer.

Translocation of the primer for (+) strand DNA synthesis. Translocation of the primer for (+) strand DNA synthesis is likely to be facilitated by the homology between DR1 and DR2 (Fig. 7.26, step 5): the capped RNA primer, which includes dr1 sequences, can anneal to both. How the primer is induced to dissociate from DR1 and associate with DR2 is unclear. A small hairpin structure that includes the 5′ end of DR1 in the (−) strand of duck hepatitis B virus DNA appears to contribute to the translocation by inhibiting *in situ* priming and, perhaps, facilitating annealing of the capped RNA fragment with the complementary sequence in DR2 (Fig. 7.27). As in the first template exchange, a particular organization of the template in the core particles is thought to facilitate the process.

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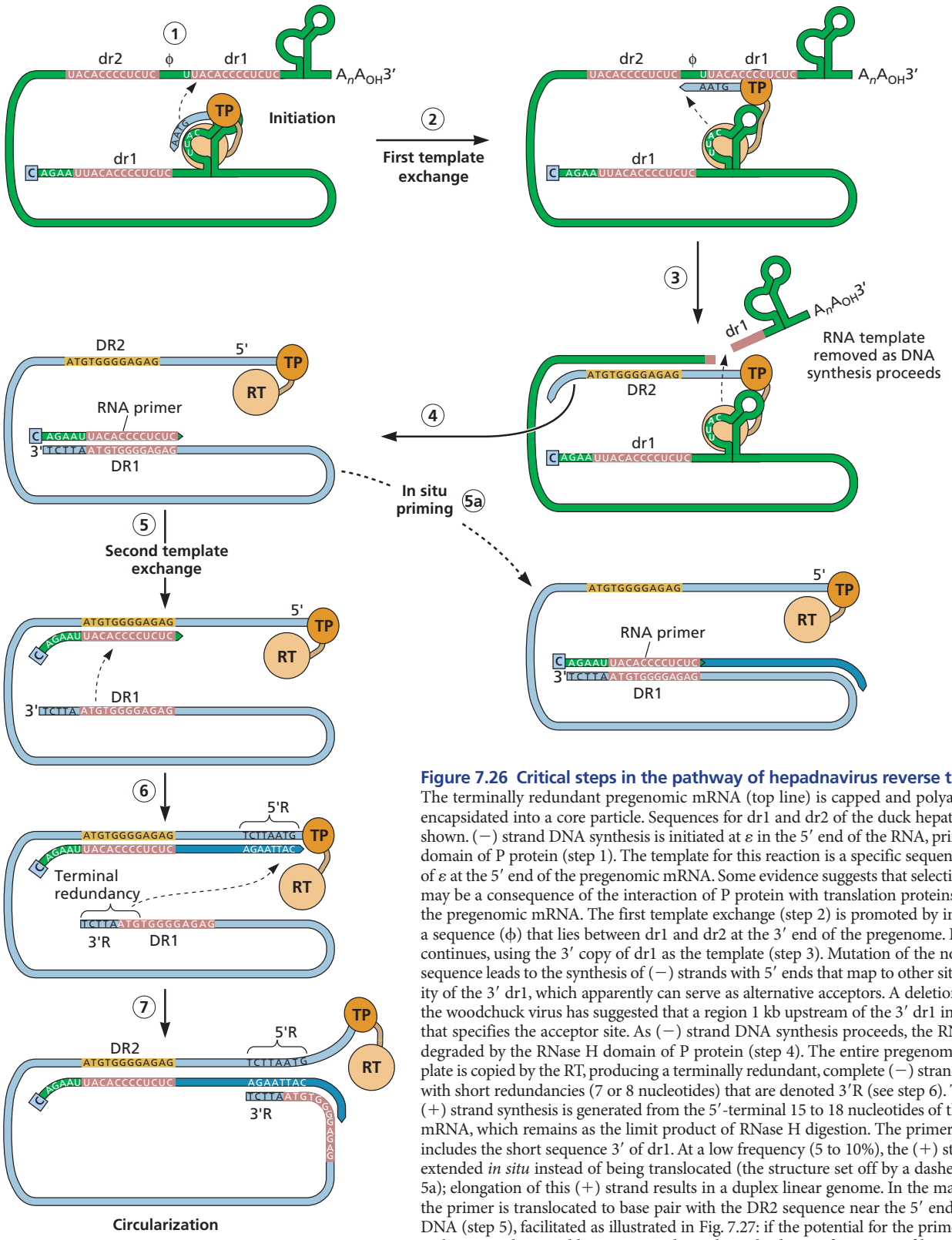


Figure 7.26 Critical steps in the pathway of hepadnavirus reverse transcription. The terminally redundant pregenomic mRNA (top line) is capped and polyadenylated and encapsidated into a core particle. Sequences for dr1 and dr2 of the duck hepatitis B virus are shown. (−) strand DNA synthesis is initiated at ε in the 5′ end of the RNA, primed by the TP domain of P protein (step 1). The template for this reaction is a specific sequence in the bulge of ε at the 5′ end of the pregenomic mRNA. Some evidence suggests that selection of this copy may be a consequence of the interaction of P protein with translation proteins at this end of the pregenomic mRNA. The first template exchange (step 2) is promoted by interaction with a sequence (ϕ) that lies between dr1 and dr2 at the 3′ end of the pregenome. DNA synthesis continues, using the 3′ copy of dr1 as the template (step 3). Mutation of the normal acceptor sequence leads to the synthesis of (−) strands with 5′ ends that map to other sites in the vicinity of the 3′ dr1, which apparently can serve as alternative acceptors. A deletion analysis with the woodchuck virus has suggested that a region 1 kb upstream of the 3′ dr1 includes a signal that specifies the acceptor site. As (−) strand DNA synthesis proceeds, the RNA template is degraded by the RNase H domain of P protein (step 4). The entire pregenome mRNA template is copied by the RT, producing a terminally redundant, complete (−) strand DNA species with short redundancies (7 or 8 nucleotides) that are denoted 3′R (see step 6). The primer for (+) strand synthesis is generated from the 5′-terminal 15 to 18 nucleotides of the pregenomic mRNA, which remains as the limit product of RNase H digestion. The primer is capped and includes the short sequence 3′ of dr1. At a low frequency (5 to 10%), the (+) strand primer is extended *in situ* instead of being translocated (the structure set off by a dashed arrow) (step 5a); elongation of this (+) strand results in a duplex linear genome. In the majority of cases, the primer is translocated to base pair with the DR2 sequence near the 5′ end of (−) strand DNA (step 5), facilitated as illustrated in Fig. 7.27: if the potential for the primer to hybridize with DR2 is disrupted by mutation, the pathway leading to formation of linear duplex DNA molecules predominates. After (+) strand synthesis is initiated, elongation proceeds (step 6). On reaching the 5′ end of (−) strand DNA, an intramolecular template exchange occurs, resulting in a circular DNA genome (step 7). This exchange is promoted by the short terminal redundancy, 5′R, in (−) strand DNA. (+) strand DNA synthesis then continues for a variable distance, resulting in the circular form of the genome found in mature virus particles. Adapted from Fig. 1 of J. W. Habig and D. D. Loeb, *J Virol* **76:**980–989, 2002, with permission.

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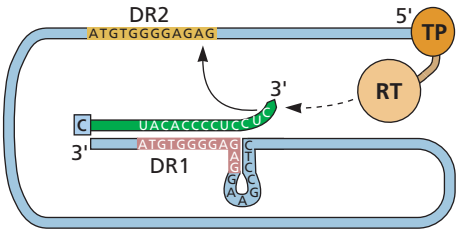


Figure 7.27 Model for (+) strand priming. Formation of a hairpin in the (−) strand DNA template displaces the 3′ end of the capped RNA fragment, preventing *in situ* priming and facilitating annealing with the homologous sequence in DR2. The ensuing translocation of the RNA primer allows initiation of (+) strand DNA synthesis. Adapted from Fig. 2 of J. W. Habig and D. D. Loeb, *J Virol* **76:**980–989, 2002.

The (+) strand synthesis primed by the translocated capped hepadnaviral RNA primer is similar to that which produces the strong-stop DNAs in retroviral reverse transcription. The (+) strand DNA synthesis begins near the 5′ end at DR2 and soon runs out of (−) strand template. As in the retroviral case, this problem is solved by a second template exchange, in this instance facilitated by the short repeat, 5′R, produced during synthesis of the (−) strand (Fig. 7.26, step 6).

The second template exchange creates a noncovalent circle. The structural requirements for the next step in hepadnaviral reverse transcription must allow displacement of the 5′ end of the (−) strand while still attached to the DNA. In addition to DR1 and DR2, *cis* interactions among other sequences at the ends and in a central region of (−) strand DNA have been implicated in this final step. It has been suggested that the simultaneous interaction of the central region with both ends may hold the termini in a position that facilitates both (+) strand primer translocation and the second template exchange. However, even with such "help," it is difficult to envision how a single protein accommodates all three DNA ends at once and catalyzes polymerization while still attached to one of them. Nevertheless, this exchange does occur with high efficiency in infected cells, and subsequent incomplete elongation of the (+) strand produces the partially duplex, noncovalent circle with variable (+) strand ends that comprises virion DNA (Fig. 7.26, step 7).

It is not clear what causes premature termination during synthesis of the (+) strand of hepadnaviral DNA. This synthesis is affected by mutations in the C protein. It has been proposed that DNA synthesis induces a change in the outer surface of the core, and that envelopment is regulated by interaction of the envelope proteins with this altered structure. Once these cores (capsids) are enveloped, DNA synthesis stops, presumably because dNTP substrates can no longer enter the particle.

Perspectives

The description of the reactions in hepadnaviral reverse transcription reveals interesting points of similarity to, and contrast with, retroviral systems (Table 7.2). Amino acid sequences and functions are conserved among retroviral RT and hepadnaviral P proteins, and both enzymes use terminal nucleic acid repeats to mediate template exchanges. However, the mechanisms by which their templates are reverse transcribed are quite distinct. Differences in the form and function of the final products of the two pathways are especially striking. A DNA circle with overlapping 5′ ends is an intermediate in the formation of a linear duplex DNA, the final product of retroviral reverse transcription. Repair of the circular intermediate is an unusual reaction, and covalently closed circle forms are dead-end products. In contrast, linear DNA is an aberrant product of hepadnaviral reverse transcription, and the covalently closed circle is the functional form for transcription.

The single-cell reproduction cycles of retroviruses and hepadnaviruses are, in a sense, permutations of one another. In comparing them to each other and to the unconventional foamy viruses (Box 7.9), it is instructive to include the cauliflower mosaic virus, a plant retroid virus that seems to combine some features of the animal viruses during reverse transcription (Fig. 7.28). This plant virus has a circular DNA genome and directs synthesis of a covalently closed episomal form, but its reverse transcription and priming mechanisms are quite analogous to those of retroviruses and retrotransposons. On the other hand, as with hepadnaviruses, RNA primers remain attached to the 5′ ends of cauliflower mosaic virus DNA. Retroid viruses appear to represent a continuum in evolution, and remind us of the varied combinations of strategies that exist in nature for replicating viral genomes and related genetic elements.

Biochemical and structural analyses reported since the last edition of this textbook have expanded our knowledge substantially and provided new insight into some of the remarkable properties of the viral RTs and the retroviral IN protein. It is now clear that these protein molecules can be scaffolds as well as catalysts, and their multiple functions appear to be enabled by a remarkable capacity for dynamic conformational change. Functional versatility is perhaps most striking in the hepadnaviral P protein, which performs all of the reactions necessary to synthesize a duplex circular DNA product from a linear RNA template while remaining covalently attached to one viral DNA end. Models derived from X-ray crystal structures of the retroviral RT and IN proteins have not only illuminated mechanistic details but have also informed efforts to develop new inhibitors that can be used in the clinic, while also providing insight into the molecular basis of drug resistance (Volume II, Chapter 9). Furthermore, identification of

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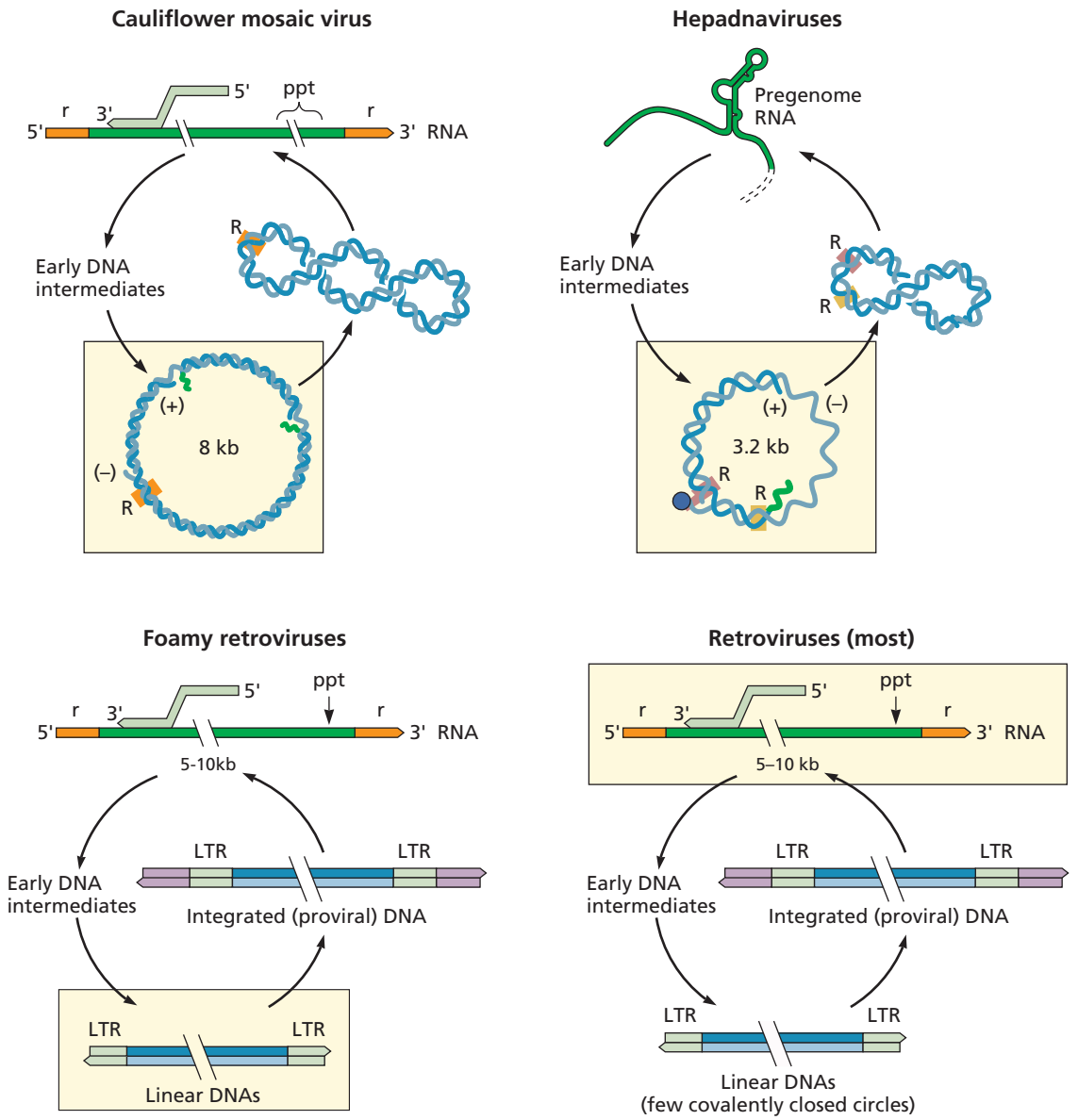


Figure 7.28 Comparison of the genome replication cycles of cauliflower mosaic viruses, hepadnaviruses, and retroviruses. The double-stranded DNA circle found in cauliflower mosaic virus particles contains three interruptions. At each interruption there is a short 5′ overlap of DNA as if formed by strand displacement synthesis. Ribonucleotides are often found attached to the 5′ ends. The (−) strand starts with either a ribo- or a deoxyribonucleoside. The 5′ ends of the (+) strand each contain 8 to 10 purine-rich matches to the viral DNA at the same location, suggesting a primer function. r, short sequence at both ends of viral RNA; R, same sequence in DNA. The shaded boxes indicate that the nucleic acids (genomes) encapsidated in particles of each virus represent different components in analogous pathways.

cellular tethering proteins and elucidation of their roles in retroviral DNA integration have led to the design of chimeric tethers that can direct integration to predetermined sites in host chromosomes. As might be expected, such progress has elicited important new questions to be addressed in the future.

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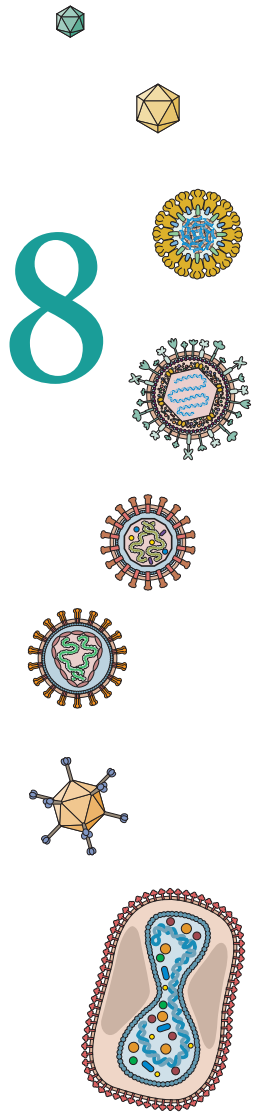
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8 Synthesis of RNA from DNA Templates

Introduction
- Properties of Cellular RNA Polymerases That Transcribe Viral DNA
- Some Viral Genomes Must Be Converted to Templates Suitable for Transcription

Transcription by RNA Polymerase II
- Regulation of RNA Polymerase II Transcription
- Common Properties of Proteins That Regulate Transcription

The Cellular Machinery Alone Can Transcribe Viral DNA Templates

Viral Proteins That Govern Transcription of Viral DNA Templates
- Patterns of Regulation
- The Human Immunodeficiency Virus Type 1 Tat Protein Autoregulates Transcription
- The Transcriptional Cascades of DNA Viruses
- Entry into One of Two Alternative Transcriptional Programs

Transcription of Viral Genes by RNA Polymerase III
- The VA-RNA I Promoter
- Regulation of VA-RNA Gene Transcription

Inhibition of the Cellular Transcriptional Machinery

Unusual Functions of Cellular Transcription Components

A Viral DNA-Dependent RNA Polymerase

Perspectives

References

LINKS FOR CHAPTER 8

⏭ *Video: Interview with Dr. Arnold Berk.*
http://bit.ly/Virology_Berk

⏭ *Movie 8.1: Initiation of transcription by RNA polymerase II.*
http://bit.ly/Virology_V1_Movie8-1

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It is possible that nature invented DNA for the purpose of achieving regulation at the transcriptional rather than at the translational level.

A. Campbell, 1967

Introduction

During the infectious cycles of viruses with DNA genomes, viral messenger RNA (mRNA) synthesis must precede production of proteins. In most cases, this step is accomplished by the host cell enzyme that produces cellular mRNA, RNA polymerase II (Table 8.1). This enzyme also transcribes the proviral DNA of retroviruses. The signals that control expression of the genes of these viruses are similar to those of cellular genes. In fact, much of our understanding of the mechanisms of cellular transcription stems from study of viral DNA templates. In contrast, viral RNA polymerases transcribe the large DNA genomes of viruses that replicate in the cytoplasm, such as poxviruses. These enzymes resemble their host cell counterparts in several respects.

The expression of viral genes in a strictly defined, reproducible sequence is a hallmark of cells infected by DNA viruses. In general, enzymes and regulatory proteins needed in smaller quantities are made during the initial period of infection, whereas structural proteins of virus particles are made only after viral DNA synthesis begins. Such orderly gene expression is primarily the result of transcriptional regulation by viral proteins. This pattern is quite different from the continual expression of all viral genes that is characteristic of the infectious cycles of many RNA viruses (Chapter 6). As discussed in this chapter, the elucidation of the molecular strategies that ensure sequential transcription of the genes of DNA viruses has identified a number of common mechanisms executed in virus-specific fashion. As a collateral dividend, we have gained insights into the cellular mechanisms that control progression through the cell cycle.

Properties of Cellular RNA Polymerases That Transcribe Viral DNA

Eukaryotes Have Three Transcriptional Systems

A general feature of eukaryotic cells is the division of transcriptional labor among three DNA-dependent RNA polymerases. These enzymes, designated RNA polymerases I, II, and III, synthesize different kinds of cellular RNA (Table 8.2). RNA polymerase II makes precursors to mRNA, as well as the precursors of small, regulatory RNA molecules (microRNAs, miRNAs; see Chapter 10). The other two enzymes produce stable RNAs, such as ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs). Synthesis of these stable "housekeeping" RNAs must be adjusted to match the rates of cell growth and division. But regulation of mRNA synthesis is crucial for orderly development and differentiation in eukaryotes, as well as for the responses of cells to their environment. The evolution of RNA polymerases with distinct transcriptional responsibilities appears to be a device for maximizing opportunities for control of mRNA synthesis, while maintaining a constant and abundant supply of the RNA species essential for the metabolism of all cells.

Despite their different functions, several of the 12 to 16 subunits of the large eukaryotic RNA polymerases are identical, while others are related in sequence to one another or to subunits of bacterial RNA polymerases. Such conservation of sequence can be attributed to the common biochemical capabilities of the enzymes. These activities include binding of ribonucleoside triphosphate substrates, binding to template DNA and to product RNA, and catalysis of phosphodiester bond formation. The structure of yeast RNA polymerase II revealed that the organization of its active center is similar to that of smaller DNA-dependent RNA polymerases, as well as of enzymes that make DNA from DNA or RNA templates (Fig. 6.4).

PRINCIPLES ***Synthesis of RNA from DNA templates***

- Transcription is the first biosynthetic reaction to occur in cells infected by double-stranded DNA viruses.
- To form a template suitable for transcription, gapped, double-stranded or single-stranded DNA genomes are converted to double-stranded DNA molecules by cellular enzymes; retroviral RNA genomes are converted to double-stranded proviral DNA that is integrated into the cellular genome by viral enzymes.
- Studies in virology led to the identification of elements in DNA that direct pre-mRNA or mRNA synthesis, including promoters and enhancers that are binding sites for components of the transcriptional machinery.
- The cellular transcriptional machinery alone is sufficient to transcribe some viral DNA templates.
- Viral proteins can stimulate transcription of their own transcriptional unit to establish a positive autoregulatory loop or activate transcription of different viral genes.
- Transcription of subsets of viral genes in distinct temporal periods (phases) is a characteristic feature of the reproductive cycles of all viruses with DNA genomes, including bacteriophages. Transitions from one phase to the next depend on viral activators and synthesis of progeny viral genomes.
- Viral proteins that regulate transcription may bind directly to viral promoter sequences or indirectly in association with cellular proteins.
- Some viruses, including the herpesviruses, establish latent infections in which transcription of lytic genes is inhibited and, in some cases, unique latency-associated transcription units are expressed.
- Most viral genes are transcribed by the cellular RNA polymerase II, but some small viral RNAs are produced by RNA polymerase III.
- Suppression of cellular transcription by viral components diverts limited cellular resources to aid viral transcription.

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Table 8.1 Strategies of transcription of viral DNA templates

Origin of transcriptional components	Virus
Host only	Retroviruses with simple genomes, caulimoviruses
Host plus one viral protein	
The viral protein transcribes late genes	Bacteriophages T3 and T7
The viral protein regulates transcription	Parvoviruses, papillomaviruses, polyomaviruses, retroviruses with complex genomes, geminiviruses
Host plus several viral proteins	Adenoviruses, bacteriophage T4, herpesviruses
Viral	Poxviruses

Transcription of cellular and viral genes requires not only template-directed synthesis of RNA but also correct interpretation of DNA punctuation signals that mark the sites at which transcription must start and stop. Initiation of transcription comprises recognition of the point at which copying of the DNA should begin, the **initiation site**, and synthesis of the first few phosphodiester bonds in the RNA. During the elongation phase, nucleotides are added rapidly to the 3′ end of the nascent RNA, as the transcriptional machinery reads the sequence of a gene. When termination sites are encountered, both the RNA product and the RNA polymerase are released from the DNA template. Purified RNA polymerases I, II, and III perform the elongation reactions *in vitro* but are incapable of specific initiation of transcription without the assistance of additional proteins.

Cellular RNA Polymerases II and III Transcribe Viral Templates

Viral mRNAs or their precursors (pre-mRNAs) are made by RNA polymerase II in cells infected by DNA viruses with both small and large genomes, such as polyomaviruses and herpesviruses, respectively. This enzyme also synthesizes the precursors to viral, as well as cellular, miRNAs. However, it can also carry out at least one reaction unique to virus-infected cells, the transcription of an RNA template by RNA polymerase II to produce hepatitis delta satellite virus genomes and mRNA (Table 8.2).

Some animal viral DNA genomes also encode small, noncoding RNAs that are made by RNA polymerase III. This phenomenon was initially observed in human cells infected by adenovirus, but RNA polymerase III transcription units are present in the genomes of other viruses (Table 8.2).

Table 8.2 Eukaryotic RNA polymerases synthesize different classes of cellular and viral RNA

	RNAs synthesized[a]	
Enzyme	**Cellular**	**Viral**
RNA polymerase I	Pre-rRNA	None known
RNA polymerase II	Pre-mRNA	Pre-mRNA and mRNA
	Pri-miRNA	Pri-miRNA
	snRNAs	HDV genome RNA and mRNA
RNA polymerase III	Pre-tRNAs	Ad2 VA-RNAs
	5S rRNA	EBV EBER RNAs
	U6 snRNA	MHV68 pre-miRNA

[a]Ad2, adenovirus type 2; EBER, Epstein-Barr virus-encoded small RNA; EBV, Epstein-Barr virus; HDV, hepatitis delta virus; MHV68, murine gammaherpesvirus 68; pri-miRNA, primary transcripts containing precursor to miRNAs; snRNA, small nuclear RNA.

Some Viral Genomes Must Be Converted to Templates Suitable for Transcription

All viral DNA molecules must enter the infected cell nucleus to be transcribed by cellular RNA polymerases, but there is considerable variation in the reactions needed to produce templates that can be recognized by the cellular machinery. Some viral genomes are double-stranded DNA molecules that can be transcribed as soon as they reach the nucleus. Transcription of specific genes is therefore the first biosynthetic reaction in cells infected by adenoviruses, herpesviruses, papillomaviruses, and polyomaviruses. Other viral DNA genomes must be converted from the form in which they enter the cell to double-stranded molecules that serve as transcriptional templates (Fig. 8.1). The hepadnaviral genome is an incomplete circular DNA molecule with a large gap in one strand that is repaired by cellular enzymes to form a fully double-stranded DNA molecule. Similarly, single-stranded genomes such as that of the adenovirus-associated virus, a parvovirus, are converted to double-stranded molecules by a cellular DNA polymerase (Chapter 9). The prerequisites for expression of retroviral genetic information are even more demanding, for the (+) strand RNA genome must be both converted into viral DNA and integrated into the cellular genome. Reverse transcription creates an appropriate double-stranded DNA template that includes the signals needed for its recognition by components of the cellular transcriptional machinery (Chapter 7).

The cellular templates for transcription by RNA polymerase II are DNA sequences packaged in chromatin, which contains the conserved histones and many other proteins. The fundamental structural unit of chromatin is the nucleosome, which comprises ~140 bp of DNA wrapped around an octamer containing two copies each of histones H2A, H2B, H3, and H4. The posttranslational modifications of the histones help distinguish highly condensed, transcriptionally silent heterochromatin from transcriptionally active genes. As the organization of DNA into nucleosomes can both block recognition of regulatory sequences and impose barriers to transcriptional elongation, numerous proteins that regulate transcription function by overcoming such obstacles.

Many viral DNA genomes transcribed by RNA polymerase II are also organized by cellular nucleosomes. Because they are integrated into the cellular genome, the proviral DNA templates for retroviral transcription are organized into chromatin indistinguishable from that of the host cell. The DNA genomes of papillomaviruses and polyomaviruses enter cells as "minichromosomes" in which the viral DNA is bound to

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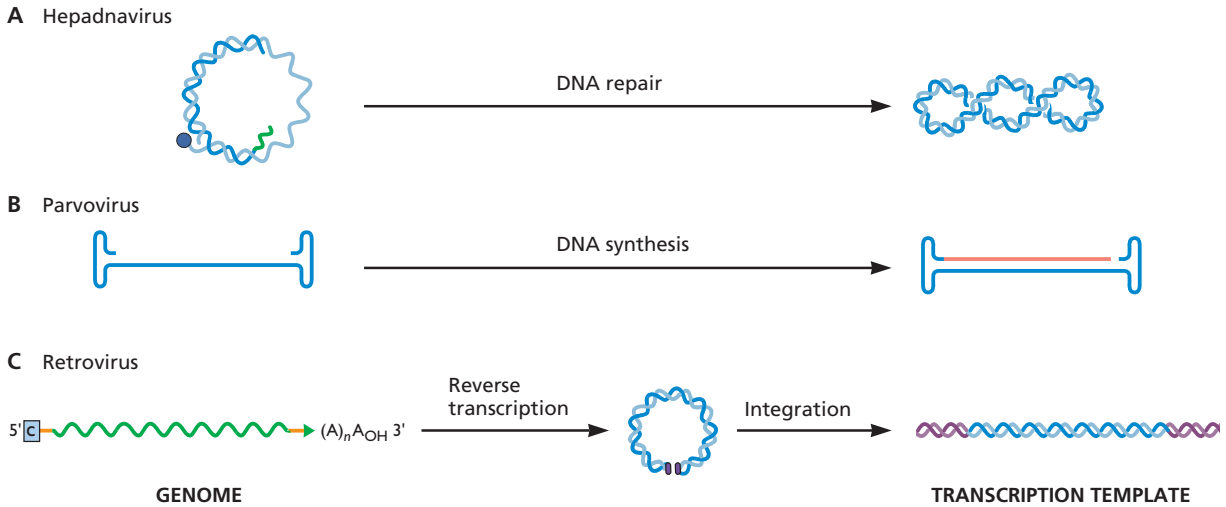


Figure 8.1 Conversion of viral genomes to templates for transcription by RNA polymerase. (A) Hepadnaviral templates for transcription are closed, circular, double-stranded DNA molecules. The mechanisms by which such DNA is formed by repair of the partially double-stranded, gapped DNA genomes are not well understood. **(B)** The single-stranded DNA genomes of parvoviruses such as adenovirus-associated virus carry an inverted terminal repetition with a free 3′ OH end. Copying of the viral genome from this primer by cellular DNA polymerase produces a double-stranded template for transcription. **(C)** Viral enzymes catalyze the conversion of retroviral (+) RNA genomes to double-stranded DNA and its subsequent integration into the host cell genome (proviral DNA) (see Chapter 7).

BOX 8.1

METHODS

Association of histones and other proteins with DNA in vivo: *the chromatin immunoprecipitation assay*

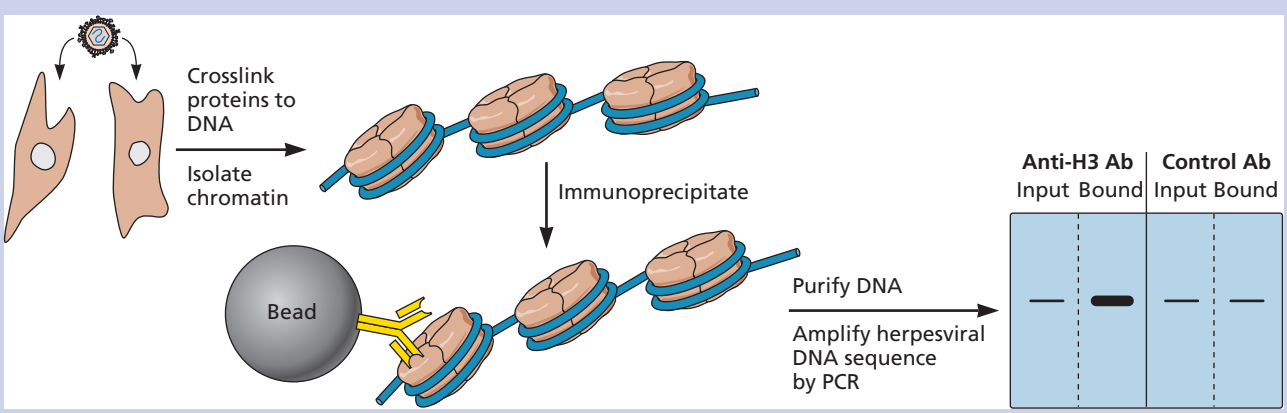
The chromatin immunoprecipitation (ChIP) assay is widely used to investigate the association of specific proteins with viral or cellular DNA within cells. Proteins are initially cross-linked to DNA by exposure of intact cells to formaldehyde. Following cell lysis and fragmentation of the DNA by sonication or enzymatic digestion, DNA fragments bound to the protein of interest are isolated by immunoprecipitation with antibodies against that protein. Enrichment for the DNA sequences of interest in the immunoprecipitate is then assessed by PCR amplification of specific sequences or by high-throughput DNA sequencing.

Application of this assay to herpes simplex virus type 1-infected cells, as illustrated, has demonstrated that histone H3 binds to immediate-early, early, and late genes in entering, but not in newly replicated, viral genomes. However, nucleosomes do not organize viral DNA into a regular structure like that of cellular chromatin, in which the histone octamers are spaced at regular intervals on DNA.

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Kwiatkowski DL, Thompson HW, Bloom DC. 2009. The polycomb group protein Bmi1 binds to the herpes simplex virus 1 latent genome and maintains repressive histone marks during latency. *J Virol* **83:** 8173–8181.

Oh J, Fraser NW. 2008. Temporal association of the herpes simplex virus genome with histone proteins during a lytic infection. *J Virol* **82:**3503–3537.



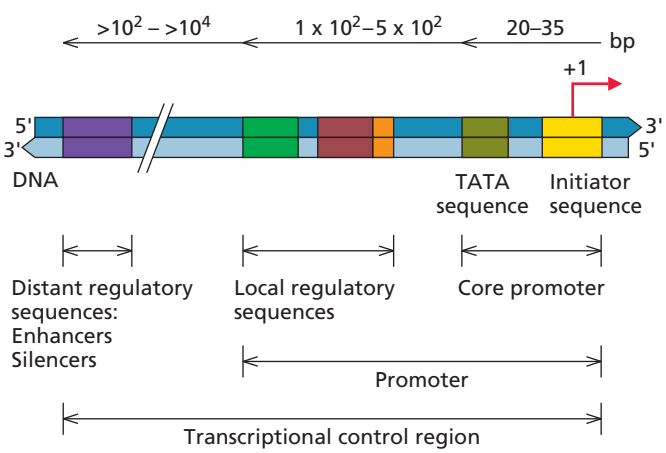
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nucleosomes, whereas histones become associated with herpesviral genomes soon after their entry into infected cell nuclei (Box 8.1). Such nucleosomal organization suggests that mechanisms analogous to those regulating transcription of cellular chromatin are likely to operate on these viral templates. Indeed, as we shall see, the properties of viral "chromatin" can result in transcriptional silencing and prevent transcription of the majority of viral genes in cells latently infected by some herpesviruses. Although transcription of viral DNA templates associated with histones is a common phenomenon, it is not universal: the initial templates for adenoviral gene expression are nucleoproteins comprising the linear, double-stranded DNA genome and the major core protein of the viral particle, protein VII.

Transcription by RNA Polymerase II

Accurate initiation of transcription by RNA polymerase II is directed by specific DNA sequences located near the site of initiation and called the **promoter** (Fig. 8.2). The promoter and the additional DNA sequences that govern transcription make up the **transcriptional control region**. These sequences of DNA viruses and retroviruses were among the first to be examined experimentally. For example, the human adenovirus type 2 major late promoter was the first from which accurate initiation of transcription was reconstituted *in vitro* (Box 8.2). Subsequently, the study of viral transcription yielded fundamental information about the mechanisms by which RNA polymerase II transcription is initiated and regulated.

Figure 8.2 RNA polymerase II transcriptional control elements. The site of initiation is represented by the red arrow drawn in the direction of transcription on the nontranscribed DNA strand, a convention used throughout this text. The core promoter comprises the minimal sequence necessary to specify accurate initiation of transcription. The TATA sequence is the binding site for TfIId (Box 8.3), and the initiator is a sequence sufficient to specify initiation at a unique site. The activity of the core promoter is modulated by local regulatory sequences typically found within a few hundred base pairs of the initiation site. The location of these sequences upstream of the TATA sequence as shown is common, but such sequences can also lie downstream of the initiation site. Distant regulatory sequences that stimulate (enhancers) or repress (silencers) transcription are present in a large number of transcriptional control regions.



Biochemical studies using model transcriptional control regions, such as the adenoviral major late promoter, established that initiation of transcription is a multistep process. The initiation reactions include promoter recognition, unwinding of the duplex DNA around the initiation site to form an open initiation complex, and movement of the transcribing complex away from the promoter (promoter clearance) (Fig. 8.3 and Movie 8.1: http://bit.ly/Virology_V1_Movie8-1). At least 40 proteins, which comprise RNA polymerase II itself and auxiliary initiation proteins, are needed to complete the intricate process of initiation. Our understanding of the functions of these proteins and of the DNA sequences that control initiation is based largely on *in vitro* systems or simple assays for detecting gene expression within cells. Application of these methods has identified a very large number of transcriptional control sequences. Fortunately, all of them can be assigned to one of the three functionally distinct regions identified in Fig. 8.2.

Core promoters of viral and cellular genes contain all the information necessary for recognition of the site of initiation and assembly of precisely organized **preinitiation complexes**. These assemblies contain RNA polymerase II and a common set of general initiation proteins required for accurate and precise initiation. A hallmark of many core RNA polymerase II promoters is the presence of a TA-rich **TATA** sequence 20 to 35 bp upstream of the site of initiation (Fig. 8.2 and 8.4), which is recognized by the TATA-binding protein (Tbp) (Box 8.3). Short sequences, termed **initiators**, which specify accurate (but inefficient) initiation of transcription in the absence of any other promoter sequences, are also commonly found (Fig. 8.4).

Many of the interactions among components of the transcriptional machinery take place before a promoter is encountered: RNA polymerase II is present in cells in extremely large assemblies that contain the initiation proteins, as well as others that are essential for transcription or its regulation. Such assemblies, termed **holoenzymes**, appear to be poised to initiate transcription as soon as they are recruited to a promoter.

Regulation of RNA Polymerase II Transcription

Numerous patterns of gene expression are necessary for eukaryotic life: some RNA polymerase II transcription units must be expressed in all cells, whereas others are transcribed only during specific developmental stages or in specialized differentiated cells. Many others must be maintained in an almost silent state, from which they can be activated rapidly in response to specific stimuli, and to which they can be returned readily. Transcription of viral genes is also regulated during the infectious cycles of most of the viruses considered in this chapter. Large quantities of viral proteins for assembly of progeny virions must be made within a finite (and often short) infectious cycle. Consequently, some viral genes must be transcribed at higher rates than others. In many cases, viral genes are transcribed in a specific and stereotyped temporal sequence. Such regulated transcription is achieved in part by

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BOX 8.2

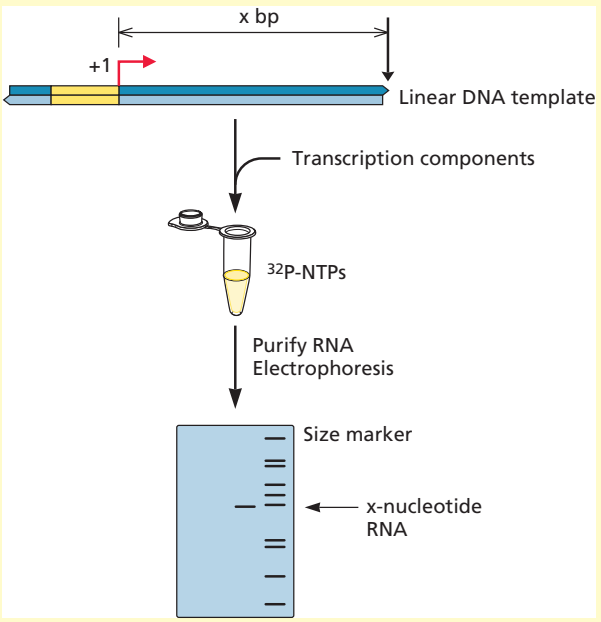
EXPERIMENTS

Mapping of a human adenovirus type 2 initiation site and accurate transcription in vitro

When cellular RNA polymerase II was identified in 1969, investigators had access only to preparations of total cellular DNA, and nothing was known about the organization of eukaryotic transcription units. Consequently, the genomes of the DNA viruses simian virus 40 and human adenovirus type 2 served as valuable resources for investigation of mechanisms of transcription. Indeed, it was detailed information about a particular adenoviral transcription unit that finally allowed biochemical studies of the mechanism of initiation. In 1978, the site at which major late transcription begins was mapped precisely, by determining the sequence of the 5′ end of the RNA transcript. This knowledge was exploited to develop a simple assay for accurate initiation of transcription, the "runoff" assay, using a linear template that includes a transcription initiation site shown in the figure. Purified RNA polymerase II produced no specific transcripts in the runoff assay, but unfractionated nuclear extracts of human cells were shown to contain all the components necessary for accurate initiation of transcription.

Weil PA, Luse DS, Segall J, Roeder RG. 1979. Selective and accurate initiation of transcription at the Ad2 major late promoter in a soluble system dependent on purified RNA polymerase II and DNA. *Cell* **18:**469–484.

Ziff EB, Evans RM. 1978. Coincidence of the promoter and capped 5′ terminus of RNA from the adenovirus 2 major late transcription unit. *Cell* **15:**1463–1475.



In this simple assay, linear DNA templates are prepared by restriction endonuclease cleavage (black arrow), a known distance, *x* bp, downstream of the initiation site (+1). When the template is incubated with the transcriptional machinery and nucleoside triphosphate (NTP) substrates, transcription initiated at position +1 continues until the transcribing complex "runs off" the linear template. Specific transcription is therefore assayed as the production of ^{32}P-labeled RNA *x* nucleotides in length. This runoff transcription assay is convenient and has been used to assess both specificity and efficiency of transcription.

means of cellular control mechanisms, for example, cellular proteins that repress transcription. In general, however, viral proteins are critical components of the circuits that establish orderly transcription of viral genes.

Recognition of Local and Distant Regulatory Sequences

Both local and distant sequences can control transcription from core promoters. However, local sequences are often sufficient for proper transcriptional regulation. These local regulatory sequences are recognized by sequence-specific DNA-binding proteins (Fig. 8.5), a property first demonstrated with the simian virus 40 early promoter. An enormous number of sequence-specific proteins that regulate transcription are now known, many first identified through analyses of viral promoters. Unfortunately, the nomenclature applied to these regulatory proteins presents serious difficulties for both writer and reader, for it is unsystematic and idiosyncratic (Box 8.4).

Efficient transcription of many viral and cellular genes also requires more distant regulatory sequences in the DNA template, which possess properties that were entirely unanticipated. The first such **enhancer**, so named because it stimulated transcription to a large degree, was discovered in the genome of simian virus 40. Enhancers are defined by their position- and orientation-independent stimulation of transcription of homologous and heterologous genes over distances as great as 10,000 bp in the genome. Despite these unusual properties, enhancers are built with binding sites for the proteins that recognize local promoter sequences.

The Simian Virus 40 Enhancer: a Model for Viral and Cellular Enhancers

The majority of viral DNA templates described in this chapter contain enhancers that are recognized by cellular DNA-binding proteins. The simian virus 40 enhancer has been studied intensively, and its properties and mechanism of action are characteristic of many enhancers, whether of viral or cellular origin.

The simian virus 40 enhancer is built from three units, termed enhancer elements, which are subdivided into

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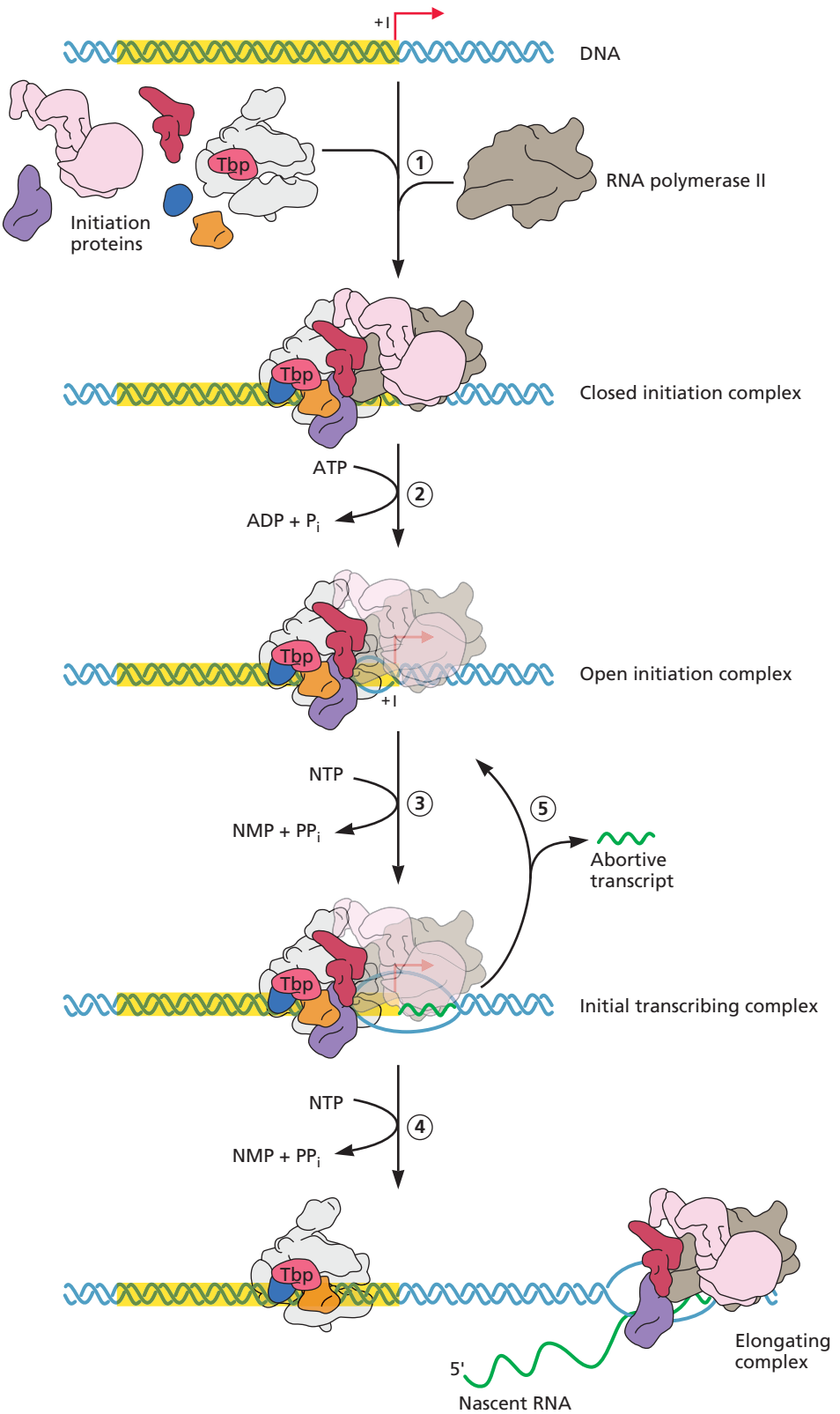


Figure 8.3 Initiation of transcription by RNA polymerase II. Assembly of the closed initiation complex (step 1) is followed by unwinding of the DNA template in the region spanning the site of initiation (step 2). RNA polymerase II then synthesizes short transcripts (less than 10 to 15 nucleotides) by template-directed incorporation of nucleotides (step 3). The initial transcribing complex is thought to be conformationally strained, because RNA polymerase II remains in contact with promoter-bound initiation proteins as it synthesizes short RNAs. The severing of these contacts allows the transcribing complex to escape from the promoter and proceed with elongation (step 4). This promoter clearance step is often inefficient, with abortive initiation (step 5) predominating. In the latter process, initial transcripts are released, reforming the open initiation complex. The initial elongating transcriptional complex contains some but not all of the proteins that form the preinitiation complex, as well as proteins that stimulate elongation (not shown). Structural data collected for RNA polymerase II and initiation proteins associated with nucleic acid has been used to produce a movie of initiation and elongation (Movie 8.1: http://bit.ly/Virology_V1_Movie8-1), adapted from A. C. Cheung and P. Cramer, *Cell* **149:**1431–1437, 2012, with permission.

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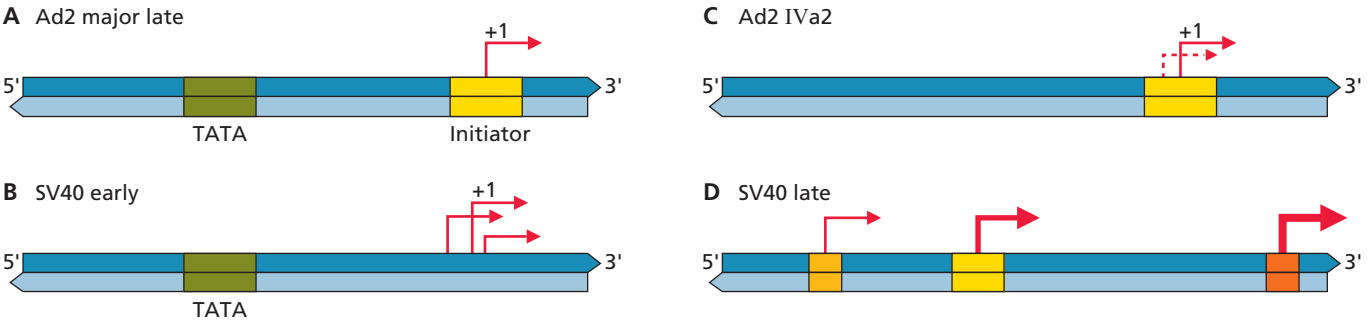


Figure 8.4 Variations in core RNA polymerase II promoter architecture. Variations in promoter architecture are illustrated using four viral promoters represented as in Fig. 8.2. The TATA or initiator sequences of the different promoters are not identical in DNA sequence. In the case of the simian virus 40 (SV40) late transcription unit **(D)**, each of the sites of initiation is included within a DNA sequence resembling those of initiators. It has not been shown experimentally that all actually function as autonomous initiator sequences. The relative frequencies with which different initiation sites in a single promoter are used are indicated by the thickness of the red arrows. Ad2, adenovirus type 2.

BOX 8.3

BACKGROUND

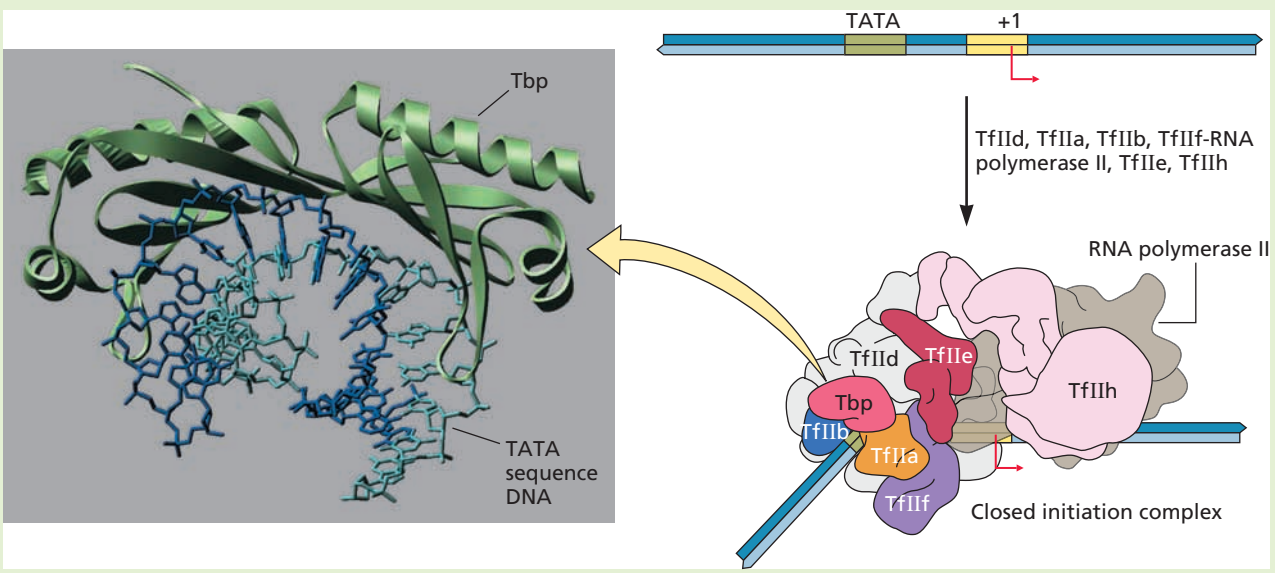
The RNA polymerase II closed initiation complex

The closed initiation complex is shown on a promoter that contains both a TATA and an initiator sequence (e.g., the adenovirus major late promoter). The TfIId protein contains a subunit that recognizes the TATA sequence (Tbp) and 8 to 10 additional subunits. The X-ray crystal structures of DNA-bound Tbp, such as that of *Arabidopsis thaliana*, bound to the adenoviral major late TATA sequence shown in the inset (courtesy of S. K. Burley, The Rockefeller University), revealed that this protein induces sharp bending of the DNA. One popular hypothesis is that such bending facilitates interactions among proteins bound to regulatory sequences located upstream of the TATA sequence and the basal transcriptional machinery. TfIId is required for transcription from all RNA polymerase II promoters. It can recognize those that lack TATA sequences by various mechanisms. TfIIh supplies DNA-dependent ATPase and helicase activities essential for transcription and a kinase that phosphorylates the C-terminal segment of the largest subunit of RNA polymerase II. The depictions of the transcription initiation proteins are based on visualization of initiation by cryo-electron microscopy.

Buratowski S, Hahn S, Guarente L, Sharp PA. 1989. Five intermediate complexes in transcription initiation by RNA polymerase II. *Cell* **56:**549–561.

He Y, Fang J, Taatjes DJ, Nogales E. 2013. Structural visualization of key steps in human transcription initiation. *Nature* **495:**481–486.

Kim JL, Nikolov DB, Burley SK. 1993. Co-crystal structure of TBP recognizing the minor groove of a TATA element. *Nature* **365:**520–527.



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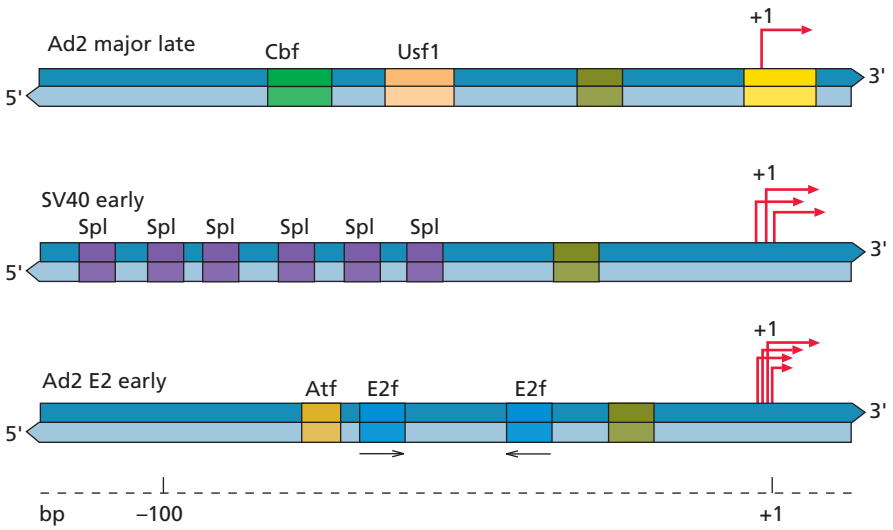


Figure 8.5 Local regulatory sequences of three viral transcriptional control regions. The TATA sequences, initiator sequences, and sites of transcription initiation are depicted as in Fig. 8.4. The local regulatory sequences of each promoter, which are recognized by the cellular DNA-binding proteins listed, are drawn to the scale shown at the bottom, where +1 is the major initiation site. The black arrows below the adenovirus type 2 (Ad2) E2 early promoter indicate the orientation of the E2 factor (E2f)-binding sites. Atf, cyclic AMP-dependent transcription factor; Cbf, CCAAT-binding factor; Sp1, stimulatory protein 1; Usf1, upstream stimulatory factor 1.

smaller sequence motifs recognized by DNA-binding proteins (Fig. 8.6). The DNA-binding proteins that interact with this viral enhancer are differentially produced in different cell types. For example, nuclear factor κb (Nf-κb) and certain members of the octamer-binding protein (Oct) family are enriched in cells of lymphoid origin, and their binding sites are necessary for enhancer activity in these cells. Other elements of the enhancer, such as the activator protein 1 (Ap1)-binding sites, confer responsiveness to cellular signaling pathways. This constellation of enhancer elements ensures transcription of the viral early gene and initiation of the viral infectious cycle in many different cellular environments. This property is exhibited by several other viral enhancers, including those present in adenoviral and herpesviral genomes and the proviral DNA of avian retroviruses. In contrast, some viral templates for RNA polymerase II transcription contain enhancers that are active only in a specific cell type, only in the presence of viral proteins, or only under particular metabolic conditions (Box 8.5).

The simian virus 40 enhancer is located within 200 bp of the transcription initiation site, but enhancers of cellular genes are typically found thousands or tens of thousands of base pairs up- or downstream of the promoters that they regulate. The most popular model of the mechanism by which these sequences exert remote control of transcription, the DNA-looping model, invokes interactions among enhancer-bound proteins and the transcriptional components assembled at the promoter, with the intervening DNA looped out. Compelling evidence in favor of this model has been collected by using the simian virus 40 enhancer (Box 8.6). These regulatory sequences can also facilitate access of the transcriptional machinery to chromatin templates. For example, the simian virus 40 enhancer contains DNA sequences that induce formation of a nucleosome-free region of the viral genome in infected cells. Enhancers can,

BOX 8.4

TERMINOLOGY

The idiosyncratic nomenclature for sequence-specific DNA-binding proteins that regulate transcription

When proteins that bind to specific promoter sequences to regulate transcription by RNA polymerase II were first identified, no rules for naming mammalian proteins (or the genes encoding them) were in place. Consequently, the names given by individual investigators were based on different properties of the protein.

- Some names indicate the function of the regulator, e.g., the glucocortoid receptor (Gr) and serum response factor (Srf).
- Some names indicate the promoter sequence to which the protein binds, e.g., cyclic AMP response element (CRE)-binding protein (Creb) and octamer-binding protein 1 (Oct-1).
- Some names are based on the promoter in which binding sites for the regulator were first identified, e.g., adenovirus E2 transcription factor (E2f).
- Some names report some very general property of the regulator, e.g., stimulatory protein 1 (Sp1 [the first sequence-specific activator to be identified]), upstream stimulatory factor (Usf), and host cell factor (Hcf).

Such inconsistency, coupled with the universal use of acronyms, can mystify rather than inform: the historical origins of the names of transcriptional regulators are not known to most readers. The subsequent recognition that many "factors" are members of families of closely related proteins compounds such difficulties.

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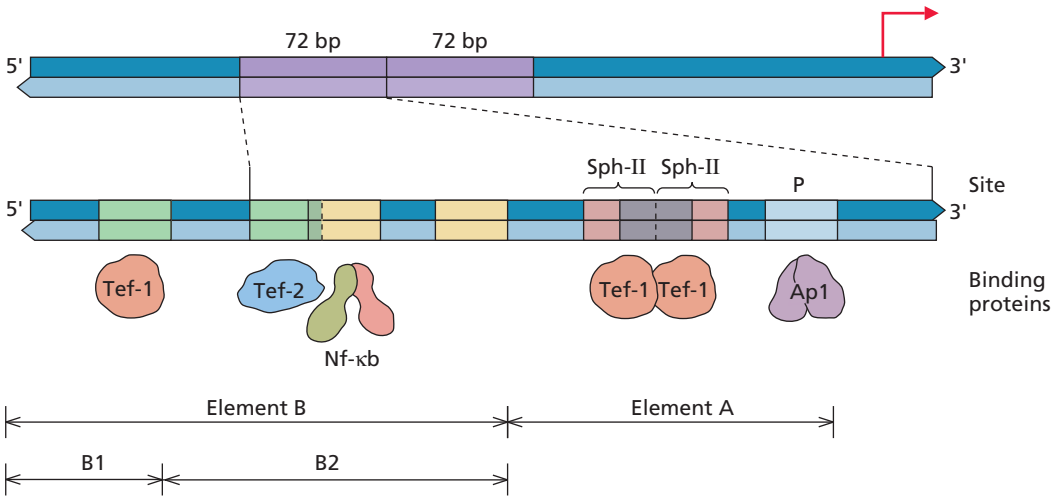


Figure 8.6 Organization of the archetypal simian virus 40 enhancer. The positions of the 72-bp repeat region containing the enhancer elements are shown relative to the early promoter at the top. Shown to scale below are functional DNA sequence units of the early promoter-distal 72-bp repeat and its 5′ flanking sequence, which forms part of enhancer element B, and the proteins that bind to them. All the protein-binding sites shown between the expansion lines are repeated in the promoter-proximal 72-bp repeat. The complete enhancer contains one copy of the enhancer element B1 and two directly repeated copies of the enhancer elements B2 and A. Some enhancer elements are built from repeated binding sites for a single sequence-specific protein. For example, cooperative binding of transcriptional enhancer factor (Tef)-1 to the two Sph-II sequences forms a functional enhancer element. Such cooperative binding renders enhancer activity sensitive to small changes in the concentration of a single protein. A second class of enhancer elements comprises sequences bound by two different proteins, as illustrated by the sequences bound by Tef-1 and Tef-2: binding is not cooperative, but these proteins interact once bound to DNA to form an active enhancer element. Ap1, activator protein 1.

BOX 8.5

DISCUSSION

Host cell metabolism can regulate viral enhancers

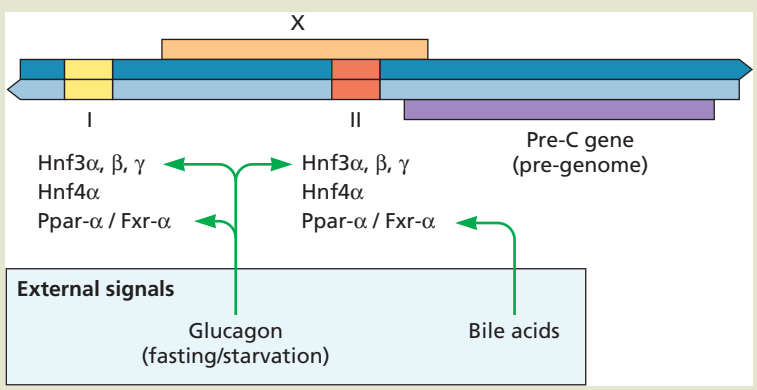
The hepatitis B virus genome contains two enhancers (I and II) that control transcription of viral genes (see figure). Enhancer I is bound by several ubiquitous transcriptional activators, as well as by activators that are present only in hepatocytes or enriched in these cells. Activation of enhancer II, which controls synthesis of the pregenome RNA and pre-C mRNA (Appendix, Fig. 11), requires the prior function of enhancer I. Enhancer II is recognized by multiple liver-enriched transcriptional activators including hepatocyte nuclear factor (Hnf) 3 family members, Hnf4, and the farnesol X and peroxisome proliferator-activated receptors (Fxr and Ppar-α, respectively).

The constellation of hepatocyte-specific or -enriched proteins that confer activity upon enhancers I and II accounts for the tropism of hepatitis B virus for the liver. However, this organ is a major metabolic hub: its many metabolic functions include synthesis of glucose in response to low concentrations of this sugar in the blood, synthesis of cholesterol and bile salts, and deamination of amino acids. Several of the proteins that govern hepatitis B virus transcription are also important regulators of metabolism, and hence sensitive to the metabolic status of the host. For example, Ppar-α is activated under conditions of fasting or starvation to promote gluconeogenesis and capture of energy by fatty acid catabolism. Consequently, this metabolic state also stimulates transcription from hepatitis B virus promoters and viral reproduction. Poor nutritional status may therefore directly promote disease caused by hepatitis B virus.

Bar-Yishay I, Shaul Y, Shlomai A. 2011. Hepatocyte metabolic signalling pathways and regulation of hepatitis B virus expression. *Liver Int* **31:**282–290.

Shlomai A, Paran N, Shaul Y. 2006. PGC-1α controls hepatitis B virus through nutritional signals. *Proc Natl Acad Sci U S A* **103:**16003–16008.

The segment of the hepatitis B virus genome containing enhancers I and II, the coding sequence for protein X, and the 59 end of the pre-C coding sequence are shown to scale. Hepatocyte-specific or -enriched proteins that bind the enhancers are listed below, as are metabolic signals that induce their activity (green arrows).



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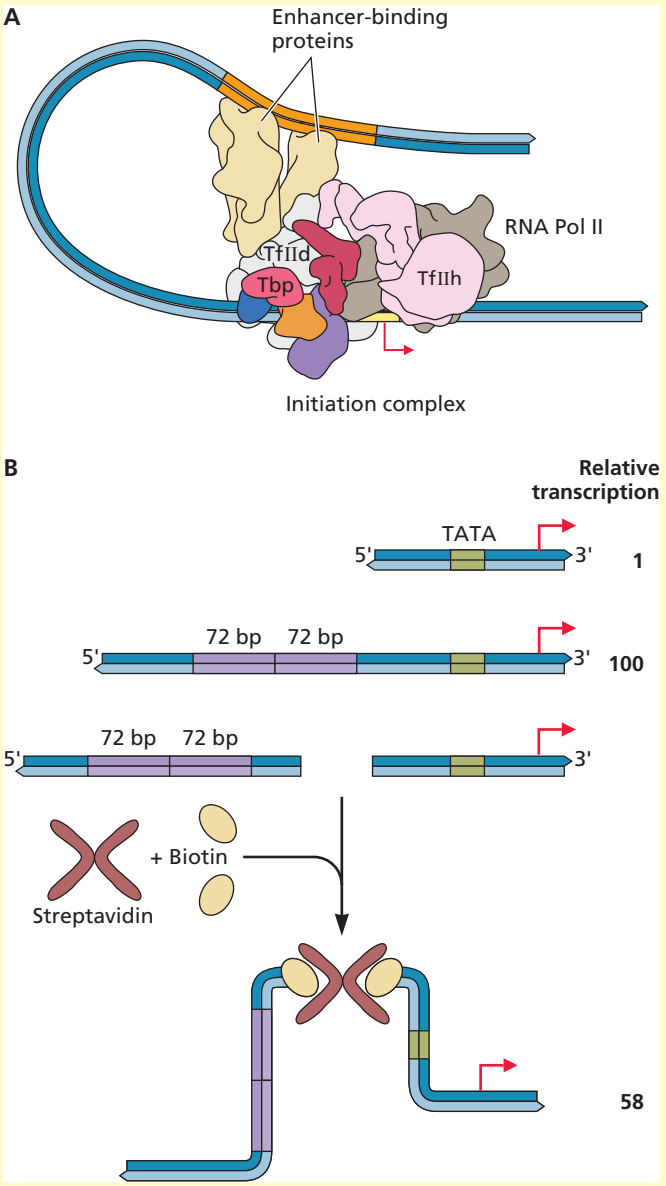
BOX 8.6

EXPERIMENTS

Mechanisms of enhancer action

(A) The DNA-looping model postulates that proteins bound to a distant enhancer (orange), here shown upstream of a gene, interact directly with the components of the transcription initiation complex, with the intervening DNA looped out. Such interactions might stabilize the initiation complex and therefore stimulate transcription. **(B)** An enhancer noncovalently linked to a promoter via a protein bridge is functional. When placed upstream of the rabbit β-globin gene promoter in a circular plasmid, the simian virus 40 enhancer stimulates specific transcription *in vitro* by a factor of 100. In the experiment summarized here, the enhancer and promoter were separated by restriction endonuclease cleavage. Under this condition, the enhancer cannot stimulate transcription. Biotin was added to the ends of each DNA fragment by incorporation of biotinylated UTP. Biotin binds the protein streptavidin noncovalently, but with extremely high affinity (K_d, 10^{-15} M). Because streptavidin can bind four molecules of biotin, its addition to the biotinylated DNA fragments allows formation of a noncovalent protein "bridge" linking the enhancer and the promoter. Under these conditions, the viral enhancer stimulates *in vitro* transcription almost as efficiently as when present in the same DNA molecule, as summarized in the column on the right. This result indicated that an enhancer can stimulate transcription when present in a separate DNA molecule (i.e., in *trans*) and ruled out models in which enhancers are proposed to serve as entry sites for RNA polymerase II. The results of this experiment are therefore consistent with the looping model shown in panel A.

Müller HP, Sogo JM, Schaffner W. 1989. An enhancer stimulates transcription in *trans* when attached to the promoter via a protein bridge. *Cell* **58**:767–777.



therefore, stimulate RNA polymerase II transcription by multiple molecular mechanisms. The primary effect of these mechanisms is to increase the probability that the gene to which an enhancer is linked will be transcribed.

Common Properties of Proteins That Regulate Transcription

Cellular, sequence-specific transcriptional regulators play pivotal roles in expression of viral genes. However, the genomes of many viruses also encode additional regulatory proteins. The cellular and viral DNA-binding proteins necessary for transcription from viral DNA templates share a number of common properties. Their most characteristic feature is modular organization: they are built from discrete structural and functional domains. The basic modules are a DNA-binding domain and an activation domain, which function as independent units. Other common properties include binding to DNA as dimers (Fig. 8.7).

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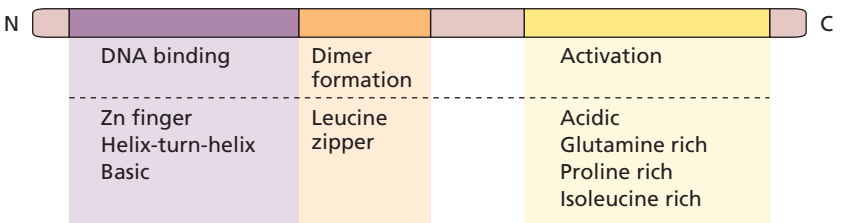


Figure 8.7 Modular organization of sequence-specific transcriptional activators. Common functional domains of eukaryotic transcriptional regulators are shown at the top, with some of the types of each domain listed below. DNA-binding and activation domains are defined by their structure (e.g., Zn finger or helix-turn-helix) and chemical makeup (e.g., acidic, glutamine rich), respectively. Transcriptional activators are often more complex than illustrated here. They can contain two activation domains, as well as regulatory domains, such as ligand-binding domains, and the various domains may be located at different positions with respect to the N and C termini of the protein.

Regulation of transcription by sequence-specific DNA-binding proteins usually requires additional proteins termed **coactivators** or **corepressors**. In general, these proteins cannot bind specifically to DNA, nor can they modulate transcription on their own. However, once recruited to a promoter by interaction with a DNA-bound protein, they dramatically augment (or damp) transcriptional responses. Coactivators can cooperate with multiple, sequence-specific activators and stimulate transcription from many promoters. A common property of many coregulators is their ability to alter the structure of nucleosomal templates, including viral templates, either directly or by interaction with appropriate enzymes. Several coactivators are histone acetyltransferases that catalyze the addition of acetyl groups to specific lysine residues in histones. This class includes p300, which was first identified by virtue of its interaction with adenoviral E1A proteins. Such histone acetyltransferases, and the deacetylases associated with corepressors, help establish the patterns of histone posttranscriptional modifications that distinguish transcriptionally active chromatin (Box 8.7). A second class of coactivators, exemplified by members of the Swi/Snf family, contain ATP-dependent chromatin-remodeling enzymes that alter the way in which DNA is bound to nucleosomes. It is thought that the coordinated action of these two classes of enzymes makes nucleosomal DNA accessible for both transcription initiation and elongation.

The ability of the RNA polymerase II system to mediate many patterns of transcription stems, in part, from the variety in both the nature of core promoters and the constellations of sequence-specific proteins and coactivators that govern their activity. Equally important is the power of the transcriptional machinery to integrate signals from multiple, promoter-bound regulators. This machinery must also be able respond to environmental cues, such as those provided by circulating hormones or growth factors. The proteins that control transcription are therefore frequently regulated by mechanisms that govern their activity, availability, or intracellular concentration. These mechanisms include modulation of the phosphorylation (or other modification) of specific amino acids, which can determine how well a protein binds to DNA, its **oligomerization** state, or the properties of its regulatory domain(s). In some cases, the intracellular location of a sequence-specific DNA-binding protein, or its association with inhibitory proteins, is modulated. Autoregulation of expression of the genes encoding transcriptional regulators is also common. This brief summary illustrates the varied repertoire of mechanisms available for regulation of transcription of viral templates by RNA polymerase II. Not surprisingly, virus-infected cells provide examples of all items on this menu, with the added zest of virus-specific mechanisms.

The Cellular Machinery Alone Can Transcribe Viral DNA Templates

In cells infected by many retroviruses, the components of the cellular transcriptional machinery described in the previous section complete the viral transcriptional program without the assistance of **any** viral proteins. The proviral DNA created by reverse transcription of retroviral RNA genomes and integration comprises a single RNA polymerase II transcription unit organized into chromatin, exactly like the cellular templates for transcription. Its transcription therefore produces a single viral RNA, which serves as both the genome and the source of viral mRNA species. Because the genomes of these retroviruses do not encode transcriptional regulators, the rate at which proviral DNA is transcribed is determined by the constellation of cellular transcription proteins present in an infected cell. This rate may be influenced by the nature and growth state of the infected cell, as well as by the organization of cellular chromatin containing the proviral DNA. Nevertheless, transcription of viral genetic information can occur throughout the lifetime of the host cell, indeed even in descendants of the cell initially infected. This strategy for transcription of viral DNA is exemplified by avian sarcoma and leukosis viruses, such as Rous-associated viruses. The long terminal repeat (LTR) of

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BOX 8.7

DISCUSSION

The histone code hypothesis

In eukaryotic cells, genomic DNA is organized and highly compacted by histones and many other proteins in chromatin. Transcriptionally active DNA is present in a less condensed form called **euchromatin**. It has been known for decades that the nucleosomal histones present in euchromatin are enriched in acetylated residues, and it is now clear that complex patterns of histone posttranslational modification govern the properties of chromatin.

Residues in the N-terminal tails of the four core histones of the nucleosome (H2A, H2B, H3, and H4) are subject to a variety of posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitinylation, and sumoylation of specific residues. Panel A of the figure shows the positions of some of the known modifications of this segment of histone H3. The large number of possible modifications of numerous residues results in a far greater number of combinations. For example, mass spectrometry has identified more than 200 combinations present in different molecules of human histone H3, just 2 of which are shown in panel B.

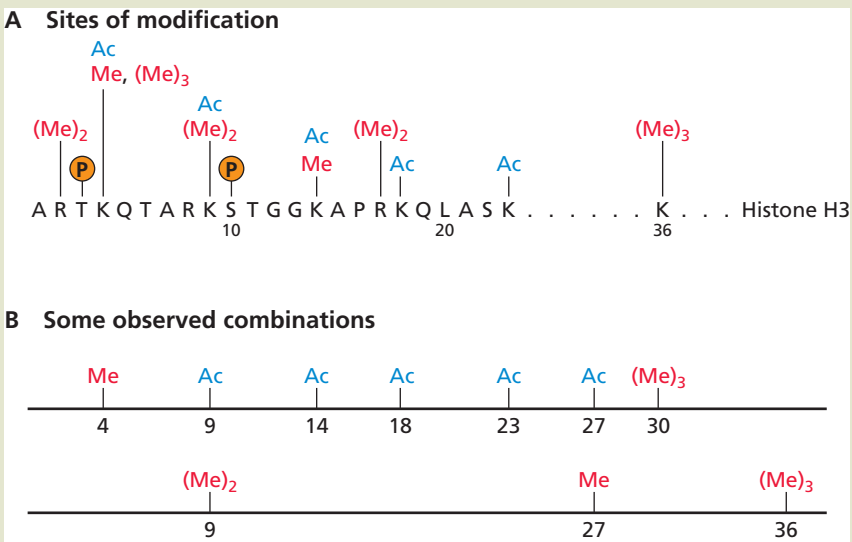
It was initially proposed that particular combinations of posttranscriptionally modified histones could identify transcriptionally active or inactive DNA. Consistent with this "histone code" hypothesis, some combinations are characteristic of transcriptionally active genes. Furthermore, modified amino acids carrying posttranslational modifications serve as recognition sites for proteins that further modify histones, modify DNA, remodel nucleosomes, or facilitate transcription by other mechanisms. Although the idea of a simple code of histone posttranslational modifications has great appeal, it is now clear that it may be more appropriate to consider this a complex "language": for example, the same modification can recruit either activators or repressors of transcription, probably depending on the cellular or local context. Furthermore, histone modifications are dynamic, changing, for example, during transcriptional elongation or from one transcriptional cycle to another.

Berger SL. 2007. The complex language of chromatin regulation during transcription. *Nature* **447:**407–412.

Garcia BA, Pesavento JJ, Mizzen CA, Kelleher NL. 2007. Pervasive combinational modification of histone H3 in human cells. *Nat Methods* **4:**487–489.

Jenuwein T, Allis CD. 2001. Translating the histone code. *Science* **293:**1074–1079.

Young NL, DiMaggio PA, Plazas-Mayorca MD, Baliban RC, Floudas CA, Garcia BA. 2009. High throughput characterization of combinatorial histone codes. *Mol Cell Proteomics* **8:**2266–2284.



these proviral DNAs contains a compact enhancer located immediately upstream of the viral promoter (Fig. 8.8). The avian and mammalian serum response proteins that bind to the enhancer also recognize a specific sequence in the promoter. The other proteins that bind to this enhancer are all members of a family defined by a "leucine zipper" motif responsible for dimerization (Fig. 8.7).

The most remarkable property of the avian retroviral transcriptional control region is that it is active in many different cell types of both the natural avian hosts and mammals. This unusual feature can be explained by the widespread distribution of the cellular proteins that bind to it. Nevertheless, transcription of proviral DNA is not an inevitable consequence of integration. Rather, it can be blocked or impaired by specific cellular proteins that induce repressive histone (and DNA) modifications, and hence epigenetic silencing of proviral transcription (Box 8.8). As discussed in Volume II, Chapter 3, such inhibition of proviral transcription is but one example of intrinsic antiviral defense mechanisms.

Because the LTRs are direct repeats of one another (Fig. 8.8), transcription directed by the 3′ LTR extends into cellular DNA and cannot contribute to the expression of retroviral genetic information. In fact, the transcriptional control region of the 3′ LTR is normally inactivated by a process called **promoter occlusion**: the passage of transcribing complexes initiating at the 5′ LTR through the 3′ LTR prevents recognition of the latter by enhancer- and promoter-binding proteins. Occasionally, transcription from the 3′ LTR **does** occur, with profound consequences for the host cell (see Volume II, Chapter 6).

Absolute dependence on cellular components for the production of viral transcripts avoids the need to devote limited viral genetic capacity to transcriptional regulatory proteins. Nevertheless, such a strategy is the exception, not the rule.

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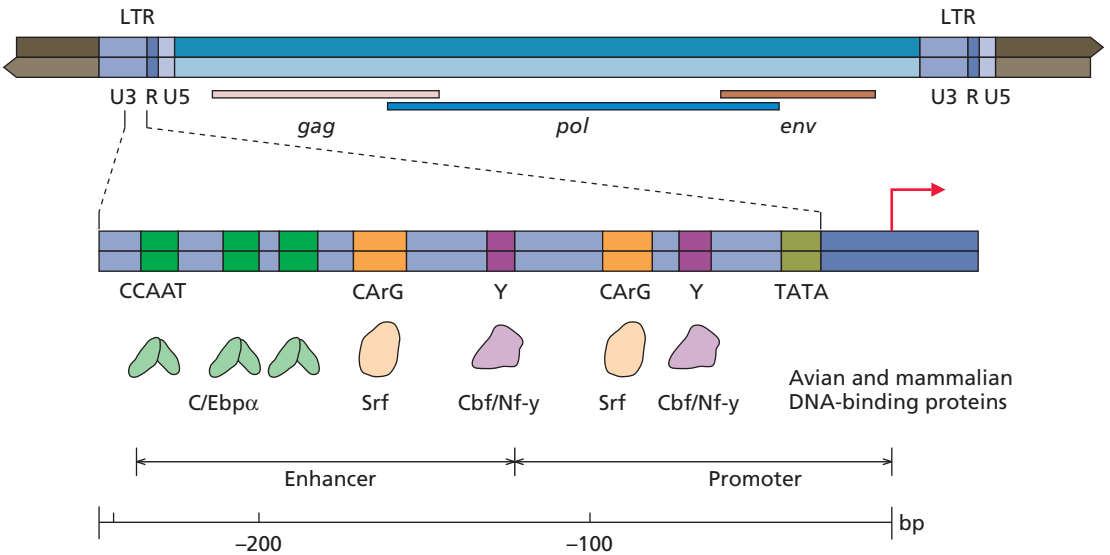


Figure 8.8 Widespread cellular transcriptional activators of an avian retrovirus. The proviral DNA of an avian leukosis virus is shown at the top. The enhancer and promoter present in the U3 regions of the LTRs are drawn to scale below. Each of the multiple CCAAT, CArG, and Y box sequences, which are required for maximally efficient transcription, is recognized by the proteins listed below, which are present in both avian and mammalian cells. Nf-y, nuclear transcription factor Y; Srf, serum response factor.

Viral Proteins That Govern Transcription of Viral DNA Templates

Patterns of Regulation

Transcription of many viral DNA templates by the RNA polymerase II machinery results in the synthesis of large quantities of viral transcripts (in some cases, more than 10^5 copies of individual mRNA species per cell) in relatively short periods. Such bursts of transcription are elicited by viral proteins that stimulate RNA polymerase II transcription and establish one of two kinds of regulatory circuits (Fig. 8.9). The first is a **positive autoregulatory loop**, epitomized by transcription of human immunodeficiency virus type 1 proviral DNA (Fig. 8.9A). A viral activating protein stimulates the rate of transcription but does not alter the complement of viral proteins made in infected cells. The second is a **transcriptional cascade**, in which different viral transcription units are activated in an ordered sequence (Fig. 8.9B). This mechanism, which ensures that different classes of viral proteins are made during different periods of the infectious cycle, is characteristic of viruses with DNA genomes. The participation of viral proteins confers a measure of control lacking when the transcriptional program is executed solely by cellular components. The following sections describe some well-studied examples of the regulatory circuits established by viral proteins.

The Human Immunodeficiency Virus Type 1 Tat Protein Autoregulates Transcription

The proteins of retroviruses with complex genomes are encoded in a single proviral transcription unit controlled by an LTR enhancer and promoter. However, in addition to the common structural proteins and enzymes, these genomes encode auxiliary proteins, including transcriptional regulators. Some of these proteins, such as the Tax protein of human T-lymphotropic virus type 1, resemble activators of other virus families, and stimulate transcription from a wide variety of viral and cellular promoters. Others, exemplified by the transactivator of transcription (Tat) of human immunodeficiency virus type 1, are unique: they recognize an RNA element in nascent transcripts.

In principle, the positive feedback loop that is established once a sufficient concentration of Tat has accumulated in an infected cell is simplicity itself (Fig. 8.9A). Cellular proteins initially direct transcription of the proviral DNA in infected cells at some basal rate: among the processed products of the primary viral transcript are the spliced mRNAs from which the Tat protein is synthesized; this protein is imported into the nucleus, where it stimulates transcription of the proviral template upon binding to its RNA recognition site in nascent viral transcripts. However, the molecular mechanisms that establish this autostimulatory loop are sophisticated and unusual. Their elucidation has been an important area of research, because the Tat protein is essential for virus propagation and represents a valid target for antiviral therapy.

Cellular Proteins Recognize the Human Immunodeficiency Virus Type 1 LTR

Cellular proteins that bind to the LTR enhancer and promoter proteins support a low rate of proviral transcription

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BOX 8.8

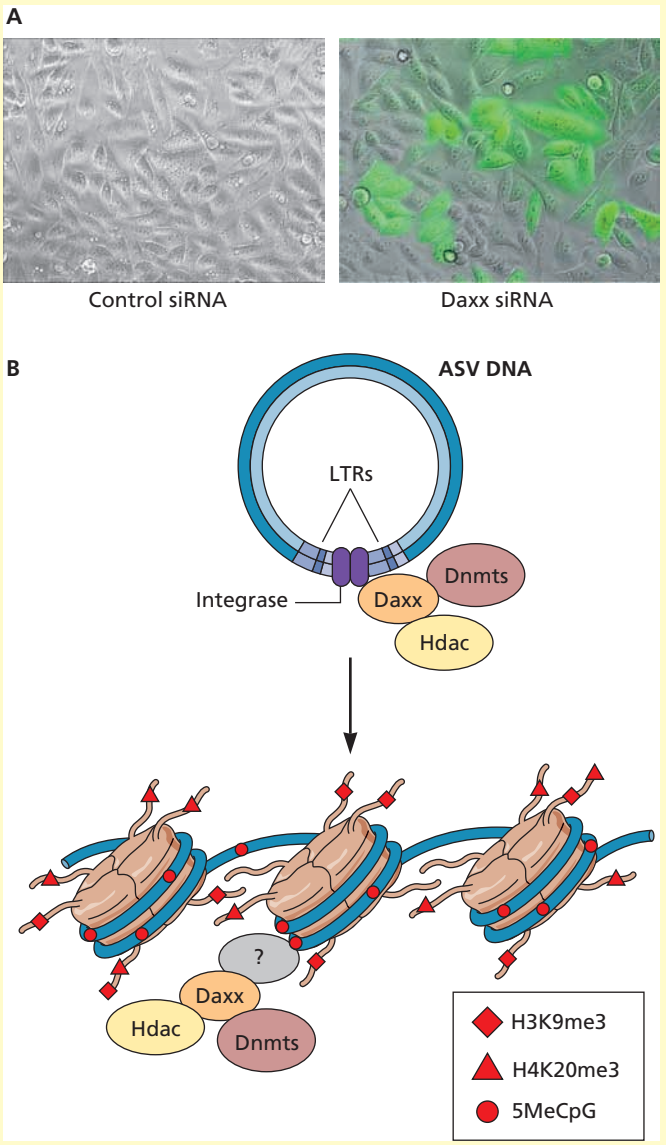
EXPERIMENTS

Epigenetic silencing of integrated proviral DNAs

It is well established that expression of exogenous genes introduced into cells in culture via retroviral vectors can gradually become inhibited, and that human immunodeficiency virus type I proviral DNA is maintained in a transcriptionally latent state in resting T lymphocytes. These phenomena illustrate the fact that integration of a proviral DNA into the host cell genome does **not** guarantee transcription of viral genetic information. Such repression is mediated, at least in part, by epigenetic mechanisms that are important for silencing of expression of cellular genes during differentiation and development. These include the addition of repressive posttranslational modifications to nucleosomal histones (see Box 8.7) and methylation of cytosine in DNA to form 5^{Me}CpG. For example, avian sarcoma proviral DNAs are subjected to rapid epigenetic silencing in mammalian cells but not in natural avian host cells. This observation indicated that one or more mammalian proteins promote an antiviral defense that represses expression of avian proviral DNAs. One such candidate protein, human death domain-associated protein 6 (Daxx), was identified by virtue of its binding to the viral integrase in a yeast two-hybrid screen and in infected cells.

Subsequent studies exploited avian sarcoma viruses that carried a green fluorescent protein (GFP) reporter gene to facilitate analysis of proviral expression and repression. It was observed that

- Daxx was not required for early events in avian sarcoma virus replication, but viral reporter gene expression was increased significantly when synthesis of Daxx in human cells was inhibited by RNA interference (RNAi), and in murine $Daxx^{-/-}$ cells
- the histone deacetylases Hdac1 and Hdac2 were associated with viral DNA in Daxx-producing but not in $Daxx^{-/-}$ cells, as assessed by chromatin immunoprecipitation
- in populations of human cells in which proviral LTR promoters were silenced and heavily methylated, knockdown of Daxx by RNAi induced expression of GFP reporter genes (panel A), as did inhibition of synthesis of specific DNA methyltransferases (Dnmts)
- in such silenced cells, Daxx and Dnmts were associated with one another (coimmunoprecipitation) and with proviral promoters (chromatin immunoprecipitation), and Daxx knockdown also substantially reduced methylation of proviral promoter DNA



The indicated siRNAs were introduced into HeLa cells that harbored avian sarcoma viral (SV) DNA that carried a silent GFP reporter gene. (A) Images of transfected cells were taken using a fluorescent microscope 96 h thereafter. siRNA-mediated knockdown of cellular Daxx protein resulted in release of epigenetic gene silencing, as shown by reactivation of expression of the reporter gene. Courtesy of Andrey Poleshko and A. Skalka, Fox Chase Cancer Center. **(B)** Models for the initiation and maintenance of retroviral silencing by Daxx via recruitment of Hdacs and Dnmts (top and bottom, respectively). Adapted from N. Shalginskikh et al., *J Virol* **87:** 2137–2150, 2013, with permission.

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Based on these and other observations, it has been proposed that Daxx associates with the viral integrase prior to proviral integration to recruit Dnmts and enzymes that catalyze formation of repressive chromatin (panel B).

Transcription of human immunodeficiency virus type 1 proviruses is also silenced in resting $CD4^+$ T lymphocytes, a reservoir of "invisible" infected cells that complicates treatment (see Volume II, Chapter 7). As discussed in the text, the sequestration in the cytoplasm of Nf-κb, which is necessary for efficient initiation of transcription, contributes to the silencing of LTR-dependent transcription, as does DNA methylation. The establishment of such latent human immunodeficiency virus proviruses has also been studied using viruses carrying genes encoding fluorescent reporter proteins. The LTRs of latent proviral DNAs were shown to be associated with histone deacetylases and nucleosomes carrying repressive posttranslational modifications. Proviral reporter gene expression upon activation of the T cells correlated not only with reversal of such repressive chromatin modification and concomitant association of RNA polymerase II with the LTR, but also with substantial increases in the nuclear concentration of p-Tefb.

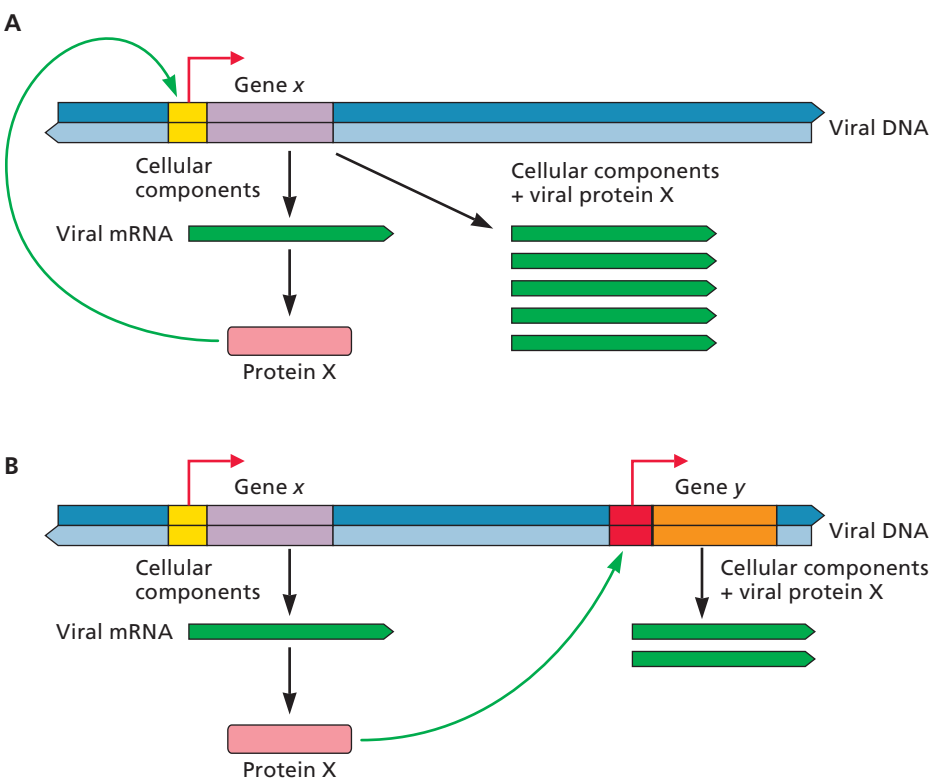
Greger JG, Katz RA, Ishov AM, Maul GG, Skalka AM. 2005. The cellular protein Daxx interacts with avian sarcoma virus integrase and viral DNA to repress viral transcription. *J Virol* **79:**4610–4618.

Shalginskikh N, Poleshko A, Skalka AM, Katz RA. 2013. Retroviral DNA methylation and epigenetic repression are mediated by the antiviral host protein Daxx. *J Virol* **87:**2137–2150.

Tyagi M, Pearson RJ, Karn J. 2010. Establishment of HIV latency in primary $CD4^+$ cells is due to epigenetic transcriptional silencing and P-TEFb restriction. *J Virol* **84:**6425–6437.

before Tat is made in infected cells. In contrast to avian retroviruses, human immunodeficiency virus type 1 propagates efficiently in only a few cell types, notably $CD4^+$ T lymphocytes and cells of the macrophage/monocyte lineage. Viral reproduction (i.e., transcription) in infected T cells in culture is stimulated by T cell growth factors, indicating that viral transcription requires cellular components available only in such stimulated T cells. Indeed, the failure of the virus to propagate efficiently in unstimulated T cells correlates with the absence of active forms of particular enhancer-binding proteins. The distribution of cellular enhancer-binding proteins is therefore an important determinant of the host range of retroviruses with both simple and complex genomes. However, the transcription of the provirus of retroviruses with

Figure 8.9 Mechanisms of stimulation of transcription by viral proteins. Cellular transcriptional components acting alone transcribe the viral gene encoding protein X. Once synthesized and returned to the nucleus, viral protein X can stimulate transcription either of the same transcription unit **(A)** or of a different one **(B)**. In either case, viral protein X acts in concert with components of the cellular transcriptional machinery.



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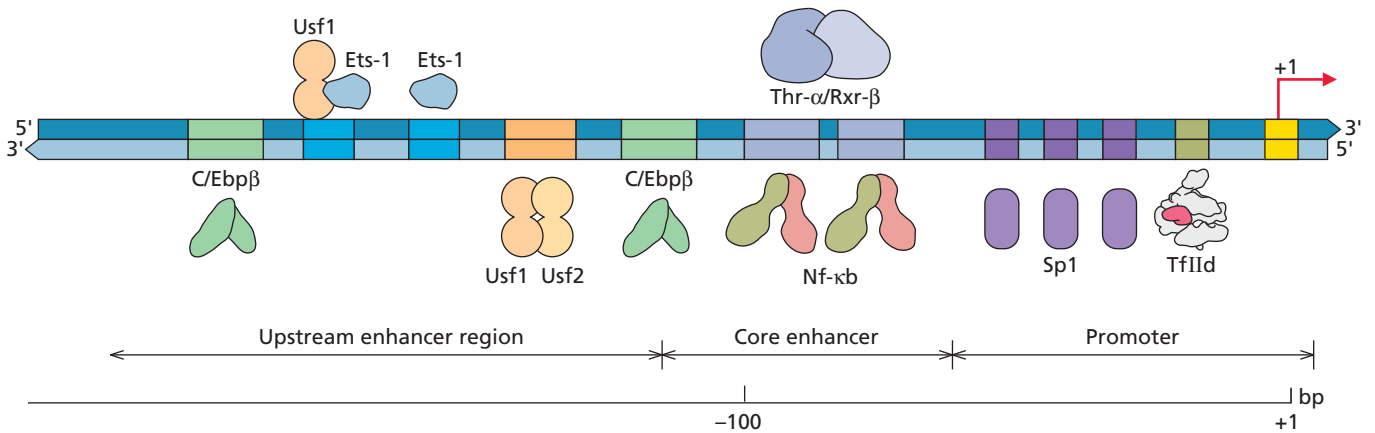


Figure 8.10 Cell-type-specific regulators bind to the transcriptional control region of human immunodeficiency virus type 1. The organization of the U3 region of the proviral LTR is shown to scale, with proteins that bind to promoter or enhancer sequences indicated above or below the DNA. Activation of C/Ebpβ (also known as NfIL6) stimulates viral gene expression in macrophages, as does T3rα-1/Rxr-β, while the other enhancer-binding proteins shown stimulate transcription in T cells. Not all the binding sites shown are well conserved in different viral isolates. Ets-1, protein C-ets1; Rxr-β, retinoic acid receptor-β; Thrα, thyroid hormone receptor-α.

simple genomes, such as the avian retroviruses, depends on proteins that are widely distributed, whereas human immunodeficiency virus type 1 transcription requires proteins that are found in only a few cell types or active only under certain conditions.

Within the human immunodeficiency virus type 1 LTR, the promoter is immediately preceded by two important regulatory regions (Fig. 8.10), termed the core and upstream enhancers, that are necessary for efficient viral transcription in both peripheral blood lymphocytes and certain T cell lines. Both the core and the upstream enhancers are densely packed with binding sites for cellular proteins, many of which are enriched in the types of cell in which the virus can reproduce. These proteins were typically identified because they stimulated transcription from the LTR in transient-expression assays. Because these assays do not reproduce physiological conditions (Box 8.9), a positive result establishes only that a certain protein **can** stimulate transcription, not that it normally does so. However, it is now clear that many of these proteins **do** stimulate viral transcription and replication in the types of cell in which human immunodeficiency virus type I can reproduce, for example, Ets-1 and C/Ebpβ in T lymphocytes and monocytes/macrophages, respectively.

Regulation of viral transcription by cellular pathways is exemplified by the critical role of the transcriptional activator Nf-κb in replication of human immunodeficiency virus type 1 in T cells (Fig. 8.11). Unstimulated T cells display no Nf-κb activity, because the protein is retained in an inactive form in the cytoplasm by binding of inhibitory proteins of the Iκb family. Growth factors that activate T cells trigger signal transduction cascades that lead to phosphorylation and subsequent degradation of these inhibitors by the cytoplasmic multiprotease complex (the **proteasome**). Consequently, Nf-κb is freed for transit to the nucleus, where it can bind to its recognition sites within the viral LTR core enhancer (Fig. 8.10). This pathway can account for the induction of human immunodeficiency virus type 1 transcription observed when T cells are stimulated. The severe, or complete, inhibition of virus reproduction (transcription) in normal human $CD4^+$ T lymphocytes caused by mutations in the Nf-κb-binding sites emphasizes the importance of activation of this cellular protein in the infectious cycle of the virus. Nevertheless, Nf-κb and the other cellular proteins that act via LTR enhancer- or promoter-binding sites do not support efficient expression of viral genes: this process depends on synthesis of the viral Tat protein, as discussed in the next section.

A characteristic feature of human immunodeficiency virus type 1 infection of individuals is a period of clinical latency in which few symptoms are manifested. The provirus can be considered dormant in infected cells that lack the constellation of active enhancer-binding proteins necessary to allow synthesis of small quantities of Tat mRNA and protein, and hence induction of the positive autoregulatory loop (Fig. 8.9A). Nevertheless, during clinical latency, virus is produced continuously in cells that contain the necessary cellular proteins, because the positive autoregulatory circuit is triggered whenever infected cells can support LTR enhancer-dependent transcription of proviral DNA: symptoms develop when the patient's immune system can no longer maintain effective countermeasures (Volume II, Chapter 7).

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BOX 8.9

WARNING

Caution: transient-expression assays do not reproduce conditions within virus-infected cells

Transient-expression assays (see figure) provide a powerful, efficient way to investigate regulation of transcription. Advantages include the following:

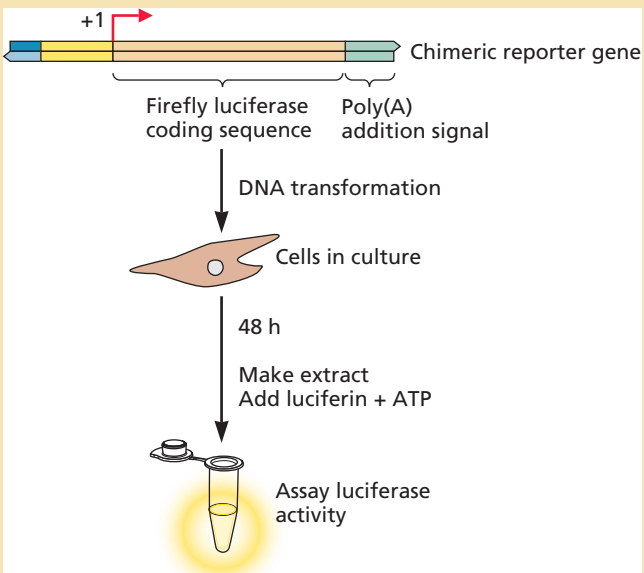
- simplicity and sensitivity of assays for reporter gene activity
- ready analysis of mutated promoters to identify DNA sequences needed for the action of the regulatory protein
- application with chimeric fusion proteins and synthetic promoters to avoid transcriptional responses due to endogenous cellular proteins
- simplification of complex regulatory circuits to focus on the activity of a single protein

Despite these advantages, transient-expression assays do not necessarily tell us how transcription is regulated in virus-infected cells, because they do not reproduce normal intracellular conditions. Important differences include the following:

- abnormally high concentrations of exogenous template DNA: concentrations of reporter genes as high as 10^6 copies per cell are not unusual. This value is significantly greater than even the maximal concentrations of viral DNA molecules attained toward the end of an infectious cycle, up to 10^3 and 10^4 copies/cells in the case of alphaherpesviruses and adenovirus, respectively
- abnormally high concentrations of the regulatory protein as a result of its deliberate overproduction
- the potential for spurious interactions of the viral protein with template, or cellular components, because of these high concentrations of template and protein
- the absence of viral components that might negatively or positively modulate the activity of the protein under study

The last three caveats apply to any experiment in which a viral protein is overproduced, for example, for investigation of its interactions with other proteins.

Because of their inherent limitations, models of regulation of viral transcription based on results obtained by exploiting the advantages of transient-expression assays require validation in infected cells.



The transient-expression assay. A segment of DNA containing the transcriptional control region of interest (yellow) is ligated to the coding sequence (orange) of an enzyme not synthesized in the recipient cells to be used, luciferase in this example, and RNA-processing signals such as those specifying polyadenylation (green box). Plasmids containing such chimeric reporter genes are introduced into cells in culture by any one of several methods, including electroporation and incubation with synthetic vesicles containing the plasmid DNA. Within a cell that takes up the plasmid, the DNA enters the nucleus, where the transcriptional control region directs transcription of chimeric RNA. The RNA is processed, exported from the nucleus, and translated by cytoplasmic polyribosomes. The activity of the luciferase enzyme is then assayed, generally 48 h after introduction of the reporter gene. This indirect measure of transcription assumes that it is **only** the activity of the transcriptional control region that determines the concentration of the enzyme. Alternatively, the concentration of the chimeric reporter RNA can be measured.

The Tat Protein Regulates Transcription by Unique Mechanisms

Tat recognizes an RNA structure. Stimulation of human immunodeficiency virus type 1 transcription by Tat requires an LTR sequence, termed the transactivation response (TAR) element, which lies within the transcription unit (Fig. 8.12A). The observation that mutations that disrupted the predicted secondary structure of TAR RNA inhibited Tat-dependent transcription suggested that the TAR element is recognized as RNA. Indeed, the Tat protein binds specifically to a trinucleotide bulge and adjacent base pairs in the stem of the TAR RNA stem-loop structure (Fig. 8.12B). Binding of Tat to this region of TAR induces a local rearrangement in the RNA, resulting in formation of a more stable, compact, and energetically favorable structure (Fig. 8.12D). Recognition of a viral transcriptional control sequence as RNA remains unique to Tat proteins.

Tat stimulates transcriptional elongation. Binding of Tat to TAR RNA stimulates production of viral RNA by as much

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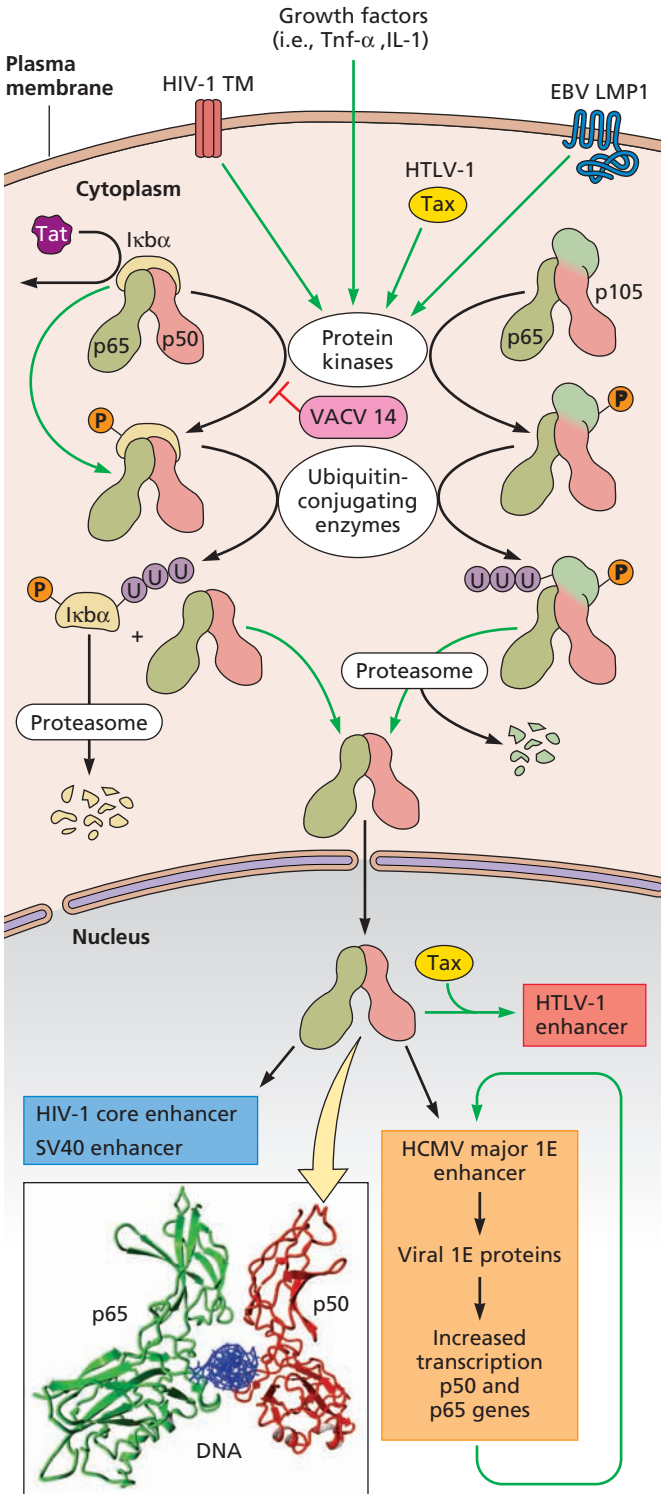


Figure 8.11 The cellular transcriptional regulator Nf-κb and its participation in viral transcription. The members of the Nf-κb–cRel protein family (p50-p65) are defined by the presence of the Rel homology region, which contains DNA-binding and dimerization motifs, and a nuclear localization signal. The p65 (Rel) protein of the p50-p65 heterodimer (left) also contains an acidic activation domain at its C terminus. p50 is synthesized as an inactive precursor, p105 (right). The p105-p65 heterodimer is one of two forms of inactive Nf-κb found in the cytoplasm (e.g., of unstimulated T cells). The second consists of mature p50-p65 heterodimers associated with an inhibitory protein such as Iκbα (left), which blocks the nuclear localization signals of the p50 and p65 proteins. The C-terminal segment of p105 functions like Iκb, with which it shares sequences, to block nuclear localization signals and retain this heterodimer in the cytoplasm. Exposure of the cells to any of several growth factors results in activation (green arrows) of protein kinases that phosphorylate specific residues of Iκb or p105. Upon phosphorylation, Iκb dissociates and is recognized by the system of enzymes that adds branched chains of ubiquitin (Ub) to proteins, a modification that targets them for degradation by the proteasome. Specific p105 cleavage by the proteasome also produces the p50-p65 dimer. Unencumbered Nf-κb dimers produced by either mechanism can translocate to the nucleus, because nuclear localization signals are now accessible. In the nucleus of uninfected cells, Nf-κb binds to specific promoter sequences to stimulate transcription via the p65 activation domain. Viral transcriptional control regions to which Nf-κb binds and some viral proteins that induce activation (green arrows) of Nf-κb are indicated. The X-ray crystal structure of a p50-p65 heterodimer bound specifically to DNA is shown in the inset. The structure is viewed down the helical axis of DNA with the two strands in blue and with the p50 and p65 subunits in red and green, respectively. The dimer makes extensive contact with DNA via protein loops. EBV, Epstein-Barr virus, HCMV, human cytomegalovirus; HIV-1, human immunodeficiency virus type 1; HTLV-1, human T-lymphotropic virus type 1; SV40, simian virus 40; VACV, vaccinia virus. NDB ID: RDR0333 F. E. Chen, D.D. Humag, Y.Q. Chen and G. Ghosh *Nature* **391:**410–413,

as 100-fold. In contrast to many cellular and viral proteins that stimulate transcription, the Tat protein has little effect on initiation. Rather, it greatly improves elongation. Complexes that initiate transcription in the absence of Tat elongate poorly, and many terminate transcription within 60 bp of the initiation site (Fig. 8.13A). The Tat protein overcomes such poor **processivity** of elongating complexes, thereby allowing efficient production of full-length viral transcripts. Consequently, in the absence of Tat, full-length transcripts of proviral DNA account for no more than 10% of the total. This property resolves the paradox of why the human immunodeficiency virus type 1 LTR enhancer and promoter are not sufficient to support efficient viral RNA synthesis.

How Tat stimulates transcriptional elongation. A search for cellular proteins that stimulate viral transcription when bound to the N-terminal region of Tat (Fig. 8.12C) identified the human Ser/Thr kinase p-Tefb (positive-acting transcription factor b), which was known to stimulate elongation of cellular transcripts. This cellular protein is essential for Tat-dependent stimulation of processive viral transcription both *in vitro* and in infected cells. One subunit of the p-Tefb heterodimer is a **cyclin**, cyclin T. Cyclins are so named because members of the family accumulate during specific periods of the cell cycle. Cyclin T regulates the activity of the second subunit of p-Tefb, cyclin-dependent kinase 9 (Cdk9). Tat and

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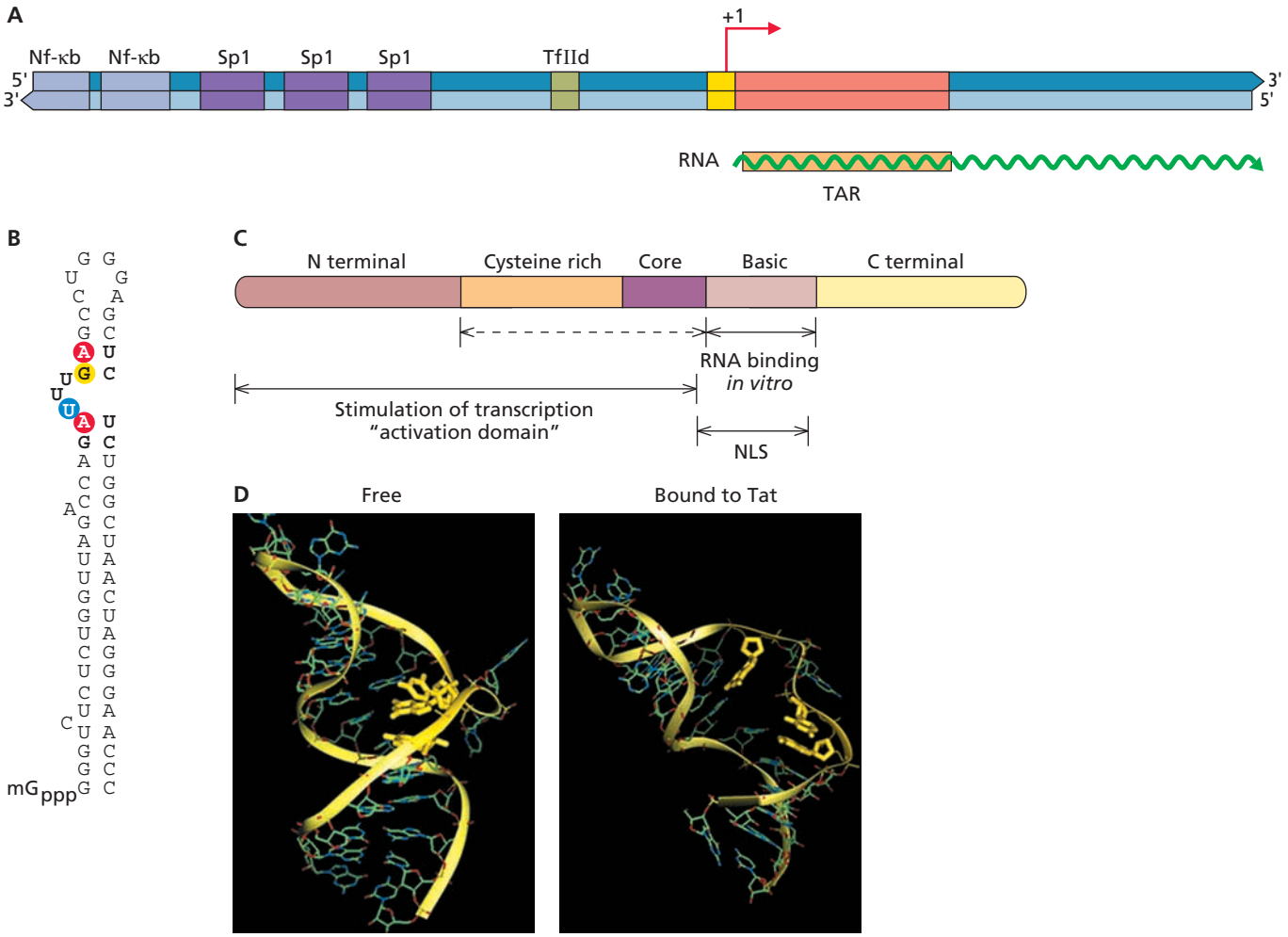


Figure 8.12 Human immunodeficiency virus type 1 TAR and the Tat protein. **(A)** The region of the viral genome spanning the site of transcription initiation is drawn to scale, with the core enhancer and promoter depicted as in Fig. 8.10. The DNA sequence lying just downstream of the initiation site (pink) negatively regulates transcription. Transcription of the proviral DNA produces nascent transcripts that contain the TAR sequence (tan box). **(B)** The TAR RNA hairpin extends from position +1 to position +59 in nascent viral RNA. Sequences important for recognition of TAR RNA by the Tat protein are colored. Optimal stimulation of transcription by Tat requires not only this binding site in TAR but also the terminal loop. **(C)** The Tat protein is made from several different, multiply spliced mRNAs (Appendix, Fig. 29B) and therefore varies in length at its C terminus. The regions of the protein are named for the nature of their sequences (basic, cysteine rich) or greatest conservation among lentiviral Tat proteins (core). Experiments with fusion proteins containing various segments of Tat and a heterologous RNA-binding domain identified the N-terminal segment indicated as sufficient to stimulate transcription. The basic region, which contains the nuclear localization signal (NLS), can bind specifically to RNA containing the bulge characteristic of TAR RNA. However, high-affinity binding, effective discrimination of wild-type TAR from mutated sequences *in vitro*, and RNA-dependent stimulation of transcription within cells require additional N-terminal regions of the protein, shown by the dashed arrow. **(D)** Major groove views of structures of a free TAR RNA corresponding to the apical stem and loop regions (but with a truncated stem) (left) and of the same RNA when bound to the Tat peptide (right) were determined by nuclear magnetic resonance methods. The bases shown in yellow are A22, U23, and G26, which are colored red, blue, and yellow, respectively, in panel B. Note the energetically favorable change in the conformation of the trinucleotide bulge region to a more stable, compact structure on binding of the Tat peptide. From F. Aboul-ela et al., *J Mol Biol* **253:**313–332, 1995, and F. Aboul-ela et al., *Nucleic Acids Res* **24:**3974–3981, 1996, with permission. Courtesy of M. Afshar, RiboTargets, and J. Karn, MRC Laboratory of Molecular Biology.

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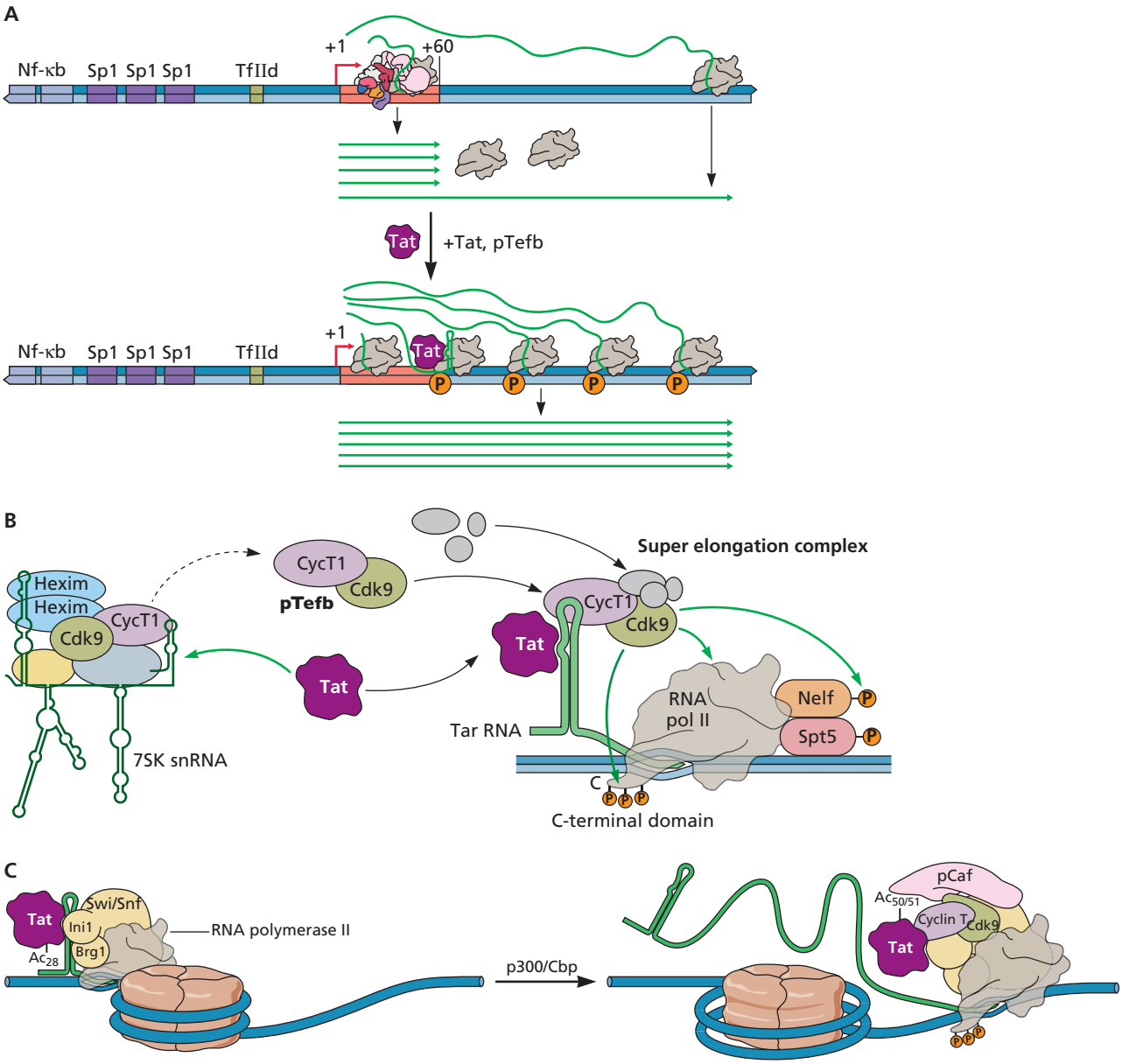


Figure 8.13 Mechanisms of stimulation of transcription by the human immunodeficiency virus type 1 Tat protein. **(A)** Model for the stimulation of elongation. The regulatory sequences flanking the site of initiation of transcription are depicted as in Fig. 8.10. In the absence of Tat, transcription complexes are poorly processive, and the great majority (9 of 10) terminate within 60 bp of the initiation site, releasing transcription components and short transcripts. Production of the Tat protein upon translation of mRNAs spliced from rare, full-length transcripts and its recruitment of p-Tefb and other regulators of elongation to nascent RNA allow transcriptional complexes to pass through the elongation block and synthesis of full-length viral RNA. **(B)** Cooperative binding to TAR of Tat and p-Tefb (via its cyclin T subunit) leads to phosphorylation (P) of the C-terminal domain of the largest subunit of RNA polymerase II by the Cdk9 kinase subunit of p-Tefb. This enzyme also phosphorylates and inactivates negative regulators of transcriptional elongation (e.g., transcription elongation factor [Spt5] and negative elongator factor complex [Nelf]). Positive regulators of elongation, such as RNA polymerase II elongation factor 2 (Ell2), are also recruited to form a super elongation complex. The net result is that transcriptional complexes become competent to carry out highly processive transcription. A second function of Tat (left) is to increase the concentration of p-Tefb available to bind to TAR in infected cells by inducing dissociation of a 7SK small nuclear RNA (snRNA)-containing ribonucleoprotein that sequesters p-Tefb from the transcriptional machinery. Hexim, hexamethylene bis-acetamide-inducible protein. Adapted from M. Ott et al., *Cell Host Microbe* **10:**426–435, 2011, with permission. **(C)** Model for nucleosome remodeling. The initial transcript (green) of proviral DNA (blue line) is depicted with Tat bound to the TAR sequence. Acetylation of Tat at Lys28 is critical for high-affinity binding to TAR and p-Tefb. The nucleosome located a short distance downstream of the initiation site blocks transcriptional elongation, and nucleosome remodeling by the Swi/Snf complex is required for efficient elongation of transcription. Specific subunits of this remodeling complex (e.g., Ini-1 and Brg-1) bind to Tat, but only once this protein is acetylated on Lys50 and Lys51 by the histone acetyltransferases Crebbp/p300 and Gcn5. These modifications also induce dissocation of Tat from TAR, presumably by neutralizing positive charge in the RNA-binding region of the protein.

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p-Tefb bind **cooperatively** to the TAR RNA stem-loop (that is, with higher affinity than either protein alone), and with greater specificity. This property is the result of the interaction of the cyclin T1 subunit of p-Tefb with nucleotides within the TAR RNA loop (Fig. 8.12B) that are not contacted by Tat but are nevertheless crucial for stimulation of transcription.

Assembly of the ternary complex containing TAR RNA, Tat, and p-Tefb promotes elongation of human immunodeficiency virus type 1 transcription in several different ways (Fig. 8.13). This process induces conformational changes that activate the Cdk9 kinase subunit of p-Tefb. Once associated with transcription complexes, the active kinase phosphorylates Ser residues within an unusual domain at the C terminus of the largest subunit of RNA polymerase II, which is hypophosphorylated when RNA polymerase II is present in preinitiation complexes (Fig 8.13B). These modifications are essential for Tat-dependent stimulation of elongation and hence complete proviral transcription. The Cdk9 kinase also modifies and inactivates negative regulators of elongation to facilitate release of paused transcriptional complexes (Fig. 8.13B). Furthermore, via its interaction with p-Tefb, Tat recruits additional proteins that increase the rate of elongation to establish a super elongation complex for viral transcription. The results of experiments in which p-Tefb was inhibited in infected cells, as well as genetic analyses, have established that p-Tefb is essential for Tat-dependent stimulation of viral transcription *in vivo*.

The Tat protein also facilitates human immunodeficiency virus transcription indirectly, by inducing release of p-Tefb from a complex in which it is sequestered in an inactive form (Fig. 8.13B). Such inhibition is mediated by Hexim1 (hexamethylene bis-acetamide-inducible protein) and requires a scaffold provided by a small cellular RNA. The mechanism by which Tat disrupts this complex is not well understood but is thought to include competition with Hexim1 for binding to a segment of the cellular RNA that is structurally similar to TAR RNA.

Tat also facilitates nucleosome remodeling. As discussed previously, integrated proviral DNA templates for transcription are organized in chromatin. Although human immunodeficiency virus type 1 proviral DNA is integrated preferentially into or near transcriptionally active genes of the host cell (Chapter 7), efficient transcription requires reorganization of nucleosomes, which are located at specific positions on this LTR. The promoter and enhancers are nucleosome free, and hence accessible to the transcriptional activators described above. In contrast, a nucleosome is located immediately downstream of the site of initiation of transcription and must be repositioned to allow transcriptional elongation. Cellular transcriptional activators, such as Nf-κb, are important for such remodeling, but this process is facilitated by Tat.

In addition to binding to the cyclin T1 subunit of p-Tefb, Tat can bind to specific subunits of ATP-dependent chromatin-remodeling enzymes of the Swi/Snf family, as well as to several histone acetyltransferases. The data currently available are consistent with a model in which binding of Tat to TAR RNA recruits not only p-Tefb but also Swi/Snf enzymes, which then alter the position or structure of the downstream nucleosome to promote elongation of viral transcription (Fig. 8.13C). Although some details are not yet clear, the inhibition of Tat-dependent transcription induced by small interfering RNA (siRNA)-mediated knockdown of specific subunits of Swi/Snf enzymes provides compelling evidence for the important contribution of this function of Tat to transcription of integrated proviral DNA.

The ability of Tat to bind to cellular proteins is governed by posttranslational modification. For example, acetylation at Lys28 promotes high-affinity binding to TAR and to p-Tefb, whereas acetylation at Lys50, within the RNA-binding region of Tat, prevents this interaction. The latter modification also provides a site for association of Tat with specific Swi/Snf subunits and a histone acetyltransferase (Fig. 8.13C).

Why such unusual transcriptional regulation? At this juncture, it is difficult to appreciate the value of the intricate transcriptional program of human immunodeficiency virus type 1 and related viruses. Those of many other viruses are executed successfully by proteins that operate, directly or indirectly, via specific DNA sequences. Binding of Tat to nascent viral RNA close to the site at which many transcriptional complexes pause or stall (Fig. 8.13A) could provide a particularly effective way to recruit the cellular proteins that stimulate processive transcription. Alternatively, it may be that regulation of transcription via an RNA sequence is a legacy from some ancestral virus-host cell interaction in an RNA world.

The Transcriptional Cascades of DNA Viruses

Common Strategies Are Executed by Virus-Specific Mechanisms

An overview of three DNA virus transcriptional programs is presented in this section to illustrate both their diversity and the common themes of the central role of virus-encoded transcriptional regulators and the coordination of transcriptional control with viral DNA synthesis.

The transcriptional strategies characteristic of the infectious cycles of viruses with DNA genomes exhibit a number of common features. The most striking is the transcription of viral genes in a reproducible and precise temporal sequence (Fig. 8.14). Prior to initiation of genome replication, during **early** phases, infected cells synthesize viral proteins necessary for efficient viral gene expression, viral DNA synthesis, or other regulatory functions. Transcription of the **late** genes, most of which encode structural proteins, requires genome replication. This property ensures coordinated production of the DNA genomes and the structural proteins from which progeny virus

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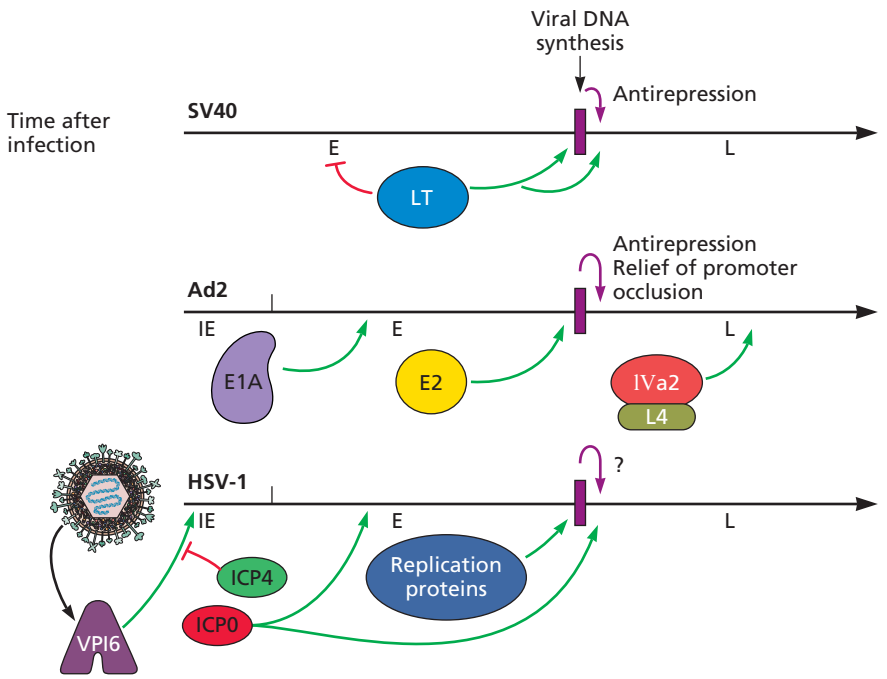


Figure 8.14 Important features of the simian virus 40 (SV40), human adenovirus type 2 (Ad2), and herpes simplex virus type 1 (HSV-1) transcriptional programs. The transcriptional programs of these three viruses are depicted by the horizontal time lines, on which the onset of viral DNA synthesis is indicated by the purple boxes. For comparative purposes **only**, the three reproductive cycles are represented by lines of equal length. The immediate-early (IE), early (E), and late (L) transcriptional phases are indicated, as are viral proteins that participate in regulation of transcription. Stimulation of transcription by these proteins and effects contingent on viral DNA synthesis in infected cells are indicated by green and purple arrows, respectively. Red bars indicate negative regulation of transcription.

particles are assembled. Another common feature is the control of the transitions from one transcriptional stage to the next by both viral proteins and genome replication. Such viral programs closely resemble those that regulate many developmental processes in animals, in both the transcription of individual genes in a predetermined sequence and the sequential action of proteins that regulate the transcription of different sets of genes.

The simplest transcriptional programs comprise only two phases. For example, the genome of simian virus 40 contains one early and one late transcription unit (Appendix, Fig. 23B), each of which encodes more than one protein. Although significantly larger, the genomes of human adenoviruses also encode multiple proteins within each of a limited number of transcription units (Appendix, Fig. 1B). This type of organization reduces the genetic information that must be devoted to transcription punctuation marks and regulatory sequences, a significant advantage when genome size is limited by packaging constraints. The price for such a transcriptional strategy is heavy dependence on the host cell's RNA-processing systems to generate multiple mRNAs by differential polyadenylation and/or splicing of a single primary transcript (Chapter 10). In contrast, the more than 80 known protein-coding sequences of herpes simplex virus type 1 are, with few exceptions, expressed as individual transcription units. Furthermore, splicing of primary transcripts is the exception. The basic distinction of early and late phases is maintained in the herpesviral transcriptional program, but temporal control of the activity of more than 80 viral promoters is obviously more complicated. In fact, the potential for finely tuned regulation is much greater when the viral genome contains a large number of independent transcription units.

Cellular enhancer- and promoter-binding proteins are sufficient to initiate the polyomaviral and adenoviral transcription programs and the synthesis in infected cells of crucial viral transcriptional regulators, large T antigen and E1A proteins, respectively. In contrast, a viral activating protein imported into cells as the virion structural component VP16 is necessary for efficient transcription of the first herpesviral genes to be expressed (called immediate-early genes) (Fig. 8.14). This simple strategy might seem to guarantee transcription of these genes in **all** infected cells. Surprisingly, however, this is not the case, because VP16 functions only in conjunction with specific cellular proteins (see next section).

As might be anticipated, the sophistication of the regulatory circuits that govern the transcriptional cascade of these DNA viruses increases with genome size. Synthesis of a single viral protein, large T antigen, in simian virus 40-infected cells leads inevitably to entry into the late phase of infection: T antigen both induces initiation of viral DNA synthesis and activates late transcription. In contrast, several transcriptional regulators control the transitions from one phase to the next during the adenoviral infectious cycle. The immediate-early E1A proteins, which regulate transcription by multiple mechanisms, are necessary for efficient transcription of all early transcription units. Among this set is the E2 gene, which encodes the proteins required for viral DNA synthesis. Accumulation of progeny viral genomes leads to relief of repression of transcription of the gene that encodes the sequence-specific DNA-binding protein IVa_2 and subsequent activation of transcription from the major late promoter (Fig. 8.14). This promoter controls synthesis of the majority of structural proteins (Appendix, Fig. 1B).

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The synthesis of progeny adenoviral DNA molecules is therefore indirectly coordinated with production of the protein components that will encapsidate them in virus particles.

As noted above, the large number of individual transcription units suggests that the regulatory scheme of herpes simplex virus type 1 may be even more elaborate. Indeed, the immediate-early viral gene products include two transcriptional regulators, ICP4 and ICP0. Like the adenoviral E1A proteins, the ICP4 protein is necessary for efficient progression beyond the immediate-early phase of infection and is regarded as the major transcriptional activator. It stimulates transcription of both early and late genes and also acts as a repressor of immediate-early transcription. Some herpesviral late genes are transcribed only following synthesis of progeny genomes, the pattern exemplified by simian virus 40 late transcription, but others attain their maximal rates of transcription during the late phase. More subtle distinctions among the large number of late genes may be made as their transcriptional regulation becomes better understood.

Examples of Viral Proteins That Stimulate Transcription

In this section, we focus on a few well-characterized viral regulators to illustrate general principles of their operation, or fundamental insights into cellular processes that have been gained through their study. These proteins all promote progression through the infectious cycle, but differ in the mechanisms by which they become associated with viral promoters, and consequently in the specificity with which they stimulate transcription. The viral protein may itself bind to a specific viral DNA sequence (the Epstein-Barr virus Zta protein) or may be recruited to promoters indirectly, either via a single cellular DNA-binding protein (herpes simplex virus type 1 VP16) or by association with several cellular activators to stimulate transcription from most promoters in the viral genome (adenovirus E1A protein).

Some viral transcriptional regulators are close relatives of cellular proteins that bind to specific DNA sequences in promoters or enhancers (Table 8.3). These viral proteins possess

Table 8.3 Properties and functions of some viral transcriptional regulators

Virus	Protein	Sequence-specific DNA binding	Properties	Function[a]
Adenovirus				
Species C human adenovirus	IVa_2	Yes	Operates with a viral L4 protein	Stimulates ML transcription
	E1A 289R, 243R	No	Bind to multiple cellular regulators of transcription	289R stimulates E gene transcription; both overcome sequestration of E2f by Rb
Herpesviruses				
Herpes simplex virus type 1	VP16	No	Binds a specific promoter sequence via cellular Oct-1 and Hcf proteins	Stimulates transcription from IE promoters
	ICP4	Yes	Typical domain organization	Stimulates transcription from E and L promoters; represses IE transcription
Epstein-Barr virus	Zta	Yes	Basic-leucine zipper protein	Activates E gene transcription; commits to lytic infection
Papillomavirus				
Bovine papillomavirus type I	E2	Yes	Typical domain organization	Stimulates transcription from viral promoters; required for genome replication
Polyomavirus				
Simian virus 40	Large T antigen	No[b]	Can bind to several transcription initiation proteins	Stimulates L gene transcription; required for genome replication
Poxvirus				
Vaccinia virus	VETF	Yes	Binds as heterodimer; DNA-dependent ATPase	Essential for recognition of E promoters by the viral RNA polymerase
Retrovirus				
Human T cell lymphotropic virus type I	Tax	No	Modulates cellular basic-leucine zipper proteins; reverses cytoplasmic sequestration of Nf-κb	Stimulates transcription from viral LTR and cellular promoters

[a]E, early; IE, immediate early; L, late; ML, major late.

[b]The sequence-specific DNA-binding activity of large T antigen (see Fig. 9.3) is not required for stimulation of transcription by this protein.

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discrete DNA-binding domains, some with sequence motifs characteristic of cellular DNA-binding proteins, and activation domains that interact with cellular initiation proteins. These properties are described in more detail for one such protein, the Epstein-Barr virus Zta protein, in the next section.

Sequence-specific DNA-binding proteins play ubiquitous roles in the transcription of cellular genes by RNA polymerase II, so it is not surprising that viral DNA genomes transcribed by this enzyme encode analogous proteins. However, viral transcriptional regulators that possess no intrinsic ability to bind specifically to DNA are equally, if not more, common (Table 8.3). Two examples, the herpes simplex virus type 1 VP16 and adenovirus E1A proteins, illustrate the diversity of mechanisms by which these viral proteins regulate transcription. The preponderance of such viral proteins, quite unexpected when they were first characterized, was a strong indication that host cells also contain proteins that modulate transcription without themselves binding to DNA. Many such proteins (e.g., coactivators) have now been recognized.

The Epstein-Barr virus Zta protein: a sequence-specific DNA-binding protein that induces entry into the productive cycle. When the gammaherpesvirus Epstein-Barr virus infects B lymphocytes, only a few viral genes are transcribed and a latent state described in "Entry into One of Two Alternative Transcriptional Programs" below is established. The products of these genes maintain the viral genome via replication from a latent phase-specific origin of replication (OriP) (Fig. 8.15A), modulate the immune system, and alter the growth properties of the cells. Virus reproduction begins with synthesis of three viral proteins that regulate gene expression. However, just one of these, the transcriptional regulator Zta (also known as ZEBRA, Z, or EB-1), is sufficient to interrupt latency and induce entry into the productive cycle.

The Zta protein exhibits many properties characteristic of the cellular proteins that recognize promoter sequences: it is a modular, sequence-specific DNA-binding protein that belongs to the basic-leucine zipper family (Table 8.3 and Fig. 8.7). Dimerization of Zta via this domain is required for its direct binding to viral promoters. The discrete activation domain, which can bind directly to cellular initiation proteins, such as subunits of transcription factor IID (TfIId), is thought to facilitate the assembly of preinitiation complexes, and hence initiation of transcription from these promoters.

The availability or activity of Zta is regulated by numerous mechanisms. In latently infected cells, transcription from the Zta promoter is blocked by binding of cellular transcriptional repressors to several sites (Fig. 8.15B). Upon B cell activation and induction of signal transduction cascades in response to external stimuli, such as binding of antigens to B cell receptors, several cellular regulators, including members of the Sp1 and Atf families, bind to and activate transcription from the Zta promoter. Synthesis of Zta augments transcription from this promoter, as the protein is a positive autoregulator. The availability of Zta mRNA for translation is also regulated, in part, by annealing of Zta pre-mRNA to the complementary transcripts of the viral EBNA-1 gene (Fig. 8.15A). The net effect of these regulatory mechanisms, which depends on the type and the proliferation and differentiation states of the Epstein-Barr virus-infected cell, determines whether active Zta protein is available. Entry into the infectious cycle appears to be an inevitable consequence of production of active Zta: this protein not only stimulates transcription from the promoters of its own gene and other early genes but also plays an important role in replication from the lytic origins.

The herpes simplex virus type 1 VP16 protein: sequence-specific activation of transcription via a cellular DNA-binding protein. The herpesviral VP16 protein, which enters infected cells in the virus particle (Fig. 8.14), has taught us much about mechanisms by which transcription by RNA polymerase II can be stimulated. Furthermore, its unusual mode of promoter recognition illustrates the importance of conformational change in proteins during formation of DNA-bound protein assemblies.

The VP16 protein lacks a DNA-binding domain. Its acidic activation domain is one of the most potent known and has been exploited to investigate mechanisms of stimulation of transcription. Chimeric proteins in which this domain is fused to heterologous DNA-binding domains strongly stimulate transcription from promoters that contain the appropriate binding sites. When part of such fusion proteins, the VP16 acidic activation domain can stimulate several reactions required for initiation of transcription (Fig. 8.16A). It can also increase the rate of transcriptional elongation and promote transcription from chromatin templates (Fig. 8.16B). These properties established that a single protein can regulate RNA polymerase II transcription by multiple molecular mechanisms.

The VP16 protein is the founding member of a class of viral regulators that possess no sequence-specific DNA-binding activity, yet activate transcription from promoters that contain a specific consensus sequence. The 5′ flanking regions of viral immediate-early genes contain at least one copy of the consensus sequence that is necessary for VP16-dependent activation of their transcription, 5′TAATGARAT3′ (where R is a purine). This sequence is bound by VP16 only in association with at least two cellular proteins, Oct-1 and host cell factor (Hcf) (Fig. 8.17). The Oct-1 protein is a ubiquitous transcriptional activator named for its recognition of a DNA sequence termed the octamer motif. This protein and VP16 can associate to form a ternary (three-component) complex on the 5′TAATGARAT3′ sequence, but the second cellular protein, Hcf, is necessary for stable, high-affinity binding. The VP16

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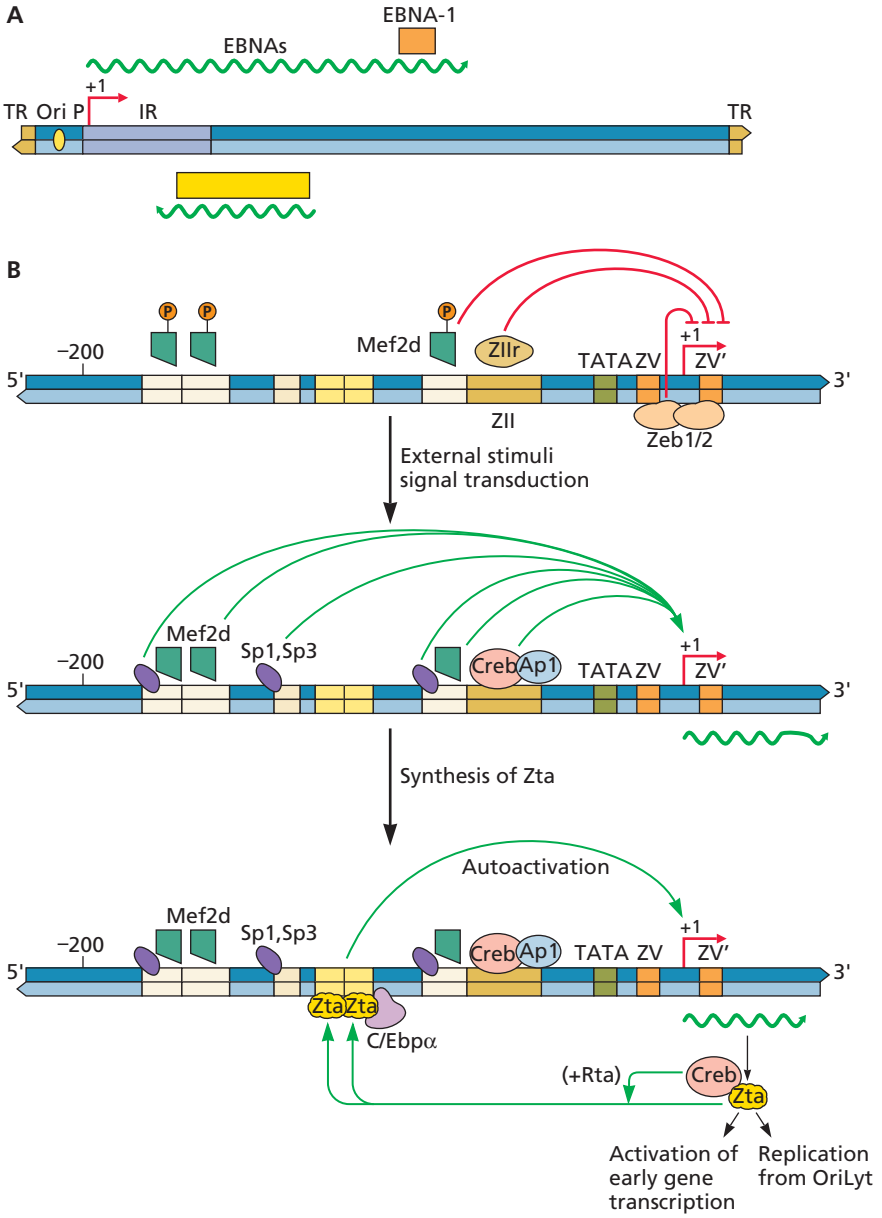


Figure 8.15 Organization and regulation of the Epstein-Barr virus Zta gene promoter. **(A)** Organization of the transcription units that contain the coding sequence for the Epstein-Barr virus nuclear antigen (EBNA) proteins (an ~100-kb transcription unit) and Zta. The locations of the genomic terminal (TR) and internal (IR) repeat sequences, the origin of replication for plasmid maintenance (OriP), and the coding sequences for the EBNA-1 and Zta proteins are indicated. The Zta protein is synthesized from spliced mRNAs processed from the primary transcripts shown. **(B)** (Top) Sequences that regulate transcription from the Zp promoter of the Zta gene are shown to scale and in the conventional 5′→3′ direction. In primary B lymphocytes, transcription from this promoter is repressed by synergistic binding of Zn-finger E-box-binding homeobox (Zeb) 1 or 2 proteins to the ZV and ZV′ sequences and of an as yet unidentified protein (designated ZII repressor, or ZIIr) to the ZII sequence. Binding of a phosphorylated form of myocyte-specific enhancer factor 2D (Mef2d) also contributes to repression by recruiting Hdacs. (Middle) Activation of B cells, for example, by reagents that induce cross-linking and activation of B cell surface receptor, induces signal transduction pathways that lead to reversal of the inhibitory modification of Mef2D, allowing recruitment of histone acetyltransferases and activation of positive regulators, such as Creb and Ap1. These proteins, in conjunction with ubiquitous activators of the Sp1 family, stimulate transcription from the Zta promoter. (Bottom) Synthesis of Zta activates transcription of early genes by reversing repressive modifications of nucleosome associated with early promoters and also promotes viral genome replication. This viral protein also establishes a positive autoregulatory circuit by binding to specific promoter sequences and cooperating with cellular C/Ebpα.

protein and Hcf form a heteromeric complex in the absence of Oct-1 or DNA. An important function of Hcf appears to be stabilization of conformational change in VP16, to allow its high-affinity binding to Oct-1 on the immediate-early promoters (Fig. 8.17). The VP16 protein interacts with Hcf and Oct-1 proteins via its N-terminal region. Its C-terminal region contains the acidic activation domain described previously. The results of chromatin immunoprecipitation experiments suggest that stimulation of immediate-early gene transcription in infected cells is mediated by several of the biochemical activities exhibited by the acidic activation domain in simplified experimental systems (Box 8.10).

One of the most remarkable features of the mechanism by which the VP16 protein is recruited to immediate-early promoters is its specificity for Oct-1. This protein is a member of a family of related transcriptional regulators defined by a common DNA-binding motif called the POU-homeodomain. The VP16 protein distinguishes Oct-1 from all other members of this family, including Oct-2, which binds to exactly the same DNA sequence as Oct-1. In fact, VP16 detects a **single** amino acid difference in the exposed surfaces of DNA-bound Oct-1 and Oct-2 homeodomains.

The incorporation of the VP16 protein into virus particles at the end of one infectious cycle appears to be an effective

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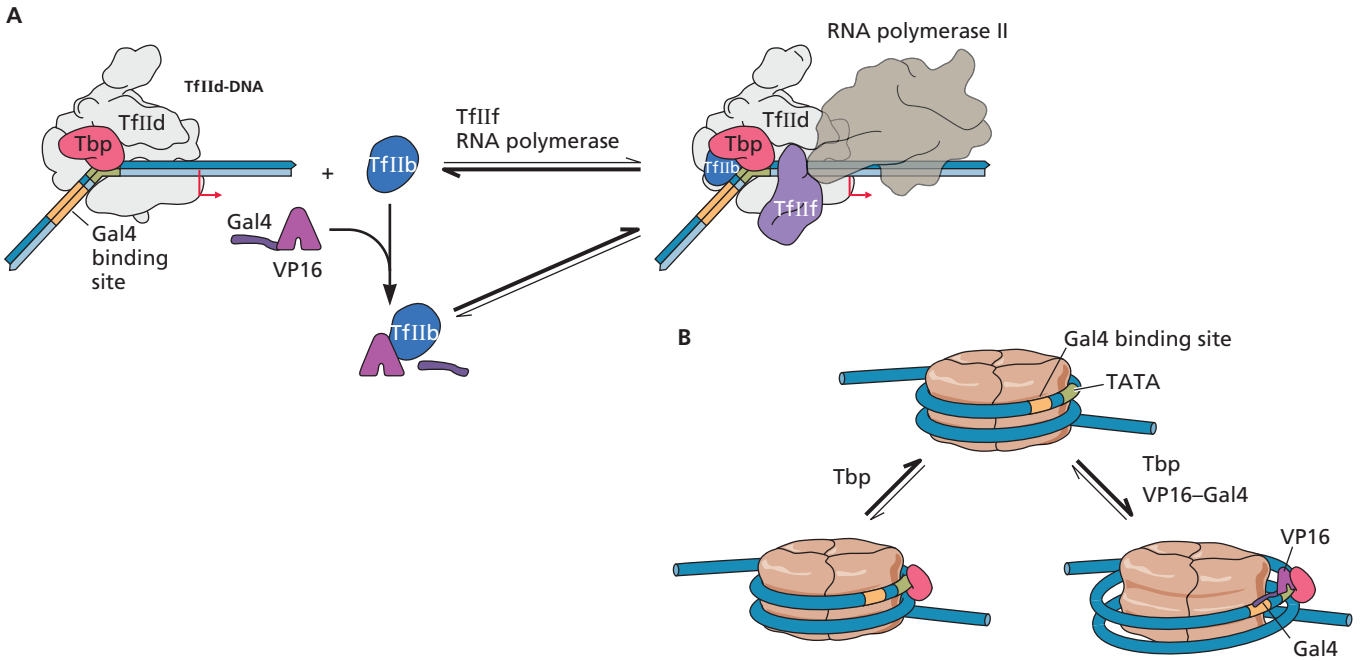


Figure 8.16 Models for transcriptional activation by the herpes simplex virus type 1 VP16 protein. **(A)** Induction of conformational change in TfIIb. In native TfIIb, the N- and C-terminal domains associate with one another such that internal segments of the protein that interact with the TfIIf-RNA polymerase II complex are inaccessible. Binding of the acidic activation domain of VP16, for example, as a chimera with the DNA-binding domain of the yeast protein Gal4, disrupts this intermolecular association of TfIIb domains, exposing its binding sites for TfIIf and RNA polymerase II. Consequently, formation of the preinitiation complex that contains TfIIb, TfIIf, and RNA polymerase II is now a more favorable reaction. **(B)** Alleviation of transcriptional repression by nucleosomes. Many activators, including the acidic activation domain of VP16, stimulate transcription from nucleosomal DNA templates to a much greater degree than they do transcription from naked DNA. This property is the result of their ability to alleviate repression of transcription by nucleosomes. Organization of DNA into a nucleosome can block access of proteins to their DNA-binding sites, as illustrated for binding of Tbp to a TATA sequence (left). Association of the acidic activation domain of VP16 with the template alters the interaction of the DNA with the nucleosome to allow Tbp access to the TATA sequence (right), presumably as a result of recruitment of ATP-dependent chromatin-remodeling enzymes and/or histone acetyltransferases (Box 8.10).

way to ensure transcription of viral genes and initiation of viral reproduction in a new host cell. Nevertheless, some features of this mechanism are not fully appreciated, in particular the benefits conferred by the indirect mechanism by which VP16 recognizes viral promoters. One advantage over direct DNA binding may be the opportunity to monitor the growth state of the host cell that is provided by the requirement for binding to Hcf: this protein regulates transcription during the cell cycle and is important for proliferation of uninfected cells. Furthermore, Hcf is a component of several chromatin-modifying complexes, and its recruitment to immediate-early promoters is required for replacement of repressive with activating modifications of the nucleosomal histones associated with these promoters. The dependence on Hcf may also contribute to the establishment of latent infections in neurons (see "Entry into One of Two Alternative Transcriptional Programs" below).

Adenoviral E1A proteins: regulation of transcription by multiple mechanisms. Two E1A proteins are synthesized from differentially spliced mRNAs during the immediate-early phase of adenovirus infection (Fig. 8.18). These two proteins share all sequences except for an internal segment (conserved region 3 [CR3]) that is unique to the larger protein. Nevertheless, they differ considerably in their regulatory potential, because the CR3 segment is primarily responsible for stimulation of transcription of viral early genes. As the larger E1A protein neither binds specifically to DNA nor depends on a specific promoter sequence, it is often considered the prototypical example of viral proteins that stimulate transcription by indirect mechanisms.

The CR3 segment of the larger E1A protein comprises an N-terminal zinc finger motif followed by 10 amino acids that are highly conserved among human adenoviruses (Fig. 8.19A). The latter region mediates binding of the E1A

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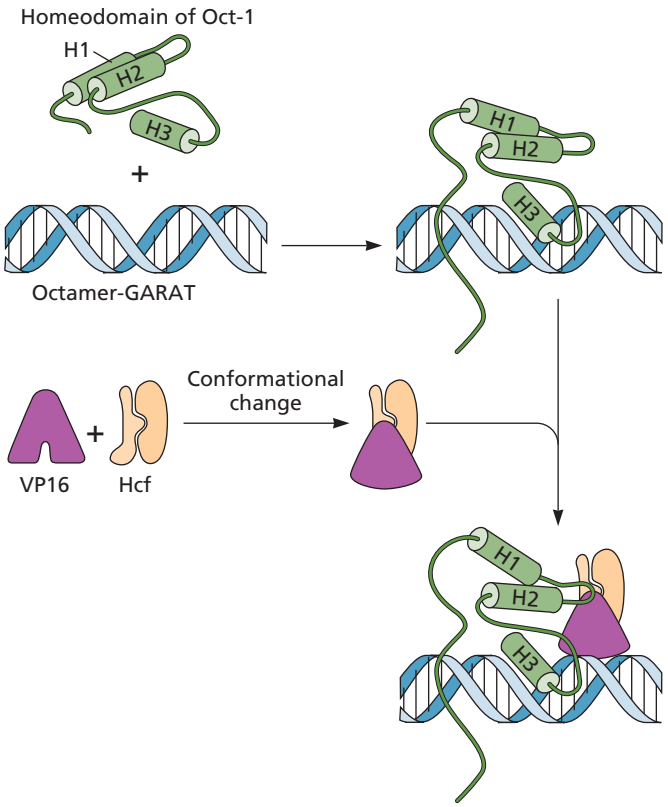


Figure 8.17 Conformational changes and recruitment of VP16 to herpes simplex virus type 1 promoters. Binding of the octamer-binding protein 1 (Oct-1) homeodomain to DNA containing the GARAT sequence and of VP16 to host cell factor (Hcf) induces conformational changes that allow specific recognition of GARAT-bound Oct-1 by VP16. This mechanism ensures that the VP16 protein is recruited only to promoters that contain the GARAT sequence, that is, viral immediate-early promoters.

protein to cellular, sequence-specific activators such as Atf-2 and Sp1, and hence association with the viral promoters. The zinc finger motif is essential for stimulation of transcription by the E1A protein in infected cells. It binds with exceptionally high affinity to a single component (Med23) of the human mediator complex (see the interview with Dr. Arnold Berk: http://bit.ly/Virology_Berk), which contains at least 20 different subunits and is essential for regulation of transcription by RNA polymerase II. The Med23-E1A interaction recruits the mediator complex to promoters at which E1A is bound to DNA-binding cellular activators. This association leads to activation of transcription in infected cells, by stimulation of preinitiation complex assembly and recruitment of the super elongation complex described previously (see "The Tat Protein Regulates Transcription by Unique Mechanisms" above). Like other viral activators we have discussed, the short E1A CR3 segment appears to stimulate multiple reactions in the transcription cycle.

Adenoviral E1A proteins activate transcription by a second mechanism, which is mediated by the conserved N-terminal regions CR1 and CR2. The CR1 or CR2 segments interact with several cellular proteins, including Rb and p300 (Fig. 8.18). The Rb protein is the product of the cellular retinoblastoma susceptibility gene, a tumor suppressor that plays a crucial role in cell cycle progression (Volume II, Chapter 6). In uninfected cells, Rb binds to cellular E2f proteins, which are sequence-specific transcriptional activators originally discovered because they bind to the human adenovirus type 2 E2 early promoter (Fig. 8.5). Such E2f-Rb complexes possess the specific DNA-binding activity characteristic of E2f, but Rb represses transcription (Fig. 8.19B). Competition for Rb by the E1A proteins disrupts the Rb-E2f association and allows transcription from E2f-dependent promoters. During the early phase of infection, E2f proteins are essential for efficient transcription of the gene that encodes the proteins required for viral DNA synthesis. Sequestration of Rb by the E1A proteins therefore ensures synthesis of replication proteins and progression into the late phase of the infectious cycle (Fig. 8.14).

The N-terminal sequences common to the two E1A proteins also bind directly to the cellular coactivators p300 and Creb-binding protein (Crebbp) (Fig. 8.18). As noted previously, these proteins are histone acetyltransferases and bind to other such enzymes to modify histones and alter the structure of transcriptionally active chromatin. The E1A proteins disrupt the interactions of p300 and Crebbp with specific activators and compete for their binding to other histone acetylases. This activity of E1A proteins has been implicated in repression of enhancer-dependent transcription and is required for induction of cell proliferation (Chapter 9).

The multiplicity of mechanisms by which the E1A proteins engage with components of the cellular transcriptional machinery is one of their most interesting features. Regulation by multiple mechanisms may prove to be a general property of viral proteins that cannot bind directly to DNA. For example, the human T-lymphotropic virus type 1 Tax protein stimulates transcription by binding to specific cellular members of the basic-leucine zipper family, and also by activating Nf-κb.

Coordination of Transcription of Late Genes with Viral DNA Synthesis

In cells infected by the viruses under consideration in this chapter, synthesis of the large quantities of structural proteins needed for assembly of progeny virus particles is restricted to the late phase of infection, following the onset of viral genome replication. This pattern, first characterized in studies of bacteriophages such as T7 and T4, is a general, if not universal, feature of the reproductive cycles of viruses with

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BOX 8.10

EXPERIMENTS

In vivo *functions of the VP16 acidic activation domain*

The acidic activation of domain of VP16 has been studied extensively as a model for transcriptional activation. It was shown to stimulate transcription by multiple mechanisms in simplified experimental systems (see text). The functions of VP16 in vivo were investigated using the chromatin immunoprecipitation assay (Box 8.1) to compare the proteins associated with herpes simplex virus type 1 immediate-early promoters in cells infected by the wild-type virus or a mutant encoding VP16 that lacks the acidic activation domain. Cross-linked DNA was immunoprecipitated with antibodies to VP16, RNA polymerase, or several other cellular proteins. The concentrations of viral promoter DNA present in such immunoprecipitates were then assessed by using PCR.

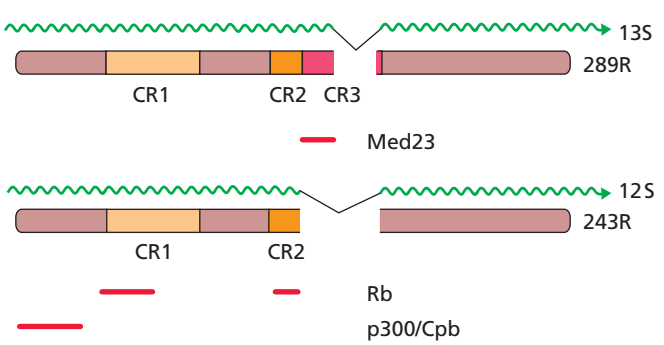
The results of these experiments provide validation for mechanisms of activation of the VP16 activation domain deduced using simplified experimental systems, notably stimulation of initiation complex assembly and induction of chromatin remodeling (see text). As summarized in the table, association of RNA polymerase II and Tbp with the viral promoters (initiation complex assembly) depended on synthesis of VP16 containing an activation domain, as did efficient recruitment of histone acetyltransferases (Crebbp and p300) and ATP-dependent remodeling proteins (Brg-1), as well as loss of histone H3 (chromatin remodeling).

Herrera FJ, Triezenberg SJ. 2004. VP16-dependent association of chromatin-modifying coactivators and underrepresentation of histones at immediate-early gene promoters during herpes simplex virus infection. *J Virol* **78:**9689–9696.

	Viral promoter DNA	
	VP16 acidic activation domain	
Promoter-bound proteins	**Not present**	**Present**
VP16	++	++
Oct-1	++	++
RNA polymerase II	–	++
Tbp	–	++
Crebbp	+	++
Brg-1	–	++
Histone H3	++	–

DNA genomes, and offers a number of potential advantages (Box 8.11). The restriction of synthesis of structural proteins to the end of a cycle of viral reproduction results from the dependence of late gene transcription on viral DNA replication: drugs or mutations that inhibit viral DNA synthesis in infected cells block efficient expression of late genes. Indeed, late genes are defined experimentally as those that are not transcribed, or are transcribed much less efficiently, when viral DNA synthesis is blocked. Despite their importance, the mechanisms by which activation of transcription can be integrated with viral DNA synthesis remain incompletely understood.

Figure 8.18 The adenoviral E1A proteins bind to multiple transcriptional regulators. Primary transcripts of the immediate-early E1A gene are alternatively spliced to produce the abundant 13S and 12S mRNAs. As such splicing does not change the translational reading frame, the E1A proteins are identical, except for an internal segment of 46 amino acids unique to the larger protein. The three most highly conserved regions are designated CR1, CR2, and CR3. The regions of the E1A proteins necessary for interaction with the Rb protein, the histone acetyltransferases p300 and Crebbp, and the mediator subunit Med23 are indicated (red lines).



Titration of cellular repressors. The most obvious consequence of genome replication in cells infected by DNA viruses is the large increase in concentration of viral DNA molecules. Even in experimental situations, infected cells contain a relatively small number of copies of the viral genome during the early phase of infection, typically 1 to 100 copies per cell depending on the multiplicity of infection (defined in Chapter 2). As soon as viral DNA synthesis begins, this number increases rapidly to values as high as hundreds of thousands of viral DNA molecules per infected cell nucleus. At such high concentrations, viral promoters can compete effectively for components of the cellular transcription machinery.

The increase in DNA template concentration also titrates cellular transcriptional repressors that bind to specific sequences of certain viral late promoters. For example, the simian virus 40 major late promoter remains inactive, because of the binding to it of a cellular repressor that belongs to the steroid/thyroid hormone receptor superfamily. Viral DNA replication increases the concentration of the late promoter

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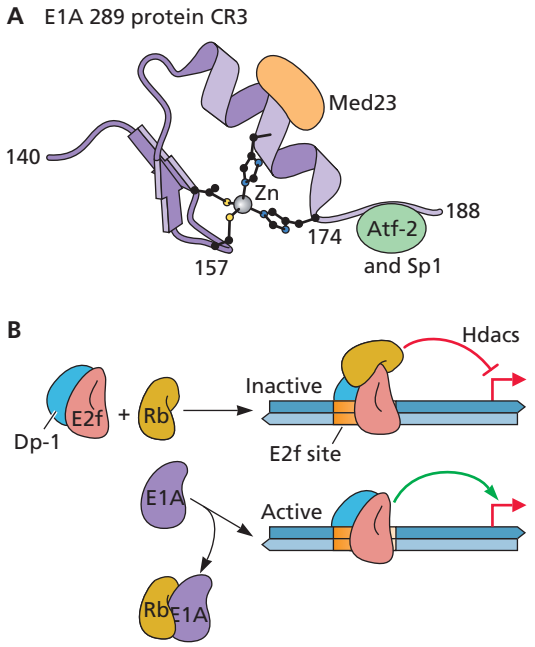


Figure 8.19 Indirect stimulation of transcription by adenoviral E1A proteins. **(A)** Interactions of the E1A CR3 sequences with components of the RNA polymerase II transcriptional machinery. The C-terminal segment of CR3 interacts with several cellular activators that bind to specific DNA sequences, as indicated by Sp1 and cyclic AMP-dependent transcription factor Atf-2, as well as with particular Taf subunits of TfIId. The Zn finger motif is required for tight binding to the Med23 subunit of the mediator complex. CR3-dependent stimulation of transcription of viral early genes is impaired in mutant cells homozygous for deletion of the *Med23* gene and when Med23 is depleted from permissive human cells by RNA interference. Such depletion also reduces association of Tbp with viral early promoters. This interaction is required for stimulation of viral early gene transcription by the super elongation complex component Cdk9. The exceptionally high-affinity binding of CR3 to Med23 may also facilitate reinitiation. **(B)** Model of competition between E1A proteins and E2f for binding to Rb protein. The E2f transcriptional activators are heterodimers of a member of the E2f protein family (described in Chapter 9) and E2f dimerization partner 1 (Dp-1). The binding of E2f to its recognition sites in specific promoters is not inhibited by association with the Rb protein, but Rb represses transcription from E2f-dependent promoters via recruitment of Hdacs (top). The CR1 and CR2 regions of the adenoviral E1A proteins made in infected (or transformed) cells bind to Rb and disrupt the E2f-Rb interaction. They also induce proteasomal degradation of Rb. Consequently, Rb is removed from association with E2f, which can then stimulate transcription.

until it exceeds that of repressor, and therefore allows this promoter to become active (Fig. 8.20). This "antirepression" mechanism directly coordinates activation of transcription of late genes with viral genome replication and is highly efficient. Consequently, it is not surprising that the same mechanism regulates transcription of the adenoviral IVa_2 gene (Fig. 8.14). The IVa_2 protein is itself a sequence-specific activator of transcription: it cooperates with a second viral protein that also binds to specific DNA sequences to stimulate the rate of initiation of transcription from the major late promoter at least 20-fold. Activation of this promoter is therefore coupled indirectly to adenovirus DNA synthesis: this process initiates a transcriptional cascade in which late promoters are activated sequentially (Fig. 8.14).

Although viral DNA synthesis is sufficient for activation of transcription of some viral late genes (e.g., the adenoviral IVa_2 gene), this process is usually facilitated by one

BOX 8.11

DISCUSSION

Some potential advantages of temporal regulation of viral gene expression

The genomes of DNA viruses come in a variety of conformations and an enormous range of sizes (Chapter 3). Nevertheless, temporal regulation of viral gene expression appears to be a universal feature of their reproductive cycles: genes encoding viral structural proteins are expressed only during the late phase, following the onset of genome replication, with earlier periods devoted to synthesis of viral enzymes and regulatory proteins. This pattern, which is also characteristic of the infectious cycles of some RNA viruses (Chapter 6), must therefore facilitate reproduction of these viruses. Possible advantages of sequential expression of viral genes and synthesis of the large quantities of structural proteins required for assembly of progeny virus particles only later in the infectious cycle may include

- the availability of viral proteins (products of early genes) that mediate efficient production of late mRNAs posttranscriptionally via effects on splicing or mRNA export from the nucleus (Chapter 10)
- reorganization of infected cell components orchestrated by early proteins, and their assembly with replicated viral DNA molecules at specialized sites for optimal transcription of late genes (Chapter 9)
- coordination of synthesis of structural proteins and viral DNA to facilitate genome encapsidation and assembly of progeny virus particles
- prevention of premature cessation of viral genome replication and transcription (these processes and encapsidation are mutually exclusive)
- postponement of competition for finite cellular resources (e.g., substrates for DNA and RNA synthesis, amino acids), potentially deleterious for both the host cell and reproduction of the virus until late in infection; by this time, the only reactions required to complete the infectious cycle are assembly and release of progeny virus particles
- restriction of synthesis of cytotoxic viral proteins that facilitate release of viral particles (Chapter 13) to the end of the infectious cycle

This strategy might therefore be considered analogous to the "just in time" inventory control method widely used in industry. This approach is defined by Wikipedia as "a production strategy that strives to improve a business return on investment by reducing in-process inventory and associated carrying costs."

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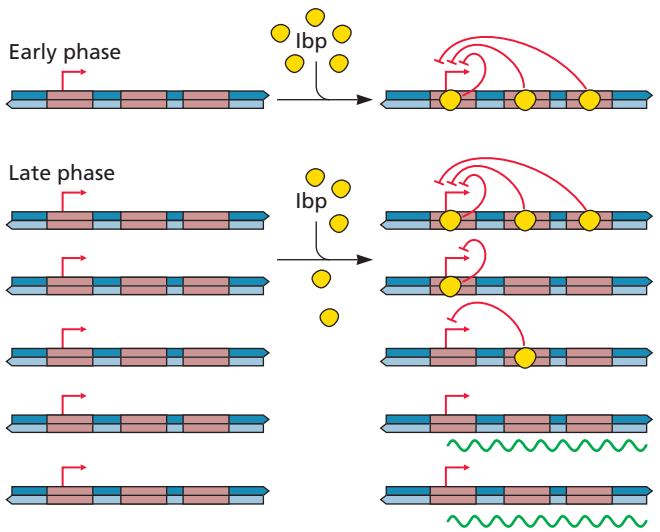


Figure 8.20 Cellular repressors regulate the activity of the simian virus 40 late promoter. The sequence surrounding the simian virus 40 major late initiation site (the thickest arrow in Fig. 8.4D) contains three binding sites for the cellular repressor termed initiator-binding protein (Ibp), which contains members of the steroid/thyroid receptor superfamily. During the early phase of infection, the concentration of Ibp relative to that of the viral major late promoter is sufficiently high to allow all Ibp-binding sites in the viral genomes to be occupied. The concentration of Ibp does not change during the course of infection. However, as viral DNA synthesis takes place in the infected cell, the concentration of the major late promoter becomes sufficiently high that not all Ibp-binding sites can be occupied. Consequently, the major late promoter becomes accessible to cellular transcription components. Although we generally speak of "activation" of late gene transcription, this DNA replication-dependent mechanism is, in fact, one of escape from repression.

or more viral proteins. For example, maximally efficient transcription from the simian virus 40 major late promoter depends on the viral early gene product large T antigen. This protein controls simian virus 40 late transcription both directly, as an activating protein (Fig. 8.14), and indirectly, as a result of its essential functions in viral DNA synthesis (Chapter 9).

Transcription of herpesviral late genes also requires viral DNA replication and synthesis of viral activators, such as ICP4 and ICP0 in the case of herpes simplex virus type 1. Transcription of viral late genes is generally regulated by inhibition or activation of initiation. However, the synthesis of progeny viral genomes can also alter termination, a regulatory mechanism illustrated by the species C human adenovirus major late transcription unit. During the early phase of infection, major late transcription terminates within a region in the middle of the transcription unit. As discussed in Chapter 10, such restricted transcription is coupled with preferential utilization of specific RNA-processing signals to produce a single major late mRNA and protein during the early phase. Viral DNA synthesis is necessary to induce full-length transcription to a termination site close to the right-hand end of the viral genome, and therefore expression of the many other major late coding sequences (Appendix, Fig. 1). The fact that only replicated viral DNA molecules can support such complete transcription suggests an unusual regulatory mechanism. One hypothesis is that alterations in template structure upon viral DNA synthesis may contribute to this process.

Availability and structure of templates. Newly replicated viral DNA molecules can enter into additional replication cycles, serve as templates for transcription, or become assembled into virus particles, with different fates predominating at different times in the infectious cycle. Transcription of all viral DNA molecules made in infected cells would seem to be a simple mechanism to ensure efficient transcription of late genes. Amazingly, however, no more than 5 to 10% of the large numbers that accumulate are transcriptionally active. In the case of simian virus 40, synthesis of viral DNA molecules is coordinated with assembly into nucleosomes, and transcriptional activity can be ascribed to establishment of an open chromatin region spanning the viral promoters and the enhancer in minichromosomes. It is not clear whether subsets of adenoviral and herpesviral DNA molecules are also marked in some way for transcriptional activity. However, one important parameter governing the concentration of transcriptional templates must be the relative concentrations of viral DNA molecules and the proteins that package them during assembly of virus particles, because packaging and transcription of genomes are mutually exclusive.

Entry into One of Two Alternative Transcriptional Programs

Studies of bacteriophage lambda led to the discovery that some viral infections result in maintenance of a quiescent viral genome for long periods in infected cells (lysogeny) rather than in viral replication (Chapter 1). Whether lambda enters this lysogenic state or the lytic cycle is determined by the outcome of the opposing actions of two viral proteins that repress transcription (Box 8.12). This regulatory mechanism, which was among the first to be elucidated in detail, emphasized the importance of repression of transcription of specific genes and established a general paradigm for transcriptional switches. Several animal viruses can establish a similar pattern of infection. For example, **latent infection** is a characteristic feature of herpesvirus infection of specific types of host cells. As in bacteriophage lambda lysogeny, latent infections are characterized by both lack of efficient expression of many viral genes and activation of a unique, latent-phase transcriptional program. Whether a herpesvirus infection is latent or lytic, as well as reentry into the productive cycle from latency (**reactivation**), is governed by mechanisms that regulate transcription.

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BOX 8.12

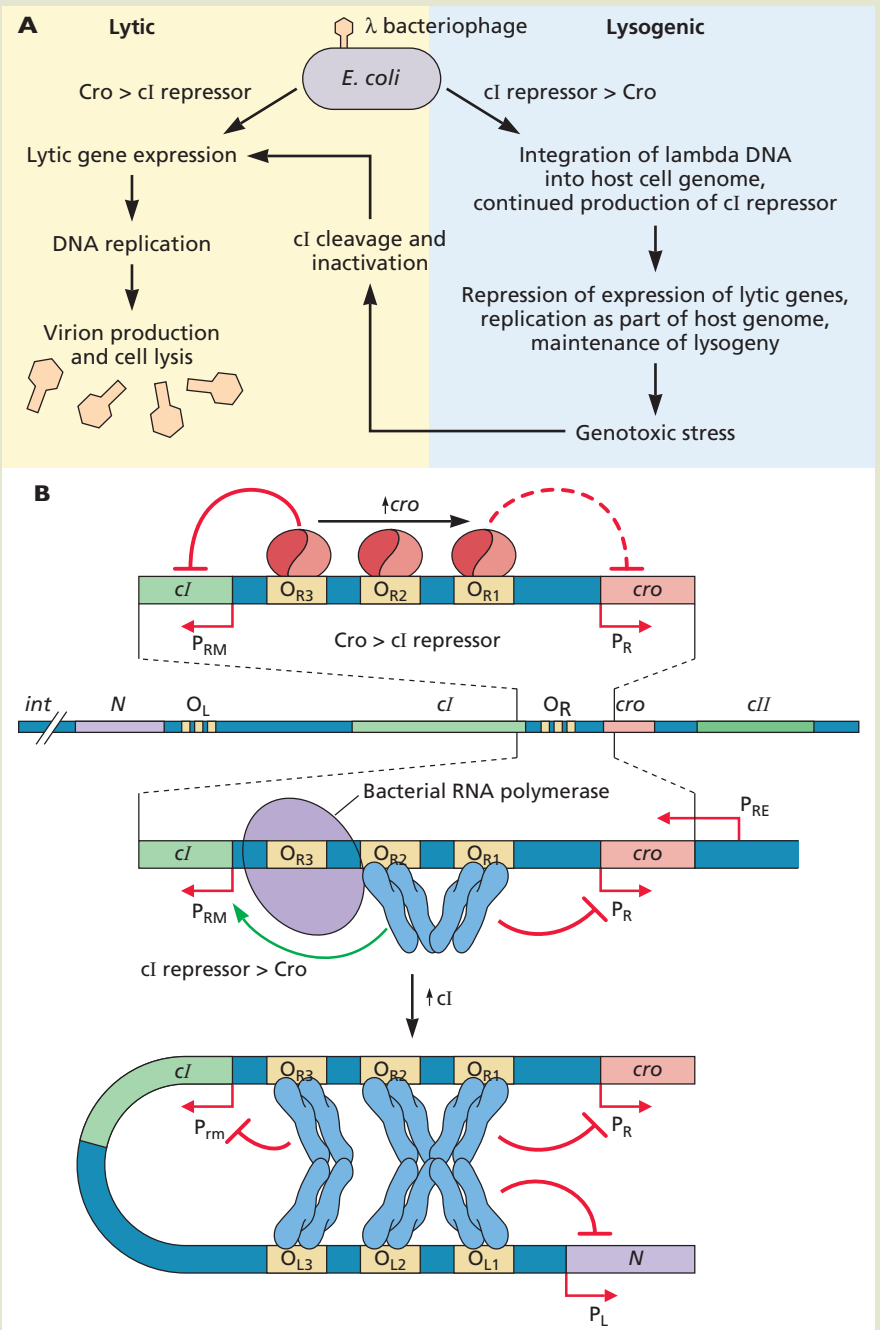
DISCUSSION

Two bacteriophage lambda repressors govern the outcome of infection

Infection of *Escherichia coli* by bacteriophage lambda leads to either synthesis of progeny virions and lysis of the host cell (lytic infection) or stable integration of the viral genome into that of the host cell (lysogenic infection) (panel A of the figure). During lysogeny, lytic genes are not expressed. Remarkably, the actions of two repressors of transcription encoded within the viral genome, the cI repressor and Cro, make a major contribution to the lytic/lysogeny "decision." When first encountered, the regulatory circuits by which these proteins govern expression of lytic and lysogenic genes can be difficult to understand: they include several promoters and multiple binding sites for the repressors. However, these circuits are crucial for survival of the bacteriophage and were among the first to be understood in detail.

The region of the lambda genome containing the *cI* repressor and *cro* genes is illustrated at the top of panel B. These coding sequences are flanked by genes encoding proteins that regulate transcription during lytic infection (e.g., N) or that are required during establishment of lysogeny (e.g., *int*, which encodes an integrase). Although both repressors bind to the operator sequences O_R and O_L adjacent to the right (P_R) and left (P_L) promoters, respectively, events at O_R are critical in determining the outcome of infection. The expanded view of the region of the genome containing O_R and P_R indicates the three binding sites for the repressors and the two promoters from which the *cI* gene is expressed, the promoters for repressor establishment and for repressor maintenance, P_{RE} and P_{RM}, respectively.

When the lambda genome enters a host cell, transcription from the P_R and P_{RE} promoters by the bacterial RNA polymerase leads to synthesis of the cI repressor and Cro. The highest-affinity binding site for the cI repressor in O_R is O_{R1}, but this dimeric protein binds cooperatively to O_{R1} and O_{R2}. As these sites overlap sequences of the P_R promoter essential for binding of *E. coli* RNA polymerase, transcription of *cro* (and other rightward lytic genes) is repressed (red bar). Transcription from P_L is blocked in the same way by binding of the cI repressor to O_{L1} and O_{L2}. The N-terminal domain of cI repressor bound to O_{R2} contacts the subunit of RNA polymerase that



(continued)

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BOX 8.12

DISCUSSION

Two bacteriophage lambda repressors govern the outcome of infection (continued)

binds to the nearby P_{RM} promoter. This interaction stimulates the formation of an open initiation complex at the P_{RM} promoter, and hence transcription of the *cI* gene (green arrow). Consequently, the concentration of cI repressor is increased to a value some 10-fold higher than that compatible with expression of lytic genes. The cI repressor has only low affinity for the O_{R3}-binding site. However, cooperative interactions occur between dimers bound to the O_L and O_R sites, to facilitate binding to O_{R3} and repression of transcription from P_{RM}. Because of such cooperative binding, whether cI repressor stimulates or blocks its own synthesis is very sensitive to concentration, and repressor concentration is maintained within a narrow range.

Although Cro binds to the same O_R sites as the cI repressors, it has the highest affinity for O_{R3}. It therefore occupies this site preferentially, and then binds to O_{R2}, to block association of RNA polymerase with the P_{RM} promoter. Consequently, the cI repressor does not attain the concentrations necessary for establishment (and maintenance) of lysogeny. Binding of Cro to O_{R2} and O_{R1} leads to weak repression of transcription from P_R (and from P_L by an analogous mechanism). This function of Cro favors lytic infection, for example, by reducing production of the cII transcriptional regulator, which promotes lysogeny by activating transcription of the *cI* gene from P_{RE}, and of the integrase gene.

It has been known for many years that environmental conditions and the activities of particular host cell gene products influence the outcome of lambda infection. The lysis/lysogeny decision was one of the first to be analyzed using a statistical-thermodynamic model of regulation of promoter activity. The results indicated that random thermal fluctuations in the rates of the reactions that comprise the regulatory circuits can lead to random phenotypic variation ("choice" between lytic and lysogenic infection) among the infected cells in a population. This conclusion is consistent with experimental observations.

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Dodd B, Shearwin KE, Egan JB. 2005. Revisited gene regulation in bacteriophage λ. *Curr Opin Genet Dev* **15:**145–152.

Ptashne M, Jeffrey A, Johnson AD, Maurer R, Meyer BJ, Pabo CO, Roberts TM, Sauer RT. 1980. How λ repressor and Cro work. *Cell* **19:**1–11.

As described in a previous section, the availability and activity of a single viral protein, Zta, determine whether Epstein-Barr virus infection is latent or lytic in B cells. This protein is necessary for transcription of viral early genes, as well as for viral DNA replication during the lytic cycle. Consequently, a latent infection ensues until the infected cell is exposed to conditions that activate transcription of the Zta gene. As Zta also represses transcription of the genes expressed in latently infected cells, it can be viewed as a simple regulatory switch. In contrast, more complex mechanisms appear to determine the outcome of infection by the alphaherpesviruses, which establish latent infections in neurons.

During latent infection of neurons by herpes simplex virus type 1, transcription of lytic genes is blocked and only a single transcription unit is expressed efficiently as latency-associated transcripts (LATs) (Fig. 8.21). As noted previously, the viral genome becomes circularized and associated with cellular nucleosomes upon entry into infected cell nuclei. In latently infected neurons, lytic genes are organized by nucleosomes that carry repressive posttranslational modifications and are associated with cellular repressors of transcription. In contrast, the LAT gene is associated with nucleosomes containing histones with modifications characteristic of actively transcribed genes. The mechanisms that lead to the silencing of lytic gene expression, the establishment of such distinct domains of "chromatin" on the viral genome, and why this process is specific to neurons are not fully understood. However, one important parameter is likely to be limited stimulation of expression of the immediate-early lytic genes by VP16: in neurons, the essential VP16 cofactor Hcf is localized largely in the cytoplasm, sequestered from viral genomes and VP16 that enter infected cell nuclei. In addition, Hcf binds to Zhangfei, a cellular protein that is a strong repressor of transcription. Zhangfei is synthesized in sensory neurons (a natural site of latency) but not in most other cell types. The synthesis of the LAT RNAs may also facilitate the establishment and maintenance of latency.

The major 2.0-kb (and 1.5-kb) LATs (Fig. 8.21), which accumulate to 40,000 to 100,000 copies in nuclei of latently infected neurons, lack poly(A) tails and are not linear molecules. Indeed, all properties observed to date indicate that they are stable introns produced by splicing of precursor RNA. The primary LAT also serves as the precursor for production of several viral miRNAs (Fig. 8.21) present at high concentrations in latently but not lytically infected neurons. Studies of properties of the RNAs synthesized from the LAT region are consistent with roles in the establishment or maintenance of latency. For example, when stably produced in neuronal cells, the LAT introns suppress replication of the viral genome and the synthesis of the immediate-early gene products that are needed for progression through the infectious cycle. They can also inhibit apoptosis and interfere with expression of interferon genes, functions that could promote the survival of latently infected neurons. Indeed, the LAT locus is required for maintenance of latently infected neurons that can support reentry into the lytic cycle. Similarly, several of the LAT-encoded miRNAs inhibit synthesis of viral transcriptional regulators such as ICP0 and ICP4 in transient-expression assays, suggesting that they might contribute to repression

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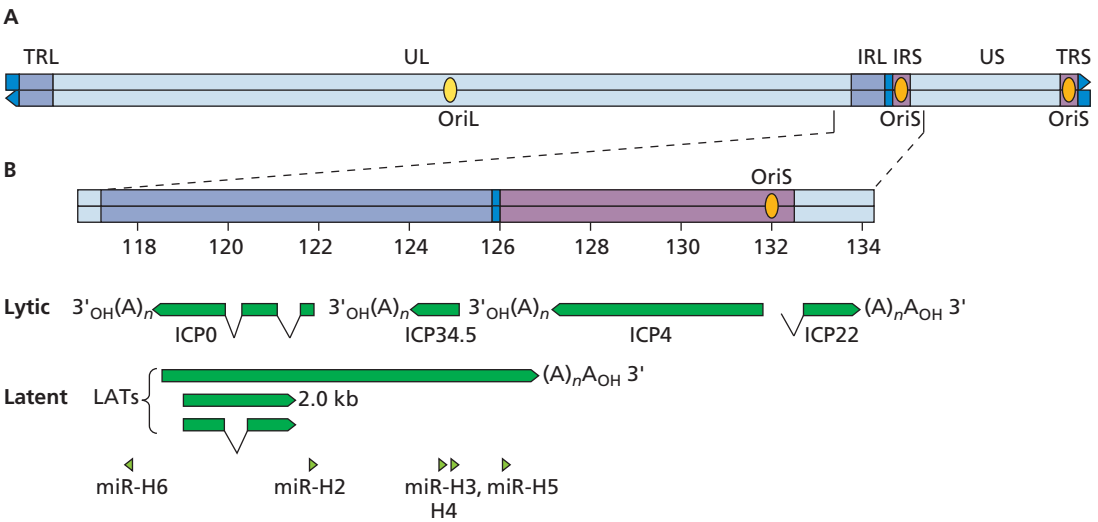


Figure 8.21 The latency-associated transcripts of herpes simplex virus type 1. (A) Diagram of the herpes simplex virus type 1 genome, showing the unique long and short segments, UL and US, respectively; the terminal repeat (TRL and TRS) and internal repeat (IRL and IRS) sequences; and the origins of replication, OriL and OriS. **(B)** Expanded map of the region shown, with the scale in kilobase pairs. This region encodes immediate-early proteins ICP0, ICP4, and ICP22, which play important roles in establishing a productive infection. Below are shown the locations of sequences encoding the major LATs. The arrows indicate the direction of transcription, and $(A)_nA_{OH}$ and ${}_{OH}A(A)_n$ 3′ poly(A) sequences. Below are shown positions of coding sequences for miRNAs miR-H2 to miR-H6, which are synthesized in high concentrations in latently infected murine trigeminal ganglion neurons. Deletions of the LAT promoter only or the promoter followed by 1.8 kbp of downstream sequence reduce the concentrations of all these miRNAs by some 2 orders of magnitude. The maintenance of the LAT region in active chromatin while lytic genes become associated with repressive nucleosomes may be facilitated by specialized DNA sequences (insulators) that flank the LAT region and demarcate different types of chromatin domains.

of lytic gene expression in latently infected cells. However, it has proved difficult to establish the specific contributions of the individual RNAs synthesized from the latency-associated region to the establishment of, maintenance of, or reactivation from latency. For example, the phenotypes exhibited by mutants carrying LAT gene deletions, such as failure to establish repressive chromatin on lytic gene promoters, were initially ascribed to the absence of LAT stable introns. However, these deletions remove the LAT promoter and hence also prevent production of the LAT-associated miRNAs. Furthermore, LAT deletions reduce the efficiency with which latency is established in some types of murine neurons (e.g., those of the trigeminal ganglia) but not in others.

How the lytic cycle transcriptional program is initiated during reactivation from latency presents a conundrum: VP16, the critical activator of this program, is a structural protein made only during the late phase of infection, which is never attained in latently infected neurons. For many years, it was thought that the need for VP16 must be circumvented. However, it is now clear that *de novo* synthesis of VP16 is induced when latently infected neurons are exposed to stresses that result in reactivation, and various lines of evidence implicate stimulation of transcription by VP16 in efficient reactivation (Box 8.13).

Transcription of Viral Genes by RNA Polymerase III

As noted previously, RNA polymerase III is dedicated to synthesis of small RNAs (typically comprising <200 nucleotides) that are made in large quantities (Table 8.2). The genomes of several of the viruses considered in this chapter contain genes that are transcribed by RNA polymerase III genomes (Table 8.4). The first, and still best-understood, example is the gene encoding human species C adenovirus virus-associated RNA I (VA-RNA I). The VA-RNA I gene specifies an RNA product that ameliorates the effects of a host cell defense mechanism (Volume II, Chapter 3) and also serves as a precursor for production of viral miRNAs. It contains a typical intragenic promoter that has been widely used in studies of initiation of transcription by RNA polymerase III.

The VA-RNA I Promoter

The human adenovirus type 5 genome contains two VA-RNA genes located very close to one another (Appendix, Fig. 1B).

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BOX 8.13

EXPERIMENTS

New insights into herpes simplex virus type 1 reactivation from studies in vivo

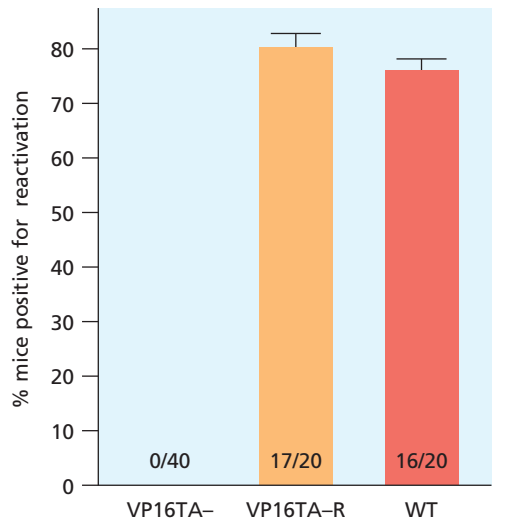
As we have seen, efficient transcription from herpes simplex virus type 1 immediate-early promoters requires VP16 brought into infected cells as a structural component of virus particles, as well as the cellular proteins Oct-1 and Hcf. In latently infected neurons, viral late genes, including that encoding VP16, are not expressed. How then can transcription of immediate-early genes occur to initiate entry into the lytic cycle during reactivation from latency? Since initial studies of this issue performed more than 25 years ago, it has been thought that VP16 does not contribute to this process. Rather, it was proposed that a different viral activator (ICP0) initiates transcription of late genes or that early gene expression and hence genome replication and late gene transcription occur prior to transcription of immediate-early genes during reactivation. These initial studies relied on ganglia explanted from mice. During preparation of these explanted neurons, their axons are cut. It is now clear that neurons damaged in this way exhibit large-scale changes in gene expression and, within 2 to 3 h, characteristic features of neuronal degeneration. These observations indicated that reactivation from latent infection in explanted neurons might not reproduce the mechanisms by which herpes simplex virus type 1 replication is reactivated *in vivo*.

More recently, the mechanism of reactivation in latently infected trigeminal ganglion neurons of herpes simplex virus type 1-infected mice has been examined by exploiting viruses carrying reporters for expression of specific viral genes (e.g., VP16) and sensitive methods for quantification of viral genomes in individual neurons. In such experiments, it was observed that

- mutations that prevent synthesis of ICP0 or viral genome replication do not impair synthesis of VP16 in neurons during reactivation in response to heat shock (mimicking fever).
- mutations that impair activation of transcription by VP16, for example, because of deletion of its C-terminal activation domain (see figure), prevent reactivation *in vivo*, but not in neurons explanted into culture
- deletion of the ICP0 TAATGARAT promoter sequence recognized by the VP16–Oct-1–Hcf complex (see text) greatly reduced the efficiency of reactivation *in vivo*, but reactivation and viral replication were restored when this sequence was reintroduced into the promoter

It has therefore been proposed that VP16 in fact induces the lytic transcriptional program during reactivation of latent infection, as it does at the beginning of a lytic infection. The mechanism(s) that induce VP16 synthesis in latently infected neurons remains to be investigated.

Thompson RL, Preston CM, Sawtell NM. 2009. De novo synthesis of VP16 coordinates the exit from HSV latency in vivo. *PLoS Pathog* **5:**e1000352. doi:10.1371/journal.ppat.1000352.



Mice were infected with a wild-type strain of herpes simplex virus type 1 (WT), a mutant defective for the transcriptional activation function of VP16 (VP16TA–), or a derivative of the mutant in which the sequences removed from the VP16 gene were restored (VP16TA–R). When latent infections had been established (40 days later), the mice were subject to hyperthermic stress to induce reactivation. Trigeminal ganglia were removed 22 h thereafter, and the concentrations of infectious virus particles were measured by plaque assay on cells in culture. The ratios on the histograms show the number of mice positive versus the number tested. Adapted from Thompson et al., *PLoS Pathog* 5:e100352, 2009, with permission.

Table 8.4 Viral RNA polymerase III transcription units

Virus	RNA polymerase III transcript	Function
Adenovirus		
Human adenovirus type 5	VA-RNA I	Blocks activation of RNA-dependent protein kinase; pre-miRNA
	VA-RNA II	Pre-miRNA
Herpesviruses		
Epstein-Barr virus	EBER-1, EBER-2	Made in latently infected cells; implicated in transformation and oncogenesis
Herpesvirus saimiri	HSVR 1–5	Degradation of certain cellular mRNAs
Murine gammaherpesvirus 68	Pre-miRNAs	Not known
Retrovirus		
Moloney murine leukemia virus	Let	Stimulation of transcription of specific cellular genes

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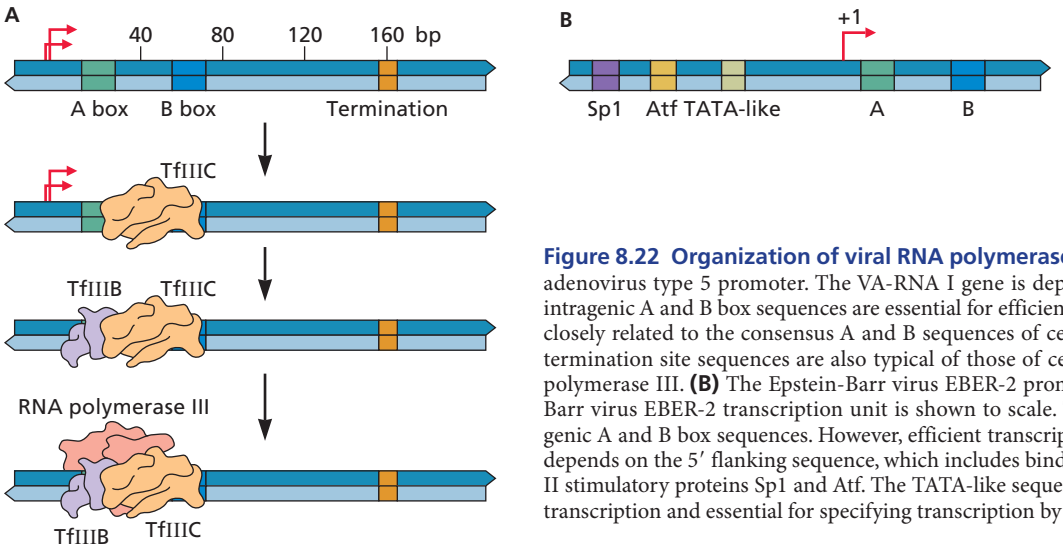


Figure 8.22 Organization of viral RNA polymerase III promoters. (A) The human adenovirus type 5 promoter. The VA-RNA I gene is depicted to scale, in base pairs. The intragenic A and B box sequences are essential for efficient VA-RNA I transcription and are closely related to the consensus A and B sequences of cellular tRNA genes. The VA-RNA termination site sequences are also typical of those of cellular genes transcribed by RNA polymerase III. **(B)** The Epstein-Barr virus EBER-2 promoter. The 5′ end of the Epstein-Barr virus EBER-2 transcription unit is shown to scale. This gene contains typical intragenic A and B box sequences. However, efficient transcription by RNA polymerase III also depends on the 5′ flanking sequence, which includes binding sites for the RNA polymerase II stimulatory proteins Sp1 and Atf. The TATA-like sequence is also important for efficient transcription and essential for specifying transcription by RNA polymerase III.

The VA-RNA I promoter is described here, for it is the more thoroughly characterized. Transcription of this gene depends on two intragenic sequences, the A and B boxes (Fig. 8.22A). As in the RNA polymerase II system, the essential promoter sequences are binding sites for accessory proteins necessary for promoter recognition. The internal sequences are recognized by the RNA polymerase III-specific initiation protein TfIIIc, which binds to the promoter to seed assembly of an initiation complex that also contains TfIIIb and the enzyme. This pathway of initiation was elucidated by using *in vitro* assays. We can be confident that this same mechanism operates in adenovirus-infected cells, because there is excellent agreement between the effects of A and B box mutations on VA-RNA I synthesis *in vitro* and in mutant virus-infected cells.

Regulation of VA-RNA Gene Transcription

The two VA-RNA genes are initially transcribed at similar rates, but during the late phase of infection, production of VA-RNA I is accelerated greatly. Such preferential transcription is the result of competition between the strong VA-RNA I and the intrinsically much weaker VA-RNA II promoters for a limiting component of the RNA polymerase III transcriptional machinery. Repression of VA-RNA I transcription may account for the similar rates at which the two genes are transcribed during the early phase. The control of transcription of VA-RNA genes emphasizes the fact that transcription by RNA polymerase III can, and must, be regulated, although the mechanisms are less elaborate than those that govern transcription by RNA polymerase II. Other viral RNA polymerase III transcription units include upstream promoter elements and hence illustrate the kinship of the RNA polymerase II and III systems (Fig. 8.22B).

Inhibition of the Cellular Transcriptional Machinery

Inhibition of cellular transcription in virus-infected cells offers several advantages. Cellular resources, such as substrates for RNA synthesis, can be devoted exclusively to the production of viral mRNAs (and, in many cases, RNA genomes), and competition between viral and cellular mRNAs for components of the translational machinery is minimized. The essential participation of cellular transcriptional systems in the infectious cycles of most viruses considered in this chapter precludes inactivation of this machinery. However, posttranscriptional mechanisms allow selective expression of adenoviral and herpesviral genes (Chapter 10). Furthermore, transcription of many cellular genes is inhibited following infection with herpes simplex virus type 1. Selective transcription of viral genes is accompanied by loss of RNA polymerase II phosphorylated at a specific amino acid, induced by the viral ICP22 protein, and proteasomal degradation of the hypophosphorylated form of the enzyme correlates with inhibition of transcription of cellular genes. Infection by poxviruses, with genomes that encode all components of a viral transcription machine, leads to rapid inhibition of synthesis of all classes of cellular RNA. Such inhibition requires viral proteins, but these have not been identified.

Reproduction of the majority of viruses with RNA genomes requires neither the cellular transcriptional machinery nor its RNA products, and is often accompanied

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by inhibition of cellular mRNA synthesis. Among the best-characterized examples is the inhibition of transcription by RNA polymerase II that is characteristic of poliovirus-infected cells. Such inhibition can be explained by the fact that the viral $3C^{pro}$ protease cleaves the Tbp subunit of TfIId at several sites. This modification eliminates the DNA-binding activity of Tbp and hence transcription by RNA polymerase II. The TATA-binding protein is also a subunit of initiation proteins that function with RNA polymerase III (TfIIIb) and RNA polymerase I. Consequently, its cleavage by $3C^{pro}$ in poliovirus-infected cells appears to be a very efficient way to prevent transcription of all cellular genes. As poliovirus yields are reduced in cells that synthesize an altered form of Tbp that is resistant to cleavage by $3C^{pro}$, it is clear that inhibition of cellular transcription is necessary for optimal virus reproduction. The RNA genomes of alphaviruses such as Sindbis virus also encode a protein that induces degradation of an essential component of the cellular transcriptional machinery, in this case one of the catalytic subunits of RNA polymerase II.

Two gene products of the rhabdovirus vesicular stomatitis virus have been implicated in inhibition of cellular transcription. Following synthesis in the cytoplasm, the leader RNA described in Chapter 6 enters the nucleus, and is primarily responsible for the rapid reduction in cellular RNA synthesis in infected cells. The question of how short RNA molecules impair DNA-dependent RNA transcription cannot yet be answered, although *in vitro* experiments suggest that binding of a cellular protein to specific sequences within the RNA may be important. The viral M protein is also a potent inhibitor of transcription by RNA polymerase II, even in the absence of other viral gene products. This activity may become important later in infection, when replication of genome RNA predominates over mRNA synthesis and less leader RNA is produced.

Unusual Functions of Cellular Transcription Components

In the preceding sections, we concentrated on the similarities among the mechanisms by which viral and cellular DNA are transcribed. Even though all mechanisms of regulation of expression of viral genes by the host cell's RNA polymerase II or RNA polymerase III cannot be described in detail, the majority are not unique to viral systems. It is therefore an axiom of molecular virology that **every** mechanism by which viral transcription units are expressed by cellular components, or by which their activity is regulated, will prove to have a normal cellular counterpart. However, virus-infected cells also provide examples of functions or activities of cellular transcription proteins that have no known cellular counterparts.

One example of such a virus-specific function is the production of hepatitis delta satellite virus RNA from an **RNA** template by RNA polymerase II, described in Chapter 6. The RNA of viroids, infectious agents of plants, is synthesized in the same manner (Volume II, Chapter 12). Such RNA-dependent RNA synthesis by RNA polymerase II is one of the most remarkable interactions of a viral genome with the cellular transcriptional machinery. No cellular analog of this reaction is yet known. Even more divergent functions of cellular transcriptional components in virus-infected cells are illustrated by the participation of the RNA polymerase III initiation proteins TfIIIb and TfIIIc in integration of the yeast retrotransposon Ty3 (see Chapter 7). Given the large repertoire of molecular and biochemical activities displayed by components of the cellular transcriptional machinery, it seems likely that other unusual activities of these cellular proteins will be discovered in virus-infected cells.

A Viral DNA-Dependent RNA Polymerase

The DNA genomes of viruses considered in preceding sections replicate in the nucleus of infected cells, where the cellular transcriptional machinery resides. In contrast, poxviruses such as vaccinia virus are reproduced exclusively in the cytoplasm of their host cells. This feat is possible because the genomes of these viruses encode the components of transcription and RNA-processing systems that produce viral mRNAs with the hallmarks of cellular mRNA, such as 5′ caps and 3′ poly(A) tails. These components, which are carried into infected cells within virus particles, include a DNA-dependent RNA polymerase with striking structural and functional resemblance to cellular RNA polymerases.

Like those of other DNA viruses, vaccinia virus genes are expressed at different times in the infectious cycle (early, intermediate, and late). Distinguishing intermediate from late genes has been difficult: both are transcribed only after viral genome replication and their promoters share sequence similarities. In fact, construction of a genome-scale map of these transcription units has been achieved only recently (Box 8.14). All viral genes are transcribed by the viral RNA polymerase. This enzyme, like the cellular RNA polymerases, is a large, multisubunit enzyme built from the products of at least eight genes. The amino acid sequences of several of these subunits (including the two largest and the smallest) are clearly related to subunits of RNA polymerase II. Like its cellular counterparts, the vaccinia viral RNA polymerase recognizes promoters by cooperation with additional proteins. For example, formation of initiation complexes on vaccinia virus early promoters is mediated by the viral

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BOX 8.14

EXPERIMENTS

The challenges of mapping vaccinia virus transcripts

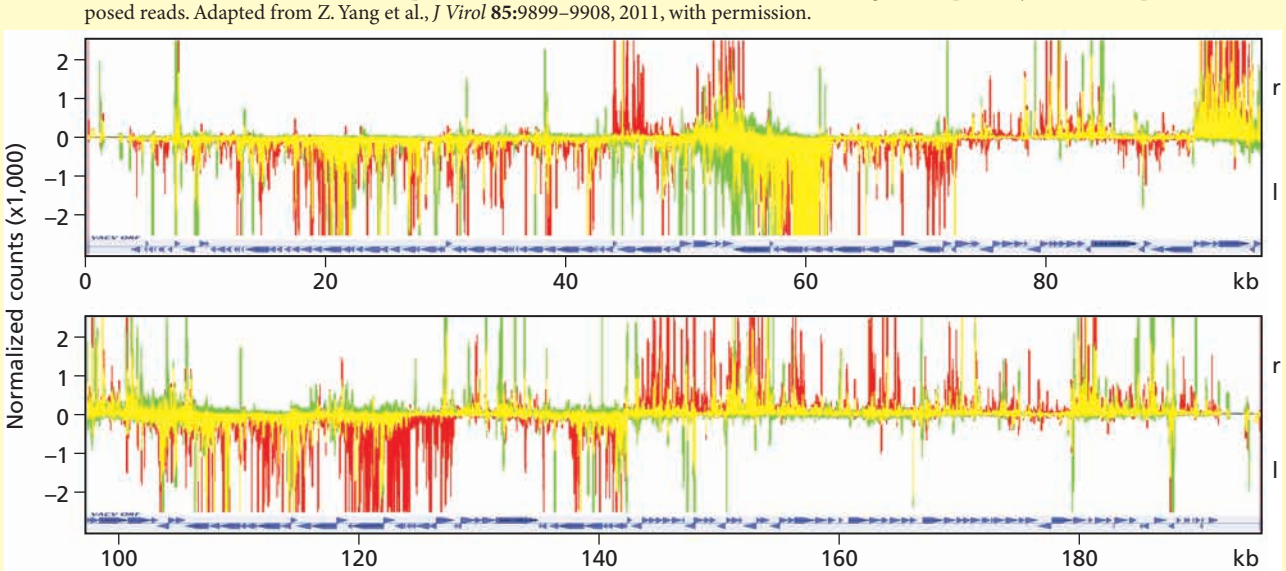
The vaccinia virus genome contains more than 200 closely spaced open reading frames that are expressed sequentially in infected cells. As discussed in the text, synthesis of early, intermediate, and late viral transcripts depends on cooperation of the viral RNA polymerase with different sets of initiation proteins. By definition, early genes are those transcribed prior to viral genome replication. Early transcripts are therefore the first to be synthesized during synchronous infection and the only viral RNAs made when infected cells are maintained in the presence of inhibitors of DNA synthesis. Consequently, 118 early genes and 93 expressed only after genome replication were readily distinguished by high-throughput sequencing and mapping of polyadenylated RNA isolated from cells infected for increasing periods or maintained in the absence or presence of an inhibitor of viral DNA synthesis. In contrast, intermediate and late genes could not be distinguished using this approach alone, for several reasons. For example, only a short period separates the onset of expression of intermediate and late genes following viral DNA synthesis. This problem is compounded by the close spacing of open reading frames and extensive read-through transcription from one gene into neighboring downstream genes. Such read-through transcription is particularly pronounced after genome replication and results in representation of virtually every nucleotide in the viral genome in the infected cell RNA population.

The dependence of late gene expression on dedicated initiation proteins was used to solve this problem and to allow intermediate and late genes to be distinguished. These experiments relied on tightly inducible expression from the viral genome of the G8R gene, which encodes the protein necessary for late transcription, under the control of the *E. coli lac* operator: in cells infected by this vaccinia virus derivative, expression of the G8R genes is 99% inhibited unless the infected cells are exposed to the inducer isopropyl-β-D-thiogalactopyranoside (IPTG). High-throughput sequencing of polyadenylated RNA isolated from cells infected by the recombinant virus in the presence or absence of IPTG identified a large number of intermediate genes (see figure). Subsequent experiments took advantage of the fact that viral genes with an intermediate promoter are expressed from plasmids when introduced into vaccinia virus-infected cells maintained in the presence of a DNA synthesis inhibitor, whereas viral late promoters are not active. These studies confirmed the identity of 53 intermediate and 38 late genes.

Yang Z, Bruno DP, Martens CA, Porcella SF, Moss B. 2010. Simultaneous high resolution analysis of vaccinia virus and host cell transcriptomes by deep RNA sequencing. *Proc Natl Acad Sci U S A* **107:** 11513–11518.

Yang Z, Reynolds SE, Martens CA, Bruno DP, Porcella SF, Moss B. 2011. Expression profiling of the intermediate and late stages of poxvirus replication. *J Virol* **85**:9899–9908.

The results of RNA sequencing are plotted as the number of reads per nucleotide along the viral genome, with read counts above and below the line representing RNAs transcribed in the rightward and leftward directions, respectively. Reads obtained for RNA made in the presence and absence of IPTG are shown in red and green, respectively. Yellow = superimposed reads. Adapted from Z. Yang et al., *J Virol* **85:**9899–9908, 2011, with permission.



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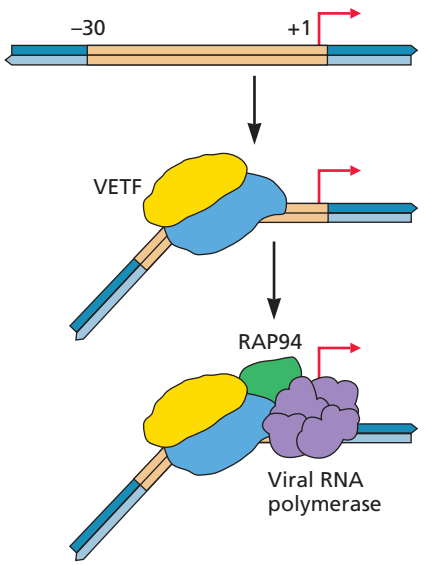


Figure 8.23 Assembly of an initiation complex on a vaccinia virus early promoter. Vaccinia virus early promoters contain an AT-rich sequence (tan) immediately upstream of the site of initiation. Vaccinia virus RNA polymerase cannot recognize these (or any other) viral promoters in the absence of other viral proteins. VETF is necessary for early promoter recognition and must bind before the viral RNA polymerase. This heteromeric protein associates specifically with early promoters and induces DNA bending. It also possesses DNA-dependent ATPase activity. VETF and the second protein necessary for early promoter specificity, RAP94, enter infected cells in virus particles. The RAP94-RNA polymerase complex associates with early promoter-bound VETF to form a functional initiation complex. Assembly of these vaccinia virus initiation complexes is therefore analogous to, although simpler than, formation of RNA polymerase II initiation complexes (Box 8.3).

proteins vaccinia virus early transcription protein (VETF) and RAP94, which are responsible for the recognition of promoter sequences and recruitment of the RNA polymerase, respectively (Fig. 8.23). These viral proteins are functional analogs of the cellular RNA polymerase II initiation proteins TfIId and TfIIf (Box 8.3). However, the vaccinia virus transcriptional machine is not analogous to its cellular counterpart in every respect. Cellular RNA polymerase II generally transcribes far beyond the sites at which the 3′ ends of mature cellular or viral mRNAs are produced by processing of the primary transcript, and does not terminate transcription at simple sequences. In contrast, transcription of the majority of vaccinia virus early genes **does** terminate at discrete sites, 20 to 50 bp downstream of specific T-rich sequences in the template. Termination requires the viral termination protein, which is also the viral mRNA-capping enzyme (see Chapter 10). The 3′ ends of the viral mRNAs correspond to sites of transcription termination. This viral mechanism is considerably simpler than the cellular counterpart.

In addition to the viral RNA polymerase, the several other proteins necessary for transcription of early genes enter host cells within vaccinia virus particles. Subsequent viral gene expression depends on viral genome replication and the ordered synthesis of viral proteins that permit sequential recognition of intermediate and late promoters. For example, transcription of intermediate genes requires synthesis of the viral RPO30 gene product (a subunit of the viral polymerase) and a second viral protein, while late transcription depends on production of several intermediate gene products. The viral genome also encodes several proteins that regulate elongation during transcription of late genes. Transcription of vaccinia virus genetic information is therefore regulated by mechanisms similar to those operating in cells infected by other DNA viruses, even though the transcriptional machinery is viral in origin.

Surprisingly, the vaccinia virus transcription system is not entirely self-contained: a cellular protein is necessary for transcription of viral intermediate genes. This protein (Vitf2) is located in the nucleus of uninfected cells but is present in both the cytoplasm and the nucleus of infected cells. As a significant number of vaccinia virus genes encode proteins necessary for transcription, such dependence on a cellular protein must confer some advantage. An attractive possibility is that interaction of the viral transcriptional machinery with a cellular protein serves to integrate the viral reproductive cycle with the growth state of its host cell. The identification of Vitf2 as a heterodimer of proteins that are produced in greatest quantities in proliferating cells is consistent with this hypothesis.

Perspectives

It is difficult to exaggerate the contributions of viral systems to the elucidation of mechanisms of transcription and its regulation in eukaryotic cells. The organization of RNA polymerase II promoters considered typical was first described for viral transcriptional control regions, enhancers were first discovered in viral genomes, and many important cellular regulators of transcription were identified by virtue of their specific binding to viral promoters. Perhaps even more importantly, efforts to elucidate the molecular basis of regulatory circuits that are crucial to viral infectious cycles have established general principles of transcriptional control. These include the importance of proteins that do not recognize DNA sequences directly and the ability of a single transcriptional regulator to modulate multiple components of the machinery. The insights into regulation of elongation by RNA polymerase II gained from studies of the human immunodeficiency virus type 1 Tat protein emphasize the intimate relationship of viral proteins with cellular components that make viral systems such rich resources for the investigation of eukaryotic transcription.

The identification of cellular and viral proteins necessary for transcription of specific viral genes has allowed many regulatory

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circuits to be traced. For example, the tissue distribution or the availability of particular cellular activators that bind to specific viral DNA sequences can account for the tropism of individual viruses, or conditions under which different transcriptional programs (latent or lytic) can be established. Furthermore, the mechanisms that allow sequential expression of viral genes are quite well established. Regardless of whether regulatory circuits are constructed of largely cellular or mostly viral proteins, these transcriptional cascades share such mechanistic features as sequential production of viral activators and integration of transcription of late genes with synthesis of viral DNA.

The models for the individual regulatory processes described in this chapter were developed initially by using convenient and powerful experimental systems. Such simplified systems (e.g., *in vitro* transcription reactions and transient-expression assays) do not reproduce the features characteristic of infected cells. Nor can they address such issues as how transcription of specific genes can be coupled with replication of the viral genome. It is therefore crucial that models be tested in virus-infected cells, even though it is more difficult to elucidate the molecular functions and mechanisms of action of transcriptional components. Many viral regulatory proteins perform multiple functions, a property that can confound genetic analysis, and the study of individual intracellular reactions, such as binding of a protein to a specific promoter sequence, is technically demanding. Nevertheless, viral *cis*-acting sequences and regulatory proteins remain more amenable to genetic analyses of their function in the natural context than do their cellular counterparts. In conjunction with increasingly powerful and sensitive methods for examining intracellular processes, continued efforts to exploit such genetic malleability will eventually establish how transcription of viral DNA templates is mediated and regulated within infected cells.

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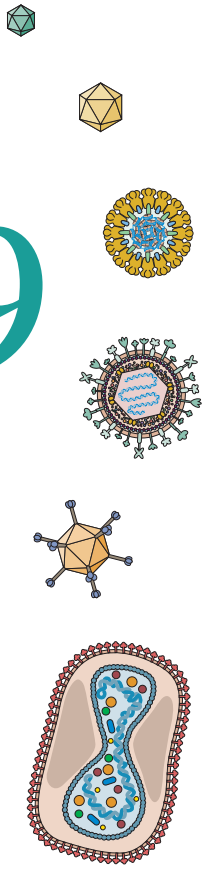
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9 Replication of DNA Genomes

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LINKS FOR CHAPTER 9

⏭ *Video: Interview with Dr. Sandra Weller*
http://bit.ly/Virology_Weller

⏭ *Mark Challberg, a cold room kind of guy*
http://bit.ly/Virology_Twiv203

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ANONYMOUS

Introduction

The genomes of DNA viruses span a considerable size range, from some 1.7 kb (circoviruses) to >2.5 Mbp (pandoraviruses), and may be single- or double-stranded DNA molecules that are linear or circular (Fig. 9.1). Whatever their physical nature, viral DNA molecules must be replicated within an infected cell to provide genomes for assembly into progeny virus particles. Such replication invariably requires the synthesis of at least one, but usually several, viral proteins. Consequently, viral DNA synthesis cannot begin immediately upon arrival of the genome at the appropriate intracellular site, but rather is delayed until viral replication proteins have attained a sufficient concentration. Initiation of viral DNA synthesis typically leads to many cycles of replication and the accumulation of large numbers of newly synthesized DNA molecules. However, longer-lasting latent infections are also common, both in nature and in the laboratory. In these circumstances, the number of viral DNA molecules made is strictly controlled.

Replication of all DNA, from the genome of the simplest virus to that of the most complex vertebrate cell, follows a set of universal rules: (i) DNA is always synthesized by template-directed, stepwise incorporation of deoxynucleoside monophosphates (dNMPs) from deoxynucleoside triphosphate (dNTP) substrates into the 3′-OH end of the growing DNA chain; (ii) each parental strand of a duplex DNA template is copied by base pairing to produce two daughter molecules identical to one another and to their parent (**semiconservative replication**); (iii) replication of DNA begins and ends at specific sites in the template, termed **origins** and **termini**, respectively; and (iv) DNA synthesis is catalyzed by DNA-dependent DNA polymerases, but many accessory proteins are required for initiation or elongation. In contrast to all DNA-dependent, and many RNA-dependent, RNA polymerases, **no** DNA polymerase can initiate template-directed DNA synthesis *de novo*. All require a **primer** with a free 3′-OH end to which dNMPs complementary to those of the template strand are added.

The genomes of RNA viruses must encode enzymes that catalyze RNA-dependent RNA or DNA synthesis. In contrast, those of DNA viruses can be replicated by the cellular machinery. Indeed, replication of the smaller DNA viruses, such as parvoviruses and polyomaviruses, requires but a single viral replication protein, and the majority of reactions are carried out by cellular proteins (Fig. 9.1). This strategy avoids the need to devote limited viral genetic coding capacity to enzymes and other proteins required for DNA synthesis. In contrast, the genomes of all larger DNA viruses encode DNA polymerases and additional replication proteins. In the extreme case, exemplified by poxviruses, the viral genome encodes a complete DNA synthesis system and is replicated in the cytoplasm of host cells.

PRINCIPLES *Replication of DNA genomes*

- As during cellular DNA replication, viral DNA is always synthesized by template-directed, stepwise incorporation of deoxynucleoside monophosphates (dNMPs) from deoxynucleoside triphosphate (dNTP) substrates into the 3′-OH end of the growing DNA chain.
  - Each parental strand of a duplex DNA template is copied by base pairing to produce two daughter molecules identical to one another and to their parent (**semiconservative replication**).
  - Replication of DNA begins and ends at specific sites in the template, termed **origins** and **termini**, respectively.
- In contrast to many RNA polymerases, no known DNA polymerase can initiate synthesis *de novo*: all require a primer with a free 3′-OH group.
- Priming of viral DNA synthesis can be via the 3′-OH terminus of RNA, a protein, or the ends of specialized structures in the genomic DNA.
- Viral DNA replication occurs either by copying of both strands at a replication fork or by copying of one strand and displacement of the other.
- The polyomavirus simian virus 40 was essential for elucidating crucial aspects of replication of viral DNA genomes, as well as identifying essential cellular replication proteins.
- Viral origins are assembly points for DNA replication machines and are recognized by dedicated origin-binding proteins.
- Viral DNA synthesis depends on a combination of viral and cellular replication proteins; in extreme cases, all replication proteins are encoded in the viral genomes.
- When viral DNA replication is carried out largely by viral proteins, cellular DNA synthesis is inhibited, probably in order to increase the pool of substrates for optimal viral replication.
- Viral DNA replication and transcription occur in discrete compartments within the cell, in which the viral proteins that participate in these processes are concentrated.
- During viral persistence, alternative replication mechanisms maintain viral genomes at low concentrations and partition them into daughter cells.
- DNA viruses are replicated with high fidelity because both cellular and viral DNA polymerases possess proofreading capability.
- Recombination drives viral diversity, and components of recombination systems may participate in viral DNA replication.

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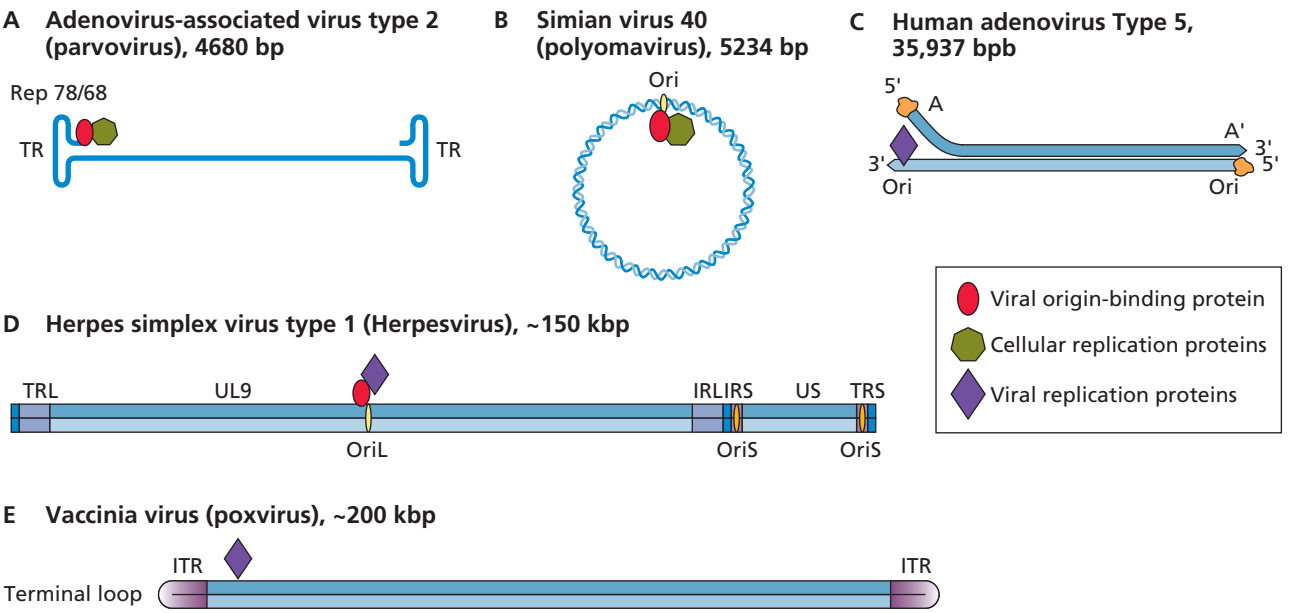


Figure 9.1 Viral and cellular proteins that synthesize viral DNA genomes. The genomes of the viruses listed are shown schematically and **not** to scale with respect to one another. The herpes simplex virus type 1 genome comprises long and short unique regions (UL and US) flanked by internal and terminal repeat sequences (IRL, IRS, TRL, TRS). When present, the positions of origins of replication (Ori) are indicated, as are the viral proteins that recognize origins, and the cellular or viral origin of the proteins that carry out DNA synthesis. ITR, inverted terminal repetition.

There is also variety in the mechanism of priming of viral DNA synthesis. In some cases, short RNA primers are first synthesized, as during replication of cellular genomes. In others, structural features of the genome or viral proteins provide primers. Despite such distinctions, the replication strategies of different viral DNAs are based on common molecular principles and one of only two mechanisms: copying of both strands of a double-stranded DNA template at a replication fork or copying of only one strand while its complement is displaced (Box 9.1). For example, the genomes of polyomaviruses and herpesviruses, which are quite different in size and structure, are replicated by the cellular replication machinery and viral replication proteins,

BOX 9.1

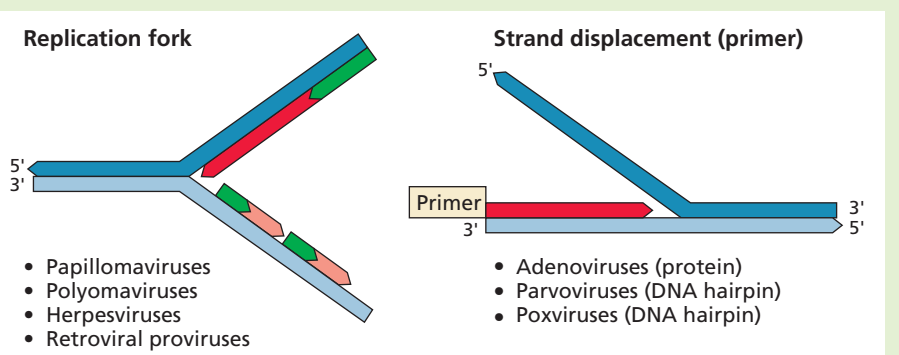
BACKGROUND

The two mechanisms of synthesis of double-stranded viral DNA molecules

Replication of double-stranded nucleic acids proceeds by **either** copying of both strands at a replication fork **or** copying of only one strand while its complement is displaced. No other replication mechanisms are known.

Among viral genomes, only those of certain double-stranded DNA viruses are synthesized via a replication fork. Replication of viral double-stranded RNAs **never** proceeds via this mechanism.

DNA synthesis via a replication fork is **always** initiated from an RNA primer. In contrast, strand displacement synthesis of viral DNA **never** requires an RNA primer.



Parental DNA, RNA primers, and newly synthesized DNA are shown in blue, green, and red/pink, respectively. The primer indicated by the tan box can be a DNA structure or a protein.

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respectively (Fig. 9.1). Nevertheless, synthesis of these two DNAs is initiated by the same priming mechanism, and the herpesviral replication machinery carries out the same biochemical reactions as the host proteins that mediate synthesis of polyomavirus DNA.

DNA Synthesis by the Cellular Replication Machinery

Our current understanding of the intricate reactions by which both strands of a typical double-stranded DNA template are copied in eukaryotic cells is based on *in vitro* studies of simian virus 40 DNA synthesis. In the next section, we discuss the cellular replication machinery that catalyzes these reactions and the molecular functions of its components that were established by such studies. Here, we briefly describe general features of eukaryotic DNA replication, and why simian virus 40 proved to be an invaluable resource for those seeking to understand this process.

Eukaryotic Replicons

General Features

The replication of large eukaryotic genomes within the lifetime of an actively growing cell depends on their organization into smaller units of replication termed **replicons** (Fig. 9.2). At the maximal rate of replication observed *in vivo*, a typical human chromosome could not be copied from a single origin as a single unit in less than 10 days! Each chromosome therefore contains many replicons, ranging in length from ~20 to 300 kbp. All but the smallest viral DNA genomes also contain two or three origins (see "Properties of Viral Replication Origins" below).

Each replicon contains an origin at which replication begins. The sites at which nascent DNA chains are being synthesized, the ends of "bubbles" seen in the electron microscope (Fig. 9.2A), are termed **replication forks**. In bidirectional replication, two replication forks are established at a single origin and move away from it as the new DNA strands are synthesized (Fig. 9.2B). However, as DNA must be synthesized in the 5′ → 3′ direction, only one of the two parental strands can be copied continuously from a primer deposited at the origin. The long-standing conundrum of how the second strand is synthesized was solved with the elucidation of the discontinuous mechanism of synthesis (Fig. 9.3A): RNA primers for DNA synthesis are synthesized at multiple sites, such that the second new DNA strand is made initially as short, discontinuous segments, termed **Okazaki fragments** in honor of the investigator who discovered them.

The discontinuous mechanism of DNA synthesis creates a special problem at the ends of linear DNAs, where excision of the terminal primer creates a gap at the 5′ end of the daughter DNA molecules (Fig. 9.3B). In the absence of a mechanism for completing synthesis of termini, discontinuous DNA

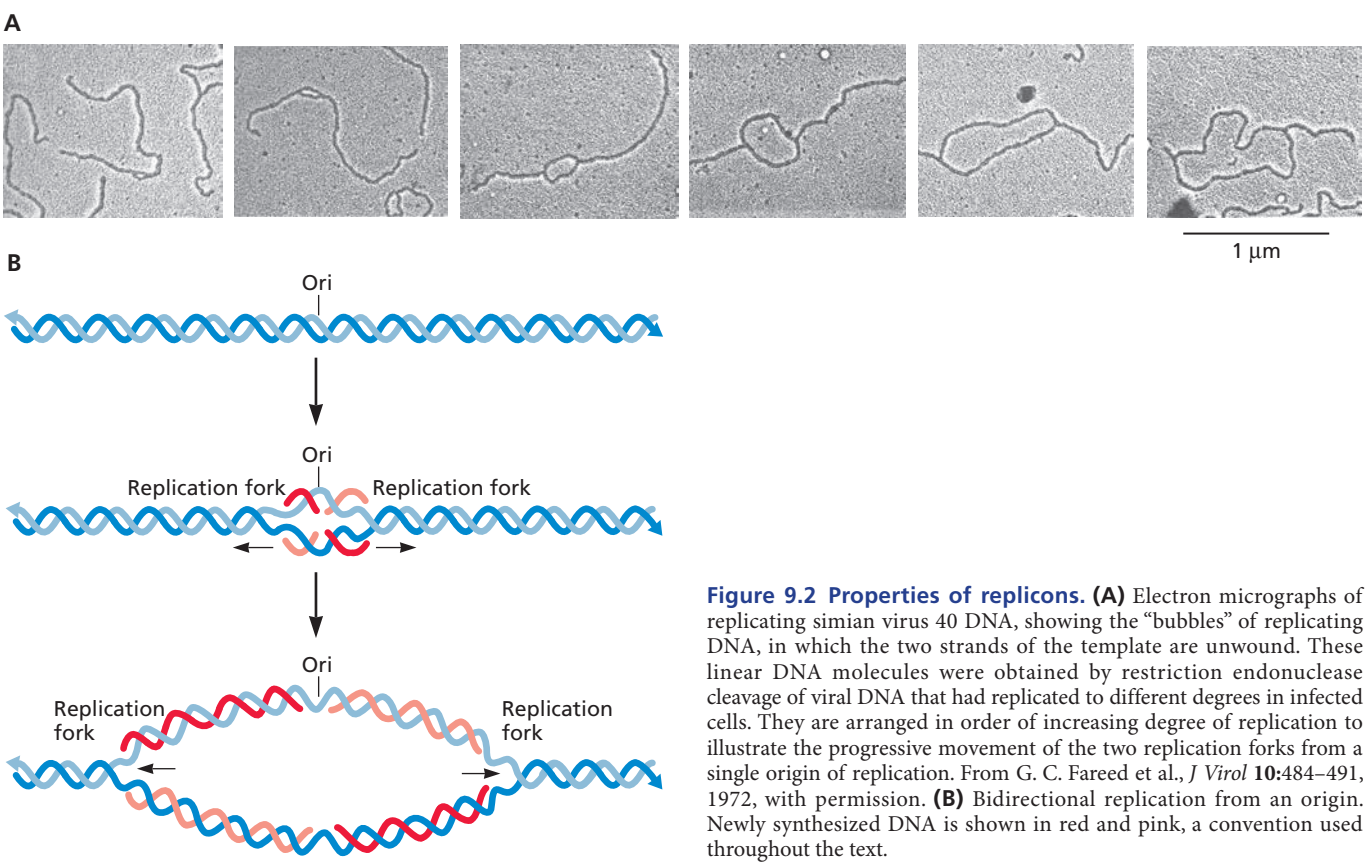


Figure 9.2 Properties of replicons. (A) Electron micrographs of replicating simian virus 40 DNA, showing the "bubbles" of replicating DNA, in which the two strands of the template are unwound. These linear DNA molecules were obtained by restriction endonuclease cleavage of viral DNA that had replicated to different degrees in infected cells. They are arranged in order of increasing degree of replication to illustrate the progressive movement of the two replication forks from a single origin of replication. From G. C. Fareed et al., *J Virol* **10:**484–491, 1972, with permission. **(B)** Bidirectional replication from an origin. Newly synthesized DNA is shown in red and pink, a convention used throughout the text.

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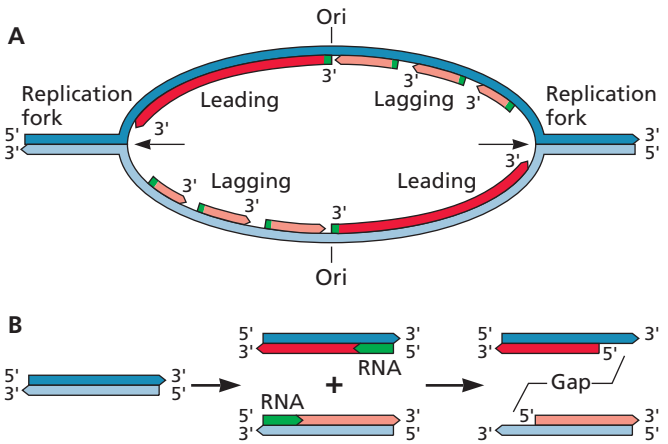


Figure 9.3 Semidiscontinuous DNA synthesis from a bidirectional origin. (A) Semidiscontinuous synthesis of the daughter strands. Synthesis of the RNA primers (green) at the origin allows initiation of continuous copying of one of the two strands on either side of the origin in the replication bubble. The second strand cannot be made in the same way (see the text). The nascent DNA population contains many small molecules termed **Okazaki fragments** in honor of the investigator who first described them. The presence of short segments of RNA at the 5′ ends of Okazaki fragments indicated that the primers necessary for DNA synthesis are molecules of RNA. With increasing time of replication, these small fragments are incorporated into long DNA molecules, indicating that they are precursors. It was therefore deduced that the second nascent DNA strand is synthesized discontinuously, also in the 5′ → 3′ direction. Because synthesis of this strand cannot begin until the replication fork has moved some distance from the origin, it is called the **lagging strand**, while the strand synthesized continuously is termed the **leading strand**. Complete replication of the lagging strand requires enzymes that can remove RNA primers, repair the gaps thus created, and ligate the individual DNA fragments to produce a continuous copy of the template strand. **(B)** Incomplete synthesis of the lagging strand. When a DNA molecule is linear, removal of the terminal RNA primer from the 5′ end of the lagging strand creates a gap that cannot be repaired by any DNA-dependent DNA polymerase.

synthesis would lead to an intolerable loss of genetic information. In chromosomal DNA, specialized elements, called **telomeres**, at the ends of each chromosome prevent loss of terminal sequences. These structures comprise simple, repeated sequences maintained by reverse transcription of an RNA template, which is an essential component of the ribonucleoprotein enzyme telomerase. As discussed subsequently, complete replication of all sequences of linear viral DNA genomes is achieved by a variety of elegant mechanisms.

Origins of Cellular Replication

It is well established that replication initiates at numerous, specific sites in eukaryotic genomes. The origins of the simple eukaryote *Saccharomyces cerevisiae* (budding yeast) can be characterized readily, because they support replication of small plasmids that are maintained as episomes. All yeast origins behave as such **autonomously replicating sequences** and can therefore be defined in detail. In contrast, this simple functional assay failed to identify analogous mammalian sequences, even when applied to DNA segments that contained origins mapped in mammalian chromosomes. Genome-wide analysis of sites of both initiation of DNA synthesis and binding of conserved replication proteins, such as the origin recognition complex (Orc), indicated that mammalian origins do not comprise specific consensus sequences (as in budding yeast). Rather, initiation sites are defined by a variety of parameters, including proximity to active promoters, presence of CG-rich sequences, and chromatin structure. The difficulties in identifying functional origins in mammalian genomes made compact viral genomes like that of simian virus 40 essential tools for elucidation of mechanisms of origin-dependent DNA synthesis.

Cellular Replication Proteins

Eukaryotic DNA Polymerases

It has been known for more than 50 years that eukaryotic cells contain DNA-dependent DNA polymerases. Mammalian cells contain several such nuclear enzymes, which are distinguished by their sensitivities to various inhibitors and their degree of **processivity**, the number of nucleotides incorporated into a nascent DNA chain per initiation reaction. These characteristics can be readily assayed in *in vitro* reactions with artificial template-primers, such as gapped or nicked DNA molecules. The requirements for viral DNA synthesis *in vitro* and genetic analyses (performed largely with yeasts) identified DNA polymerases α, δ, and ε as the enzymes that participate in genome replication. Other DNA polymerases are restricted to mitochondria or act only during repair of damaged DNA (e.g., DNA polymerase β). Only DNA polymerase α is associated with priming activity, because it is bound tightly to a heteromeric **primase**.

One of the most striking properties of these DNA polymerases is their obvious evolutionary relationships to prokaryotic and viral enzymes. All template-directed nucleic acid polymerases share several sequence motifs and probably a similar core architecture (Chapters 6 and 7), indicating that important features of the catalytic mechanisms are also common to all these enzymes.

Other Proteins Required for DNA Synthesis in Mammalian Cells

Analogy with well-characterized bacterial DNA replication machines indicated that several proteins in addition to DNA polymerase and primase would be required for mammalian DNA synthesis. Identification of such proteins awaited the development of cell-free systems for origin-dependent initiation. This feat was first accomplished for synthesis of adenoviral DNA, a breakthrough soon followed by origin-dependent replication of simian virus 40 DNA *in vitro*. Because cellular components are largely responsible for simian virus 40 DNA synthesis, development of this system proved to be the watershed in the investigation of eukaryotic DNA replication: it allowed the identification

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of previously unknown cellular replication proteins and elucidation of their mechanisms of action.

Mechanisms of Viral DNA Synthesis

In this section, we first describe the contribution of studies of simian virus 40 genome replication, for subsequent comparison to the variety of virus-specific solutions to the mechanistic problems associated with each step in DNA synthesis.

Lessons from Simian Virus 40

The Origin of Simian Virus 40 DNA Replication

The simian virus 40 (SV40) origin was the first viral control sequence to be located on a physical map of the viral genome, in which the reference points were restriction endonuclease cleavage sites (Box 9.2). We now possess a detailed picture of this viral origin (Fig. 9.4) and of the binding sites for the viral origin recognition protein, large T antigen (LT). A 64-bp sequence, the **core origin**, which lies between the sites at which early and late transcription begin, is sufficient for initiation of DNA synthesis in infected cells. This sequence contains four copies of a pentanucleotide-binding site for LT, flanked by an AT-rich element and a 10-bp imperfect palindrome (Fig. 9.4). Additional sequences within this busy control region of the viral genome increase the efficiency of initiation of DNA synthesis from this core origin.

Mechanism of Simian Virus 40 DNA Synthesis

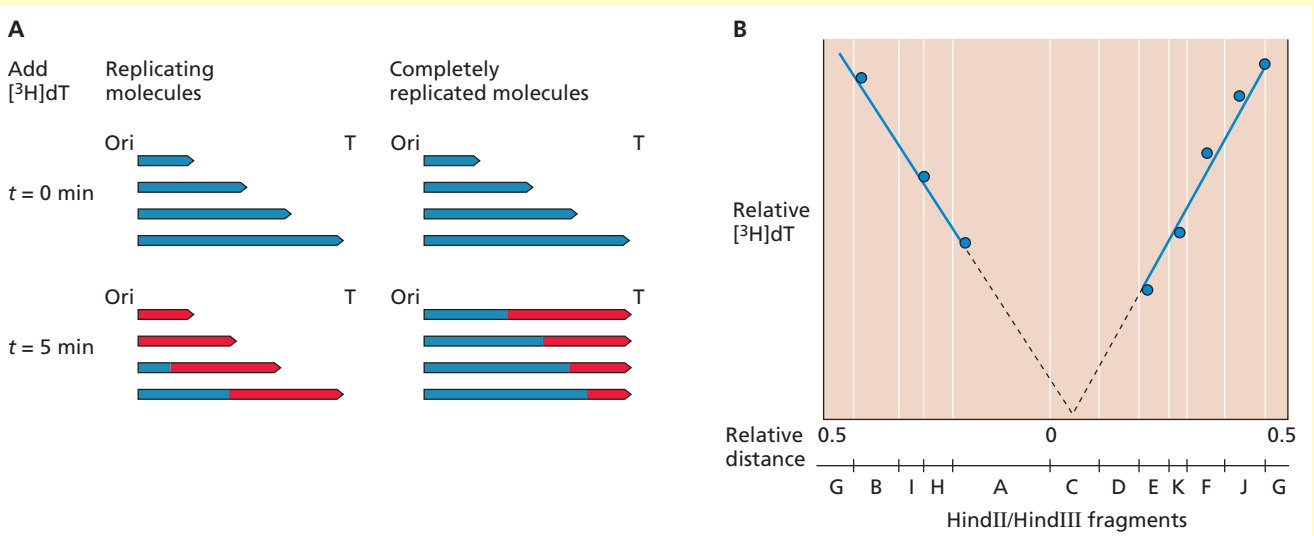
Origin recognition and unwinding. The first step in SV40 DNA synthesis is the recognition of the origin by LT, the major early gene product of the virus. This viral protein can bind to pentanucleotide repeat sequences in the core origin to form a hexamer (Fig. 9.4). However, initiation of viral DNA synthesis also requires the sequences that flank the minimal origin. When bound to ATP, LT assembles to form a double hexamer on the origin and elicits structural distortions in the flanking sequences. In concert with cellular replication protein A (Rp-A), which possesses single-stranded-DNA-binding activity, the intrinsic 3′ → 5′ helicase activity of LT then harnesses the energy of ATP hydrolysis to unwind DNA bidirectionally from the core

BOX 9.2

EXPERIMENTS

Mapping of the simian virus 40 origin of replication

As illustrated in panel **A** of the figure (left), exposure of simian virus 40-infected monkey cells to [^{3}H]thymidine ([^{3}H]dT) for a period less than the time required to complete one round of replication (e.g., 5 min) results in labeling of the growing points of replicating DNA. If replication proceeds from a specific origin (Ori) to a specific termination site (T), the DNA replicated last will be labeled preferentially in the population of completely replicated molecules (panel **A**, right). The distribution of [^{3}H]thymidine among the fragments of completely replicated viral DNA generated by digestion with restriction endonucleases HindII and HindIII is shown in panel **B**. The simian virus 40 genome is represented as cleaved within the G fragment, and relative distances are given with respect to the junction of the A and C fragments. The observation of two decreasing gradients of labeling that can be extrapolated (dashed lines) to the same region of the genome confirmed that simian virus 40 replication is bidirectional (Fig. 9.2B) and allowed location of the origin on the physical map of the viral genome. Modified from K. J. Danna and D. Nathans, *Proc Natl Acad Sci U S A* **69:**3097–3100, 1972, with permission.



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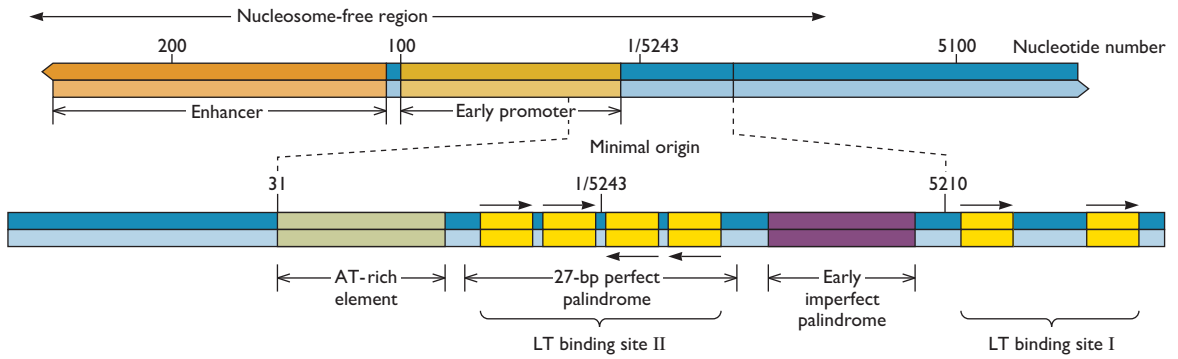


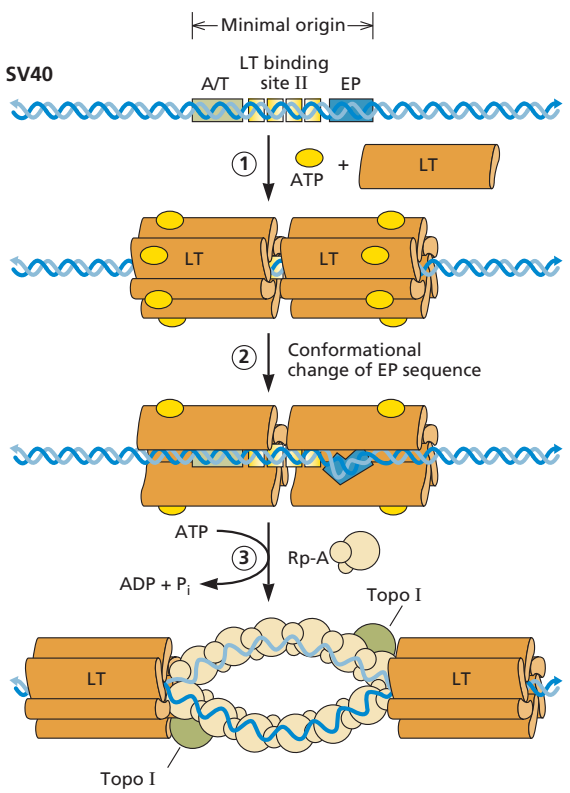
Figure 9.4 The origin of simian virus 40 DNA replication. The positions in the simian virus 40 genome of the minimal origin necessary for DNA replication *in vivo* and *in vitro* and of the enhancer and early promoter (see Chapter 8) are indicated. The pentameric LT-recognition sequences are shown in yellow. The AT-rich element and early imperfect palindrome, as well as LT-binding site II, are essential for replication. A second LT-binding site (site I) stimulates replication modestly *in vivo*. Other sequences, including the enhancer and Sp1-binding sites of the early promoter, increase the efficiency of viral DNA replication at least 10-fold. The activation domains (see Chapter 8) of transcriptional regulators that bind to these sequences might help recruit essential replication proteins to the origin. Alternatively, the binding of transcriptional activators might induce remodeling of chromatin in the vicinity of the origin. This possibility is consistent with the fact that, as indicated at the top, the region of the genome containing the origin and transcriptional control regions is nucleosome free in a significant fraction (~25%) of minichromosomes in infected cells.

origin (Fig. 9.5). Assembly of LT at the SV40 origin resembles assembly reactions at well-characterized bacterial origins, such as *Escherichia coli* OriC, or the origin of phage λ, in which multimeric protein structures assemble on AT-rich sequences. Furthermore, formation of hexamers around DNA is a property common to several viral and cellular replication proteins.

Leading-strand synthesis. Binding of DNA polymerase α-primase to both LT and Rp-A at the SV40 origin sets the stage for the initiation of leading-strand synthesis (Fig. 9.6). The primase synthesizes the RNA primers of the leading strand at each replication fork, while DNA polymerase α extends them to produce short fragments. The 3′-OH ends of these DNA fragments are then bound by cellular replication factor C (Rf-C), proliferating-cell nuclear antigen (Pcna), and DNA polymerase ε. Pcna is the processivity factor for DNA polymerase ε: it is required for synthesis of long DNA chains from a single primer. This mammalian protein is the functional analog of the β subunit of *E. coli* DNA polymerase III and phage T4 gene 45 product. These remarkable **sliding clamp** proteins form closed rings that track along the DNA template and serve as movable platforms for DNA polymerases. Subsequent binding of the replicative DNA polymerase completes assembly of a multiprotein assembly capable of leading-strand synthesis by continuous copying of the parental template strand.

Lagging-strand synthesis. The first Okazaki fragment of the lagging strand is synthesized by the DNA polymerase α-primase complex (Fig. 9.6, step 4). The lagging strand is synthesized by DNA polymerase δ, and transfer of the 3′ end of the first Okazaki fragment to this enzyme is thought to proceed as on the leading strand. The lagging-strand template is then copied **toward** the origin of replication**.** Consequently, synthesis of the lagging

Figure 9.5 Model of the recognition and unwinding of the simian virus 40 origin. In the presence of ATP, two hexamers bind to the origin via the pentanucleotide LT-binding sites (step 1). Binding of LT hexamers protects the flanking AT-rich (A/T) and early palindrome (EP) sequences of the minimal origin from DNase I digestion and induces conformational changes, for example, distortion of the early palindrome (step 2). Stable unwinding of the origin requires the cellular, single-stranded-DNA-binding protein replication protein A (Rp-A), which binds to LT. LT helicase activity, in concert with Rp-A and topoisomerase I, progressively unwinds the origin (step 3).



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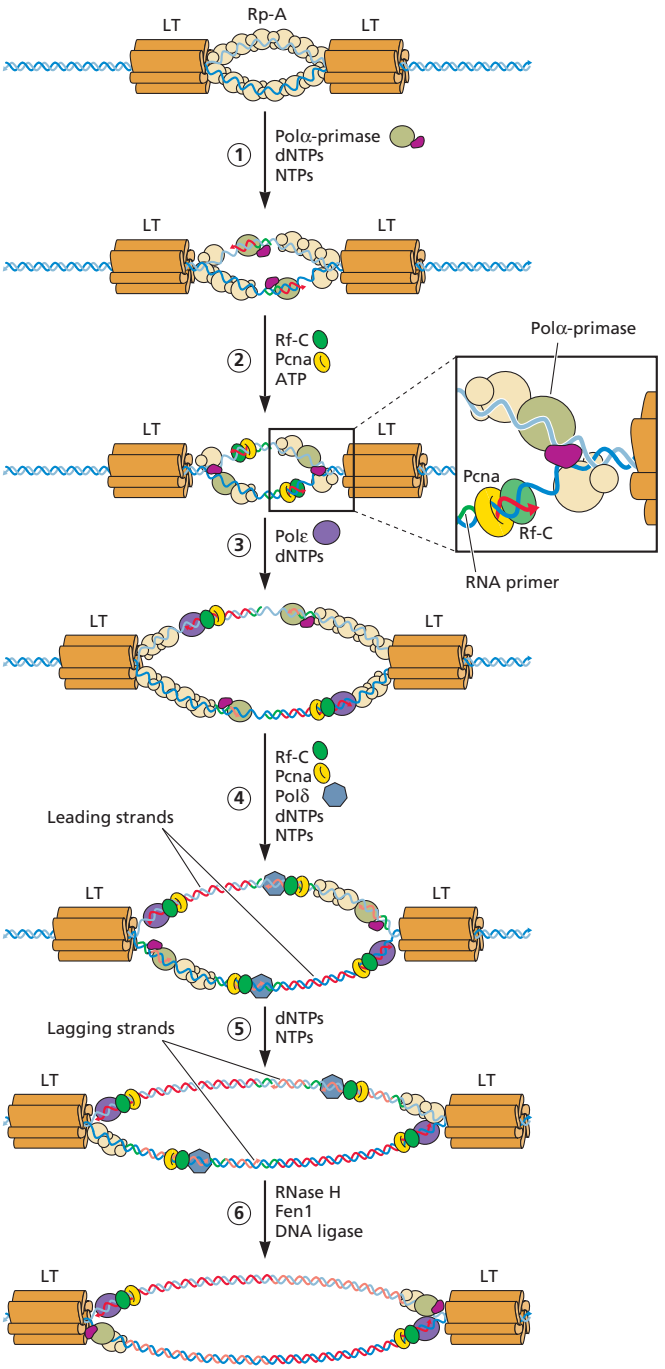


Figure 9.6 Synthesis of leading and lagging strands. The DNA polymerase (Pol) α-primase responsible for the synthesis of Okazaki fragments binds specifically to both replication protein A (Rp-A) and large T antigen (LT) assembled at the origin in the presynthesis complex. Once bound, the enzymes synthesize leading-strand RNA primers that are subsequently extended as DNA (step 1). The 3′-OH group of the nascent RNA-DNA fragment (~30 nucleotides in total length) is then bound by replication factor C (Rf-C) in a reaction that requires ATP but not its hydrolysis. Rf-C allows ATP-dependent opening of the proliferating-cell nuclear antigen (Pcna) ring and its loading onto the template (step 2). This reaction induces dissociation of DNA polymerase α-primase. Replicative DNA polymerase (usually ε) then binds to the Pcna/Rf-C complex (step 3). Because the clamp-loading protein Rf-C binds to the 5′-OH ends of the DNA fragments, it places the processivity protein at the replication forks. This replication complex is competent for continuous and highly processive synthesis of the leading strands (steps 4 and 5). Lagging-strand synthesis begins with synthesis of the first Okazaki fragment by DNA polymerase α-primase (step 3). Processive DNA polymerase (δ) is recruited as during leading-strand synthesis and produces a lagging-strand segment (step 5). The multiple DNA fragments produced by discontinuous lagging-strand synthesis are sealed by removal of the primers by RNase H (an enzyme that specifically degrades RNA hybridized to DNA) and the 5′ → 3′ exonuclease Fen1, repair of the resulting gaps by DNA polymerase δ, and joining of the DNA fragments by DNA ligase I (step 6).

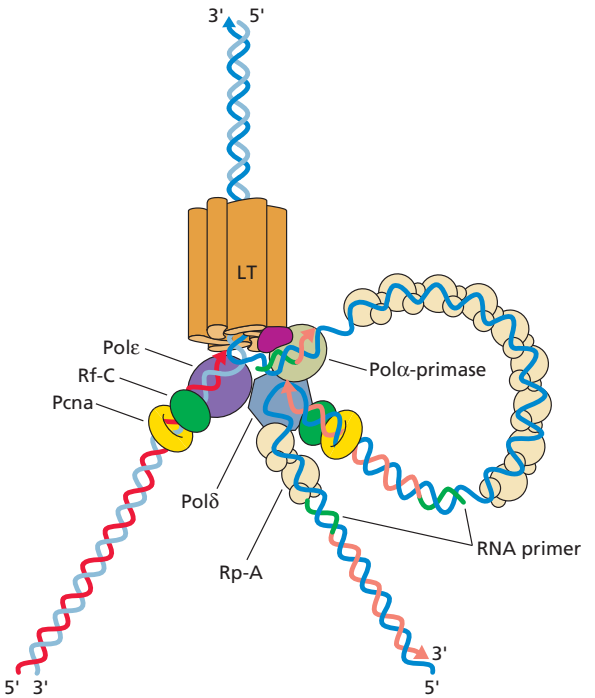


Figure 9.7 A model of the simian virus 40 replication machine. A replication machine containing all proteins necessary for both continuous synthesis of the leading strand and discontinuous synthesis of the lagging strand would assemble at each replication fork. Spooling of a loop of the template DNA strand for discontinuous synthesis would allow the single complex to copy the two strands in opposite directions. Pol, polymerase.

strand requires initiation by DNA polymerase α-primase at many sites progressively farther from the origin. The mechanisms by which leading- and lagging-strand synthesis are coordinated are not fully understood. If the replication machinery tracked along an immobile DNA template, the complexes responsible for leading- and lagging-strand synthesis would have to move in opposite directions. A more attractive alternative is that the DNA template is spooled through an immobile replication complex that contains all the proteins necessary for synthesis of both daughter strands. This mechanism would allow simultaneous copying of the template strands in opposite directions at each fork (Fig. 9.7). Consistent with this idea, replication

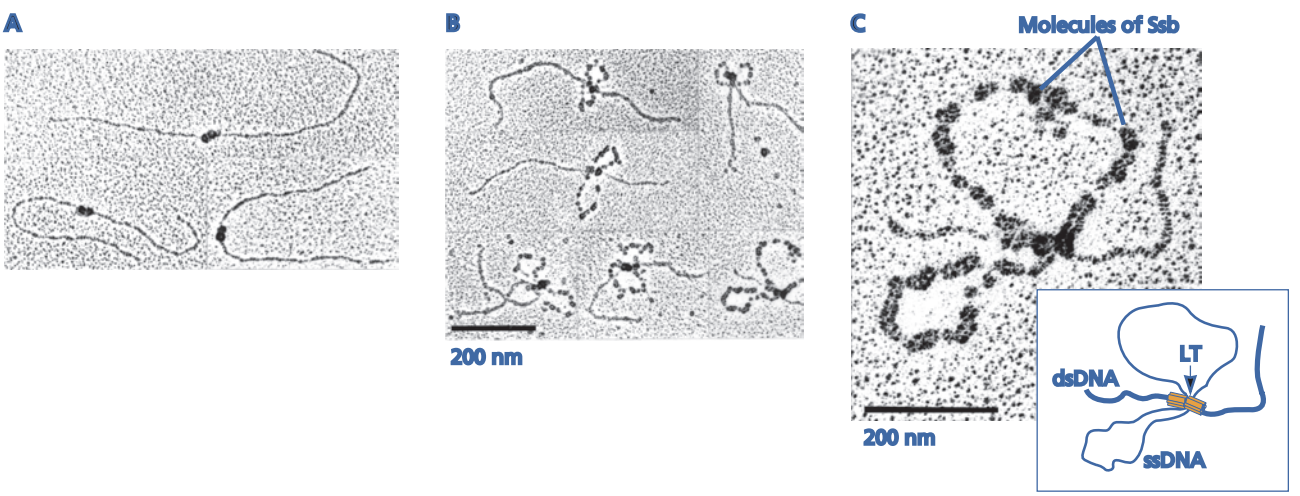
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BOX 9.3

EXPERIMENTS

Unwinding of the simian virus 40 origin leads to spooling of DNA

Visualization by electron microscopy of structures formed during LT-dependent unwinding from the simian virus 40 origin *in vitro* suggested that the two hexamers remain in contact as DNA is unwound. LT was incubated with origin-containing DNA and ATP for 15 min in the presence of *E. coli* single-stranded binding protein (Ssb) to stabilize unwound DNA. Proteins were then cross-linked to DNA and samples processed for negative-contrast electron microscopy. **(A)** LT bound to the origin, as a characteristic bilobed structure (the double hexamer shown in Fig. 9.5); **(B)** unwinding intermediates; **(C)** the intermediate at the bottom right in panel B at higher magnification. This intermediate contains a bilobed LT complex connecting the two replication forks, so the single-stranded DNA (ssDNA), which is marked by the Ssb molecules bound to it, is looped out as "rabbit ears." The formation of such structures containing a dimer of the LT hexamer, in which each monomer is bound to a replication fork, stimulates the helicase activity of LT. This property supports the view that the DNA template is spooled through an immobile replication machine (see the text). dsDNA, double-stranded DNA. From R. Wessel et al., *J Virol* **66:**804–815, 1992, with permission. Courtesy of H. Stahl, Universität des Saarlandes.



of chromosomal DNA occurs at fixed sites in the nucleus, and proteins that interact with the replicative helicase and both leading-strand and lagging-strand DNA polymerases have been identified. Furthermore, structures indicative of DNA spooling have been observed in the electron microscope during the initial, LT-dependent unwinding of the SV40 origin (Box 9.3).

Base pairing of dNTP substrates with template DNA requires the unwinding of double-stranded DNA genomes like that of SV40. LT is the helicase responsible for unwinding DNA at the origin, and remains associated with the replication forks, unwinding the template during elongation (Fig. 9.6).

Termination and resolution. Because the circular SV40 DNA genome possesses no termini, its replication does not lead to gaps in the strands made discontinuously. Nevertheless, additional cellular proteins are needed for the production of two daughter molecules from the circular template. These essential components are the cellular enzymes topoisomerases I and II, which alter the topology of DNA. These enzymes, which differ in their catalytic mechanisms and functions in the cell, reverse the winding of one duplex DNA strand around another (**supercoiling**). Because they remove supercoils, topoisomerases are said to **relax** DNA. In a closed circular DNA molecule, the unwinding of duplex DNA at the origin and subsequently at the replication forks is necessarily accompanied by supercoiling of the remainder of the DNA (Fig. 9.8A). If not released, the torsional stress so introduced would act as a brake on movement of the replication forks, eventually bringing them to a complete halt. Topoisomerase I associates with LT and is required for progression of SV40 replication forks and for viral reproduction. A single cycle of SV40 DNA synthesis produces two interlocked (catenated) circular DNA molecules that can be separated only when one DNA molecule is passed through a double-strand break in the other. The break is then resealed. Topoisomerase II catalyzes this series of reactions (Fig. 9.8B).

Replication of chromatin templates. The SV40 genome is associated with cellular nucleosomes both in virus particles and

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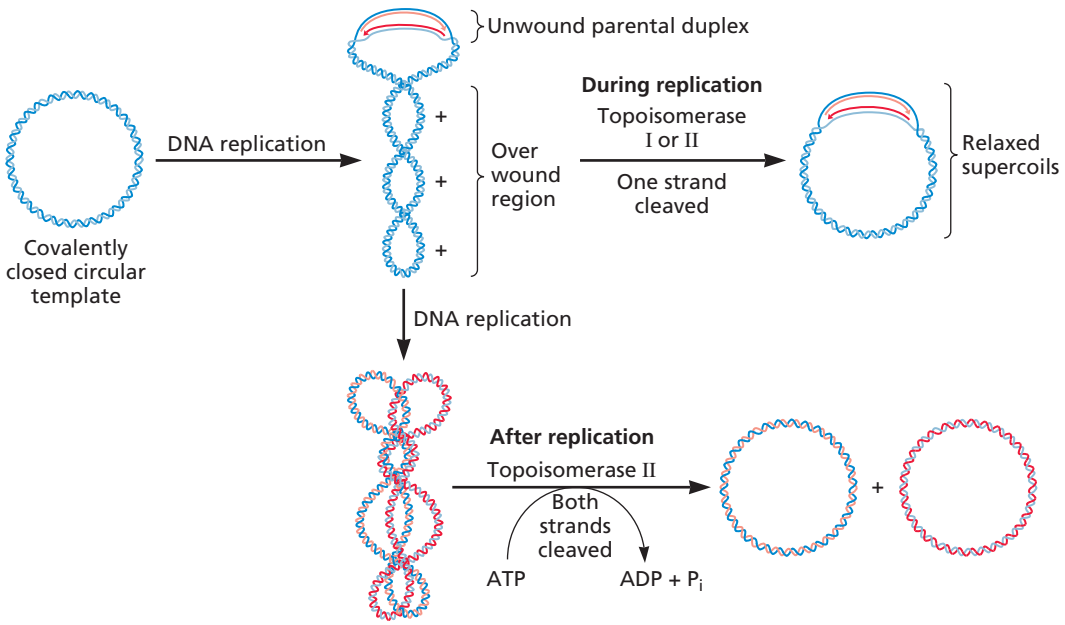


Figure 9.8 Function of topoisomerases during simian virus 40 DNA replication. Unwinding of the template DNA at the origin and two replication forks leads to overwinding (positive supercoiling) of the DNA ahead of the replication forks (middle). Either topoisomerase I or topoisomerase II can remove the supercoils to relieve such overwinding and allow continued movement of the replication fork. However, LT binds to topoisomerase I, and substitutions that impair this interaction inhibit LT-dependent DNA synthesis *in vitro* and viral reproduction in infected cells. The products of genome replication are interlocked daughter molecules (below). Their separation requires topoisomerase II, which makes a double-strand break in DNA, passes one double strand over the other to unwind one turn, and reseals the DNA in reactions that require hydrolysis of ATP.

in infected cell nuclei. It is therefore replicated as a minichromosome, in which the DNA is wrapped around nucleosomes. This arrangement raises the question of how the replication machinery can copy a DNA template that is bound to nucleosomal histones. A similar problem is encountered during the replication of many viral RNA genomes, when the template RNA is packaged by viral RNA-binding proteins in a large ribonucleoprotein. The mechanisms by which replication complexes circumvent such barriers to movement are not understood in detail. Nevertheless, numerous proteins that couple ATP hydrolysis to remodeling of nucleosomal DNA have been identified (see Chapter 8). The organization of the SV40 genome into a minichromosome also implies that viral DNA replication must be coordinated with binding of newly synthesized DNA to cellular nucleosomes. In fact, new nucleosomes are deposited at viral replication forks, a reaction that is catalyzed by the essential human protein chromatin assembly factor 1.

Summary. Analysis of simian virus 40 replication *in vitro* identified essential cellular replication proteins, led to molecular descriptions of crucial reactions in the complicated process of DNA synthesis, and provided new insights into chromatin assembly. The detailed understanding of the reactions completed by the cellular DNA replication machinery laid the foundation for elucidation of the mechanisms by which other animal viral DNA genomes are replicated, and of some of the intricate circuits that regulate DNA synthesis and its initiation.

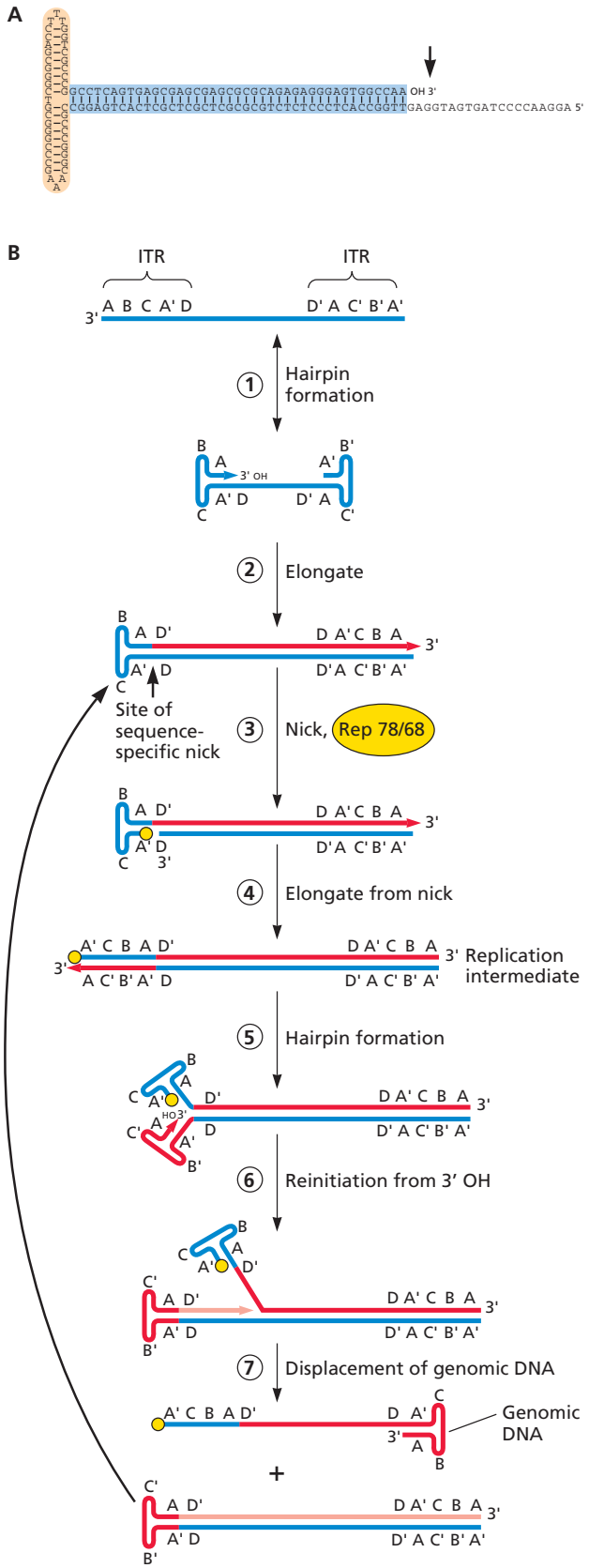
Replication of Other Viral DNA Genomes

The replication of all viral DNA genomes within infected cells comprises reactions analogous to those necessary for simian virus 40 DNA synthesis, namely, origin recognition and assembly of a presynthesis complex, priming of DNA synthesis, elongation, termination, and often resolution of the replication products. However, the mechanistic problems associated with each of these reactions are solved by a variety of virus-specific mechanisms. Synthesis of viral DNA molecules is initiated not only by RNA priming, but also by unusual mechanisms in which DNA and even protein molecules function as primers. As we shall see, these latter priming strategies circumvent the need for discontinuous synthesis of daughter DNA molecules.

Synthesis of Viral RNA Primers by Cellular or Viral Enzymes

The standard method of priming is synthesis of a short RNA molecule by a specialized primase. As we have seen, cellular DNA polymerase α-primase synthesizes all RNA primers needed for replication of both template strands of polyomaviral

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genomes. A similar mechanism operates at papillomavirus origins and those of some herpesviruses, such as that directing replication of the episomal Epstein-Barr viral genome in latently infected cells (see " Different Viral Origins Regulate Replication of Epstein-Barr Virus" below). The integrated proviral genomes of retroviruses are also replicated via RNA primers, which are synthesized by the cellular primase at the origin of the cellular replicon in which the provirus resides. In actively dividing cells, proviral DNA is therefore replicated once per cell cycle by the cellular replication machinery. Viral primases can also synthesize RNA primers. This mechanism is characteristic of genome replication during productive infection by herpesviruses, such as herpes simplex virus type 1.

Priming via DNA: Specialized Structures in Viral Genomes

Self-priming of viral DNA synthesis via specialized structures in the viral genome is a hallmark of all *Parvoviridae*, among the smallest DNA viruses that replicate in animal cells. This virus family includes the dependoviruses, such as adenovirus-associated viruses, and the autonomous parvoviruses, such as minute virus of mice. This mechanism is illustrated here with adenovirus-associated virus, which has a small (<5-kb) genome of single-stranded, linear DNA that carries **inverted terminal repetitions** (ITRs). Genomic DNA is of both (+) and (−) polarity, for both strands are encapsidated, but in separate virus particles. Palindromic sequences within the central 125 nucleotides of the ITR base pair to form T-shaped structures (Fig. 9.9A). Formation of this

Figure 9.9 Replication of parvoviral DNA. (A) Sequence and secondary structure of the adenovirus-associated virus type 2 inverted terminal repetition (ITR). A central palindrome (tan background) is flanked by a longer palindrome (light blue background) within the ITR. When these bases pair at the 3′ end of the genome, a T-shaped structure in which the internal duplex stem terminates in a free 3′-OH group (arrow) is formed. **(B)** Model of adenovirus-associated virus DNA replication. The ITRs are represented by 3′ABCA′D5′ and 5′A′B′C′AD′3′. Formation of the 3′-terminal hairpin provides a template-primer (step 1). Elongation from the 3′-OH group of the hairpin allows continuous synthesis (red) to the 5′ end of the parental strand (step 2). To complete copying of the parental strand, a nick to generate a new 3′ OH is introduced at the specific **terminal resolution site** (marked by the arrow) by the viral Rep 78/68 proteins (step 3). Elongation from the nick results in copying of sequences that initially formed the self-priming hairpin to form the double-stranded replication intermediate (step 4). However, the parental strand then contains newly replicated DNA (red) at its 3′ end. As a result, the ITR of the parental strand is no longer the initial sequence but rather its complement. This palindromic sequence is therefore present in populations of adenovirus-associated virus DNA molecules in one of two orientations. Such sequence heterogeneity provided an important clue for elucidation of the mechanism of viral DNA synthesis. The newly replicated 3′ end of the replication intermediate can form the same terminal hairpin structure (step 5) to prime a new cycle of DNA synthesis (step 6) with displacement of a molecule of single-stranded genomic DNA, and the formation of the incompletely replicated molecule initially produced (step 7). The latter molecule can undergo additional cycles of replication as in steps 3 and 4.

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structure at the 3′ end of single-stranded viral DNA provides an ideal template-primer for initiation of viral DNA synthesis (Fig. 9.9B). Experimental evidence for such **self-priming** includes the dependence of adenovirus-associated virus DNA synthesis on self-complementary sequences within the ITR. Following recognition of the free 3′-OH end of the viral DNA primer, the single template strand of an infecting genome can be copied by a continuous mechanism, analogous to leading-strand synthesis during replication of double-stranded DNA templates. In subsequent cycles of replication, the same 3′-terminal priming structures form in the duplex replication intermediate produced in the initial round of synthesis (Fig. 9.9B). Adenovirus-associated virus DNA synthesis is therefore always continuous and requires cellular DNA polymerase δ, Rf-C, and Pcna, but not DNA polymerase α-primase.

On the other hand, a specialized mechanism **is** necessary to complete replication of each strand, because the initial product retains the priming hairpin and is largely duplex DNA in which parental and daughter strands are covalently connected (Fig. 9.9.B, step 2). Complete copying is initiated by nicking of this intermediate within the parental DNA strand at a specific site. The new 3′-OH end liberated in this way then primes continuous synthesis to the end of the DNA molecule (Fig. 9.9B, step 4). The nick is introduced by the related viral proteins Rep 78 and Rep 68 (Rep 78/68). These proteins are site- and strand-specific endonucleases, which bind to, and cut at, specific sequences within the ITR. During this terminal resolution process, Rep 78/68 becomes covalently linked to the cleaved DNA at the sites that will become the 5′ termini of the fully replicated molecule and single-stranded daughter genome. This covalent linkage is maintained during genome encapsidation and assembly of virus particles, but the subsequent fate of genome-linked Rep 78/68 is not known. Following the synthesis of a duplex of the genomic DNA molecule (the **replication intermediate**), formation of the 3′-terminal priming hairpin allows continuous synthesis of single-stranded genomes by a strand displacement mechanism, with re-formation of the replication intermediate (Fig. 9.9B, steps 6 and 7).

Rep 78 and Rep 68 are similar to simian virus 40 LT in several respects and can be considered origin recognition proteins (Table 9.1). They are the only viral gene products necessary for parvoviral DNA synthesis. In addition to recognizing and cleaving the terminal resolution site, these proteins provide the ATP-dependent, 3′ → 5′ helicase activity needed for unwinding of the replicated ITR and re-formation of the priming hairpin (Fig. 9.9B, step 5). However, the cellular helicase Mcm (minichromosome maintenance complex) is also required for adenovirus-associated virus DNA synthesis *in vitro* and in infected cells.

Whether priming of DNA synthesis via complementary sequences in the genome is a unique feature of parvoviral replication is not yet clear. Single-stranded, linear genomes of other viruses, such as the widespread geminivirus of plants, are not replicated in this way, but by a rolling-circle mechanism (Box 9.4). However, a self-priming and strand displacement mechanism is consistent with some, but not all, properties of replication of the large, double-stranded DNA genomes of poxviruses, such as vaccinia virus (Box 9.5).

Table 9.1 Viral origin recognition proteins

Virus	Protein(s)	Origin-binding properties	Other activities and functions
Parvovirus			
Adenovirus-associated virus	Rep 78/68	Binds to specific sequences in ITR as hexamer	Site- and strand-specific endonuclease; ATPase and helicase; transcriptional regulator
Papovaviruses			
Simian virus 40	LT	Binds cooperatively to origin site II to form double hexamer; distorts origin	ATPase and helicase; binds to cellular Rp-A and polymerase α-primase; represses early and activates late transcription; binds cellular Rb protein to induce progression through the cell cycle
Bovine papillomavirus type 5	E1	Binds strongly and cooperatively only in presence of E2 protein	ATPase and helicase
	E2	Binds to specific sequences in origin as dimer	Regulates transcription by binding to viral enhancers
Adenovirus			
Human adenovirus type 5	Pre-TP–DNA polymerase	Binds to minimal origins	Primes continuous synthesis of both strands of viral genome
Herpesviruses			
Herpes simplex virus type 1	UL9	Binds cooperatively to specific sites in viral origins; distorts DNA	ATPase and helicase; binds UL29 protein, UL8 subunit of viral primase, and UL42 processivity protein
Epstein-Barr virus	EBNA-1	Binds to multiple sites in OriP as dimer	Stimulates transcription from viral promoters

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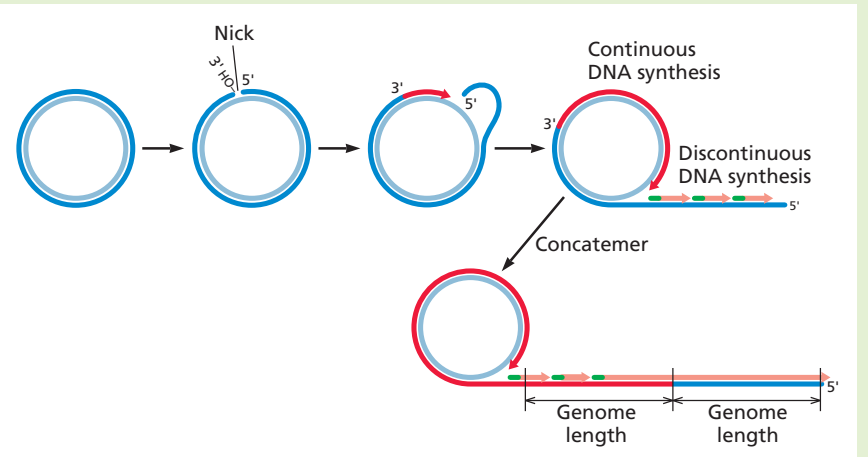
BOX 9.4

BACKGROUND

Rolling-circle replication

The rolling-circle replication mechanism of DNA synthesis was discovered during studies of the replication of the single-stranded DNA genome of bacteriophage ϕX174. However, it also operates during replication of double-stranded genomes, such as that of bacteriophage λ.

Rolling-circle replication is initiated by introduction of a nick that creates a 3′-OH end in one strand of a double-stranded, circular DNA. One strand of the template is copied continuously, and multiple times, while the displaced strand is copied discontinuously. As shown in the figure, this mechanism produces genome concatemers.



Protein Priming

Initiation of DNA synthesis via a protein primer is a relatively rare mechanism, restricted to some bacteriophages (e.g., ϕ29 and PRD1) and to hepadnaviruses and adenoviruses among those DNA viruses that infect animal cells. The replication of some viral RNA genomes is also initiated from a protein primer, notably the VPg protein of poliovirus discussed in Chapter 6. Here, we use adenoviral replication to illustrate the mechanism of protein priming.

The 5′ ends of adenoviral genomes that enter infected nuclei are covalently linked to the terminal protein (TP). The precursor to this protein (Pre-TP) serves as the primer for viral DNA synthesis. The adenoviral DNA polymerase covalently links the α-phosphoryl group of dCMP to the hydroxyl group of a specific serine residue in Pre-TP (Fig. 9.10). The 3′-OH group of the protein-linked dCMP then primes synthesis of daughter viral DNA strands by the viral DNA polymerase. Once the first few nucleotides have been incorporated, the DNA polymerase must disassociate from Pre-TP to allow elongation of the daughter DNA strand. The structure of the ϕ29 DNA polymerase bound to its priming terminal protein suggests that such dissociation is the result of conformational change induced by displacement of the priming domain from the catalytic site in the polymerase (Box 9.6). The nucleotide is added to Pre-TP **only** when this protein primer is assembled with the DNA polymerase into preinitiation complexes at the origins of replication. As the origins lie at the ends of the linear genome, each template strand is then copied continuously from one end to the other by strand displacement (Fig. 9.10). The parental template strand displaced initially is copied by the same mechanism, following annealing of an ITR sequence to re-form the duplex DNA sequence present at the ends of parental DNA. This unusual strand displacement mechanism therefore results in semiconservative replication, even though the two parental strands of viral DNA are not copied at the same replication fork.

Properties of Viral Replication Origins

Origins of replication contain the sites at which viral DNA synthesis begins and can be defined experimentally as the minimal DNA segment necessary for initiation of replication in cells or *in vitro* reactions. Viral origins of replication support initiation of DNA synthesis by a variety of mechanisms, including some with no counterpart in cellular DNA synthesis. Nevertheless, they are discrete DNA segments that contain sequences recognized by viral origin recognition proteins to seed assembly of multiprotein complexes, and they exhibit a number of common features.

Number of Origins

In contrast to papillomaviral and polyomaviral DNAs, the genomes of the larger DNA viruses contain not one, but two or three origins. As noted above, the two identical adenoviral origins at the ends of the linear genome are the sites of assembly of preinitiation complexes (Fig. 9.10). The genomes of herpesviruses, such as Epstein-Barr virus and herpes simplex virus type 1, contain three origins of replication. Different functions can be ascribed to the different Epstein-Barr virus origins: a single origin (OriP) allows maintenance of episomal genomes in latently infected cells (see "Different Viral Origins Regulate Replication of Epstein-Barr Virus" below), while the two others (OriLyt)

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BOX 9.5

DISCUSSION

Self-priming or RNA priming of vaccinia viral DNA synthesis?

The two strands of the large DNA genome of vaccinia virus are covalently connected by terminal, unpaired loops at the ends of inverted terminal repeated sequences (Appendix, Fig. 25). The potential of these terminal sequences to form hairpins suggests the possibility for self-pairing and continuous synthesis of viral DNA, initiated following introduction of a nick near one or both termini to form a 3′-OH primer (see the figure). This model is consistent with the observation that the products of DNA synthesis are concatemers of the viral genome (see the figure), and with the changes in the sedimentation properties of viral DNA that are indicative of nicking that occur following entry into host cells. In addition, the terminal sequences of the viral genome confer optimal replication upon linear minichromosome templates. However, vaccinia virus-infected cells support replication of exogenous circular plasmids that contain **no** viral DNA. Such origin-independent replication takes place at the sites of viral genome replication, specialized cytoplasmic replication factories, and requires viral replication proteins. These properties suggest an alternative model for vaccinia viral DNA synthesis.

Indeed, early studies reported the detection of short fragments of newly synthesized vaccinia viral DNA that were covalently linked to RNA and became incorporated into larger DNA molecules. The production of these viral Okazaki fragments suggests a mechanism that includes RNA priming and discontinuous DNA synthesis during genome replication. Viral enzymes that catalyze reactions pivotal to this mechanism have been identified only quite recently. For example, the D5R protein, long known to be a helicase, contains an N-terminal primase that catalyzes DNA-dependent synthesis of RNA with little template specificity. Viral replication also requires a DNA ligase, the enzyme that joins DNA fragments following discontinuous DNA synthesis, encoded by either the viral A50R gene, or, in actively growing cells, cellular DNA ligase 1.

These apparently contradictory observations illustrate the difficulties of establishing how large viral DNA genomes are replicated. They have yet to be reconciled (or explained), and there is currently no generally accepted model of vaccinia viral DNA synthesis.

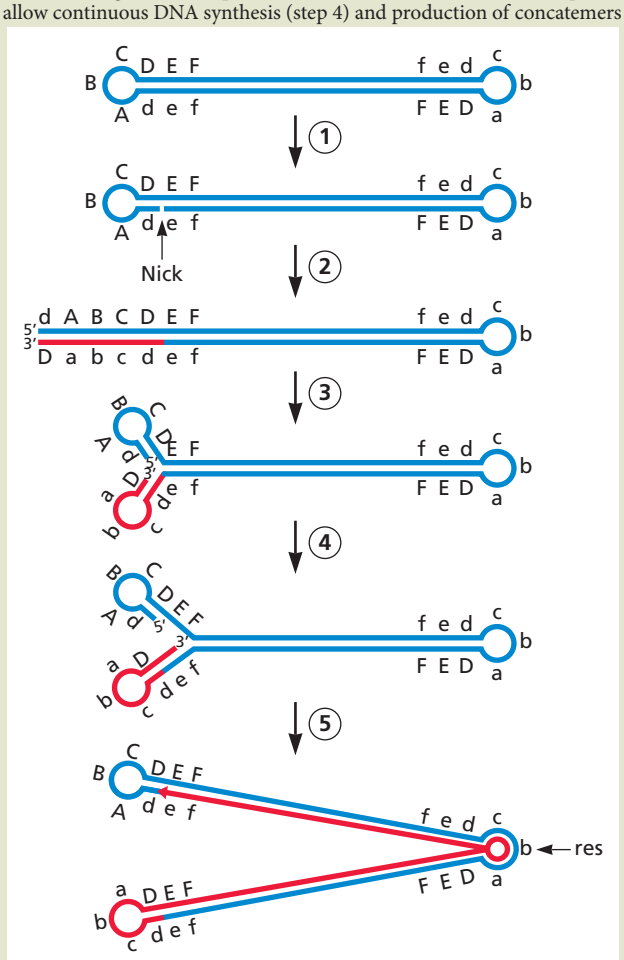
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A self-priming model for vaccinia viral DNA synthesis. The double-stranded DNA is not depicted to scale, to emphasize the inverted terminal repetitions and terminal loops. Complementary sequences within these regions are indicated by upper- and lower-case letters. Viral DNA synthesis would be initiated by introduction of a nick that creates a 3′-OH end (step 1). Following synthesis of DNA to the end of the genome (step 2), re-formation of the terminal hairpins by base pairing (step 3) would allow continuous DNA synthesis (step 4) and production of concatemers (step 5).



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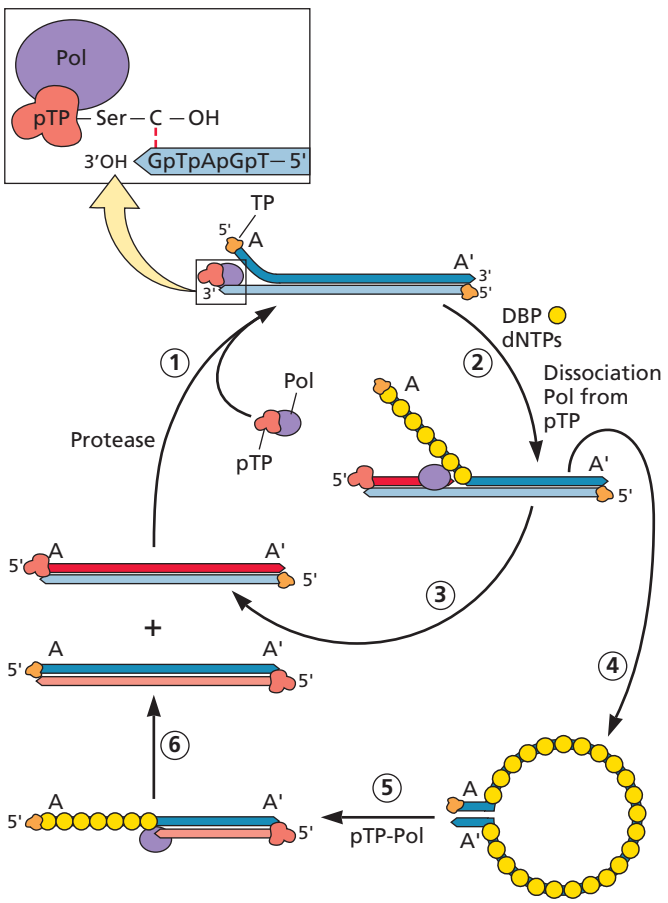


Figure 9.10 Replication of adenoviral DNA. Assembly of the viral preterminal protein (pTP) and DNA polymerase (Pol) into a preinitiation complex at each terminal origin of replication activates covalent linkage of dCMP to a specific serine residue in pTP by the DNA polymerase (step 1). The free 3′-OH group of pTP-dCMP primes continuous synthesis in the 5′ → 3′ direction by Pol (step 2). This reaction also requires the viral E2 single-stranded-DNA-binding protein (DBP), which coats the displaced second strand of the template DNA molecule, and a cellular topoisomerase. As the terminal segments of the viral genome comprise an inverted repeat sequence (A and A′), there is an origin at each end, and both parental strands can be replicated by this displacement mechanism (step 3). Reannealing of the complementary terminal sequences of the parental strand initially displaced forms a short duplex stem identical to the terminus of the double-stranded genome (step 4). The origin re-formed in this way directs a new cycle of protein priming and continuous DNA synthesis (steps 5 and 6). The pTP is cleaved by the viral protease to the terminal protein (TP) during maturation of viral particles.

support replication of the genome during productive infection. The herpes simplex virus type 1 genome contains two copies of OriS and one of OriL (Fig. 9.11). The two types of origin possess considerable nucleotide sequence similarity, but differ in their organization and can be distinguished functionally. For example, OriL is activated when differentiated neuronal cells are exposed to a glucocorticoid hormone, but OriS is repressed. As glucocorticoids are produced in response to stress, a condition reactivates latent herpes simplex virus type 1 infection, it has been suggested that replication from OriL may be particularly important during the transition to a productive infection.

Viral Replication Origins Share Common Features

Even though the origins of replication of double-stranded DNA viruses are recognized by different proteins and support different mechanisms of initiation, they exhibit a number of common features (Fig. 9.12). The most prominent of these is the presence of AT-rich sequences. In general, AT base pairs contain only two hydrogen bonds, whereas GC pairs interact via three such bonds. The less stable AT-rich sequences are thought to facilitate the unwinding of origins that is necessary for initiation of viral DNA synthesis on double-stranded templates. Another general feature is the close relationship between origin sequences and those that regulate transcription. For example, sequences adjacent to the polyomaviral and adenoviral core origins that increase replication efficiency include binding sites for transcriptional activators. Other viral origins, those of papillomaviruses and parvoviruses and OriLyt of Epstein-Barr virus, contain binding sites for viral proteins that are **both** transcriptional regulators and essential replication proteins. And all three herpes simplex virus type 1 origins lie between promoters for viral transcription. Assembly of viral preinitiation complexes on adenoviral origins is stimulated by direct interactions with cellular transcriptional activators that bind to adjacent sequences (Fig. 9.12). In other cases, such cellular proteins may promote viral DNA synthesis indirectly via alterations in the properties of nucleosomes with which the viral genomes are associated (see Chapter 8).

Recognition of Viral Replication Origins

The paradigm for viral origin recognition is the simian virus 40 LT protein. We therefore describe its properties as the prelude to discussion of other viral proteins with similar functions.

Properties of Simian Virus 40 LT

Functions and organization. The LT proteins of polyomaviruses provide functions essential for viral DNA synthesis, viral gene expression, and optimization of the intracellular environment (Table 9.1). As we have seen, simian virus 40 LT is both necessary and sufficient for recognition of the viral origin and also supplies the helicase activity that drives origin unwinding and perhaps movement of the replication fork. The LT proteins make a major contribution to the species specificity of polyomaviruses. Although the genomes of simian virus 40 and mouse polyomavirus are closely related in organization and sequence, they replicate only in simian and murine cells, respectively. Such host specificity is largely the result of species-specific binding of LT to the largest subunit of DNA polymerase α of the host cell in which the virus will replicate. Although the precise mechanism remains to be

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BOX 9.6

DISCUSSION

Model for the transition between initiation and elongation during protein-primed DNA synthesis

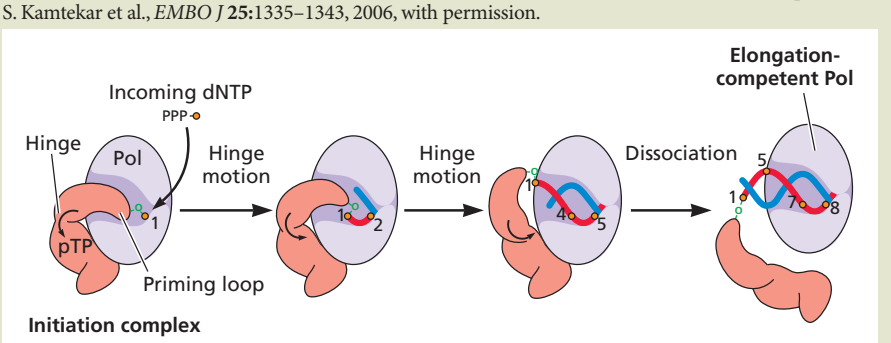
Association of the adenoviral DNA polymerase with the Pre-TP primer is necessary for catalysis of covalent linkage of the priming dCMP to Pre-TP (see the text). However, this interaction must be reversed following initiation to allow processive elongation by the enzyme. Clues about how this transition occurs have come from structural studies of bacteriophage ϕ29 replication proteins.

Replication of the linear, double-stranded ϕ29 genome is initiated by protein priming from origins at the ends of the genome. The phage DNA polymerase (Pol) and priming terminal protein (TP) form a heterodimer and the enzyme catalyzes linkage of the priming nucleotide to TP, just as in adenoviral DNA synthesis (see Fig. 9.10). The structure of the ϕ29 Pol-TP heterodimer has been determined by X-ray crystallography. In this complex, the TP priming domain lies in the site occupied by the DNA template-primer in a model of the elongating enzyme. The loop that contains the serine to which the priming nucleotide is attached lies closest to the Pol active site. The priming domain is connected to a domain (the intermediate domain) that makes extensive contacts with the DNA polymerase via a hinge.

The results of modeling studies indicate that up to 6 or 7 nucleotides can be added to the nascent DNA while TP maintains close contacts with DNA polymerase: motion about the hinge allows displacement of the priming domain while the intermediate domain maintains contact with Pol (see figure). However, this mechanism cannot accommodate further translocation of the priming domain. Rather, the intermediate domain of TP and Pol must dissociate, presumably as a result of additional structural changes. Consequently, the DNA polymerase is released for elongation, as illustrated for incorporation of eight dNMPs in the figure.

Kamtekar S, Berman AJ, Wang J, Lazaro JM, de Vega M, Blanco L, Salas M, Steitz TA. 2006. The ϕ29 DNA polymerase: protein primer structure suggests a model for the initiation to elongation transition. *EMBO J* **25:**1335–1343.

The 39-OH group of the priming nucleotide attached to TP and nascent DNA are shown in green and red, respectively, with newly incorporated dNMPs indicated by orange circles. Adapted from S. Kamtekar et al., *EMBO J* **25:**1335–1343, 2006, with permission.

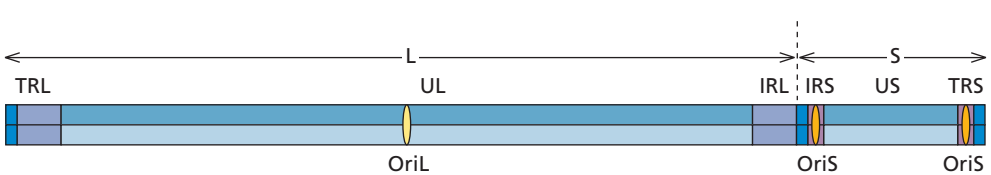


determined, assembly of preinitiation complexes competent for unwinding of the origin does not take place when the LT of one polyomavirus binds to the origin of another.

LT proteins also ensure that the cellular components needed for simian virus 40 DNA synthesis are available in the host cell. By binding and sequestering specific cellular proteins, LT perturbs mechanisms that control cell proliferation and can induce infected cells to enter S phase (see "Viral Proteins Can Induce Synthesis of Cellular Replication Proteins" below). LT also regulates its own synthesis and activates late gene expression.

Sequences of simian virus 40 LT that are necessary for its numerous activities have been mapped by analysis of the effects of specific alterations in the protein on virus replication in infected cells, DNA synthesis *in vitro*, or the individual biochemical activities of the protein. The properties of such altered proteins indicate that LT contains discrete structural and functional domains, such as the minimal domain for specific binding to the viral origin (Fig. 9.13). However, the activities of such functional regions defined by genetic and biochemical methods may be influenced by distant sites, as discussed in the next section.

Figure 9.11 Features of the herpes simplex virus type 1 genome. The long (L) and short (S) regions of the viral genome that are inverted with respect to one another in the four genome isomers are indicated at the top. Each segment comprises a unique sequence (UL or US) flanked by internal and terminal repeated sequences (IR and TR). The locations of the two identical copies of OriS, in repeated sequences, and of the single copy of OriL are indicated.



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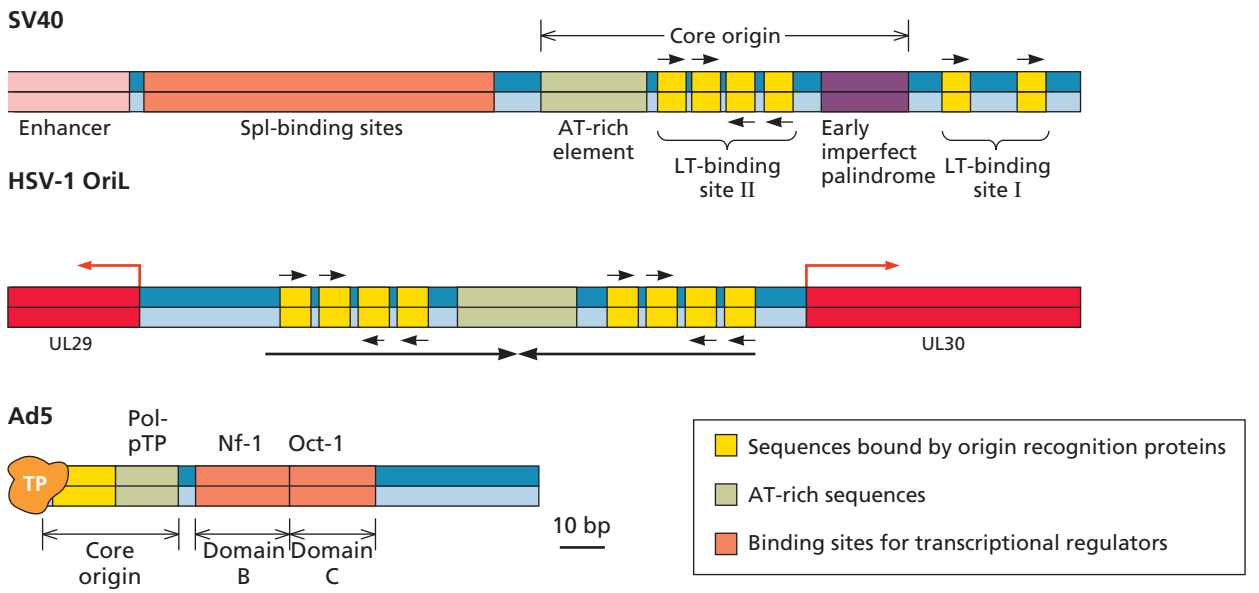
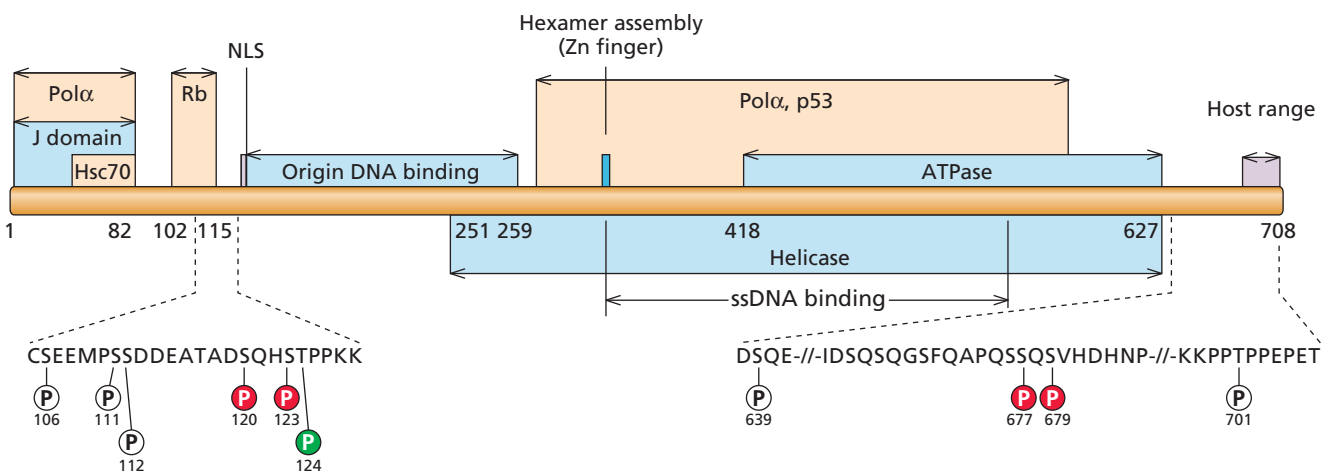


Figure 9.12 Common features of viral origins of DNA replication. The simian virus 40 (SV40) origin, herpes simplex virus type 1 (HSV-1) OriL (Fig. 9.11), and adenovirus type 5 (Ad5) origin (Fig. 9.10) are illustrated to scale, emphasizing the common features shown in the key. Sites of initiation of transcription are indicated by jointed red arrows and palindromic DNA sequences by black arrows. The two copies of herpesviral OriS (Fig. 9.11) are very similar in sequence to OriL. The terminal sequence of the adenoviral origin designated the core origin functions inefficiently in the absence of the adjacent binding site for the transcriptional activator nuclear factor 1 (Nf-1).

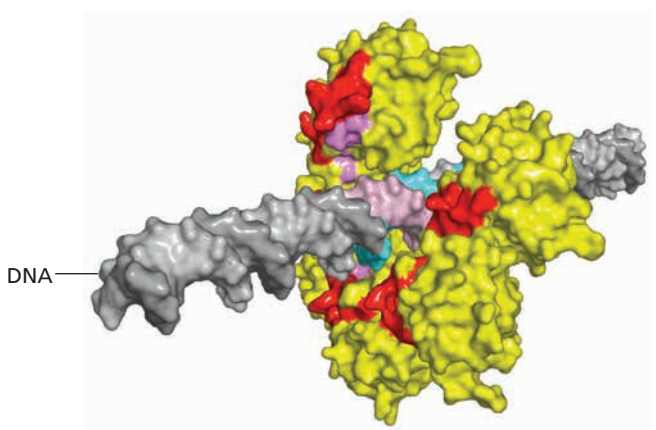
Figure 9.13 Functional organization of simian virus 40 LT. LT is represented to scale. Indicated are the sequences required for binding to the DNA polymerase α-primase complex (Polα), to the cellular chaperone Hsc70, to the cellular retinoblastoma (Rb) and p53 proteins, to the origin of replication (origin DNA binding), and to single-stranded (ss) DNA. Also shown are segments necessary for the helicase and ATPase activities, hexamer assembly at the origin, the nuclear localization signal (NLS), and a C-terminal sequence necessary for production of viral particles but not viral DNA synthesis (host range). The region that binds to Hsc70 lies within an N-terminal segment termed the J domain, because it shares sequences and functional properties with the *E. coli* protein DnaJ, a chaperone that assists the folding and assembly of proteins and is required during reproduction of bacteriophage λ. The chaperone functions of the J domain and Hsc70 are essential for replication in infected cells and seem likely to assist assembly or rearrangement of the preinitiation complex. Below are shown the two regions of the protein in which sites of phosphorylation are clustered, indicating modifications that have been shown to inhibit (red) or activate (green) the replication activity of LT.



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During initiation of viral DNA synthesis, LT first binds specifically to double-stranded pentanucleotide repeat sequences in the origin. It must then interact with single-stranded DNA nonspecifically during origin distortion and unwinding and when the protein couples the hydrolysis of ATP to translocate along DNA at replication forks. Structural studies of various forms of individual domains have provided important insights into the different interactions of LT with DNA. For example, X-ray crystallography of the origin-binding domain bound to the core origin identified numerous contacts between residues in LT and specific origin sequences. In the absence of DNA, the origin-binding domain forms a gapped, hexameric spiral with a large central channel lined by these residues, as well as the distinct but overlapping set that mediates binding to single-stranded DNA (Fig. 9.14). Comparison of the high-resolution structures of various forms of this domain, and of the helicase domain, indicates that both can undergo substantial conformational change. How such reorganizations within individual domains are integrated and coordinated during the intricate process of LT-dependent initiation of DNA synthesis is not clear, in part because the inherent flexibility of full-length LT has limited structural studies. Although important questions remain to be addressed, elegant studies of LT-dependent unwinding of single DNA molecules have established that LT translocates along single-stranded DNA in the 3′ → 5′ direction to unwind DNA by steric exclusion (Box 9.7).

Figure 9.14 Structure of origin-binding domain of simian virus 40 large T antigen, determined by X-ray crystallography. The structure of the origin-binding domain (amino acids 131 to 260) hexamer is shown in surface representation. In this model of the hexamer bound to DNA, the DNA is gray, with the palindromic LT-binding sequences (Fig. 9.4) in cyan and magenta. The DNA-binding regions of LT are colored red and purple. The results of mutational analysis indicate that in the double hexamer of the full-length protein, the origin-binding domains in the two hexamers interact with one another. Adapted from G. Meinke et al., *J Virol* **80:**4304–4312, 2006, with permission. Courtesy of Andrew Bohm, Tufts University School of Medicine.



Regulation of LT activity. The viral early gene encoding LT is transcribed efficiently as soon as the viral chromosome enters the nucleus (Chapter 8). The spliced LT mRNA is the predominant product of processing of these early transcripts. Although production of LT is not regulated during the early phase of infection in simian cells in culture, its activity is tightly controlled.

Specific posttranslational modifications regulate the ability of LT to support viral DNA synthesis. For example, the combination of phosphorylation of Thr124 with lack of phosphorylation of Ser120 and Ser123 stimulates binding of LT to origin site II, promotes assembly of the double hexamer (Fig. 9.5), and is essential for unwinding of DNA from the origin. As Thr124 does not lie within the minimal origin-binding domain (Fig. 9.13), such regulation of DNA-binding activity is thought to be the result of conformational change induced by phosphorylation at this site. The best candidate for the protein kinase that phosphorylates Thr124 is cyclin-dependent kinase 2 (Cdk2) associated with cyclin A.

Viral Origin Recognition Proteins Share Several Properties

Other viral origin recognition proteins share with simian virus 40 LT the ability to bind specifically to DNA sequences within the cognate origin of replication. They also interact with other replication proteins (although these may be viral or cellular), and several possess the biochemical activities exhibited by LT (Table 9.1). For example, the herpes simplex virus type 1 protein UL9, which recruits viral rather than cellular replication proteins, binds cooperatively to specific origin sequences and distorts adjacent AT-rich sequences of the viral origins (Fig. 9.11). It also possesses an ATP-dependent helicase activity that unwinds DNA in the 3′ → 5′ direction. The adenovirus-associated virus Rep 78/68 protein possesses these same activities but is also the site-specific endonuclease that is essential for terminal resolution (Fig. 9.9). The domain that mediates sequence-specific binding adjacent to the terminal resolution site includes a large region very similar in architecture to the origin-binding domains of simian virus 40 LT and the papillomavirus E1 protein (Fig. 9.15). Such structural homology is remarkable, as there is no amino acid identity among the three viral proteins.

In many respects, the herpesviral UL9 protein is a typical origin-binding protein (Table 9.1). However, it is required only during the initial stage of viral DNA synthesis, as are the viral origins. The UL9 protein is cleaved by the cellular protease cathepsin B following the onset of viral DNA synthesis. Such cleavage may contribute to a switch from origin-dependent to origin-independent replication by preventing UL9-dependent initiation of DNA synthesis at the origins.

Although recognition of viral origins of replication by a single viral protein is common, it is not universal. The

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BOX 9.7

EXPERIMENTS

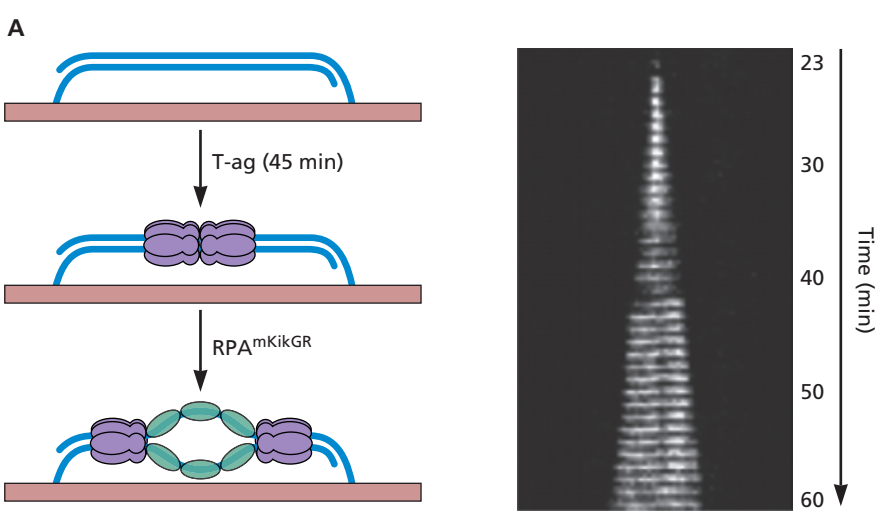
The mechanism by which simian virus 40 LT unwinds and translocates along DNA

Despite decades of study, fundamental questions about the mechanism by which simian virus 40 LT unwinds DNA during genome replication remain unanswered. These include whether LT functions as a double hexamer throughout replication, as, for example, suggested by the studies described in Box 9.3, and how it translocates along DNA during unwinding. Structural studies of a hexamer of the LT helicase domain established that the central channel can expand sufficiently to accommodate double-stranded DNA, and identified side channels through which single-stranded DNA might be extruded, consistent with translocation on double-stranded DNA. However, the structurally related papillomavirus E1 helicase, as well as the cellular helicase minichromosome maintenance complex (Mcm), translocate in the 3′ → 5′ direction along single-stranded DNA to unwind double-stranded templates by steric exclusion.

In one approach to examine the first question (panel A of the figure, left), DNA containing the simian virus 40 origin was attached at both ends to the surface of a microfluidic flow cell. LT was drawn into the cell and allowed to assemble at the origin. The single-stranded-DNA-binding protein Rp-A fused to a green fluorescent-like protein (designated *-Rp-A) was then introduced, and fluorescent images were recorded for 60 min. Symmetrically growing linear tracks of the fluorescent Rp-A protein were observed (panel A, right), consistent with spatial separation of LT hexamers during unwinding from the origin. The observation that tethering both ends of the origin-containing template reduced neither the extent nor the rate of replication compared to tethering of just one end was inconsistent with an LT double hexamer drawing double-stranded DNA into its central chamber to unwind it: in this case, replication of doubly tethered templates would proceed more slowly. Why uncoupling of LT hexamers was observed in these but not in previous (Box 9.3) experiments remains to be explained and the form in which LT unwinds DNA in infected cells to be established.

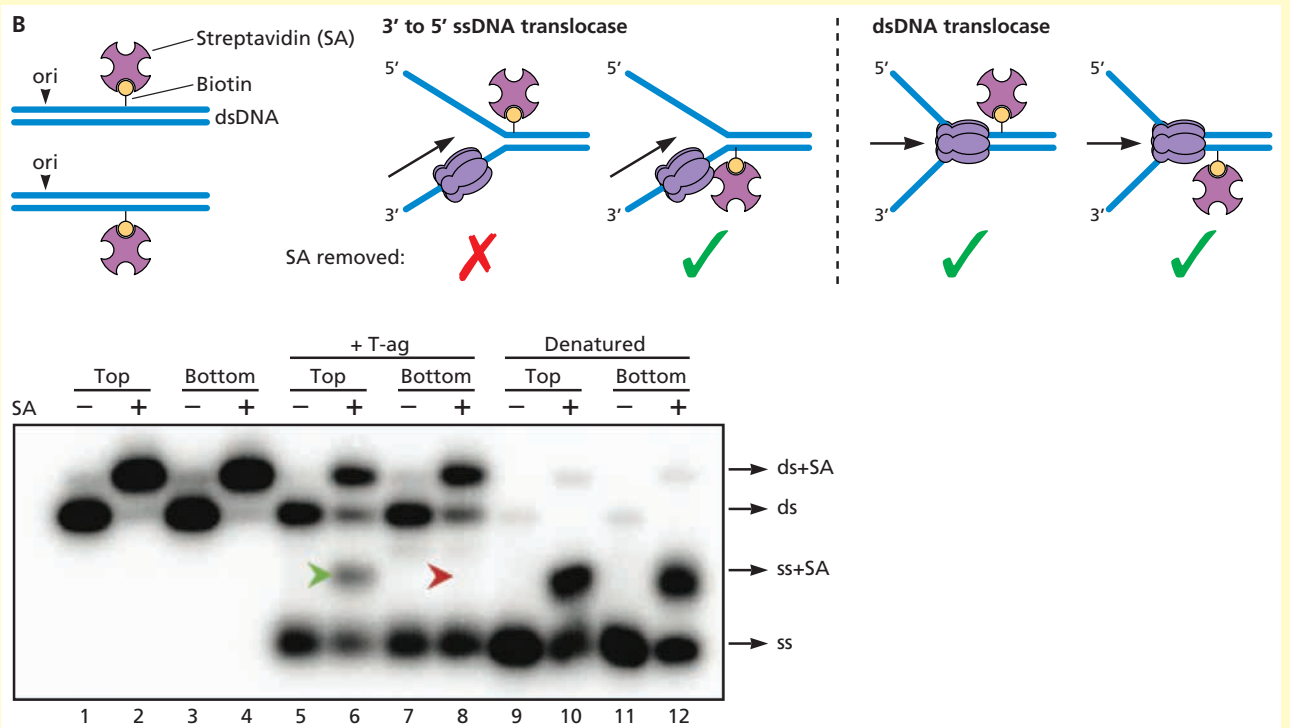
To investigate the mechanism of translocation, a small, linear, origin-containing DNA template was modified by addition of a molecule of biotin to a specific site on the top or the bottom strand. As shown in panel B (left), biotin attached to the top strand can be displaced only if LT translocates along double-stranded DNA. Radioisotopically labeled versions of the templates were incubated with excess streptavidin, and then with LT and Rp-A for 30 min. Strand displacement was examined as the appearance of single-stranded DNA decreased in mobility by binding of streptavidin to the biotin tag. Control experiments established that denaturation of the templates resulted in complete release of both the biotin-tagged strands. LT-dependent displacement of a biotin-tagged single strand was observed **only** when biotin was attached to the bottom strand (panel B, right). These results indicate that, following assembly at the viral origin, simian virus 40 LT translocates in the 3′ → 5′ direction on the leading-strand template. This mechanism is therefore a common feature of viral and cellular helicases that operate during DNA synthesis in mammalian cells.

Yardimci H, Wang X, Lowland AB, Zudner DZ, Hurwitz J, van Oijen AM, Walter JC. 2012. Bypass of a protein barrier by a replicative DNA helicase. *Nature* **492:**205–209.



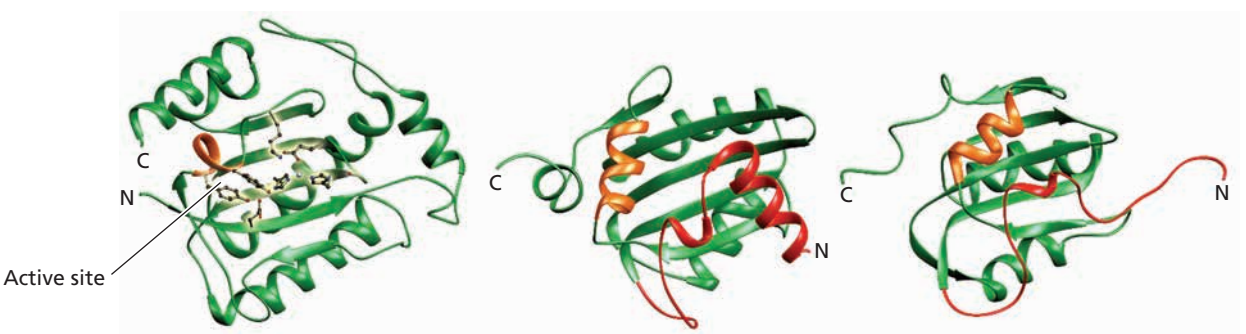
Visualization of formation of a replication bubble during DNA unwinding by LT. (A) The experimental strategy is depicted at the left, and the results obtained upon binding of fluorescent Rp-A during LT unwinding from the origin of a single template molecule are shown on the right. The symmetrical increase in length in the unwound DNA (bound by fluorescent Rp-A) as a function of time indicates that LT hexamers uncouple and move apart after initiation of unwinding. *(continued)*

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Visualization of formation of a replication bubble during DNA unwinding by LT. *(continued)* **(B)** As depicted at the top, LT can displace a biotin-tagged upper strand of an origin-containing template only if the protein translocates on double-stranded DNA. However, only the bottom biotin-tagged strand was released, as detected by the decrease in mobility after binding of streptavidin, when DNA unwinding was carried out by LT and Rp-A (right).

Figure 9.15 Structural homology among DNA-binding domains of viral origin recognition proteins. The X-ray crystal structures of the adenovirus-associated virus type 5 Rep 68 DNA-binding endonuclease domain and the bovine papillomavirus E1 and simian virus 40 LT origin-binding domains are shown in ribbon form. Each protein contains a central antiparallel β-sheet flanked by three α-helices. However, the Rep protein includes a cleft on one surface of the β-sheet that contains the endonuclease active site (residues shown in ball-and-stick). In the other two viral proteins, no cleft is present, as this region is occupied by N-terminal extensions (red) and helices shifted with respect to the position in Rep (orange). Adapted from A. Hickman et al., *Mol Cell* **10:**327–337, 2002, with permission. Courtesy of Alison Hickman, National Institutes of Health.



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papillomavirus E1 proteins possess the same activities as simian virus 40 LT (Table 9.1), to which they are related in sequence, organization, and structure (Fig. 9.15). Nevertheless, the E1 protein cannot support papillomaviral DNA replication in infected cells: a second viral protein, the E2 transcriptional regulator, is also necessary. The minimal origin of replication of papillomaviral genomes includes adjacent binding sites for both the E1 and E2 proteins (Fig. 9.16A). The E1 protein binds to origin DNA with only low specificity. In contrast, when the E1 and E2 proteins bind cooperatively, the specificity and affinity of the E1-DNA interaction are increased significantly. Once a specific E1-E2 protein complex has assembled on the origin, hydrolysis of ATP to E1 appears to induce a conformational change that leads to dissociation of E2, allowing additional molecules of E1 to bind (Fig. 9.16B). The final product is an E1 double hexamer assembled on single-stranded DNA.

The adenoviral origins of replication are also recognized by two viral proteins, the preterminal protein and viral DNA polymerase. In this case, the proteins associate as they are synthesized in the cytoplasm and, once within the nucleus, bind specifically to a conserved sequence within the minimal origins of replication (Fig. 9.12).

Viral DNA Synthesis Machines

Larger viral DNA genomes encode DNA polymerases and other essential replication proteins. A particularly simple viral replication apparatus is that of adenoviruses, which comprises the Pre-TP primer and DNA polymerase and only one other protein, a single-stranded-DNA-binding protein. The latter protein stimulates initiation and is essential during elongation, when it coats the displaced strands of the template DNA molecule (Fig. 9.10). Cooperative binding of this protein to single-stranded DNA stimulates the activity of the viral DNA polymerase as much as 100-fold and induces highly processive DNA synthesis. Remarkably, no ATP hydrolysis is required. Rather, the DNA-binding protein multimerizes via a C-terminal hook (Fig. 9.17A), and the formation of long protein chains by cooperative, high-affinity binding to single-stranded DNA provides the driving force for ATP-independent unwinding of the duplex template (Fig. 9.17B). Other single-stranded-DNA-binding proteins, such as the herpes simplex virus type 1 UL8 protein and cellular replication protein A, may destabilize double-stranded DNA helices by a similar mechanism.

Other viral replication systems include a larger number of accessory replication proteins (Table 9.2). Herpes simplex virus type 1 genes that encode essential replication proteins have been discovered by both genetic methods and a DNA-mediated transformation assay that identifies the gene products necessary for plasmid replication directed by a viral origin (Fig. 9.18). Replication from a herpes simplex virus

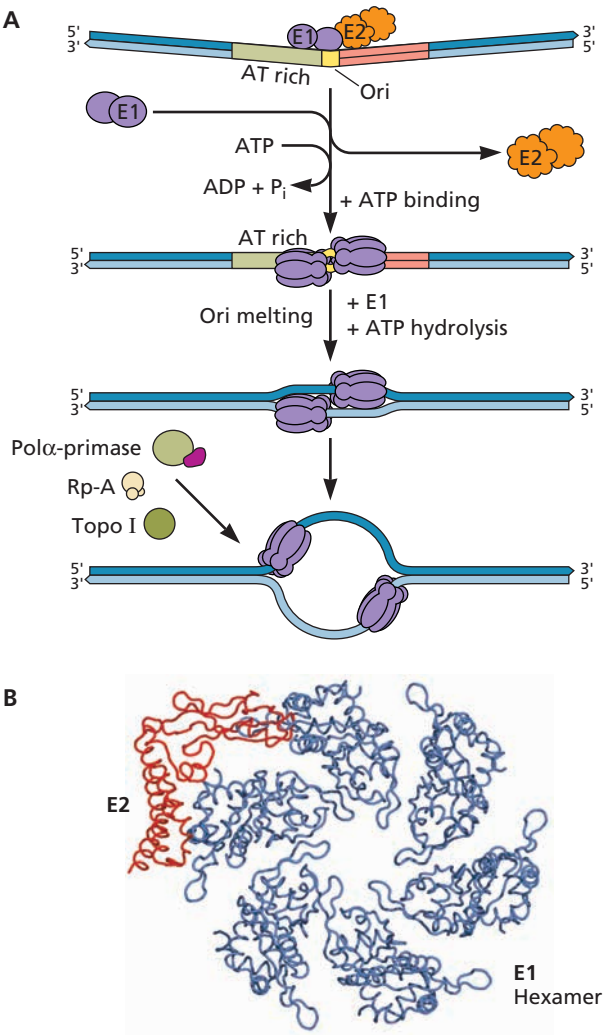


Figure 9.16 Origin loading of the papillomaviral E1 initiation protein by the viral E2 protein. (A) Schematic model. The sequence features of the minimal origin of replication of bovine papillomavirus type 1 are depicted as in Fig. 9.12. This origin contains an essential binding site for the viral E2 protein, a sequence-specific transcriptional regulator. The model of the origin loading of the viral E1 by the E2 protein is based on *in vitro* studies of the interactions of these proteins with the origin. The E1 and E2 proteins, which are both homodimers, bind cooperatively to the viral origin, with specificity and affinity far greater than that exhibited by the E1 protein alone. When ATP is hydrolyzed (presumably by the ATPase of the E1 protein), the $(E1)_2(E2)_2$-Ori complex is destabilized, the E2 dimers are displaced, and additional E1 molecules bind, initially forming double trimers. Upon further ATP hydrolysis and unwinding of origin DNA by an assembly intermediate, E1 double hexamers assemble, each encircling a single strand of DNA. **(B)** X-ray crystal structures of the E2 activation domain (red) and the E1 ATPase/helicase domain (blue) are shown in ribbon form. This overlay of E2 and the E1 hexamer illustrates how association with E2 blocks the E1 surface that mediates hexamer assembly. Hence, E2 must dissociate prior to E1 assembly. Consistent with this model, the E1 and E2 proteins form a 1:1 complex in the absence of ATP, but in the presence of ATP E1 assembles into a high-molecular-mass form that contains no E2. Adapted from E. Abbate et al., *Genes Dev* **18:**1981–1986, 2004, with permission. Courtesy of Eric Abbate and Michael Botchan, University of California, Berkeley.

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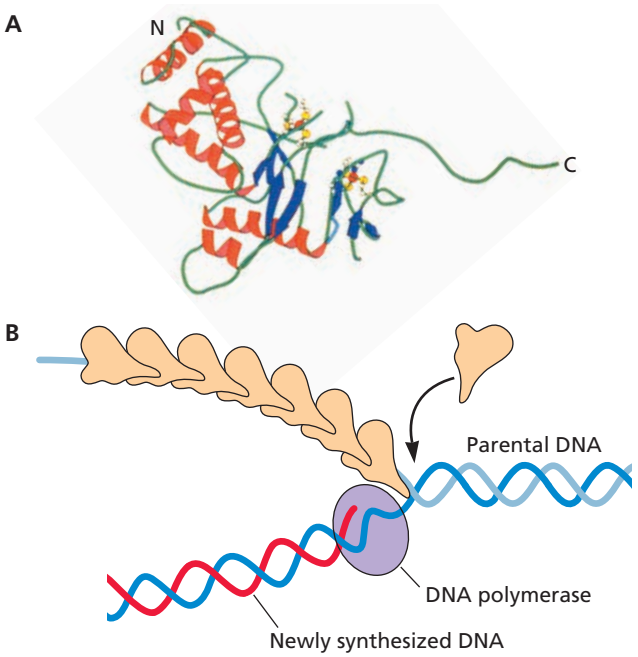


Figure 9.17 Crystal structure of the adenoviral single-stranded-DNA-binding protein. (A) Ribbon diagram of the C-terminal nucleic acid-binding domain (amino acids 176 to 529) of the human adenovirus type 5 protein, showing the two sites of $Zn^{2}+$ (red atom) coordination. The most prominent feature is the long (<40-Å) C-terminal extension. This C-terminal extension of one protein molecule invades a cleft between two α-helices in its neighbor in the protein array formed in the crystal. Deletion of the C-terminal 17 amino acids of the DNA-binding protein fragment eliminates cooperative binding of the protein to DNA, indicating that the interaction of one molecule with another via the C-terminal hook is responsible for cooperativity in DNA binding. From P. A. Tucker et al., *EMBO J* **13:**2994–3002, 1994, with permission. Courtesy of P. C. van der Vliet, Utrecht University. **(B)** Model of unwinding of double-stranded adenoviral DNA by cooperative interactions among the viral single-stranded-DNA-binding protein.

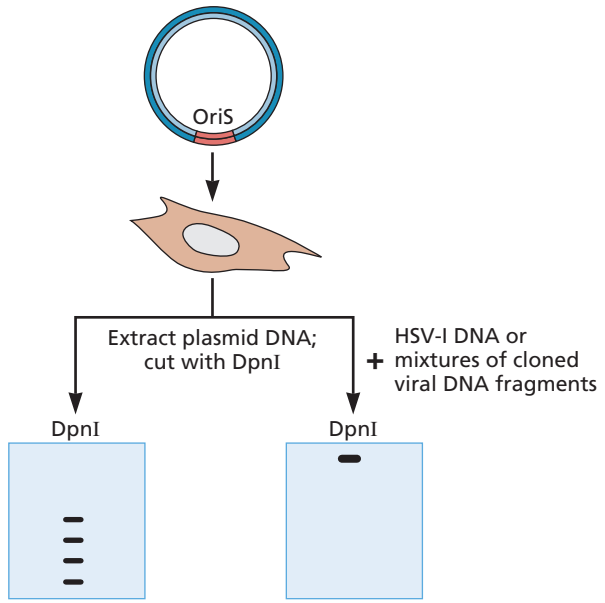


Figure 9.18 DNA-mediated transformation assay for essential herpes simplex virus type 1 replication proteins. A plasmid carrying a viral DNA fragment spanning OriS is introduced into monkey cells permissive for herpesvirus replication. In the absence of viral proteins (left), the plasmid DNA is not replicated and retains the methyl groups added to A residues in a specific sequence by the *E. coli dam* methylation system. As these sequences include the recognition site for the restriction endonuclease DpnI, which cleaves only such methylated DNA, the unreplicated plasmid DNA is sensitive to DpnI cleavage. When all viral genes encoding proteins required for OriS-dependent replication are also introduced into the cells (right), the plasmid is replicated. Because the newly replicated DNA is **not** methylated at DpnI sites, it cannot be cleaved by this enzyme. Resistance of the plasmid to DpnI cleavage therefore provides a simple assay for plasmid replication, and for the identification of viral proteins required for replication from OriS.

Table 9.2 Replication systems of large DNA viruses

Function	Viral protein(s)	
	Herpes simplex virus type 1	Vaccinia virus
Common components		
DNA polymerase and associated 3′→5′ exonuclease	UL30	E9L
Primase/helicase	UL5, UL8, and UL52 Heterotrimer	D5R
Processivity factor	UL42	A20R
Single-stranded-DNA-binding protein	UL2	I3L
Apparently unique components		
Origin recognition protein	UL9	–
Type I topoisomerase	–	H6R
DNA ligase	–	A50R

type 1 origin requires five proteins in addition to the viral DNA polymerase and origin recognition protein. These proteins carry out the same reactions as essential components of the cellular replication machinery, as do the proteins necessary for synthesis of vaccinia viral DNA (Table 9.2). Although these herpesviral proteins provide an extensive repertoire of replication functions, they are not sufficient for viral DNA synthesis *in vitro*. While all the additional viral and/or cellular proteins needed to reconstitute herpesviral DNA synthesis have not been identified, cellular topoisomerase II is essential for replication in infected cells.

Resolution and Processing of Viral Replication Products

Several of the viral DNA replication mechanisms described in preceding sections yield products that do not correspond to the parental viral genome. As we have seen, replication of simian virus 40 DNA yields two interlocked, double-stranded,

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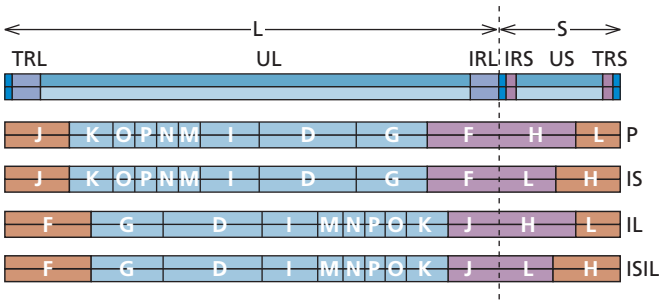


Figure 9.19 Isomers of the herpes simplex virus type 1 genome. The organization of the unique and repeated sequences of the viral genome are depicted at the top, as in Fig. 9.11. This orientation is defined as the prototype (P) genome isomer. The other three isomers differ, with respect to the P form, in the orientation of S (IS), in the orientation of L (IL), or in both S and L (ISIL). These differences are illustrated using HindIII fragments. The unusual isomerization of this viral genome was deduced from the presence of fragments that span the terminal or internal inverted repeat sequences at 0.5 and 0.25 molar concentrations, respectively, in such HindIII digests, and examination of partially denatured DNA in the electron microscope.

circular DNA molecules that must be separated by cellular topoisomerase II. Such resolution is required whenever circular templates (e.g., papillomavirus or episomal Epstein-Barr virus DNA) are replicated as monomers. In other cases, replication yields multimeric DNA molecules, from which linear genomes of fixed length must be processed for packaging into virus particles. This situation is exemplified by the herpes simplex virus type 1 genome (Fig. 9.19).

The products of herpesviral DNA synthesis are head-to-tail **concatemers** containing multiple copies of the viral genome. It is well established that the linear viral genomes that enter infected cell nuclei at the start of a productive infection are converted rapidly to "endless" molecules in which the DNA termini are joined together. This reaction requires cellular DNA ligase IV, which normally mediates joining of nonhomologous DNA ends during a cellular repair process, but it is not clear if unit-length circles (as found in latently infected cells) or linear concatemers are produced (Box 9.8). This distinction is of more than esoteric interest, as the configuration of the parental DNA has profound implications about the mechanism of viral DNA synthesis. When a genome is circular, concatemers can be synthesized by the rolling-circle mechanism (Box 9.4), as during initial replication of the double-stranded DNA genome of bacteriophage λ. In contrast, recombination is required to produce longer-than-unit-length genomes during replication of a linear template (see "Recombination of Viral Genomes" below).

Linear herpes simplex virus type 1 DNA molecules with termini identical to those of the infecting genome are liberated from concatemeric replication products by cleavage at specific sites within the *a* repeats (Fig. 9.11). Such cleavage is coupled with encapsidation of viral DNA molecules during assembly of virus particles (Chapter 13).

Exponential Accumulation of Viral Genomes

The details of the mechanisms by which DNA genomes are replicated vary considerably from one virus family to another. Nevertheless, each of these strategies results in efficient viral DNA synthesis. Production of 10^3 to 10^4 viral genomes, or more, per infected cell is not uncommon, as the products of one cycle of replication are recruited as templates for the next. Such exponential viral DNA synthesis sets the stage for assembly of a large burst of progeny virus particles. In this section, we discuss regulatory mechanisms that ensure efficient viral DNA synthesis.

Viral Proteins Can Induce Synthesis of Cellular Replication Proteins

With few exceptions, virus reproduction is studied by infecting established cell lines that are susceptible and permissive for the virus of interest. Such immortal or transformed cell lines proliferate indefinitely and differ markedly from the cells in which viruses reproduce in nature. For example, highly differentiated cells, such as neurons or the outer cells of an epithelium, do not divide and are permanently in a specialized resting state, termed the **G_0 state**. Many other cells in an organism divide only rarely, or only in response to specific stimuli, and therefore spend much of their lives in G_0. Such cells do not contain many of the components of the replication machinery and are also characterized by generally low rates of synthesis of RNAs and proteins. Virus reproduction entails the synthesis of large quantities of viral nucleic acids (and proteins), often at a high rate. Consequently, the resting state does not provide a hospitable environment. Nevertheless, viruses often reproduce successfully within cells infected when they are in G_0. In some cases, such as replication of the genomes of several herpesviruses in neurons, the DNA synthesis machinery is encoded within the viral genome. Infection by other viruses stimulates resting or slowly growing cells to abnormal activity, by disruption of cellular circuits that restrain cell proliferation. This strategy is characteristic of polyomaviruses and adenoviruses.

Functional Inactivation of the Rb Protein

Loss or mutation of both copies of the cellular retinoblastoma (*rb*) gene is associated with the development of tumors of the retina in children and young adults. Because it is the **loss** of normal function that leads to tumor formation, *rb* is defined as a **tumor suppressor gene**. The Rb protein is an important component of the regulatory program that ensures that cells grow, duplicate their DNA, and divide in an orderly manner (Volume II, Chapter 6). In particular, the Rb protein

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BOX 9.8

DISCUSSION

Circularization or concatemerization of the herpes simplex virus type 1 genome in productively infected cells?

It has been known for some time that the linear herpes simplex virus type 1 DNA that enters nuclei of productively infected cells rapidly adopts a new conformation in which the termini are fused: restriction endonuclease cleavage of viral DNA recovered shortly after infection established that cleavage products generated from free ends decrease in concentration as those characteristic of joined ends increase. As shown in the figure, joined ends could arise by either circularization or concatemerization (as long as infected cell nuclei contain multiple copies of the genome). The origin of joined ends has been difficult to determine, for the following reasons:

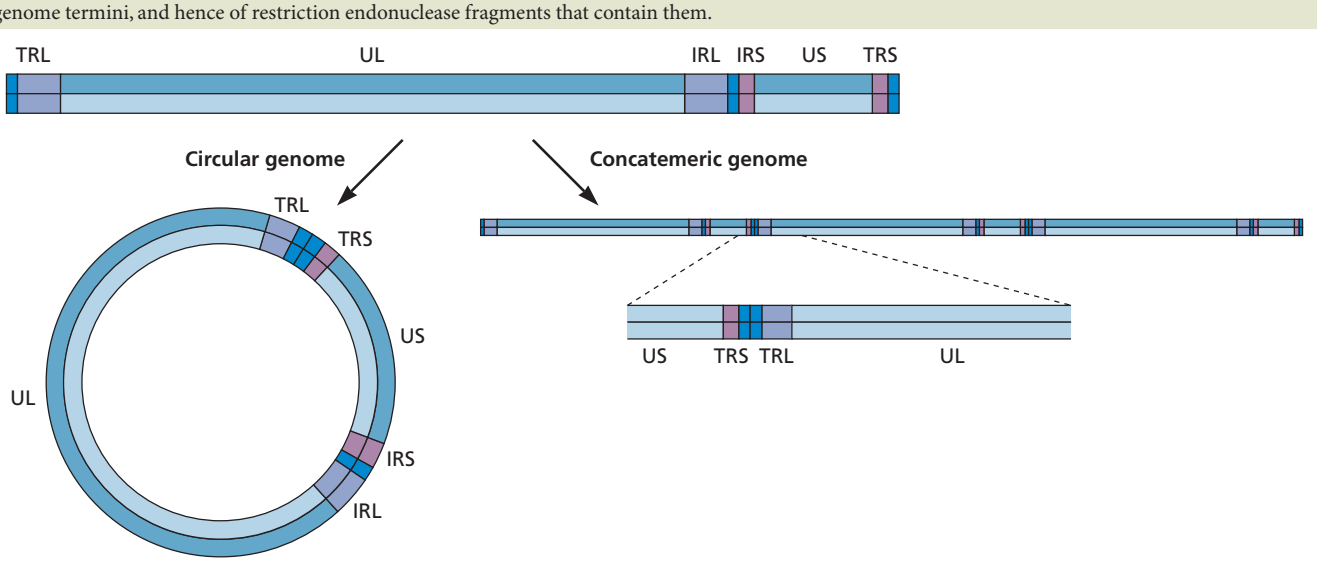
- Formation of **either** unit-length circles or concatemers results in loss of free termini.
- The presence of an internal, inverted copy of the joined terminal repeats precludes the use of an assay based on detection of joined termini.
- Conventional methods for separation and identification of linear and circular DNA molecules by electrophoresis cannot be applied to the large herpesviral genome.
- The large size of the herpesviral genome renders it very sensitive to damage during extraction from infected cells.
- Under conditions that facilitate detection of entering viral DNA, high multiplicity of infection, the majority of infecting DNA molecules are neither transcribed nor replicated.

Consequently, application of different experimental approaches has led to reports that entering viral genomes become circular and that they form concatemers, and these divergent conclusions have yet to be reconciled.

Jackson SA, DeLuca NA. 2003. Relationship of herpes simplex virus genome configuration to productive and persistent infections. *Proc Natl Acad Sci U S A* **100:**7871–7876.

Strang BL, Stow ND. 2005. Circularization of the herpes simplex virus type 1 genome upon lytic infection. *J Virol* **79:**12487–12494.

The herpes simplex virus type 1 genome is depicted as in Fig. 9.11. As shown below, either circularization or formation of concatemers leads to loss of genome termini, and hence of restriction endonuclease fragments that contain them.



controls entry into the period of the cell cycle in which DNA is synthesized, the **S phase**, from the preceding (G_1) phase. Our current appreciation of the critical participation of this protein in the control of cell cycle progression, and of the mechanism by which it operates, stems from the discovery that Rb binds directly to the two adenoviral E1A proteins (see Chapter 8) and functionally analogous proteins of papillomaviruses and polyomaviruses.

In the G_1 phase of uninfected cells, the Rb protein is bound to transcriptional regulators of the E2f family. These complexes, which bind to specific promoters via the DNA-binding activity of E2f, function as repressors of transcription (Fig. 9.20A). Binding of adenoviral E1A proteins, simian virus 40 LT, or E7 proteins of highly oncogenic human papillomaviruses to Rb releases E2f from this association and sequesters Rb. The E2f proteins therefore become available to stimulate transcription of cellular genes for proteins that participate directly or indirectly in DNA synthesis or in control of cell cycle progression (Fig. 9.20A; Volume II, Chapter 6).

Many of the genes that encode DNA polymerases, accessory replication proteins, and enzymes that catalyze synthesis of dNTPs contain E2f-binding sites in their transcriptional

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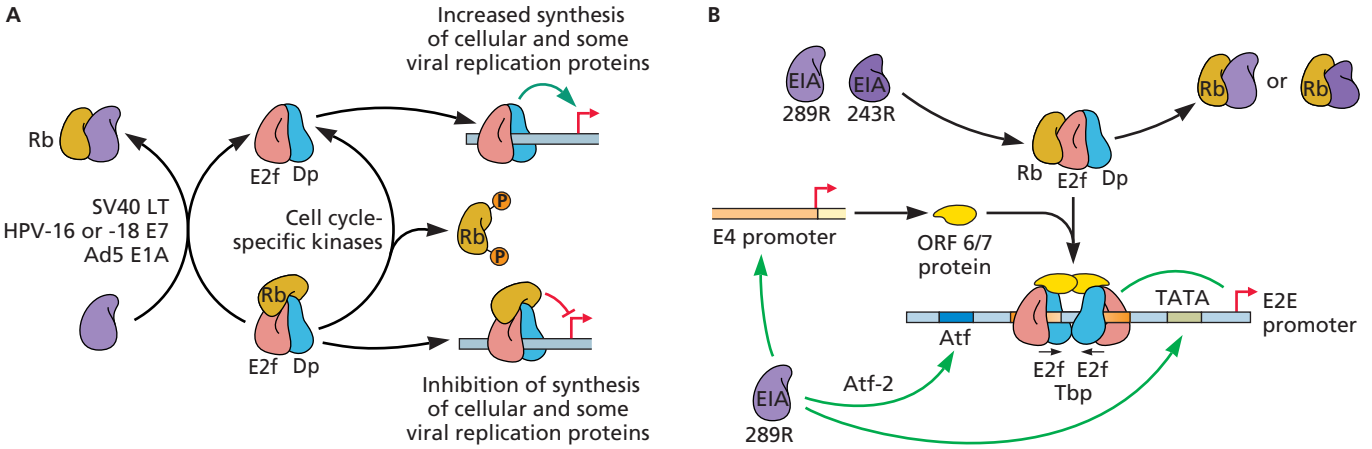


Figure 9.20 Regulation of production of cellular and viral replication proteins. (A) Model for the abrogation of the function of the Rb protein by viral proteins. E2f transcriptional regulators are heterodimeric proteins, each containing one E2f and one Dp (E2f dimerization partner) subunit. E2f dimers stimulate transcription of cellular genes encoding replication proteins, histones, and proteins that allow passage through the cell cycle (green arrow). Binding of Rb protein does not prevent promoter recognition by E2f. However, Rb protein represses transcription (red bar). Phosphorylation of Rb protein at specific sites induces its dissociation from E2f and activates transcription of cellular genes expressed in S phase. The adenoviral E1A proteins, simian virus 40 LT, and the E7 proteins of certain human papillomaviruses (for example, types 16 and 18) bind to the region of Rb protein that contacts E2f to disrupt Rb-E2f complexes and activate E2f-dependent transcription. **(B)** Stimulation of transcription from the adenoviral E2 early promoter by E1A proteins. The E2E promoter-binding sites for the cellular Atf (activating transcription factor), E2f, and TfIId (transcription factor IId) proteins are necessary for E2E transcription in infected cells. The inversion of the two E2f sites (arrows) and their precise spacing are essential for assembly of an E2f-DNA complex unique to adenovirus-infected cells, in which the viral E4 Orf6/7 protein is bound to each E2f heterodimer. Binding of the E4 protein promotes cooperative binding of E2f and increases the lifetime of E2f-DNA complexes. The availability of the cellular E2f and viral E4 Orf6/7 proteins is a result of the action of immediate-early E1A proteins: either the 243R or 289R protein can sequester unphosphorylated Rb to release active E2f from Rb-E2f complexes, and the 289R protein stimulates transcription from the E4 promoter. This larger E1A protein can also stimulate transcription from the E2E promoter directly.

control regions. This property normally restricts synthesis of these gene products to when they are needed in S phase. However, the sequestration of Rb by simian virus 40 LT allows production of the cellular proteins necessary for viral DNA synthesis, regardless of the proliferation state of the host cell. As LT is the only viral protein needed for viral DNA synthesis, its production seems likely to maximize the efficiency of genome replication, by coordinating initiation of this process with entry of the host cell into S phase. Such integration is reinforced by the phosphorylation of LT (on Thr124) by a kinase, Cdk2-cyclin A, which is present **only** during this period, because this modification is essential for initiation of viral DNA synthesis.

A major consequence of activation of E2f in adenovirus-infected cells is stimulation of production of the three viral replication proteins. The viral DNA polymerase, Pre-TP primer, and DNA-binding protein are encoded within the E2 gene, which is transcribed from an early promoter that contains two binding sites for E2f (Fig. 9.20B). In fact, these critical cellular regulators derive their name from the E2 promoter-binding sites, which are necessary for efficient E2 transcription during the early phase. As noted above, the viral E1A proteins disrupt Rb-E2f complexes to release E2f, but also stimulate transcription from the E2 promoter by two other mechanisms (Fig. 9.20B). The E1A-dependent regulatory mechanisms presumably operate synergistically, to allow synthesis of the viral mRNAs that encode replication proteins in quantities sufficient to support numerous cycles of viral DNA synthesis.

The mechanism by which LT and E1A proteins counter Rb (and Rb family members) to induce cell cycle progression are well established, as is their importance in transformation of nonpermissive rodent cells (Volume II, Chapter 6). Furthermore, it has been shown that the smaller E1A protein is necessary for efficient adenoviral DNA synthesis in quiescent human cells, and that this protein displaces Rb and related proteins from cellular promoters, including those that contain binding sites for E2f.

Synthesis of Viral Replication Machines and Accessory Enzymes

The DNA genomes of several viruses, exemplified by those of herpes simplex virus type 1 and the poxvirus vaccinia virus, encode large cohorts of proteins that participate in viral genome replication directly (the proteins that mediate viral DNA synthesis described previously) or indirectly (accessory enzymes). These enzymes are viral analogs of cellular proteins that catalyze synthesis of dNTP substrates, such as thymidine kinase and ribonucleotide reductase, or participate in repair

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Table 9.3 Viral enzymes of nucleic acid metabolism

Virus	Protein	Functions
Herpesvirus		
Herpes simplex virus type 1	Thymidine kinase (UL23 protein, ICP36)	Phosphorylates thymidine and other nucleosides; essential for efficient reproduction in animal hosts
	Ribonucleotide reductase ($\alpha_2\beta_2$ dimer of UL39 and U40 proteins)	Reduces ribose to deoxyribose in ribonucleotides; essential in nondividing cells
	dUTPase (UL50 protein)	Hydrolyzes dUTP to dUMP, preventing incorporation of dUTP into DNA and providing dUMP for conversion to dTMP
	Uracil DNA glycosylase	Corrects insertion of dUTP or deamination of C in viral DNA
	Alkaline nuclease (UL12 protein)	Required for production of infectious DNA
Poxvirus		
Vaccinia virus	Thymidine kinase	Phosphorylates thymidine; required for efficient virus reproduction in animal hosts
	Thymidylate kinase	Phosphorylates TMP
	Ribonucleotide reductase, dimer	Reduces ribose to deoxyribose in ribonucleotides; essential in nondividing cells
	dUTPase	Hydrolyzes dUTP to dUMP (see above)
	DNase	Has nicking-joining activity; present in virion cores
	D4R protein	Uracil DNA glycosylase

of DNA (Table 9.3). In general, such proteins are dispensable for replication in proliferating cells in culture, because cellular enzymes supply the substrates for DNA synthesis. However, herpes simplex viruses that lack thymidine kinase or ribonucleotide reductase genes cannot reproduce in neurons: such terminally differentiated cells are permanently withdrawn from the cell cycle and do not make enzymes that produce substrates for DNA synthesis.

Timely synthesis of herpes simplex virus type 1 replication proteins is the result of the viral transcriptional cascade described in Chapter 8. Expression of the early genes that encode these viral proteins is regulated by immediate-early proteins. These regulatory proteins operate transcriptionally (e.g., ICP0 and ICP4) or posttranscriptionally (e.g., ICP27) to induce synthesis of viral replication proteins at concentrations sufficient to support efficient replication of viral genomes.

Viral DNA Replication Independent of Cellular Proteins

One method guaranteed to ensure replicative success of a DNA virus, regardless of the proliferation state of the host cell, is to encode **all** of the necessary proteins in the viral genome. On the other hand, this mechanism is genetically expensive, which may be the reason why it is restricted to the viruses with the largest DNA genomes, such as the poxvirus vaccinia virus. The genome of this virus, which is replicated in the cytoplasm, encodes a DNA polymerase, several accessory replication proteins, and enzymes for synthesis of dNTPs (Table 9.3). None of the latter appear to be essential for virus reproduction in actively growing cells. However, several of them, such as the thymidine kinase, are necessary for efficient virus propagation in quiescent cells or in animal hosts, where they presumably contribute to synthesis of nucleotide substrates for genome replication.

Delayed Synthesis of Structural Proteins Prevents Premature Packaging of DNA Templates

Provided that daughter viral DNA molecules are available to serve as templates, each cycle of genome replication increases the number of DNA molecules that can be copied in the subsequent cycle. One process that sequesters potential templates is encapsidation of genomes during assembly of new virus particles. However, particle assembly is delayed with respect to initiation of viral DNA synthesis, in part because transcription of late genes that encode structural proteins depends on genome replication (Chapter 8). The increase in the pool of replication templates therefore seems likely to make an important contribution to rapid amplification of genomes.

Inhibition of Cellular DNA Synthesis

When viral DNA replication is carried out largely by viral proteins, cellular DNA synthesis is often inhibited, presumably to increase the availability of substrates for viral genome replication. Indeed, infection by the larger DNA viruses (herpesviruses and poxviruses) induces severe inhibition of cellular DNA synthesis. This process is also blocked when adenoviruses infect proliferating cells in culture. Although inhibition of cellular DNA synthesis in cells infected by these DNA viruses was described in some of the earliest studies of their infectious cycles, very little is known about the mechanisms that shut down this cellular process.

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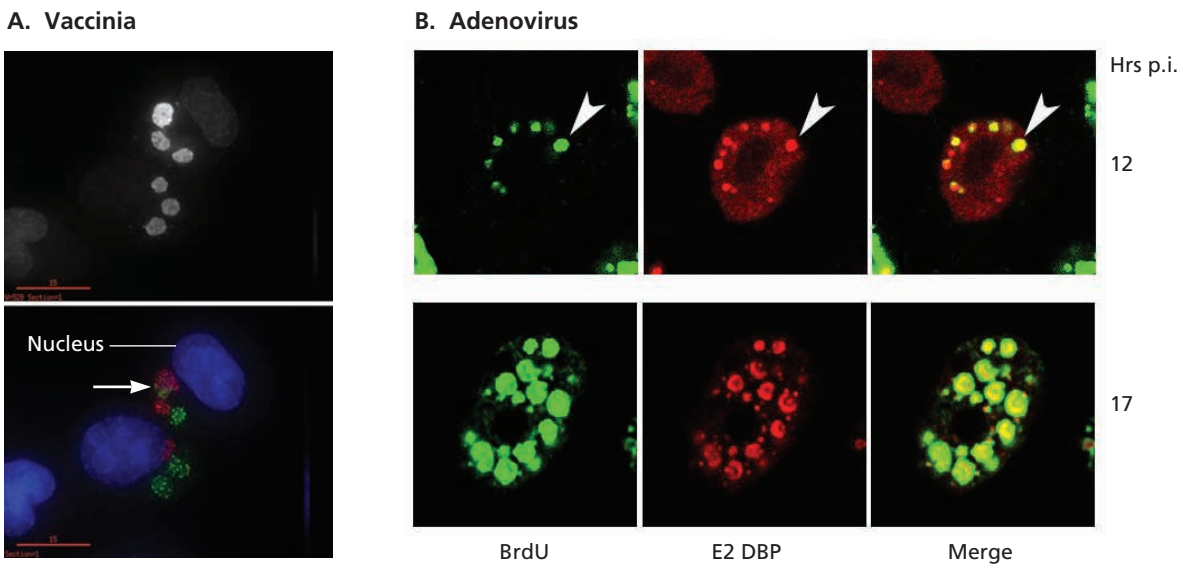
There is some evidence that inhibition of cellular DNA synthesis is an active process rather than an indirect result of passive competition between viral and cellular DNA polymerases for the finite pools of dNTP substrates. For example, infection of proliferating cells by adenovirus or betaherpesviruses such as human cytomegalovirus induces cell cycle arrest, as does synthesis of the Epstein-Barr virus Zta protein, a sequence-specific transcriptional regulator and origin-binding protein. In the latter case, arrest is the result of **increased** concentrations of cellular proteins that negatively regulate progression through the cell cycle, such as the Rb protein.

Viral DNAs Are Synthesized in Specialized Intracellular Compartments

A common, probably universal, feature of cells infected by viruses with DNA genomes is the presence of virus-specific territories that are the sites of viral DNA synthesis. Vaccinia virus DNA is replicated in the cytoplasm, in discrete **viral factories** that contain viral genomes and lie near infected cell nuclei. Visualization of viral genomes in living cells and *in situ* hybridization suggests that each viral factory is established by a single infectious particle (Fig. 9.21A). These compartments contain the viral replication proteins, all the viral enzymes and other proteins necessary for synthesis of viral mRNAs, and cellular translation proteins. Consequently, the viral proteins that participate in replication of the vaccinia virus genome are produced within the specialized compartments in which they will operate.

The replication of viral DNA genomes within infected cell nuclei also takes place in specialized compartments, which can be visualized as distinctive, infected cell-specific foci containing viral proteins. Such structures, known as **replication centers** or **replication compartments**, have been best characterized in human cells infected by adenovirus or herpes simplex virus type 1 (Fig. 9.21B). They contain newly synthesized viral DNA and the viral proteins necessary for viral DNA

Figure 9.21 Discrete sites of viral replication. (A) Cytoplasmic vaccinia virus factories. Monkey cells stably synthesizing the DNA-binding bacteriophage λ Cro repressor fused to enhanced green fluorescent protein (Cro-EGFP) were infected with a 1:1 mixture of vaccinia viruses carrying in their genomes the coding sequence for either bacteriophage T7 RNA polymerase or *E. coli* LacZ. Direct fluorescent imaging of living cells indicated that Cro-EGFP labeled both cellular DNA in the cytoplasm and cytoplasmic viral DNA, via nonspecific binding to DNA. The top panel shows such as a fluorescent image recorded from 1 to 6.5 h after infection, by which time the initial structures have increased significantly in size. The infected cells were then examined by fluorescent *in situ* hybridization (FISH) with probes specific for the T7 RNA polymerase (red) or LacZ (green) genes, and stained with 4′,6-diamidino-2-phenylindole (blue). The processing necessary for FISH denatures Cro-EGFP and eliminates its fluorescence. As shown in the bottom panel, replication of individual incoming viral genomes encoding either T7 RNA polymerase or LacZ takes place in distinct factories. However, some genome mixing is evident (white arrow) as individual factories coalesce or fuse. Adapted from Y.-C. Lin and D. H. Evans, *J Virol* **84:**2432–2443, 2010,with permission. Courtesy of D. H. Evans, University of Alberta, Edmonton, Canada. **(B)** Adenoviral replication centers visualized in infected cells exposed to the dNTP analog bromodeoxyuridine (BrdU) for 1 h at the times postinfection (p.i.) indicated to mark newly synthesized DNA. Such DNA (green) and the E2 single-stranded-DNA-binding protein (DBP) (red) were detected by indirect immunofluorescence. As illustrated, replication centers develop from small foci (white arrows in top panel) to larger ring-like structures as the infectious cycle progresses. Adapted from D. Gautam and E. Bridge, *J Virol* **87:**8687–8696, 2013, with permission. Courtesy of E. Bridge, University of Miami.



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synthesis and increase in size as viral genome replication takes place. As is the case for vaccinia virus cytoplasmic factories, each replication center formed in alphaherpesvirus-infected cells originates from a single viral genome. However, the number of such genomes that can be expressed and replicated in a single infected cell is strictly limited (Box 9.9). A characteristic feature of the replication centers established in cells infected by herpesviruses is the recruitment to these sites of numerous cellular DNA repair and recombination proteins (Fig. 9.22). Several of the latter, including DNA mismatch

BOX 9.9

EXPERIMENTS

Counting the number of herpesviral genomes that can be expressed and replicated

Conventional methods for visualization of viral DNA molecules in infected cells, such as indirect immunofluorescence or *in situ* hybridization, detect **all** viral genomes and cannot distinguish functional genomes from those that are nonfunctional. Consequently, whether all viral genomes that enter permissive host cells can be expressed and replicated to produce progeny virus particles is a long-standing question. This issue has now been addressed for an alphaherpesvirus exploiting the properties of mixing of light of different wavelengths.

For these experiments, isogenic derivatives of pseudorabies virus that direct the synthesis of a red, cyan, or yellow fluorescent protein were constructed. Porcine kidney epithelial cells were infected with an equal mixture of the three viruses at increasing multiplicities of infection (MOIs), and the color profiles of infected cells visualized 6 h after infection by using epifluorescence microscopy. The color spectra of thousands of cells per condition were determined and plotted according to their position on a triangle plot, as shown in panel A of the figure. In this plot, each vertex of the triangle represents a pure color, each side represents a mixture of two colors, and mixtures of the three colors observed in individual cells are represented by the points within the triangle.

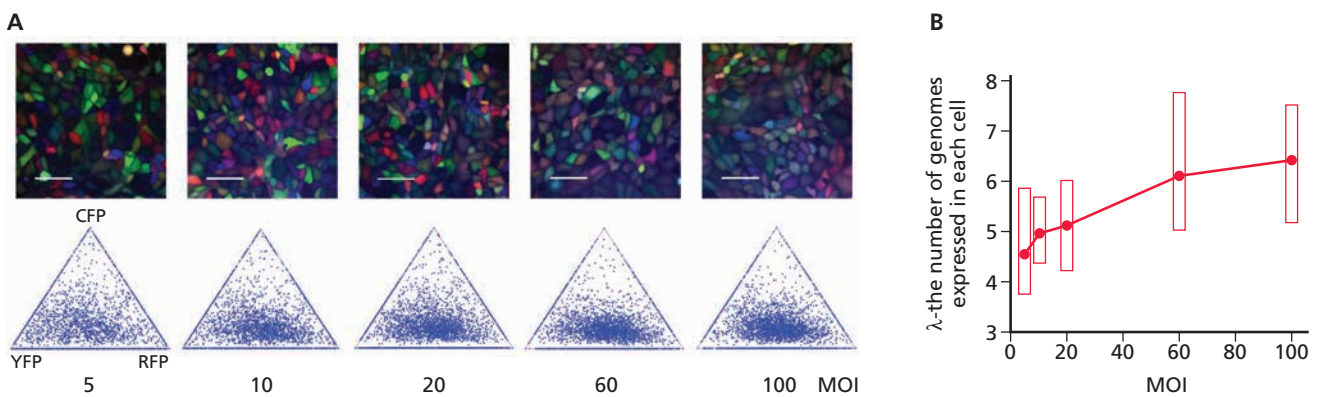
As the MOI is increased, each cell should be infected by an increasing number of genomes. Consequently, the number of cells with mixed colors should increase as those exhibiting a single color (red, cyan, or yellow) decrease. While this pattern was observed, a significant number of cells exhibited single or double colors even at the highest MOIs (panel A). The number of fluorescent proteins (0 to 3) per cell was determined from the colors of individual cells. This parameter was then used in conjunction with a mathematical model to estimate the average number of genomes expressed in an infected cell (l). Strikingly, when l was examined as a function of MOI, the number of expressed genomes expressed did not increase linearly. Rather, this value approached the low limit of <10 genomes per cell (panel B). This result was independent of the viral promoter from which the genes encoding fluorescent proteins were expressed and of whether the reporter proteins were made early or late after infection. It was also shown that the genomes that are expressed are also those that are replicated.

These experiments establish that the number of herpesviral genomes that support viral reproduction is strictly limited, presumably by properties of the host cell. The number of active genomes correlates closely with the number of viral replication centers that are established in infected cell nuclei and the number of genomes that are packaged into virus particles. Although the mechanisms responsible for this limitation of active genomes are not yet known, one possibility is that most infecting DNA genomes are repressed by intrinsic nuclear defense systems.

Kobiler O, Brodersen P, Taylor MP, Ludmir EB, Enquist LW. 2011. Herpesvirus replication compartments originate with single incoming viral genomes. *MBio* **2:**e00278-11. doi:10.1128/mbio.00278-11.

Kobiler O, Lipman Y, Therkelsen K, Daubechies I, Enquist LW. 2010. Herpesviruses carrying a Brainbow cassette reveal replication and expression of limited numbers of incoming genomes. *Nat Commun* **1:**146. doi:10.1038/ncomms1145.

(A) Confluent porcine kidney epithelial cells were infected with mixtures of equal concentrations of infectious particles of pseudorabies viruses that direct expression of red, cyan, or yellow fluorescent protein (RFP, CFP, and YFP, respectively) at the MOIs indicated. Representative color profiles visualized by epifluorescence microscopy are shown at the top with triangle plots for >3,000 cells per condition shown below. Bar = 100 μm. **(B)** The values of λ calculated from two experiments (each with three separate replicate wells) plotted as a function of MOI. The range of λ values among the replicates is represented for each point by the bar. Adapted from O. Kobiler et al., *Nat Commun* **1:**146, 2010, with permission.



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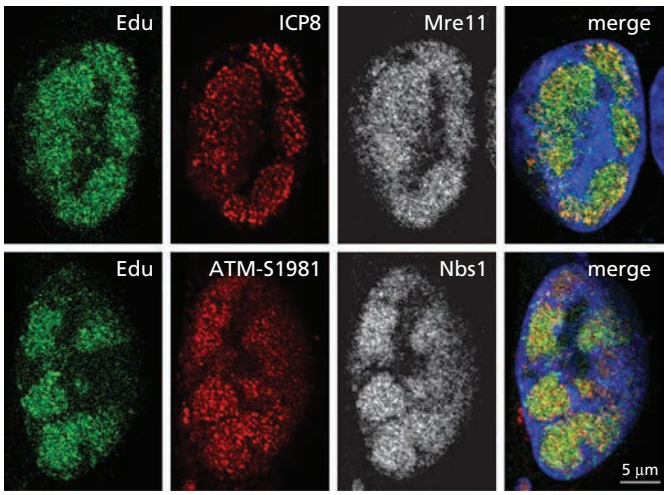


Figure 9.22 Association of cellular DNA damage response proteins with herpesviral replication centers. The cellular DNA damage response proteins Nbs1, Mre11, and activated (phosphorylated) Atm kinase (ATM-S1981) and the viral single-stranded-DNA-binding protein ICP8 (UL42) were detected by indirect immunofluorescence 8 h after herpes simplex virus type 1 infection of HeLa cells. Edu indicates viral DNA detected by incorporation of ethynyl deoxyuridine, biotinylation by "click" chemistry, and reaction with anti-biotin antibodies. Courtesy of M. D. Weitzman, University of Pennsylvania.

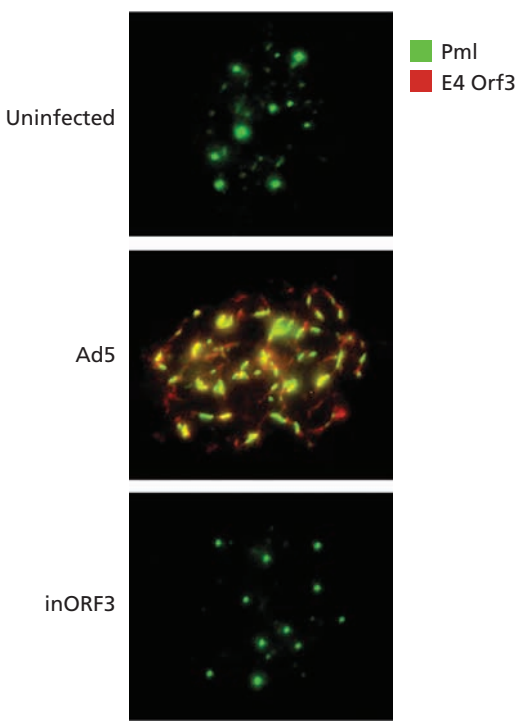
repair proteins and components of a signal transduction pathway activated in response to DNA damage, are required for maximally efficient viral replication.

The localization of the templates for viral DNA synthesis as well as the replication proteins at a limited number of sites undoubtedly facilitates efficient genome replication. This arrangement increases the local concentrations of proteins that must interact with one another, or with viral origin sequences or replication forks, favoring such intermolecular interactions by the law of mass action. In addition, the high local concentrations of replication templates and proteins are likely to allow efficient recruitment of the products of one replication cycle as templates for the next. Viral replication centers also serve as foci for viral gene expression, presumably in part by concentrating templates for transcription with the proteins that carry out or regulate this process. For example, the herpes simplex virus type 1 immediate-early ICP4 and ICP27 proteins, as well as the host cell's RNA polymerase II, are recruited to these nuclear sites.

Viral replication centers do not assemble at random sites, but rather are formed by viral colonization of specialized niches within mammalian cell nuclei. When they enter the nucleus, infecting adenoviral or herpes simplex virus type 1 genomes, and those of papillomaviruses and polyomaviruses, localize to preexisting nuclear bodies that contain the cellular promyelocytic leukemia proteins (Pmls). These are therefore called **Pml bodies**, or nuclear domains 10, a name derived from the average number present in most cells. Viral proteins then induce reorganization of Pml bodies as viral replication centers are established (Fig. 9.23). The human adenovirus type 5 E4 Orf3 protein induces disruption of these structures, with relocalization of some components, such as specific Pml isoforms, to viral replication centers and of others to the cytoplasm for degradation. The herpes simplex virus type 1 ICP0 protein is responsible for similar reorganization of Pml bodies and degradation of several Pml body proteins. This viral protein is an E3 ubiquitin ligase, which catalyzes addition of polyubiquitin chains to proteins, thereby targeting them for destruction by the proteasome (Box 9.10).

The association of replication centers of different DNA viruses with constituents of the same intranuclear bodies suggests that reorganization of host cell nuclei facilitates viral DNA synthesis. The discovery that the genomes of nuclear DNA viruses home to Pml bodies stimulated characterization of their components, but much remains to be learned about their molecular functions. There is evidence that Pml bodies

Figure 9.23 Reorganization of Pml bodies by the adenoviral E4 Orf3 protein. Monkey cells were infected with a wild-type adenovirus type 5 (Ad5) or a mutant that cannot direct synthesis of the E4 Orf3 protein (inORF3). This viral protein (red) and Pml protein (green) were examined by indirect immunofluorescence. In the presence of the E4 Orf3 protein, Pml foci are rearranged to track like structures that contain this viral protein. Adapted from A. J. Ullman et al., *J Virol* **81:**4744–4752, 2007, with permission. Courtesy of P. Hearing, Stony Brook University.



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BOX 9.10

BACKGROUND

Ubiquitinylation of proteins: a posttranslational modification that can target for destruction

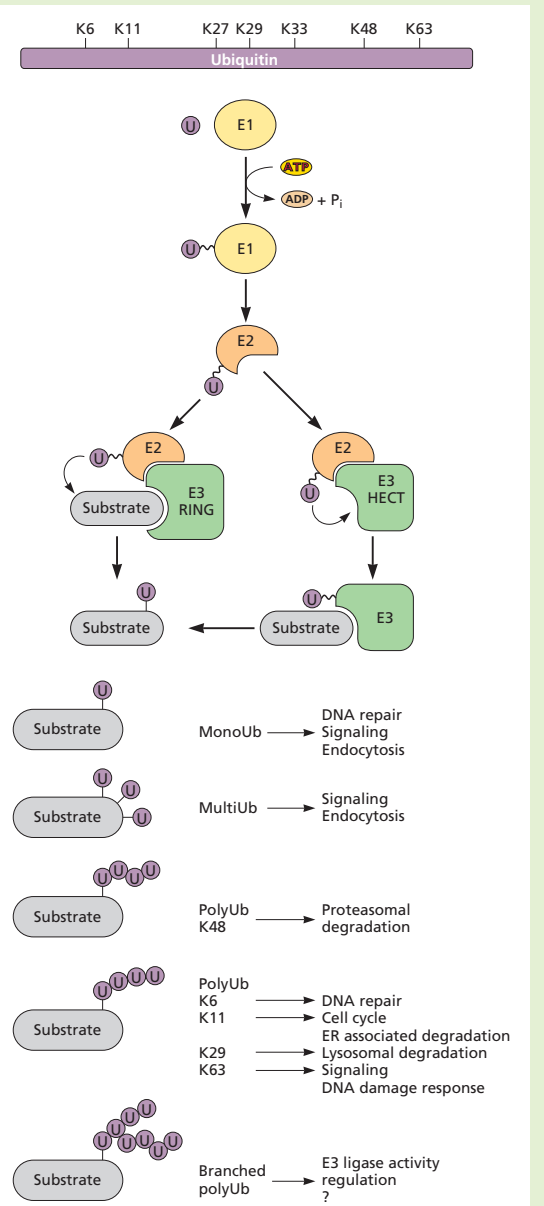
Covalent linkage of the small (76 amino acids) protein ubiquitin to Lys residues is a posttranslational modification that is ubiquitous (hence the protein name) and conserved in eukaryotes. Reversible addition to proteins of small chemical groups, e.g., during phosphorylation or acetylation, requires but a single enzyme, such as a protein kinase. In contrast, ubiquitinylation depends on the sequential activation of three enzymes, a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and an E3 ubiquitin ligase that catalyzes transfer of ubiquitin from the E2 enzyme to a Lys residue of the substrate. The human E1-activating enzyme Ubal cooperates with multiple E2s and a very large number of E3s, which determine substrate specificity. As summarized in the figure, these ubiquitin ligases are divided into two groups on the basis of the presence of a RING (really interesting new gene) or a HECT (homologous to E6-Ap carboxy terminus) domain.

Ubiquitin itself contains multiple Lys residues to which an additional molecule of the small protein modifier can be linked. Indeed, the substrates of E3 ubiquitin ligases may be polyubiquitinylated via different types of linkages among ubiquitin moieties, or monoubiquitinylated. As illustrated, the nature and site of the modification determines whether the substrate protein is targeted for degradation by the proteasome (polyubiquitinylation at K48 of ubiquitin molecules) or its activity regulated (e.g., monoubiquitinylated). The reversible addition of other small proteins discovered subsequently, such as Sumo (small ubiquitin-like modifier) proteins and ubiquitin-like protein-Nedd8, can also regulate the location or activity of proteins.

The genomes of members of various families encode proteins that are themselves E3 ubiquitin ligases or that form these enzymes with distinct specificities upon association with components of cellular E3 ubiquitin ligases. The former class includes herpes simplex virus type 1 ICP0, which induces polyubiquitinylation and degradation of Pml and other Pml body components described in the text, and human herpesvirus 8 proteins (K3 and K5) that target major histocompatibility class I proteins and other components of immune defenses for proteasomal degradation. Viral proteins that redirect the activities of cellular E3 ubiquitin ligases are more numerous. This set includes the human adenovirus type 5 E1B 55-kDa and E4 Orf6 proteins, which coopt the cellular proteins Cul5, EloB and C, and Rbx1 to mark components of the MRN complex (see the text) and the human tumor suppressor p53 (Volume II, Chapter 6) for degradation; retroviral Vif proteins, which cooperate with the same set of cellular proteins to block an innate host defense (Volume II, Chapter 7); and the human papillomavirus type 16 and 18 E6 proteins, which induce degradation of p53 (and other proteins) by recruiting the cellular E3 ubiquitin ligase E6-Ap.

Gustin JK, Moses AV, Früh K, Douglas JL. 2011. Viral takeover of the host ubiquitin system. *Front Microbiol* **2:**161. doi:10.3389/fmicb.2011.00161.

Kerscher O, Felberbaum R, Hockstrasser M. 2006. Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu Rev Cell Dev Biol* **22:**159–180.



The sequential action of the enzymes required to covalently link ubiquitin to a Lys residue in a substrate protein and the two major classes of E3 ubiquitin ligases are shown. As indicated, the nature of the modification determines its impact on the target protein.

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represent a form of intrinsic antiviral defense (Volume II, Chapter 3). For example, exposure of cells to antiviral cytokines (interferons) increases both the number and size of Pml bodies. However, other advantages conferred by the degradation or dispersal of Pml body proteins are likely to be virus specific. The human papillomavirus type 18 E6 protein induces proteasomal degradation of a Pml isoform (Pml-IV) that causes primary human cells to become senescent, a state in which cellular proteins required for replication of the viral genome are not made. In contrast, herpesviral DNA synthesis may require cellular repair and recombination proteins that become relocalized from Pml bodies to viral replication centers.

Limited Replication of Viral DNA Genomes

Synthesis of large numbers of genomes is the typical pattern when DNA viruses infect cells in culture. Nevertheless, several can establish long-term relationships with their hosts and host cells, in which the number of genomes produced is limited. Various mechanisms that achieve copy number control are described in this section.

Integrated Parvoviral DNA Can Replicate as Part of the Cellular Genome

The adenovirus-associated viruses reproduce only in cells coinfected with a helper adenovirus or herpesvirus. Although the latter viruses are widespread in hosts infected by adenovirus-associated viruses, the chances that a particular host cell will be infected simultaneously by two viruses are very low. The strategy of exploiting other viruses to provide essential functions would therefore appear to impose an obstacle to reproduction of individual adenovirus-associated virus particles. In fact, this is not the case, for this viral genome can survive in the absence of a helper virus by an alternative mechanism: its genome becomes integrated into that of the host cell and is replicated as part of a cellular replicon.

This program for long-term survival of the adenovirus-associated virus genome depends on expression of its regulatory region (Rep) (Appendix, Fig. 19). The two larger proteins encoded by this region, Rep 78/68, are multifunctional and control all phases of the viral life cycle (Table 9.1). When helper virus proteins, such as adenoviral E1A, E1B, and E4 proteins, allow synthesis of large quantities of Rep 78/68, adenovirus-associated virus DNA is replicated by the mechanism described previously. In the absence of helper functions, only small quantities of Rep 78/68 are made, there is little viral DNA synthesis, and the genome becomes integrated into that of the host cell. Integration is also mediated by Rep 78/68.

One of the most unusual features of the integration reaction is that it occurs preferentially near one end of human chromosome 19. It was believed for many years that integration required the recognition of the viral ITR origin (Fig. 9.9) by Rep 78/68. However, the observation that integration of DNA molecules containing only the ITR was exceedingly inefficient led to the identification of a viral sequence that increased the frequency of site-specific integration by up to 100-fold in established lines of human cells. This sequence, which can function as an origin, overlaps the p5 promoter (Fig. 9.24). The Rep 78/68 protein can bind simultaneously to both viral DNA and the related human chromosomal 19 DNA sequences that are required for integration, at least *in vitro*. The current model of integration therefore proposes that its specificity is the result of such simultaneous binding to the two DNA molecules by multimeric Rep 78/68.

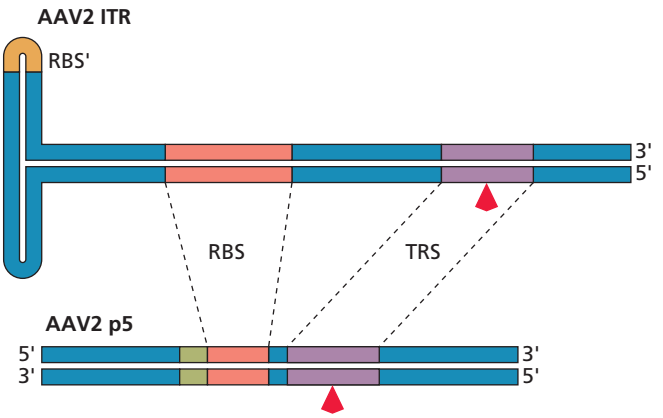


Figure 9.24 Common features of the adenovirus-associated virus type 2 ITR and the p5 sequences important for integration. The Rep 78/68-binding sites are shown in pink, and the terminal resolution sites (TRS) are shown in purple. The p5 origin TRS, like that of the ITR origin, has been shown to be cleaved by the viral protein (red arrowheads). Adapted from D. L. Glauser et al., *J Virol* **79:**12218–12230, 2005, with permission.

In the absence of Rep protein, as in cells infected by typical adenovirus-associated virus vectors (Chapter 3), viral genomes commonly persist as episomal concatemers. It is thought that double-stranded, circular genomes form initially, for example, upon annealing of complementary single-stranded genomes, and then undergo recombination to give rise to concatemers. The long-term persistence of these forms in cells that do not divide is likely to be an important reason for the therapeutic success of some adenovirus-associated virus vectors.

Site-specific integration of the viral genome also occurs in cells infected by human herpesvirus 6 and chicken Marek's disease virus, the result of recombination between the repeated sequences that comprise telomeres and related sequences in viral DNA (Box 9.11). Such integration accounts for persistence and congenital transmission of human herpesvirus 6 DNA and is important for transformation and tumor formation by the oncogenic Marek's disease virus.

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BOX 9.11

DISCUSSION

Integration into host cell telomeres as a mechanism of herpesvirus latency?

A characteristic property of herpesviruses is the establishment of latent infections in specific cell types, for example, neurons and B cells in the case of alphaherpesviruses and Epstein-Barr virus, respectively. Although linear in virus particles, viral genomes persist in such latently infected cells as circular episomes, either because the cells do not divide (neurons) or as a result of coordination of replication and segregation of viral genomes with the host cell cycle (B cells). Studies of the betaherpesvirus human herpesvirus 6 have prompted consideration of an alternative mode of herpesviral latency.

Primary infection with human herpesvirus 6, which is widespread in the human population, occurs early in life, and in 25 to 35% of cases is associated with development of fever and a characteristic rash in babies (roseola infantum). The virus establishes latency following primary infection, and reactivation from this state can cause serious disease, particularly in immunocompromised individuals. The first indications of an unusual mechanism of persistence of this herpesviral genome were reports that ~1% of the human population in several different countries carry chromosomally integrated herpesvirus 6 DNA, often in multiple cell types including cells of the germ line.

The human herpesvirus 6 genome contains a unique sequence bounded by direct repeats. These direct repeats are in turn flanked by multiple copies of short sequences that are either identical to the 6-bp repeat sequence that comprises human telomeric DNA or imperfect copies of the telomere repeat sequence (see the figure). Analysis of integrated viral DNA by fluorescent *in situ* hybridization in cells recovered from patients revealed integration sites in host cell telomere sequences in all cases examined. This conclusion has been confirmed by direct sequencing of junctions between viral and host cell DNA recovered by PCR from both patients' cells and cells infected in culture. It is thought that integration is the result of homologous recombination between the telomere repeat sequences in the viral genome and present at the ends of human chromosomes. While the mechanism remains to be established, the presence of telomere repeat-related sequences in the viral genome is not sufficient: such sequences are also present in the genome of human herpesvirus 7, but integration of this herpesviral genome has never been observed. This difference could be explained if integration of human herpesvirus 6 DNA is mediated by the viral U94 protein (a unique gene product), which has sequence homology to the adenovirus-associated virus Rep 78/68 endonuclease/helicase and can complement a Rep 78/68 deletion mutant.

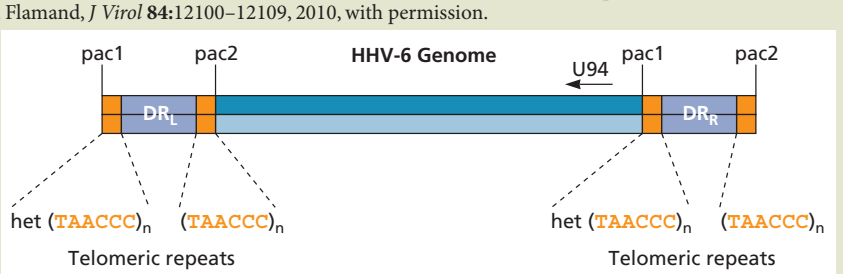
As yet, there is no consensus as to whether integration of herpesvirus 6 DNA into telomeres represents a form of latency or is an epiphenomenon of DNA replication and represents a dead end for the viral genome.

Arbuckle JH, Medveczky MM, Luka J, Hadley SH, Luegmayr A, Ablashi D, Lund TC, Tolar J, De Meirleir K, Montoya JG, Komaroff AL, Ambros PF, Medveczky PG. 2010. The latent human herpesvirus-6A genome specifically integrates in telomeres of human chromosomes in vivo and in vitro. *Proc Natl Acad Sci U S A* **107:**5563–5568.

Arbuckle JH, Pantry SN, Medveczky MM, Prichett J, Loomis KS, Ablashi D, Medveczky PG. 2013. Mapping the telomere integrated genome of human herpesvirus 6A and 6B. *Virology* **442:**3–11.

Morissette G, Flamand L. 2010. Herpesviruses and chromosomal integration. *J Virol* **84:**12100–12109.

Diagram of the human herpesvirus 6 (HHV-6) genome showing the position of the direct repeats (DR_R and DR_L), the perfect or imperfect telomere repeat sequences (TRS and hetTRS, respectively), and the coding sequence for the U94 protein. Adapted from G. Morissette and L. Flamand, *J Virol* **84:**12100–12109, 2010, with permission.



Different Viral Origins Regulate Replication of Epstein-Barr Virus

During herpesviral latent infections, the viral genome is stably maintained at low concentrations, often for long periods (Volume II, Chapter 5). Furthermore, replication of viral and cellular genomes can be coordinated. This pattern is characteristic of human B cells latently infected by Epstein-Barr virus. Many such cell lines have been established from patients with Burkitt's lymphoma, and this state is the usual outcome of infection of B cells in culture. Characteristic features of latent Epstein-Barr virus infection include expression of only a small number of viral genes, the presence of a finite number of viral genomes, and replication from a specialized origin. Because replication from this origin, which is not active in productively infected cells, is responsible for maintenance of episomal viral genomes, it is termed the **origin for plasmid maintenance** (OriP).

The Epstein-Barr virus genome is maintained in nuclei of latently infected cells as a stable circular **episome**, present at 10 to 50 copies per cell. For example, one immortal Burkitt's lymphoma cell line (Raji) has carried ~50 copies per cell of episomal viral DNA for more than 40 years and many passages. When Epstein-Barr virus infects a B cell, the linear viral genome circularizes by a mechanism that is not well understood. The circular viral DNA is then amplified during S phase of the host cell to the final concentration noted above. Such replication is by the cellular DNA polymerases and accessory proteins that synthesize simian virus 40 DNA. However, this process also requires OriP (Fig. 9.25A) and the

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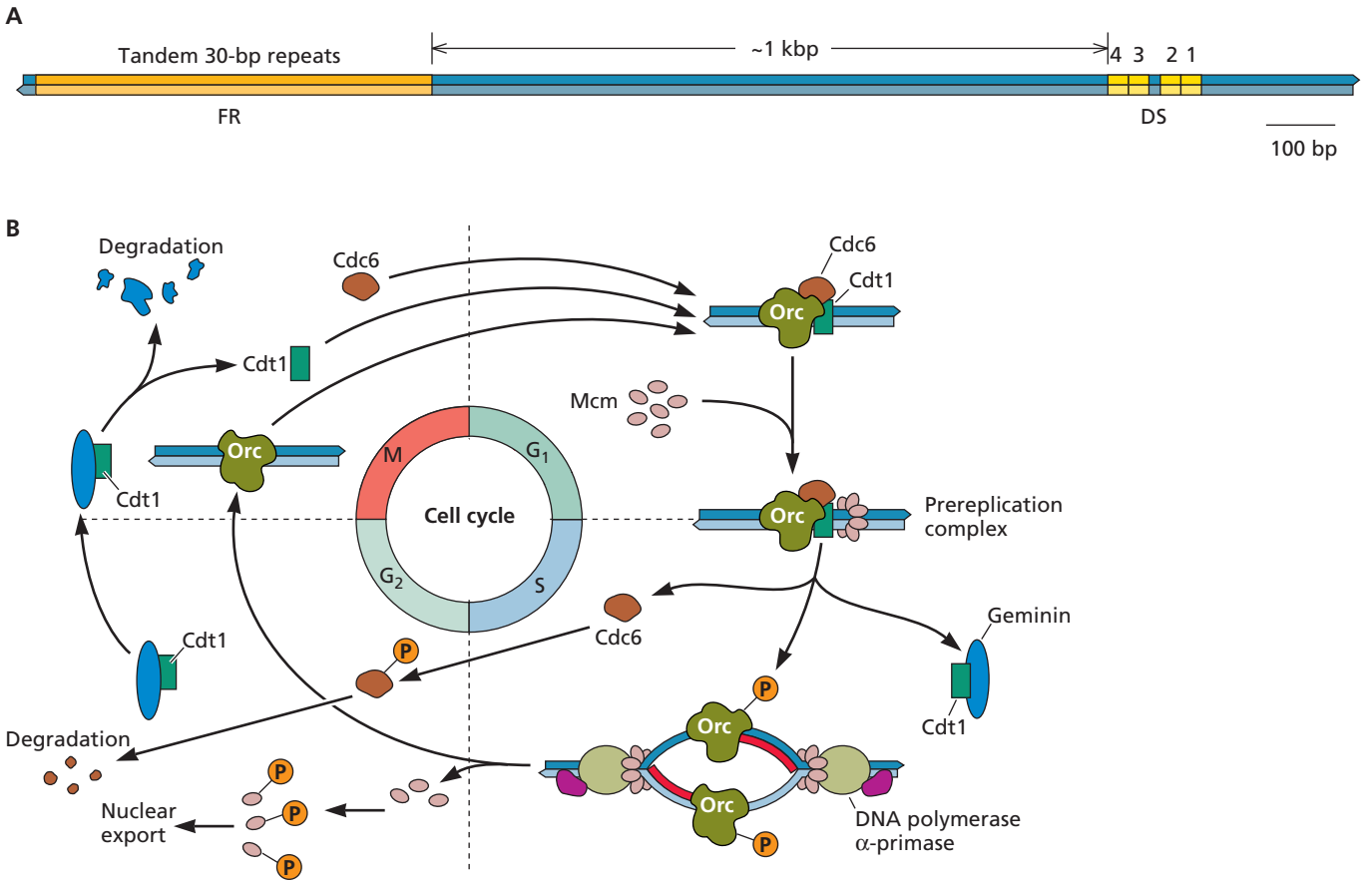


Figure 9.25 Licensing of replication from Epstein-Barr virus OriP. **(A)** Organization of EBNA-binding sites, shown to scale. The dyad symmetry (DS) sequence, which comprises two pairs of binding sites (1 to 4) for EBNA-1 dimers, is the site of initiation of DNA synthesis. The activity of the DS origin is regulated by sequences adjacent to the EBNA-1-binding sites that are recognized by cellular telomere-binding proteins (see the text) and stimulated by the family of repeat (FR) sequence. Proteins that bind to telomere repeat-related sequences include Trf2 (see text) and telomere-associated poly(ADP-ribose) polymerase. The latter, as well as a second poly(ADP-ribose) polymerase, interfere with preinitiation complex assembly at OriP by catalyzing addition of polymers of ADP-ribose to EBNA-1. Binding of EBNA-1 to multiple FR sequences is necessary for maintenance of episomal viral DNA in latently infected B cells. **(B)** The multiprotein origin recognition complex (Orc) is present throughout the cell cycle and is associated with replication origins. However, initiation of DNA synthesis requires loading of the hexameric minichromosome maintenance complex (Mcm), which provides helicase activity. It is the recruitment of Mcm that is regulated during the cell cycle to limit the initiation of DNA synthesis to S phase. This reaction requires two proteins, Cdc6 and Cdt1. The concentrations and activities of both are tightly controlled during the cell cycle. As cells complete mitosis and enter G_1, Cdc6 and Cdt1 accumulate in the nucleus, where they associate with DNA-bound Orc. These interactions permit loading of Mcm at the G_1-to-S-phase transition, and subsequently of components of the DNA synthesis machinery, such as Rp-A and DNA polymerase α-primase. The latter step requires phosphorylation of specific components of the prereplication complex by cyclin-dependent kinases that accumulate during the G_1-to-S-phase transition (Volume II, Chapter 6). Reinitiation of DNA synthesis is prevented by several mechanisms. A cyclin-dependent kinase that accumulates during the G_2 and M phases phosphorylates both Mcm proteins and Cdc6. This modification induces nuclear export of the former and degradation of the latter. In addition, the protein called geminin is present in the nucleus from S until M phase (when it is degraded). This protein binds to Cdt1, sequestering it from interaction with Cdc6 and Orc. As a consequence of such regulatory mechanisms, the prereplication complex can form **only** in the G_1 phase, ensuring firing of the origin once per cell cycle. The association of Mcm with OriP during G_1 and S but not during G_2 and the inhibition of OriP-dependent replication by overproduction of a protein that prevents recruitment of Mcm provide strong support for the conclusion that synthesis of viral DNA genomes in latently infected cells is governed by the mechanisms that ensure once-per-cell-cycle firing of cellular origins.

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viral protein that binds specifically to it, Epstein-Barr virus nuclear antigen 1 (EBNA-1) (Table 9.1), which is always synthesized in latently infected cells. Amplification of the episomal viral genome is limited to a few cycles. Subsequently, viral DNA genomes are duplicated once per cell cycle during S phase and partitioned evenly to daughter cells during mitosis (Box 9.12). The EBNA-1 protein and OriP are sufficient for both once-per-cell-cycle replication and the orderly segregation of viral genomes when host lymphocytes divide.

The availability of cellular replication proteins only in late G_1 and S can account for the timing of Epstein-Barr virus replication in latently infected cells. However, this property **cannot** explain why each genome is replicated only once in each cell cycle, just as each cellular replicon: OriP and cellular origins fire **once and only once** in each S phase. The mechanisms that control once-per-cycle firing of eukaryotic origins, a process termed **replication licensing**, were initially elucidated in budding yeasts, which contain compact origins of replication. Mammalian homologs of the yeast origin recognition complex (Orc) and proteins that regulate initiation of DNA synthesis, such as Mcm, have been identified in all other eukaryotes examined. The human Orc proteins, which are associated with OriP and can bind to EBNA-1, are necessary for OriP-dependent replication, as is the hexameric Mcm helicase, which became associated with OriP during the G_1 phase. Several mechanisms ensure that the essential Mcm helicase is available **only** at the G_1-to-S-phase transition, and hence limit origin firing to once per cell cycle (Fig. 9.25B). For example, recruitment of Mcm to the origin requires cell division control protein 6 homolog (Cdc6) and DNA replication factor Cdt1. These proteins accumulate in the nucleus during S phase, but are subsequently degraded (Cdc6) or sequestered (Cdt1).

Initiation of Epstein-Barr virus DNA synthesis is also regulated temporally and takes place late during S phase. For reasons that are not yet clear, initiation early in S phase is detrimental to both replication efficiency and the maintenance of episomal viral genomes. One parameter important for such temporal regulation is phosphorylation of telomere repeat-binding protein 2 (Trf2) early during S phase by Chk2, a kinase implicated in control of replication timing in yeast. Trf2 binds to three copies of a telomere repeat-related sequence in OriP and interacts with both Orc and histone deacetylases. Consequently, it is thought to coordinate nucleosome remodeling at OriP with recruitment of the proteins essential for initiation of DNA synthesis from this origin. Phosphorylation of Trf2 by Chk2 inhibits these functions and is thought to contribute to preventing too-early initiation of OriP-dependent replication.

Orderly segregation of episomal viral DNA molecules during mitosis (Box 9.12) requires binding of EBNA-1 to its high-affinity sites in the family of repeat (FR) sequences of OriP (Fig. 9.25A). Direct observation of episomal viral genomes by *in situ* hybridization has established that these DNA molecules become tethered to the cellular sister chromatids that are separated during mitosis. Tethering of viral DNA chromosomes, and their subsequent partitioning, is mediated by an N-terminal EBNA-1 sequence that contains two domains that bind directly to AT-rich DNA. In metaphase chromosomes, regions of less condensed (that is, accessible) AT-rich DNA are found between segments that are highly condensed. Any derivative of EBNA-1 that contains two such AT-hook domains (even if these are derived from cellular proteins) binds to chromosomes and supports maintenance of OriP-containing episomes in a host cell population.

As a latent infection is established, the Epstein-Barr virus genome becomes increasingly methylated at C residues present in CG dinucleotides. Sequences that must function in latently infected cells, such as OriP, generally escape this modification, but how methylation specificity is established is not known. Such DNA methylation is associated with repression of transcription and contributes to inhibition of viral gene expression. The viral genome also becomes packaged by cellular nucleosomes and is therefore replicated as a circular minichromosome, much like that of simian virus 40. Replication of the Epstein-Barr virus genome once per cell cycle persists unless conditions that induce entry into the viral productive cycle are encountered. The critical step for this transition is activation of transcription of the viral genes that encode the transcriptional activators Zta and Rta (Chapter 8). These proteins induce expression of the early genes that encode the viral DNA polymerase and other proteins necessary for replication from OriLyt. In addition, Zta appears to be the viral OriLyt recognition protein. Consequently, once this protein is made in an Epstein-Barr virus-infected cell, its indirect and direct effects on viral DNA synthesis ensure a switch from OriP-dependent to OriLyt-dependent replication, and progression through the infectious cycle.

Limited and Amplifying Replication from a Single Origin: the Papillomaviruses

Papillomaviruses reproduce in the differentiating cells of an epithelium, with distinct modes of viral DNA synthesis associated with cells in various differentiation states (Fig. 9.26). Entry of a papillomaviral genome into the nucleus of an undifferentiated, proliferating basal cell initiates a period of amplification of the circular genome, just as during the early stages of latent infection by Epstein-Barr virus. Replication continues until a moderate number of viral genomes (~50 to 100) has accumulated. A maintenance replication pattern, in which the viral genomes are duplicated on average once per cell cycle, is then established as the cells differentiate and move toward the cell surface. The mechanism that governs the switch from amplification to maintenance replication is not known.

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BOX 9.12

EXPERIMENTS

Visualization of duplication and partitioning of Epstein-Barr virus plasmid replicons in living cells

It is well established that episomal Epstein-Barr virus genomes are maintained in populations of proliferating host cells. However, the mechanisms responsible for such maintenance, which is governed by the partition of replicated genomes to daughter cells, are not well understood. In one approach to address this issue, duplication and partition of Epstein-Barr virus replicons were visualized in individual cells by live-cell imaging.

The plasmid replicon used in these experiments contained all sequences of OriP and many copies of the binding site for the Lac repressor (panel A of the figure). Its replication and partitioning were observed in HeLa cells that stably produce the viral EBNA-1 protein, and the Lac repressor fused to two copies of the red fluorescent protein and to a nuclear localization signal (Lac-RFP). This protein was prevented from binding to replicons until visualization was desired by inclusion in the medium of an inducer of the Lac repressor, isopropyl-β-D-1-thiogalactopyranoside. Two clones of the modified HeLa cells that maintained on average 3 to 4 copies/cell of the plasmid were identified for use in subsequent experiments.

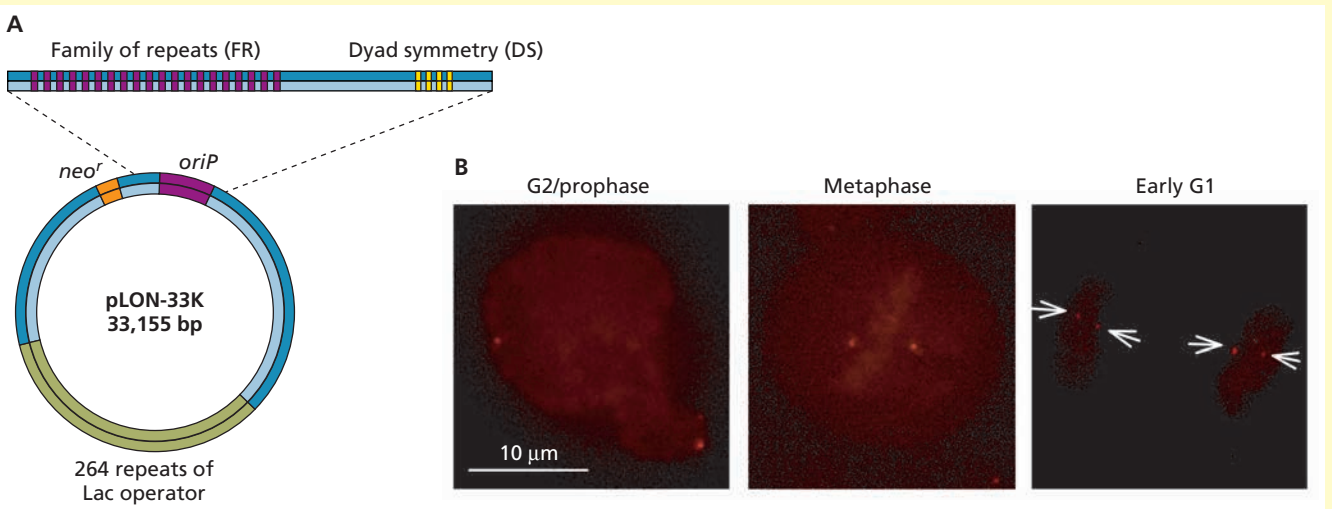
To examine the fate of OriP-containing plasmids, the cells described above were synchronized by blocking cell cycle progression at the beginning of S phase. The plasmid replicons were then visualized via binding of Lac-RFP and live-cell imaging at various times during cell cycle progression after release from the block (panel B). Of 370 plasmids observed in this way,

- 84% were duplicated in S phase, with close colocalization of the daughter plasmids.
- 88% of colocalized pairs of replicated plasmids partitioned accurately (i.e., one plasmid per daughter cell) during the subsequent mitosis.
- 16% of the plasmids failed to replicate and partitioned randomly.

These observations revealed the previously unknown colocalization of newly synthesized OriP-containing episomes and the coupling of the dependence of subsequent, nonrandom partitioning of the episomes during mitosis upon such colocalization.

Nanbo A, Sugden A, Sugden B. 2007. The coupling of synthesis and partitioning of EBV's plasmid replicon is revealed in live cells. *EMBO J* **26:**4252–4262.

(A) Schematic of the replicon, indicating the presence of OriP, binding sites for the Lac repressor, and a neomycin resistance gene (neo^r). This gene allows selection of cells in which the plasmid is present. **(B)** Examples of plasmid segregation (bright dots) during mitosis. This cell contains two pairs of plasmids (as determined by the fluorescence intensity) that segregate as mitosis proceeds from metaphase and partition equally to the daughter cells. Adapted from A. Nanbo et al., *EMBO J* **26:**4252–4262, 2007, with permission. Panel B courtesy of B. Sugden, University of Wisconsin, Madison.



The single viral origin and the viral E1 and E2 proteins that bind to specific origin sequences (Fig. 9.16; Table 9.1) are necessary for both the initial amplification of the papillomavirus genome and its maintenance for long periods at a more or less constant concentration. Initial studies of bovine papillomavirus indicated that such maintenance replication is not the result of strict, once-per-cell-cycle replication of viral DNA. Rather, replication of individual viral episomes occurs at random, taking place on average once per cell cycle. Subsequent studies of human papillomavirus DNA replication in different epithelial cell lines, including those derived from naturally infected cervical epithelia, have established that the viral genome can be replicated by both random and strict, once-per-cell-cycle mechanisms. Which mode of replication

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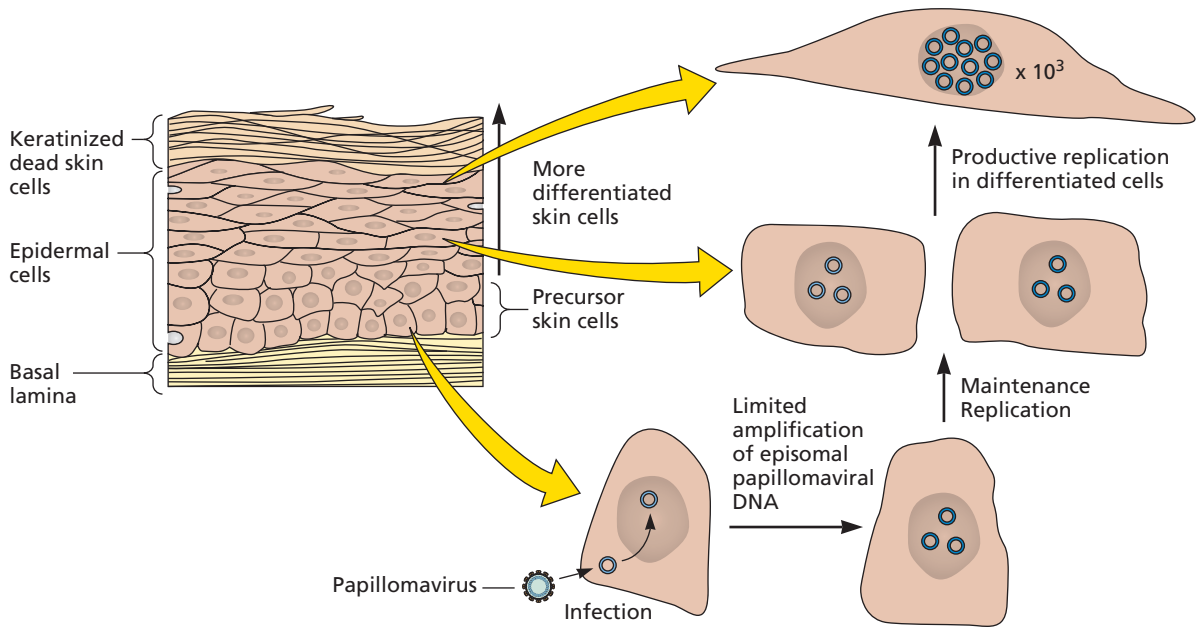


Figure 9.26 Regulation of papillomaviral DNA replication in epithelial cells. The outer layers of the skin are shown as depicted in Fig. 2.5. The virus infects proliferating basal epithelial cells, to which it probably gains access after wounding. The double-stranded, circular viral DNA genome is imported into the infected cell nucleus and initially amplified to a concentration of 50 to 100 copies per cell. This concentration of viral DNA episomes is maintained by further limited replication as the basal and parabasal cells of the epithelium divide **(maintenance replication)**. As cells move to the outer layers of the epidermis and differentiate, productive replication of the viral genome to thousands of copies per cell takes place.

prevails is determined by both the nature of the host cell and the concentration of the viral E1 protein (Box 9.13).

Stable maintenance of the viral genome requires an additional sequence, called the **minichromosome maintenance element**, which is composed of multiple binding sites for the E2 protein. When bound by the viral protein, the minichromosome maintenance element is attached to mitotic chromosomes and remains associated with them during all stages of mitosis. This association is mediated by binding of E2 to the cellular bromodomain-containing protein 4 (Brd 4), an acetylated histone H4-binding protein that interacts with mitotic chromosomes. Such tethering seems to be a mechanism that is shared among a number of viruses. The Brd 4 protein has also been implicated in binding to mitotic chromosomes of episomal DNA of human herpesvirus 8, a herpesvirus that is associated with various human tumors (Volume II, Chapter 6). Brd 4 and the related Brd 2 and Brd 3 proteins have been shown to facilitate integration of the DNA of the gammaretroviruses murine leukemia virus and feline leukemia virus by binding to the retroviral integrase protein and tethering the preintegration complex to host chromosomes.

Remarkably, the final stage of papillomaviral DNA replication, production of high concentrations of the viral genome for assembly into progeny virus particles, is restricted to nondividing, differentiated epithelial cells, such as terminally differentiated keratinocytes (Fig. 9.26). Induction of the DNA damage response mediated by the Atm (ataxia telangiectasia mutated) kinase is required for such genome amplification. The viral E1 and E7 proteins contribute to activating this response and induce the accumulation of Atm and other proteins that mark sites of DNA damage, such as Chk2 and components of the Mre11-Rad50-Nbs1 (MRN) complex, with the viral replication proteins at discrete nuclear foci. These sites are also associated with cellular proteins that participate in homologous recombination. However, it is not clear whether recombination is necessary for genome amplification or to resolve concatemeric replication products into unit-length circular genomes.

Origins of Genetic Diversity in DNA Viruses

Fidelity of Replication by Viral DNA Polymerases

Proofreading Mechanisms

Cellular DNA replication is a high-fidelity process with an error rate of only about one mistake in every 10^9 nucleotides incorporated. Such fidelity, which is essential to maintain the integrity of the genome, is based on accurate base pairing during genome replication and after this process is complete. Nonstandard base pairs between template and substrate deoxyribonucleotide bases can form quite readily, but DNA

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BOX 9.13

EXPERIMENTS

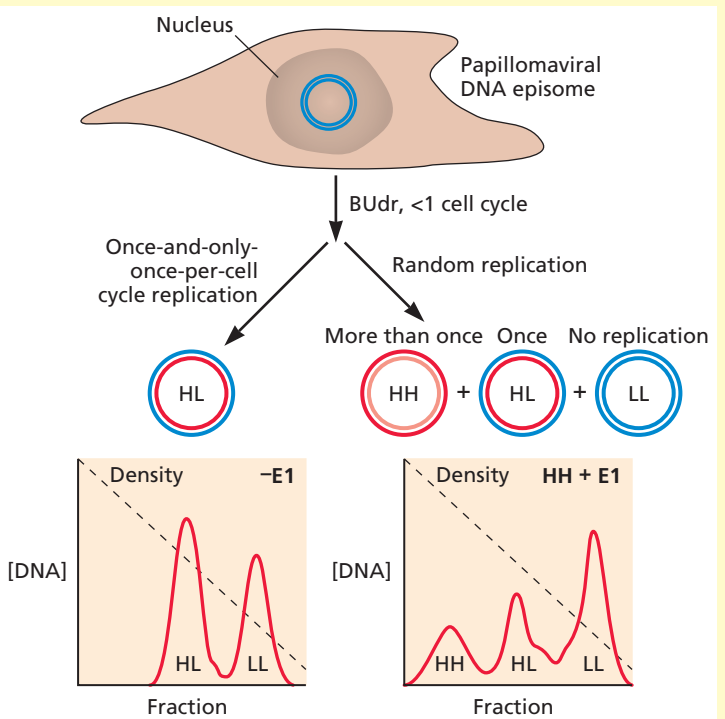
Distinguishing once-per-cell-cycle from random replication of human papillomavirus DNA

In once-per-cell-cycle replication, each molecules of episomal viral DNA is replicated just once during S phase. In random replication, some DNA molecules are replicated several times in a single cell cycle, some are replicated once, and some do not replicate. As illustrated in the figure, these mechanisms can be distinguished by the densities of the DNA molecules synthesized when cells are incubated with the dense analog of thymidine bromodeoxyuridine (BUdR) for a period of less than the time required to complete one cell cycle. Newly synthesized DNA into which BUdR is incorporated is heavy (H), whereas parental DNA is lighter (L).

Results obtained when this method was applied to W12 cervical keratinocytes that contain human papillomavirus type 16 DNA are shown schematically in the figure. In these cells, viral DNA replication is by the once-per-cell-cycle mechanism: no HH DNA could be detected (left). When a vector for expression of the viral E1 protein was introduced, random replication of the viral DNA ensued (right).

How E1 induces this switch has not been established. However, it might override licensed once-per-cell-cycle replication mediated by cellular proteins such as Mcm. This proposal is consistent with reports that maintenance replication does not, in fact, require E1. Alternatively, overproduction of E1 might circumvent mechanisms that limit E1-dependent replication to once per cell cycle, such as regulation of its nuclear localization by phosphorylation by the S-phase-specific kinase cyclin E-Cdk2.

Hoffman R, Hirt B, Bechtold V, Beard P, Raj K. 2006. Different modes of human papillomavirus DNA replication during maintenance. *J Virol* **80:**4431–4439.



synthesis does not proceed if the terminal nucleotide, or the preceding region of the primer-template, is mismatched. In such circumstances, the mismatched base in the primer strand is excised by a 3′ → 5′ exonuclease present in all replicative DNA polymerases until a perfectly base-paired primer-template is created (Fig. 9.27). Replicative DNA polymerases are therefore self-correcting enzymes, removing errors made in newly synthesized DNA. Mispaired bases that are not eliminated by such proofreading activity are corrected subsequently by mismatch repair. During this process, errors presently in the newly synthesized strand are corrected using the information present in the template strand.

The cellular DNA polymerases that replicate small viral DNA genomes possess proofreading exonucleases. Infection by these viruses (e.g., papillomaviruses and polyomaviruses) does not result in inhibition of cellular protein synthesis, and indeed may induce expression of cellular replication proteins. As the cellular mechanisms of mismatch repair are available to operate on progeny viral genomes, replication of the genomes of these small DNA viruses is likely to be as accurate as that of the genomes of their host cells.

The small, single-stranded DNA genomes of viruses like the parvoviruses and circoviruses are also synthesized by cellular DNA polymerases with proofreading activity. Nevertheless, the rates of mutation of such genomes are considerably higher than those of double-stranded DNA genomes, on the order of 10^{-6} substitutions/site/genome, and these viruses evolve rapidly. Such lower fidelity may result from the inability of the mismatch repair system to detect and correct errors when newly synthesized DNA is single-stranded.

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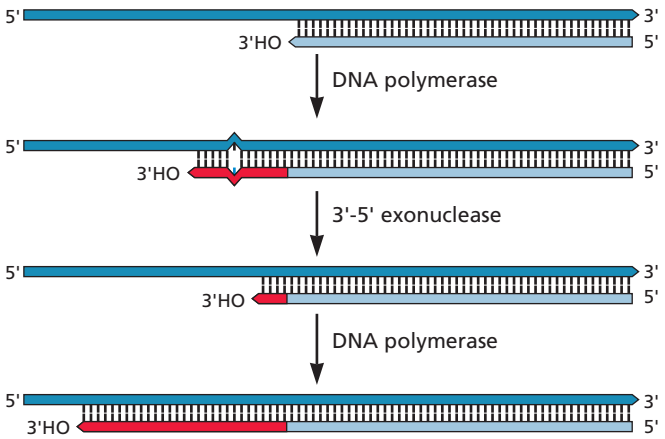


Figure 9.27 Proofreading during DNA synthesis. If permanently fixed into the genome, mispaired bases would result in mutation. However, the majority are removed by the proofreading activity of replicative DNA polymerases. A mismatch at the 3′-OH terminus of the primer-template during DNA synthesis activates the 3′ → 5′ exonuclease of all replicative DNA polymerases, which excises the mismatched region to create a perfect duplex for further extension. In the best-characterized case, DNA polymerase I of *E. coli*, the rate of extension from a mismatched nucleotide is much lower than when a correct base pair is formed at the 3′ terminus of the nascent strand. This low rate of extension allows time for spontaneous unwinding (breathing) of the new duplex region of the DNA and transfer of the 3′ end to the 3′ → 5′ exonuclease site for removal of the mismatched nucleotide. Because preferential excision of mismatched nucleotides is the result of differences in the **rate** at which the polymerase can add the next nucleotide, this mechanism is called **kinetic proofreading**.

Proofreading by Viral DNA Polymerases

The question of how accurately viral DNA is replicated by viral DNA polymerases, such as those of adenoviruses, herpesviruses, and poxviruses, has received relatively little attention. However, each of these viral enzymes possesses an intrinsic 3′ → 5′ exonuclease that preferentially excises mismatched nucleotides from duplex DNAs *in vitro*, and mutations that impair the exonuclease activity of the herpes simplex virus type 1 DNA polymerase greatly increase the mutation rate.

Relatively little is known about the effects of infection by the larger DNA viruses on the production or function of cellular mismatch repair proteins that normally back up proofreading. Because expression of cellular genes and cellular DNA synthesis are generally inhibited in cells infected by these viruses, it is possible that mismatch repair proteins are not present in the concentrations necessary for effective surveillance and repair of newly synthesized viral DNA. Indeed, infection of primary human fibroblasts by human cytomegalovirus (a betaherpesvirus) reduces the activity of an enzyme important for excision of alkylated bases. More detailed information about the rates at which viral DNA polymerases introduce errors during DNA synthesis *in vitro*, and the rates of mutation of viral DNA genomes during productive infection, would help to establish whether cellular repair systems help to maintain the integrity of these viral genomes. Similarly, the contributions of viral enzymes that could prevent or repair DNA damage, such as the dUTPase and uracil DNA glycosylase of herpesviruses and poxviruses (Table 9.3), remain to be established.

Inhibition of Repair of Double-Strand Breaks in DNA

Exposure of mammalian cells to ultraviolet (UV) or infrared light, as well as stalling or collapse of replication forks, can produce double-strand breaks in the DNA genome. Such lesions are potentially lethal, so it is not surprising that they elicit powerful and sensitive damage-sensing and response systems. Proteins that recognize double-stranded DNA ends initiate signaling to effector proteins that both halt progression through the cell cycle (to allow time for repair) and repair the broken ends. The DNA ends are sealed by either nonhomologous end joining or homologous recombination repair (Fig. 9.28). Nonhomologous end joining is an error-prone process in which broken DNA ends are simply joined together after trimming. This important repair pathway is blocked in cells infected by several DNA viruses.

The products of adenoviral DNA synthesis are unit-length copies of the linear viral genome that require no processing prior to packaging (Fig. 9.10). However, accumulation of these viral DNA molecules requires inactivation of nonhomologous end joining. In the absence of the viral E4 Orf3 and Orf6 proteins, newly synthesized viral DNA molecules are joined end to end to form concatemers far too large to be packaged into progeny virus particles. Accumulation of such multimeric DNA molecules depends on cellular proteins that function in nonhomologous end joining, including DNA ligase IV and the MRN complex, which normally accumulates at sites of DNA damage (Fig. 9.28). In adenovirus-infected cells, the protein components of this complex become redistributed within nuclei by the E4 Orf3 protein and are then degraded. The E4 Orf6 and the viral E1B 55-kDa proteins assemble with several cellular proteins to form an infected cell-specific E3 ubiquitin ligase that modifies both MRN components and DNA ligase IV, thereby targeting them for proteasomal degradation. When neither this virus-specific E3 ligase nor the E4 Orf3 protein is present, the cellular repair proteins accumulate in viral replication centers and viral DNA synthesis is inhibited.

Inhibition of nonhomologous end joining is an obvious prerequisite for replication and packaging of linear adenoviral DNA molecules. However, proteasomal degradation of MRN complex components is also induced by LT and increases the yield of simian virus 40. As this virus possesses a circular, double-stranded DNA genome, the reason for this increase is not obvious.

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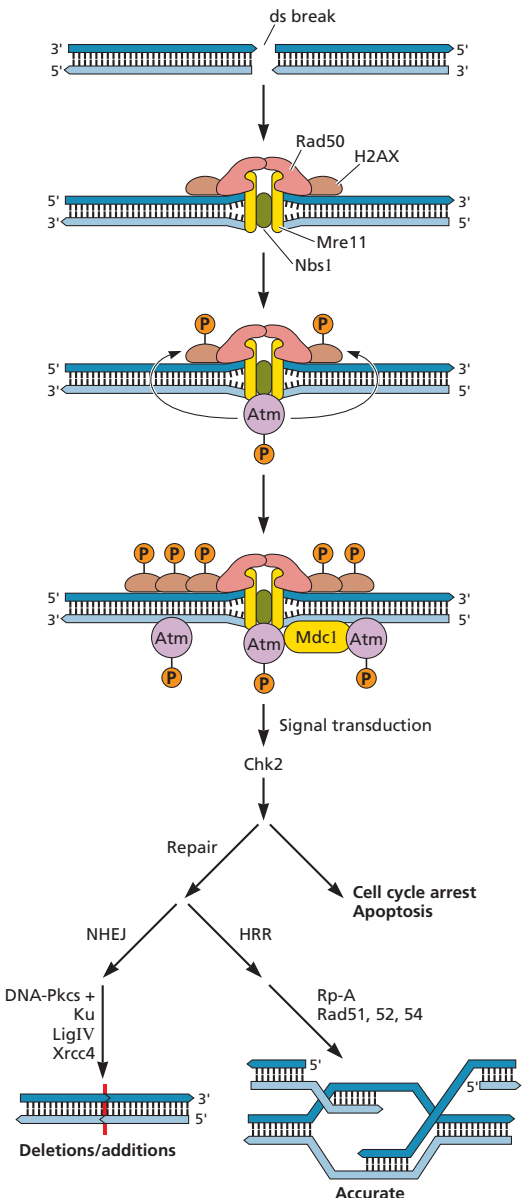


Figure 9.28 Detection of double-strand breaks in DNA. Induction of a double-strand break in the DNA genome triggers rapid accumulation of the MRN complex at the break. This complex contains two copies each of the Mre11 and Rad50 proteins, which move from the cytoplasm into the nucleus, and one of the Nbs1 protein. Mre11 possesses 3′ → 5′ exonuclease, single-stranded DNA endonuclease, and helicase activities. It is thought that these activities unwind the DNA ends at the site of the break, allowing recruitment of the large protein kinase ataxia telangiectasia mutated, Atm. This kinase then becomes activated, perhaps by conformational change and autophosphorylation, and phosphorylates substrates such as the variant histone H2aX. This modification allows amplification of the signal via binding of additional MRN complexes and of mediator of DNA damage checkpoint protein 1, Mdc1. Both this protein and Nsb1 bind phosphorylated H2aX. The Mdc1 protein transduces the signal via additional protein kinases (e.g., Chk2) and other proteins to induce such responses as cell cycle arrest and DNA repair. The two major repair pathways are nonhomologous end joining (NHEJ) and homologous recombination repair (HRR).

Recombination of Viral Genomes

General Mechanisms of Recombination

Genetic recombination is an important source of genetic variation in populations. It also makes a major contribution to repair of breaks in a DNA genome (Fig. 9.28) and can rescue replication when this process has stalled at unfavorable sequences in the template. Much of our understanding of the mechanisms of recombination is based on studies of bacterial viruses, such as bacteriophage λ. Similar principles apply to recombination of DNA genomes of animal viruses.

Two types of recombination are generally recognized: site specific and homologous. In **site-specific recombination**, exchange of DNA takes place at short DNA sequences that are specifically recognized by proteins that catalyze recombination, such as the λ and retroviral integrases. These sequences may be present in only one or both of the DNA sequences that are recombined in this way. Much more common during reproduction of DNA viruses is **homologous recombination**, the exchange of genetic information between **any** pair of related DNA sequences.

Origin-Independent, Recombination-Dependent Replication

In previous sections, we focused on viral genome replication initiated by binding of specialized proteins to origins of replication to induce unwinding of the template and establishment of replication forks. However, early studies of the replication of the genomes of bacteriophages T4 and λ identified an alternative mechanism (Box 9.14). This replication mechanism does not require recognition of viral origin sequences, but rather depends on viral recombination proteins. For example, mutations in the bacteriophage T4 genes that encode such proteins lead to arrest of viral DNA synthesis. In such recombination-dependent replication, recombination proteins catalyze the invasion of double-stranded DNA by a single DNA strand with a 3′-OH terminus, hence providing a primer for DNA synthesis (Fig. 9.29).

As we have seen, the replication of herpes simplex virus genomes exhibits several properties consistent with such a recombination-dependent replication mechanism: viral DNA synthesis becomes independent of the origins and origin-binding protein late in infection; certain cellular DNA repair and recombination proteins become associated with viral replication centers, and inhibition of their synthesis impairs virus reproduction; and the viral genome encodes proteins like those that form the bacteriophage λ recombinase (see next section). Furthermore, herpes simplex virus DNA replication is accompanied by a high degree of recombination between repeated sequences in the genome. Indeed, conversion of the genome from one of its four isomers to another (Fig. 9.19) occurs by the time that newly replicated DNA can first be detected in infected cells. These properties suggest that

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BOX 9.14

DISCUSSION

Replication and recombination/repair are two sides of the same coin: earliest insights from bacteriophage λ

In the early 1970s, studies of the replication of bacteriophage λ showed that mutants defective in viral recombination genes (*redα*$^-$ or *redβ*$^-$, *gam*$^-$) synthesize DNA at only half to one-third the wild-type rate. Furthermore, the concatemers typical of late DNA synthesis were on average shorter than usual, and viral bursts were only 30 to 40% of wild-type values. The role of Gam was explained by its inhibition of the cellular RecBCD nuclease, which would be expected to destroy free concatemer ends. However, the role of Red proteins was not so readily apparent. Furthermore, the fact that viral *red*$^-$ mutants failed to plate at all on certain cells, for example, those that were deficient in host DNA polymerase I or ligase, suggested a critical role for recombination and repair functions in λ DNA replication.

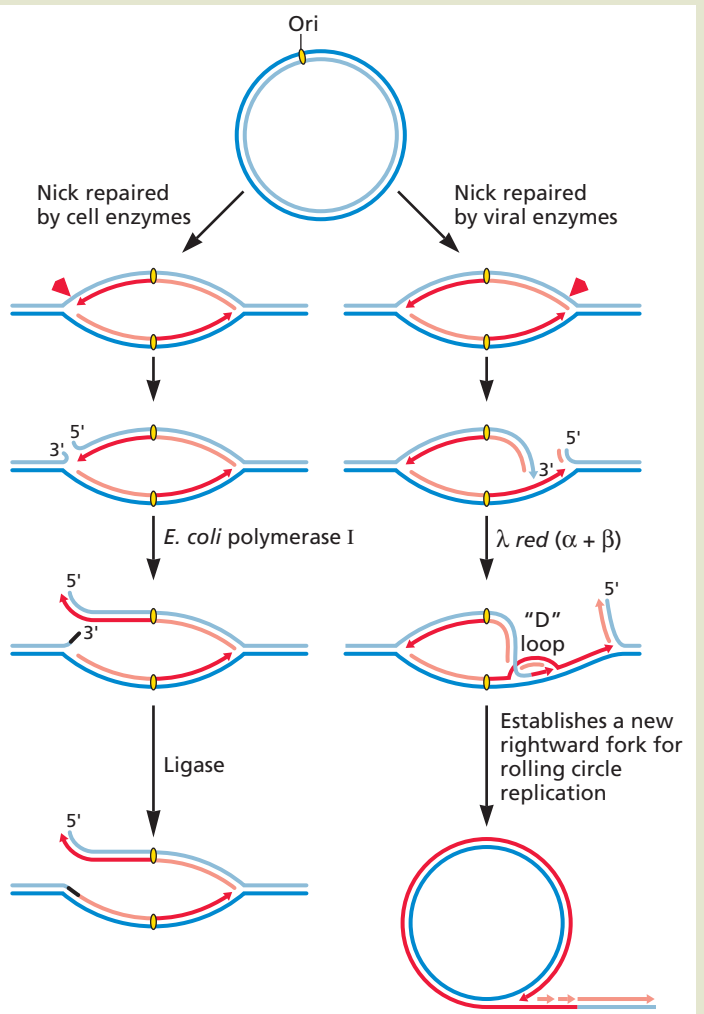
An elegant series of genetic and biochemical experiments led to a model (shown here) for the transition from circle to rolling-circle replication, which proposed a mechanism by which viral recombination or host DNA repair proteins might produce new replication forks when encountering damage induced by a single-strand break.

It was suggested at the time that the principles illustrated in this model might be applicable to cellular DNA metabolism. The idea that recombination could generate a replication origin was novel at the time, but current schemes for the repair of stalled replication forks in both bacterial and eukaryotic cells incorporate the very same ideas elaborated from studies of λ more than 30 years ago. Furthermore, it has been reported recently that homologous recombination alone can support efficient replication of the 2.85-Mbp genome of the archaeon *Haloferax volcanic*.

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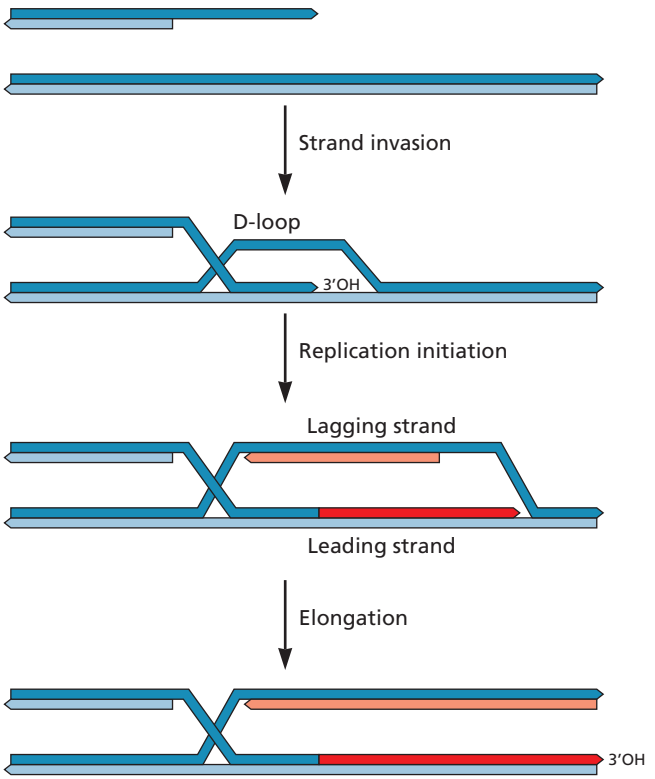


Figure 9.29 General model for initiation of recombination-dependent replication. Replication of linear viral DNA genomes by the standard mechanism of leading- and lagging-strand synthesis from RNA primers produces DNA molecules with single-stranded ends carrying a 3′-OH terminus (see Fig. 9.2B). Such a process is thought to occur during one or a few initial cycles of bacteriophage T4 DNA synthesis. The action of cellular repair proteins that are recruited to double-strand breaks in DNA can also create such single-stranded 3′ ends. The crucial reaction for initiation of recombination-dependent replication is invasion of a homologous sequence in another viral DNA molecule to form a D-loop. This process is exactly analogous to the initial step of general homologous recombination and is catalyzed by repair proteins. The 3′-OH terminus of the invading strand provides a primer for initiation of leading-strand synthesis. Once the D-loop is sufficiently enlarged (equivalent to a replication bubble), lagging-strand synthesis takes place (via synthesis of RNA primers). Continued elongation of the two daughter strands leads to replication to the end of a linear viral DNA template. The newly synthesized strand with the terminating 3′OH at the end can invade homolgous sequences in another viral DNA molecule repeat the process and generate large, branched concatemeric viral DNA molecules.

recombination may be an essential reaction during herpes simplex virus type 1 DNA synthesis. However, it remains to be established whether recombination promotes initiation of viral DNA synthesis or stimulates replication indirectly, for example, by processing of replication intermediates.

Viral Genome Recombination

The integration of adenovirus-associated virus DNA into a specific region of chromosome 19, and its excision when conditions are appropriate, is the result of **site-specific** recombination reactions mediated by the Rep 78/68 viral proteins, which bind to specific sequences in both viral and host DNA molecules. In contrast, although there are some host sequence preferences, integration of retroviral DNA (Chapter 7) is sequence specific only for the viral DNA.

All viral DNA genomes undergo homologous recombination. Because the initial step in recombination, pairing of homologous sequences with one another, depends on random collision, it is concentration dependent. Recombination is therefore favored by the large numbers of viral DNA molecules present in productively infected cells, and their concentration within specialized replication compartments. Furthermore, the structures of replication intermediates, or the nicking of viral DNA during replication or packaging that yields DNA ends, can facilitate recombination. The formation of nuclear replication compartments can also result in the concentration of cellular proteins that participate in recombination (and repair) with viral genomes, as, for example, observed in cells infected by herpes simplex virus type 1. The ease with which viral DNA sequences can recombine is an important factor in the evolution of these viruses. It is also of great benefit to the experimenter, facilitating introduction of specific mutations into the viral genome or construction of viral vectors (see Chapter 3).

As viral genomes do not generally encode homologous recombination proteins, it is thought that this process is catalyzed by host cell enzymes. One exception is the herpes simplex virus type 1 alkaline nuclease (Table 9.3). This enzyme is a 5′ →3′ exonuclease with homology to the Red α component of the bacteriophage λ recombinase (Box 9.14). In conjunction with the viral single-stranded-DNA-binding protein (ICP8), the alkaline nuclease can mediate the exchange of strands between two DNA molecules *in vitro*. Recombination mediated by these viral proteins during infection is important for production of normal, infectious genomes: the viral DNA synthesized and packaged in cells infected by mutants that do not direct synthesis of the active nuclease contains structural abnormalities and is poorly infectious.

Although recombination among animal viral DNA sequences has been widely exploited in the laboratory, the mechanisms have not received much attention. One important exception is the homologous recombination of DNA sequences of some herpesviruses, including herpes simplex virus type 1, that is responsible for isomerization of the genome. Populations of viral DNA molecules purified from herpes simplex virus type 1 virus particles contain four isomers of the genome, defined by the relative orientations of the two unique sequence segments (L and S) with respect to one another (Fig. 9.19). All four isomers are present at equimolar concentrations when viral DNA is isolated from a single plaque, suggesting that a single virus particle containing just one genome isomer gives rise to all four by recombination

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between repeated DNA sequences. Recombination between the inverted repeats that flank unique sequences in the viral genome promotes inversion of the L and S segments. Such homologous recombination takes place during viral DNA synthesis and requires the viral replication machinery.

Despite some 30 years of study, the function of the unusual isomerization of the genome of herpes simplex virus type 1 and certain other herpesviruses remains enigmatic. Isomerization is not absolutely essential for virus reproduction in cells in culture, because viruses "frozen" as a single isomer by deletion of internal inverted repeats are viable. On the other hand, the reduced yield of such mutants, and the presence of the inverted repeat sequences in **all** strains of herpes simplex virus type 1 examined, emphasize the importance of the repeated sequences in natural infections. It may be that these sequences themselves fulfill some beneficial function (as yet unknown). Recombinational isomerization would then be a secondary result of the presence of multiple, inverted copies of these sequences in the viral genome. Alternatively, as discussed previously, isomerization might be a consequence of an important role for recombination in replication of the viral genome. The value of the unusual isomerization of the herpes simplex virus type 1 genome may become clearer as understanding of the mechanism of viral DNA synthesis improves.

Perspectives

Our understanding of mammalian replication proteins and the intricate reactions they carry out during DNA synthesis would still be rudimentary were it not for pioneering studies that focused on the simian virus 40 origin of replication, and the discovery that this relatively simple viral DNA sequence could support origin-dependent replication *in vitro* when cellular proteins are supplemented with a single viral protein, LT.

Knowledge of the mechanism of synthesis of this small viral DNA genome also provided the conceptual framework within which to appraise the diversity in replication of other viral DNA genomes. One parameter that varies considerably is the degree of dependence on the host cell's replication machinery. In contrast to those of papillomaviruses, parvoviruses, and polyomaviruses, the genomes of the larger DNA viruses (herpesviruses and poxviruses) encode the components of a complete DNA synthesis system, as well as accessory enzymes responsible for the production of dNTP substrates. Nevertheless, replication of **all** viral DNA genomes requires proteins that carry out the reactions first described for simian virus 40 DNA synthesis, namely, an origin recognition protein(s), one or more DNA polymerases, proteins that promote processive DNA synthesis, origin-unwinding and helicase proteins, and, usually, proteins that synthesize, or serve as, primers.

Strategies for replication of viral DNA genomes range from simple, continuous synthesis of both strands of a double-stranded DNA template (adenovirus) to baroque (and not well-understood) mechanisms that produce DNA concatemers (herpesviruses). These diverse strategies represent alternative mechanisms for circumventing the inability of all known DNA-dependent DNA polymerases to initiate DNA synthesis *de novo*. In some cases, initiation of viral DNA synthesis requires RNA primers and the lagging strand is synthesized discontinuously, but in others, all daughter DNA strands are synthesized continuously from protein or DNA sequence primers.

Efficient reproduction of DNA viruses requires the production of large numbers of progeny viral DNA molecules for assembly of viral particles in relatively short periods. One parameter important for such genome amplification is the efficient production of the proteins that mediate or support DNA synthesis, be they viral or cellular in origin. Viral DNA replication at specialized intracellular sites, a common feature of cells infected by these viruses, is also likely to contribute. Further exploration of this incompletely understood phenomenon should shed new light on host cell biology, in particular the structural and functional compartmentalization of the nucleus. The cues that set the stage for alternative modes of limited replication that are characteristic of some DNA viruses also remain incompletely understood. Elucidation of the mechanisms that result in close integration of viral DNA synthesis with the physiological state of the host cell seems certain to continue to provide important insights into both host cell control mechanisms and the long-term relationships these viruses can establish with their hosts.

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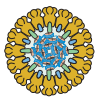
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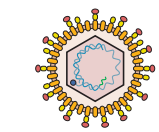
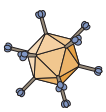
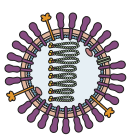
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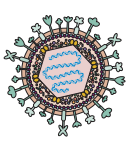
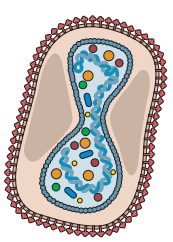
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10 Processing of Viral Pre-mRNA

Introduction

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Perspectives

References

LINKS FOR CHAPTER 10

⏭ *Video: Interview with Dr. Phillip Sharp*
http://bit.ly/Virology_Sharp

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Introduction

Viral messenger RNAs (mRNAs) are synthesized by either viral or cellular enzymes and may be made in the nucleus or the cytoplasm of an infected cell. Regardless of how and where they are made, all must be translated by the protein-synthesizing machinery of the host cell. A series of covalent modifications, collectively known as **RNA processing** (Fig. 10.1), facilitate recognition of mRNAs by the protein synthesis machinery and translation of the coding sequences by cellular ribosomes. Most RNA-processing reactions were discovered in viral systems, primarily because virus-infected cells provide large quantities of specific mRNAs for analysis.

Two modifications important for efficient translation are the addition of m^7GpppN to the 5′ end (**capping**) and the addition of multiple A nucleotides to the 3′ end (**polyadenylation**) (Fig. 10.1). The enzymes that perform these chemical additions may be encoded by viral or cellular genes. When an RNA is produced in the nucleus, another chemical rearrangement, called **splicing**, is possible. During splicing, short blocks of noncontiguous coding sequences (**exons**) are joined precisely to create a complete protein-coding sequence for translation, while the intervening sequences (**introns**) are discarded (Fig. 10.1). Splicing therefore dramatically alters the precursor mRNA (pre-mRNA) initially synthesized. As no viral genome is known to encode even part of the intricate machinery needed to catalyze splicing reactions, splicing of viral pre-mRNAs is accomplished by cellular gene products. Some viral pre-mRNAs undergo a different type of internal chemical change, in which a single base is replaced by another or one or more nucleotides are inserted at specific positions. Such **RNA editing** reactions introduce nucleotides that are not encoded in the genome, and consequently may change the sequence of the encoded protein.

When a viral RNA is produced in the nucleus, it must be exported to the cytoplasm for translation (Fig. 10.1). Such export of mature viral and cellular mRNAs is considered to be part of mRNA processing, even though the RNA is not known to undergo any chemical change during transport. Viral mRNAs invariably leave the nucleus by cellular pathways, but the cargo transported by nuclear export pathways may be altered in virus-infected cells. Once within the cytoplasm, an mRNA has a finite lifetime before it is recognized and degraded by ribonucleases. The susceptibilities of individual mRNA species to attack by these destructive enzymes vary greatly, and can be modified in virus-infected cells.

These RNA-processing reactions (Fig. 10.1) not only produce functional mRNAs but also provide numerous opportunities for posttranscriptional control of gene expression. Regulation of RNA processing can increase the coding capacity of the viral genome, determine when specific viral proteins are made during the infectious cycle, and facilitate selective expression of viral genetic information. An additional component of the varied repertoire of posttranscriptional mechanisms that regulate viral and cellular gene expression has been recognized more recently. Cellular and viral genomes encode small RNAs that induce mRNA degradation or inhibition of translation upon base pairing to an mRNA. This phenomenon is known as RNA silencing or **RNA interference**. RNA interference by cellular RNAs is an important component of antiviral defense, and both cellular and viral small RNAs can modulate virus-host cell interactions.

In this chapter, we focus on these RNA-processing reactions to illustrate both critical viral regulatory mechanisms and the seminal contributions of viral systems to the elucidation of essential cellular processes.

PRINCIPLES *Processing of viral pre-mRNA*

- Viral mRNAs must be translated by the host cell machinery.
- Addition of a modified nucleotide to the 5′ end of an mRNA, "capping," ensures efficient translation, protects the mRNA from exonucleases, and prevents activation of antiviral responses.
- Addition of poly(A) to the 3′ end of an mRNA enhances translation and mRNA stability.
- Viral mRNA splicing is mediated by host cell components; no viral genome encodes any splicing components.
- Alternative splicing and editing of viral pre-mRNAs expand coding capacity and can regulate viral gene expression.
- The export of viral mRNAs is mediated by the host cell machinery and, in most cases, is indistinguishable from export of analogous cellular RNAs.
- Reproduction of some viruses requires production of unspliced or partially spliced mRNAs; virus-encoded proteins or sequences allow the nuclear export of these molecules.
- The genomes of several viruses encode proteins that regulate one or more RNA-processing reactions and are important for temporal regulation of viral gene expression or inhibition of the production of cellular mRNAs.
- Inhibiting the production of cellular mRNAs can favor viral mRNA translation.
- Cellular micro-RNAs may inhibit or facilitate reproduction of a variety of viruses; virally encoded miRNAs may promote viral replication, persistence, or latency or inhibit the host response.

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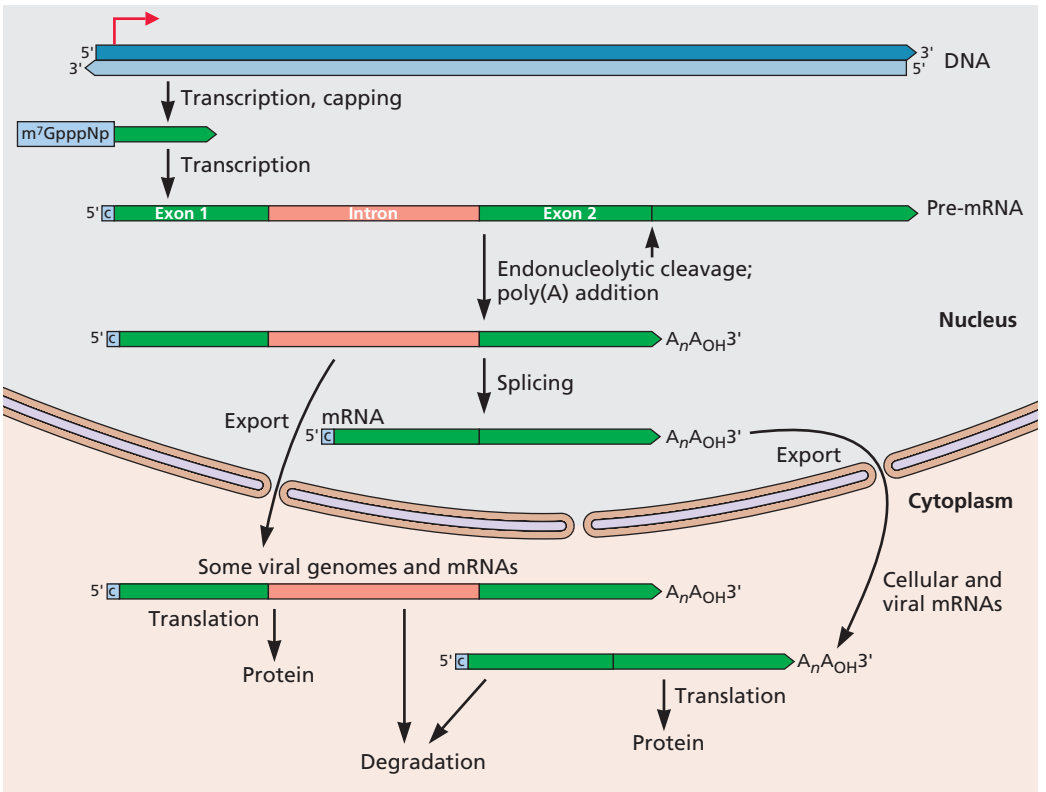


Figure 10.1 Processing of a viral or cellular mRNA synthesized by RNA polymerase II. The reactions by which mature mRNA is made from a typical RNA polymerase II transcript are shown. The first such reaction, capping, takes place cotranscriptionally. For clarity, the exons of a hypothetical, partially processed (i.e., polyadenylated but unspliced) pre-mRNA are depicted, even though polyadenylation and splicing are often coupled and many splicing reactions are cotranscriptional. Most cellular and viral pre-mRNAs synthesized by RNA polymerase II are processed by this pathway (right). However, some viral mRNAs that are polyadenylated but not spliced, or are incompletely spliced, are also exported to the cytoplasm (left).

Covalent Modification during Viral Pre-mRNA Processing

Capping the 5′ Ends of Viral mRNA

The first mRNAs shown to carry the 5′-terminal structure termed the **cap** were those of reovirus and vaccinia virus (Box 10.1). These viral mRNAs are made and processed by virus-encoded enzymes, but subsequent research established that the great majority of viral and cellular mRNAs possess the same cap structure, m^7GpppN, where N is any nucleotide (Fig. 10.2A). This structure protects mRNAs from 5′ exonucleolytic attack and is essential for the efficient translation of most mRNAs, as it is recognized by translation initiation proteins. The principal exceptions are the uncapped mRNAs of certain (+) strand viruses, notably picornaviruses and the flavivirus hepatitis C virus, which are translated by the cap-independent mechanism described in Chapter 11. Cytoplasmic RNA molecules with uncapped 5′-triphosphate termini can be recognized by components of intrinsic defense systems of the host cell (Volume II, Chapter 3). Capping of viral mRNAs blocks such recognition and consequently mitigates induction of cellular antiviral defenses.

Although most viral mRNAs carry a 5′-terminal cap, there is considerable variation in how this modification is made. Three mechanisms can be distinguished: *de novo* synthesis by cellular enzymes, synthesis by viral enzymes, and acquisition of preformed 5′ cap structures from cellular pre-mRNAs or mRNAs.

Synthesis of Viral 5′ Cap Structures by Cellular Enzymes

Viral pre-mRNA substrates for the cellular capping enzyme are invariably made in the infected cell nucleus by cellular RNA polymerase II. The formation of cap structures on the 5′ ends of such pre-mRNAs, the first step in their processing, is a cotranscriptional reaction that takes place when the nascent RNA is only 20 to 30 nucleotides in length. Phosphorylation of paused RNA polymerase II at specific serines in the C-terminal domain of the largest subunit is the signal for binding of the capping enzyme (see below) and capping of the

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BOX 10.1

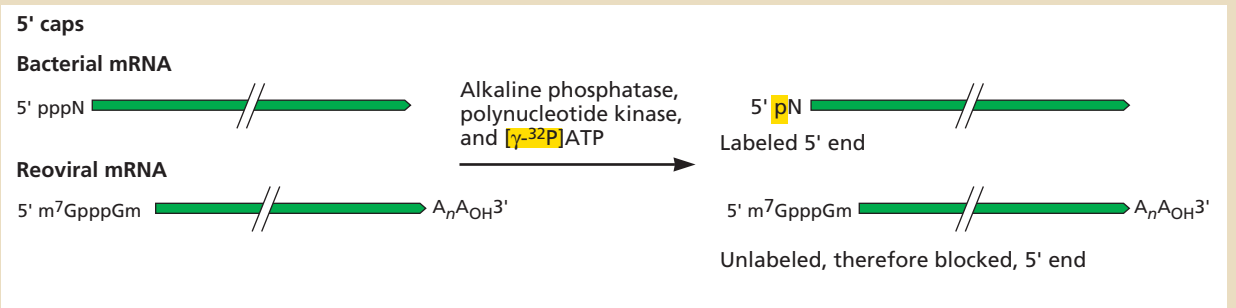
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Identification of 5′ cap structures on viral mRNAs

The first clues that the termini of mRNAs made in eukaryotic cells possess special structures came when viral mRNAs did not behave as predicted from the known structure of bacterial mRNAs. The figure summarizes one of the experiments that identified 5′ cap structures. The 5′ end of reoviral (or vaccinia virus) mRNA, in contrast to that of a bacterial mRNA, could not be labeled by polynucleotide kinase and [γ-^{32}P]ATP (yellow) after alkaline phosphatase treatment. This property established that the 5′ end did not carry a simple phosphate group, but rather was blocked. The structure of the 5′ blocking group (termed the **cap**) was elucidated subsequently by differential labeling of specific chemical groups of the viral mRNA, such as methyl and terminal phosphate groups, followed by digestion of the mRNA with nucleases with different specificities.

Furuichi Y, Morgan M, Muthukrishnan S, Shatkin AJ. 1975. Reovirus messenger RNA contains a methylated, blocked 5′-terminal structure: m^{7}G(5′)ppp(5′)G^{m}pCp-. *Proc Natl Acad Sci U S A* **72:**362–366.

Wei CM, Moss B. 1975. Methylated nucleotides block 5′-terminus of vaccinia virus messenger RNA. *Proc Natl Acad Sci U S A* **72:**318–322.



nascent RNA. The intimate relationship between the cellular capping enzyme and RNA polymerase II ensures that all transcripts made by this enzyme are capped at their 5′ ends.

The 5′ cap structure is assembled by the action of several enzymes. In mammalian cells, a single protein, commonly called **capping enzyme**, contains both activities required for synthesis of a 5′ cap (Fig. 10.2B). Following the action of capping enzyme, the terminal residues are modified by methylation at specific positions. The cap 1 structure, m^{7}GpppNm, is common in viral and mammalian mRNAs. However, the sugar of the second nucleotide can also be methylated by a cytoplasmic enzyme to form the cap 2 structure (Fig. 10.2B). Methylation of the guanine base added during capping is important for recognition of mRNA by the translation machinery, whereas 2′-0 methylation of the sugar(s) blocks the inhibition of translation induced by interferons, major components of the initial antiviral defenses (Volume II, Chapter 3).

Synthesis of Viral 5′ Cap Structures by Viral Enzymes

When viral mRNAs are made in the cytoplasm of infected cells, their 5′ cap structures are, of necessity, synthesized by viral enzymes. These enzymes form cap structures typical of those present on cellular mRNA, although with some variations; for example, alphaviral mRNAs carry the cap 0 structure (Fig. 10.2B). Like their cellular counterparts, viral capping enzymes are intimately associated with the RNA polymerases responsible for mRNA synthesis. In the simplest case, exemplified by the vesicular stomatitis virus L protein, the several enzymatic activities required for synthesis of the mRNA and a 5′ cap structure are supplied by a single viral protein. The large (>2,000-amino-acid) L protein contains discrete domains that catalyze RNA and cap synthesis and subsequent methylation. This arrangement presumably facilitates coordination of capping with RNA synthesis. More-complex viruses encode dedicated capping enzymes, such as the λ-2 protein of reovirus particles and the VP4 protein of bluetongue virus (both members of the *Reoviridae*). The latter protein catalyzes all of the four reactions required for synthesis of the cap 1 structure, and its active sites are organized as a capping "assembly line" (Fig. 10.3). One of the first capping enzymes to be analyzed in detail was the vaccinia virus enzyme, which displays striking functional similarities to its host cell counterpart: it binds directly to the viral RNA polymerase and adds 5′ cap structures cotranscriptionally to nascent viral transcripts that are ~30 nucleotides in length.

Most viral capping enzymes cooperate with viral RNA-dependent RNA polymerases that can synthesize both (−) and (+) strand RNAs, but cap only (+) strand RNAs. The mechanisms that coordinate capping activity with viral mRNA synthesis are not fully understood. In some cases, sequence or structural features of the (+) strand RNA may be recognized by capping enzymes. For example, the methyltransferase of

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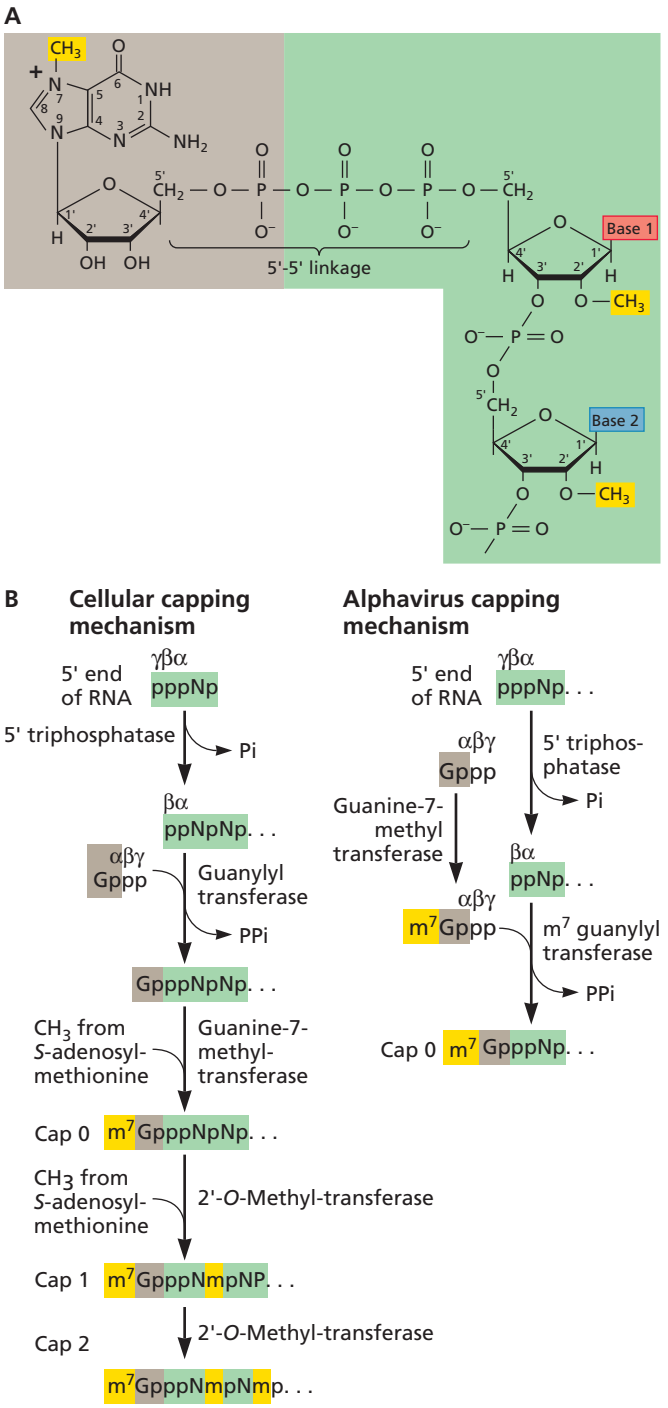


Figure 10.2 The 59 cap structure and its synthesis by cellular or viral enzymes. (A) In the cap structure shown, cap 2, the sugars of the two transcribed nucleotides (green) adjacent to the terminal m^7G (gray) contain 2′-*O*-methyl groups (yellow). The first and second nucleotides synthesized are methylated in the nucleus and in the cytoplasm, respectively. **(B)** The enzymes and reactions by which this cap is synthesized by cellular enzymes are listed (left) and compared to the synthesis of the caps of Semliki Forest virus (a togavirus) mRNAs by viral enzymes in the cytoplasm of infected cells (right).

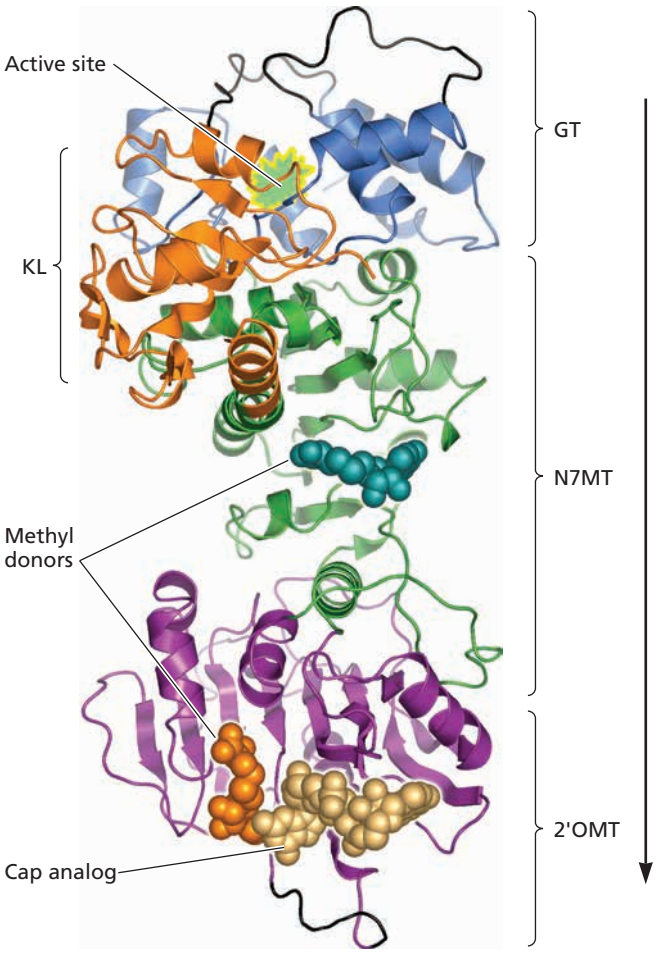


Figure 10.3 A unimolecular assembly line for capping. The structure of the bluetongue virus VP4 protein determined by X-ray crystallography is shown in ribbon form, with each of the four domains in a different color. Localization of the binding sites for substrates and products (e.g., a cap analog) identified the 2′-*O*-methyltransferase (2′OMT, purple), guanine-7-methyltransferase (N7MT, green), and guanylyltransferase (GT, blue) domains. The latter may also contain the RNA 5′-triphosphatase active site. The linear layout of the active sites in the sequence in which capping reactions take place (Fig. 10.2B) allows efficient coordination of these reactions. The KL domain (orange), which is located on one side of the otherwise linear protein, contains no active sites and is thought to mediate interactions with other proteins, such as the viral RNA-dependent RNA polymerase. Adapted from G. Sutton et al., *Nat Struct Mol Biol* **14:**449–451, 2007, with permission. Courtesy of Polly Roy, London School of Hygiene and Tropical Medicine.

the flavivirus West Nile virus binds specifically to a stem-loop structure at the 5′ end of (+) strand RNA. Substitutions of specific residues within this region inhibit cap methylation and viral replication. In other cases, such as the alphaviruses Sindbis virus and Semliki Forest virus, activation of capping enzymes may be the result of proteolytic processing. The viral P1234 polyprotein is responsible for the initial synthesis of (−)

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strand RNA from the (+) strand viral genome (Chapter 6). This polyprotein includes the sequences of the RNA polymerase and the capping enzyme, but the latter is inactive. Cleavage of the polyprotein, which is necessary for synthesis of viral mRNAs (see Fig. 6.15), also releases the capping enzyme.

Acquisition of Viral 5′ Cap Structures from Cellular RNAs

The 5′ cap structures of orthomyxoviral and bunyaviral mRNAs are produced by cellular capping enzymes, but in a unique manner: the 5′ caps of these viral mRNAs are acquired when viral cap-dependent endonucleases cleave cellular transcripts to produce the primers needed for viral mRNA synthesis, a process called **cap snatching** (see Fig. 6.17). The 5′-terminal segments and caps of influenza virus mRNAs are obtained from cellular pre-mRNA in the nucleus. On the other hand, bunyaviral mRNA synthesis is primed with 5′-terminal fragments cleaved from mature cellular mRNAs in the cytoplasm.

Synthesis of 3′ Poly(A) Segments of Viral mRNA

Like the 5′ cap structure, a 3′ poly(A) segment was first identified in a viral mRNA (Box 10.2). This 3′-end modification was soon found to be a common feature of mRNAs made in eukaryotic cells, including most viral mRNAs. Like the 5′ cap, the 3′ poly(A) sequence stabilizes mRNA, and also increases the efficiency of translation. Those RNAs that are not endowed with a 3′ poly(A) tail, such as reoviral and arenaviral mRNAs, may survive by virtue of 3′-terminal stem-loop structures that block nucleolytic attack. Such structures are also present at the 3′ ends of cellular, poly(A)-lacking mRNAs that encode histones. The addition of 3′ poly(A) segments to viral pre-mRNAs, like capping of their 5′ ends, can be carried out by either cellular or viral enzymes. However, cellular and viral polyadenylation mechanisms can differ markedly.

Polyadenylation of Viral Pre-mRNA by Cellular Enzymes

Viral pre-mRNAs synthesized in infected cell nuclei by RNA polymerase II are invariably polyadenylated by cellular enzymes. Transcription of a viral or cellular gene by this enzyme proceeds beyond the site at which poly(A) will be added. The 3′ end of the mRNA is determined by endonucleolytic cleavage of its pre-mRNA at a specific position. Such cleavage is also required for termination of transcription. Poly(A) is then added to the new 3′ terminus, while the RNA downstream of the cleavage site is degraded (Fig. 10.4). Cleavage and polyadenylation sites are identified by specific sequences, first characterized in simian virus 40 and adenovirus pre-mRNAs, including the highly conserved and essential polyadenylation signal, 5′AAUAAA3′. The first reaction in polyadenylation is recognition of this sequence by the protein termed Cpsf (cleavage and polyadenylation specificity protein), an interaction that is stabilized by other proteins (Fig. 10.4). Poly(A) polymerase is then recruited and, following cleavage of the pre-mRNA, synthesizes a poly(A) segment of 200 to 250 nucleotides in a two-stage process. Like

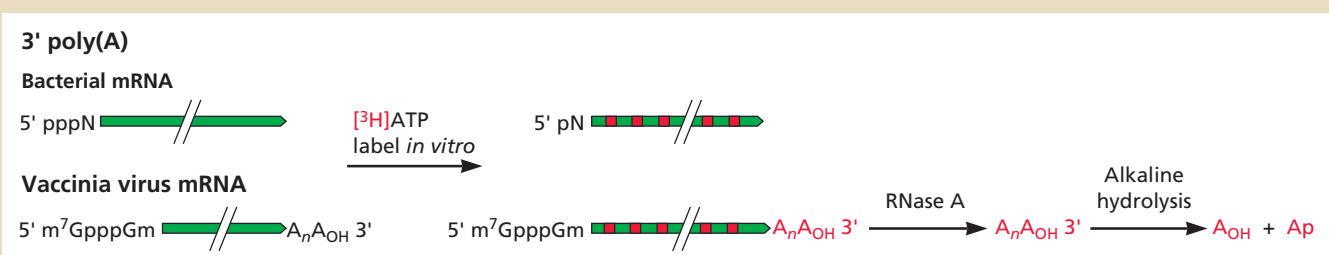
BOX 10.2

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Identification of poly(A) sequences on viral mRNAs

Polyadenylation of viral mRNAs was first indicated by the observation that an RNA chain resistant to digestion by RNase A, which cleaves after U and C, was produced when vaccinia virus mRNA, but not bacterial mRNA, was treated with this enzyme following labeling with [^{3}H]ATP (see the figure). The presence of a tract of poly(A) was confirmed by the specific binding of vaccinia virus mRNA, but not of bacterial mRNA, to poly(U)-Sepharose under conditions that allowed annealing of complementary nucleic acids. The position of the poly(A) sequence in viral mRNA was determined by analysis of the products of alkaline hydrolysis, when phosphodiester bonds are broken to produce nucleotides with 5′ hydroxyl and 3′ phosphate (Ap) groups. The liberation of A residues carrying 3′ hydroxyl groups by this treatment indicated that the poly(A) was located at the 3′ end of the mRNA.

Kates J. 1970. Transcription of the vaccinia virus genome and occurrence of polyriboadenylic acid sequences in messenger RNA. *Cold Spring Harb Symp Quant Biol* **35:**743–752.



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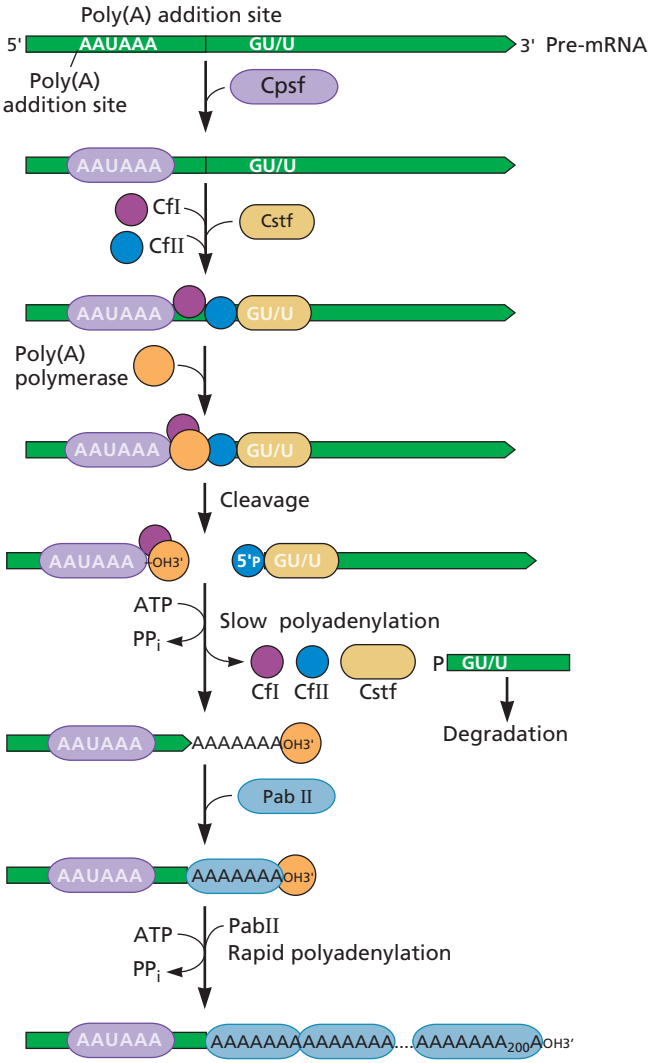


Figure 10.4 Cleavage and polyadenylation of vertebrate pre-mRNAs. The 3′ end of mature mRNA is formed 10 to 30 nucleotides downstream of the essential polyadenylation signal, 5′AAUAAA3′. However, this sequence is **not** sufficient to specify poly(A) addition. For example, it is found within mRNAs at internal positions that are never used as polyadenylation sites. Sequences at the 3′ side of the cleavage site, notably a U- or GU-rich sequence located 5 to 20 nucleotides downstream, are required. In many mRNAs (particularly viral mRNAs), additional sequences 5′ to the cleavage site are also important. The cleavage and polyadenylation specificity protein (Cpsf), which contains four subunits, binds to the 5′AAUAAA3′ poly(A) addition signal. Cleavage stimulatory protein (Cstf) then interacts with the downstream U/GU-rich sequence to stabilize a complex that also contains the two cleavage proteins, CfI and CfII. Binding of poly(A) polymerase is followed by cleavage at the poly(A) addition site by a subunit of Cpsf, and CfI, CfII, Cstf, and the downstream RNA cleavage product are then released. The polymerase slowly adds 10 to 15 A residues to the 3′-OH terminus produced by the cleavage reaction. Poly(A)-binding protein II (PabII) then binds to this short poly(A) sequence and, in conjunction with Cpsf, tethers poly(A) polymerase to the poly(A) sequence. This association facilitates rapid and processive addition of A residues until a poly(A) chain of ~200 residues has been synthesized.

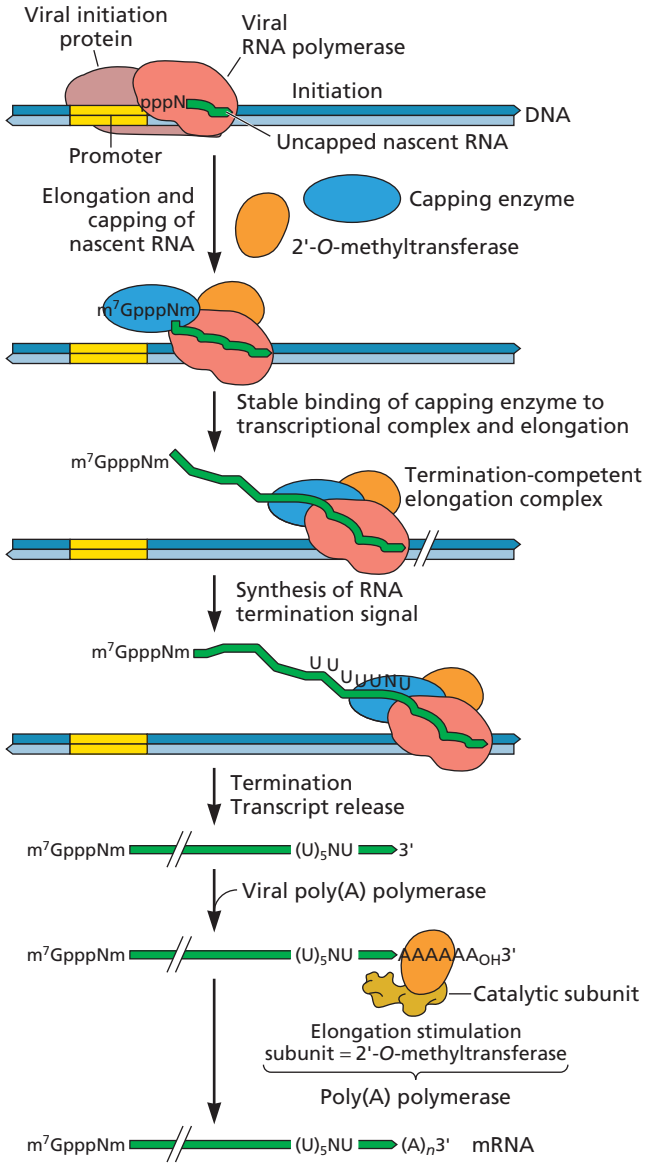


Figure 10.5 The vaccinia virus capping enzyme and 2′-*O*-methyltransferase process both the 5′ and 3′ ends of vaccinia virus mRNAs. After capping the 5′ ends of nascent viral mRNA chains ~30 nucleotides in length (step 1), the capping enzyme remains bound to the nascent RNA chain and to the RNA polymerase as the latter enzyme transcribes the template DNA. The viral 2′-*O*-methyltransferase, which produces a cap 1 structure, also binds to the viral RNA polymerase and stimulates elongation during transcription of viral intermediate and late genes (step 2). This protein is also a subunit of the viral poly(A) polymerase. Termination of transcription (step 3), which takes place 30 to 50 nucleotides downstream of the RNA sequence 5′UUUUNU3′, is mediated by the termination protein/capping enzyme and the viral nucleoside triphosphate phosphohydrolase I, which is a single-stranded-DNA-dependent ATPase. A fraction of the 2′-*O*-methyltransferase molecules act as an elongation stimulation protein for the viral poly(A) polymerase, analogous to cellular poly(A)-binding protein II. This viral enzyme, like its cellular counterpart (Fig. 10.4), adds poly(A) to the 3′ ends of the mRNA in a two-step process (steps 4 and 5).

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capping, polyadenylation at the 3′ ends of an mRNA appears to be coordinated with synthesis of the pre-mRNA: binding of both Cpsf and Cstf (cleavage stimulatory protein) (Fig. 10.4) to the C-terminal domain of the largest subunit of RNA polymerase II is essential for polyadenylation *in vivo*.

Polyadenylation of Viral Pre-mRNAs by Viral Enzymes

Synthesis of poly(A) tails by viral enzymes can occur either posttranscriptionally, like polyadenylation of cellular mRNAs, or during viral mRNA synthesis.

Formation of 3′ ends by termination of transcription. A poly(A) polymerase synthesizes the 3′ poly(A) sequence of vaccinia viral early mRNAs in a two-step process remarkably like that catalyzed by the cellular enzyme (compare Fig. 10.4 and Fig. 10.5). Nevertheless, this viral system for formation of the 3′ ends of mRNA is distinctive in two major respects. The 3′ ends of vaccinia virus early mRNAs are formed by termination of transcription by the viral DNA-dependent RNA polymerase at specific sites (Fig. 10.5), a mechanism with no known counterpart in cellular mRNA synthesis systems. The vaccinia virus capping enzyme is one protein that is required for termination of transcription. Furthermore, all the proteins needed for such termination of transcription and synthesis of poly(A) are also components of the viral capping machinery (Fig. 10.5). These dual-function viral RNA-processing proteins seem likely to facilitate coordination of the reactions by which viral mRNAs are produced.

Polyadenylation during viral mRNA synthesis. The posttranscriptional synthesis of the poly(A) segments of vaccinia virus early mRNAs resembles the cellular mechanism in several respects. In contrast, the poly(A) sequences of other mRNAs made by viral RNA polymerases are produced during synthesis of the mRNA. In the simplest case, exemplified by (+) strand picornaviruses, a poly(U) sequence present at the 5′ end of the (−) strand RNA template is copied directly into a poly(A) sequence of equivalent length. The mRNAs of (−) strand RNA viruses like vesicular stomatitis virus and influenza virus are polyadenylated by reiterative copying of short stretches of U residues in the (−) strand RNA template, a mechanism described in Chapter 6.

Splicing of Viral Pre-mRNA

Discovery of Splicing

Between 1960 and the mid-1970s, the study of putative nuclear precursors of mammalian mRNAs established that these RNAs are larger than the mRNAs translated in the cytoplasm and heterogeneous in size. They were therefore named **heterogeneous nuclear RNAs** (hnRNAs). Such hnRNAs were shown to carry both 5′-terminal cap structures and 3′ poly(A) sequences, leading to the conclusion that both ends of the hnRNA were preserved in the smaller, mature mRNA. Investigators were faced with the conundrum of deducing how smaller mRNAs could be produced from larger hnRNAs while both ends of the hnRNA were retained.

The puzzle was solved by two groups of investigators, led by Phillip Sharp and Richard Roberts, who shared the 1993 Nobel Prize in physiology or medicine (See the interview with Dr. Phillip Sharp: http://bit.ly/Virology_Sharp). These investigators showed that adenoviral major late mRNAs are encoded by four **separate** genomic sequences (Box 10.3). The distribution of the mRNA-coding sequences into four separate blocks in the genome, in conjunction with the large size of major late mRNA precursors, implied that these mRNAs were produced by excision of noncoding sequences from primary transcripts (introns), with precise joining of coding sequences (exons). The demonstration that nuclear major late transcripts contain the introns confirmed that the mature mRNAs are formed by **splicing** of noncontiguous coding sequences in the pre-mRNA. This mechanism had great appeal, because it could account for the puzzling properties of hnRNA. Indeed, it was shown within a matter of months that splicing of pre-mRNA is not an obscure, virus-specific device: splicing occurs in all eukaryotic cells, and the great majority of mammalian pre-mRNAs, like the adenoviral major late mRNAs, comprise exons interspersed among introns.

The organization of protein-coding sequences into exons separated by introns has profound implications for the evolution of the genes of eukaryotes and their viruses. Introns are generally much longer than exons, and only short sequences at their ends are necessary for accurate splicing (see "Mechanism of Splicing" below). Consequently, introns provide numerous sites at which DNA sequences can be broken and rejoined without loss of coding information, and greatly increase the frequency with which random recombination reactions can create new functional genes by rearrangement of exons. Evidence of such "exon shuffling" can be seen in the modular organization of many modern proteins. Such proteins comprise combinations of a finite set of structural and functional domains or motifs, or multiple repeats of a single protein domain, each often encoded by a single exon. The presence of introns is also thought to have facilitated the recombination of viral and cellular genetic information.

Any viral transcript that is synthesized by cellular RNA polymerase II can potentially be spliced. Indeed, splicing is the rule for the transcripts of parvo-, papilloma-, polyoma-, and adenoviral genomes, as well as those of integrated proviral DNAs of retroviruses. Furthermore, alternative splicing of these viral transcripts is an important mechanism for expanding the coding capacity of such viral genomes. Although the (+) strand RNAs of influenza A virus are synthesized by

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BOX 10.3

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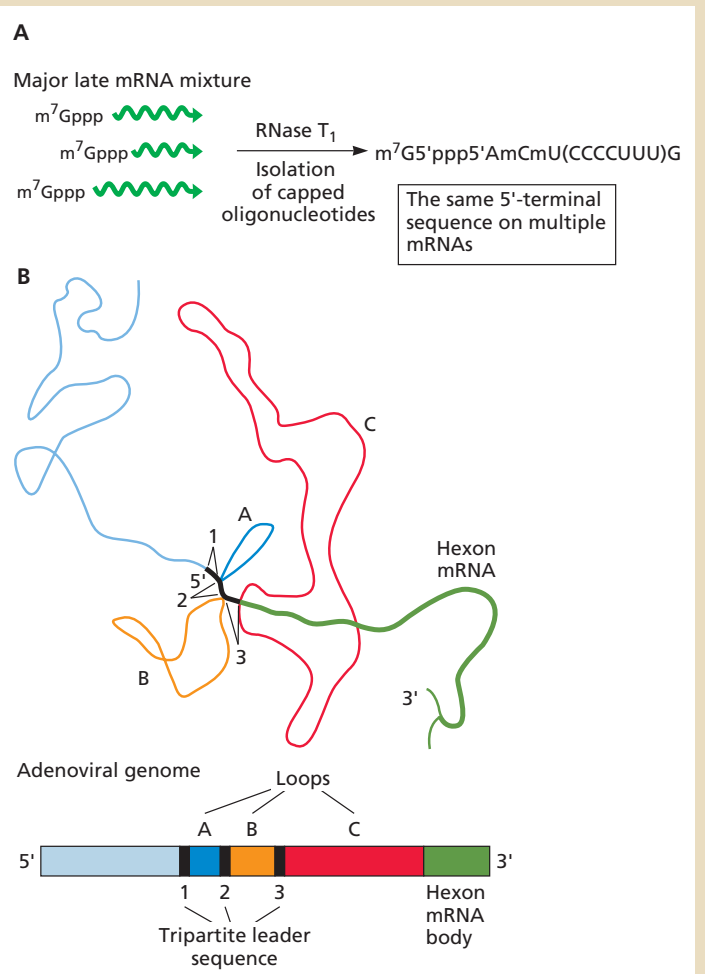
Discovery of the spliced structure of adenoviral major late mRNAs

(A) Digestion of adenoviral major late mRNAs with RNase T_1, which cleaves after G, and isolation of the capped 5′ oligonucleotides indicated that the **same** 11-nucleotide sequence was present at the 5′ ends of several different mRNAs. This observation was surprising and puzzling. Hybridization studies indicated that these 5′ ends were **not** encoded adjacent to the main segments of major late mRNAs. Direct visualization of such mRNAs hybridized to viral DNA provided convincing proof that their coding sequences are dispersed in the viral genome. **(B)** Schematic diagram of one major late mRNA (hexon mRNA) hybridized to a complementary adenoviral DNA fragment extending from the left end of the genome to a point within the hexon-coding sequence. Three loops of unhybridized DNA (thin lines), designated A, B, and C, bounded or separated by three short segments (1, 2, and 3) and one long segment (hexon mRNA) of DNA-RNA hybrid (thick lines) were observed. Other adenoviral late mRNAs yielded the same sets of hybridized and unhybridized viral DNA sequences at their 5′ ends, but differed in the length of loop C and the length and location of the 3′-terminal RNA-DNA hybrid. It was therefore concluded that the major late mRNAs contain a common 5′-terminal segment (segments 1, 2, and 3) built from sequences encoded at three different sites in the viral genome and termed the tripartite leader sequence. This sequence is joined to the mRNA body, a long sequence complementary to part of the hexon-coding sequence in the example shown. Panel B adapted from S. M. Berget et al., *Proc Natl Acad Sci U S A* **74:**3171–3175, 1977, with permission.

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Chow LT, Gelinas RE, Booker TR, Roberts RJ. 1977. An amazing sequence arrangement at the 5′ ends of adenovirus 2 messenger RNA. *Cell* **12:**1–8.

Gelinas RE, Roberts RJ. 1977. One predominant undecanucleotide in adenovirus late messenger RNAs. *Cell* **11:**533–544.



a viral RNA polymerase (Chapter 6), some are nevertheless spliced. For example, the (+) M RNA serves as the mRNA for the matrix protein, but is also spliced to produce the mRNA that specifies the M2 ion channel protein (Appendix, Fig. 15). Such splicing, which may account for the synthesis of influenza A virus RNAs in infected cell nuclei, is an exception to the coordination among the cellular components that synthesize and process pre-mRNAs. The production of a stable intron in neurons latently infected by herpes simplex virus type 1 (Box 10.4) is a second example of a virus-specific permutation of the RNA-processing reactions of the host cell.

Mechanism of Splicing

Sequencing of DNA copies of a large number of cellular and viral mRNAs and of the genes that encode them identified short consensus sequences at the 5′ and 3′ **splice sites**, which are joined to each other in mature mRNA (Fig. 10.6A). The conserved sequences lie largely within the introns. The dinucleotides GU and AG are found at the 5′ and 3′ ends, respectively, of almost all introns. Mutation of any one of these four nucleotides eliminates splicing, indicating that all are essential. Elucidation of the mechanism of splicing came with the development of *in vitro* systems in which model pre-mRNAs

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BOX 10.4

DISCUSSION

Cell-type-specific production of a stable viral intron

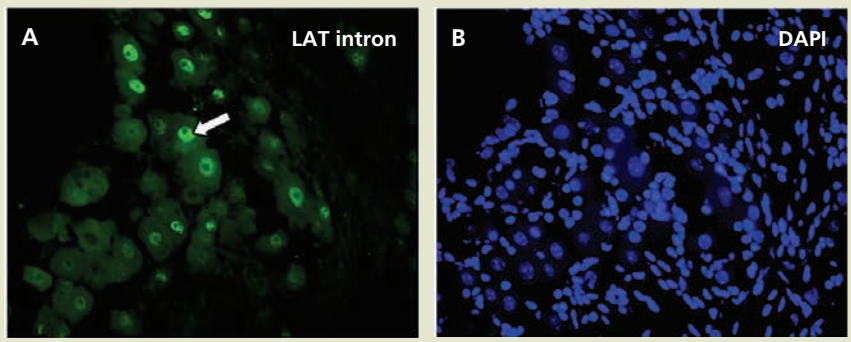
The most abundant RNA detected in neurons latently infected with herpes simplex virus type 1, the 2.0-kb major latency-associated transcript (LAT), is an excised intron: it is not linear, lacks a 3′ poly(A) sequence, and contains a branch point like those of the intron lariats excised during mRNA splicing (Fig. 10.6B).

Processing of LAT precursor RNA is much more efficient in the sensory ganglia, in which herpes simplex virus type 1 establishes latent infections, than in other cell types (see the figure). Furthermore, in contrast to typical introns, which are degraded rapidly, the LAT intron is remarkably stable, with a half-life of >24 h.

These properties imply that the LAT RNA intron fulfills a beneficial function during establishment or maintenance of latency or during subsequent reactivation and entry into the viral lytic cycle. However, the functions of this RNA remain unknown.

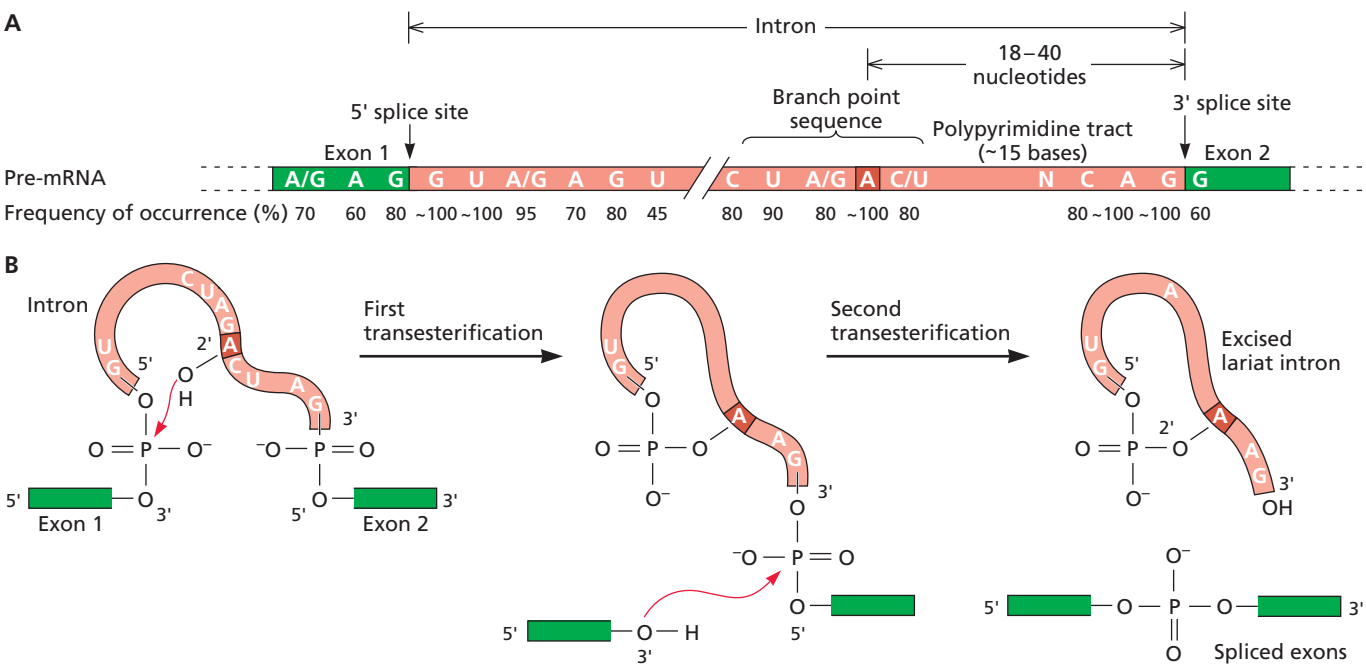
Gussow AM, Giordani NV, Tran RK, Imai Y, Kwiatkowski DL, Rall GF, Margolis TP, Bloom DC. 2006. Tissue-specific splicing of the herpes simplex virus type latency-associated transcript (LAT) intron in LAT transgenic mice. *J Virol* **80:**9414–9423.

Zabolotny JM, Krummenacher C, Fraser NW. 1997. The herpes simplex virus type 1 2.0-kilobase latency-associated transcript is a stable intron which branches at a guanosine. *J Virol* **71:**4199–4208.



Trigeminal neurons isolated from LAT transgenic mice were hybridized to a digoxigenin-containing complementary RNA specific for spliced LAT RNA, incubated with fluorescein-labeled anti-digoxigenin antibodies **(A)**, stained with 4′,6-diamidino-2-phenylindole (DAPI) **(B)**, and examined by fluorescent microscopy. The white arrow indicates a neuron in which LAT RNA splicing was efficient. The many nuclei that do not contain LAT RNA are those of glial cells. Adapted from A. M. Gussow et al., *J Virol* 80:9414–9423, 2006, with permission.

Figure 10.6 Splicing of pre-mRNA. (A) Consensus splicing signals in cellular and viral pre-mRNAs. The most conserved sequences are found at the 5′ and 3′ splice sites at the junctions of exons (green) and introns (pink) and at the 3′ ends of introns. The intronic 5′GU3′ and 5′AG3′ dinucleotides at the 5′ and 3′ ends, respectively, of introns and branch point A (highlighted) are present in all but rare mRNAs made in higher eukaryotes. **(B)** The two transesterification reactions of pre-mRNA splicing. In the first reaction, the 2′ hydroxyl group of the conserved A residue in the intronic branch point sequence makes a nucleophilic attack on the phosphodiester bond at the 5′ side of the GU dinucleotide at the 5′ splice site to produce the intron-3′ exon lariat and the 5′ exon. A second nucleophilic attack by the newly formed 3′ hydroxyl group of the 5′ exon on the phosphodiester bond at the 3′ splice site then yields the spliced exons and the intron lariat.



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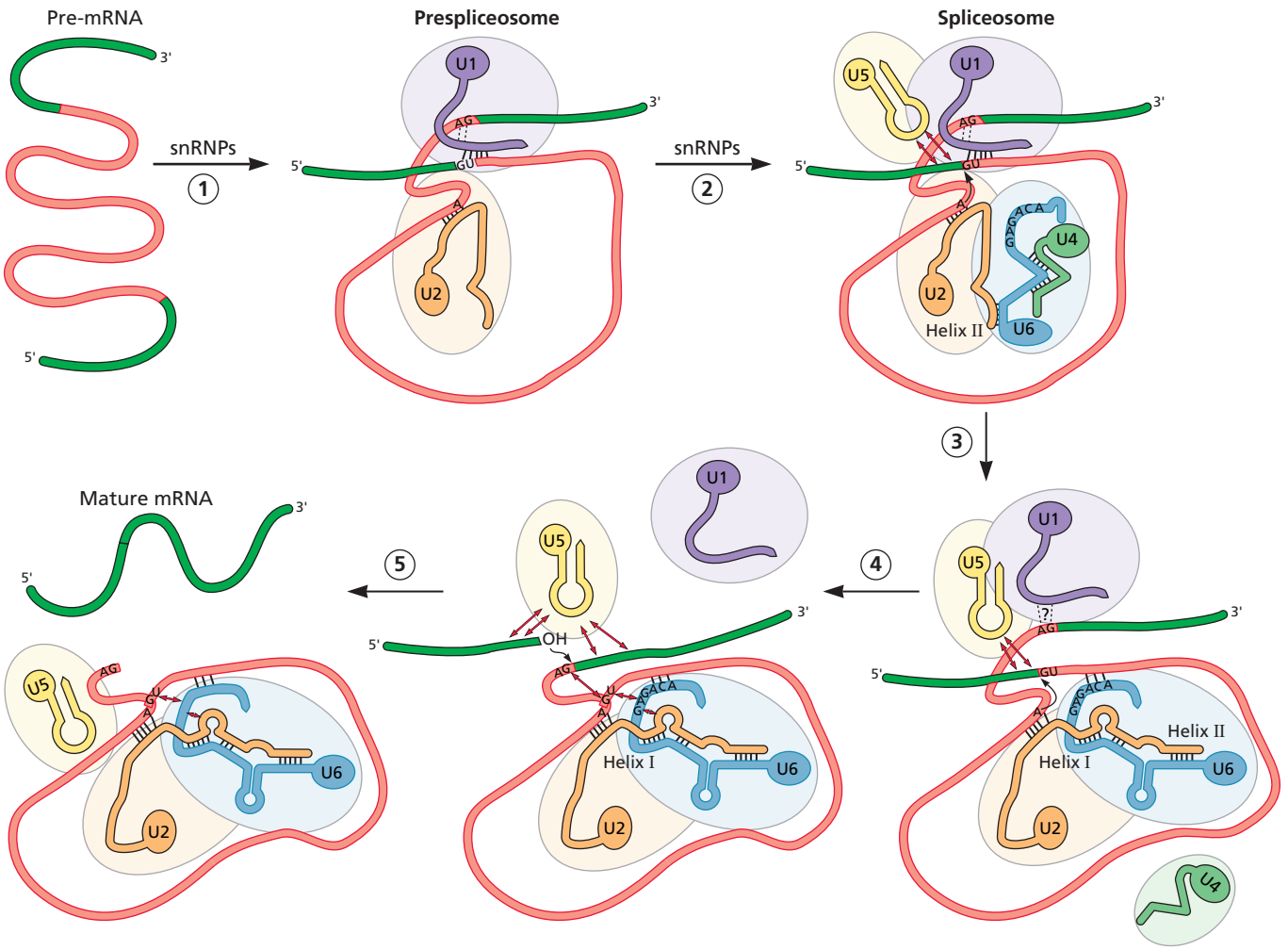


Figure 10.7 RNA-RNA interactions organize substrates and catalysts during splicing. Base pairs are indicated by dashes and experimentally observed or presumed contacts among the RNA molecules by the two-headed arrows. The U1 and U2 snRNAs initially base pair with the 5′ splice site and branch point sequence, respectively, in the pre-mRNA (step 1). The other snRNPs then enter the assembling spliceosome (step 2). The U4 and U6 snRNAs, which are present in a single snRNP, are base paired with one another over an extended complementary region. This snRNP binds to the U5 snRNP, and the snRNP complex associates with that containing the pre-mRNA and U1 and U2 snRNAs to form the spliceosome. RNA rearrangements then activate spliceosomes for catalysis of splicing (step 3). U4 snRNA dissociates from U6 snRNA, which forms hydrogen bonds with both U2 snRNA and the pre-mRNA. The interaction of U6 snRNA with the 5′ splice site displaces U1 snRNA (step 4). One of the U2 sequences hydrogen bonded to U6 snRNA (helix I) is adjacent to the U2 snRNA sequence that is base paired with the pre-mRNA branch point region. The interactions of U2 and U6 snRNAs with each other and with the pre-mRNA therefore juxtapose the branch point and 5′ splice site sequences for the first transesterification reaction (Fig. 10.6B). The U5 snRNA base pairs to sequences in both the 5′ and 3′ exons to align them for the second transesterification reaction (step 5). The many proteins that participate in spliceosome assembly and activation or that package the pre-mRNA and snRNAs are not shown. Adapted from T. W. Nilsen, *Cell* **78:**1–4, 1994, with permission.

(initially of viral origin) are accurately spliced. Pre-mRNA splicing occurs by two transesterification reactions, in which one phosphodiester bond is exchanged for another without the need for an external supply of energy. The first reaction yields two products, the 5′ exon and the intron-3′ exon **lariat**. In the second, the two exons are joined and the intron lariat is released (Fig. 10.6B).

From a chemical point of view, the splicing of pre-mRNA is a simple process. However, each splicing reaction must be completed with a high degree of accuracy to ensure that coding information is not lost or altered, and the chemically active hydroxyl groups must also be brought into close proximity to the phosphodiester bonds they will attack (Fig. 10.6B). Many genes contain a large number of introns separating multiple exons, which must be spliced in the correct order. It is presumably for such reasons that pre-mRNA splicing occurs in the large structure called the **spliceosome**, which contains both many proteins and several small RNAs.

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Five small nuclear RNAs (snRNAs) participate in splicing: the U1, U2, U4, U5, and U6 snRNAs. In vertebrate cells, these RNAs vary in length from 100 to 200 nucleotides and are associated with proteins to form **small nuclear ribonucleoproteins** (snRNPs). The RNA components of the snRNPs recognize splice sites and other sequences in cellular and viral pre-mRNAs. Indeed, they participate in multiple dynamic interactions with the pre-mRNA and with each other both during the initial ordered assembly of the spliceosome and in splicing. These base-pairing interactions juxtapose first the 5′ splice site and the branch point for the first transesterification reaction and then the 5′ and the 3′ exons (Fig. 10.7). However, RNAs of the snRNPs do much more than simply organize the pre-mRNA sequences into a geometry suitable for transesterification. It has long been suspected that the spliceosome might be an RNA enzyme (or **ribozyme**), and compelling evidence for catalysis by U6 snRNAs during splicing has been reported recently (Box 10.5).

Although the snRNAs play essential roles in splicing as both guides and catalysts, the spliceosome also contains ~150 non-snRNP proteins. One class comprises proteins that package the pre-mRNA substrate. Many other splicing proteins contain both RNA-binding and protein-protein interaction domains. Such proteins bind to pre-mRNA sequences within or adjacent to exons to facilitate spliceosome assembly or regulate splicing or exon recognition. Other proteins important for splicing are RNA-dependent helicases, which are thought to catalyze the multiple rearrangements of hydrogen bonding among different snRNAs and the pre-mRNA substrate (Fig. 10.7). Such helicases are generally ATP dependent, and spliceosome assembly and rearrangement depend on energy supplied by ATP hydrolysis.

Splicing of pre-mRNAs is commonly cotranscriptional, and components of the splicing machinery associate with the hyperphosphorylated form of the C-terminal domain of the largest subunit of RNA polymerase II during elongation of

BOX 10.5

DISCUSSION

Catalysis of pre-mRNA splicing by RNA

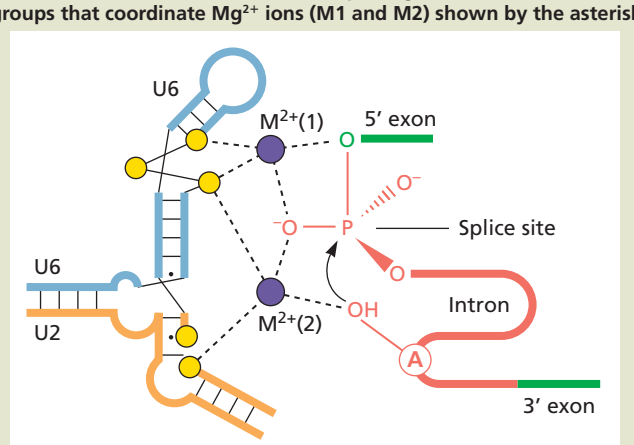
The catalytic activity of RNA was established with the discovery of *Escherichia coli* RNase P, which contains a small RNA molecule essential for catalysis, and self-splicing RNAs. The first such RNA to be described was a short (414-nucleotide) intron in pre-rRNA of *Tetrahymena*. Specific nucleotides in the introns coordinate metal ions to catalyze the same phosphoryl transfer reactions that accomplish pre-mRNA splicing. This precedent, the formation of a lariat intermediate during both pre-mRNA splicing and that of some self-splicing introns, and the essential role of snRNAs in pre-mRNA splicing suggested that the latter process is also catalyzed by RNA. Consistent with this view, U2 and U6 snRNAs synthesized *in vitro* base pair to form a stable structure with the configuration thought to be present at the active site of the spliceosome. In the absence of any protein, these RNAs are sufficient to catalyze a reaction analogous to the first transesterification during pre-mRNA splicing. Nevertheless, direct evidence that U6 catalyzes pre-mRNA splicing has been reported only recently.

In these experiments, sulfur was substituted for single oxygen atoms at 20 positions in U6 snRNA that were known to be important for pre-mRNA splicing or analogous to catalytic residues in self-splicing introns. Splicing reactions require Mg^{2+}, which binds efficiently to oxygen but not to sulfur. These modified U6 snRNAs were then assembled into spliceosomes and model splicing substrates added. Five of the substitutions inhibited splicing in the presence of Mg^{2+} ions. However, splicing was restored when metal ions that bind sulfur, Mn^{2+} or Cd^{2+}, were supplied. Analysis of which reactions were blocked by individual substitutions and other experiments indicated that one of the two metal ions is coordinated to the nucleophilic hydroxyl group that initiates the first transesterification reaction, and the second is coordinated to the leaving group (M2 and M1, respectively, in the figure). These studies provide direct evidence for RNA-mediated catalysis during pre-mRNA splicing.

Fica SM, Tuttle N, Novak T, Li NS, Lu J, Koodathingal P, Dai Q, Staley JP, Piccirilli JA. 2013. RNA catalyses nuclear pre-mRNA splicing. *Nature* **503:**229-234.

Valadkhan S, Manley JL. 2001. Splicing-related catalysis by protein-free snRNAs. *Nature* **413:**701–707.

The hairpin structure of U6 snRNA and its base pairing to U2 snRNA are shown, with the phosphoryl groups that coordinate Mg^{2+} ions (M1 and M2) shown by the asterisks.



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transcription. Peptide mimics of the C-terminal domain, or antibodies raised against it, inhibit pre-mRNA splicing in cells in culture or *in vitro*. Furthermore, nontranscribed sequences within RNA polymerase II promoters can dictate whether a particular exon is retained or removed during splicing. As we have seen, association of components of the 5′ capping and 3′ polyadenylation systems with this domain of RNA polymerase is necessary for these processing reactions. The synthesis of pre-mRNA and its complete processing are therefore coordinated as a result of association of specific proteins needed for each processing reaction with the C-terminal domain of RNA polymerase II. Such a transcription and RNA-processing machine is analogous to that of vaccinia virus, described above, but much more elaborate.

Alternative Splicing

Many viral and cellular pre-mRNAs contain multiple exons. Splicing of many such transcripts removes all introns and joins all exons in the order in which they are present (Fig. 10.8A). However, numerous cellular and many viral pre-mRNAs yield more than one mRNA as a result of the splicing of different combinations of exons, a process termed **alternative splicing**. Several different types of alternative splicing can be defined (Fig. 10.8B). Alternative splicing, which can comprise selection among large numbers of exons in a pre-mRNA, is governed by multiple parameters, including splice site and regulatory sequences in the pre-mRNA and the constellation of splicing proteins present in particular cell types (Box 10.6).

As alternative splicing generally leads to the synthesis of mRNAs that differ in their protein-coding sequences, its most obvious advantage is that it can expand the limited coding capacity of viral genomes. The early genes of polyomaviruses and adenoviruses each specify two or more proteins as a result of splicing of primary transcripts at alternative 5′ or 3′ splice sites. Alternative splicing can also be important for temporal regulation of viral gene expression or the control of a crucial balance in the production of spliced and unspliced mRNAs.

Although described separately in previous sections, capping, polyadenylation, and splicing of a pre-mRNA by cellular components are not independent. Rather, one processing reaction governs the efficiency or specificity of another. For example, interaction of the nuclear cap-binding protein with the 5′ end of a pre-mRNA facilitates both removal of the 5′-terminal intron and efficient cleavage at the 3′ poly(A) addition site. Similarly, the presence of a 3′ poly(A) addition signal generally stimulates removal of the intron closest to it.

Alternative Processing of Viral Pre-mRNA

Cellular Differentiation Regulates Production of Papillomaviral Late Pre-mRNAs

The late proteins of bovine papillomavirus type 1 are synthesized efficiently only in highly differentiated keratinocytes. Productive replication of viral DNA and assembly and release of virus particles are also restricted to these outer cells in an epithelium. Alterations in polyadenylation and splicing are crucial for production of the mRNA encoding the major capsid proteins, such as the L1 mRNA (Fig. 10.9A). *In situ* hybridization studies have shown that this mRNA is made only in fully differentiated cells (Fig. 10.9B). Production of the late mRNAs requires utilization of both polyadenylation and 3′ splice sites that are not recognized in undifferentiated cells. The 3′ splice sites for papillomavirus late mRNAs are suboptimal, and their recognition is governed by *cis*-acting suppressor or enhancer sequences in the pre-mRNA (Fig. 10.9C). It has therefore been proposed that terminal differentiation of keratinocytes is accompanied by changes in the activity or abundance of the cellular proteins that bind to these sequences. Similarly, binding of U1 snRNP to a pseudo-5′ splice site appears to suppress recognition of the poly(A) addition site for

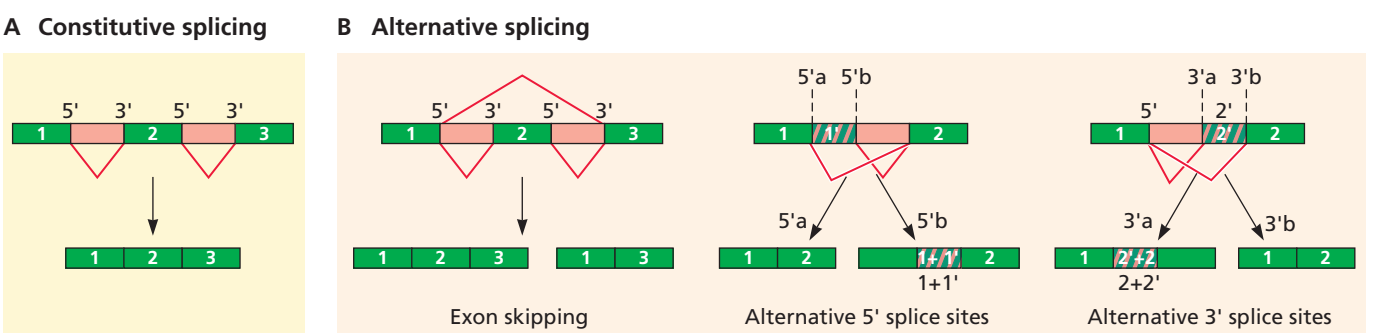


Figure 10.8 Constitutive and alternative splicing. (A) In constitutive splicing, all exons (green) are joined sequentially and all introns (pink) are excised. **(B)** Alternative splicing occurs by several mechanisms. In exon skipping, the 3′ splice site of exon 2 is sometimes ignored, so that this exon is not included in some fraction of the spliced mRNA molecules, whereas in intron retention, a set of splice sites is ignored. Alternatively, one of two 5′ splice sites (5′a and 5′b) in exon 1 or one of two 3′ splice sites (3′a and 3′b) in exon 2 are recognized. Recognition of different 5′ and 3′ splice sites produces alternatively spliced simian virus 40 early and adenoviral major late (Fig. 10.11) mRNAs, respectively.

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BOX 10.6

BACKGROUND

Discrimination among splice sites for alternative splicing of pre-mRNA

Many mammalian, and some viral, pre-mRNAs are alternatively spliced. This process, which can yield large numbers of mRNAs from a primary transcript (see, for example, Fig. 10.11), requires the splicing machinery to discriminate between alternative 5′ or alternative 3′ splice sites, to skip an exon or to retain an intron (that is, ignore particular combinations of 5′ and 3′ splice sites) (Fig. 10.8). Detailed studies of alternative splicing of specific pre-mRNAs or model substrates and genome-wide analyses have identified multiple features of the substrate that cooperate to govern alternative splicing. These parameters include the following:

- Splice site "strength," that is, match to the consensus 5′ and 3′ splice site sequences (Fig. 10.6A)
- The presence of stable RNA secondary structures that interfere with recognition of individual splice sites
- The lengths of exons and introns. In mammalian cells, interactions among splicing components at a 5′ splice site and the upstream 3′ splice site are responsible for recognition of most exons. Such "exon definition" is more efficient when exons are short. Furthermore, exons flanked by long introns are much more likely to be alternatively spliced than those flanked by short introns.
- The presence in both exons and introns of binding sites for proteins that facilitate or suppress recognition of neighboring splice sites, and hence alternative splicing. Examples of these sequences, termed exonic or intronic splicing enhancers or suppressors, are described in the text. The impact of such a regulatory element can be context dependent, determined by distance from adjacent splice site(s), the number of copies of the sequence, and the constellation of other splicing regulatory elements in the vicinity (see the figure).

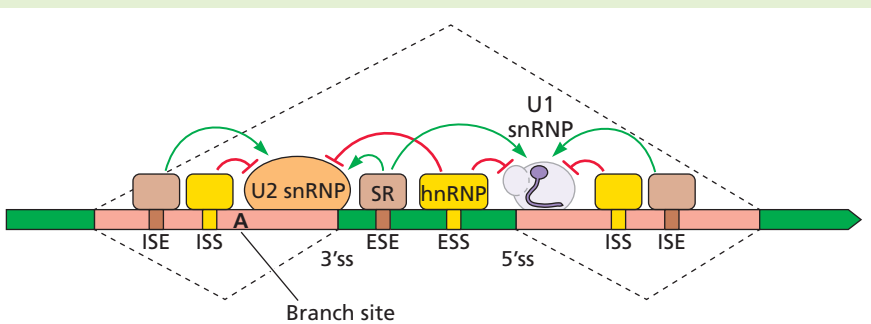
The concentration of proteins that recognize splicing regulatory sequences in particular cells or tissues is an important determinant of alternative splicing, resulting in tissue-specific synthesis of different isoforms of many mammalian mRNAs. The alternative splicing of pre-mRNAs can also be coupled to transcription, by recruitment of particular splicing proteins by binding to RNA polymerase II, and as a result of the rate at which splice sites and splicing regulatory sequences are made in a nascent pre-mRNA.

Hertel KJ. 2008. Combinatorial control of exon recognition. *J Biol Chem* **283:**1211–1215.

Kornblihtt AR, Schor IE, Alló M, Dujardin G, Petrillo E, Muñoz MJ. 2013. Alternative splicing: a pivotal step between eukaryotic transcription and translation. *Nat Rev Mol Cell Biol* **14:**153–165.

Wang Z, Burge CB. 2008. Splicing regulation: from a parts list of regulatory elements to an integrated splicing code. *RNA* **14:**802–813.

Schematic illustration of splicing regulatory sequences and some of the proteins that recognize them. ESE, exonic splicing enhancer; ESS, exonic splicing suppressor; ISE, intronic splicing enhancer; ISS, intronic splicing suppressor; SR, serine- and arginine-rich splicing proteins.



production of bovine papillomavirus type 1 late mRNAs until the keratinocyte host cell is fully differentiated (Fig. 10.9D).

Production of Spliced and Unspliced RNAs Essential for Virus Replication

The expression of certain coding sequences in retroviral genomes (Gag and Pol) (Appendix, Fig. 29) and orthomyxoviruses (M1 and NS1) (Appendix, Fig. 15) depends on an unusual form of alternative splicing that produces both spliced and unspliced mRNAs. This phenomenon has been well studied in retrovirus-infected cells.

In cells infected by retroviruses with simple genomes, such as avian leukosis virus, a full-length, unspliced transcript of proviral DNA serves as both the genome and the mRNA for the capsid proteins and viral enzymes, while a singly spliced mRNA specifies the viral envelope protein (Fig. 10.10A). Retrovirus production depends rather critically on the maintenance of a proper balance in the proportions of unspliced and spliced RNAs: modest changes in splicing efficiencies cause replication defects (Fig. 10.10A). This phenomenon has been used as a genetic tool to select for mutations that affect splicing control. Such mutations arise in different splicing signals at the 3′ splice site and alter the efficiency of either the first or second step in the splicing reaction. Features that maintain the proper splicing balance include suboptimal recognition of the 3′ splice site, and a splicing enhancer in the adjacent exon. A negative regulatory sequence located more than 4000 nucleotides upstream of the 3′ splice site is also important. This sequence, which is bound by both U1 snRNP and specific cellular proteins (Fig. 10.10A), has been proposed to act as a "decoy" 5′ splice site: it forms a complex with the 3′ splice site for production of Env mRNA, but one that does

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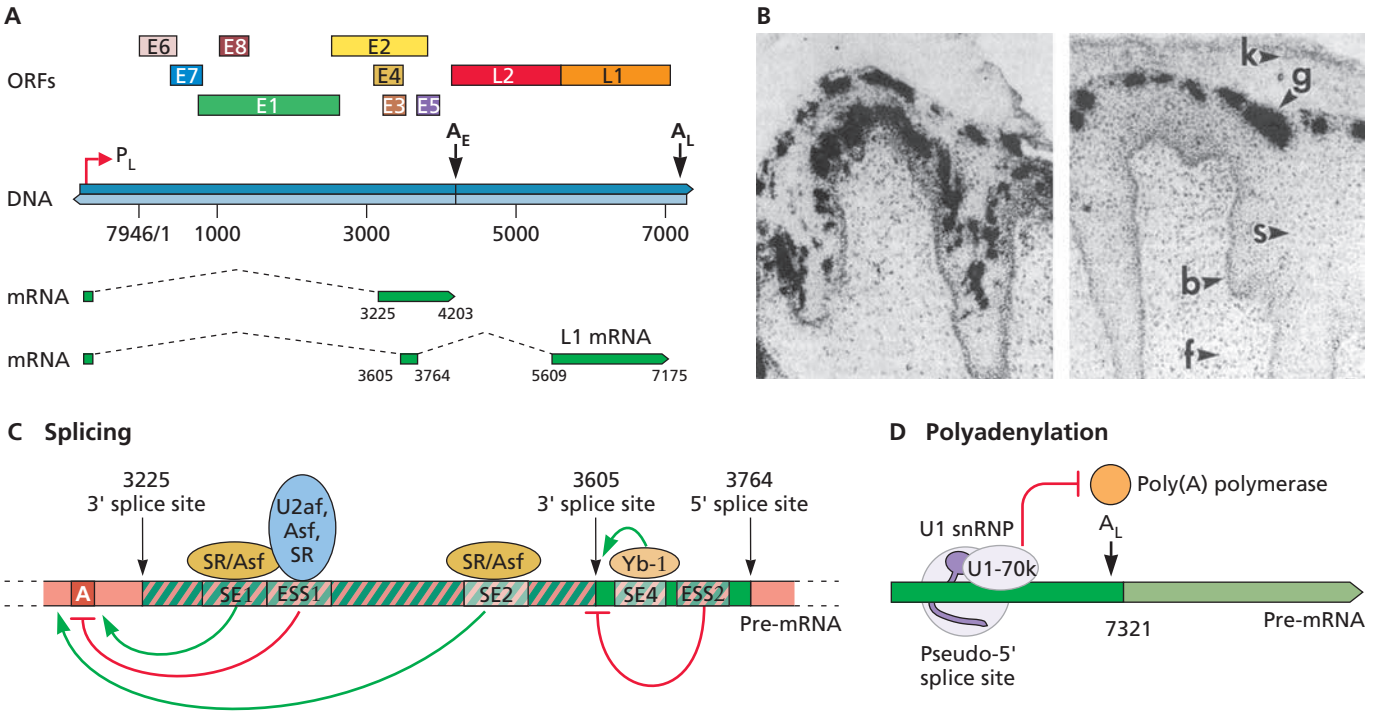
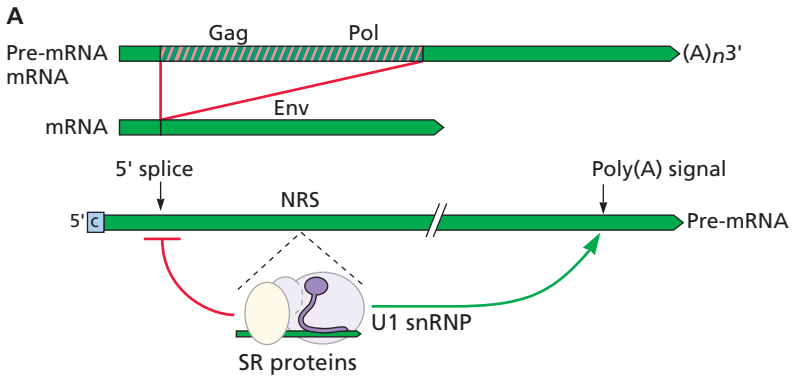


Figure 10.9 Alternative polyadenylation and splicing control the production of bovine papillomavirus type 1 late mRNAs. **(A)** The circular bovine papillomavirus type 1 genome is represented in linear form, with open reading frames (ORFs) shown above. Two of the many mRNAs made from transcripts from the late promoter (P_L) are shown to illustrate the changes in recognition of splice sites and of poly(A) addition sites necessary to produce the L1 mRNA. Synthesis of this mRNA depends on recognition of a 3′ splice site at position 3605, rather than that at 3225, which is used during the early phase of infection. Polyadenylation of pre-mRNAs must also switch from the early (A_E) to the late (A_L) polyadenylation site. **(B)** *In situ* hybridization of bovine fibropapillomas to probes that specifically detect mRNAs spliced at the 3225 3′ splice site (left) or at the 3605 site (right). The cell layers of the fibropapilloma are indicated in the right panel. Abbreviations: k, keratin horn; g, granular cell layer; s, spinous cell layer; b, basal cell layer; f, fibroma. Note the production of late mRNA spliced at the 3605 3′ splice site only in the outermost layer (g) of fully differentiated cells. From S. K. Barksdale and C. C. Baker, *J Virol* **69:**6553–6556, 1995, with permission. Courtesy of C. C. Baker, National Institutes of Health. **(C)** Mechanisms that regulate splicing to produce L1 mRNA, which are specific to highly differentiated keratinocytes of the granular cell layer. The sequences that control alternative splicing at the 3225 and 3605 3′ splice sites are located between these splice sites. The splicing enhancers, SE1, SE2, and SE4, are recognized by cellular SR (serine- and arginine-rich) and other splicing proteins such as Asf (alternative splicing factor). The SE1 enhancer and the adjacent sequence that inhibits splicing at the 3605 3′ splice site, termed exonic splicing suppressor (ESS1), are thought to facilitate recruitment of U2-associated protein (U2af) and recognition of the branch point sequence upstream of the 3225 3′ splice site. SE2 is located very close to the 3605 3′ splice site and may block access to the branch point for splicing at this site until keratinocytes differentiate. The hnRNP A1 protein that binds to a sequence that inhibits recognition of the downstream 3′ splice site of the late pre-mRNA is not present in differentiated keratinocytes. **(D)** Inhibition of polyadenylation at the A_E site by the binding of U1 snRNP to a pseudo-5′ splice site located nearby in the primary transcript (see the text). Such inhibition is the result of binding of the U1 snRNP 70k subunit to poly(A) polymerase.

not participate in splicing reactions. The splicing of human immunodeficiency virus type 1 pre-mRNA is necessarily much more complicated, as >40 alternatively spliced mRNAs are made in infected cells. Nevertheless, alternative splicing is also regulated by specific sequences that promote or repress recognition or utilization of splice sites and by the degree of conformity of 3′ splice sites to the optimal sequence.

The long terminal repeats at each end of proviral DNAs include a poly(A) addition signal (Fig. 10.10B). Transcription of some proviral DNAs, such as that of Rous sarcoma virus, initiates downstream of the polyadenylation signal in the 5′ long terminal repeat sequence so that a poly(A) addition site is present only at the 3′ ends of pre-mRNAs. However, many other retroviral transcripts carry complete signals for this modification at both their 5′ and 3′ ends, but poly(A) is added to only the 3′ ends of these pre-mRNAs. At least two mechanisms ensure that the correct poly(A) addition signal of human immunodeficiency virus type 1 is recognized. Sequences present only at the 3′ end of the pre-mRNA stimulate polyadenylation *in vitro* and in cells in culture, by facilitating binding of Cpsf to the nearby 5′AAUAAA3′ sequence. In addition, recognition of the 5′ poly(A) signal is suppressed by the 5′ splice site lying immediately downstream (Fig. 10.10B).

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Unspliced:spliced RNA	Viral propagation
2:1	Wild type
1:1	Reduced
1:2	Undetectable

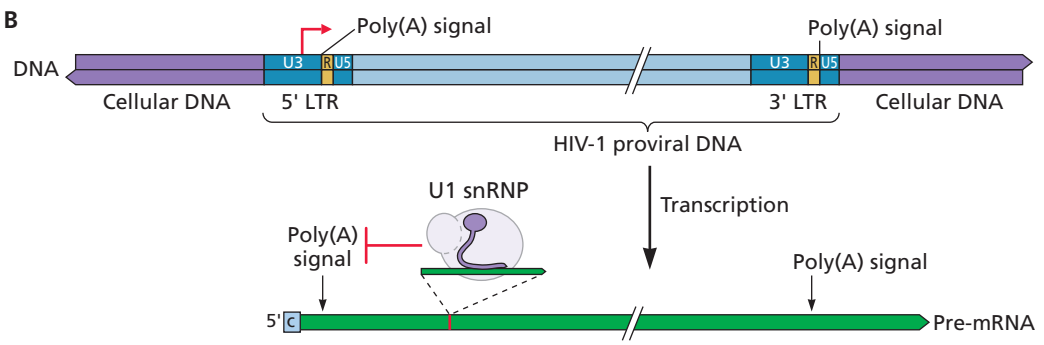


Figure 10.10 Control of RNA-processing reactions during retroviral gene expression. (A) Balanced production of spliced and unspliced mRNAs is illustrated for avian leukosis virus. A single 3′ splice site is recognized in about one-third of the primary transcripts to produce spliced mRNA encoding the Env protein. The Gag and Pol proteins are synthesized from unspliced transcripts. Even a 2-fold reduction in the ratio of unspliced to spliced mRNA impairs virus reproduction (right). Shown below is the negative regulatory sequence (NRS) located within Gag-coding sequences, which is bound by U1 snRNP and SR proteins. This sequence is believed to act as a "decoy" 5′ splice site to inhibit splicing (red bar). It also stimulates polyadenylation (green arrow) by an unknown mechanism. **(B)** Suppression of poly(A) site recognition. Utilization of the 5′ polyadenylation site in primary transcripts of human immunodeficiency virus type 1 proviral DNA is inhibited by binding of U1 snRNP to the major 5′ splice site located 195 nucleotides downstream. The ability of the U1 snRNP protein U1a to bind to both poly(A) polymerase and Cpsf suggests that the U1 snRNP might inhibit their activity.

Temporal Regulation of Synthesis of Adenoviral Major Late mRNAs

The production of adenoviral major late mRNAs epitomizes complex alternative splicing and polyadenylation at multiple sites in a pre-mRNA, which in this case can give rise to at least 15 different mRNAs. These mRNAs fall into five families (L1 to L5) defined by which of five polyadenylation sites is recognized (Fig. 10.11). The frequency with which each site is used must therefore be regulated to allow production of all major late mRNAs. High-efficiency polyadenylation at the L1 site during the early phase of infection prevents synthesis of L2 to L5 mRNAs. In contrast, during the late phase of adenovirus infection, each of the five polyadenylation sites directs 3′-end formation with approximately the same efficiency. The mechanism(s) responsible for such balanced recognition of multiple poly(A) addition sites is not fully understood, but alteration in the activities of cellular polyadenylation proteins as infection proceeds allows the switch from polyadenylation at only the L1 site (see "Posttranscriptional Regulation of Viral or Cellular Gene Expression by Viral Proteins" below).

All major late mRNAs contain the 5′-terminal tripartite leader sequence. The splicing reactions that produce this sequence from three small exons (Box 10.3) take place before polyadenylation of the primary transcript. The final splicing reaction joins the tripartite leader sequence to one of many mRNA sequences (Fig. 10.11). Each primary transcript therefore yields only a **single** mRNA, even though it contains the sequences for many, and most of its sequence is discarded. It remains a mystery why the majority of adenoviral late mRNAs are made by this bizarre mechanism. However, one contributing factor may be that it ensures that each major late mRNA molecule carries the 5′-terminal tripartite leader sequence, which is important for efficient translation late in the infectious cycle (Chapter 11).

Editing of Viral mRNAs

The term **RNA editing** describes the process by which nucleotides not specified in the genome are introduced into mRNAs, first reported in 1980 for a mitochondrial mRNA of

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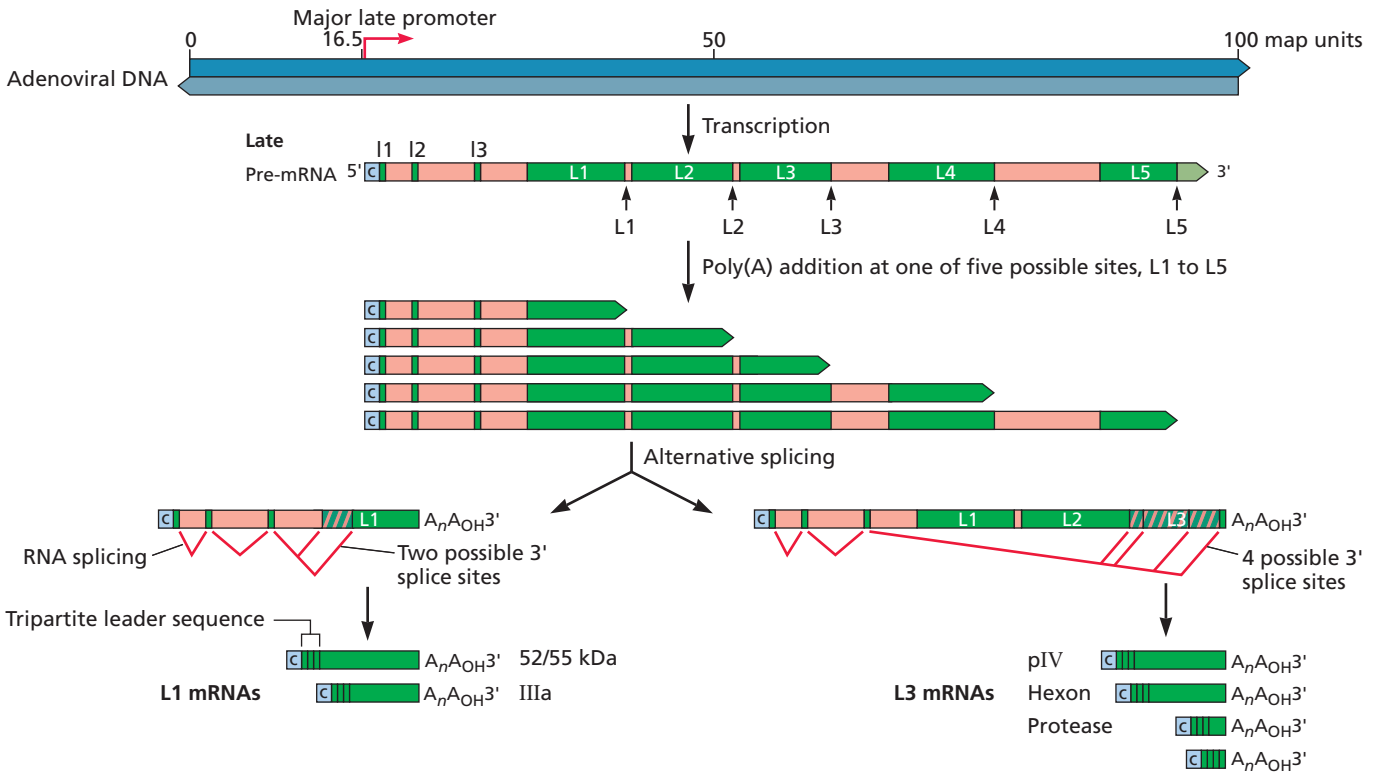


Figure 10.11 Alternative polyadenylation and splicing of adenoviral major late transcripts. During the late phase of adenovirus infection, major late primary transcripts extend from the major late promoter almost to the right end of the genome. They contain the sequences for at least 15 mRNAs and are polyadenylated at one of five sites, L1 to L5, as a result of decreased activity of Cstf (see the text). The tripartite leader sequence, present at the 5′ ends of all late mRNAs, is assembled by the splicing of three short exons, l1, l2, and l3. This sequence is then ligated to alternative 3′ splice sites. Such joining of the spliced tripartite leader sequence to an mRNA sequence has been reported to take place after polyadenylation of pre-mRNA. Polyadenylation therefore appears to determine which 3′ splice sites can be utilized during the final splicing reaction.

trypanosomes. Since this modification was discovered, RNA editing has been identified in many different eukaryotes, as well as in some viral systems.

Viral mRNAs are edited by either insertion of nucleotides not directly specified in the template during synthesis or alteration of a base *in situ*, changing the sequence and function of the protein specified by edited mRNA. Consequently, RNA editing has the potential to make an important contribution to regulation of viral gene expression.

For the most part, viral mRNAs are edited during or following their synthesis from viral RNA genomes. These reactions are described in Chapter 6. However, like their cellular counterparts, transcripts of viral DNA templates can also be edited by Adar1 (adenosine deaminase acting on RNA 1), which deaminates adenine bases to generate inosineI. Transcripts of the K12 region of the human herpesvirus 8 genome are edited efficiently at just one site, both in infected cells and by Adar1 *in vitro*. This modification appears to regulate the function of the kaposin protein that is encoded within the K12 region: this protein exhibits transforming activity only when it is made from the unedited coding sequence, and editing of this site predominates in productively infected cells.

Editing by Adar1 also increases the efficiency of reproduction of human immunodeficiency virus type 1. Edited A residues have been identified at several positions in the viral RNA genome, including the 5′ untranslated region and just downstream of the Rev-response element that directs export of unspliced and partially spliced viral transcripts from the nucleus (see "The Human Immunodeficiency Virus Type 1 Rev Protein Directs Export of Intron-Containing mRNAs" below). Mutational studies have shown that editing of the latter sequence stimulates production of unspliced viral RNA and specific viral proteins, but the consequences of editing of other genomic sequences are not yet known.

Editing as a Powerful Antiviral Defense Mechanism

Adar1 can facilitate reproduction of several viruses by editing-independent inhibition of Pkr, an important component of antiviral defenses mounted in response to exposure of cells to interferon (Volume II, Chapter 3). However, cellular

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editing enzymes can also inhibit virus reproduction. This phenomenon is exemplified by the enzymes known as Apobec3s, which edit RNA by deamination of cytidine to uridine. Inhibition of the activity of such enzymes is important for the successful reproduction of several viruses, including hepatitis B virus and human immunodeficiency virus type 1 (Volume II, Chapter 7).

Export of RNAs from the Nucleus

Any mRNA made in the nucleus must be transported to the cytoplasm for translation. Other classes of RNA, including small cellular and viral RNAs made by RNA polymerase III, also enter the cytoplasm permanently (e.g., transfer RNAs [tRNAs]) or transiently (snRNAs). The export of viral mRNAs is mediated by the host cell machinery and, in most cases, is indistinguishable from export of analogous cellular RNAs. In this section, we describe the cellular export machinery and the mechanisms that ensure export of some atypical viral mRNA substrates.

The Cellular Export Machinery

The substrates for mRNA export are not naked RNA molecules, but rather ribonucleoproteins. Indeed, export of RNA molecules (with the exception of tRNAs) is directed by sequences present in the proteins associated with them. Like proteins entering the nucleus, RNA molecules travel between nuclear and cytoplasmic compartments via the nuclear pore complexes described in Chapter 5. Numerous genetic, biochemical, and immunocytochemical studies have demonstrated that specific nucleoporins (the proteins from which nuclear pore complexes are built) participate in nuclear export. Export of RNA molecules also shares several mechanistic features with import of proteins into the nuclei: substrates for nuclear export or import are identified by specific protein signals, and some soluble proteins, including the small guanosine nucleotide-binding protein Ran, function in both import and export. And RNA export, like protein import, is mediated by receptors that recognize nuclear export signals and direct the proteins, and ribonucleoproteins that contain them, to and through nuclear pore complexes.

Export of Viral mRNA

All viral mRNAs made in infected cell nuclei carry the same 5′- and 3′-terminal modifications as cellular mRNAs that are exported. Furthermore, many viral mRNAs, like their cellular counterparts, are produced by splicing of intron-containing precursors. Cellular pre-mRNAs that contain introns and splice sites and have not been spliced ordinarily are retained in the nucleus, at least in part because they remain associated with spliceosomes. Furthermore, a protein complex that marks mature mRNAs for export is assembled on the RNA only during splicing, and efficient export requires cooperation among multiple adapter proteins that are deposited as a pre-mRNA is processed. However, reproduction of retroviruses, herpesviruses, and orthomyxoviruses requires production of mRNAs that are not spliced at all, because either the intron is retained or the mRNAs contain no introns or splice sites. Efforts to address the question of how these unusual mRNAs leave the nucleus provided important insights into the molecular mechanisms that mediate export of macromolecules, including the identification of RNA export receptors.

The Human Immunodeficiency Virus Type 1 Rev Protein Directs Export of Intron-Containing mRNAs

The human immunodeficiency virus type 1 Rev protein is the best understood of the viral proteins that modulate mRNA export from the nucleus. This protein and related proteins of other lentiviruses promote export of the unspliced (and partially spliced) viral mRNAs. Rev binds specifically to an RNA sequence termed the **Rev-responsive element** that lies within an alternatively spliced intron of viral pre-mRNA (Fig. 10.12). The Rev-responsive element is some 350 nucleotides in length and forms several stem-loops (Fig. 10.13A), one of which contains a high-affinity binding site for the arginine-rich RNA-binding domain of Rev (Fig. 10.13B). This site is formed by conformational change in the RNA following the initial interaction with Rev. Subsequently, Rev monomers oligomerize cooperatively on the RNA (Fig. 10.13C). Export of RNAs that contain the Rev-responsive element depends on the formation of these RNA-bound oligomers, and a leucine-rich nuclear export signal present in Rev.

When oligomeric Rev is assembled on the RNA, the nuclear export signals of the protein become organized on one surface (Fig. 10.13C). One cellular protein that binds to the nuclear export signal of Rev is exportin-1 (Xpo1, also known as Crm-1). This protein, which binds simultaneously to Rev and the GTP-bound form of Ran, is the **receptor** for Rev-dependent export of the human immunodeficiency virus type 1 RNAs bound to it. The viral protein functions as an **adapter**, directing viral, intron-containing mRNAs to a preexisting cellular export receptor. Translocation of the complex containing viral RNAs, Rev, and cellular proteins through the nuclear pore complex to the cytoplasm requires specific nucleoporins and other proteins (Fig. 10.14). In the cytoplasm, hydrolysis of GTP bound to Ran by a Ran-specific GTPase-activating protein present only in the cytoplasm induces dissociation of the export machinery. Rev then shuttles back into the nucleus via a typical nuclear localization signal, where it can pick up another cargo RNA molecule.

Perhaps the most interesting aspect of Rev-dependent RNA export is the exit of mRNAs by a pathway that normally does not handle such cargo, but rather exports small RNA species (and proteins) of the host cell. The Rev nuclear export

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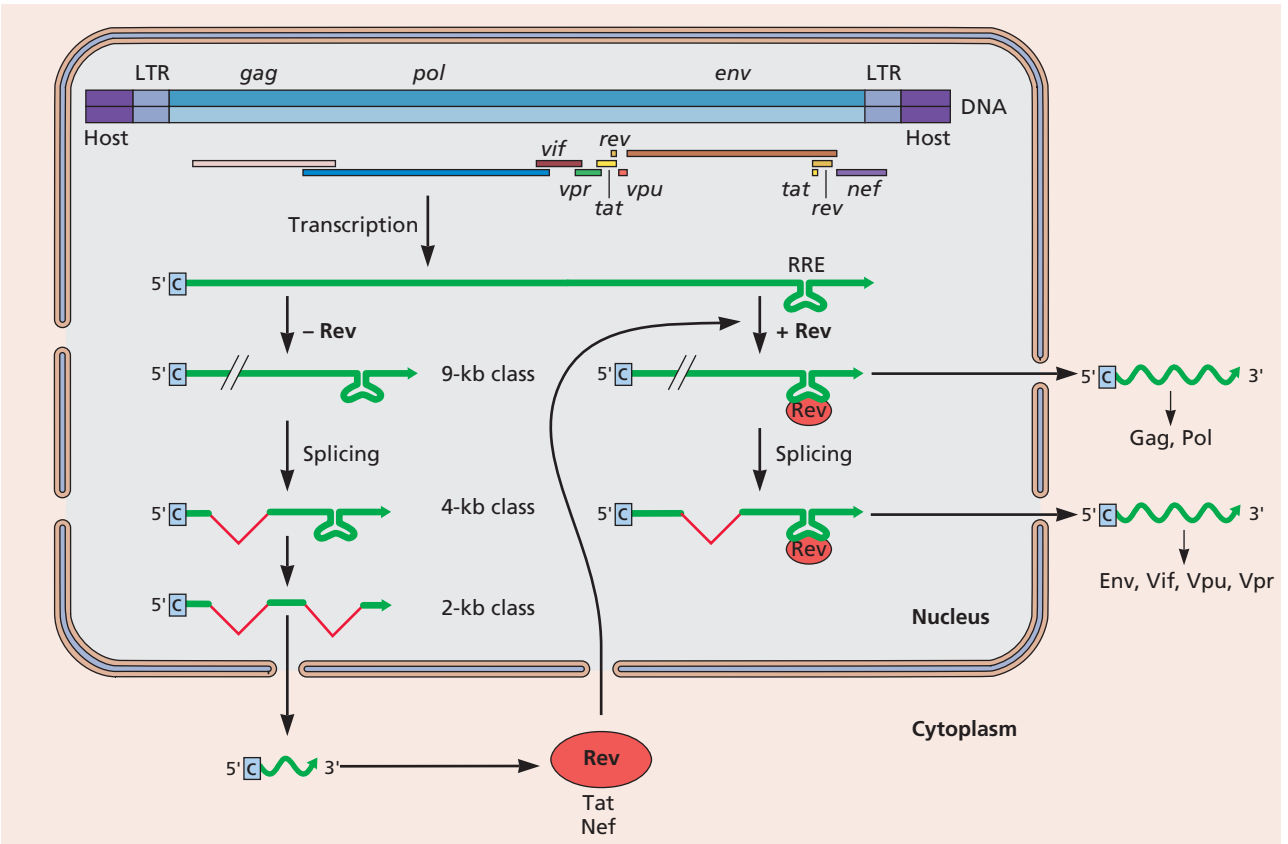


Figure 10.12 Regulation of export of human immunodeficiency virus type 1 mRNAs by the viral Rev protein. Before the synthesis of Rev protein in the infected cell, only fully spliced (2-kb class) viral mRNAs are exported to the cytoplasm (left). These mRNAs specify viral regulatory proteins, including Rev. The Rev protein enters the nucleus, where it binds to an RNA structure, the Rev-responsive element (RRE) present in unspliced (9-kb class) and singly spliced (4-kb class) viral mRNAs. This interaction induces export to the cytoplasm of the RRE-containing mRNAs, from which viral structural proteins and enzymes are made (right). The Rev protein therefore alters the pattern of viral gene expression as the infectious cycle progresses.

signal is similar to, and can be functionally replaced by, that of the cellular protein TfIIIa. This protein binds specifically to 5S rRNA and is required for export of this cellular RNA from the nucleus. Peptides containing the Rev nuclear export signal inhibit export of 5S rRNA (and other small RNAs), but not of mRNAs. The human immunodeficiency virus type 1 Rev protein therefore circumvents the normal restriction on the export of intron-containing pre-mRNAs from the host cell nucleus by diverting such viral mRNAs to a cellular pathway that handles intronless RNAs. Export of unspliced viral RNA via the Xpo1 pathway is required for efficient assembly of virus particles in the cytoplasm, for reasons that are not yet clear.

RNA Signals Can Mediate Export of Intron-Containing Viral mRNAs by Cellular Proteins

The genomes of retroviruses with simple genomes do not encode proteins analogous to Rev, even though unspliced viral RNAs must reach the cytoplasm. These unspliced viral mRNAs contain specific sequences that promote export. Because they must function by means of cellular proteins, such sequences were termed **constitutive transport elements** (CTEs). The first such sequence was found in the 3′ untranslated region of the genome of Mason-Pfizer monkey virus.

Even low concentrations of RNA containing the Mason-Pfizer monkey virus CTE inhibit export of mature mRNAs when microinjected into *Xenopus* oocyte nuclei, but CTE RNA does **not** compete with Rev-dependent export. This observation indicated that this retroviral RNA sequence is recognized by components of a cellular mRNA export pathway. A search for such proteins led to the first identification of a mammalian protein mediating mRNA export, the human nuclear export factor 1 (Nxf1, also known as Tap). This protein binds specifically to the CTE and is essential for export from the nucleus of the unspliced viral RNAs and spliced cellular mRNAs.

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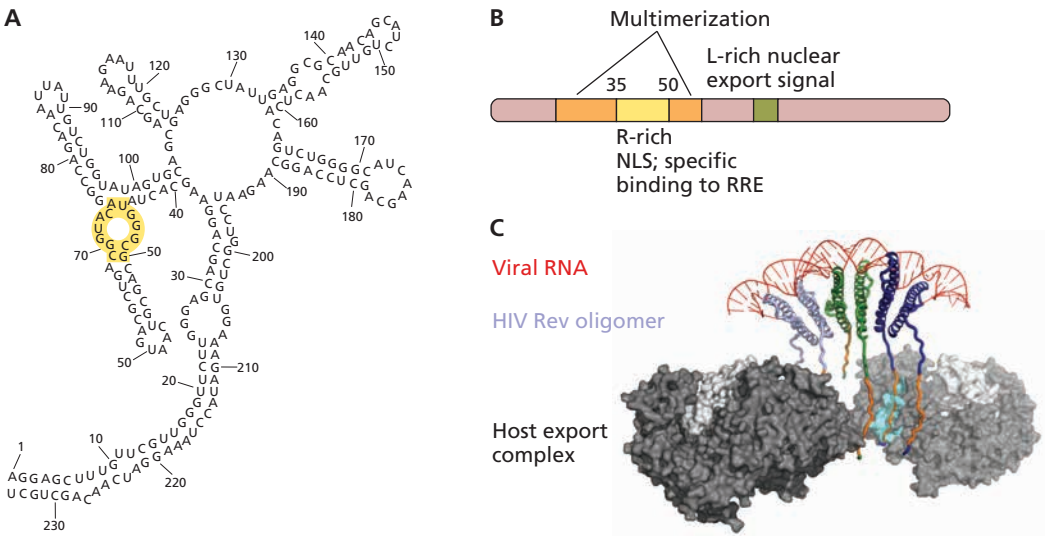


Figure 10.13 Features of the Rev-responsive element and Rev protein. (A) Predicted secondary structure of the 234-nucleotide Rev-responsive element, with the high-affinity binding site for Rev shaded in yellow. **(B)** The functional organization of the Rev protein. **(C)** Model of a Rev hexamer (three Rev dimers: light blue, green, and dark blue) assembled on the RRE, based on the crystal structure of a Rev dimer lacking the disordered C-terminal 46 amino acids and carrying alterations at positions 12 and 60 to prevent aggregation, and the observation that six Rev monomers assemble on the RRE. Modeling of the nuclear export signal-containing C termini not present in the structure indicated that they project away from the RNA-binding domain of Rev. They are shown modeled into the binding sites (cyan) for leucine-rich nuclear export signals in Xpo1 bound to Ran-GTP complex. Adapted from M. D. Daugherty et al., *Nat Struct Mol Biol* **11:**1337–1342, 2010, with permission. Courtesy of A. D. Frankel, University of California, San Francisco.

The pathway of Nxf1-dependent mRNA export has not yet been fully elucidated, but the Ran protein does **not** participate. The direct and specific binding of Nxf1 to the CTE of unspliced retroviral RNAs bypasses a cellular process that ensures that export is normally coupled with transcription and pre-mRNA processing (Fig. 10.15). This protein can bind only nonspecifically and with low affinity to cellular pre-mRNAs, but is recruited by export adapters, including several SR splicing proteins, a subunit of Cpsf, and a protein complex called Trex1 (transcription and export complex 1) that is deposited during splicing. Such indirect recruitment of Nxf1 to an mRNA, which couples export to RNA synthesis and processing, is circumvented in the case of retroviral pre-mRNAs containing CTEs: these unspliced RNAs are recognized directly by Nxf1, allowing their export from the nucleus.

Control of the Balance between Export and Splicing

The relative efficiencies of splicing and export maintain a finely tuned balance in the production of spliced and unspliced retroviral RNAs. This balance is of critical importance as even a 2-fold change prevents viral reproduction (Fig. 10.10A). On one hand, splicing of viral pre-mRNA must be inefficient to allow export of the essential, intron-containing mRNAs. Indeed, increasing the efficiency of splicing of human immunodeficiency virus type 1 pre-mRNA, by replacing the natural, suboptimal splice sites with efficient ones, leads to complete splicing of all pre-mRNA molecules before Rev can recognize and export the unspliced mRNA to the cytoplasm. On the other hand, when unspliced RNAs remain in the nucleus (e.g., before Rev is made in infected cells), they are eventually spliced to completion. Efficient export is therefore required to place unspliced mRNA into the cytoplasm for translation or incorporation into virus particles.

Export of Single-Exon Viral mRNAs

Most of the viral mRNAs made in nuclei of cells infected by hepadnaviruses, herpesviruses, or orthomyxoviruses are not spliced. In contrast to the retroviral mRNAs described in previous sections, these viral mRNAs do not contain introns. Rather, the viral genes that encode them contain no such sequences, and consequently the RNAs **cannot** be spliced. We therefore designate such mRNAs as **single-exon mRNAs** to distinguish them from those that retain introns.

Single-exon mRNAs are rare in uninfected mammalian cells, numbering only a few hundred. The majority encode regulatory proteins, such as signal components of signal transduction pathways and cytokines. Viral and cellular single-exon mRNAs cannot become associated with export adapters during spliceosome assembly and splicing, but nonetheless must be transported efficiently to the cytoplasm. The export of such viral mRNA is promoted by specific RNA sequences or viral proteins, analogous to the retroviral CTE and Rev protein, respectively.

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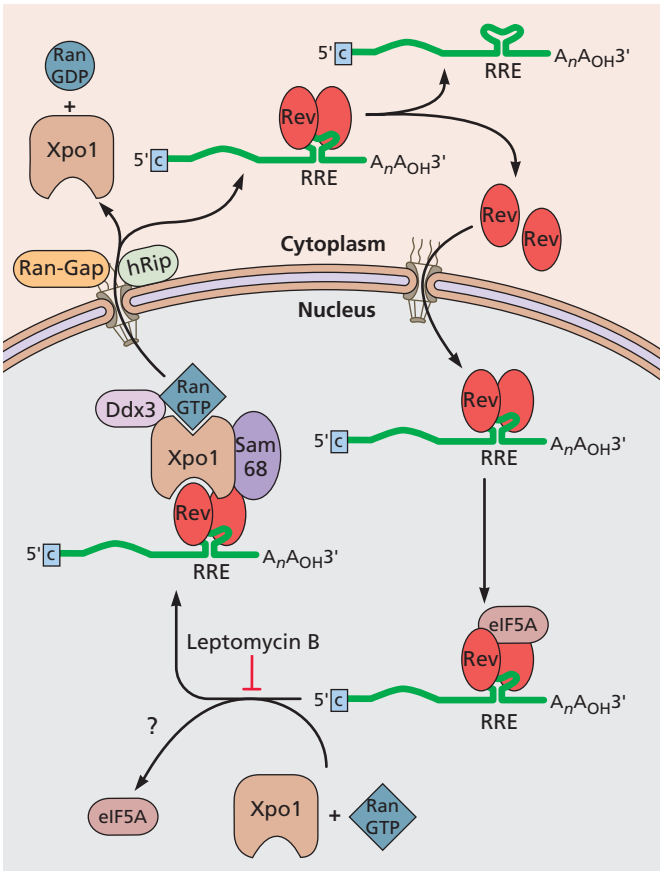


Figure 10.14 Mechanism of Rev protein-dependent export. The cellular nuclear proteins exportin-1 (Xpo1), the GTP-bound form of Ran (Ran-GTP), and the 68-kDa Src-associated protein in mitosis (Sam68) have been implicated in Rev-dependent mRNA export, for example, by analysis of the effects of dominant negative forms of the proteins. In the presence of Ran-GTP, Rev binds to Xpo1. This protein is related to the import receptors described in Chapter 5, and interacts with nucleoporins. The complex containing Rev, Xpo1, and Ran-GTP bound to the Rev-responsive element in RNA is translocated through the nuclear pore complex to the cytoplasm via interactions of Xpo1 with nucleoporins, such as Can/Nup14 and Nup98. Translocation may be facilitated by the action of Ddx3, an ATP-dependent RNA helicase. The Sam68 protein can bind to the Rev nuclear export signal, but does not appear to shuttle between nucleus and cytoplasm. It may therefore act prior to docking of the viral RRE-containing RNA complex at the nuclear pore. The human Rev-interacting protein (hRip) appears to act following translocation, as it is essential for efficient release of Rev-associated RNA into the cytoplasm. Hydrolysis of GTP bound to Ran to GDP induced by the cytoplasmic Ran GTPase-activating protein (Ran-Gap) is presumed to dissociate the export complex, releasing viral RNA for translation or assembly of virus particles, and Ran, Xpo1, and other proteins for reentry into the nucleus.

Specific RNA sequences that promote export of single-exon mRNAs are exemplified by the conserved posttranscriptional regulatory element (PRE) of hepadnaviral mRNAs. The PRE is recognized by export adapters such as components of the Trex1 complex and is analogous to export-promoting sequences subsequently identified in cellular single-exon mRNAs, including those encoding interferon α1 and interferon β1. The viral sequence is sufficient to facilitate cytoplasmic accumulation of heterologous mRNAs, and has therefore been included in many vectors for gene expression in mammalian cells (Box 10.7).

Efficient export of herpesviral single-exon mRNAs depends on a viral protein, ICP27 in the case of herpes simplex virus type 1. Like the human immunodeficiency virus type 1 Rev protein, ICP27 shuttles between the nucleus and cytoplasm and binds to viral RNA, in this case via distinct N- and C-terminal RNA-binding domains. Although ICP27 contains a leucine-rich nuclear export signal, it also binds to Nxf1, and various lines of evidence indicate that it serves as a virus-specific adapter for export via the Nxf1 pathway. The influenza A virus NS1 protein, which can bind to both Nxf1 and viral mRNAs, may serve a similar function for export of viral mRNA made later in the infectious cycle.

Posttranscriptional Regulation of Viral or Cellular Gene Expression by Viral Proteins

The genomes of several viruses encode proteins that regulate one or more RNA-processing reactions. These proteins are critical for temporal regulation of viral gene expression, or inhibit the production of cellular mRNAs.

Temporal Control of Viral Gene Expression

Regulation of Alternative Polyadenylation by Viral Proteins

During infection by adenoviruses, herpesviruses, and papillomaviruses, the frequencies of utilization of alternative poly(A) addition sites within specific viral pre-mRNAs change. As discussed previously, the polyadenylation of bovine papillomavirus type 1 late mRNA is activated by a specific complement of cellular proteins found only in fully differentiated cells of the epidermis. In contrast, viral proteins have been implicated in regulation of polyadenylation in cells infected by the larger DNA viruses.

Despite its name, the adenoviral major late promoter is active during the early phase of infection, prior to the onset of viral DNA synthesis. The major late pre-mRNAs made during this period are polyadenylated predominantly at the L1 mRNA site, even though they also contain the L2 and L3 3′ processing sites (Fig. 10.11). Such selective recognition of this polyadenylation signal depends on Cstf, which binds to the U/GU-rich sequence 3′ to the cleavage site (Fig. 10.3). As infection continues, the activity of this cellular protein decreases. It has been shown experimentally that synthesis of the viral L4 33-kDa protein is essential for the switch to the late pattern of gene expression (Box 10.8). It is not yet known whether this protein modulates the activity of Cstf

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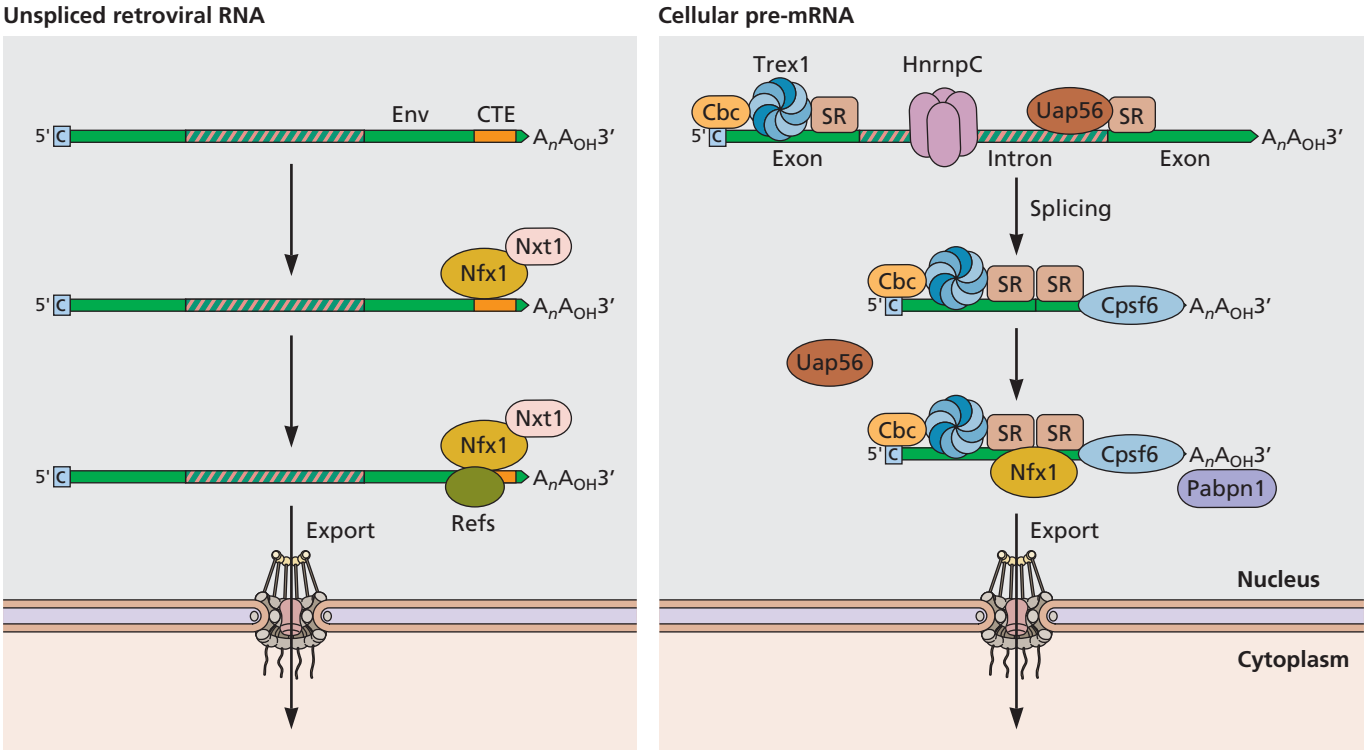


Figure 10.15 Export of unspliced RNA of retroviruses with simple genomes and cellular mRNAs from the nucleus. Export of unspliced, primary transcripts of many retroviruses depends on the constitutive transport element (CTE) in the RNA (left). This sequence is recognized by the cellular Nxf1 subunit of the export receptor dimer Nxf1-Nxt1, which is then bound by proteins that mark mRNAs as appropriate substrates for export, such as Ref. A variety of experimental approaches, including genetic studies in yeast, have indicated that Nxf1 is an essential component of the major pathway for export of cellular mRNAs. However, Nxf1 does not bind to cellular mRNAs with high affinity, but rather becomes associated with them indirectly via interactions with specific proteins, such as several SR proteins. Mature mRNAs are exported from the nucleus as associated with numerous proteins, i.e., as ribonucleoproteins, including those recognized by RNA export receptors. As indicated at the right, such export adapters become associated with mammalian pre-mRNAs, most of which contain introns and must be spliced, as primary transcripts are synthesized and processed. In this way, mRNA export is coupled to transcription and processing reactions. Several SR splicing proteins, the cap-binding complex (Cbc), a subunit of Cpsf, and the multiple-subunit protein Trex1, which is deposited on mammalian pre-mRNAs during splicing, interact with Nxf1. These adapters cooperate to direct efficient export via Nxf1-Nxt1. The export substrate is shown as a compact structure, in which the 5′ and 3′ ends are held in proximity by association of Cbc with the nuclear poly(A)-binding protein Pabn1 (see Chapter 11). Entry into this pathway also requires binding to nascent transcripts made by RNA polymerase II of HnRnpC. This interaction prevents export via the Xpo1 receptor. The direct interaction of Nxf1 with retroviral CTEs (left) therefore bypasses the mechanism(s) that couples splicing of cellular mRNAs with their export. Adapted from M. Müller-McNicoll and K. M. Neugebauer, *Nat Rev Genet* **14:**275–287, 2013, with permission.

or other components of the polyadenylation machinery. The recognition of the other four polyadenylation sites present in major late pre-mRNA synthesized during the late phase (Fig. 10.11) is much less dependent on Cstf. It is therefore likely that these poly(A) addition signals compete more effectively with the L1 site for components of the polyadenylation machinery later in the infectious cycle.

Viral Proteins Can Regulate Alternative Splicing

Some viral proteins that regulate pre-mRNA splicing alter the balance among alternative splicing reactions at specific points in the infectious cycle. For example, the ratios of alternatively spliced mRNA products of several adenoviral pre-mRNAs change with the transition into the late phase of infection. This phenomenon has been studied most extensively using the L1 mRNAs. The L1 pre-mRNA can be spliced at one of two alternative 3′ splice sites. However, only the L1 mRNA that specifies the 52/55-kDa protein is made prior to the onset of viral DNA synthesis, because binding of cellular SR proteins to a negative regulatory sequence located immediately upstream of the branch point for the L1 IIIa mRNA blocks its recognition (Fig. 10.16). Such inhibition is overcome by a viral early protein encoded within the E4 transcription unit, which induces dephosphorylation of the SR proteins. Overproduction of the SR protein Sf2 in adenovirus-infected cells impairs synthesis of the L1 IIIa mRNA, as well as viral reproduction.

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BOX 10.7

METHODS

Increasing expression of transgenes in mammalian cells using the woodchuck hepatitis virus posttranscriptional regulatory element

Initial studies demonstrated that efficient expression of genes of the hepadnavirus hepatitis B virus depends on an RNA sequence. This sequence, termed the posttranscriptional regulatory element (PRE), acts in *cis* to allow transport to the cytoplasm of the single-exon viral mRNAs. It can be considered functionally equivalent to an intron: the PRE can stimulate expression of β-globin cDNA, normally very low because of the absence from the transcripts of an intron and splice site, and conversely, inclusion of an intron in the viral surface protein mRNA (in the absence of the PRE) stimulates production of this protein. Subsequently, a similar PRE in the genome of woodchuck hepatitis virus was identified and characterized. This sequence, called WPRE, shares two of its three elements with the hepatitis B virus PRE (see the figure) and is more effective in inducing export to the cytoplasm of a single-exon viral mRNA.

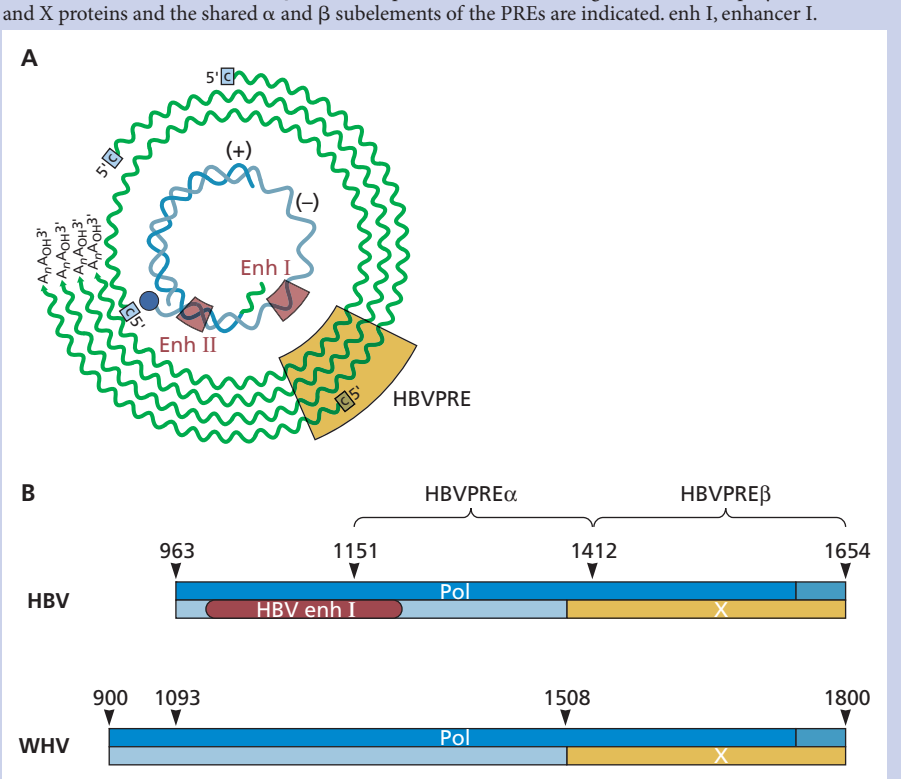
These properties prompted inclusion of the woodchuck hepatitis virus PRE in vectors for expression of transgenes in mammalian cells: to circumvent the large size of many genes of humans (and other mammals), as well as the prevalence of alternative splicing of primary transcripts, such transgenes are typically cDNAs that contain no introns. For example, the presence of WPRE in the 3′ untranslated regions of intronless reporter genes in either retroviral or lentiviral vectors increased synthesis of the reporter proteins 5- to 8-fold. Such stimulation was promoter independent, but was observed only when the WPRE was in the sense orientation.

Donello JE, Loeb JE, Hope TJ. 1998. Woodchuck hepatitis virus contains a tripartite posttranscriptional regulatory element. *J Virol* **72:**5085–5092.

Huang ZM, Yen TS. 1995. Role of the hepatitis B virus posttranscriptional regulatory element in export of intronless transcripts. *Mol Cell Biol* **15:**3864–3869.

Zufferey R, Donello JE, Trono D, Hope TJ. 1999. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol* **73:**2886–2892.

(A) The hepdnaviral genome and transcripts are depicted as in the Appendix, Fig. 11, with the position of the PRE indicated. (B) The hepatitis B virus (HBV) and woodchuck hepatitis virus (WHV) PREs are compared. The positions of the reading frames for the polymerase (Pol) and X proteins and the shared α and β subelements of the PREs are indicated. enh I, enhancer I.



This observation indicates that dephosphorylation of cellular SR proteins makes a major contribution to posttranscriptional regulation of adenoviral gene expression. However, efficient production of the L1 IIIa mRNA depends on a splicing enhancer and the viral L4 33-kDa protein (Fig. 10.16).

Regulation of mRNA Export

Even though all are encoded within a single proviral transcription unit, the regulatory and structural proteins of human immunodeficiency virus type 1 are made sequentially in infected cells, as a result of regulation of mRNA export by the Rev protein: this protein regulates a switch in viral gene expression from early production of viral regulatory proteins to a later phase, in which components of virus particles are made (Fig. 10.12).

Viral proteins that modulate mRNA export may play secondary, but nonetheless crucial, roles in temporal regulation of viral gene expression. For example, the transcriptional program described in Chapter 8 results in efficient transcription of herpes simplex virus late genes only following initiation of viral DNA synthesis in infected cells. Because all but one of the late mRNAs contains a single exon, their entry into the cytoplasm and the synthesis of viral late proteins require ICP27. Consequently, this viral posttranscriptional regulator is essential for putting the viral transcriptional program into effect. Similarly, the complete panoply of adenoviral major

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BOX 10.8

EXPERIMENTS

A single adenoviral protein controls the early-to-late switch in major late RNA processing

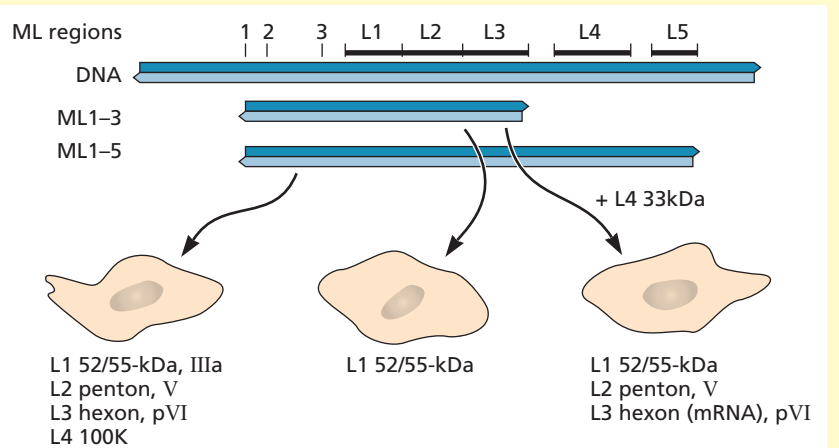
In efforts to develop cell lines that stably produce adenoviral late proteins, plasmids containing various segments of the major late (ML) transcription unit under the control of an inducible promoter were introduced into human cells. As summarized in the figure, the plasmid ML1-5 supported very efficient expression of all the ML-coding sequences and synthesis of the full set of the ML proteins. In contrast, **only** the L1 52/55-kDa protein was synthesized efficiently in cells containing the plasmid carrying the L1, L2, and L3 sequences. Examination of the cytoplasmic concentrations of processed ML mRNAs showed that only the L1 52/55-kDa mRNA was made efficiently in cells containing this truncated plasmid, as is also the case during the early phase of infection. These observations implied that one or more viral proteins encoded in the L4 or L5 region induce the early-to-late switch in processing of ML pre-mRNA. In fact, synthesis of the L4 33-kDa protein in cells containing the ML1-3 plasmid allowed production of the L1 IIIa and the L2 and L3 proteins. This viral protein stimulated the synthesis of fully processed hexon mRNA, but did not alter the nuclear concentration of the pre-mRNA. It was therefore concluded that the L4 33-kDa protein is necessary and sufficient to switch processing of the ML pre-mRNA from the early to the late pattern.

The subsequent discovery of a promoter that directs transcription of the L4 region of the adenoviral genome solved the puzzle of how the major late-encoded L4 33-kDa protein became available to induce the late pattern of viral gene expression.

Farley DC, Brown JL, Leppard KN. 2004. Activation of the early-late switch in adenovirus type 5 major late transcription unit expression by L4 gene products. *J Virol* **78:**1782–1791.

Wright J, Leppard KN. 2013. The human adenovirus 5 L4 promoter is activated by cellular stress response protein p53. *J Virol* **87:**11617–11625.

The major late (ML) coding regions (L1 to L5) of the adenovirus type 5 genome are shown to scale at the top, with the regions in the ML1-3 and ML1-5 plasmids introduced into human cells shown below. 1, 2, and 3 indicate the positions of the three segments of the tripartite leader sequence. The proteins made in cells containing these plasmids, and the ML1-3 plasmid plus a vector directing synthesis of the viral L4 33-kDa protein, are indicated below.



late gene products can be produced only when the viral L4 33-kDa protein induces the switch to the late pattern of processing of these pre-mRNAs (Box 10.8).

Viral Proteins Can Inhibit Cellular mRNA Production

All viral mRNAs are translated by the protein synthesis machinery of the host cell. Inhibition of production of cellular mRNAs can therefore favor this essential step in viral replication. Several mechanisms of selective inhibition of cellular RNA processing operate in virus-infected cells (Fig. 10.17).

Inhibition of Polyadenylation and Splicing

The influenza virus NS1 protein can inhibit both polyadenylation and splicing of cellular pre-mRNAs. A C-terminal segment of this viral protein is required for inhibition of polyadenylation and contains binding sites for both Cpsf and poly(A)-binding protein II (PabII) (Fig. 10.4). Its interaction with Cpsf inhibits polyadenylation of cellular mRNAs in experimental systems. When NS1 is not made, infected cells produce larger quantities of cellular mRNAs that encode interferons and other proteins with antiviral activities. Inhibition of processing of such cellular mRNAs may therefore contribute to the circumvention of host cellular defenses, a critical function of the NS1 protein (Volume II, Chapter 3).

In addition to its other activities, herpes simplex virus ICP27 inhibits splicing of cellular pre-mRNAs. This protein inhibits splicing in *in vitro* reactions, probably because its direct interaction with components of the spliceosome blocks an early step in spliceosome assembly. Genetic analyses have shown that disruption of cellular RNA processing by ICP27 leads to inhibition of cellular protein synthesis, and that this function is distinct from the requirement for the protein for efficient production of viral late mRNAs. Because herpesviral genes generally lack introns, inhibition of splicing is an effective strategy for the selective inhibition of cellular gene expression.

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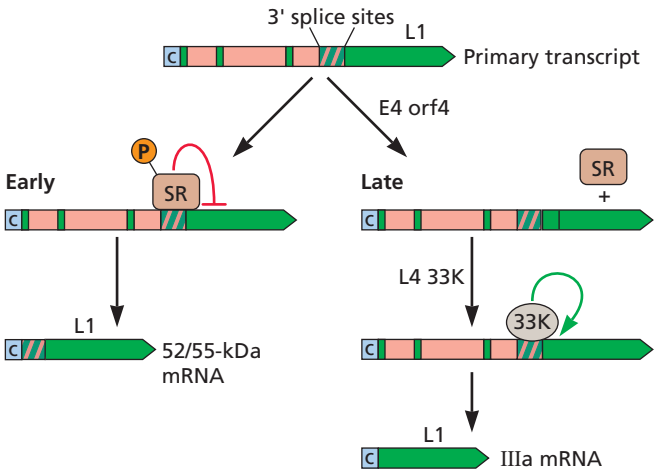


Figure 10.16 Regulation of alternative splicing of adenoviral major late L1 pre-mRNA. The polyadenylated L1 pre-mRNA contains alternative 3′ splice sites, for the 52/55-kDa protein and protein IIIa. (Left) During the early phase of infection, only the 3′ splice site for the 52/55-kDa protein is utilized, because binding of SR proteins to the pre-mRNA blocks recognition of the 3′ splice site for production of the mRNA for protein IIIa. (Right) An E4 protein induces dephosphorylation of these cellular proteins by protein phosphatase 2. This modification inhibits binding of the SR proteins to the pre-mRNA. However, efficient utilization of the IIIa mRNA 3′ splice site (during the late phase) requires the viral L4 33-kDa protein, which activates splicing via an infected cell-specific splicing enhancer. This L4 protein also stimulates splicing at other suboptimal 3′ splice sites in major late pre-mRNAs, such as those that produce the L2 mRNAs for proteins V and pre-VII.

Inhibition of Cellular mRNA Export

To facilitate production of viral mRNAs. In contrast to the other viruses considered in this section, adenovirus infection disrupts cellular gene expression by inhibition of export of cellular mRNAs from the nucleus. Synthesis and processing of cellular pre-mRNAs are unaffected, but these RNAs are not exported and are degraded within the nucleus (Fig. 10.17). Consequently, during the late phase of infection, the great majority of newly synthesized mRNAs entering the cytoplasm are viral in origin. When selective viral mRNA export is prevented by mutations in the viral genome, both the quantities of late proteins made in infected cells and virus yield are reduced substantially. These same phenotypes are seen in herpes simplex virus-infected cells when the ICP27 protein is defective for the inhibition of pre-mRNA splicing. These properties emphasize the importance of posttranscriptional inhibition of cellular mRNA production for efficient virus reproduction.

The preferential export of late mRNAs in adenovirus-infected cells requires two viral early proteins, the E1B 55-kDa and E4 Orf6 proteins, which associate with one another and with proteins present in cellular E3 ubiquitin ligases to form a virus-specific enzyme. E3 ubiquitin ligases typically add chains of ubiquitin to mark proteins for degradation by the proteasome. Although assembly of the adenovirus-specific enzyme is required, it is not known how it regulates mRNA export.

The selectivity of mRNA export in adenovirus-infected cells is especially puzzling, because the viral mRNAs possess all the characteristic features of cellular mRNAs, are made in the same way, and are exported via the Nxf1 pathway. One hypothesis is that the viral E1B-E4 protein complex recruits nuclear proteins needed for export of mRNA to the specialized sites within the nucleus at which the adenoviral genome is replicated and transcribed. As a result of such sequestration, viral mRNAs would be exported preferentially.

To block antiviral responses. The genomes of many RNA viruses encode all the enzymes necessary for synthesis and processing of viral mRNAs in the cytoplasm. Infection by some of these viruses results in inhibition of export of cellular RNAs from the nucleus. This response can reduce competition of cellular with viral mRNAs for components of the translational machinery. However, such inhibition can also facilitate virus reproduction indirectly, by impairing host antiviral responses, as observed in cells infected by rhabdoviruses and picornaviruses.

The vesicular stomatitis virus M protein inhibits export of cellular mRNAs (as well as small RNAs) by binding to a cellular nucleoporin (Nup98) and the cellular export protein Rae1, which normally shuttles between the nucleus and cytoplasm and binds to Nxf1 (Fig. 10.17). The consequent disruption of cellular mRNA export probably reduces host cell protein synthesis. This response also appears to block an important antiviral defense, as expression of the cellular Rae1 and Nup98 genes is induced by interferon, a potent antiviral cytokine (see Volume II, Chapter 3). The observation that specific alterations in the M protein that prevent inhibition of export of interferon β mRNA reduce viral reproduction is consistent with this interpretation.

Picornaviruses also disrupt trafficking from the nucleus to the cytoplasm. The poliovirus 2A protease induces relocation of particular nuclear proteins to the cytoplasm (Fig. 10.17). Such redistribution correlates with loss of structure from the central channel of the nuclear pore, and cleavage of specific nucleoporins (e.g., Nup153). The small leader (L) protein of encephalomyocarditis virus, a member of the cardiovirus group within the *Picornaviridae*, binds to Ran-GTPase, an essential component of Ran-dependent nuclear export and import pathways, and induces hyperphosphorylation of several of the nucleoporins that are cleaved by the poliovirus 2A protease. The phenotypes of mutants with deletions in the L gene suggest that inhibition of trafficking between the nucleus and cytoplasm both tempers the interferon antiviral response and contributes to inhibition of cellular protein synthesis.

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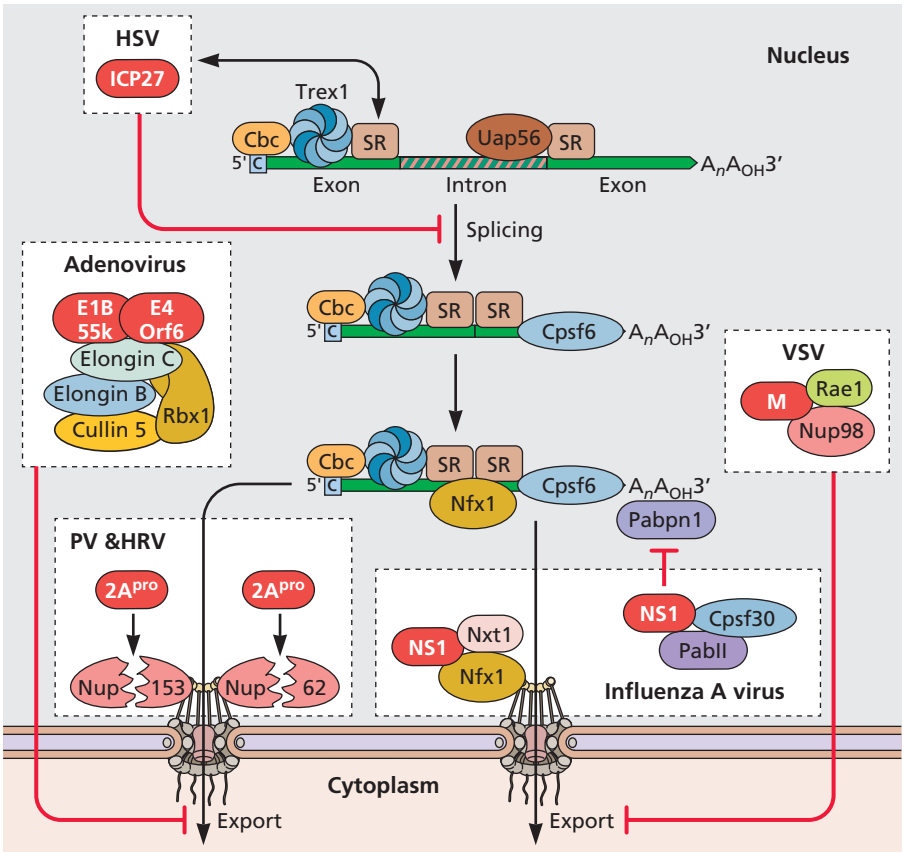


Figure 10.17 Inhibition of cellular pre-mRNA processing by viral proteins. The integration of synthesis and processing of cellular pre-mRNA with export of the mature mRNA to the cytoplasm is depicted as in Fig. 10.15 (right). Some viral proteins inhibit splicing (herpes simplex virus type 1 [HSV-1] ICP27), polyadenylation (influenza A virus NS1), or export (vesicular stomatitis virus [VSV] M and influenza A virus NS1) by interaction with the cellular proteins required for these processes. Inhibition of export of processed cellular mRNAs in cells infected by human adenovirus depends on assembly of the virus-specific E3 ubiquitin ligase that contains the viral E1B 55-kDa and E4 Orf6 proteins, but the mechanism of inhibition has not been elucidated. Export from the nucleus is inhibited in cells infected by picornaviruses as a result of degradation of specific nucleoporins by the viral 2A protease ($2A^{Pro}$), poliovirus (PV), and human rhinovirus (HRV) or hyperphosphorylation of these same nucleoproteins induced by the leader (L) protein of encephalomyocarditis virus (not shown).

Regulation of Turnover of Viral and Cellular mRNAs in the Cytoplasm

Individual mRNAs may differ in the rate at which they are translated, and also in such properties as cytoplasmic location and stability. Indeed, the intrinsic lifetime of an mRNA can be a critical parameter in the regulation of gene expression.

In the cytoplasm of mammalian cells, the lifetimes of specific mRNAs can differ by as much as 100-fold. This property is described in terms of the time required for 50% of the mRNA population to be degraded under conditions in which replenishment of the cytoplasmic pool is blocked, the **half-life** of the mRNA. Many mRNAs are very stable, with half-lives exceeding 12 h. As might be anticipated, these mRNAs encode proteins needed in large quantities throughout the lifetimes of all cells, such as ribosomal proteins. At the other extreme are unstable mRNAs with half-lives of <30 min. This class includes mRNAs specifying regulatory proteins that are synthesized in a strictly controlled manner in response to cues from external or internal environments of the cell, such as cytokines, and proteins that regulate cell cycle progression. The short lifetimes of these mRNAs ensure that synthesis of their products can be shut down effectively once they are no longer needed. Specific sequences that signal the rapid turnover of the mRNAs in which they reside have been identified, such as a 50- to 100-nucleotide AU-rich sequence within the 3′ untranslated region. Mammalian mRNAs are degraded by the pathways summarized in Fig. 10.18, in which deadenylation of the 3′ end of the mRNA triggers either removal of the 5′ cap for degradation by the 5′ → 3′ exoribonuclease Xrn1 or 3′ → 5′ degradation by the conserved, multiprotein exosome. These reactions take place in dynamic cytoplasmic foci, termed P (processing) bodies, that are enriched in proteins that mediate mRNA degradation or inhibit translation, mRNAs that are translationally silent (often deadenylated), and micro-RNAs (see next section).

The stabilities of viral mRNAs have not been examined in much detail, in part because many viral infectious cycles are completed within the normal range of mRNA half-lives and many viral mRNAs carry the 5′ caps and 3′ poly(A) tails that protect against degradation. When these features are absent, the 5′ and/or 3′ ends of viral mRNAs often form structures that block exonucleolytic attack,

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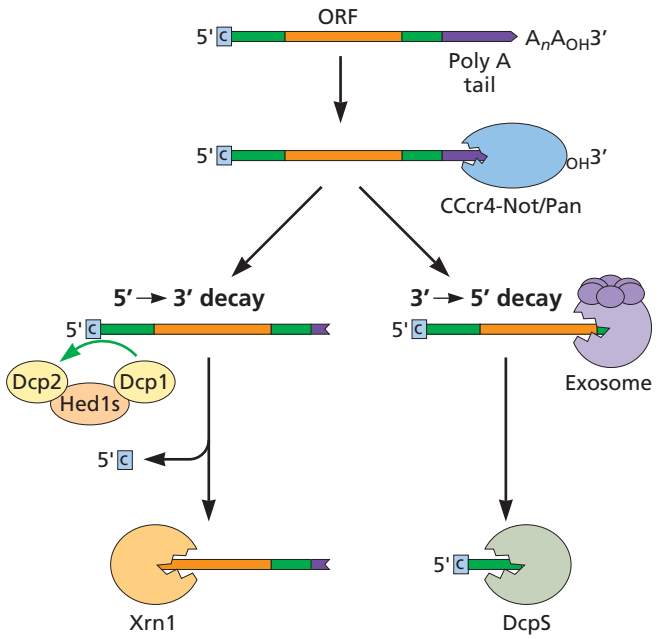


Figure 10.18 Mechanisms of cellular mRNAs of deadenylation-dependent degradation. A major pathway of mRNA degradation in the cytoplasm depends on initial deadenylation. In the case of short-lived mRNAs, this process can be initiated by binding of specific proteins to 5′AUUUUA3′ sequences. Regardless, shortening of the poly(A) tail is a two-step process catalyzed by different deadenylases, the Pan and Ccr4-Not complexes. In subsequent 5′ → 3′ decay (left), shortening of the poly(A) tail to <110 nucleotides triggers decapping by the enzyme-decapping protein 2 (Dcp2). This reaction is stimulated by Dcp1, which interacts with Dcp2 via the Hedls (human enhancer of decapping large subunit) protein. The exact way in which the decapping enzyme is recruited to target mRNAs, and the contribution of shortening of the poly(A) tail, are not fully understood. The decapped mRNA is then degraded by 5′ → 3′ exonucleases, such as Xrn1. Alternatively (right), the deadenylated mRNA can be degraded in the 3′ → 5′ direction by the exosome, which contains multiple exo- and endonucleases, including a processive 3′ → 5′ exoribonuclease, and decapping by Dcp5. Adapted from V. K. Nagarajan et al., *Biochim Biophys Acta* **1829:** 590–603, 2013, with permission.

such as stem-loop structures in the 3′ untranslated regions of arenavirus, bunyavirus, and flavivirus mRNAs. Viral RNAs can also include binding sites for cellular proteins that increase their stability. Viral proteins that induce RNA degradation make an important contribution to selective expression of viral genes in cells infected by large DNA viruses. Regulation of the stability of specific viral or cellular mRNAs has also been implicated in the permanent changes in cell growth properties (transformation) induced by some viruses. Furthermore, RNA-mediated induction of degradation of specific mRNAs, a widespread phenomenon known as RNA interference, is thought to contribute to host antiviral defense mechanisms, and degradation of genomic RNA even contributes to the pathogenesis of flaviviruses (Box 10.9).

Regulation of mRNA Stability by Viral Proteins

Cellular proteins that participate in mRNA degradation are removed or relocalized in cells infected by several viruses. For example, subunits of the deadenylation (Pan3) and decapping (Dcp1a) enzymes and Xrn1 (Fig. 10.18) are degraded in poliovirus-infected cells, most likely by the viral protease $3C^{pro}$, and P bodies are disrupted. These structures are also dismantled in cells infected by the flavivirus West Nile virus, and by the adenovirus E4 Orf3 protein. How these changes facilitate virus reproduction has not yet been established, although destruction of the 5′ → 3′ exonuclease might stabilize poliovirus mRNA, which lacks a protective 5′ cap (see Chapter 6). The genomes of other viruses encode proteins that accelerate RNA decay.

The first such protein to be described, the virion host shutoff protein (Vhs) of herpes simplex virus type 1, reduces the stability of mRNAs in infected cells. As its name implies, Vhs is a structural protein: it is present at low concentrations in the tegument and hence delivered to infected cells at the start of the infectious cycle. It remains in the cytoplasm, where it mediates degradation of some cellular mRNAs to facilitate viral gene expression, presumably by reducing or eliminating competition from cellular mRNAs during translation. The Vhs protein is an endoribonuclease that targets mRNA by virtue of its binding to translation initiation proteins, such as eIF4H and the cap-binding complex eIF4F. Following endonucleolytic cleavage by Vhs near the 5′ end, mRNA is degraded by Xrn1. Although recruited to mRNAs by different mechanisms, the human herpesvirus 8 SOX and severe acute respiratory syndrome coronavirus nsp1 proteins also induce cleavage of mRNA to allow exonucleolytic degradation (Fig. 10.19).

Vhs cannot distinguish viral mRNAs from their cellular counterparts, and induces degradation of both. Although more Vhs protein is made in infected cells once its coding sequence is expressed during the late phase of infection, the protein is sequestered in the tegument of assembling virus particles by interaction with the viral VP16 protein. As a result, the activity of Vhs decreases as the infection cycle progresses. This mechanism presumably contributes to the efficient synthesis of viral proteins characteristic of the late phase of infection. In contrast, the coronavirus nsp1 protein induces selective degradation of cellular mRNA, because viral mRNAs are protected from endonucleolytic cleavage by the common leader sequence present at their 5′ ends (Chapter 6).

The genomes of poxviruses, such as vaccinia virus, also contain the coding sequence for enzymes that induce degradation of viral and cellular mRNAs. These proteins, D9 and D10, are not, however, RNases, but rather decapping enzymes that share a motif with their cellular counterpart and hydrolyze the cap to release m^7GDP (Fig. 10.19). It is clear from the results of genetic experiments that the D10

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BOX 10.9

DISCUSSION

Coopting a cellular mechanism of RNA degradation for viral pathogenesis

The family *Flaviviridae* includes important agents of human disease, many of which are spread by arthropod vectors, notably yellow fever virus, West Nile virus, Japanese encephalitis virus, and dengue virus. In infected cells, the (+) strand RNA genome is not only translated and replicated, but also serves as the precursor for subgenomic flaviviral RNAs (sfRNAs) 300 to 500 nucleotides in length. These RNAs correspond to most of the 3′ untranslated regions of the (+) strand RNAs and are produced by incomplete degradation of the full-length (+) strand RNA by the cellular 5′ → 3′ exonuclease Xrn1 (following removal of the cap by an unknown mechanism) (panel A of the figure). The introduction of mutations that prevent production of sfRNAs (by disrupting the RNA structures that block the progress of exonuclease described below) reduced the ability of West Nile virus to kill cells in culture. Such mutant viruses also failed to induce encephalitis, and death, in young mice. The essential role of sfRNAs in pathogenesis focused attention on the features of the 3′ untranslated regions of the genomic RNA that confer resistance to Xrn1.

A combination of phylogenetic studies, *in silico* prediction, and experimental analysis of RNA structure using chemical probes indicated that the 3′ untranslated regions of these flaviviruses are rich in secondary (and higher-order) structures (panel B of the figure). When present in short model RNAs, each of the stem-loops at the 5′ ends of the 3′ untranslated regions was shown to be resistant to Xrn1 digestion *in vitro*. They were also required for accumulation of specific sfRNAs in infected cells.

The tertiary structure of the first stem-loop inferred from the results of mutagenesis and RNA-folding experiments was confirmed by X-ray crystallography. This structure contains a three-way junction, and two of its three RNA helices form a ring-like structure with the 5′ end of the RNA passing through its center and "tied" in place by base pairing and other interactions with bases in the ring (panel B). Simple unwinding of RNA helices could not make the 5′ end of the RNA accessible, explaining how this structure blocks degradation by Xrn1, which proceeds in the 5′ → 3′ direction.

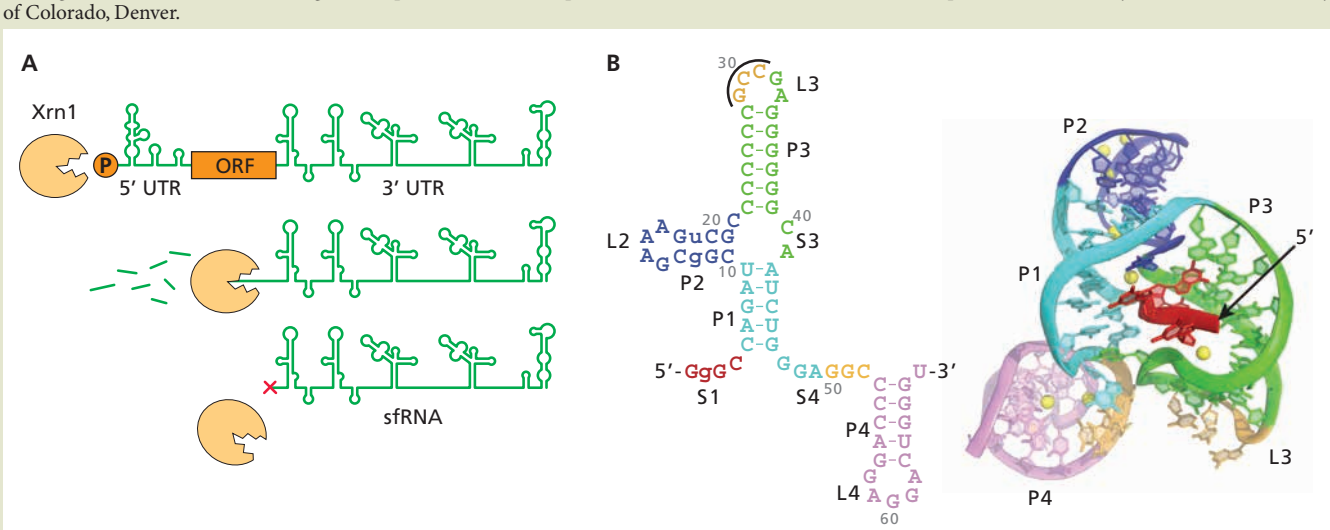
Because they are relatively short and highly conserved among the arthropod-borne flaviviruses, it has been suggested that it might be possible to develop therapeutic agents that target the structures that confer Xrn1 resistance,. Furthermore, mutations that prevent formation of sfRNAs should attenuate these viruses and may facilitate development of vaccines.

Chapman EG, Costantino DA, Rabe JL, Moon SL, Wilusz J, Nix JC, Kieft JS. 2014. The structural basis of pathogenic subgenomic flavivirus RNA (sfRNA) production. *Science* **344:**307–310.

Chapman EG, Moon SL, Wilusz J, Kieft JS. 2014. RNA structures that resist degradation by Xrn1 produce a pathogenic Dengue virus RNA. *eLife* **3:**e01892. doi:10.7554/eLife.01892.

Pijlman GP, Funk A, Kondratieva N, Leung J, Torres S, van der Aa L, Liu WJ, Palmenberg AC, Shi PY, Hall RA, Khromykh AA. 2008. A highly structured, nuclease-resistant, noncoding RNA produced by flaviviruses is required for pathogenicity. *Cell Host Microbe* **11:**579–591.

(A) Model for production of sfRNAs. (B) Organization and sequence of conserved RNA stem-loop 1, in this case of Murray Valley encephalitis virus, and structure (right) determined by X-ray crystallography at 2.5-Å resolution. In the structural model, the different elements are color coded as in the secondary structure representation shown at the left. Note the interaction between bases at the 5′ end of the RNA and those present in helices P1 and P3. ORF, open reading frame; UTR, untranslated region. Adapted from E. G. Chapman et al., *Science* **344:**307–310, 2014, with permission. Courtesy of J. S. Kieft, University of Colorado, Denver.



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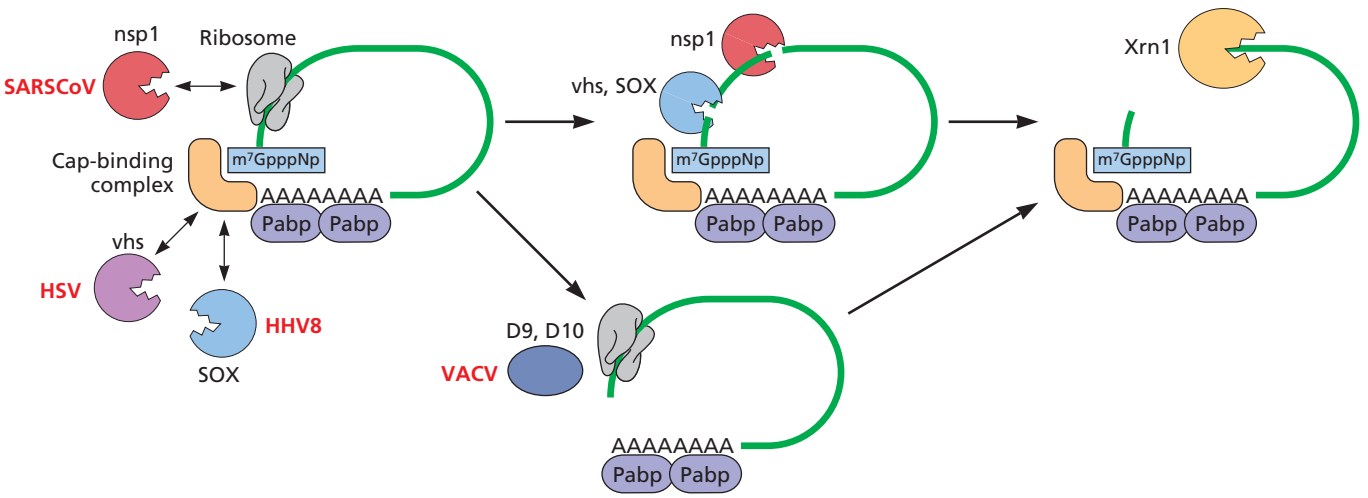


Figure 10.19 Viral proteins initiate mRNA degradation by different mechanisms. The genomes of alpha- and gammaherpesviruses encode endonucleases that initiate degradation of mRNA in infected cells, exemplified by the herpes simplex virus type 1 (HSV-1) vhs and human herpesvirus 8 (HHV8) SOX proteins. However, the former is a nuclease of the Fen1 family, whereas SOX is related to members of a different endonuclease family. Vhs is recruited to mRNA by interaction with the cap-binding complex, prior to ribosome binding. SOX also associates with mRNA before the ribosome, but the cellular components with which it interacts have not yet been identified. These viral proteins cleave the mRNA, within the 5′ untranslated region in the case of vhs, to allow subsequent 5′ → 3′ exonucleolytic degradation by Xrn1. The severe acute respiratory syndrome coronavirus (SARSCoV) nsp1, which is not obviously related to any viral or cellular nuclease, functions to cleave mRNA in a similar manner, but targets this substrate by interaction with the 40S ribosomal subunit. In contrast, the D9 and D10 proteins of the poxvirus vaccinia virus (VACV) share sequence motifs with cellular decapping enzymes and remove this 5′ protective structure to initiate mRNA degradation. Pabp, poly(A)-binding protein. Adapted from M. M. Gaglia et al., *J Virol* **86:**9527–9530, 2012.

protein induces rapid turnover of viral and cellular mRNAs, and hence facilitates inhibition of cellular protein synthesis in infected cells. It has been suggested that turnover of viral mRNAs may facilitate the production of specific sets of viral proteins during the successive phases of the infectious cycle, with the D9 and D10 enzymes acting early and late in infection, respectively.

mRNA Stabilization Can Facilitate Transformation

Stabilization of specific viral mRNAs appears to be important in the development of cervical carcinoma associated with infection by high-risk human papillomaviruses, such as types 16 and 18. The E6 and E7 proteins of these viruses induce abnormal cell proliferation (Volume II, Chapter 6). In benign lesions, the circular human papillomavirus genome is not integrated. The E6 and E7 mRNAs that are synthesized from such templates contain destabilizing, AU-rich sequences in their 3′ untranslated regions and possess short half-lives. In cervical carcinoma cells, the viral DNA is integrated into the cellular genome. Such reorganization of viral DNA frequently disrupts the sequences encoding the E6 and E7 mRNAs, such that their 3′ untranslated regions are copied from cellular DNA sequences (Fig. 10.20). These hybrid mRNAs therefore lack the destabilizing AU-rich sequences and are more stable. The increase in the stability of the viral mRNAs accounts, at least in part, for the higher concentrations of the papillomaviral transforming proteins in tumor cells.

Production and Function of Small RNAs That Inhibit Gene Expression

Small Interfering RNAs, Micro-RNAs, and Their Synthesis

In the early 1990s, attempts to produce more vividly purple petunias by creation of transgenic plants carrying an additional copy of the gene for the enzyme that makes the purple pigment often resulted in white flowers. It is now clear that this seemingly esoteric observation represented the first example of a previously unknown mechanism of post-transcriptional regulation of gene expression, called **RNA interference** or RNA silencing. We now know that RNA-based silencing of gene expression is widespread and ancient (Box 10.10). Our understanding of the mechanisms and functions of RNA interference, as well as its exploitation as an experimental tool, has advanced at a remarkably rapid pace. Indeed, Andrew Fire and Craig Mello were awarded the Nobel Prize in physiology or medicine in 2006, just 8 years after the publication of their groundbreaking study of the mechanism of RNA interference.

RNA interference is mediated by small RNA molecules (typically 19 to 25 nucleotides in length) that function in antiviral defense or regulate gene expression. The two main

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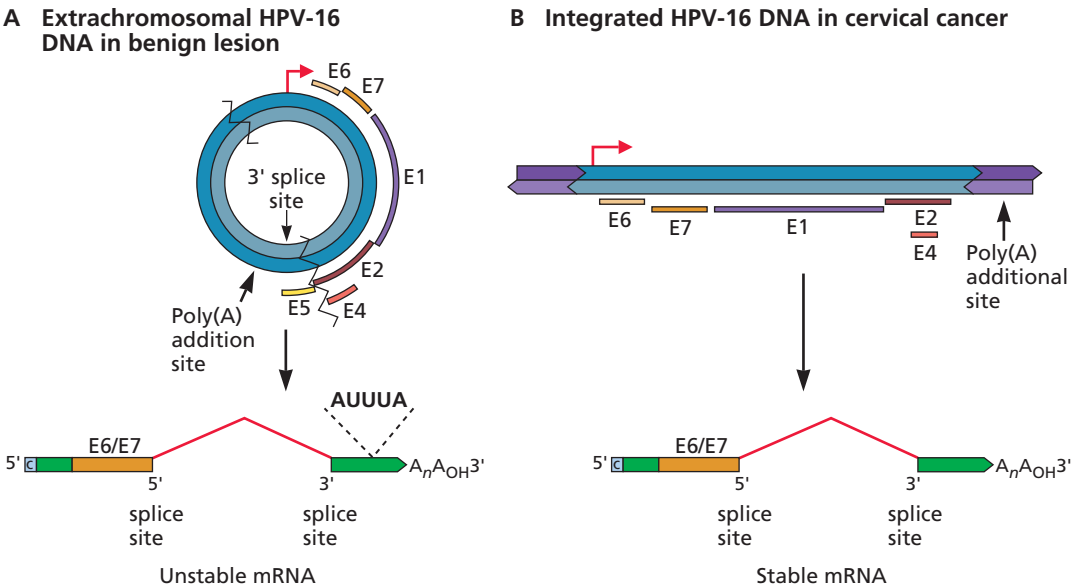


Figure 10.20 Stabilization of human papillomavirus type 16 (HPV-16) mRNAs upon integration of the viral genome into cellular DNA. (A) In benign lesions, the viral genome is maintained as an extrachromosomal, circular episome. Transcription of such viral DNA and pre-mRNA processing produce various alternatively spliced mRNAs containing the E6 and/or E7 protein-coding sequences, but, as illustrated, all contain destabilizing 5′AUUUA3′ sequences in their 3′ untranslated regions. **(B)** In cervical carcinoma cells, the viral genome is integrated into cellular DNA (purple) such that the viral genome is disrupted upstream of the E6/E7 mRNA 3′ splice site. The mRNAs encoding these viral proteins are therefore made by using 3′ splice and polyadenylation sites transcribed from adjacent cellular DNA, and they lack the destabilizing sequence.

types of these regulatory RNA molecules present in eukaryotes are distinguished by how they are synthesized. **Small interfering RNAs** (siRNAs), such as those first discovered in plants, are initially processed by endonucleolytic cleavage of double-stranded RNAs by cytoplasmic Dicer enzymes. The double-stranded RNA precursors are formed by base pairing of transcripts that contain complementary sequences, such as the (+) and (−) strand RNAs synthesized in cells infected by many viruses with RNA genomes. MicroRNAs (miRNAs) can be processed from RNAs synthesized by RNA polymerase III or from introns within pre-mRNAs. However, their precursors are generally capped and polyadenylated transcripts synthesized by RNA polymerase II, in which self-complementary regions form imperfect hairpin structures (Fig. 10.21). The sequences that encode miRNAs are often clustered, an arrangement that allows synthesis of transcripts containing multiple miRNA sequences. Such transcripts are initially processed by endonucleolytic cleavage in the nucleus to liberate pre-miRNAs, imperfect hairpins of 60 to 80 nucleotides. Further processing of pre-miRNAs occurs following export to the cytoplasm, where they are cleaved by Dicer enzymes.

In the case of both siRNAs and miRNAs, the products of Dicer cleavage are largely double-stranded, with two unpaired bases at the 3′ ends. These RNAs are then unwound from one 5′ end (Box 10.11), and one strand becomes tightly associated with a member of the argonaute (Ago) family of proteins in the effector ribonucleoprotein, termed the RNA-induced silencing complex, Risc. In these complexes, the small RNA acts as a "guide," identifying the target mRNA by base pairing to specific sequences within it prior to cleavage of the mRNA or inhibition of its translation. Perfect base pairing with the target mRNA usually results in mRNA cleavage. Such cleavage requires Ago2, the only one of the four human Ago proteins found in Risc that possesses endoribonuclease activity. However, inhibition of translation by miRNAs is often followed by deadenylation and decay of the mRNA.

The introduction of small, double-stranded RNAs analogous to the products formed by Dicer has proved to be a very valuable experimental tool. Such exogenous RNAs are incorporated into Riscs with high efficiency, allowing the experimenter to inhibit expression of particular genes by targeting siRNAs to degrade the corresponding mRNA. However, mammalian cells synthesize miRNAs rather than siRNAs. Some cellular miRNAs have a significant impact on virus-host cell interactions, and the genomes of several DNA viruses and retroviruses contain sequences coding for miRNAs.

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BOX 10.10

BACKGROUND

An ancient antiviral defense guided by RNA: the CRISPR system

Bacteria (and archaea) are infected by numerous viruses with DNA genomes, and are also exposed to foreign DNA as a result of transduction and conjugation. It has been estimated that bacteriophages represent the most abundant biological entities on the planet and that they are responsible for destruction of 4 to 50% of bacterial populations. Not surprisingly, these organisms have developed a variety of defense mechanisms, such as the well-characterized bacterial restriction-modification systems that destroy foreign DNA (the sources of the restriction endonucleases so widely used in research). The most recent to be discovered, the clustered regularly interspersed short palindromic repeat (CRISPR) system, is a powerful mechanism that provides acquired immunity to exogenous DNAs via short RNAs.

As its name indicates, a definitive feature of this system is the presence in the genome of arrays of short repeated sequences interspersed with nonrepetitive spacers (panel A of the figure). Such an array was first described in 1987 in the *E. coli* genome, and the name CRISPR was coined in 2000 following identification of such arrays in the genomes of other bacteria and archaea. The number of CRISPR loci, the number of repeat-spacer units per locus, and the lengths of repeats and spacers vary considerably among bacterial and archaeal species. However, the presence of palindromic sequences, and hence the ability to form hairpins, is observed in most CRISPR repeat sequences. Such CRISPR loci are adjacent to a set of conserved protein-coding genes. These genes vary in number, position, and orientation with respect to CRISPR loci (e.g., panel A), but include a core set encoding the proteins necessary for defense against foreign DNAs.

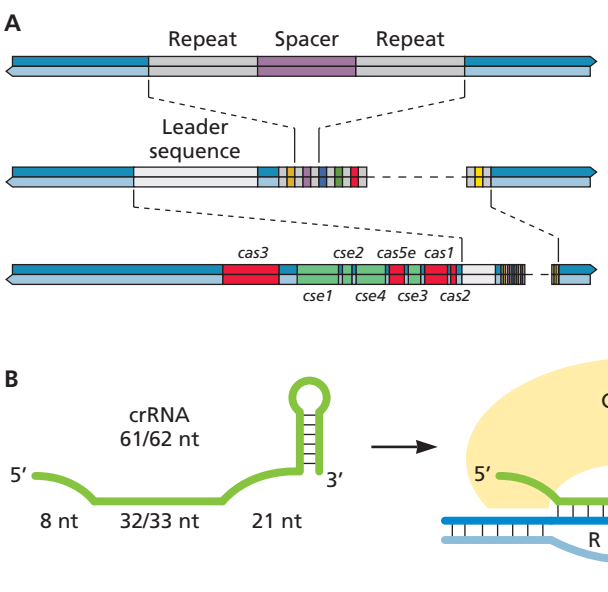
The breakthrough in understanding the function of CRISPR arrays came from computational studies of the origin of the spacer sequences: among those represented in sequence databases, most matched sequences of bacteriophage genomes or plasmids. Furthermore, species containing a spacer derived from a particular invader proved to be resistant to that invader. These observations led to the hypothesis that CRISPR loci provide a form of acquired immunity. The demonstration that strains of *Streptococcus thermophilus* selected for resistance to specific bacteriophages carried new, phage-derived sequences in the 5′ end of their CRISPR locus provided direct experimental evidence for this hypothesis.

Subsequently, multiple experimental approaches confirmed that the CRISPR system provides defense against invading genetic elements, and elucidated the mechanism summarized in panel B. When a foreign DNA, such as a bacteriophage genome, enters a bacterial cell, some fraction is fragmented. Short fragments that match conserved sequence motifs adjacent to the CRISPR spacers, termed protospacer adjacent motifs (PAMs), then become integrated into the CRISPR locus. Proteins necessary for this process have been identified, but the molecular mechanisms are not well understood. Following transcription, CRISPR RNAs are processed by a multiprotein complex (Cascade) to produce CrRNAs (~60 nucleotides), each of which carries a repeat-derived sequence at its 5′ end, a 3′ hairpin, and a unique, spacer-derived internal sequence (panel B). When the spacer of a CrRNA base pairs with a complementary sequence in an invading DNA molecule, an R-loop in which one strand of the foreign DNA is single-stranded is formed. CRISPR-associated endonucleases, such as Cas1 in *E. coli*, cleave the DNA, which then becomes extensively degraded. The integration of the sequences of the invading DNA into the host cell genome, from which they can be mobilized in the form of CrRNAs, provides a form of "memory" and acquired immunity. Remarkably, bacteriophages that infect *Vibrio cholerae* were discovered subsequently to encode a CRISPR/Cas system that counteracts host chromosomal sequences that inhibit bacteriophage reproduction.

Karginov FV, Hannon GJ. 2010. The CRISPR system: small RNA-guided defense in bacteria and archaea. *Mol Cell* **37:**7–19.

Seed KD, Lazinski DW, Calderwood SB, Camilli A. 2013. A bacteriophage encodes its own CRISPR/Cas adaptive response to evade host innate immunity. *Nature* **494:**489–491.

Westra ER, Swarts DC, Staals RH, Jore MM, Brouns SJ, van der Oost J. 2012. The CRISPRs, they are a-changin': how prokaryotes generate adaptive immunity. *Annu Rev Genet* **46:**311–339.



(A) The organization of CRISPR repeat and spacer sequences is illustrated at the top, and the position of the *E. coli* CRISPR locus with respect to CRISPR-associated genes (cas and cse genes) below. Adapted from F. V. Karginov and G. J. Hannon, *Mol Cell* **37:**7–19, 2010, with permission. **(B)** The structure of a CrRNA, following processing of CRISPR transcripts that contain multiple repeat-spacer units, is summarized at the top. As shown below, perfect base pairing of CrRNA with a complementary sequence adjacent to a PAM exposes a strand of the DNA for endonucleolytic attack by a CRISPR-associated endonuclease, such as Cas1. Adapted from E. R. Westra et al., *Annu Rev Genet* **46:**311–339, 2012, with permission.

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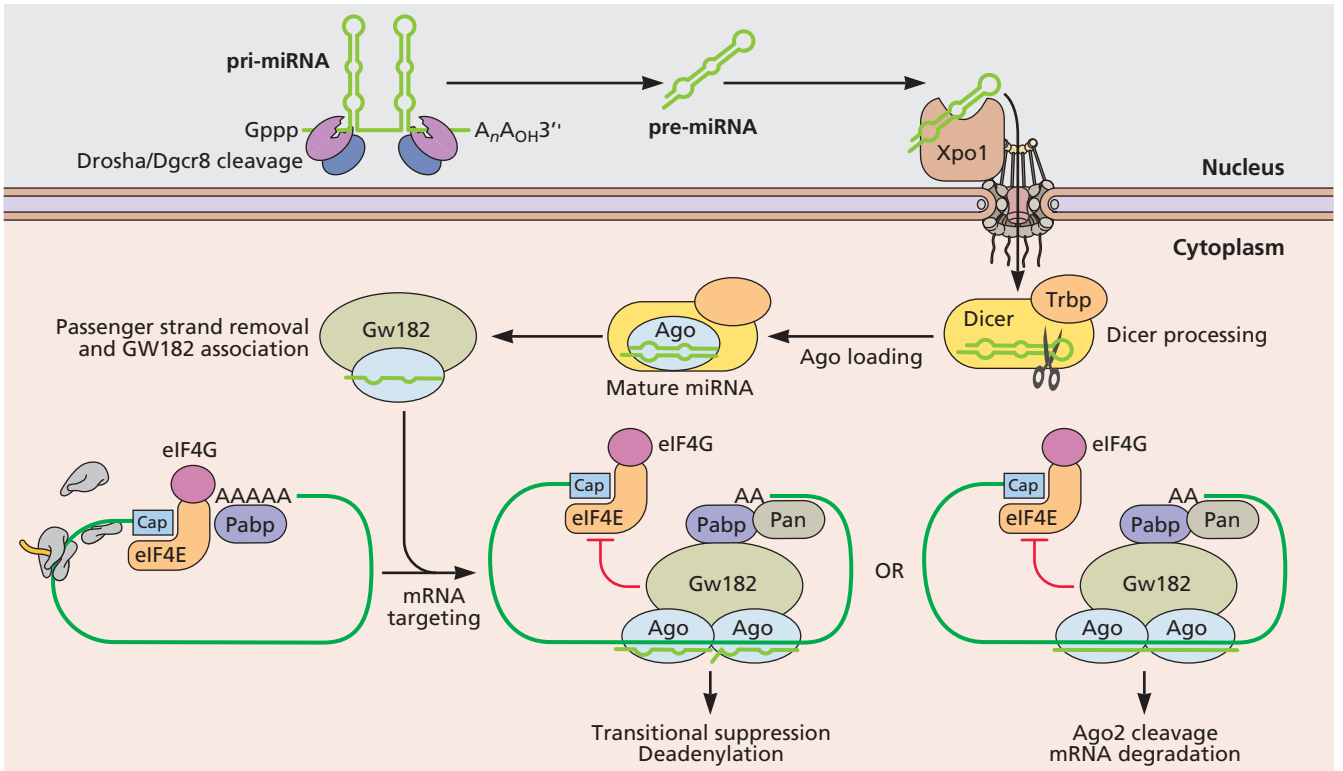


Figure 10.21 Synthesis and function of miRNAs. The precursors of miRNAs (pri-miRNAs), which are typically transcripts made by RNA polymerase II, undergo initial processing in the nucleus. Such transcripts are cleaved by the microprocessor, which comprises the ribonuclease Drosha and DiGeorge syndrome critical region 8 (Dgcr8) protein that is necessary for binding to the RNA substrate. The pre-miRNAs thus produced are then exported to the cytoplasm via the export receptor exportin-5. In the cytoplasm, further processing by the enzyme Dicer associated with a double-stranded-RNA-binding protein such as human TAR RNA-binding protein (Trbp) liberates 22-nucleotide, double-stranded RNAs with unpaired nucleotides at the 3′ ends. Upon unwinding of these duplexes, one RNA strand becomes tightly associated with an argonaute (Ago) protein (and others) in the RNA-induced silencing complex (Risc). The other strand is degraded. When human RISC contains Ago2, an mRNA to which its miRNA base pairs perfectly can be cleaved by this endonuclease (left). More generally (right), Ago proteins (Ago1-4 in human cells) interact with proteins of the Gw182 family that are required for RNA-mediated silencing, and induce inhibition of translation. Such proteins recruit deadenylases [e.g., poly(A)-binding protein (Pabp)-dependent poly(A)-specific ribonuclease (Pan)] that initiate degradation of mRNA by deadenylation, decapping, and 5′ → 3′ exonucleolytic degradation (Fig. 10.18).

Cellular miRNAs in Virus-Infected Cells

Micro-RNAs made in particular cell types have been reported to inhibit reproduction of a variety of viruses, including hepatitis B and C viruses, herpesviruses, human immunodeficiency virus type 1, influenza virus, and papillomaviruses. For example, at least six different cellular miRNAs can target human immunodeficiency virus type 1. However, cellular miRNAs can also dictate the outcome of virus infection and even facilitate virus reproduction. Examples of these phenomena are described below.

Cellular miRNA-155 Promotes Viral Oncogenesis

When Epstein-Barr virus infects primary B cells, it establishes a latent state characterized by limited expression of viral genetic information and maintenance replication of the viral genome (Chapters 8 and 9). The infected cells are immortalized and transformed and, *in vivo*, give rise to various B-cell malignancies. This process depends on viral gene products (Volume II, Chapter 6). However, induction of synthesis of cellular miRNA-155 is also important. This miRNA, which is present at high concentrations in human B-cell lymphomas and tumors, is encoded by a gene first identified as a common integration site of the retrovirus avian leukosis virus (Volume II, Chapter 6). The concentration of miRNA-155 is increased substantially in B cells transformed by Epstein-Barr virus in culture. Inhibition of its activity in such cells, by introduction of excess short, complementary RNA (an RNA "sponge"), inhibited cell proliferation and induced apoptosis. Although a number of mRNA targets of miRNA-155 have been identified, the mechanism by which this small RNA promotes oncogenic transformation has not yet been elucidated.

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BOX 10.11

DISCUSSION

How the guide strand of siRNAs is identified

During formation of RNA-induced silencing complexes, one strand of the double-stranded siRNA, the guide strand, is retained while the second (often called the passenger strand) is destroyed. siRNAs contain many different sequences, raising the question of how guide and passenger strands are distinguished.

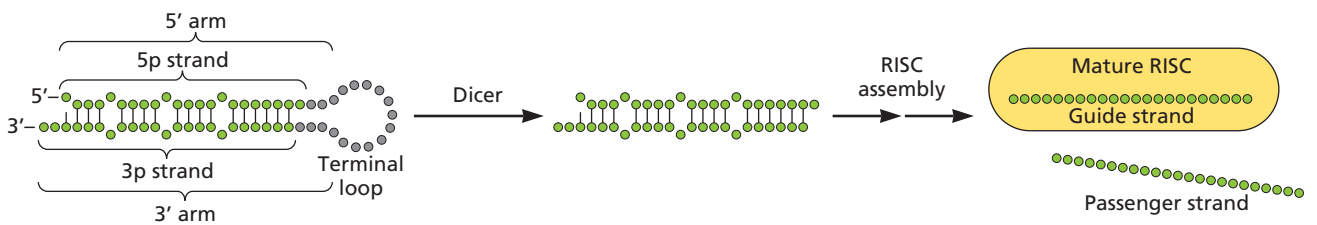
The answer came from efforts to identify siRNAs that are most effective in inducing mRNA cleavage when introduced into cells. It was observed that, in such siRNAs, the 5′ end of the guide RNA forms thermodynamically less stable base pairs than the 3′ end. Naturally occurring siRNAs and miRNAs exhibit this same asymmetry. As base pairs at the ends of double-stranded nucleic acids transiently break and re-form (they are said to "breathe"), this property might favor recognition of the transiently single-stranded 5′ end of the guide strand. Regardless, the less stable base pairs at the 5′ end of the guide strand favor unwinding from that end, which requires Dicer and RNA-binding proteins, such as TAR RNA-binding protein (Trbp). Subsequent studies demonstrated that the latter proteins bind to the siRNA end that contains the most-stable base pairs and therefore determines the orientation of the Dicer–RNA-binding protein heterodimer on the duplex siRNA.

Khvorova A, Reynolds A, Jayasena SD. 2003. Functional siRNAs and miRNAs exhibit strand bias. *Cell* **115:**209–216.

Schwarz DS, Hutvágner G, Du T, Xu Z, Aronin N, Zamore PD. 2003. Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115:**199–208.

Tomari Y, Matranga C, Haley B, Martinez N, Zamore PD. 2004. A protein sensor for siRNA asymmetry. *Science* **306:**1377–1380.

Processing and assembly into Risc of a guide strand of a pre-miRNA and release (for rapid degradation) of the passenger strand are illustrated. Which strand functions as the guide is independent of the original orientation in the pre-miRNA, but determined by properties of the small RNA duplex produced by Dicer, such as thermodynamic asymmetry, and the identity of the nucleotide at the 5′ end.



Cellular miRNA-122 Promotes Liver-Specific Reproduction of Hepatitis C Virus

The flavivirus hepatitis C virus is a widespread human pathogen that can establish chronic infection of the liver and is associated with the development of cirrhosis and hepatocellular carcinoma. The liver-specific reproduction of this virus is facilitated by cellular miRNA-122, the most abundant miRNA in hepatocytes: inhibition of the function of this miRNA impairs accumulation and expression of (+) strand viral RNA in hepatocytes in culture and in infected chimpanzees. The stimulation of hepatitis C virus reproduction by miRNA-122 is **not** the result of inhibition of synthesis of cellular proteins that directly or indirectly block the infectious cycle but rather of unusual virus-specific adaptations of base pairing with miRNAs: the miRNA both stabilizes (+) strand viral RNAs and impairs their translation, freeing them to serve as templates for genome replication (Box 10.12).

Viral Micro-RNAs

The first viral miRNAs were identified in 2004, by cloning and sequencing of small RNA molecules made in cells latently infected by Epstein-Barr virus. Subsequently, miRNAs of a number of other viruses have been described. Such RNAs are typically identified by combining computational methods that screen viral genomes for sequences with the properties of pre-miRNAs with assays for detection, such as high-throughput sequencing of low-molecular-weight RNAs isolated from infected cells. miRNA databases—e.g., miRBase (http://www.mirbase.org/index.shtml) and VIRmiRNA http://crdd.osdd.net/servers/virmirna/ hundreds of viral miRNAs, but the functions of the great majority are not yet known. We therefore describe a few well-characterized examples to illustrate what are likely to be general roles of such viral gene products.

Polyomavirus miRNAs That May Promote Persistence of Infected Cells

The genomes of simian virus 40 and the closely related human polyomaviruses JC virus and BK virus contain the sequence for a single pre-miRNA, which is transcribed as part of the late pre-mRNA and, unusually, processed to produce two miRNAs (Fig. 10.22). The miRNAs induce cleavage and degradation of the mRNA for the early gene product, large T antigen (LT). Mutations designed to disrupt the simian virus 40 pre-miRNA secondary structure prevented both viral miRNA synthesis and LT mRNA degradation and reduced the

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BOX 10.12

DISCUSSION

A cellular miRNA that protects the hepatitis C virus genome from degradation and promotes its replication

miRNAs typically interact with target sequences in the 3′ untranslated regions of mRNAs. However, miRNA-122 base pairs with two complementary sequences present in the 5′ untranslated region of (+) strand hepatitis C virus, and in so doing protects the genome, which lacks a 5′ cap or 3′ poly(A) sequence, from degradation by Xrn1: mutated genomic RNA that lacks the miRNA-122 binding sites is less stable than the wild type, unless production of Xrn1 is also inhibited. Protection of the (+) strand RNA from degradation correlates with recruitment of a Risc-like complex to its 5′ end.

Removal of Xrn1 is not sufficient to restore replication of mutant viral genomes that lack the miRNA-122 binding sites, indicating that the cellular miRNA contributes to efficient reproduction of hepatitis C virus by one or more additional mechanisms. When the concentration of miRNA-122 was increased in infected cells, the steady-state concentrations of both viral mRNA and viral protein also increased, regardless of the presence or absence of Xrn1. Subsequent kinetic analyses of the accumulation of newly synthesized viral mRNA and protein established that the miRNA stimulated viral mRNA synthesis, but not its translation. Such stimulation is the result of competition for binding to the 5′ end of the (+) mRNA between miRNA-122 and a cellular protein that facilitates translation of this mRNA, Pcbp2 [poly(rC)-binding protein 2].

Antagonists of miRNA-122, such as antisense oligonucleotides, block virus reproduction with no harmful effects in animal models, and are currently in clinical trials in humans.

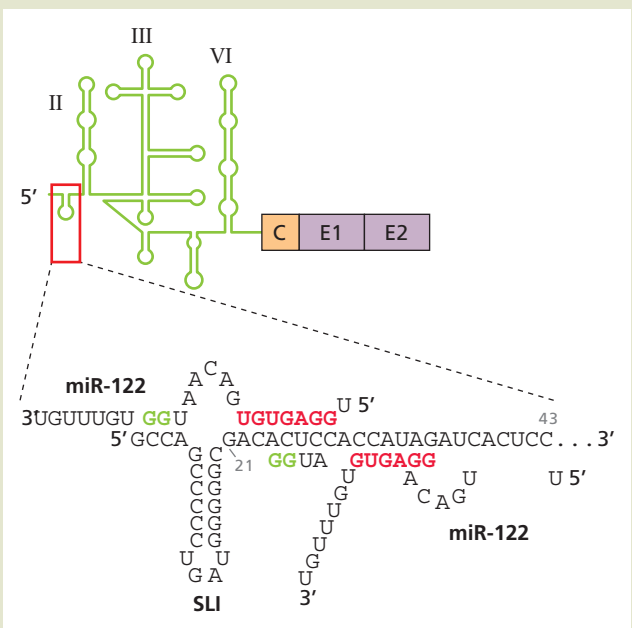
Gottwein E. 2013. Roles of microRNAs in the life cycles of mammalian viruses. *Curr Top Microbiol Immunol* **371:**201–227.

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Shimakami T, Yamane D, Jangra RK, Kempf BJ, Spaniel C, Barton DJ, Lemon SM. 2012. Stabilization of hepatitis C virus RNA by an Ago2-miR-122 complex. *Proc Natl Acad Sci U S A* **109:**941–946.

The organization of the 5′ end of the (+) strand hepatitis C virus genome is summarized at the top, but not to scale. The secondary structures shown are consistent with the results of structural and mutational studies. The expansion illustrates the base pairing of cellular miRNA-122 (miR-122) with viral sequences on either side of stem-loop 1 (SL1), with the miRNA sequence shown in red. Adapted from E. Gottwein, *Curr Top Microbiol Immunol* **371:**201–227, 2013, with permission.



susceptibility of infected cells to killing by cytotoxic T cells specific for LT. However, no effects of such mutations on simian virus 40 reproduction in cells in culture or *in vivo* could be discerned. More-recent studies show that these miRNAs limit LT production and genome replication of an archetypical strain of BK virus that has not acquired rearrangement of sequences that control viral gene expression (Box 10.13). It is therefore possible that this function can promote the establishment of persistent BK virus infection in cells of the urinary tract.

Latency-Associated miRNAs of Herpesviruses

It is striking that pre-miRNA-coding sequences that are expressed in latently infected cells have been identified in regions of several alpha-, beta-, and gammaherpesviral genomes. For example, some 12 miRNAs are made in cells latently infected by human herpesvirus 8, which is a causative agent of Kaposi's sarcoma and B-cell lymphoma (Volume II, Chapter 6). The latency-associated miRNAs are processed from three overlapping transcripts synthesized from viral promoters active in latently infected cells. Ectopic expression of the viral miRNA coding region reduced substantially the concentrations of eight cellular mRNAs for proteins that participate in regulation of proliferation, immune responses, and apoptosis, such as that encoding the cyclin-dependent kinase inhibitor p21, which blocks cell cycle progression (Volume II, Chapter 6). Other human herpesvirus 8 miRNAs

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BOX 10.13

WARNING

BK virus miRNAs and viral early gene expression: impact of sequence variations acquired during laboratory adaptation

Mutations that prevent synthesis of the viral miRNAs had no effect on the reproduction of simian virus 40 or mouse polyomavirus. However, subsequent experiments with BK virus suggest that these failures may be the result of noncoding sequence rearrangements present in the laboratory-adapted strains used in these studies.

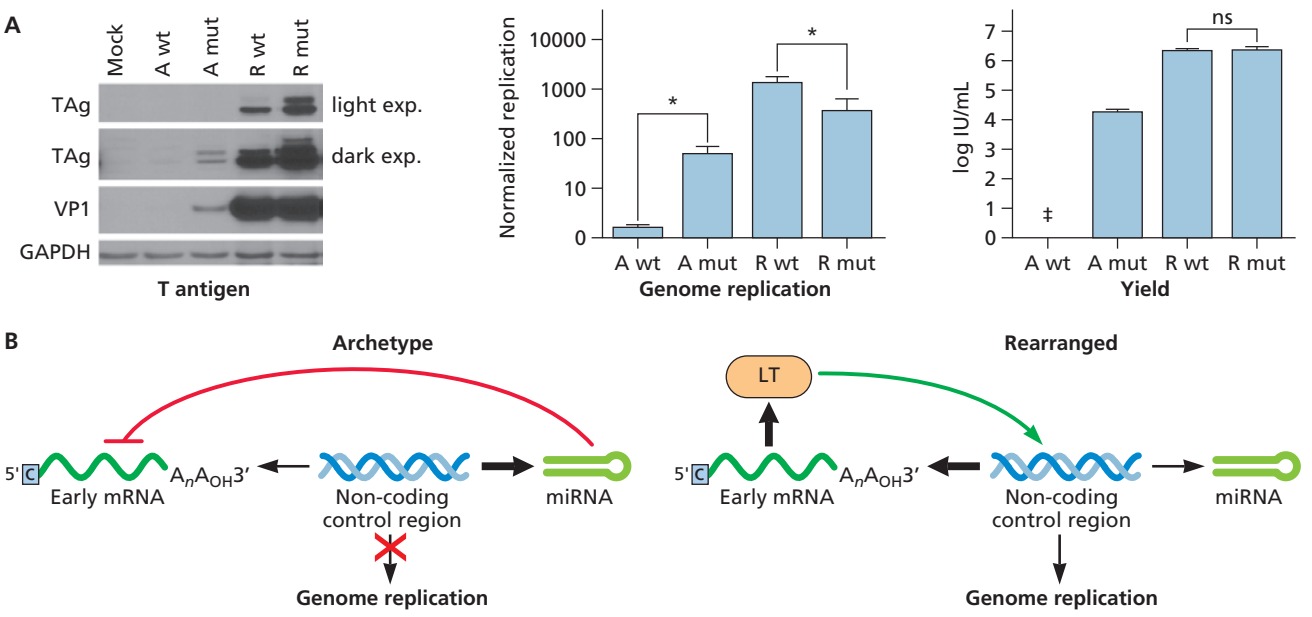
The human polyomavirus BK virus is associated with persistent infection in healthy individuals and diseases of the kidney in immunosuppressed transplant patients. The organization of the coding and control sequences in the BK virus genome is like that of the closely related simian virus 40 (Appendix, Fig. 23). A strain of the virus isolated from healthy people, designated the archetype strain, does not produce progeny virus particles in primary renal proximal tubule epithelia cells in culture, and both LT and viral DNA synthesis are extremely inefficient. In contrast, variants with deletions and duplications within the noncoding control region reproduce efficiently in such cells. As the viral miRNAs restrict production of LT (Fig. 10.22), the origin recognition protein necessary for viral DNA synthesis, their role in regulating viral replication was compared in archetype and variant viruses.

Mutations that prevent processing of the miRNAs, but do not alter the overlapping coding sequence of LT (Fig. 10.22), were introduced into the genomes of these viruses, and the inhibition of miRNA synthesis was confirmed. In the absence of miRNAs, accumulation of LT (and its mRNA), viral DNA synthesis, and production of virions were increased significantly in primary renal epithelial cells infected by archetype BK virus (panel A of the figure). In contrast, although LT concentration was also increased somewhat and viral genome replication decreased, the mutations did not alter the yield of the variant BK virus. It was also established that the viral miRNAs can be made during the early phase of infection. Furthermore, their production is impaired, while early promoter activity is increased, by the rearrangements in the variant viral genome.

These observations led to a model in which the combination of the action of the viral miRNAs and a weak early promoter severely limit LT synthesis during the early phase of archetype BK virus infection (panel B). Consequently, the genome cannot replicate to a degree sufficient for assembly of progeny virus particles. These control parameters are reversed (strong early promoter, inefficient miRNA synthesis) by alterations in variant BK virus genomes, resulting in accumulation of high concentrations of LT and efficient genome replication and virus particle production. Limitation of LT synthesis by viral miRNAs during the early phase of infection *in vivo* is likely to be important for establishing the persistent infection characteristic of archetype BK virus.

Broekema NM, Imperiale MJ. 2013. miRNA regulation of BK polyomavirus replication during early infection. *Proc Natl Acad Sci U S A* **110:**8200–8205.

(A) Accumulation of LT (TAg) examined by immunoblotting, genome accumulation, and virus yield in viral DNA synthesis in primary renal proximal tubule epithelial cells infected by wild-type (wt) archetype BK virus (A) or a variant with a rearranged noncoding region (R) or derivations carrying mutations that prevent production of the viral miRNAs (mut). GAPDH, cellular glyceraldehyde-3-phosphate dehydrogenase (internal control); VP1, virion protein 1; ns, not significant; ‡, below the limit of detection; *, $P < 0.05$. **(B)** Model of control of archetype BK virus replication by miRNAs. Adapted from N. M. Broekema and M. J. Imperiale, *Proc Natl Acad Sci U S A* **110:**8200–8205, 2013, with permission. Panel A courtesy of M. Imperiale, University of Michigan Medical School.



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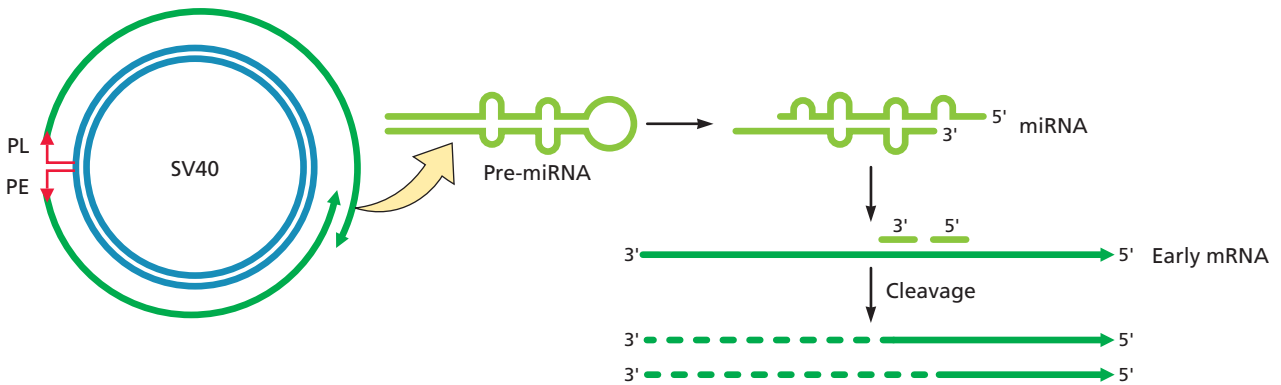


Figure 10.22 The miRNAs of simian virus 40. The circular simian virus 40 genome is shown at the left, with the positions of the early (P_E) and late (P_L) promoters and the primary transcripts indicated. As shown, the 3′ ends of early and late pre-mRNAs are encoded by opposite strands of the same sequence. Downstream of its polyadenylation site (arrowhead), the late pre-RNA contains a pri-miRNA sequence that is processed to a 57-nucleotide pre-miRNA and then to two miRNAs, designated 3′ and 5′. Both are perfectly complementary to specific sequences in the early mRNAs that encode LT and induce its cleavage.

act as functional homologs of cellular miRNAs that promote cell survival and proliferation or prevent synthesis of the protein that activates expression of lytic genes, thereby helping to maintain latent infection.

Inhibition of Antiviral Defenses

The genomes of several herpesviruses encode miRNAs that have been implicated in blocking intrinsic and immune antiviral defenses. Examples include four Epstein-Barr virus miRNAs that impair production of specific proapoptotic proteins (Volume II, Chapter 3); several beta- and gamma-herpesviral miRNAs that protect infected cells against natural killer cells (Volume II, Chapter 3); and human herpesvirus 8 miRNA-K5 and -K9, which target components of signaling pathways that induce production of interferons and proinflammatory cytokines in response to infection.

Viral Gene Products That Block RNA Interference

siRNAs provide antiviral defenses in plants and invertebrates (Volume II, Chapter 3). As might therefore be anticipated, the genomes of viruses that replicate in these organisms encode proteins that suppress RNA silencing, such as the tomato bushy stunt virus and Flock house virus double-stranded-RNA-binding proteins p19 and B2, respectively. Viral inhibition of the production or function of miRNAs in mammalian cells appears to be rare, so far reported only for adenoviruses and poxviruses, such as vaccinia virus. The enormous quantities of the small virus-associated (VA) RNAs that accumulate in adenovirus-infected cells compete with cellular pre-miRNAs for binding to the active site of Dicer. Production of cellular miRNAs is impaired, as Dicer processes the viral RNAs to produce viral miRNAs (of unknown function). The VA RNAs, which are synthesized by RNA polymerase III in the nucleus, also block export of the Dicer mRNA to the cytoplasm by binding to Xpo5, a function that contributes to optimal reproduction of the virus. In contrast, degradation of cellular miRNAs is induced in vaccinia virus-infected cells. The viral poly(A) polymerase, which is both necessary and sufficient, targets cellular miRNAs for destruction by catalyzing the addition of short (<10 nucleotides) oligo(A) segments to their 3′ ends. This mechanism may represent a virus-specific variant of the recently described cellular system in which pre-miRNAs are targeted for destruction by addition of 3′ oligo(U).

Perspectives

Many of the molecular processes required for reproduction of animal viruses, including such virus-specific reactions as synthesis of genomic RNAs and mRNAs from an RNA template, were foretold by the properties of the bacteriophages that parasitize bacterial cells. In contrast, the covalent modifications necessary to produce functional mRNAs in eukaryotic cells were without precedent when discovered in viral systems. Study of the processing of viral RNAs has yielded much fundamental information about the mechanisms of capping, polyadenylation, and splicing. More recently, viral systems have provided equally important insights into export of mRNA from the nucleus to the cytoplasm. Perhaps the most significant lesson learned from the study of viral mRNA processing is the importance of these reactions in the regulation of gene expression.

Viral RNA processing can be regulated passively, by differences in the concentrations or activities of specific cellular components in different cell types, or actively by viral gene

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products. Several mechanisms by which viral gene products or RNAs can regulate or inhibit polyadenylation or splicing reactions, export of mRNA from the nucleus, or mRNA stability have been quite well characterized. However, our understanding of regulation of viral gene expression via RNA-processing reactions is far from complete: the mechanisms of action of several critical viral regulatory proteins have not been fully elucidated, and many of the specific mechanisms deduced by using experimental systems have yet to be confirmed in virus-infected cells. Similarly, much remains to be learned about the benefits for virus reproduction of the increasing numbers of viral proteins now known to destroy or relocalize components of the cellular mRNA degradation machinery, or that are analogs of such components.

Since we prepared the previous edition of this book, the catalog of viral miRNAs has grown enormously and noncanonical functions of viral miRNAs have been discovered. Furthermore, we can now document some fascinating instances of cellular miRNAs that shape the outcome of virus-host cell interactions. It therefore seems likely that the continued exploration of the molecular and physiological functions of viral miRNAs, currently unknown in most cases, will transform our understanding of the interplay among viruses and their hosts.

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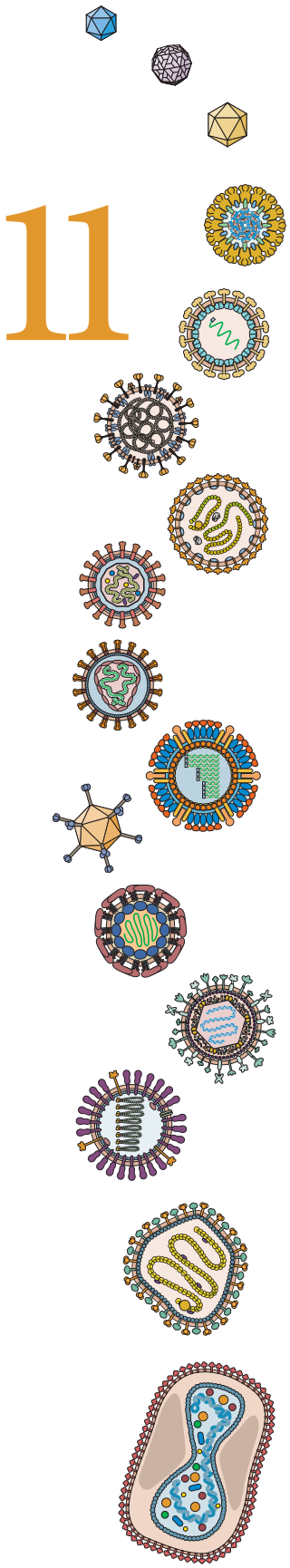
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11 Protein Synthesis

Introduction

Mechanisms of Eukaryotic Protein Synthesis
- General Structure of Eukaryotic mRNA
- The Translation Machinery
- Initiation
- Elongation and Termination

The Diversity of Viral Translation Strategies
- Polyprotein Synthesis
- Leaky Scanning
- Reinitiation
- Suppression of Termination
- Ribosomal Frameshifting
- Bicistronic mRNAs

Regulation of Translation during Viral Infection
- Inhibition of Translation Initiation after Viral Infection
- Regulation of eIF4F
- Regulation of Poly (A)-Binding Protein Activity
- Regulation of eIF3
- Interfering with RNA
- Stress-Associated RNA Granules

Perspectives

References

LINKS FOR CHAPTER 11

⏭ *Video: Interview with Dr. Ian Mohr*
http://bit.ly/Virology_Mohr

⏭ *California virology*
http://bit.ly/Virology_Twiv97

⏭ *Hantavirus protein replaces eIF4F*
http://bit.ly/Virology_1-22-09

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Translation is that which transforms everything so that nothing changes.

GÜNTER GRASS

Introduction

No viral genome encodes a complete translational apparatus (Box 11.1). Consequently, translation of viral messenger RNAs (mRNAs) is wholly dependent on the host cell. To allow efficient production of viral proteins, the translational machinery is usually modified to ensure that viral mRNAs are preferentially translated. Viral mRNAs are also translated in noncanonical ways to maximize their coding potential and allow the production of multiple proteins from a single mRNA.

Studies of virus-infected cells have contributed considerably to our understanding of protein synthesis and its regulation. Before the advent of recombinant DNA technology, infected cells were a rich source of large quantities of relatively pure mRNAs for *in vitro* studies of protein synthesis. The 5′ cap structure was identified on a viral RNA, and new translation initiation mechanisms, such as internal ribosomal entry, were discovered during studies of infected cells. Our understanding of how the activity of the multisubunit cap-binding complex can be regulated originated from the finding that one of its subunits is cleaved in infected cells.

Translation is a universal process in which proteins are synthesized from the amino to the carboxy terminus from mRNA templates read in the 5′ → 3′ direction. Each amino acid is specified by a genetic code consisting of three bases, a **codon**, in the mRNA. Translation takes place on **ribosomes**, and **transfer RNAs (tRNAs)** are the adapter molecules that link specific amino acids with individual codons in the mRNA. This chapter explores the basic mechanisms by which translation occurs in eukaryotic cells, the many ways by which viral mRNAs are translated to expand the limited coding capacity in genomes of limited size, and how translation is regulated in infected cells.

Mechanisms of Eukaryotic Protein Synthesis

General Structure of Eukaryotic mRNA

With the exception of organelle and certain viral mRNAs, eukaryotic mRNAs begin with a 5′ 7-methylguanosine (m^7G) **cap structure** (Fig. 11.1; see also Fig. 10.2). It is joined to the second nucleotide by a 5′-5′ phosphodiester linkage, in contrast to the 5′-3′ bonds found in the remainder of the mRNA. The unique cap structure directs pre-mRNAs to processing and transport pathways, regulates mRNA turnover, and is required for efficient translation by the 5′-end-dependent mechanism. Eukaryotic mRNAs contain **5′ untranslated regions**, which may vary in length from 3 to >1,000 nucleotides, although they are typically 50 to 70 nucleotides long. Such 5′ untranslated regions often contain secondary structures (e.g., hairpin loops [see Fig. 6.2]) formed by base pairing of the RNA. These double-helical regions must be unwound to allow passage of 40S ribosomal subunits during translation.

Translation begins and ends at **initiation codons** and **termination codons**, respectively. The termination codon is followed by a **3′ untranslated region**, which can regulate initiation, translation efficiency, and mRNA stability. At the very 3′ end of the mRNA is a stretch of adenylate residues known as the **poly(A) tail**, which is added to nascent pre-mRNA. The poly(A) tail is necessary for efficient translation, and for interactions among proteins that bind both ends of the mRNA.

Most bacterial and archaeal mRNAs are **polycistronic**: they encode several proteins, and each open reading frame is separated from the next by an untranslated spacer region. The vast majority of eukaryotic mRNAs are **monocistronic**; i.e., they encode only a single protein (Fig. 11.1). A small number of eukaryotic mRNAs are functionally polycistronic, and there are different strategies for synthesizing multiple proteins from a single mRNA. Members of the virus family *Dicistroviridae* are unique because the virus particles contain true bicistronic mRNAs.

PRINCIPLES ***Protein synthesis***

- No viral genome encodes the complete translational apparatus.
- The majority of viral mRNAs are translated by 5′-end-dependent mechanisms, but there is appreciable variation in this process, including mimicry of the initiator transfer RNA (tRNA) in the viral genome.
- Some viral RNAs are translated by a 5′-end-independent mechanism in which ribosomes bind internally to internal ribosome entry sites (IRESs).
- IRESs require RNA-binding proteins for activity.
- (+) strand RNA genomes that lack caps and poly(A) tails require a 3′-cap-independent translational enhancer for protein synthesis.
- A variety of unusual translation mechanisms expand the coding capacity of viral genomes and allow the synthesis of multiple polypeptides from a single RNA genome.
- Alterations in the cellular translational apparatus are commonplace in virus-infected cells.
- RNA granules are cytoplasmic aggregates that are assembled in response to viral infection to sequester RNAs, and many virus infections inhibit their formation or function.

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BOX 11.1

TRAILBLAZER

Viral contributions to the translational machinery

Analysis of the nucleic acid of the largest DNA viruses challenges the belief that no viral genomes encode any part of the translational machinery. The 330- to 380-kbp DNA genome of viruses that infect the unicellular green alga *Chlorella* encode 10 to 15 tRNAs. These viral tRNAs are produced in infected cells, and some of them are aminoacylated, suggesting that they function during protein synthesis. These viral genomes also encode a homolog of elongation protein 3 that is synthesized in infected cells. DNA genomes of other giant viruses, including *Mimivirus*, *Pandoravirus*, and *Cafeteria roenbergensis* virus, encode multiple tRNAs, aminoacyl-tRNA synthetases, and a variety of initiation, elongation, and termination proteins, some of which have been shown to be functional.

These remarkable observations suggest that parts of the cellular translational machinery might be replaced by viral gene products. Support for this hypothesis comes from the observation that mimivirus-encoded translation termination proteins are synthesized by two recoding events: translational read-through and frameshifting. Although the amino acid sequences of these proteins are clearly eukaryotic, the regulatory features are specific to bacteria.

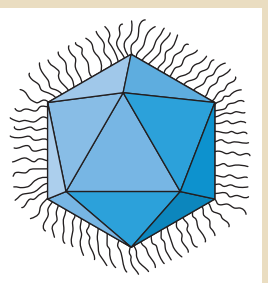
Why viral genomes encode gene products that participate in protein synthesis is not known. One possibility is that they modify the translation apparatus to favor the production of viral proteins. For example, the use of viral tRNAs may compensate for the low abundance of some tRNAs in host cells, allowing more efficient reproduction.

Jeudy S, Abergel C, Claverie JM, Legendre M. 2012. Translation in giant viruses: a unique mixture of bacterial and eukaryotic termination schemes. *PLoS Genet* **8:**e1003122. doi:10.1371/journal.pgen.1003122.

Nishida K, Kawasaki T, Fujie M, Usami S, Yamada T. 1999. Aminoacylation of tRNAs encoded by *Chlorella* virus CVK2. *Virology* **263:**220–229.

Raoult D, Audic S, Robert C, Abergel C, Renesto P, Ogata H, La Scola B, Suzan M, Claverie JM. 2005. The 1.2-megabase genome sequence of *Mimivirus*. *Science* **306:**1344–1350.

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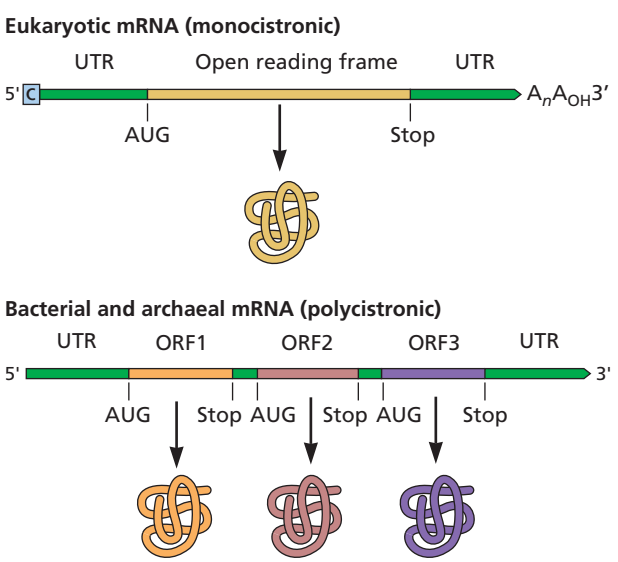


The Translation Machinery

Ribosomes

Mammalian ribosomes, the sites of protein synthesis, are composed of two subunits designated according to their sedimentation coefficients, 40S and 60S (Fig. 11.2A). The 40S subunit comprises an 18S rRNA molecule and 30 proteins, while the 60S subunit contains 3 rRNAs (5S, 5.8S, and 28S rRNAs) and 50 proteins. Actively growing mammalian cells may contain as many as 10 million ribosomes.

Figure 11.1 Structure of eukaryotic and bacterial/archaeal mRNAs. UTR, untranslated region; AUG, initiation codon; ORF, open reading frame; Stop, termination codon. Adapted from G. M. Cooper, *The Cell: a Molecular Approach* (ASM Press, Washington, DC, and Sinauer Associates, Sunderland, MA, 1997).



The mRNA moves past three sites on the ribosome, called A (aminoacyl or acceptor), P (peptidyl), and E (exit). The initiator tRNA enters at the P site, but all subsequent charged tRNAs enter the A site. The peptide bond is formed at the P site, while exit of the uncharged tRNA takes place at the E site.

Remarkably, the catalytic activity of ribosomes resides in RNA, not protein. After removal of 95% of the ribosomal proteins, the 60S ribosomal subunit can still catalyze the formation of peptide bonds; the peptidyltransferase center, where peptide bonds are formed, contains only RNA. The ribosome is the largest known RNA catalyst, providing evidence for an RNA world in which RNA, not proteins, carried out chemical reactions. The protein components of ribosomes help fold the rRNAs properly, so that they can fulfill their catalytic function, and to position the tRNAs.

tRNAs

tRNAs are adapter molecules that align each amino acid with its corresponding codon on the mRNA. Each tRNA is 70 to 80 nucleotides in length and folds into a highly base-paired L-shaped structure (Fig. 11.2B). This shape is thought to be required for the appropriate interaction between tRNA and the ribosome during translation. The adapter function of tRNAs is carried out by two distinct regions of the molecule. At their 3′ ends, all tRNAs have the sequence 5′-CCA-3′, to

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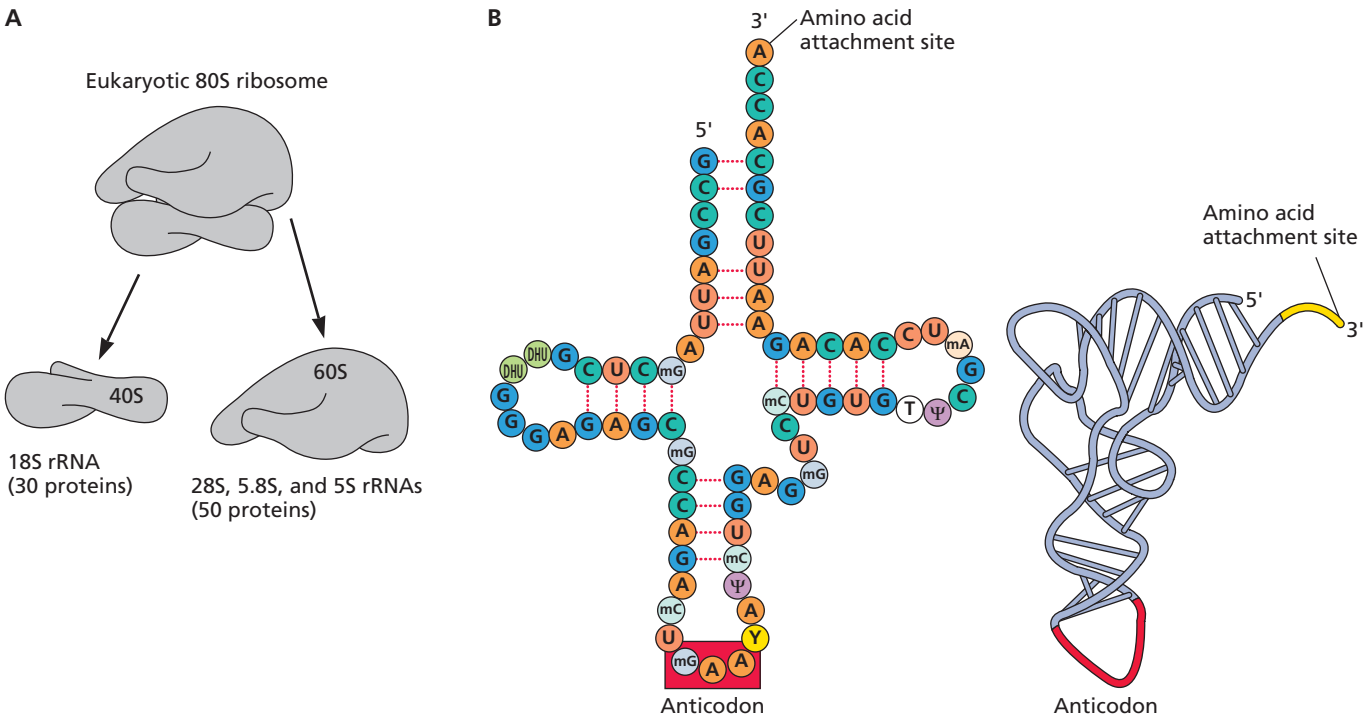


Figure 11.2 Ribosomes and tRNAs. (A) Model of a eukaryotic ribosome. The 80S ribosome consists of 60S and 40S subunits, which are made of ribosomal proteins and rRNAs. **(B)** Structure of tRNA. The model on the left shows how base pairing among the nucleotides of the tRNA results in a cloverleaf-like structure. Modified bases include methylguanosine (mG), methylcytosine (mC), dihydrouridine (DHU), ribothymidine (T), a modified purine (Y), and pseudouridine (Ψ). On the right is a folded representation showing the L-shaped structure. Adapted from G. M. Cooper, *The Cell: a Molecular Approach* (ASM Press, Washington, DC, and Sinauer Associates, Sunderland, MA, 1997).

which amino acids are covalently linked by **aminoacyl-tRNA synthetase**. Each of these enzymes recognizes a single amino acid and the correct tRNA. At the opposite end of the tRNA is the **anticodon loop**, which base pairs with the mRNA template. The accuracy of protein synthesis is maintained by two different proofreading mechanisms: faithful incorporation of amino acids depends on the specificity of codon-anticodon base pairing, as well as on the correct attachment of amino acids to tRNAs by aminoacyl-tRNA synthetases.

Translation Proteins

Many nonribosomal proteins are required for eukaryotic translation. Some form multisubunit assemblies containing as many as 11 different proteins, while others function as monomers. Translation can be separated experimentally into three distinct stages: initiation, elongation, and termination. The proteins that participate at each stage are named eukaryotic initiation, elongation, and termination proteins. These proteins are named in the same way as their bacterial and archaeal counterparts, with the prefix "e" to designate those of eukaryotic origin. The amino acid sequences of these proteins are conserved from yeasts to mammals, indicating that the mechanisms of translation are similar throughout eukaryotes.

Initiation

The majority of regulatory mechanisms function during initiation, because it is the rate-limiting step in the translation of most mRNAs (see "Regulation of Translation during Viral Infection" below). At least 11 initiation proteins participate in this energy-dependent process. The end result is formation of a complex containing the mRNA, the ribosome, and the initiator Met-tRNA$_i$, in which the reading frame of the mRNA has been set. The 80S ribosome, which is the predominant species in cells, must be dissociated, because it is the 40S subunit that participates in initiation. Three initiation proteins, eIF1A, eIF3, and eIF6, promote such dissociation.

There are two mechanisms by which ribosomes bind to mRNA in eukaryotes. In 5′-end-dependent initiation, by which the majority of mRNAs are translated, the initiation complex binds to the 5′ cap structure and moves, or scans, in a 3′ direction until the initiating AUG codon is encountered. In contrast, during 5′-end-independent initiation, the initiation complex binds at, or just upstream of, the initiation codon.

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Internal ribosome entry sites were first discovered in picornavirus mRNAs, and are now known to be present in some cellular mRNAs.

5′-End-Dependent Initiation

How ribosomes assemble at the correct end of mRNA. The first step in the 5′-end-dependent initiation pathway is recognition of the m^7G cap by the cap-binding protein, eIF4E (Fig. 11.3). eIF4G acts as a scaffold between the cap structure and the 40S subunit, which associates with the mRNA via an interaction of eIF3 with the C-terminal domain of eIF4G. This important adapter molecule was first discovered as the target of proteolytic cleavage in poliovirus-infected cells, a modification that results in the inhibition of host protein synthesis. After binding near the cap, the 40S ribosomal subunit, which is part of a **preinitiation complex** that includes Met-tRNA$_i$ and other initiation proteins, moves in a 3′ direction on the mRNA in a process called **scanning**. Such movement depends upon a conformation of the 40S ribosomal subunit that allows processive motion, and unwinding of double-stranded structures to permit the RNA to thread through the ribosome and expose codon triplets (see "The role of mRNA secondary structure in translation" below). Scanning is a combination of a series of forward and backward movements with overall net movement in the 5′ → 3′ direction. When the preinitiation complex reaches the AUG initiation codon, an event detected by the second two bases of Met-tRNA$_i$ with the assistance of eIF1 and eIF1A, GTP is hydrolyzed and initiation proteins are released, allowing the 60S ribosomal subunit to associate with the 40S subunit to form the 80S initiation complex.

Role of the poly(A) tail in initiation. The presence of a poly(A) tail can stimulate mRNA translation. This effect is a consequence of interactions between proteins associated with the 5′ and 3′ ends of the mRNA, which promote 40S subunit

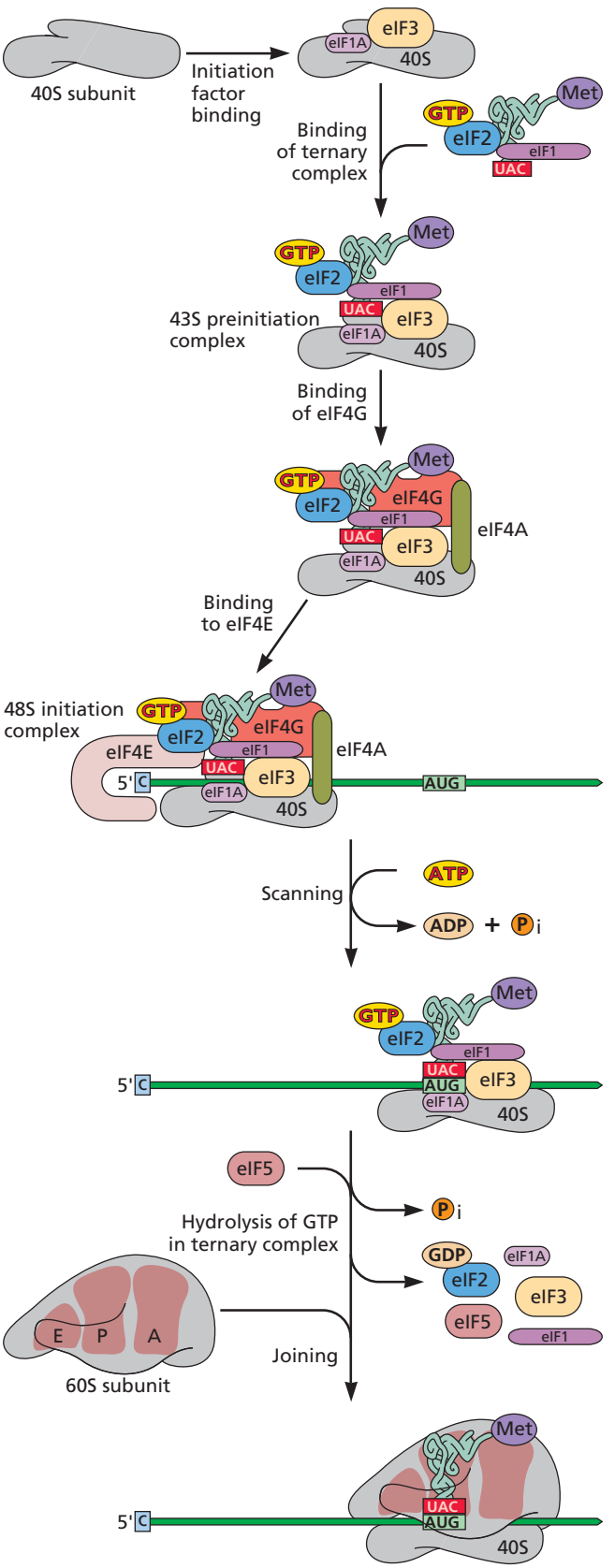
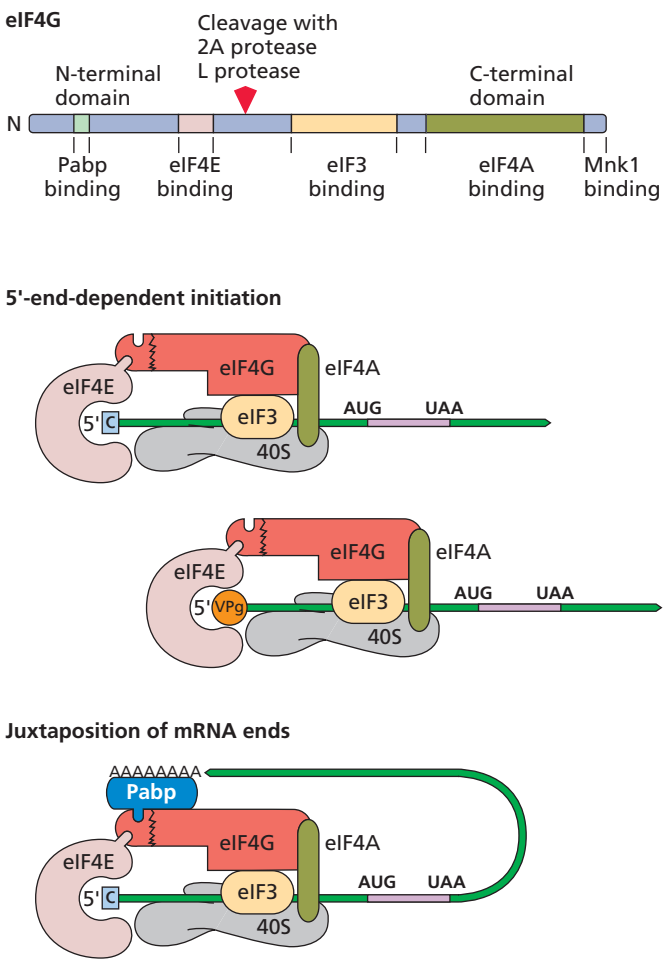


Figure 11.3 5′-cap-dependent assembly of the initiation complex. Initiation proteins eIF3 and eIF1A bind to free 40S subunits to prevent their association with the 60S subunit, while interaction of eIF6 (not shown) with the larger subunit prevents it from associating with the 40S subunit. eIF4F, which consists of three proteins, eIF4A, eIF4E, and eIF4G, binds the cap via the eIF4E subunit, and the ribosome binds a ternary complex containing eIF2, GTP, and Met-tRNA$_i$, forming a 43S preinitiation complex. The ribosome then binds eIF4G via eIF3. Alternatively, eIF4G may first join the 43S preinitiation complex and then bind the mRNA via eIF4E bound to the cap. The 40S subunit then scans down the mRNA until the AUG initiation codon is reached. eIF1 and eIF1A are required for selection of the correct AUG initiation codon. eIF5 triggers GTP hydrolysis, eIF2 bound to GDP is released along with other initiation proteins, and the 60S ribosomal subunit joins the complex. The ribosomal A site binds the aminoacylated tRNA; the P site binds the peptidyl-tRNA, and the uncharged tRNA leaves at the E site. Adapted from G. M. Cooper, *The Cell: a Molecular Approach* (ASM Press, Washington, DC, and Sinauer Associates, Sunderland, MA, 1997).

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recruitment. Such interactions were first demonstrated in the yeast *Saccharomyces cerevisiae*, in which poly(A)-binding protein Pabp1 is required for efficient mRNA translation. Stimulation of translation by poly(A) occurs by enhancing the binding of 40S ribosomal subunits to mRNA. Pabp1 interacts with the N terminus of eIF4G (Fig. 11.4). Alteration of this binding site on eIF4G destroys stimulation of translation by poly(A). These results have led to a model in which Pabp1, bound to the poly(A) tail, associates with eIF4G bound to the 5′ cap, stabilizing the interaction and assisting in recruitment of 40S subunits (Fig. 11.4). A consequence of these interactions is that the 5′ and 3′ ends of the mRNA are brought into close proximity.

Figure 11.4 5′-end-dependent initiation. (Top) Schematic of eIF4G. Adapted from S. J. Morley et al., *RNA* **3:**1085–1104, 1997, with permission. (Middle) Model of initiation complex assembly. eIF4F is brought to the mRNA 5′ end by interaction of eIF4E with the cap structure (top) or genome-linked VPg (middle). The N terminus of eIF4G binds eIF4E, and the C terminus binds eIF4A. The 40S ribosomal subunit binds to eIF4G indirectly via eIF3. (Bottom) 5′-end-dependent initiation is stimulated by the poly(A)-binding protein Pabp1, which interacts with eIF4G. This interaction may bring the mRNA ends together and facilitate formation of the initiation complex at the 5′ end. Adapted from M. W. Hentze, *Science* **275:**500–501, 1997, with permission.



Some viral mRNAs, such as those of certain plant viruses, lack a 5′-terminal cap and 3′ poly(A) sequence. Nevertheless, the ends of these mRNAs are brought together by base pairing between discrete sequences in the 5′ and 3′ untranslated regions. Translation of mRNA of the flavivirus dengue virus, which has a 5′ cap structure but lacks a 3′ poly(A) sequence, may also depend on complementarity between sequences in the untranslated regions.

The juxtaposition of mRNA ends might be a mechanism to ensure that only intact mRNAs that contain a 5′ cap and 3′ poly(A) are translated. Such structures could also stabilize mRNA, by preserving the interaction among the translation initiation proteins associated with the ends, and hence sequestering them to attack by exonucleases. Translation reinitiation might also be stimulated by such an arrangement: once the ribosome terminates translation, it might be repositioned at the AUG initiation codon rather than dissociating from the mRNA template.

VPg-dependent ribosomal recruitment. The 40S ribosomal subunit appears to be brought to the mRNAs of members of the *Potyviridae* and the *Caliciviridae* via interactions with VPg, the small protein linked to the first base of the RNA (Fig. 6.11). VPg of the plant virus turnip mosaic virus (*Potyviridae*) binds eIF4E, thereby recruiting eIF4G, eIF3, and the 40S ribosomal subunit to the mRNA (Fig. 11.4). In cells infected with members of the *Caliciviridae*, VPg binds both eIF4E and eIF3. Such interactions may also facilitate selective translation of viral mRNAs, although the mechanisms involved have not been elucidated.

The role of mRNA secondary structure in translation. Translation efficiency is reduced by the presence of a stable secondary structure in the mRNA 5′ untranslated region. There are at least two reasons for this effect. If an RNA stem-loop structure is adjacent to the 5′ cap, it can inhibit binding of the 40S ribosomal subunit. In addition, the presence of secondary structure blocks ribosome movement toward the initiation codon.

The ATP-dependent RNA helicase activity of eIF4A, assisted by eIF4B, unwinds intramolecular regions of double-stranded RNA (dsRNA) near the 5′ end of the mRNA, allowing the 43S preinitiation complex to bind. The helicase may also migrate in a 3′ direction, unwinding dsRNA and enabling movement of ribosomes. mRNAs with less secondary structure in the 5′ untranslated region have a reduced requirement for RNA helicase activity during translation, and hence are less dependent on the cap structure, which brings the helicase to the mRNA. Dependence of translation on the cap can be measured experimentally by determining the effect on protein synthesis of cap analogs, such as m^7GDP and m^7GTP. These compounds inhibit 5′-end-dependent

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initiation competitively by binding to eIF4E. For example, the 5′ untranslated region of alfalfa mosaic virus (a plant bromovirus) RNA segment 4 is largely free of secondary structure, and translation of this mRNA is quite resistant to inhibition by cap analogs.

Choosing the initiation codon. The selection of the initiating AUG codon depends on both its position in the mRNA and the surrounding nucleotide sequence. For >90% of mRNAs, translation initiates at the 5′-proximal AUG codon. If the 5′-proximal AUG codon is mutated so that it cannot serve as an initiation codon, translation starts at the next downstream AUG. Insertion of an AUG codon upstream of the initiating codon leads to initiation at the more 5′-proximal site. The efficiency of initiation is influenced by the nucleotide sequence surrounding this codon. Studies of the effects of mutating these sequences have shown that the consensus sequence 5′-GCC**A**CCAUGG-3′ is recognized most efficiently in mammalian cells: the presence of a purine at the −3 position (boldface) is most important. However, only 5% of eukaryotic mRNAs contain this ideal consensus sequence: most have suboptimal sequences that result in less-efficient translation. This finding indicates that not all mRNAs must be translated at maximal efficiency, but rather only at levels appropriate for the function of the protein product. If a very poor match to this consensus sequence is present, the AUG codon may be passed over by the ribosome and initiation may occur farther downstream (see "The Diversity of Viral Translation Strategies" below).

Methionine-independent initiation. The structural proteins of some viruses begin not with methionine but with glutamine (CAA), proline (CCU), or alanine (GCU or GCA). Initiation of synthesis of these viral proteins does not require Met-tRNA$_i$ or the ternary complex, because the viral mRNA mimics the structure of tRNA (Fig. 11.5A). The tRNA-like structure occupies the P site of the ribosome, allowing initiation to take place within the A site. These mRNAs require no translation initiation proteins, and can bind ribosomes and induce them to enter the elongation phase of translation. Methionine-independent initiation of the mRNA of turnip yellow mosaic virus (a *Tymovirus*) is accomplished in a similar way, except that the tRNA-like structure is located in the 3′ untranslated region of the viral RNA (Fig. 11.5B). The tRNA-like structure is aminoacylated with valine, which is incorporated as the first amino acid of the viral polyprotein.

Ribosome shunting. Stable RNA secondary structures in 5′ untranslated regions may inhibit scanning of 40S ribosomes. In some RNAs, such hairpin structures are not inhibitory because ribosomes bypass them. This process, called **ribosome shunting**, may be dependent or independent

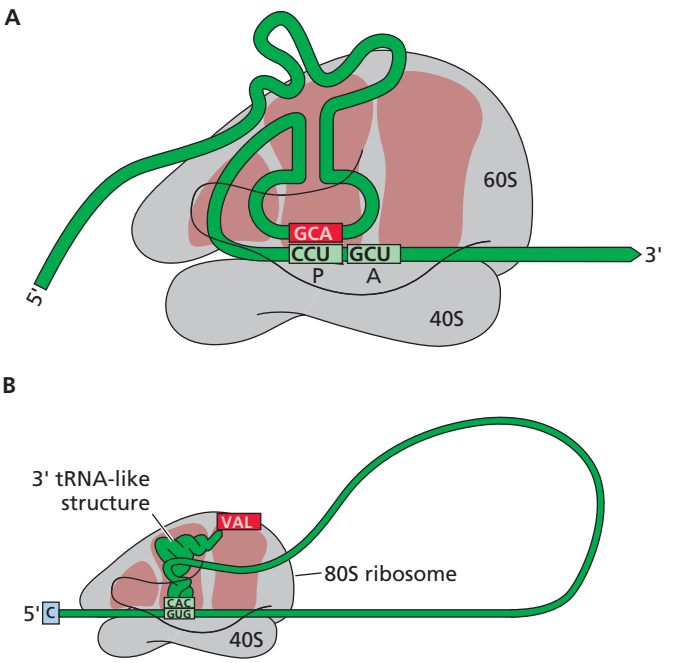
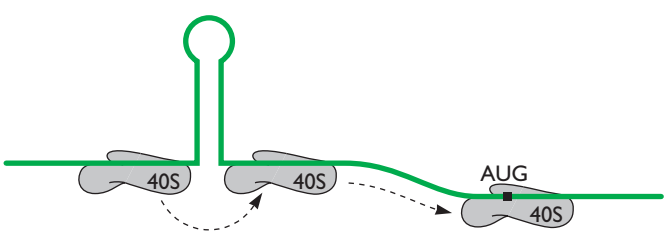


Figure 11.5 Two mechanisms of methionine-independent initiation. (A) The viral mRNA of picornavirus-like viruses of insects mimics the structure of tRNA, which occupies the P site of the ribosome, allowing initiation to take place within the A site. Adapted from M. Bushell and P. Sarnow, *J Cell Biol* **158:**395–399, 2002, with permission. **(B)** A tRNA-like structure in the 3′ untranslated region of turnip yellow mosaic virus RNA, aminoacylated with valine, occupies the P site of the ribosome. Adapted from S. Barends et al., *Cell* **112:**123–129, 2003, with permission.

of viral proteins. Shunting on the 35S cauliflower mosaic virus RNA requires translation of a very short upstream open reading frame on the same viral mRNA. Upon termination of translation of this open reading frame, ribosomes bypass a 480-nucleotide stem-loop structure that includes 8 AUG codons, and resume scanning just beyond the structure (Fig. 11.6). The ability to bypass this structure is thought to be a consequence of initiation proteins retained

Figure 11.6 Hypothetical model of ribosome shunting. The 40S ribosomal subunit binds to the mRNA by a cap-dependent mechanism and then bypasses regions of the mRNA with secondary structure to reach the AUG initiation codon. Shunting elements, such as the loops in the figure, and viral or cellular proteins may direct ribosome movement.



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on the 40S subunit after translation, together with a temporary loss of other proteins that allow discontinuous scanning over the stem-loop base. In contrast, shunting on other viral mRNAs, including those of adenoviruses, paramyxoviruses, reoviruses, and hepadnaviruses, takes place in the absence of viral proteins.

5′-End-Independent Initiation

The internal ribosome entry site. The mRNAs of picornaviruses differ from most host cell mRNAs: they lack the 5′-terminal cap structure, and the 5′ untranslated regions are highly structured and contain multiple AUG codons. Infection of host cells by many picornaviruses results in the inhibition of translation of cellular mRNAs. These observations led to the hypothesis that translation of the mRNA of (+) strand picornaviruses was initiated by an unusual mechanism. It was suggested that the ribosome bound internally, rather than at the mRNA 5′ end. In an important experiment, the 5′ untranslated region of poliovirus mRNA was shown to promote internal binding of the 40S ribosomal subunit, and was termed the **internal ribosome entry site (IRES)** (Box 11.2).

An IRES has been identified in the mRNAs of all picornaviruses, in other viral mRNAs including those of pestiviruses and hepatitis C virus, and in some cellular mRNAs. Viral IRESs have been placed in five groups, depending on a variety of criteria, including primary sequence and secondary structure conservation, the location of the initiation codon, and activity in different cell types. There is very little nucleotide sequence conservation among members of the different groups, with the exception of a 25-nucleotide-long oligopyrimidine tract at the 3′ end of the IRES. Viral IRESs contain extensive regions of RNA secondary structure (Fig. 11.7). Although such secondary structure is not strictly conserved, it is of extreme importance for ribosome binding.

The discovery of the IRES makes even more puzzling the rarity of eukaryotic mRNAs that contain multiple long open reading frames (Fig. 11.1). In principle, all the open reading frames of a polycistronic mRNA can be translated in a eukaryotic cell as long as each frame is preceded by an IRES. Nevertheless, only one such naturally occurring polycistronic mRNA has been identified in eukaryotes, and it is not known if an IRES is present. Bicistronic mRNAs produced in the laboratory have been used in the expression of cloned genes (Box 11.3).

The mechanism of internal initiation. Different sets of translation initiation proteins are required for the function of various IRESs. Internal ribosome binding on the hepatitis A virus IRES requires all the initiation proteins, including eIF4E. At the other extreme, the intergenic IRES of cricket paralysis virus requires **none** of them. However, the activity of most IRESs depends on a subset of initiation proteins. Initiation on the type 1, 2, and 5 IRESs requires all except eIF4E, and either the carboxy-terminal two-thirds or the central one-third fragment of eIF4G. Both fragments contain binding sites for eIF3 and eIF4A (Fig. 11.8) and function better than the full-length protein in IRES-directed protein synthesis. In poliovirus-infected cells, eIF4G is cleaved, reducing the translation of most cellular mRNAs.

Initiation of translation via an IRES comprises binding of the 40S ribosomal subunit, followed by scanning to the initiation codon. Depending on the IRES, the 40S subunit may bind directly to the RNA or may be recruited to the IRES by means of interaction with translation initiation proteins (Fig. 11.8). For example, the cleavage products of eIF4G bind directly to the type 1 or type 2 IRES, and the 40S ribosomal subunit is recruited to the IRES via interaction with eIF3.

The IRESs of hepatitis C virus (Fig. 11.7C), some pestiviruses, and teschoviruses function very differently from those of picornaviruses. The formation of the 48S initiation complex on the mRNA is independent of eIF4A, eIF4B, and eIF4F. Purified 40S ribosomal subunits bind directly to stem-loop IIId of the hepatitis C virus IRES, and single point mutations in this structure abolish both the interaction and internal initiation. Translation requires formation of a 3-nucleotide base pair between a loop in the IRES and a helix in 18S rRNA. Addition of only Met-tRNA$_i$, eIF2, and GTP is required to assemble the 48S complexes. A dramatic conformational change in the 40S ribosomal subunit occurs when it binds the hepatitis C virus IRES, clamping the mRNA in place and setting the AUG initiation codon within the P site of the ribosome. The IRES also contacts the E site of the ribosome, where the deacylated tRNA is harbored after translocation of the 80S ribosome. Initiation of translation from the IRES of hepatitis C virus and related viruses therefore resembles initiation of translation of bacterial mRNAs.

The intergenic IRESs of picornavirus-like viruses of insects are bound by the 40S ribosome independent of initiation proteins, and translation does not begin at an AUG codon. The secondary structure of the IRES of these viruses mimics an uncharged tRNA, and mutations that destabilize the fold abrogate translation. The tRNA-like structure is recognized and bound by the 40S ribosomal subunit, placing the initiation codon within the A site instead of the P site (Fig. 11.5A). Initiation is therefore dependent on elongation proteins eEF1A and eEF2 and the appropriate aminoacylated tRNAs. Consequently, initiation from these IRESs is inhibited by the ternary complex (Met-tRNA$_i$–eIF2–GTP) and a high concentration of Met-tRNA$_i$. Furthermore, in cells infected with these viruses, recycling of eIF2-GDP is blocked and the concentration of the ternary complex is low, both consequences of eIF2α phosphorylation by the host as an antiviral defense. Cellular mRNA translation is inhibited, but the activity of the intergenic IRES is not reduced, because the ternary complex is not needed.

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BOX 11.2

EXPERIMENTS

Discovery of the IRES

The hypothesis that poliovirus mRNA is translated by internal ribosome binding was first tested by examining the translation of mRNAs containing two open reading frames (ORFs) separated by the poliovirus 5′ untranslated region (figure, panel A). The second ORF was efficiently translated only if it was preceded by the picornavirus 5′ untranslated region. It was concluded that ribosomes bind within the viral 5′ untranslated region, thereby permitting translation of the second ORF. The segment of the 5′ untranslated region that directs internal ribosome entry was called the IRES.

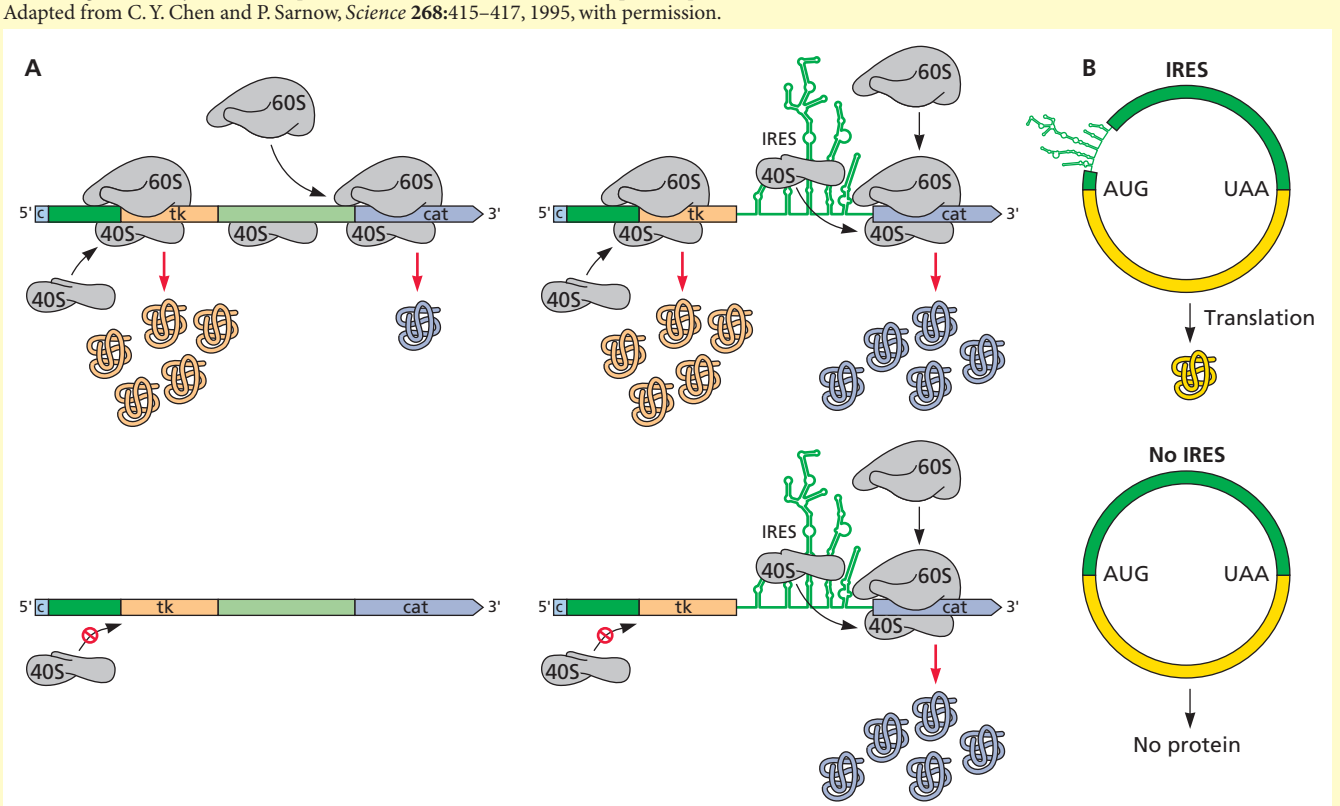
It had long been known that covalently closed circular mRNAs cannot be translated by 5′-end-dependent initiation. Translation by internal ribosome binding, however, should not require a free 5′ end. To test this hypothesis, circular mRNAs with and without an IRES were created. The circular mRNA was translated only if an IRES was present (figure, panel B). This experiment formally proved that translation initiation directed by an IRES occurs by internal binding of ribosomes and does not require a free 5′ end.

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Jang SK, Kräusslich HG, Nicklin MJ, Duke GM, Palmenberg AC, Wimmer E. 1988. A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *J Virol* **62:**2636–2643.

Pelletier J, Sonenberg N. 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* **334:**320–325.

Assays for an IRES. (A) Bicistronic mRNA assay. Plasmids were constructed that encode bicistronic mRNAs encoding the thymidine kinase (tk) and chloramphenicol acetyltransferase (cat) proteins separated by a spacer (light green) or a poliovirus IRES (dark green). Plasmids were introduced into mammalian cells by transformation. In uninfected cells containing either plasmid (top lines), both tk and cat proteins were detected, although without an IRES, cat synthesis was inefficient. Translation of cat from this plasmid probably occurs by reinitiation. In poliovirus-infected cells, 5′-end-dependent initiation is blocked (stop sign), and no proteins are observed without an IRES. cat protein is detected in infected cells when the IRES is present, demonstrating internal ribosome binding. Adapted from J. Pelletier and N. Sonenberg, *Nature* **344:**320–325, 1988, with permission. **(B)** Circular mRNA assay for an IRES. Circular mRNAs containing an ORF (yellow) were produced and translated *in vitro*. No protein product was observed unless an IRES was included in the circular mRNA. Adapted from C. Y. Chen and P. Sarnow, *Science* **268:**415–417, 1995, with permission.



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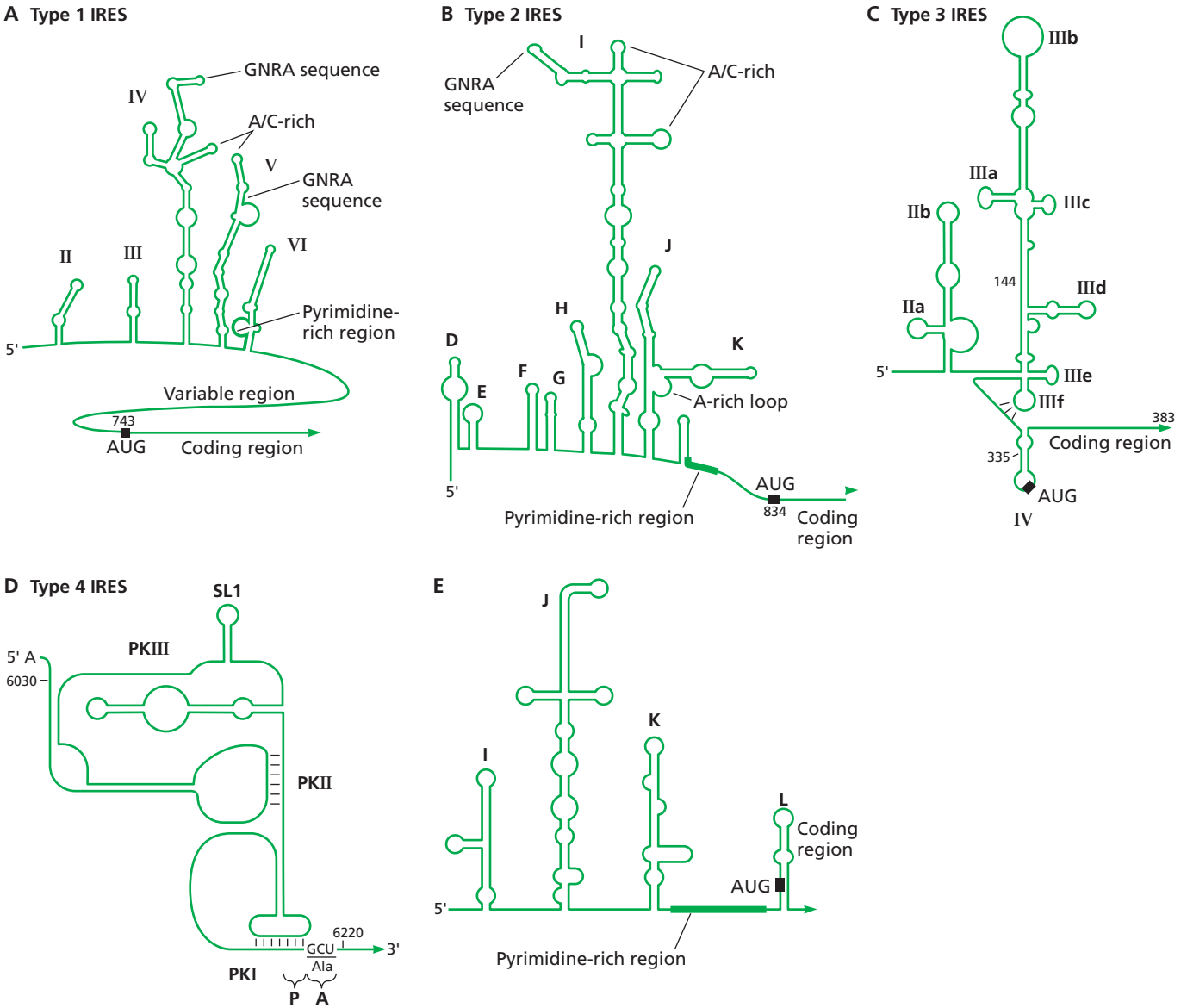


Figure 11.7 Five types of IRES. The 5′ untranslated regions from genome RNAs of poliovirus **(A)**, encephalomyocarditis virus **(B)**, hepatitis C virus **(C)**, cricket paralysis virus **(D)**, and Aichi virus **(E)** are shown. Predicted secondary and tertiary RNA structures (RNA pseudoknots) are shown. Nearly every picornavirus IRES can be placed into one type, with the exception of hepatitis A virus. The poliovirus IRES is a type 1 IRES, which is found in the genomes of enteroviruses and rhinoviruses. The ribosome probably enters the IRES at domains V and VI and scans to the AUG initiation codon, which is located 50 to 100 nucleotides past the 3′ end of the IRES. The type 2 IRES is found in the genomes of aphthoviruses and cardioviruses. The 3′ end of the hepatitis C virus IRES (type 3) extends beyond the AUG initiation codon (black box). The IRES of picornavirus-related viruses of insects (type 4), such as cricket paralysis virus, mimics a tRNA and occupies the P site in the 40S ribosomal subunit. Translation initiates with a non-AUG codon from the A site. The fifth class of IRES is exemplified by the 5′ untranslated region of Aichi virus, which comprises four domains. GNRA, a four-base hairpin loop sequence comprised of guanine, any base, a purine, and adenine; PK, pseudoknot; SL, stem-loop. (A and B) Adapted from S. R. Stewart and B. L. Semler, Semin. Virol. **8:**242–255, 1997, with permission. (C) Adapted from S. M. Lemon and M. Honda, Semin. Virol. **8:**274–288, 1997, with permission. (D) Adapted from E. Jan and P. Sarnow, J. Mol. Biol. **324:**889–902, 2002, with permission.

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BOX 11.3

BACKGROUND

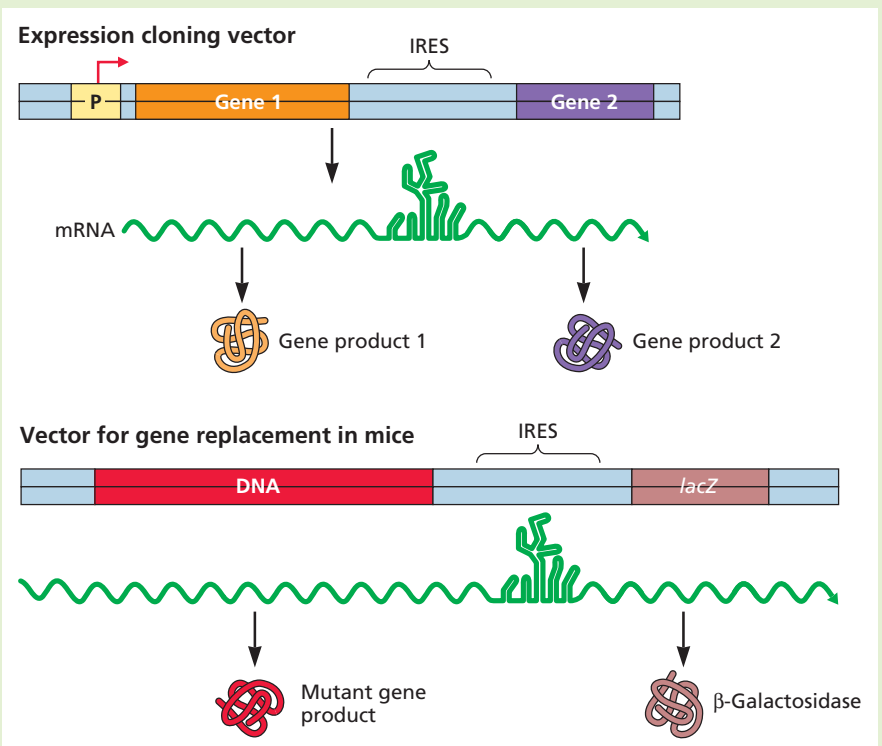
Use of the IRES in expression vectors

The IRES has been used widely in the expression of exogenous genes in eukaryotes. One strategy is to produce mRNAs in the cytoplasm by using a bacteriophage DNA-dependent RNA polymerase, such as T7 RNA polymerase. Such mRNAs are poorly translated because they are not capped; inclusion of an IRES in the 5′ untranslated region allows them to be translated efficiently.

Another application of IRESs is in gene therapy, where the ability to introduce multiple therapeutic genes is desirable. An example is the treatment of ischemic disease by coproduction of vascular endothelial growth factor and angiopoietin: synergistic effects are obtained. To accomplish this goal, an IRES is incorporated between two transgenes (figure, top panel). A single mRNA is produced from which two proteins are translated.

IRESs have also been used in the isolation of mutant mice by homologous recombination in embryonic stem cells. Bicistronic vectors are designed to produce mRNA encoding the altered protein and β-galactosidase, separated by an IRES (figure, bottom panel). Because β-galactosidase is encoded on the same mRNA as the targeted gene product, it serves as a marker for expression of the mutated gene.

Renaud-Gabardos E, Hantelys F, Morfoisse F, Chaufour X, Garmy-Susini B, Prats AC. 2015. Internal ribosome entry site-based vectors for combined gene therapy. *World J Exp Med* **5:**11–20.



(Top) Design of plasmids for expression of two genes. DNA encoding the first gene is followed by an IRES and then a second gene. A single mRNA is produced from a promoter when this plasmid DNA is introduced into cells. The first gene is translated by 5′-end-dependent translation, and that of the second by internal ribosome entry. **(Bottom)** Vector for gene replacement in mice. In this example, the goal is to replace the gene with a mutant version. The targeting plasmid consists of mutant DNA followed by an IRES and the *lacZ* gene. The flanking light blue bars represent sequences from the mouse gene that mediate homologous recombination. After replacement of the endogenous gene with this synthetic version, mRNA that encodes the mutant gene product as well as the β-galactosidase protein will be produced. The latter can be detected in tissues by staining with the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

As discussed above, translation of cellular mRNAs is enhanced by the juxtaposition of mRNA ends (Fig. 11.4). Translation of viral mRNAs by internal initiation is also stimulated by this arrangement. An example is the 5′ and 3′ ends of the RNA genome of foot-and-mouth disease virus, which are brought together by RNA-RNA interactions (Fig. 11.9A). This mechanism is distinct from the protein-RNA interactions that bring together RNA ends of cellular mRNAs.

Other host cell proteins that contribute to IRES function. In addition to canonical translation proteins, activity of IRESs requires other cellular RNA-binding proteins. These were first discovered because the poliovirus IRES functions poorly in reticulocyte lysates, in which most capped mRNAs are translated efficiently (Box 11.4). Addition of a cytoplasmic extract from other cells to reticulocyte lysates restores efficient translation from this IRES. These observations led to the suggestion that ribosome binding to the IRES requires more than translation initiation proteins. Such proteins were first identified by their ability to bind to the IRES and to restore its function in the reticulocyte lysate.

The requirements for RNA-binding proteins differ among various IRESs, and no single host cell protein that is essential for the function of all of them has been identified.

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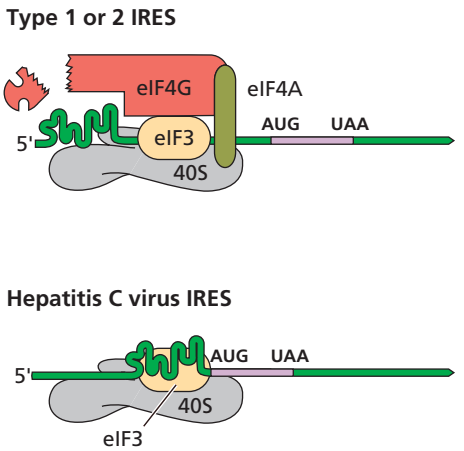


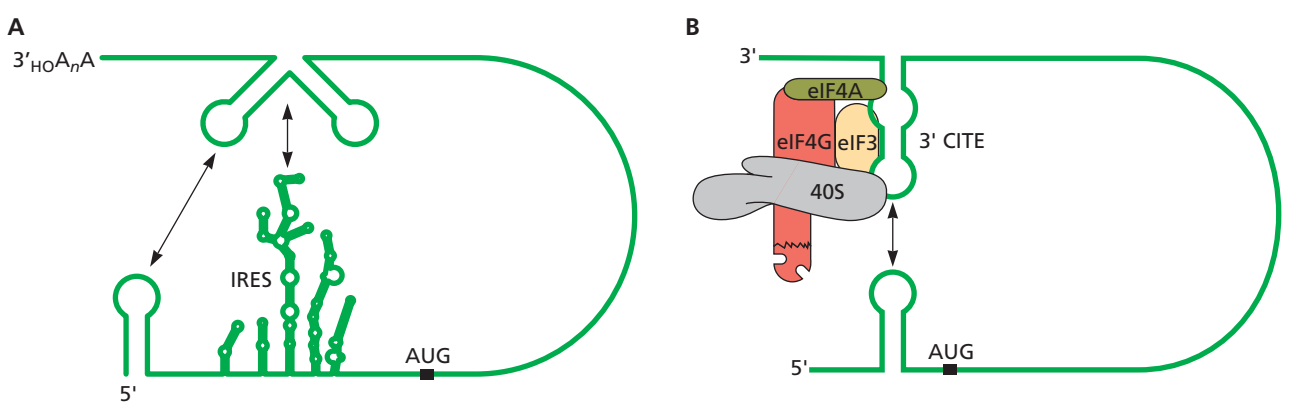
Figure 11.8 5′-end-independent initiation. (Top) Initiation on the type 1 or 2 IRES does not depend on the presence of a cap structure, but requires the C-terminal fragment of eIF4G to recruit the 40S ribosomal subunit via its interaction with eIF3. eIF4G probably binds directly to the IRES. (Bottom) The ribosomal 40S subunit binds to the hepatitis C virus IRES without the need for translation initiation proteins. eIF3 also binds the IRES and is thought to be necessary for recruitment of the 60S ribosomal subunit.

All type 1 IRESs require the cytoplasmic RNA-binding protein poly(rC)-binding protein 2 (Pcbp2) for activity. This protein was originally identified by its ability to bind stem-loop IV of the poliovirus IRES (Fig. 11.7A). Mutations in the poliovirus 5′ untranslated region that abolish binding of Pcbp2 lead to decreased translation *in vitro*. Depletion of Pcbp2 from human translation extracts inhibits translation dependent on the IRESs of poliovirus, Coxsackievirus B, and rhinovirus, but not on those of encephalomyocarditis virus or foot-and-mouth disease virus. Translation activity of the IRESs was restored by addition of purified Pcbp2. This protein binds to, and functions cooperatively during internal initiation with, serine/arginine-rich splicing factor 3 (SRp20), a protein that is essential for constitutive splicing and regulation of alternative splice site selection. Cleavage of Pcbp2 is thought to enable a switch from translation to replication during poliovirus infection (Chapter 6).

The encephalomyocarditis virus IRES is highly active in the absence of RNA-binding proteins, while the rhinovirus and foot-and-mouth disease virus IRESs require polypyrimidine-tract-binding protein (Ptb), also called heterogeneous nuclear ribonucleoprotein I (hnRnpI), a negative regulator of alternative pre-mRNA splicing. The poliovirus and Aichi virus IRESs also require Ptb for activity. This predominantly nuclear protein is redistributed to the cytoplasm during poliovirus infection. Ptb binds to sequences upstream of the pyrimidine-rich sequence of the poliovirus IRES, and to both the 5′ and 3′ untranslated regions of hepatitis C virus RNA. Yet other RNA-binding proteins are required for the activities of the foot-and-mouth disease virus, rhinovirus, and poliovirus IRESs.

There is no evidence that such RNA-binding proteins facilitate recruitment of 43S preinitiation complexes to the IRES. Rather, it appears that these proteins act as RNA chaperones to maintain the IRES in a secondary and tertiary structure that is appropriate for binding to ribosomes and translation initiation proteins. In support of this hypothesis is the observation that all are RNA-binding proteins that can form multimers that contact the IRES at multiple points; these proteins protect some IRESs from enzymatic degradation. They bind at numerous sites, consistent with a role in constraining three-dimensional flexibility. The binding site for Ptb on the poliovirus IRES overlaps that of eIF4G, leading to

Figure 11.9 Long-range RNA-RNA interactions aid translation. (A) Activity of the IRES of foot-and-mouth disease virus is enhanced by interactions with sequences at the 3′ end of the viral RNA. **(B)** The 3′-cap-independent translational enhancer (3′ CITE) found in some plant viral RNAs binds eIF4F, allowing recruitment of the 40S ribosomal subunit. A long-range interaction of this sequence with the 5′ end of the viral RNA positions the 40S ribosomal subunit at the initiation codon.



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BOX 11.4

METHODS

Translation in vitro*: the reticulocyte lysate and wheat germ extract*

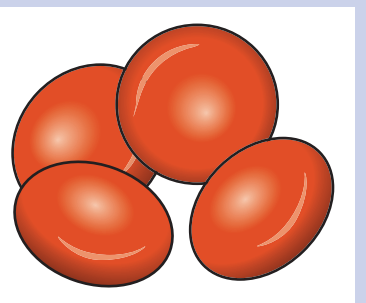
Our present understanding of the fundamentals of translation initiation, elongation, and termination, as well as viral translation strategies, would not be possible without the technique of *in vitro* translation in cell extracts. In this method, cells are lysed and the nuclei are removed by centrifugation. The mRNA is added to the lysate, and the mixture is incubated to allow translation to proceed.

The ideal extract for *in vitro* translation has two important properties: high translation efficiency and low protein synthesis in the absence of exogenous mRNA. By the early 1970s, cell extracts prepared from Krebs II ascites tumor cells or rabbit reticulocytes (immature red blood cells that primarily produce hemoglobin; they lack nuclei) were found to translate protein with high efficiency, but the presence of endogenous mRNAs that were also translated complicated the analysis of proteins made from added mRNA. In 1973, a cell extract from commercial wheat germ that had low background protein synthesis, and in which exogenous mRNAs were translated very efficiently, was developed. A few years later, the background in a reticulocyte lysate was eliminated by treatment with micrococcal nuclease, which destroyed the endogenous mRNA. This nuclease requires calcium for its activity, and it was therefore a simple matter of adding a calcium chelator, EGTA, to the reaction to prevent the degradation of exogenously added mRNA.

Wheat germ extract and reticulocyte lysate are still widely used in studies of translation, because the cells are abundant, inexpensive, and excellent sources of initiation proteins. Micrococcal nuclease followed by calcium chelation has been successfully used to make mRNA-dependent extracts from many mammalian cell types, although the translation efficiency of such systems does not approach that of wheat germ or reticulocyte lysates. Unfortunately, it has not been possible to prepare translation extracts from normal mammalian tissues consistently, a failure that has hampered the study of regulation of tissue-specific translation in virus-infected and uninfected cells.

Pelham HR, Jackson RJ. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur J Biochem* **67:**247–256.

Roberts BE, Patterson BM. 1973. Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell-free system from commercial wheat germ. *Proc Natl Acad Sci U S A* **70:**2330–2334.



repositioning of the protein, which may explain the need for this chaperone.

3′-cap-independent translational enhancers. The (+) strand RNA genomes of a number of plant viruses that lack both 5′ caps and 3′ poly(A) tails require a 3′-cap-independent translational enhancer (3′ CITE) for protein synthesis. These structures have been placed into several different classes, but all recruit ribosomes by binding directly or via eIF4G or eIF4E. Because they are located in the 3′ noncoding region of the viral RNA, long range RNA-RNA interactions are required to place ribosomes or translation proteins at the 5′ end, where translation begins. An example is the genome of barley yellow dwarf virus, in which complementary sequences located in the 5′ untranslated region and 3′ CITE form an RNA-RNA bridge by a kissing-loop interaction (Fig. 11.9B). Simultaneous binding of the 3′ CITE to eIF4F and the 5′ untranslated region recruits the 40S ribosomal subunit to the RNA 5′ end.

Some viral genomes that lack caps and poly(A) tails utilize both a 5′ IRES and 3′ CITE for translation. For example, in the mRNA of blackcurrant reversion virus, a member of the *Picornavirales*, long-range RNA-RNA interactions between these elements are required for translation. This interaction might represent yet another way to maximize translation efficiency by juxtaposing the 5′ and 3′ ends of uncapped and unpolyadenylated RNAs.

Elongation and Termination

During elongation, the ribosome selects aminoacylated tRNA according to the sequence of the mRNA codon, and catalyzes the formation of a peptide bond between the nascent polypeptide and the incoming amino acid. The 40S ribosomal subunit is responsible for both decoding and selection of the cognate tRNA. The RNA of the 60S subunit catalyzes the peptidyltransferase reaction without any soluble nonribosomal proteins or a source of energy. Elongation is assisted by three proteins that maintain the speed and accuracy of translation. In the 80S initiation complex, the Met-tRNA$_i$ is bound to the P site of the ribosome (Fig. 11.10). Elongation of the peptide chain begins with addition of the next amino acid encoded by the triplet that occupies the A site. An important component of this process is elongation factor eEF1A, which is bound to aminoacylated tRNA, a molecule of GTP, and the nucleotide exchange protein eEF1B.

Interaction between the codon and the anticodon leads to a conformational change in the ribosome called **accommodation**, the hydrolysis of GTP and the release of eEF1A-GDP. Accommodation maintains the fidelity of translation, because it can occur only upon proper codon-anticodon base pairing and is required for GTP hydrolysis. If an incorrect tRNA enters the A site, accommodation does not occur and the aminoacylated tRNA is rejected. The large ribosomal subunit catalyzes the formation of a peptide bond between the amino acids

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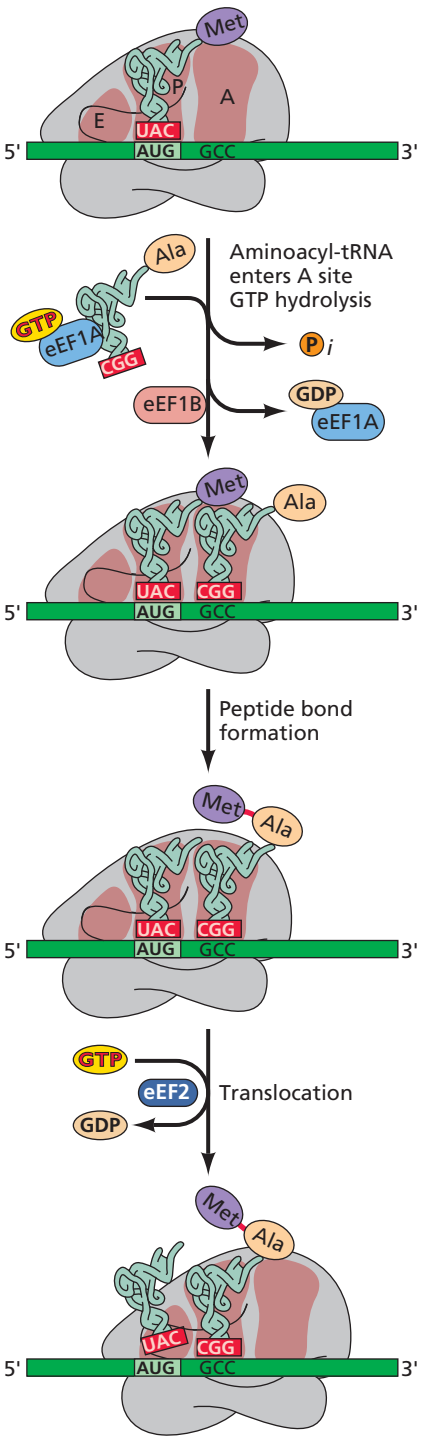


Figure 11.10 Translation elongation. There are three tRNA-binding sites on the ribosome, called peptidyl (P), aminoacyl or acceptor (A), and exit (E). After the initiating Met-tRNA$_i$ is positioned in the P site, the second aminoacyl-tRNA (alanyl-tRNA is shown) is brought to the A site by eEF1A bound to GTP. After GTP hydrolysis, eEF1A is released. The guanine nucleotide exchange protein eEF1B exchanges GDP of eEF1A-GDP with GTP, allowing eEF1A to interact with a tRNA synthetase and bind a newly aminoacylated tRNA. The peptide bond is then formed; this reaction is followed by movement of the ribosome 3 nucleotides along the mRNA, a step that requires GTP hydrolysis and eEF2. The peptidyl (Met-Ala) tRNA moves to the P site, and the uncharged tRNA moves to the E site. The A site is now empty, ready for another aminoacyl-tRNA. Adapted from G. M. Cooper, *The Cell: a Molecular Approach* (ASM Press, Washington, DC, and Sinauer Associates, Sunderland, MA, 1997).

occupying the P and A sites. The 80S ribosome then moves 3 nucleotides along the mRNA. Translocation is dependent upon eEF2 and hydrolysis of GTP. This motion moves the uncharged tRNA to the exit (E) site and the peptidyl-tRNA to the P site, enabling a new aminoacylated tRNA to enter the A site and subsequent release of the uncharged tRNA. This cycle is repeated until the ribosome encounters a stop codon. mRNAs are usually bound by many ribosomes (**polysomes**), with each ribosome separated from its neighbors by ~100 to 200 nucleotides.

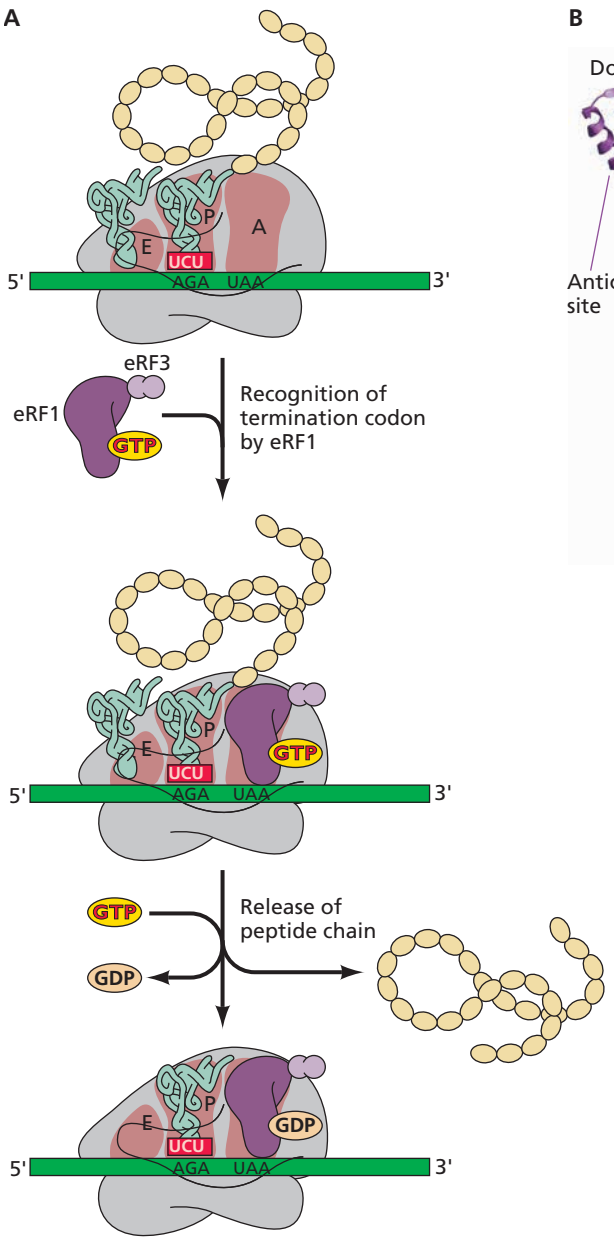
Termination is a modification of the elongation process: once the stop codon enters the A site of the ribosome, it is recognized by the 40S subunit, and the 60S subunit cleaves the ester bond between the protein chain and the last tRNA. Recognition of the three stop codons (UAA, UAG, and UGA) by the 40S ribosomal subunit is facilitated by the release proteins eRF1 and eRF3 (Fig. 11.11). The structure of eRF1 mimics that of tRNA, allowing the release protein to occupy the A site of the ribosome. The N terminus of eRF1 recognizes all three stop codons. Once bound in the A site, eRF1 and eRF3 cooperate to induce a rearrangement of the 80S ribosome, translocation of the P-site codon, and release of the polypeptide. The interaction between eRF1 and the ribosome stimulates the GTPase activity of eRF3, which is bound to the C terminus of eRF1. GTP hydrolysis is required for release of the nascent polypeptide.

In addition to accommodation, the E site is an important determinant of the fidelity of protein synthesis. When the E site is occupied by a deacylated tRNA, the affinity of the A site for aminoacyl-tRNA is low. Consequently, incorrect tRNAs are readily rejected. When the E site is empty, the affinity of the A site for aminoacyl-tRNA is significantly higher, making rejection of incorrect tRNAs less likely. An occupied E site also prevents tRNA slippage; when this site is empty, increased ribosomal frameshifting occurs.

Although stop codons are the major determinants of translation termination, other sequences can affect the efficiency of this process. The nucleotide immediately downstream of the stop codon can influence chain termination and ribosome dissociation. In eukaryotes, the preferred termination signals are UAA(A/G) and UGA(A/G).

After release of the polypeptide chain, the 60S ribosomal subunit and tRNA are released from the mRNA by the cooperation of eIF1, eIF1A, and eIF3 (Fig. 11.12). It has

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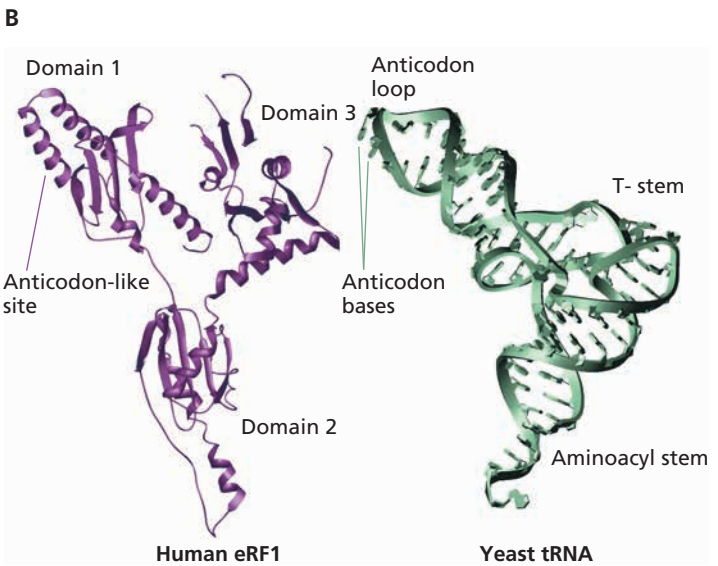


Figure 11.11 Translation termination. (A) Overview of termination. When a termination codon is encountered at the A site, it is usually recognized by a release factor (eRF) instead of a tRNA. The peptide chain is then released, followed by dissociation of tRNA and ribosome from the mRNA. eRF3, which is bound to the C terminus of eRF1, is a GTPase that is required for release of the protein. Adapted from G. M. Cooper, *The Cell: a Molecular Approach* (ASM Press, Washington, DC, and Sinauer Associates, Sunderland, MA, 1997). **(B)** Atomic structure of eRF1 and yeast $tRNA^{Phe}$. The structure of eRF1 mimics that of tRNA, providing a mechanism for recognition of termination codons.

been suggested that 40S ribosomal subunits preferentially engage in new rounds of translation initiation on the same mRNA. This hypothesis is supported by the finding that eIF3, which remains bound to the 40S ribosomal subunit after termination, also binds eIF4G (Fig. 11.13). Other observations that are consistent with this model include the ability of eRF3 to bind Pabp1 (Fig. 11.13) and the stimulation of 60S ribosomal subunit joining by this protein. As a result, ribosomes may shuttle from the 3*f* end of the mRNA back to the 5*f* end, beginning the synthesis of another molecule of the protein.

The Diversity of Viral Translation Strategies

A variety of unusual translation mechanisms expand the coding capacity of viral genomes and allow the synthesis of multiple polypeptides from a single RNA (Fig. 11.14). All were discovered in virus-infected cells and subsequently shown to operate during translation of cellular mRNAs. Nontranslational solutions for maximizing the number of proteins encoded in viral genomes are discussed in other chapters and include the synthesis of multiple subgenomic mRNAs, mRNA splicing, and RNA editing.

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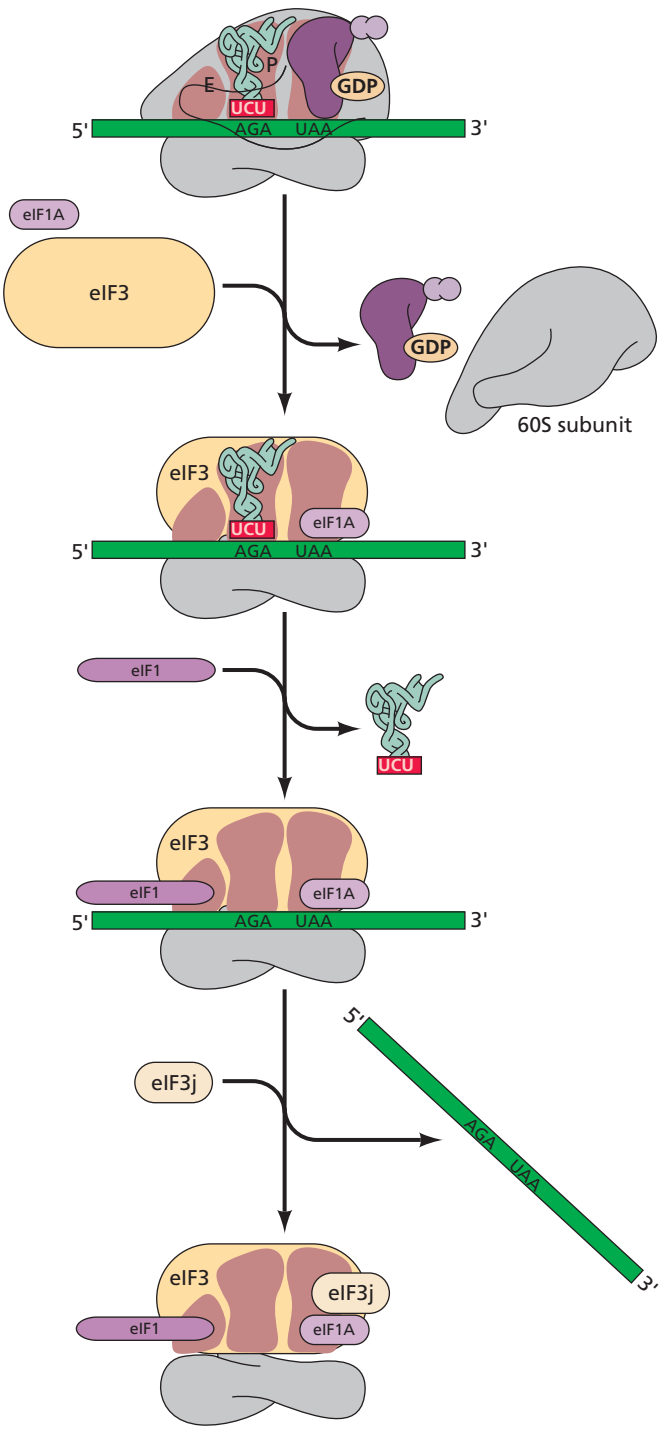


Figure 11.12 Ribosome recycling. After peptide release, eIF1A and eIF3 cause dissociation and release of the 60S ribosomal subunit. Release of the P-site deacylated tRNA is promoted by eIF1 and is followed by dissociation of mRNA mediated by eIF3j binding.

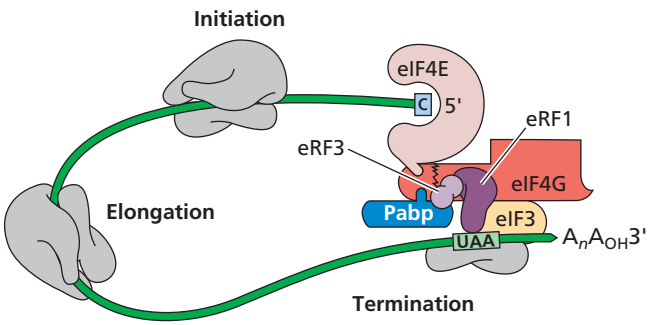


Figure 11.13 Juxtaposition of mRNA ends. Shown is a juxtaposition of mRNA ends by interactions of termination and initiation proteins, Pabp1, and the mRNA 5′ and 3′ ends. eRF3 binds both eRF1 and Pabp1. Adapted from N. Uchida et al., *J Biol Chem* **277:**50286–50292, 2002, with permission.

Polyprotein Synthesis

One strategy allowing for the production of multiple proteins from an RNA genome is to synthesize from a single mRNA a polyprotein precursor, which is then proteolytically processed to form functional viral proteins. A dramatic example of protein processing occurs in picornavirus-infected cells: nearly the entire (+) strand RNA is translated into a large polyprotein (Fig. 11.15A). Processing of this precursor is carried out by two virus-encoded proteases, $2A^{pro}$ and $3C^{pro}$, which cleave between Tyr and Gly and between Gln and Gly, respectively. In both cases, flanking amino acids control the efficiency of cleavage so that not all Tyr-Gly and Gln-Gly pairs in the polyprotein are processed. These two proteases are active in the nascent polypeptide and release themselves by self-cleavage. Consequently, the polyprotein is not observed in infected cells because it is processed as soon as the protease-coding sequences have been translated. After the proteases have been released, they cleave other polyprotein molecules.

Protein production can be controlled by the rate and extent of polyprotein processing. In addition, alternative utilization of cleavage sites can produce proteins with different activities. For example, the poliovirus protease $3C^{pro}$ does not process the capsid protein precursor P1 efficiently. Rather, the $3C^{pro}$ precursor, $3CD^{pro}$, is required for processing of P1. By regulating the quantity of $3CD^{pro}$ produced, the extent of capsid protein processing can be controlled. Because $3CD^{pro}$ and $3C^{pro}$ process Gln-Gly pairs in the remainder of the polyprotein with the same efficiency, an interesting question is why $3CD^{pro}$, which also contains $3D^{pol}$ protein, is further processed to produce $3C^{pro}$ (Fig. 11.15A). The answer is that $3CD^{pro}$ protein, while active as a protease, does not possess RNA polymerase activity and consequently some molecules must be cleaved to allow RNA replication.

Some viral precursor proteins are processed by cellular proteases. The genome of flaviviruses contains an open

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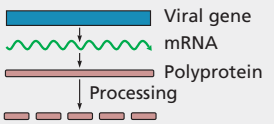
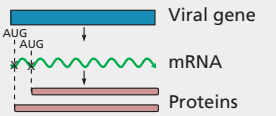
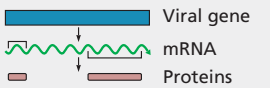
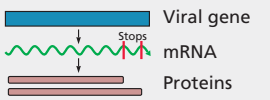
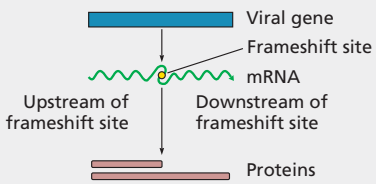
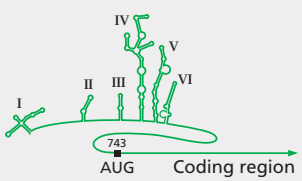
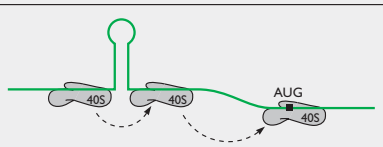
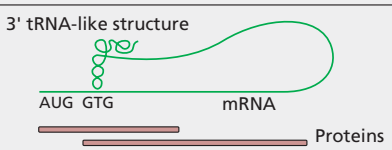
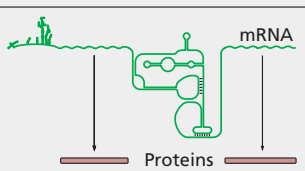
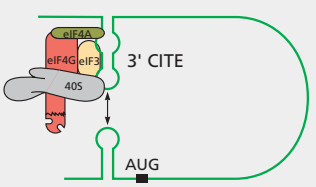
Mechanism of translation	Examples	
Polyprotein synthesis	Picornaviruses Flaviviruses Alphaviruses Retroviruses	
Leaky scanning	Sendai virus P/C mRNA Influenza B virus RNA 6 Human immunodeficiency virus type 1 Env/Vpu Human T-lymphotropic virus Tax, Rex Simian virus 40 VP2, VP3 Simian virus 40 agnoprotein	
Reinitiation	Influenza B virus RNA 7 Cytomegalovirus gp48 mRNA	
Suppression of termination	Alphavirus nsP4 Retrovirus Gag-Pol	
Ribosomal frameshifting	Coronavirus ORF1a-ORF1b Human astrovirus type 1 ORF1a-ORF1b Retrovirus Gag-Pol	
Internal ribosome entry	Picornaviruses Flaviviruses	
Ribosome shunting	Adenovirus Cauliflower mosaic virus	
Internal initiation mediated by tRNA-like structure in the 3' untranslated region	Turnip yellow mosaic virus	
Bicistronic mRNAs	Cricket paralysis virus *Rhopalosiphum padi* virus	
3′-cap-independent translational enhancer	Pea enation mosaic virus Barley yellow dwarf virus	

Figure 11.14 The diversity of viral translation strategies.

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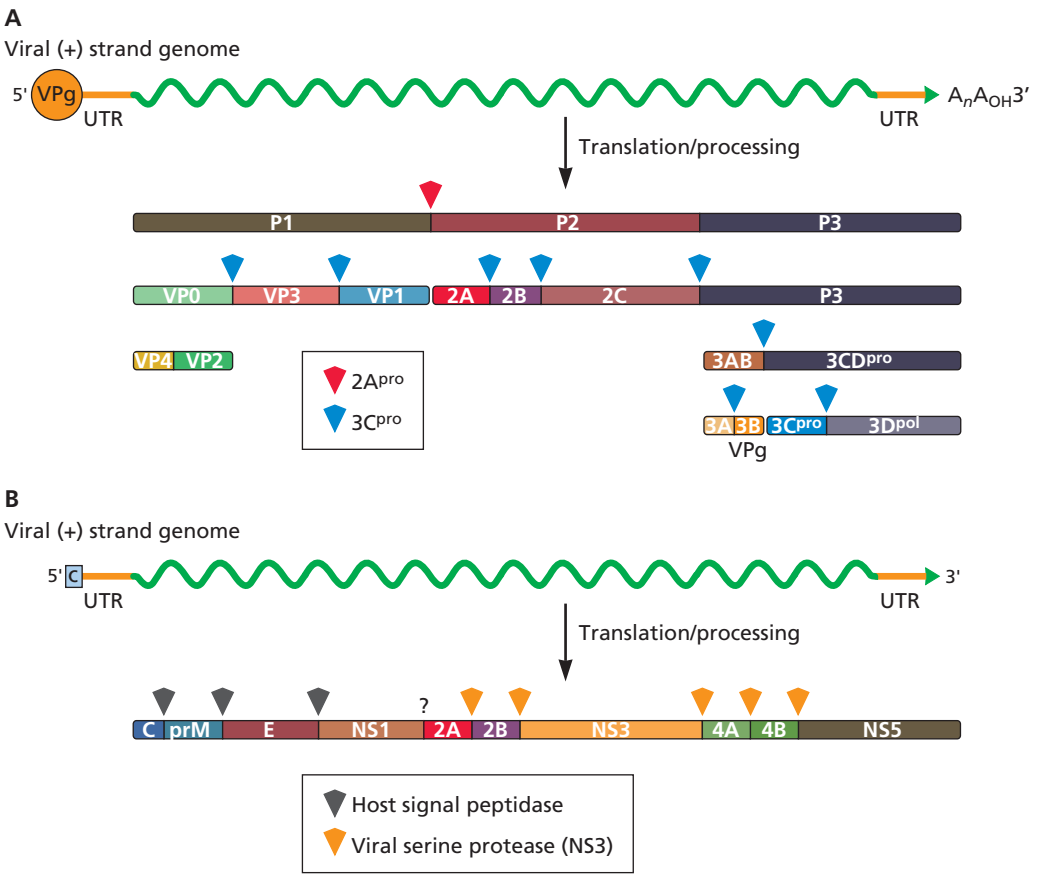


Figure 11.15 Polyprotein processing of picornaviruses and flaviviruses. (A) Processing map of protein encoded by the poliovirus genome. The viral RNA is translated into a long precursor polyprotein that is processed by two viral proteases, $2A^{pro}$ and $3C^{pro}$, to form viral proteins. Cleavage sites for each protease are shown. **(B)** Cleavage map of protein encoded in the flavivirus genome. Processing of the flavivirus precursor polyprotein is carried out either by the host signal peptidase or by the viral protease NS3. UTR, untranslated region.

reading frame of >10,000 bases (Fig. 11.15B). This mRNA is translated into a polyprotein precursor that is processed by a viral serine protease and by host signal peptidase. The latter enzyme is located in the endoplasmic reticulum (ER), where it removes the signal sequence from proteins translocated into the lumen (Chapter 12). The viral proteins processed by the cellular signal peptidase must therefore be inserted into the ER.

Leaky Scanning

Although the vast majority of eukaryotic mRNAs are monocistronic (Fig. 11.1), leaky scanning allows some viral mRNAs to be functionally polycistronic, i.e., to encode more than one protein. In the scanning model of mRNA translation, 40S ribosomal subunits bind close to the mRNA 5′ end and initiate translation at the first AUG. In a mechanism called **leaky scanning**, some ribosomes bypass the first AUG codon and continue scanning to an alternative downstream AUG. Leaky scanning can allow the synthesis of multiple isoforms of a protein with common C termini, or distinct proteins, by translation of overlapping or nonoverlapping open reading frames, respectively. Translation of overlapping reading frames also occurs in many other viral mRNAs, and is the most frequent mechanism for translation of polycistronic mRNAs of RNA viruses.

The P/C gene of Sendai virus is the model for genes that encode mRNAs with such translational flexibility (Fig. 11.16). P protein is translated from an open reading frame beginning with an AUG codon at nucleotide 104. C proteins are produced from a different reading frame, which begins at nucleotide 81, and are completely different from P proteins. No less than four C proteins (called C′, C, Y1, and Y2) are produced by translation beginning at four in-frame initiation codons. The first start site is an unusual ACG codon, and the third, fourth, and fifth are AUG codons; the result is a nested set of proteins with a common C terminus.

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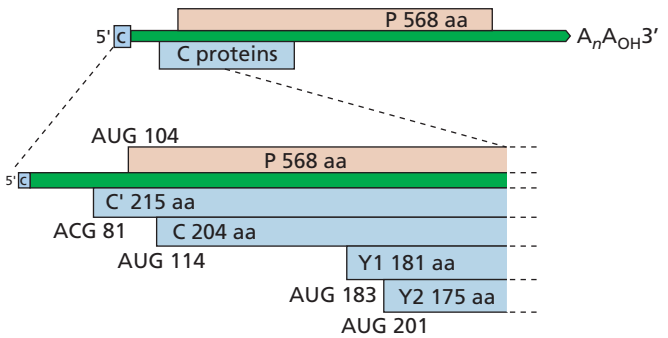


Figure 11.16 Leaky scanning and mRNA editing in the Sendai virus P/C gene. P and C protein open reading frames are shown as brown and blue boxes, respectively. An enlargement of the 5′ end of the mRNA is shown below, indicating the different start sites for four of the C proteins. aa, amino acids. Adapted from J. Curran et al., *Semin Virol* **8:**351–357, 1997, with permission.

The first three initiation sites on P/C mRNA are likely to be arranged to permit translation by leaky scanning. The first start site, $ACG^{81/C'}$, is surrounded by a good initiation context but is inefficient because of the unusual start codon. Some ribosomes bypass this initiator codon and initiate at the second, $AUG^{104/P}$ (CGCAUGG). Although the second is an AUG codon, the context is poor, and some ribosomes find their way to the third initiation codon, $AUG^{114/C}$, which has a better context (AAGAUGC). Consistent with this hypothesis, mutagenesis of $ACG^{81/C'}$ to AUG abolishes initiation at $AUG^{104/P}$ and $AUG^{114/C}$. When successive initiation codons are used in leaky scanning, they are increasingly efficient as start sites.

The last two C protein initiation codons, $AUG^{183/Y1}$ and $AUG^{201/Y2}$, are not likely to be translated by leaky scanning because they are in the poorest contexts of the five. Furthermore, mutagenesis of $ACG^{81/C'}$ to AUG has no effect on synthesis of Y1 and Y2 proteins. Translation of Y1 and Y2 proteins is initiated by ribosome shunting. An interesting question is how the different mechanisms for translation of P/C mRNA are coordinated such that, for example, shunting does not dominate at the expense of translation of upstream AUG codons. The answer to this question is not known, but Y protein synthesis relative to that of the other C proteins varies in different cell lines. This result suggests that cellular proteins might regulate ribosome shunting on P/C mRNA, although no such protein has been identified.

Leaky scanning may be promoted by mechanisms other than a suboptimal sequence surrounding the first AUG codon. Proximity of an AUG codon to the mRNA 5′ end (<30 nucleotides) or to a downstream AUG codon (within ~10 nucleotides) decreases efficiency of initiation.

Reinitiation

Upon termination of translation, the ribosome dissociates into 40S and 60S subunits and falls away from the mRNA. Ribosomes that translate a short open reading frame may remain associated with the mRNA and can reinitiate on a downstream AUG, resulting in two proteins from a single mRNA (Fig. 11.17). When the ribosome completes translation of the short open reading frame, it cannot reinitiate until it reacquires initiation proteins, including the ternary complex, as it moves downstream.

Reinitiation after translation of a long open reading frame is rare, and requires specialized signals in the mRNA or *trans*-acting proteins. Reinitiation of translation of longer, overlapping reading frames occurs on mRNA of influenza B virus RNA 7, which encodes two proteins, M1 protein and BM2 protein (Fig. 11.17). M1 protein is translated from the 5′-proximal AUG codon, while the BM2 protein AUG initiation codon is part of the termination codon for M1 protein (UAAUG).

Suppression of Termination

Although translational suppression in eukaryotic mRNAs is extremely rare, suppression of termination occurs during translation of many viral mRNAs as a means of producing a second protein with an extended C terminus. The Gag and Pol genes of Moloney murine leukemia virus are encoded in a single mRNA and separated by an amber termination codon, UAG (Fig. 11.18). The efficiency of suppression is about 4 to 10%. The Gag-Pol precursor is subsequently processed proteolytically to liberate the Gag and Pol proteins. Without this suppression mechanism, the viral enzymes reverse transcriptase and integrase could not be produced. In a similar way, translational suppression of a different termination codon, UGA, is required for the synthesis of nsP4 of alphaviruses (Fig. 11.18). In this example, the efficiency of synthesis is about 10% of that of the normally terminated nsP3 protein. Because nsP4 encodes the RNA-dependent RNA polymerase, suppression is essential for viral RNA replication.

Most translational suppression takes place when normal tRNAs misread termination codons. The misreading of the amber codon in Moloney murine leukemia virus Gag protein for a Gln codon is an example. Suppressor tRNAs that can recognize termination codons and insert a specific amino acid are rare. One example is a suppressor tRNA that inserts selenocysteine, the 21st amino acid, in place of a UGA codon.

The nucleotide sequence 5′ or 3′ of the termination codon can influence the efficiency of translational suppression. Two adenosines just 5′ to the stop codon stimulate read-through of many plant virus mRNAs. Downstream stimulators of suppression comprise either nucleotides adjacent to the codon or RNA secondary structures that begin ~8 nucleotides from the termination codon. In Sindbis virus, efficient suppression of the UGA codon requires only a single C residue 3′ of the termination codon. In contrast, read-through of the UAG codon in Moloney murine leukemia virus mRNA requires a purine-rich sequence 3′ to the termination codon, as well as a

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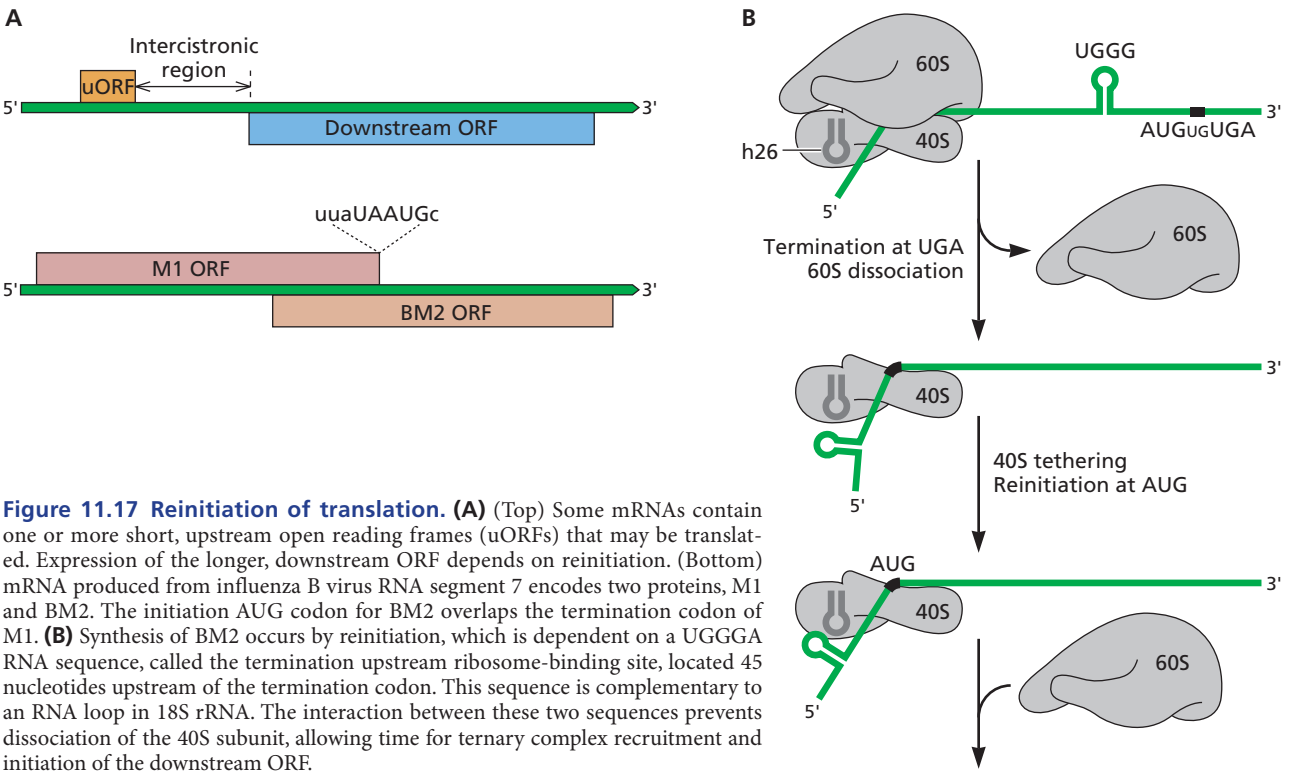
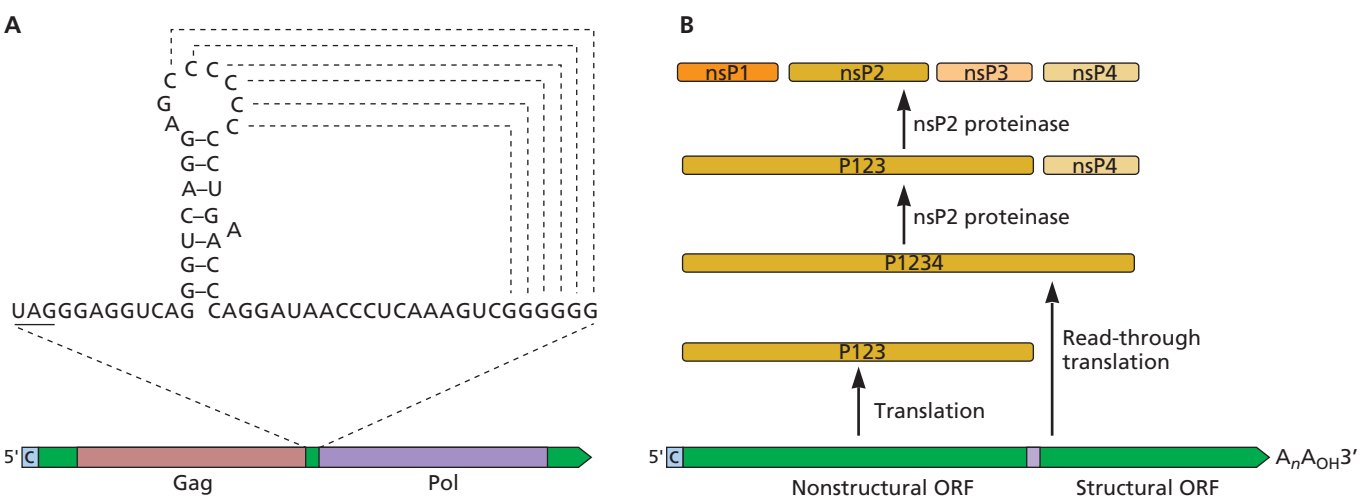


Figure 11.17 Reinitiation of translation. (A) (Top) Some mRNAs contain one or more short, upstream open reading frames (uORFs) that may be translated. Expression of the longer, downstream ORF depends on reinitiation. (Bottom) mRNA produced from influenza B virus RNA segment 7 encodes two proteins, M1 and BM2. The initiation AUG codon for BM2 overlaps the termination codon of M1. **(B)** Synthesis of BM2 occurs by reinitiation, which is dependent on a UGGGA RNA sequence, called the termination upstream ribosome-binding site, located 45 nucleotides upstream of the termination codon. This sequence is complementary to an RNA loop in 18S rRNA. The interaction between these two sequences prevents dissociation of the 40S subunit, allowing time for ternary complex recruitment and initiation of the downstream ORF.

pseudoknot structure farther downstream (see Chapter 6 for a description of pseudoknots).

The effect of bases at the 3′ side of the stop codon may influence suppression by regulating competition between release factor and near-cognate tRNAs that bind the stop codon. Secondary RNA structures may govern suppression by modulating mRNA-protein or mRNA-rRNA interactions, by sterically interfering with release factor function, or by blocking unwinding by ribosome-associated helicases. It has been suggested that the pseudoknot of Moloney murine leukemia

Figure 11.18 Suppression of termination codons of alphaviruses and retroviruses. (A) Structure of the termination site between Gag and Pol of Moloney murine leukemia virus. The stop codon that terminates synthesis of Gag is underlined; it is followed by a pseudoknot that is important for suppression of termination. Adapted from J. H. Strauss and E. G. Strauss, *Microbiol Rev* **58:**491–562, 1994. **(B)** Suppression of termination during the synthesis of alphavirus P123 to produce nsP4, the RNA-dependent RNA polymerase. The termination codon is shown on the RNA as a box.



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virus RNA causes the ribosome to pause, allowing the suppressor tRNA to compete with eRF1 at the suppression site. Maximal read-through efficiency also requires the interaction of viral reverse transcriptase with eRF1.

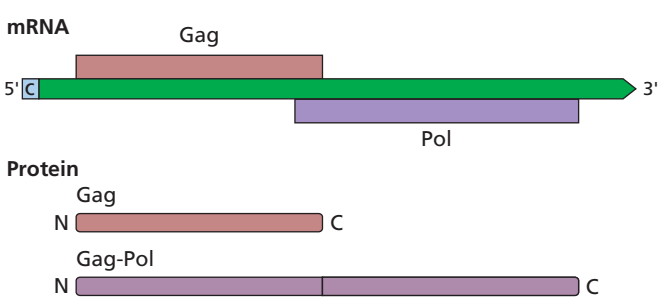
Suppression of termination is far more prevalent during translation of RNAs of RNA viruses than mRNAs of DNA viruses or cells. The RNA sequences and structures required for suppression are not found in most cellular mRNAs. For example, there is a strong bias against cytidine residues at the 3′ end of UGA termination codons in cellular mRNAs. Furthermore, suppression by tRNAs charged with selenocysteine has been found in <50 eukaryotic mRNAs.

Ribosomal Frameshifting

During ribosomal frameshifting, in response to signals in mRNA, ribosomes move into a different reading frame and continue translation. This mechanism was discovered in cells infected with Rous sarcoma virus and has since been described for many other viruses, including human immunodeficiency virus type 1, severe acute respiratory syndrome coronavirus, and herpes simplex virus. Frameshifting also occurs during translation of archaeal, bacterial, and eukaryotic mRNAs. This process may occur by shifting the reading frame 1 base toward the 5′ end (−1 frameshifting) or the 3′ end (+1 frameshifting) of the mRNA.

Frameshifting not only enables production of two proteins from one mRNA, but also can regulate their ratio. In the genome of retroviruses, the *gag* and *pol* genes may be separated by a stop codon (Fig. 11.18), or they may be in different reading frames, with *pol* overlapping *gag* in the −1 direction (Fig. 11.19). During synthesis of Rous sarcoma virus Gag, ribosomes frameshift before reaching the Gag stop codon and continue translating Pol, such that a Gag-Pol fusion is produced at about 10% of the frequency of Gag. Alteration of the frameshifting ratio by mutagenesis can be deleterious to viral replication.

Figure 11.19 Frameshifting on a retroviral mRNA. The structure of open reading frames is illustrated. Rous sarcoma virus mRNA encodes Gag and Pol proteins in reading frames that overlap by −1. Normal translation and termination produce the Gag protein; ribosomal frameshifting to the −1 frame results in the synthesis of a Gag-Pol fusion protein.



Studies on the requirements for frameshifting in retroviruses and coronaviruses have identified two essential components: a "slippery" homopolymeric sequence, which is a heptanucleotide stretch with two homopolymeric triplets of the form X-XXY-YYZ (e.g., in Rous sarcoma virus A-AAU-UUA); and an RNA secondary structure, usually a pseudoknot, 5 to 8 nucleotides downstream. The pseudoknot is thought to impede forward movement of the ribosome over the slippery sequence, creating tension in the mRNA that is relieved by disengagement of tRNAs followed by slippage and realignment to the −1 reading frame.

The tandem shift model for frameshifting has received substantial experimental support. In this model, two tRNAs in the zero reading frame (X-XXY-YYZ) slip back by 1 nucleotide during the frameshift to the −1 phase (XXX-YYY). Each tRNA base pairs with the mRNA in the first 2 nucleotides of each codon (Fig. 11.20). The peptidyl-tRNA is transferred to the P site, the −1 frame codon is decoded, and translation continues to produce the fusion protein. In this model, slippage occurs before peptide transfer, with the peptidyl- and aminoacyl-tRNAs bound to the P and A sites. However, it is possible that the shift occurs after peptide transfer but before translocation of the tRNAs, or when the aminoacyl-tRNA occupies the A site. These models cannot be distinguished by mutagenesis or by the sequence of the protein products.

Bicistronic mRNAs

Some viral mRNAs are bicistronic: they have two nonoverlapping open reading frames, and translation of each occurs by internal initiation. Examples include the mRNAs of members of the *Dicistroviridae*, including cricket paralysis virus and *Rhopalosiphum padi* (aphid) virus (Fig. 11.14). The upstream open reading frame begins with an AUG codon and is preceded by an IRES similar to those of picornaviruses. The downstream open reading frame, which encodes the viral capsid proteins, is translated independently from a completely different IRES. The 40S ribosomal subunit binds directly to the intergenic region that is partially folded to mimic a tRNA (Fig. 11.5A). The tRNA-like structure occupies the P site of the ribosome, and initiation occurs from the A site at a nonmethionine codon. The genome of canine picodicistrovirus is also bicistronic, but both open reading frames are translated from picornavirus-like IRESs.

Regulation of Translation during Viral Infection

Alterations in the cellular translation apparatus are commonplace in virus-infected cells. As part of the antiviral defense, or in response to stress caused by virus infection, the cell initiates measures designed to inhibit protein synthesis and limit virus production. Many viral genomes encode proteins or nucleic acids that neutralize this response, restore trantslation,

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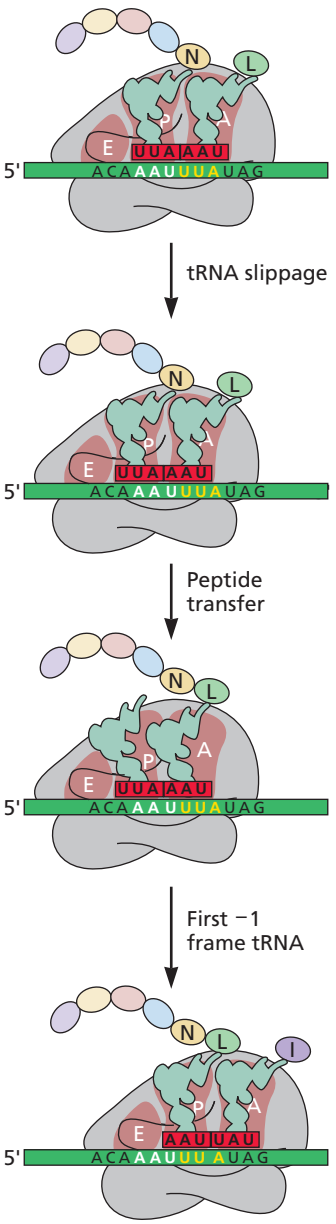


Figure 11.20 A model for −1 frameshifting. Slippage of the two tRNAs occurs after aminoacyl-tRNA enters the A site but before peptide transfer. Slippage allows the tRNAs to form only two base pairs with the mRNA. The site shown is that of Rous sarcoma virus. One-letter amino acid codes are used. Adapted from P. J. Farabaugh, *Microbiol Rev* **60:** 103–134, 1996.

and maximize virus reproduction. In addition, many viral gene products modify the host translation machinery to favor synthesis of viral proteins over those of the cell. As a result, not only can the entire synthetic capability of the cell be turned to the production of new virus particles, but translation of cellular antiviral proteins is restricted. These cellular and viral modifications of the translation apparatus may affect initiation, elongation, or termination. Some viral proteins inactivate eIF5B, eEF1A, or eEF2 to regulate 60S ribosome subunit recruitment and elongation; modulation of termination was discussed above.

Inhibition of Translation Initiation after Viral Infection

Phosphorylation of eIF2α

Translation initiation can be regulated by phosphorylation of the α subunit of the translation initiation protein eIF2 by four different cellular protein kinases that respond to virus infection or metabolic stress. One of these is the double-stranded-RNA-dependent protein kinase Pkr, which is induced by interferons (IFNs) produced as part of the rapid innate immune response of vertebrates to viral infection (discussed in Volume II, Chapter 3). IFNs diffuse to neighboring cells, bind to cell surface receptors, and activate signal transduction pathways that result in transcription of hundreds of cellular genes and the establishment of an **antiviral state**. IFN production by infected cells induces antiviral proteins in neighboring cells, thereby preventing viral reproduction and spread.

Pkr is a serine/threonine protein kinase composed of an N-terminal regulatory domain and a C-terminal catalytic domain (Fig. 11.21; see also Volume II, Chapter 3). Small quantities of an inactive form of Pkr are present in most uninfected mammalian tissues. Transcription of its gene is induced 5- to 10-fold by interferon. Pkr is activated by the binding of dsRNA to two dsRNA-binding motifs at the N terminus of the protein (Fig. 11.22). Such dsRNA is produced in cells infected by either DNA or RNA viruses. Binding to dsRNA leads to formation of Pkr dimers and autophosphorylation. This modification is thought to stabilize the dimer, which can then phosphorylate eIF2α in the absence of dsRNA. A cell protein, Pkr activator (Pact), may activate this protein kinase independently of dsRNA (Fig. 11.22).

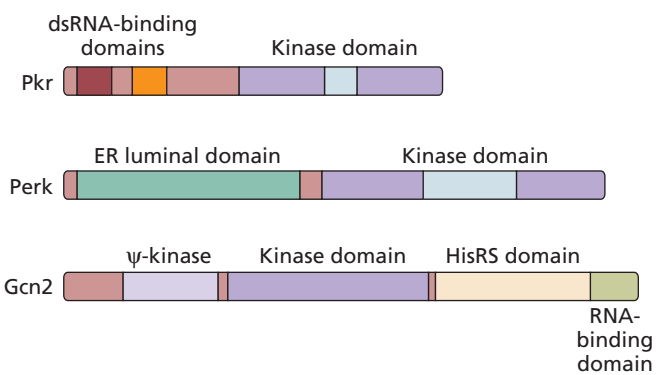


Figure 11.21 Schematic structures of three eIF2α kinases. Ψ-kinase, pseudokinase domain; HisRS domain, histidyl-tRNA synthetase-like domain. Adapted from C. G. Proud, *Semin Cell Dev Biol* **16:** 3–12, 2005, with permission.

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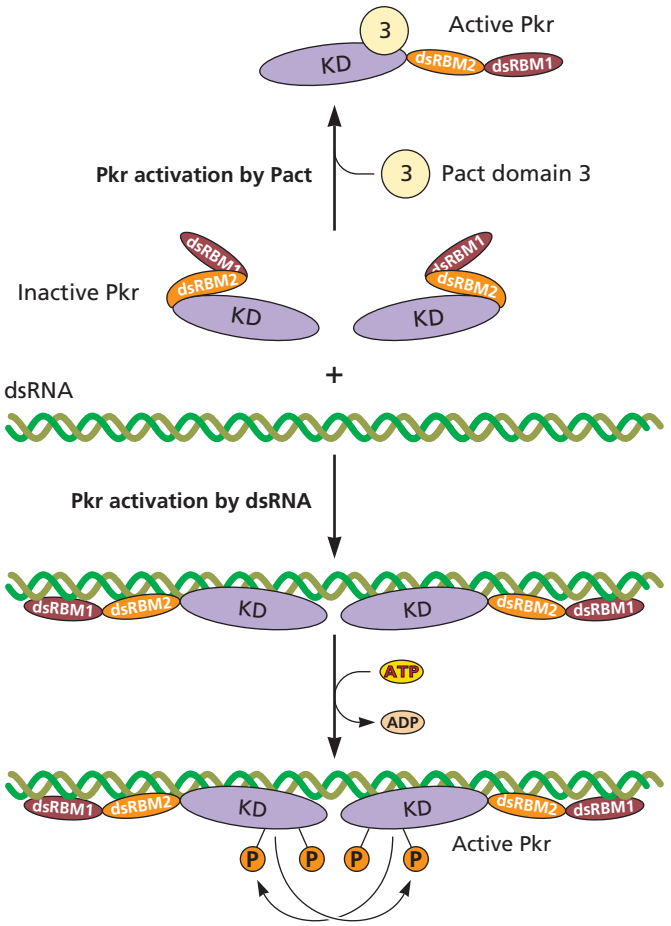


Figure 11.22 Model of activation of Pkr. Pkr is maintained in an inactive monomer by the interaction between a Pact domain 3-binding sequence in Pkr and dsRBM2. Pkr is activated when it binds Pact or dsRNA. When two or more molecules of inactive Pkr bind to one dsRNA molecule, cross-phosphorylation occurs because of the physical proximity of the molecules. Phosphorylation is thought to cause a conformational change in the kinase domain (KD) to allow phosphorylation of other substrates, including eIF2α. dsRBM, double-stranded-RNA-binding motif. Adapted from J. W. B. Hershey et al. (ed.), *Translational Control* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1996), with permission.

Two other eIF2α protein kinases regulate translation during virus infection. In mammalian cells, general control nonderepressible 2 protein (Gcn2p) is activated during amino acid starvation when uncharged tRNA binds a histidyl-tRNA synthetase-like domain in the protein (Fig. 11.21). During infection with Sindbis virus, vesicular stomatitis virus, or adenovirus, Gcn2p is activated upon binding of viral RNA, leading to phosphorylation of eIF2α and restriction of virus reproduction. Consistent with a role in mediating antiviral responses, Sindbis virus reproduction is more efficient in cells lacking Gcn2p. Pkr-like ER kinase (Perk), a transmembrane protein of the ER, is a component of the unfolded protein response. Its luminal domain senses the equilibrium between unfolded and misfolded proteins and chaperone proteins. Under conditions of intracellular stress, such as occurs during infection of cells with enveloped viruses, Perk oligomerizes within the membrane, is activated, and phosphorylates eIF2α in the cytoplasm.

The initiation protein eIF2α is part of the ternary complex that also contains GTP and Met-tRNA$_i$ (Fig. 11.3). After GTP hydrolysis, the bound GDP must be exchanged for GTP to permit the binding of another molecule of Met-tRNA$_i$. This exchange is carried out by eIF2B (Fig. 11.23). When the α subunit of eIF2 is phosphorylated, eIF2-GDP binds eIF2B with such high affinity that it is effectively trapped; recycling of eIF2 stops, and ternary complexes are depleted. eIF2B is less abundant than eIF2, and phosphorylation of about 10 to 40% of eIF2 (depending on the cell type and the relative concentrations of eIF2 and eIF2B) results in the complete sequestration of eIF2B, leading to a block in protein synthesis. As viral translation is also impaired, the production of new virus particles is diminished.

Viral Regulation of Pkr

Most viral infections induce activation of eIF2α kinases and consequent phosphorylation of eIF2α. As global inhibition of translation would be a threat to successful viral reproduction, viral genomes encode one or more proteins that prevent eIF2α phosphorylation in different ways (Fig. 11.24).

RNA antagonists of Pkr. The 166-nucleotide adenovirus VA-RNA I, which accumulates to massive concentrations (up to 10^9 copies per cell) late in infection following transcription of the viral gene by RNA polymerase III, is a potent inhibitor of Pkr. An adenovirus mutant that cannot express the VA-RNA I gene grows poorly. In cells infected with this mutant virus, eIF2α becomes extensively phosphorylated, causing global translational inhibition. VA-RNA I binds the dsRNA-binding region of Pkr and blocks activation. It has been suggested that binding of VA-RNA I to Pkr prevents the interaction with authentic dsRNA and hence prevents activation of the kinase. The Epstein-Barr virus genome also encodes small RNAs that inhibit Pkr activation.

dsRNA-binding proteins. The vaccinia virus genome encodes a protein (E3L) that sequesters dsRNA. This protein contains the same dsRNA-binding motif as Pkr; it binds dsRNA and prevents it from activating the kinase. Deletion of the gene encoding the E3L protein renders the virus more sensitive to IFN and causes production of larger quantities of active Pkr in infected cells. The influenza virus NS1 protein and the reovirus σ3 protein also sequester dsRNA.

Inhibition of kinase function. The genomes of several viruses encode proteins that directly inhibit the kinase activity of Pkr or Perk, and some do so by acting as pseudosubstrates.

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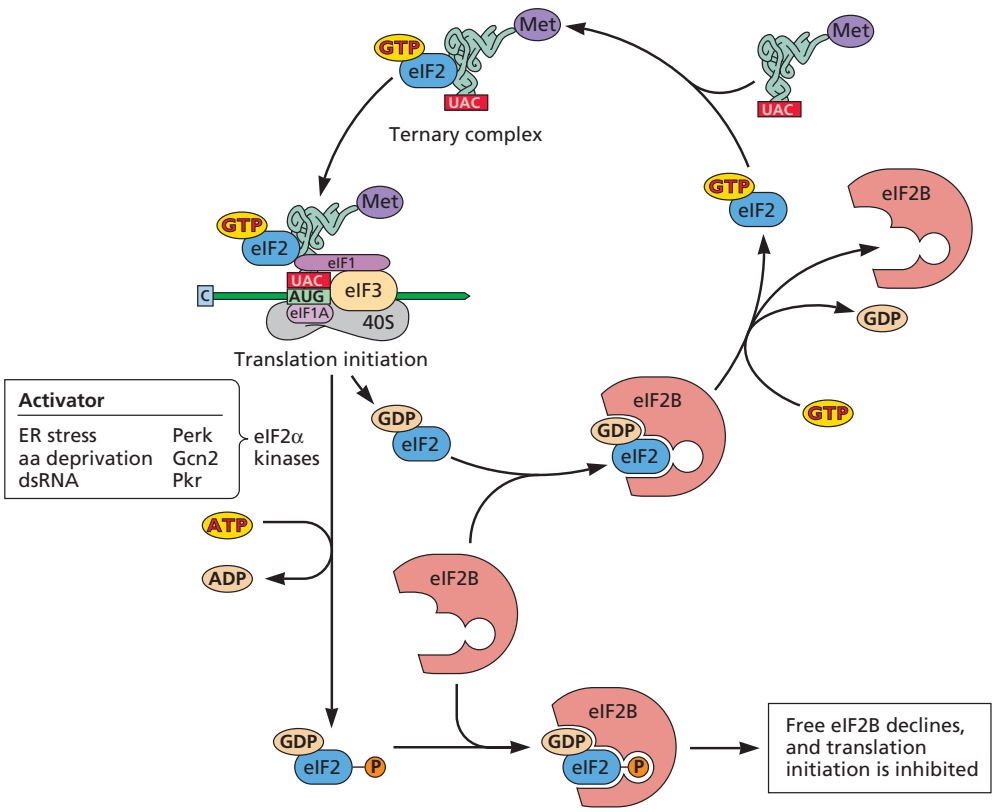


Figure 11.23 Effect of eIF2α phosphorylation on catalytic recycling. eIF2-GTP and tRNA-Met$_i$ form the ternary complex required for translation initiation. During initiation, GTP is hydrolyzed to GDP, and in order for initiation to continue, eIF2 must be recharged with GTP. Such recycling is accomplished by eIF2B, which exchanges GTP for GDP on eIF2. When eIF2 is phosphorylated on the α subunit, it binds irreversibly to eIF2B, preventing the latter from carrying out its role in recycling active eIF2. As a result, the concentration of eIF2-GTP declines and translation initiation is inhibited. aa, amino acid.

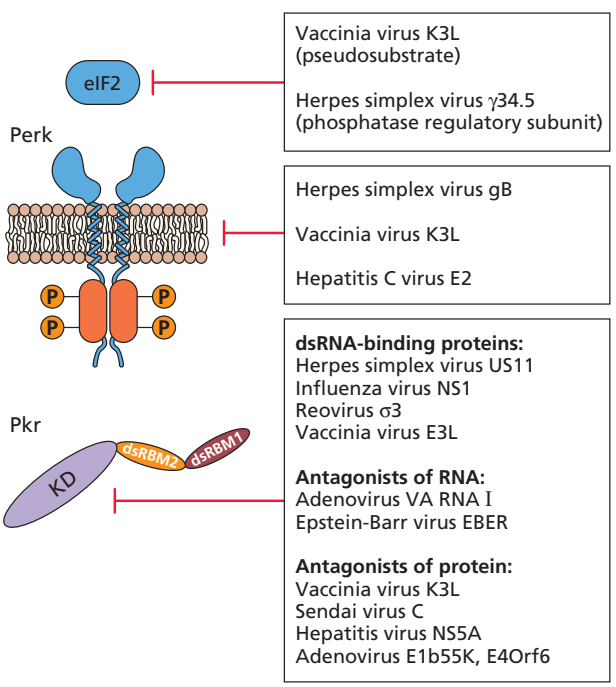


Figure 11.24 Some viral proteins and RNAs that counter inactivation of eIF2. Vaccinia virus K3L and herpes simplex virus γ34.5 interfere with phosphorylation of eIF2α by acting as a pseudosubstrate or by removing the phosphate from the protein, respectively. Other viral proteins that directly inhibit eIF2α kinases Perk and Pkr are shown. Viral proteins that prevent double-stranded activation of Pkr are also listed. dsRBM, double-stranded-RNA-binding motif; KD, kinase domain.

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For example, vaccinia virus K3L protein has amino acid homology to the N terminus of eIF2α. The protein binds tightly to Pkr within the catalytic cleft and blocks autophosphorylation. The growth of vaccinia virus mutants lacking the K3L gene is severely impaired by IFN. The herpes simplex virus type 1 genome encodes proteins that bind to Pkr and Perk and directly inhibit kinase activity. Us11 binds to Pkr and blocks its activation, while the viral glycoprotein gB associates with the luminal domain of Perk and prevents its activation and subsequent phosphorylation of eIF2α.

Cellular proteins can also function as inhibitors of eIF2α kinase function. Influenza virus infection activates a cellular protein, $p58^{IPK}$, that binds Pkr and prevents autophosphorylation. In cells lacking this protein, eIF2α phosphorylation is increased and viral mRNA translation is reduced.

Dephosphorylation of eIF2α. Another mechanism for reversing the consequences of Pkr activation is dephosphorylation of its target. In herpes simplex virus-infected cells, Pkr is activated but eIF2α is not phosphorylated. During infection with viruses lacking the viral ICP34.5 gene, Pkr is activated and eIF2α becomes phosphorylated, causing global inhibition of protein synthesis. This viral protein associates with a type 1a protein phosphatase and acts as a regulatory subunit, redirecting the enzyme to dephosphorylate eIF2α (Volume II, Fig. 5.15). The effects of activated Pkr are reversed, ensuring continued protein synthesis. In a similar fashion, the E6 protein of human papillomavirus activates a phosphatase, leading to dephosphorylation of eIF2α.

Host and virus evolution. The inhibitory effect of eIF2α phosphorylation on viral reproduction has led to the acquisition of viral genes that antagonize this function. In turn, the *pkr* gene has been selected to evade the effects of viral inhibitors. Phylogenetic analysis of the *pkr* gene in primates indicates that it has undergone bursts of positive selection. Some of the observed amino acid substitutions prevent binding of Pkr by the vaccinia virus antagonist, the K3L protein. How such mutations become fixed in the viral genome can be illuminated by experiments in cell culture. The vaccinia virus K3L protein does not efficiently antagonize Pkr of human cells. Serial propagation of the virus in cell culture leads to amplification of the *k3l* gene, causing a 7 to 10% increase in genome size. These amplifications are transient; when amino acid changes are selected that increase the antagonism of K3L for Pkr, genome reduction takes place. The expanding and contracting viral genes that antagonize host defenses have been characterized as "genomic accordions."

Beneficial Effects of eIF2α Phosphorylation on Viral Reproduction

Inhibition of host translation by phosphorylation of eIF2α can be beneficial for virus reproduction because viral mRNAs can be selectively translated, and the host IFN response may be repressed. Consistent with this reasoning, eIF2α phosphorylation is not blocked in cells infected with some viruses. Translation of some viral mRNAs, such as those of the picornavirus-like viruses of insects discussed previously in this chapter, does not require eIF2α, because the secondary structure of the IRES of these viruses mimics an uncharged tRNA. Translation of other viral mRNAs does require Met-$tRNA_i$, yet can proceed when eIF2α is phosphorylated. Translation of classical swine fever virus mRNA is not inhibited by eIF2α phosphorylation, because eIF5B, independent of eIF2, can promote Met-$tRNA_i$ binding to the ribosome. A different mechanism is exemplified by Sindbis virus: translation of subgenomic mRNAs is not inhibited by eIF2α phosphorylation. Delivery of Met-$tRNA_i$ to the ribosome is accomplished by other cellular proteins, including ligatin, a protein that normally participates in cellular localization of phosphoglycoproteins. This unusual mechanism depends on placement of the AUG codon in the P site of the ribosome, an activity mediated by a stem-loop structure ~25 nucleotides downstream of the initiation codon.

Regulation of eIF4F

The eIF4F protein plays several important roles during 5′-end-dependent initiation, including recognition of the cap, recruitment of the 40S ribosomal subunit, and unwinding of RNA secondary structure. It is not surprising, therefore, that several viral proteins modify the activity of this protein. The cap-binding subunit eIF4E is frequently a target, probably because its activity can be modulated in at least two ways and because it is present in limiting quantities in cells. The cap-binding complex can also be inactivated by cleavage of eIF4G.

Cleavage of eIF4G

Poliovirus infection of mammalian cells in culture results in dramatic inhibition of cellular protein synthesis. By 2 h after infection, polyribosomes are disrupted and translation of nearly all cellular mRNAs declines (Fig. 11.25). Translationally competent extracts from infected cells can readily translate poliovirus mRNA but not capped mRNAs. Studies of these extracts demonstrated that they lack functional eIF4F: eIF4G is cleaved proteolytically. As the N-terminal domain of eIF4G binds eIF4E, which in turn binds the 5′ cap of cellular mRNAs, such cleavage prevents eIF4F from recruiting 40S ribosomal subunits (Fig. 11.26). Poliovirus mRNA is uncapped and is translated by internal ribosome binding, a process that does not require intact eIF4G. In fact, IRES-mediated initiation function appears to require the C-terminal fragment of eIF4G, which, as discussed above, is necessary to recruit 40S ribosomal subunits to the IRES. Consequently, cleavage of eIF4G not only inhibits translation of cellular mRNAs but also is a strategy for stimulating IRES-dependent translation. Cleavage of eIF4G is carried out by viral proteases

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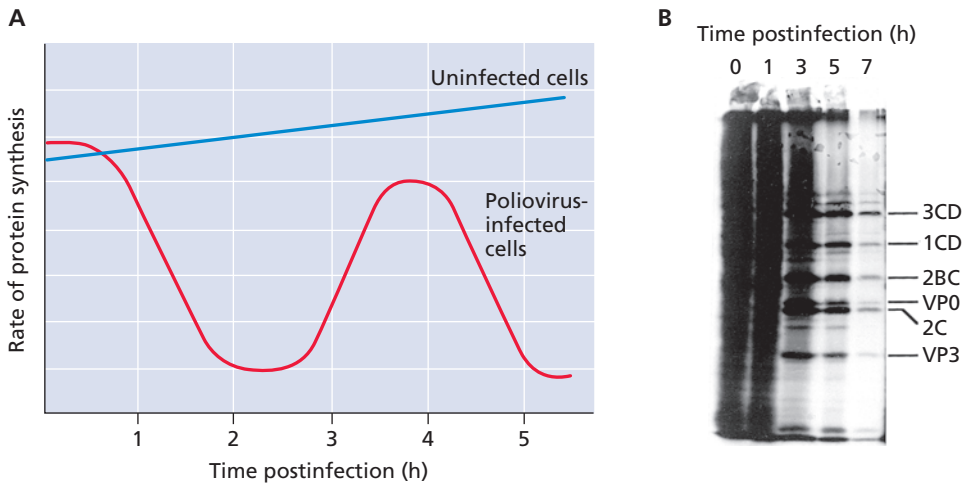


Figure 11.25 Inhibition of cellular translation in poliovirus-infected cells. (A) Rate of protein synthesis in poliovirus-infected and uninfected cells. During poliovirus infection, host cell translation is inhibited by 2 h after infection and is replaced by translation of viral proteins. Adapted from H. Fraenkel-Conrat and R. R. Wagner (ed.), *Comprehensive Virology* (Plenum Press, New York, NY, 1984), with permission. **(B)** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled proteins at different times after poliovirus infection. In this experiment, host translation was shut off by 5 h postinfection and was replaced by the synthesis of viral proteins, some of which are labeled at the right.

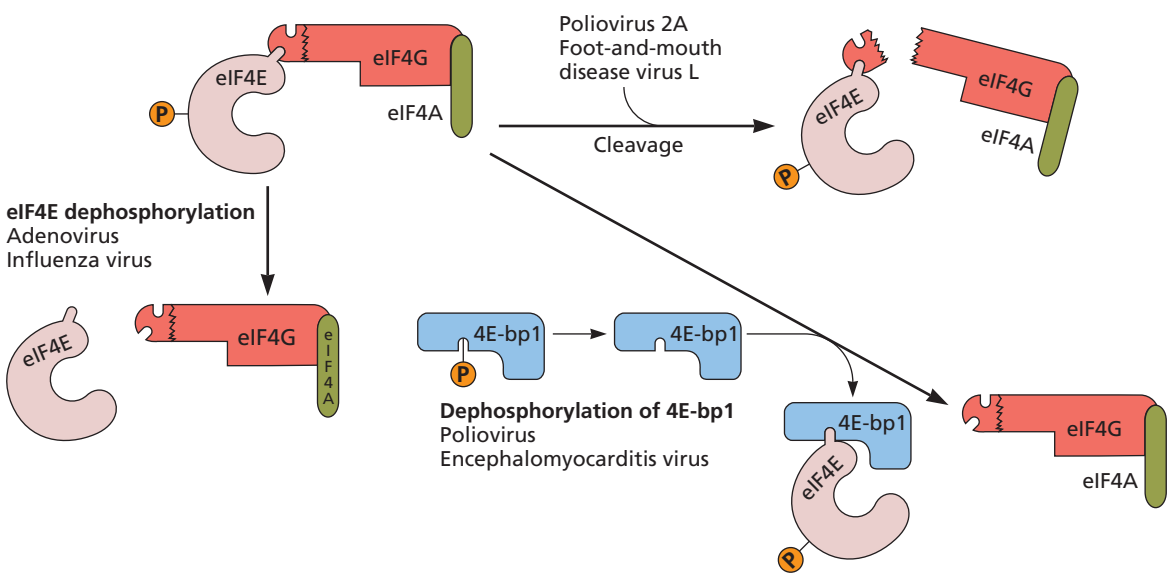
such as 2A^{pro} of poliovirus, rhinovirus, and Coxsackievirus and the L protease of foot-and-mouth disease virus.

Modulation of eIF4E Activity by Phosphorylation

Two protein kinases that are associated with eIF4G, mitogen-activated protein kinase interacting serine/threonine kinase 1 and 2 (Mnk1 and Mnk2), phosphorylate Ser209 of eIF4E. Inhibition of cellular translation during mitosis and heat shock correlates with reduced phosphorylation of eIF4E. It has been suggested that phosphorylation of eIF4E allows tighter binding to the 5′-terminal cap. However, the effect of phosphorylation on the function of eIF4E is unclear.

A decrease in eIF4E phosphorylation may be responsible for the inhibition of mRNA translation in cells infected

Figure 11.26 Regulation of eIF4F activity. The illustration shows regulation of eIF4F activity, and inhibition of translation, by dephosphorylation of eIF4E, interaction with two eIF4E-binding proteins, and proteolytic cleavage of eIF4G.



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with some viruses. For example, cellular protein synthesis is inhibited at late times in adenovirus-infected cells, a result of virus-induced underphosphorylation of eIF4E. The viral L4 100-kDa protein binds to the C terminus of eIF4G, preventing binding of Mnk1, and hence presumably blocks phosphorylation of eIF4E. Adenoviral late mRNAs continue to be translated because they possess a reduced requirement for eIF4E. The majority of these viral mRNAs contain the tripartite leader (Fig. 10.12), a common 5′ noncoding region that mediates translation by ribosome shunting. Initiation by this mechanism is less dependent on eIF4F, presumably because the shunting of part of the 5′ untranslated region reduces the requirement for RNA-unwinding (helicase) activity associated with initiation by cap binding and scanning. Furthermore, adenovirus late mRNAs efficiently recruit the small quantities of phosphorylated eIF4E present late in infection, a feature of mRNAs with little RNA secondary structure near the 5′ cap. The tripartite leader therefore confers selective translation of viral over cellular mRNAs under conditions in which eIF4E is underphosphorylated. Adenovirus-induced translation inhibition not only boosts viral late mRNA translation, but also enhances cytopathic effects and consequently release of virus from cells.

Phosphorylation of eIF4E also regulates the innate immune response to infection. Mice that produce only a form of eIF4E that cannot be phosphorylated are less susceptible to infection with a number of RNA and DNA viruses. The animals produce more IFN, because nonphosphorylated eIF4E leads to reduced production of the inhibitor Ikbα, a regulator of Nf-κb. The genomes of herpesviruses and poxviruses encode proteins that promote phosphorylation of eIF4E, presumably to antagonize Nf-κb activation and reduce IFN production.

Modulation of eIF4E Activity by Binding Proteins

Three related low-molecular-weight cellular proteins, 4E-bp1, 4E-bp2, and 4E-bp3, bind to eIF4E and inhibit translation following 5′-end-dependent scanning, but not by internal ribosome entry (Fig. 11.26). The first was found to be identical to a previously described protein, called phosphorylated heat- and acid-stable protein regulated by insulin (Phas-I). This protein was known to be an important substrate for phosphorylation in cells treated with insulin and growth factors. Phosphorylation of 4E-bp *in vitro* blocks its association with eIF4E. When bound to 4E-bp, eIF4E cannot bind to eIF4G, and active eIF4F is not formed. eIF4G and 4E-bp proteins compete for binding to eIF4E. Treatment of cells with hormones and growth factors leads, via signal transduction pathways, to the phosphorylation of 4E-bp and its release from eIF4E. Translation of mRNAs with extensive secondary structure in the 5′ untranslated region is preferentially sensitive to the phosphorylation state of 4E-bp.

Some viral infections lead to alteration of the phosphorylation state of 4E-bp (Fig. 11.26). In contrast to the shutoff that occurs in poliovirus-infected cells, inhibition of cellular protein synthesis in cells infected with another picornavirus, encephalomyocarditis virus, occurs late in infection and is not mediated by cleavage of eIF4G. Rather, infection with this virus induces dephosphorylation of 4E-bp1. As a result, translation of cellular mRNAs is inhibited, but, because the viral mRNA contains an IRES, its translation is unaffected.

Phosphorylation of 4E-bp is carried out by a serine/threonine kinase, mammalian target of rapamycin kinase complex 1 (mTorC1). This complex regulates protein synthesis in response to a variety of signals (Fig. 11.27). Presence of growth factors, oxygen, glucose, and energy lead to increased translation as a result of phosphorylation of 4E-bp1 and ribosomal protein S6.

Many viral mRNAs are capped and therefore depend upon eIF4F for translation. As would be expected, mTorC1 is activated during infection, leading to increased protein synthesis under conditions (e.g., virus-induced stress) that would otherwise limit translation. Examples include inhibition of the tuberous sclerosis complex (Tsc) by the human papillomavirus E6 protein and stimulation of phosphatidylinositol 3-kinase (Pi3k) by the adenovirus E4 Orf1 protein.

Members of the *Herpesviridae* stimulate protein synthesis via multiple mechanisms. Both herpes simplex virus type 1 and human cytomegalovirus stimulate mTorC1 via inhibition of Tsc. The Us3 protein of herpes simplex virus type 1, a serine/threonine kinase, functions as an Akt mimic and phosphorylates Tsc2. In contrast, the human cytomegalovirus UL38 protein inactivates Tsc2 through direct binding. The vGpcr (viral G-protein coupled receptor) protein of Kaposi's sarcoma-associated herpesvirus and the LMP-2A protein of Epstein-Barr virus inactivate signaling pathways upstream of mTorC1. In contrast to many other viruses, human cytomegalovirus infection does not inhibit cellular protein synthesis, and furthermore, the abundance of eIF4F increases. These effects are a consequence in part of the viral UL38 protein, which activates mTorC1.

The protein 4E-bp1 is degraded by the proteasome in cells infected with herpes simplex virus type 1, but this action is not sufficient to promote assembly of eIF4F. Binding of eIF4E to eIF4G is stimulated by the viral ICP6 protein, which shares a domain with cellular chaperone protein hsp27 that controls eIF4F formation.

Modulation of eIF4E by miRNA

In response to enterovirus infection, synthesis of miR-141, a micro-RNA (miRNA) that targets mRNA encoding eIF4E, is induced as a result of the synthesis of a cellular transcription protein. Consequently, translation by 5′-end-dependent initiation is impaired. Translation of viral mRNAs is unaffected, because they are initiated by an IRES-dependent mechanism that does not require eIF4E. As expected, silencing of miR-141 reduces the production of infectious virus particles.

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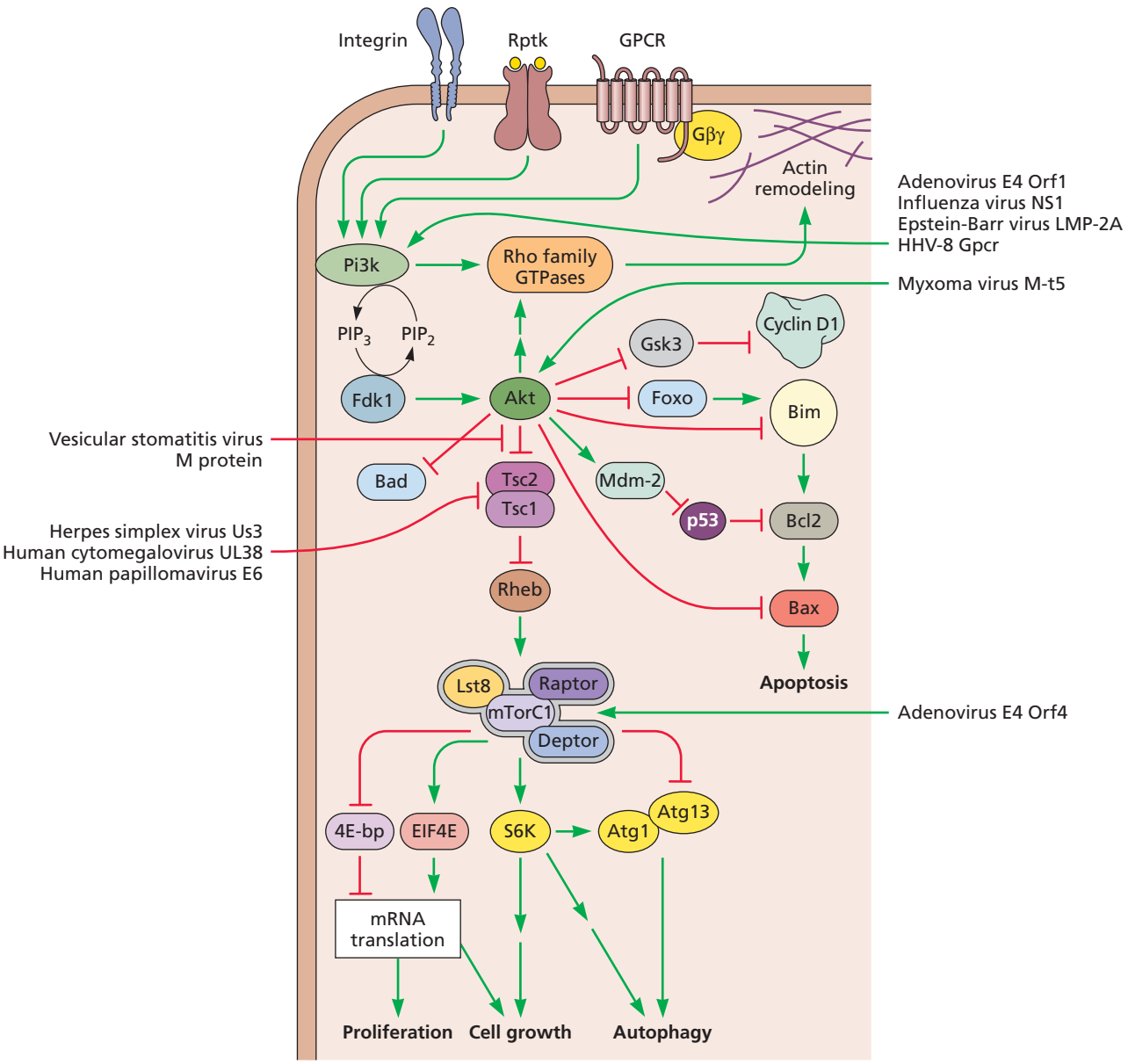


Figure 11.27 The mammalian Pi3k-Akt-mTor signaling route. The core features of this signaling transduction system are illustrated. Binding of ligand to any one of several types of plasma membrane receptors initiates signaling to Pi3k associated with the inner surface of the plasma membrane and phosphorylation and activation of this kinase. Once activated, phosphoinositol 3-kinases phosphorylate phosphoinositol present on membrane lipids to produce phosphoinositol 3,4,5-triphosphate (PIP_3). These modified lipids are bound by particular domains of other proteins, such as phophoinositide-dependent kinase 1 (Pdk1), which then transmit the signal to Akt. Synthesis of PIP_3 also leads to activation of small G proteins of the Rho (Ras homology) family that control actin polymerization and depolymerization, such as Rac (Ras-related C3 botulism toxin substrate 1) and Cdc42 (cell division control protein 42 homolog). Shown are consequences of Akt activation that promote cell growth and proliferation via activation of the mTor kinase present in mTorC1. Activated mTor facilitates translation by multiple mechanisms and also induces autophagy. Viral proteins that activate (green) or inhibit (red) are shown. Atg, autophagy-related protein; Bad, Bcl2-associated death protein; Bax, apoptosis-regulator Bcl2-associated protein; Bcl2, apoptosis-regulator Bcl2 (B cell CLL/lymphoma 2); Bim, Bcl2-interacting mediator of cell death; Deptor, Dep domain-containing mTor-interacting protein; 4E-bp, eukaryotic initiation factor 4E-binding protein; Foxo, forkhead box protein 1; Gpcr, G-protein coupled receptor; Gsk3, glycogen synthase kinase 3; HHV, human herpesvirus; Lst8, target of rapamycin complex subunit Lst8 homology; Mdm2, E3 ubiquitin ligase Mdm2 (double minute protein 2); Raptor, regulatory-associated protein of mTorC1; Rheb, Ras-homology enriched in protein; Rptk, receptor protein tyrosine kinase; S6k, ribosomal protein S6 kinase; Tsc, tuberous sclerosis protein.

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A Viral Protein That Replaces eIF4F

The nucleocapsid (N) protein of hantaviruses can replace all components of eIF4F. N protein substitutes for eIF4E by binding the mRNA cap, and can bind directly to the 43S preinitiation complex, replacing eIF4G. N also replaces the helicase activity of eIF4A. A heptanucleotide sequence in the 5′ untranslated region of viral mRNAs is sufficient for preferential N-dependent translation of viral over nonviral mRNAs. These activities presumably ensure efficient translation of viral mRNAs.

A Viral Cap-Binding Protein

Influenza virus infection leads to the inhibition of cellular mRNA translation, in part via dephosphorylation of eIF4E. How capped viral mRNAs are translated in infected cells was revealed by the finding that the viral polymerase, consisting of PB1, PB2, and PA subunits, binds with high affinity to cap structures. The PB2 subunit of the polymerase binds eIF4G, which is required for viral mRNA translation. The viral NS1 participates in translation by binding eIF4G and poly(A)-binding protein, possibly bringing together the 5′ and 3′ ends of the viral mRNAs to ensure more efficient translation.

Regulation of Poly (A)-Binding Protein Activity

The poly (A)-binding protein plays a crucial role in mRNA translation, bringing together the ends of the mRNA (Fig. 11.4). In cells infected with enteroviruses, lentiviruses, and caliciviruses, viral proteases cleave this protein, while it is sequestered by the rubella virus capsid protein. These events are believed to contribute to inhibition of host cell translation. However, poly(A)-binding protein is required for IRES-mediated translation initiation. Cleavage of this protein in enterovirus-infected cells (and sequestration by the rubella virus capsid) may contribute to the inhibition of translation needed for the switch to viral RNA synthesis.

The 3′ ends of rotaviral mRNAs are not polyadenylated and therefore cannot interact with poly(A)-binding protein. Instead, these 3′ untranslated regions contain a conserved sequence that binds the viral protein nsP3. This protein also occupies the poly(A)-binding protein binding site of eIF4G, bringing together the 5′ and 3′ ends of mRNA. Host translation is inhibited because nsP3 displaces poly(A)-binding protein from eIF4G. These interactions are believed to favor translation of viral mRNAs. However, nsP3 is not required for translation of viral mRNAs or for virus reproduction.

Redistribution of poly(A)-binding protein is another mechanism for selective translation of viral mRNAs in infected cells. The herpes simplex virus type 1 ICP27 and UL47 proteins and the Kaposi's sarcoma-associated herpesvirus proteins SOX and K8.1 cause redistribution of this poly(A)-binding protein to the nucleus. This effect likely contributes to shutoff of host cell translation: in cells infected with viruses lacking the gene encoding the SOX protein, poly(A)-binding protein is not routed to the nucleus, and host translation is unimpaired. In contrast, poly(A)-binding protein is not found in the nucleus of cells infected with human cytomegalovirus, in which host cell translation is unaffected. The redistribution of poly(A)-binding protein and eIF4F to cytoplasmic replication factories in poxvirus-infected cells likely contributes to inhibition of host translation and favoring of viral mRNA translation.

Regulation of eIF3

Some viruses encode proteins that bind eIF3 and impair 5′-end-dependent translation. The spike glycoprotein of severe acute respiratory syndrome coronavirus, the rabies virus M protein, and the measles virus N proteins all bind subunits of eIF3. The eIF3α and eIF3β subunits are cleaved by the viral protease in cells infected with the picornavirus foot-and-mouth disease virus, further contributing to inhibition of host protein synthesis caused by cleavage of eIF4G. It is not known how viral mRNAs are translated under these conditions.

An antiviral mechanism comprises three IFN-induced human genes, *ISG54*, *ISG56*, and *ISG60*, which encode proteins (P54, P56, and P60) that bind subunits of eIF3 and prevent translation. The P56 protein binds the e subunit of eIF3, while P54 binds to the c and e subunits. Both P54 and P56 interfere with stabilization of the ternary complex (Met-tRNA$_i$–eIF2–GTP), and P54 also inhibits formation of the 48S initiation complex (Fig. 11.3). Both 5′-end-dependent and internal initiation are inhibited by P56.

Interfering with RNA

Cellular protein synthesis may also be interrupted by virus-induced alteration of cellular mRNAs. Among RNA viruses, influenza viral and hantaviral endonucleases cleave cellular mRNAs to provide primers for viral RNA synthesis (Chapter 6). This process leads to destabilization of cellular mRNAs and inhibition of translation. The nsp1 protein of severe acute respiratory syndrome coronavirus has a similar effect by binding 40S ribosomes and degrading cellular mRNAs (Fig. 10.19). In cells infected with vesicular stomatitis virus, nuclear export of cellular mRNAs is suppressed.

DNA viruses such as poxviruses encode decapping enzymes that destabilize cellular mRNAs, while the herpes simplex virus type 1 virion shutoff protein is an endonuclease that binds eIF4A and eIF4B, leading to increased mRNA turnover (Fig. 10.19). The SOX protein of Kaposi's sarcoma-associated herpesvirus also induces degradation of cellular mRNA, but by a different mechanism: it recruits the cellular Xrn1 exonuclease to polysomes. The SOX protein bypasses the regulatory steps of deadenylation and decapping typically required for activation of Xrn1. Instead, SOX first internally

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cleaves mRNAs, which are then degraded by Xrn1. Some cellular mRNAs are protected from SOX cleavage by a sequence within the 3′ untranslated region.

In response to the production of viral dsRNAs, the cellular antiviral response includes production of RNase L, which is activated by the products of 2′-5′-oligoadenylate synthetase and degrades both rRNA and mRNA (Volume II, Chapter 3). Viral genomes encode a variety of proteins that bind dsRNAs and inhibit the RNase L pathway, preventing degradation of mRNAs. The murine hepatitis virus ns2 gene encodes a protein that cleaves 2′,5′-oligoadenylate chains to limit activation of RNase L. AU-rich binding proteins bind to sequences in the 3′ noncoding region of mRNAs to modulate their stability. These proteins also bind to the 5′ noncoding region of enteroviruses, but viral mRNA degradation is blocked because they are degraded by the viral 3CD protease.

Stress-Associated RNA Granules

Another mechanism by which mRNA translation can be impaired in virus-infected cells is by sequestering of mRNA from the translation apparatus in processing (P) bodies and stress granules. P bodies and stress granules are two nonmembranous cytoplasmic aggregates composed of mRNA, cellular miRNAs, mRNA-binding proteins, 40S ribosomal subunits, and many proteins that participate in mRNA translation. These granules are believed to form when translation is inhibited by intracellular and extracellular stresses such as nutrient deprivation or viral infection. A critical trigger for their formation is phosphorylation of eIF2α. When stress conditions are alleviated, the mRNAs found in these aggregates may be deadenylated and degraded, or returned to the pool of translated RNAs. Stress granules and P bodies may interact and exchange proteins and mRNAs with each other and with the cytoplasm.

Stress granules contain hundreds of RNA-binding proteins, and >100 cellular genes encode proteins that participate in their assembly. Two components of stress granules include T cell-restricted intracellular antigen-1 (Tia-1) and the Rasgap SH3 domain-binding protein 1 (G3bp1) (Fig. 11.28). Reduction in concentrations of either protein impairs formation of stress granules, and overproduction of either component stimulates formation of these aggregates. Stress granule formation occurs during infection with different viruses, and in most cases, they are suppressed at some point in the infectious cycle. Stress granules form early in cells infected with poliovirus. Late in viral infection, the viral proteinase $3C^{pro}$ cleaves G3bp1, disassembling stress granules, an event required for efficient viral reproduction. The presence of a noncleavable form of G3bp1 prevents the disassembly of stress granules and impairs viral reproduction. The NS1 protein of influenza A viruses prevents formation of stress granules by antagonizing Pkr. Stress granule components may also be redirected to other cellular sites in virus-infected cells. For example, the nsP3 protein of Semliki Forest virus sequesters G3bp1 into viral replication complexes. Removal of the nsP3 sequences that are important for interaction with this cellular protein impair viral production, suggesting a role for the protein in viral reproduction.

P bodies are a second type of non-membrane-bound aggregate in the cytoplasm that is enriched for components of the RNA decay machinery. These aggregates, which are the sites of RNA deadenylation and mRNA repression, are composed of proteins such as the decapping enzymes and proteins that mediate mRNA deadenylation. Virus replication may also lead to alteration of P bodies and redirection of their components. The enterovirus $3C^{pro}$ proteinase cleaves several P-body components including Xrn1, Dcp1a, and Pan3, disrupting P-body formation. Influenza virus infection leads to dispersal of P bodies via the viral NS1 protein, which binds a cellular protein required for P-body assembly. Infection with some viruses leads to co-opting of P-body components. In cells infected with the flavivirus West Nile virus, a number of P-body proteins are sequestered in viral replication complexes as the number of P bodies diminish. Some of these cellular proteins may be required for viral RNA synthesis. Subgenomic flavivirus RNA (sfRNA) is a fragment of the 3′ untranslated region produced when exonucleolytic decay by Xrn1 stalls at a pseudoknot. The sfRNA enters P bodies, where it inhibits Xrn1 activity.

Perspectives

From the smallest to the largest, all viral genomes encode proteins that recruit the host cell translational machinery for production of proteins needed for viral reproduction. These viral proteins control or modify cellular translation proteins, ribosomes, and the signaling pathways that regulate their activities. The result is not only production of viral proteins, but also suppression of intrinsic immune defenses. Among all the viruses studied, every step of the translation process appears to be modified. The study of such modifications has revealed a great deal about how proteins are made and how this process is regulated.

Very early in infection, intrinsic defense responses are mounted, and protein synthesis is inhibited in an attempt to limit viral reproduction. Should infection proceed, cellular stress responses, which cause further reduction in translation, are activated. As viral proteins and RNAs are produced, modifications to the cellular translation apparatus take place to favor the production of viral proteins. The interplay of cellular and viral modifications is an important determinant of the outcome of infection. Studies of ancient viral and cellular proteins that participate in translation reveal an evolutionary arms race as viral proteins change to overcome host defenses, and cellular proteins change in response. The results reveal the remarkable plasticity of protein function, and how genes and genomes have been shaped by challenges from viruses.

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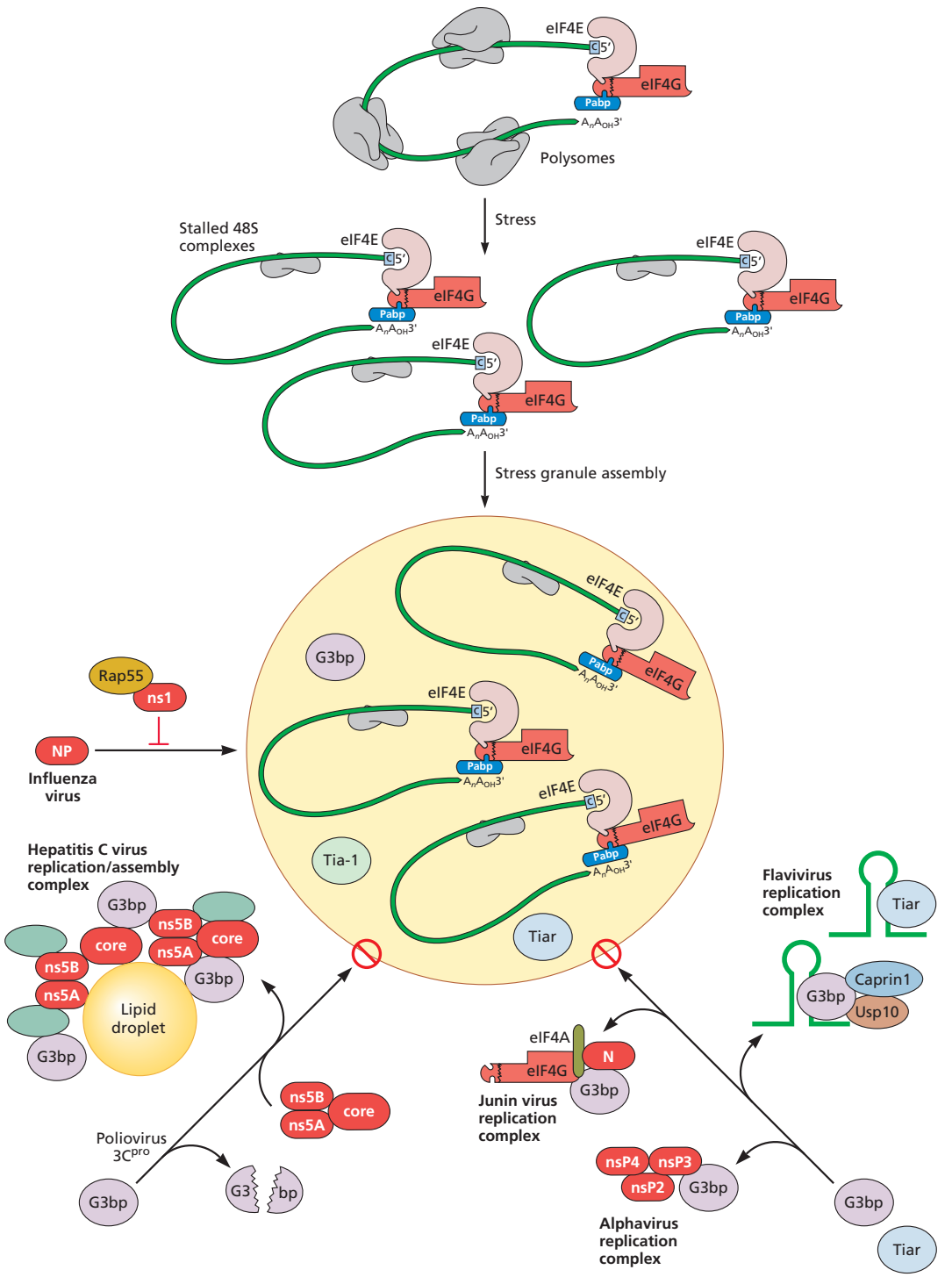


Figure 11.28 Inhibition of stress granule assembly by viral proteins. When protein synthesis is inhibited as a response to stress, stalled translational complexes are routed to stress granules. Three marker proteins for stress granules, T cell intracellular antigen-1 (Tia-1), Tia-1-related protein (Tiar), and G3bp, are shown. Infection by some viruses, such as West Nile virus, dengue virus, and poliovirus, may antagonize the formation of stress granules by interfering with the function of Tiar, Tia-1, or G3bp. RNA-associated protein 55 (Rap55) is a component of stress granules that is bound by influenza virus NS1 protein. G3bp may be cleaved by poliovirus $3C^{pro}$, or sequestered into the replication complexes that form in cells infected with hepatitis C virus, Junin virus, alphaviruses, or flaviviruses, blocking stress granule assembly. Caprin1 and Usp10 bind G3bp and may also have a role in stress granule formation.

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Viral reproduction cycles often include inhibition of translation of cellular mRNAs. The vast majority of virus-induced modifications affect the initiation step of protein synthesis. Indeed, our detailed understanding of this step of translation has been a consequence of unraveling the effects of viral infection. Although elongation and termination require far fewer cellular proteins, there are nonetheless examples of viral modulation of these steps as well. We also have a growing appreciation of how viral infection affects the stability of cellular mRNAs, through cellular mRNA decay pathways. A description of these controls can be found in Chapter 10.

An intriguing recent addition to our knowledge of translational control in virus-infected cells concerns RNA-containing granules. They regulate the mRNA cycle, metabolism, and gene expression and are an important point of control during virus infection. The field of virus-RNA granule interactions is young, and many questions remain. The impact of RNA granule formation on virus reproduction, the effect of virus proteins on granules, and the roles of granule proteins in viral reproduction have barely been explored. As it has not been possible to purify RNA granules from cells, other approaches to understanding how they are built and how they function must be developed. An intriguing hypothesis is that the formation of stress granules is part of an integrated response that includes intrinsic antiviral mechanisms. Emerging evidence indicates that intrinsic immunity and stress responses are linked at many levels. An example is Pkr, which is an IFN response protein but might also sense the formation of stress granules. Stress granule proteins are localized with proteins such as Rig-I-like receptors that activate IFN responses. How stress responses and intrinsic immunity interact at multiple levels may well be a major goal of future research in this field.

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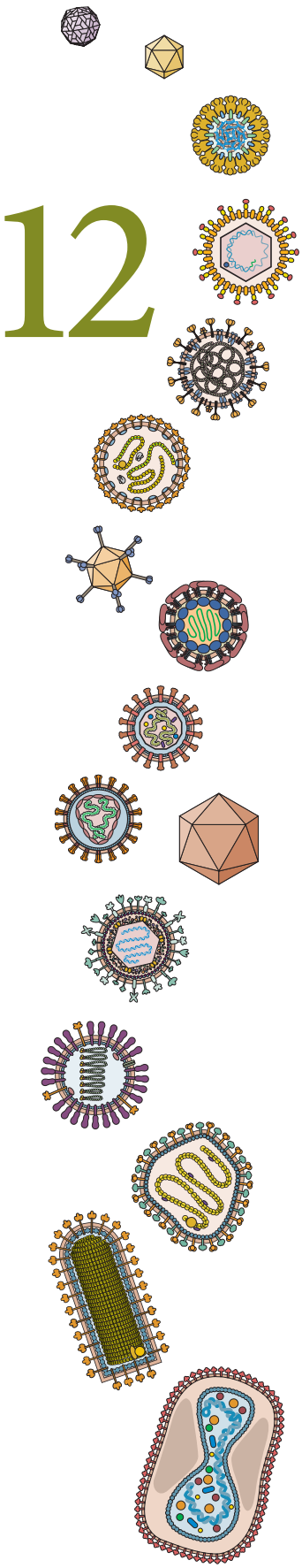
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12 Intracellular Trafficking

Introduction

Assembly within the Nucleus
- Import of Viral Proteins for Assembly

Assembly at the Plasma Membrane
- Transport of Viral Membrane Proteins to the Plasma Membrane
- Sorting of Viral Proteins in Polarized Cells
- Disruption of the Secretory Pathway in Virus-Infected Cells
- Signal Sequence-Independent Transport of Viral Proteins to the Plasma Membrane

Interactions with Internal Cellular Membranes
- Localization of Viral Proteins to Compartments of the Secretory Pathway
- Localization of Viral Proteins to the Nuclear Membrane

Transport of Viral Genomes to Assembly Sites
- Transport of Genomic and Pregenomic RNA from the Nucleus to the Cytoplasm
- Transport of Genomes from the Cytoplasm to the Plasma Membrane

Perspectives

References

LINKS FOR CHAPTER 12

- *Video: Interview with Dr. Ari Helenius*
  http://bit.ly/Virology_Helenius
- *Movie 12.1: Mouse salivary glands infected with a virulent strain of dually fluorescent derivative of pseudorabies virus*
  http://bit.ly/Virology_V1_Movie12-1

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He who is everywhere is nowhere.

SENECA

Introduction

Successful viral reproduction requires the intracellular assembly of progeny virions from their protein, nucleic acid, and, in many cases, membrane components. In preceding chapters, we have considered molecular mechanisms that ensure the synthesis of the macromolecules from which virus particles are constructed in the host cell. Because of the structural and functional compartmentalization of eukaryotic cells, components of these particles are generally produced at multiple intracellular locations, and must be brought together for assembly. Intracellular trafficking and sorting of viral nucleic acids, proteins, and glycoproteins to the appropriate sites is therefore an essential prelude to the assembly of all animal viruses.

From our point of view, animal cells are very small, with typical diameters of 10 to 30 μm. However, in the microscopic world inhabited by viruses, an animal cell is large: the distances over which virion components must be transported within a cell are roughly equivalent to a mile on the macroscopic, human scale. The properties of the intracellular milieu prevent viral particles, genomes, or subassemblies from reaching the appropriate intracellular destinations during entry or egress within reasonable periods simply by diffusion (Box 12.1). Their movement therefore requires transport systems and a considerable expenditure of energy, supplied by the host cell. The cellular highways most commonly used for movement of viral components for assembly are those formed by microtubules (as is also true during entry). These filaments are polarized and highly organized within the cell, with (−) ends at the microtubule-organizing center (near the nucleus) and (+) ends at the cell periphery. They are traveled by cellular (−) end- and (+) end-directed motor proteins that carry cargo and convert the chemical energy of ATP into kinetic energy.

The intracellular trafficking of viral macromolecules must be appropriately directed so that the building blocks of virus particles are delivered to the correct assembly site. Assembly of viral particles can occur at any one of several intracellular addresses, depending on whether the particles are enveloped or naked and on the site and mechanism of genome replication (e.g., Fig. 12.1 and 12.2). All viral envelopes are derived from one of the host cell's membranes, which are modified by insertion of viral proteins. Many virus particles assemble at the plasma membrane, but some envelopes are derived from membranes of internal compartments. Consequently, assembly of enveloped viruses requires delivery of some viral proteins to the appropriate membrane, as well as transport of other proteins and the nucleic acid genome to that membrane. Other assembly sites are the cell nucleus and within the cytoplasm. These strategies impose less complex trafficking problems than does assembly of enveloped viruses at membrane sites, but additional mechanisms may be required for egress of progeny particles from the cell. In some cases, genome-containing nucleocapsids are formed in infected cell nuclei but assembly is completed at a cellular membrane. Such spatial and temporal separation of assembly reactions depends on appropriate coordination among multiple transport processes.

The need for movement of proteins and nucleic acids from one cellular compartment to another, or for insertion of proteins into specific membranes, is not unique to viruses. The majority of cellular RNA species are exported from

PRINCIPLES *Intracellular trafficking*

- Progeny genomes, structural proteins, and enzymes of virus particles must be concentrated at the intracellular site at which assembly takes place.
- The movement of viral components requires transport systems and a considerable expenditure of energy, supplied by the host cell.
- When viruses with DNA genomes are assembled in the nucleus, cytoplasmic proteins must be actively imported.
- The particles of many viruses that reproduce in animal cells include a lipid envelope derived from a host cell membrane host cell, and therefore assembly takes place at a cell membrane.
- All proteins destined for insertion into the plasma membrane enter the ER as they are translated, and signal sequences within the amino termini of such proteins guide this process.
- The ER lumen is the site of many essential protein modification and folding reactions.
- Elaborate quality control mechanisms in the ER ensure that proteins that are misfolded are transported to the cytoplasm and degraded.
- Viral glycoproteins may be proteolytically processed in the Golgi network, a reaction essential for the formation of infectious particles.
- Viral components are sorted to specialized surfaces in polarized cells, including epithelial cells and neurons.
- The matrix or tegument proteins of enveloped viruses, which lie between the inner surface of the membrane and the capsid, do not enter the secretory pathway, but are synthesized in the cytoplasm and directed to membrane assembly sites by specific signals.
- The envelopes of a variety of viruses are acquired from internal membranes of the infected cell, rather than from the plasma membrane.

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BOX 12.1

DISCUSSION

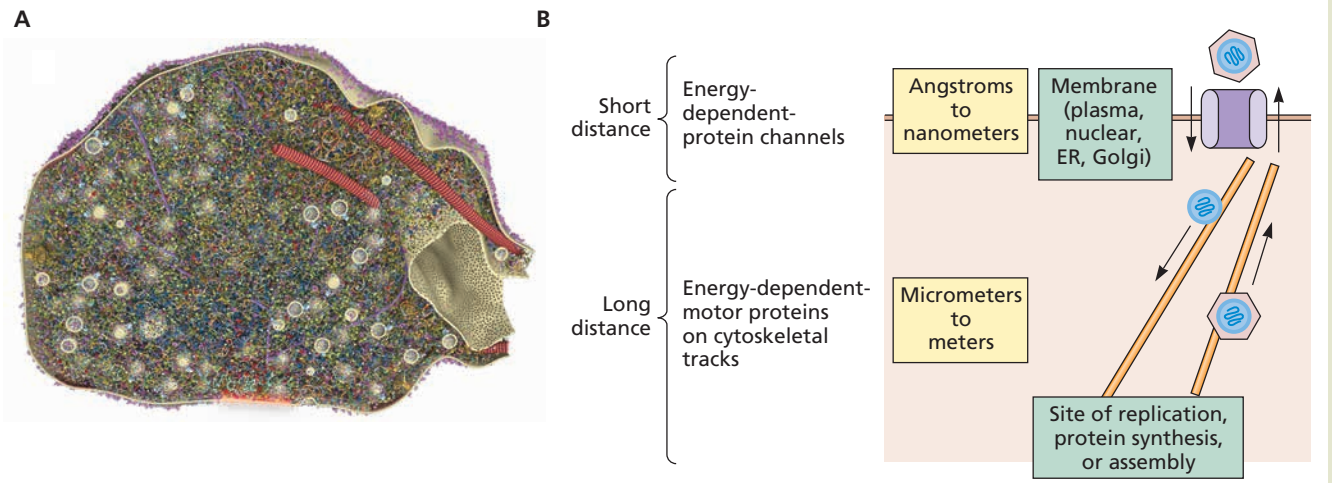
Getting from point A to point B in heavy traffic

Within a cell, directional movement and coordination of such movements in space and time are very complicated processes. Distributions of high-molecular-weight reactants and products are rarely controlled by concentration gradients and diffusion, as they are *in vitro*. Indeed, the inside of a cell is so tightly packed with organelles and cytoskeletal structures (panel A in the figure) that it is simply inappropriate to think of the contents of the cytoplasm, the nucleus, or organelle lumens as "gels" or "suspensions."

Directional movement in cells is achieved by two general processes (panel B). Short-distance movement across membranes or in and out of capsids is measured in angstroms to nanometers and is accomplished primarily via protein channels. Movement through such channels (transporters, translocons, pores, and portals) generally requires energy supplied by hydrolysis of ATP (or other nucleoside triphosphates).

Long-distance movement of proteins, viral particles or their components, and organelles inside cells is measured in micrometers to meters. Such movement invariably requires energy and is mediated by molecular motors moving on cytoskeletal tracks; myosins move cargo on actin fibers, while dynein and kinesin move cargo on microtubules.

(A) A three-dimensional model of a section through a rat synaptic bouton (the site of neurotransmitter release at the end of an axon terminal) showing 60 proteins, and the plasma membrane (light beige). This model was constructed by combining the results of several complementary approaches: quantitative immunoblotting and quantitative mass spectrometry to measure the number of molecules of each protein; electron microscopy to determine the number, size, and positions of organelles; and super-resolution fluorescence microscopy to localize the proteins. Previously determined molecular structures of the proteins and their interactions were also used. Adapted from B. G. Wilhelm et al., *Science* **344:**1023–1028, 2014, with permission. Courtesy of S. Rizzoli, European Neuroscience Institute, Germany. **(B)** Summary of properties of short- and long-range transport of viral components in infected cells.



the nucleus, in which transcription takes place. Similarly, cellular proteins are made by translation of messenger RNAs (mRNAs) in the cytoplasm and must then be transported to their sites of operation. Eukaryotic cells are therefore constantly engaged in transport of macromolecules among their compartments via intracellular trafficking systems. The cellular systems that sort macromolecules to particular intracellular sites are just as indispensable for viral reproduction as the cellular biosynthetic machineries responsible for transcription, DNA synthesis, or translation. Indeed, the advances in our understanding of cellular trafficking mechanisms can be traced to initial studies of viral membrane or nuclear proteins. In the following sections, the cellular transport pathways required during viral reproduction are described in the context of the site at which virion assembly takes place.

Assembly within the Nucleus

Assembly of the majority of viruses with DNA genomes, including adenoviruses, papillomaviruses, and polyomaviruses, takes place within infected cell nuclei, the site of viral DNA synthesis. All structural proteins of these nonenveloped viruses are imported into the nucleus following synthesis in the infected cell cytoplasm (Fig. 12.1), allowing complete assembly within this organelle. In contrast, assembly of the structurally more elaborate herpesviruses, which harbor a DNA-containing nucleocapsid assembled within the nucleus, is completed at extranuclear sites. So too is that of some enveloped RNA viruses with genomes that are replicated in nuclei, such as orthomyxoviruses. In these cases, only a subset of viral structural proteins must be imported into the nucleus.

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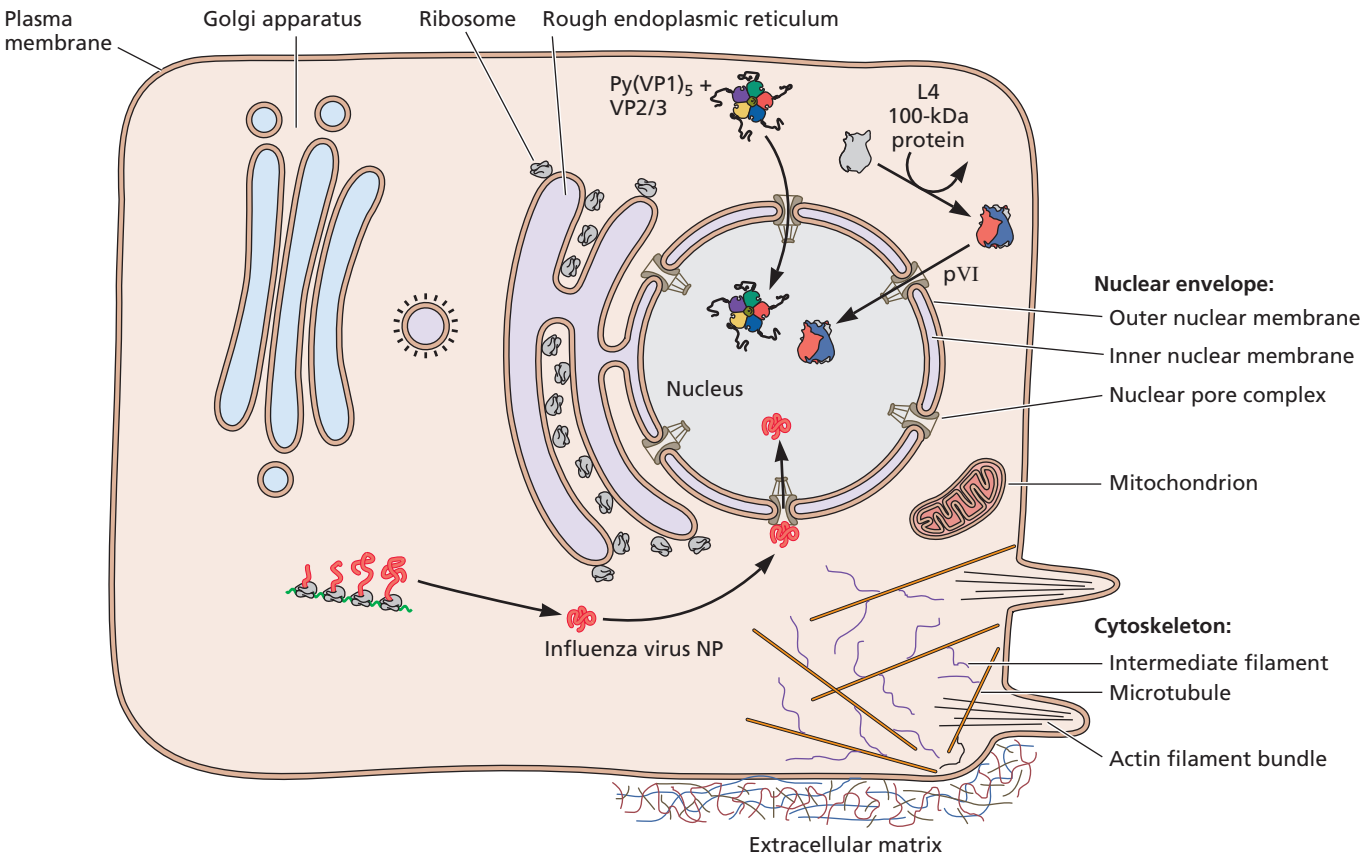


Figure 12.1 Localization of viral proteins to the nucleus. The nucleus and major membrane-bound compartments of the cytoplasm, as well as components of the cytoskeleton, are illustrated schematically and not to scale. Viral proteins destined for the nucleus are synthesized by cytoplasmic polyribosomes, as illustrated for the influenza virus NP protein. They engage with the cytoplasmic face of the nuclear pore complex and are translocated into the nucleus by the protein import machinery of the host cell. Some viral structural proteins enter the nucleus as preassembled structural units, as shown for polyomavirus [Py] VP1 pentamers associated with one molecule of either VP2 or VP3 and adenovirus hexon trimers formed with the assistance of the viral L4 100-kDa protein chaperone. Interaction of hexon trimers with the import receptors importin α/β is mediated by a second structural protein, protein pre-VI (pVI).

As far as we know, all viral structural proteins that enter the nucleus do so via the normal cellular pathways of nuclear protein import. These same pathways are responsible for import of both viral genomes (or nucleoproteins) and viral nonstructural proteins that function in the nucleus early in the infectious cycle (Chapter 5). Proteins destined for the nucleus carry nuclear localization signals (see Fig. 5.22), which are recognized by components of the cellular nuclear import machinery for subsequent transport into the nucleus.

Import of Viral Proteins for Assembly

The primary sequences of many such viral proteins destined for nuclear import contain putative nuclear localization sequences, which are characterized by clusters of basic amino acids. The majority of these sequences that have been verified experimentally conform to the simple or bipartite nuclear localization signals described in Chapter 5 (Fig. 5.22). However, some have noncanonical sequences, for example, the herpes simplex virus type 1 structural protein VP19C, or form only once the folded protein has assembled into structural units, as in porcine parvovirus VP2: this three-dimensional nuclear localization motif is present only in VP2 trimers, the major structural unit of the capsid.

A typical mammalian cell contains on the order of 3,000 to 4,000 nuclear pore complexes, each with a very high translocation capacity, with 10^3 translocation events/s. However, nuclear import also depends on the limited supply of soluble transport proteins. As large quantities of viral structural proteins must enter the nucleus prior to assembly, there is potential for competition among viral and cellular proteins for access to receptors or the nuclear pore complex proteins that mediate transport. Such competition is minimized in

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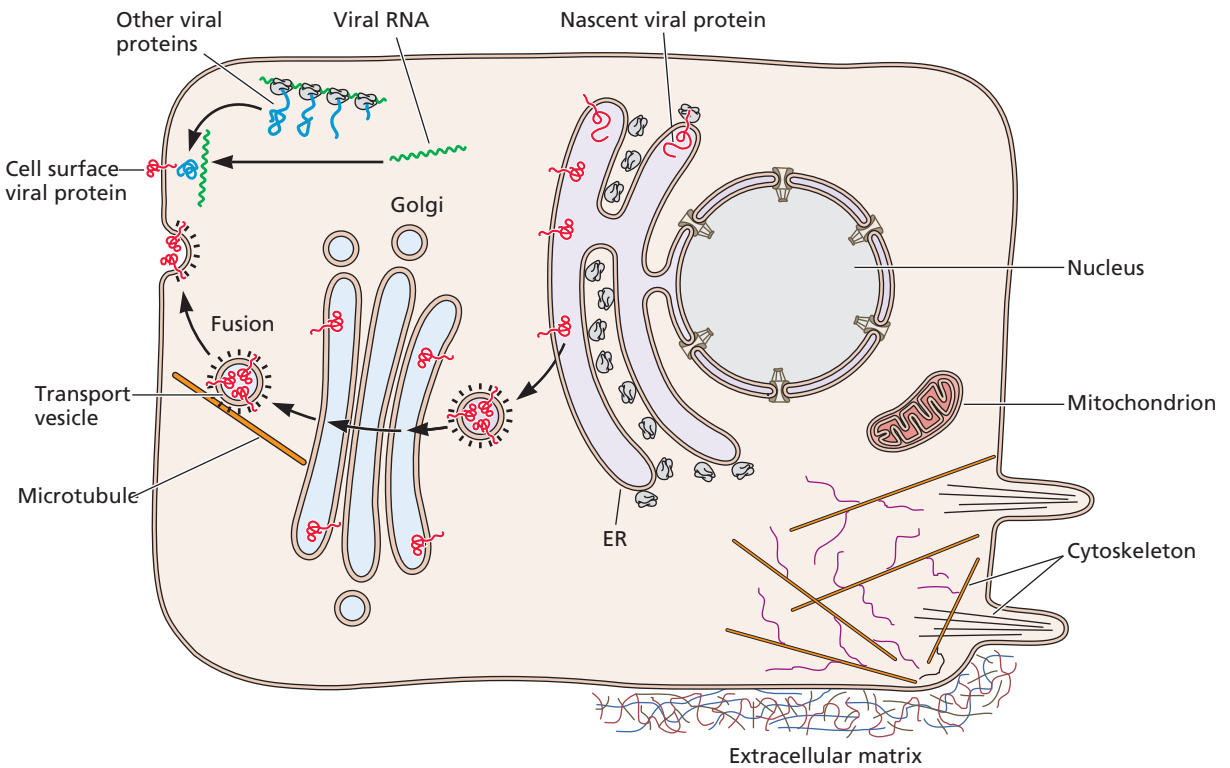


Figure 12.2 Localization of viral proteins to the plasma membrane. Viral envelope glycoproteins (red) are cotranslationally translocated into the ER lumen and folded and assembled within that compartment. They travel via transport vesicles to and through the Golgi apparatus and then to the plasma membrane. The internal proteins of the particle (blue) and the genome (green) are also directed to plasma membrane sites of assembly.

cells infected by the larger DNA viruses, such as adenoviruses and herpesviruses: by the time structural proteins are made during the late phase of infection, cellular protein synthesis is severely inhibited. The proteins of viruses that do not induce inhibition of cellular protein synthesis, such as those of the polyomaviruses, must enter the nucleus despite continual transport of cellular proteins. Whether import of viral proteins is favored in such circumstances, for example, by the presence of high-affinity nuclear localization signals, is not known.

Many viral structural proteins that enter infected cell nuclei form multimeric capsid components. Import of structural units of virus particles can depend on prior assembly in the cytoplasm to form the nuclear localization signal (see above) or to ensure efficient import. Pentamers of the major capsid protein (VP1) of simian virus 40 and polyomavirus specifically bind a common C-terminal sequence of either VP2 or VP3, the minor capsid proteins (Appendix, Fig. 23B). Such heteromeric assemblies are the substrates for import into the nucleus. Indeed, efficient nuclear localization of polyomavirus VP2 and VP3 proteins can occur only in cells in which VP1 is also made. Assembly of the heteromeric complex facilitates import of the minor structural proteins, even though each contains a nuclear localization signal. The increased density of these signals may allow more effective competition for essential components of the import pathway, or the nuclear localization signals may be more accessible in the complex.

Despite such potential advantages as increased efficiency of import of viral proteins and transport of the structural proteins in the appropriate stoichiometry, import of preassembled capsid components is not universal. For example, adenoviral hexons, trimers of viral protein II, are found only in the nucleus of the infected cell. Assembly of trimers requires a viral chaperone, the L4 100-kDa protein. However, when these two proteins are made in the absence of other adenoviral gene products, hexons do not enter the nucleus. This process requires a second structural protein, pre-VI (pVI), which interacts with hexons and cellular import receptors (Fig. 12.1).

Assembly at the Plasma Membrane

The particles of many viruses that reproduce in animal cells include a lipid envelope derived from a membrane of the host cell, although this structural feature is rare among

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BOX 12.2

DISCUSSION

Does host cell architecture shape virus structure?

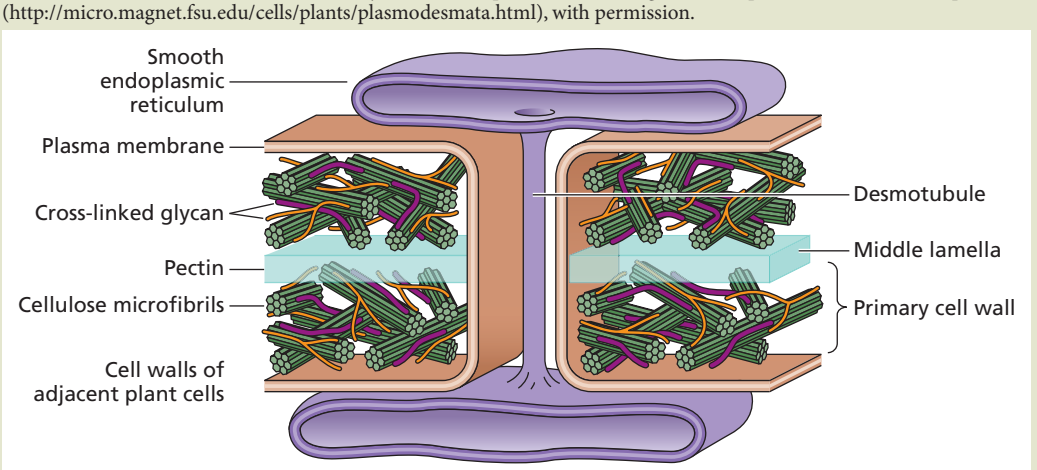
Many viruses that are important human pathogens, including hepatitis B and C viruses, human immunodeficiency virus type 1, and influenza A virus, are enveloped. In fact, the particles of >50% of the virus families that reproduce in animal cells include a lipid membrane, regardless of the nature of the viral genome. Furthermore, acquisition of the envelope and release of these viruses from the host cell are frequently accomplished in a single step. In contrast, the particles of only some 10% of plant virus families are enveloped (3 of 29 listed in the *Ninth Report of the International Committee on Taxonomy of Viruses* [2012]). Two of these families, *Bunyaviridae* and *Rhabdoviridae*, also include viruses that replicate in animal cells, but with significant differences in assembly and release.

In mammalian cells, rhabdoviruses, such as vesicular stomatitis virus, acquire their envelope, and are concomitantly released, by budding through the plasma membrane. However, plant rhabdoviruses form upon budding of internal components either into the endoplasmic reticulum (lettuce necrotic yellow virus) or through the inner nuclear membrane (potato yellow dwarf virus), and in both cases accumulate at these intracellular sites. In similar fashion, bunyavirus particles are released from infected animal cells via the secretory pathway following formation within Golgi compartments, but are not released from plant cells. For example, tomato spotted wilt virus particles accumulate in vesicles derived from Golgi and endoplasmic reticulum membranes until the cells are ingested by insect vectors (thrips) during feeding. In infected salivary gland cells of the insect host, tomato spotted wilt virus particles are formed and secreted from the plasma membrane like bunyavirus particles in mammalian cells.

Formation of an envelope provides an effective means of direct or indirect release from animal cells of progeny virus particles, which can then infect other cells in the organism via their accessible plasma membranes. In contrast, plant cells are surrounded by a structure that imposes formidable barriers to exit and entry by these mechanisms, the cell wall. This thick and rigid structure is built from microfibrils of cellulose organized into a network with the polysaccharides pectin and cross-linking glycans (see the figure). Neighboring cell walls are penetrated by the numerous microchannels (plasmodesmata) by which a plant cell is connected to its neighbors. Consequently, the acquisition of an envelope is of little benefit to viruses that reproduce in plant cells. Rather, the genomes of all plant viruses encode movement proteins that induce alterations of plasmodesmata to allow direct passage of virus particles (or genomes) from one cell to another (Box 13.14). Furthermore, the great majority of plant viruses are transmitted among host plants not by release into the environment but by vectors, most commonly insects.

Kormelink R, Garcia ML, Goodin M, Sasaya T, Haenni AL. 2011. Negative-strand RNA viruses: the plant-infecting counterparts. *Virus Res* **162:**184–202.

Two adjacent plant cells showing the plasma membrane components of the cell wall and a plasmodesma through the plasma membrane and its internal tube-like structure, the dermotubule derived from the endoplasmic reticulum. Plasmodesmata directly connect one plant cell to its neighbors. Adapted from Molecular Expressions (http://micro.magnet.fsu.edu/cells/plants/plasmodesmata.html), with permission.



plant viruses (Box 12.2) . Assembly of the majority of such enveloped viruses takes place at the plasma membrane. Before such virus particles can form, viral integral membrane proteins must be transported to this cellular membrane. The first stages of the pathway by which viral and cellular proteins are delivered to the plasma membrane were identified more than 35 years ago, and the process is now understood quite well. Viruses with envelopes derived from the plasma membrane also contain internal proteins, which may be membrane associated, and, of course, nucleic acid genomes. These internal components must also be sorted to appropriate plasma membrane sites for assembly (Fig. 12.2).

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Transport of Viral Membrane Proteins to the Plasma Membrane

Viral membrane proteins reach their destinations by the highly conserved, cellular **secretory pathway**. Many of the steps in the pathway have been studied by using viral membrane glycoproteins, such as the vesicular stomatitis virus G and influenza virus hemagglutinin (HA) proteins. These viral proteins offer several experimental advantages: they are synthesized in large quantities; their synthesis is initiated in a controlled fashion following infection; and their transport can be studied readily by genetic, biochemical, and imaging methods.

Entry into the first staging post of the secretory pathway, the endoplasmic reticulum (ER), is accompanied by membrane insertion of integral membrane proteins. Viral envelope proteins generally span the cellular membrane into which they are inserted only once, and therefore contain a single transmembrane domain. In viral proteins, transmembrane segments (described in Chapter 5) usually separate large extracellular from smaller cytoplasmic domains (Fig. 12.3). The former include the binding sites for cellular receptors, crucial for initiation of the infectious cycle, whereas the latter are important in virus assembly. Viral membrane proteins are usually oligomers (Chapter 4). Most interactions among the subunits of viral membrane proteins are noncovalent, but some examples of association via covalent interchain disulfide bonds are known. Oligomer assembly takes place during transit from the cytoplasm to the cell surface, as does the proteolytic processing necessary to produce some mature (functional) envelope glycoproteins from the precursors that enter the secretory pathway. For example, the human immunodeficiency virus type 1 Env protein and influenza virus HA0 precursor of pathogenic strains of avian influenza virus are cleaved within Golgi compartments.

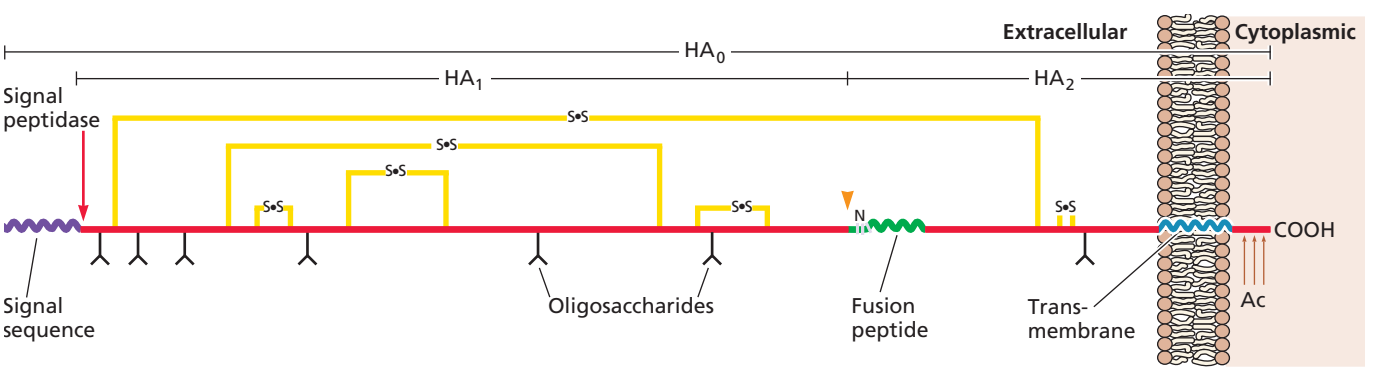
Viral (and cellular) proteins that travel the secretory pathway also possess distinctive structural features, including disulfide bonds and covalently linked oligosaccharide chains (Fig. 12.3). These characteristic covalent modifications (as well as oligomerization) take place as proteins travel through a series of specialized compartments that provide the chemical environments and enzymatic machinery necessary for their maturation, as illustrated in Fig. 12.4 for the influenza A virus HA0 protein. The first such compartment, the ER, is encountered by viral membrane proteins as they are synthesized.

Translocation of Viral Membrane Proteins into the Endoplasmic Reticulum

All proteins destined for insertion into the plasma membrane, or the membranes of such intracellular organelles as the Golgi apparatus, enter the ER as they are translated (Fig. 12.2). This membranous structure appears as a basketwork of tubules and sacs extending throughout the cytoplasm (Fig. 12.5A). The ER membrane demarcates a geometrically convoluted but continuous internal space, the **ER lumen**, from the remainder of the cytoplasm. The ER lumen is characterized by a chemically distinctive environment and is topologically equivalent to the outside of the cell. Proteins that enter the ER during their synthesis are therefore sequestered from the cytoplasmic environment as they are made.

Polyribosomes engaged in synthesis of proteins that will enter the secretory pathway become associated with the cytoplasmic face of the ER membrane soon after translation begins. Areas of the ER to which polyribosomes are bound form the **rough ER** (Fig. 12.5B). The association of polyribosomes with the ER membrane is directed by a short sequence in the nascent protein, termed the **signal peptide**. It is now taken for granted that the primary sequences of proteins

Figure 12.3 Primary sequence features and covalent modifications of the influenza virus HA protein. The primary sequence of the HA0 protein is depicted by the red line in the center, with the orange arrowhead indicating the site of the proteolytic cleavages that produce the HA1 and HA2 subunits from HA0 of pathogenic avian strains. The fusion peptide, the N-terminal signal sequence that is removed by signal peptidase in the ER, and the C-terminal transmembrane domain are hydrophobic. Disulfide bonds, one of which maintains covalent linkage between the HA1 and HA2 proteins following HA0 cleavage, are indicated, as are sites of N-linked glycosylation (oligosaccharides) and palmitoylation (Ac).



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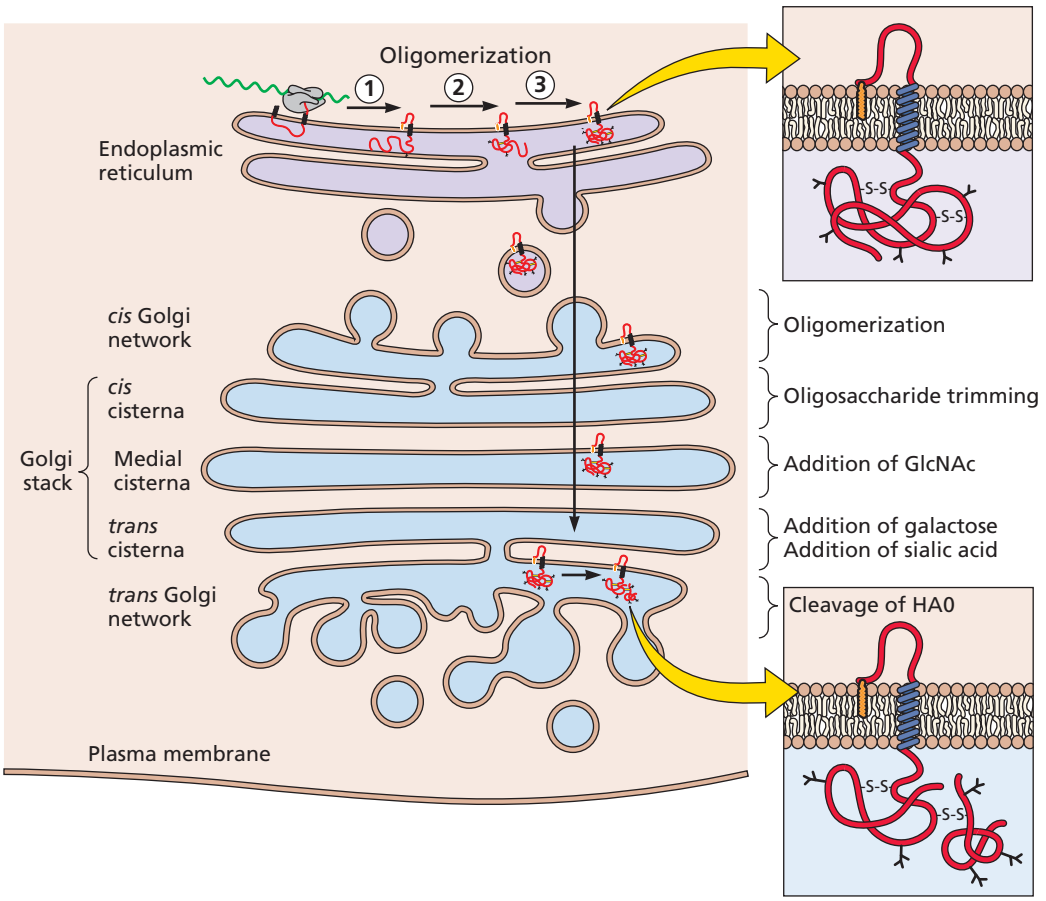


Figure 12.4 Maturation of influenza virus HA0 protein during transit along the secretory pathway. The modifications that occur during transit of the influenza virus HA0 protein through the various compartments of the secretory pathway are illustrated. In the ER, these are translocation and signal peptide cleavage (1), disulfide bond formation, and addition of N-linked core oligosaccharides (2), as the protein folds (3). The cytoplasmic domain acquires palmitate (orange) while the protein travels to the plasma membrane, but it has not been established when this modification takes place. For simplicity, the protein is depicted as a monomer, although oligomerization also takes place in the ER lumen. Note that the protein domain initially introduced into the ER lumen, in this case the N-terminal portion of the protein (type I orientation), corresponds to the extracellular domain of the cell surface protein.

include "zip codes" specifying the cellular addresses at which the proteins must reside to fulfill their functions, such as the nuclear localization signals discussed in the previous section. The signal peptides of proteins that enter the ER lumen were the first such zip codes to be identified, and established this paradigm some 35 years ago. Signal peptides are commonly found at the N termini of proteins destined for the secretory pathway. They are usually about 20 amino acids in length and contain a core of 15 hydrophobic residues. Signal peptides are often removed enzymatically during protein translocation into the ER by a protease located in the lumen, signal peptidase.

Translation of a protein that will enter the ER begins in the normal fashion and continues until the signal peptide emerges from the ribosome (Fig. 12.6). This signal then directs binding of the translation machinery to the ER membrane by means of two components: the signal peptide is recognized by the **signal recognition particle (SRP)**, which in turn binds to the cytoplasmic domain of an integral ER membrane protein termed the **SRP receptor**. Binding of the signal recognition particle to the ribosome temporarily halts translation, to allow the stalled translation complex to bind to the ER membrane. Following the initial docking of the complex at the membrane, the ribosome becomes tightly bound to the membrane and engaged with a protein translocation channel, which forms a gated, aqueous pore through the ER membrane. This interaction is coordinated with release of the signal recognition particle, association of the signal peptide with the

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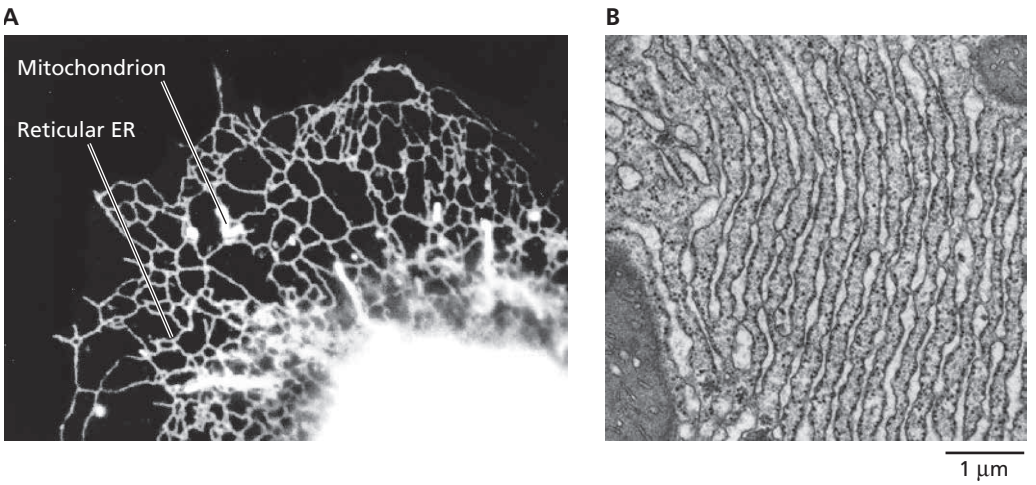
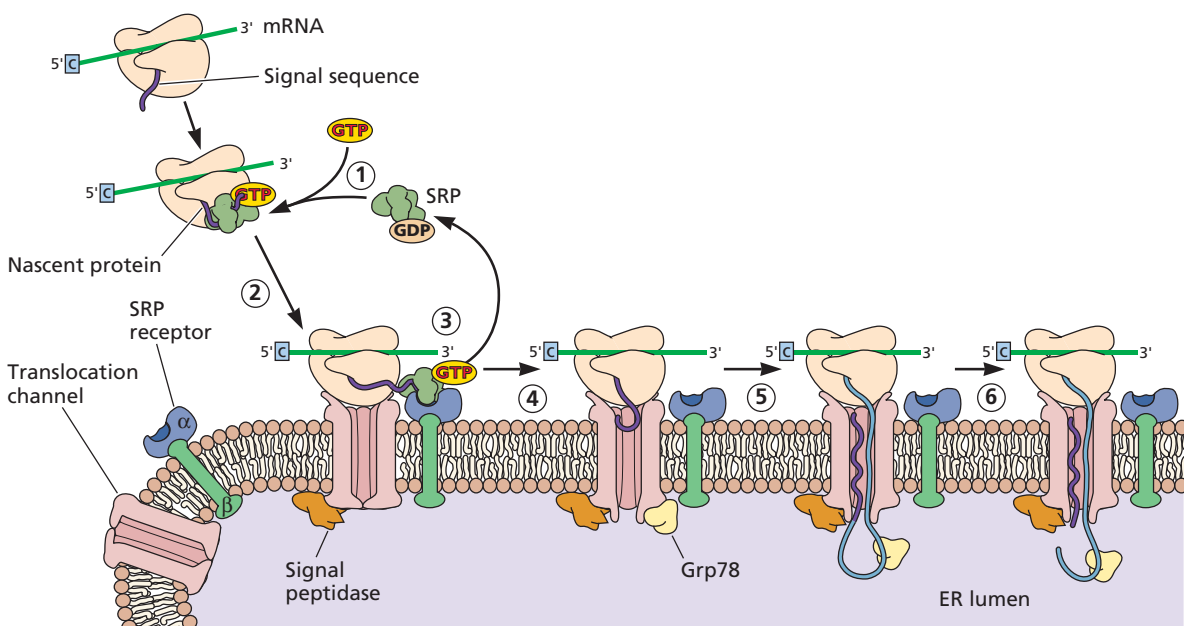


Figure 12.5 The endoplasmic reticulum. **(A)** The ER of a mammalian cell in culture. The reticular ER, which extends throughout the cytoplasm, was visualized by fluorescence microscopy of fixed African green monkey kidney epithelial cells stained with the lipophilic fluorescent dye 3,3′-dihexyloxacarbocyanine iodide. This dye also stains mitochondria. The ER membrane accounts for over half of the total membrane of a typical animal cell and possesses a characteristic lipid composition. Courtesy of M. Terasaki, University of Connecticut Health Center. **(B)** Electron micrograph of the rough ER in rat hepatocytes. Note the many ribosomes associated with the cytoplasmic surface of the membrane. From R. A. Rodewald, Biological Photo Service.

Figure 12.6 Targeting of a nascent protein to the ER membrane. Translation of an mRNA encoding a protein that will enter the ER lumen proceeds until the signal peptide (purple) emerges from the ribosome. The signal recognition particle (SRP), which contains a small RNA molecule and several proteins, binds to both the signal peptide and the ribosome to halt or pause translation, upon binding of GTP to one of the protein subunits (step 1). The nascent polypeptide-SRP-ribosome complex then binds to the SRP receptor in the ER membrane (step 2). This interaction triggers hydrolysis of GTP bound to SRP and to its receptor; release of SRP (step 3); and close association of the ribosome with, and binding of the hydrophobic signal peptide to, the heterotrimeric protein translocation channel (step 4). These interactions trigger opening of the cytosolic end of the channel. The luminal end of the translocation channel is also initially closed. Translation is then resumed, and the seal maintained at the luminal end of the channel early in translocation is reversed by binding of the chaperone Grp78. The growing polypeptide chain is transferred through the membrane as its translation continues (step 5). In some cases, signal peptidase removes the signal peptide cotranslationally (step 6). A lateral gate in the channel opens within the membrane for transfer of the transmembrane domain(s) of translocated proteins into the ER membrane.



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translocation channel, and resumption of translation. Because the ribosome remains bound to the membrane upon release of the signal recognition particle, continued translation facilitates movement of the growing polypeptide chain through the membrane. Such coupling of translation and translocation ensures that the protein crosses the membrane as an unfolded chain that can be accommodated within the translocation channel. Movement of the growing polypeptide through the membrane channel is facilitated by binding of the luminal chaperone Grp78 (Bip) to the nascent protein.

When a protein entering the ER is destined for secretion from the cell, translocation continues until the entire polypeptide chain enters the lumen. During translocation, the signal peptide is proteolytically removed by signal peptidase, releasing the soluble protein into the ER. In contrast, translocation of integral membrane proteins with a single transmembrane domain, such as viral envelope proteins, halts when a hydrophobic **stop transfer signal** is encountered in the nascent protein. This sequence may be the signal peptide itself or a second, internal hydrophobic sequence. In proteins that span the membrane multiple times, the number, location, and orientation of stop and start transfer signals within a protein determine the topology with which it is organized in the ER membrane. The programming of insertion of proteins into the ER membrane by signals built into their primary sequences ensures that every molecule of a particular protein adopts the identical topology in the membrane. As this topology is maintained during the several membrane budding and fusion reactions by which proteins reach the cell surface, the way in which a protein is inserted into the ER membrane determines its orientation in the plasma membrane.

Reactions within the ER

The folding and initial posttranslational modification of proteins that enter the secretory pathway take place within the ER. The lumen contains many enzymes that catalyze chemical modifications, such as disulfide bond formation and glycosylation, or that promote folding and oligomerization.

Glycosylation. Viral envelope proteins that travel the secretory pathway, like their cellular counterparts, are generally modified by the addition of oligosaccharides to either asparagine (N-linked glycosylation) or serine or threonine (O-linked glycosylation). Initial assembly of a typical oligosaccharide, its transfer to a protein, and its subsequent maturation by removal and addition of sugar residues require a large suite of enzymes. Consequently, the great majority of viral glycoproteins are glycosylated by host cell components, but the structural proteins of some large DNA viruses are modified in this way by viral enzymes (Box 12.3) .

The presence of oligosaccharides on a protein can be detected as changes in the protein's electrophoretic mobility, following exposure of cells to inhibitors of glycosylation, or of cell extracts to enzymes that cleave the oligosaccharide (Fig. 12.7A). The first steps in N-linked glycosylation take place as a polypeptide chain emerges into the ER lumen. Oligosaccharides rich in mannose preassembled on a lipid carrier are added to asparagine residues by an oligosaccharyltransferase (Fig. 12.7B). Subsequently, several sugar residues are trimmed from N-linked core oligosaccharides in preparation for additional modifications that take place as the protein travels from the ER to the plasma membrane.

Sites of N-linked glycosylation are characterized by the sequence NXS/T (where X is any amino acid except proline), but not every potential glycosylation site is modified. Even a single specific site within a protein is not necessarily modified with 100% efficiency. Each glycoprotein population therefore comprises a heterogeneous mixture of **glycoforms**, varying in whether a particular site is glycosylated, as well as in the composition and structure of the oligosaccharide present at each site. As many viral and cellular proteins contain a large number of potential N-linked glycosylation sites, particular proteins can exist in an extremely large number of glycoforms. This property complicates investigation of the physiological functions of oligosaccharide chains present on glycoproteins. Nevertheless, glycosylation has been assigned a wide variety of functions.

As essential components of receptors and ligands, oligosaccharides participate in many molecular recognition reactions. These processes include binding of certain hormones to their cell surface receptors; interactions of cells with one another; binding of virus particles, such as those of influenza A virus and herpesviruses, to their host cells; and later steps in virus entry. Some sugar units serve as signals, targeting proteins to specific locations, in particular to lysosomes. Glycosylation has also been suggested to fulfill more general functions, such as protecting proteins (and virus particles) that circulate in body fluids from degradation and host immune defenses. Many proteins contain such a large number of glycosylation sites that carbohydrate can contribute >50% of the mass of the mature protein, for example, the poliovirus receptor and the respiratory syncytial virus G protein. The hydrophilic oligosaccharides are present on the surface of such proteins, where they can form a sugar "shell," masking much of the proteins' surfaces, including epitopes recognized by antiviral antibodies (Box 12.4).

Studies of viral glycoproteins have established that glycosylation can be absolutely required for proper folding. For example, elimination (by mutagenesis) of all sites at which the vesicular stomatitis virus G or influenza virus HA0 proteins are glycosylated blocks the folding of these proteins and their exit from the ER (see "Protein folding and quality control" below). Before a protein folds, its hydrophobic amino acids, which are ultimately buried in the interior, are exposed. Such exposed hydrophobic patches on individual unfolded polypeptide chains tend to interact with one another nonspecifically, leading to aggregation. The hydrophilic oligosaccharide chains are thought to counter this tendency.

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BOX 12.3

EXPERIMENTS

Self-glycosylation: virus-encoded enzymes for formation of glycoproteins

The paradigm for production of viral envelope glycoproteins is addition of N- (and O-) linked oligosaccharides as the proteins travel the secretory pathway. Such modification is the result of sequential action of several host cell glycosidases and glycolsyltransferases located in the ER and Golgi compartments (Fig. 12.4). Consequently, the genomes of enveloped viruses (some of which are quite small) typically do not encode such enzymes. One striking exception is provided by phycodnaviruses.

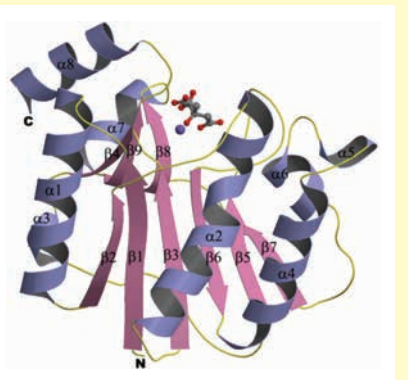
These viruses, such as *Paramecium bursaria* chlorella virus 1 (PBCV-1), have large, double-stranded DNA genomes encoding >350 proteins and share evolutionary history with other large DNA viruses, including poxviruses. The major capsid protein, Vp54, and two minor structural proteins are glycosylated. Sequencing of the PBCV-1 genome identified five potential glycosyltransferases, and several observations indicate that these enzymes, rather than the host cell machinery, modify the viral proteins:

- Sugars typically present in N- (and O-) linked oligosaccharides synthesized by cellular enzymes (e.g., *N*-acetylglucosamine) could not be detected in Vp54 glycans.
- Polyclonal antibodies against virus particles do not react with cellular glycoproteins and recognize Vp54 before, but not after, chemical removal of its oligosaccharides.
- Vp54 proteins specified by viral mutants resistant to the inhibitory effects of such antibodies exhibit differences in electrophoretic migration when glycosylated, but not following removal of glycans.
- The Vp54 genes of all such mutants are identical in sequence to the wild-type gene, but a subset carry substitutions or deletions in a gene encoding a candidate glycosyltransferase, α64r.
- The N-terminal domain of the α64r protein is structurally similar to glycosyltransferases that transfer sugars from a UDP carrier.

These and other observations establish that viral enzymes glycosylate the PBCV-1 Vp54 protein (at six Asn and Ser residues). The mechanisms of glycosylation, and when this process occurs during the infectious cycle, remain to be established.

Graves MV, Bernadt CT, Cerny R, Van Etten JL. 2001. Molecular and genetic evidence for a virus-encoded glycosyltransferase involved in protein glycosylation. *Virology* **285:**332–345.

Van Etten JL, Gurnon JR, Yanai-Balser GM, Dunigan DD, Graves MV. 2010. Chlorella viruses encode most, if not all, of the machinery to glycosylate their glycoproteins independent of the endoplasmic reticulum and Golgi. *Biochim Biophys Acta* **1800:**152–159.



Crystal structure of the glycosyltransferase domain of the viral A64R protein with $Mn2^+$ and citrate ions bound. These ions are shown in ball-and-stick representation with N, C, O, and Mn atoms colored blue, gray, red, and purple, respectively. This structure, which comprises a central β-sheet flanked by α-helices, is very similar to that of the catalytic domains of one of two groups of cellular glycosyltransferases. Such structural conservation is striking, as the sequence conservation is very low (<14%) and no relationship of the viral to cellular enzymes could be established by sequence analysis. Adapted from Y. Zhang et al., *Structure* **15:**1031–1039, 2007, with permission. Courtesy of M. Rossmann, Purdue University.

Disulfide bond formation. A second chemical modification that generally is restricted to proteins entering the secretory pathway, and essential for the correct folding of many, is the formation of intramolecular disulfide bonds between pairs of cysteine residues (Fig. 12.3). These bonds can make important contributions to the stability of a folded protein. However, they rarely form in the reducing environment of the cytoplasm. The more oxidizing ER lumen provides an appropriate chemical environment for disulfide bond formation. This compartment contains high concentrations of protein disulfide isomerase and other enzymes that catalyze the formation, reshuffling, or even breakage of disulfide bonds under appropriate redox conditions. As formation of the full and correct complement of disulfide bonds in a protein is often the rate-limiting step in its folding, these enzymes are important catalysts of this process.

The cellular enzymes that promote formation of disulfide bonds are present in the ER lumen. Consequently, this modification typically is limited to proteins that enter, or protein domains exposed to, this compartment. Remarkably, however, several viral membrane proteins present in mature virus particles of the poxvirus vaccinia virus and other viruses with large DNA genomes have stable disulfide bonds in their **cytoplasmic** domains: the genomes of these viruses encode all the enzymes necessary to catalyze the formation of disulfide bonds in the cytoplasm (Box 12.5).

Protein folding and quality control. A number of other cellular proteins assist the folding of the extracellular domains of viral membrane glycoproteins as they enter the lumen of the ER. In contrast to the enzymes described above, these proteins do not alter covalent structures. Rather, their primary function is to facilitate folding, largely by preventing improper associations among unfolded, or incompletely folded, polypeptide chains, such as the nonspecific, hydrophobic interactions described above. Such **molecular chaperones** play essential roles in the folding of individual polypeptides and in the oligomerization of proteins.

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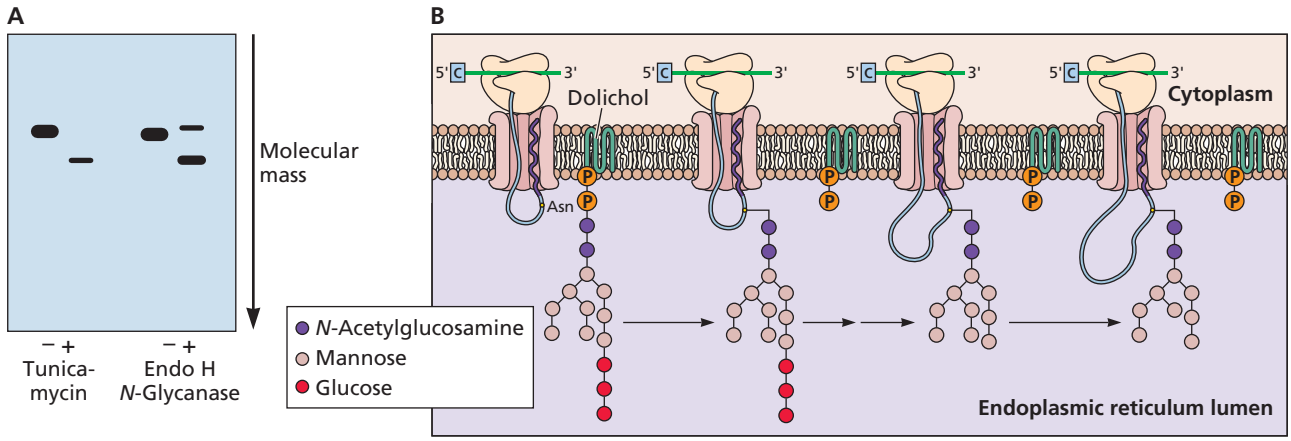


Figure 12.7 Detection and structure of N-linked oligosaccharides. **(A)** Detection of N-linked oligosaccharides using inhibitors or specific enzymes. Addition to cells of tunicamycin, an inhibitor of the first step in synthesis of the oligosaccharide precursor, prevents N-linked glycosylation, so that the mobility of glycoproteins is altered (left). *In vitro* treatment of glycoproteins with enzymes that cleave within the oligosaccharide, such as endoglycosidase H (Endo H) or *N*-glycanase, can also alter glycoprotein mobility (right). Glycosylation of a protein can also be assayed by incorporation of radioactively labeled monosaccharides. **(B)** The branched, mannose-rich oligosaccharide added via an N-glycosidic bond to asparagine residues of proteins is initially assembled on the lipid carrier dolichol phosphate (left). This common precursor is transferred to N-linked glycosylation sites as proteins are translocated into the ER. While within the ER, three glucose residues and one mannose residue are trimmed from the core oligosaccharide.

BOX 12.4

DISCUSSION

The evolving sugar "shield" of human immunodeficiency virus type 1

Mutational studies have implicated N-linked glycosylation at specific sites in the envelope proteins of several viruses in protection against host neutralizing antibodies. The Env protein of human immunodeficiency virus type 1 (HIV-1) provides a dramatic example of this phenomenon.

The SU (gp120) subunit of the HIV-1 Env protein carries a large number of oligosaccharide chains, which form a dense shell that masks much of the protein's surface (see the figure). These oligosaccharides govern several properties of HIV-1. For example, the tropism of the virus for CCr5 or CXCr4 coreceptors correlates with specific patterns of glycosylation in the variable loops of the SU subunit. However, a major function of such modification is to block access of host anti-HIV-1 antibodies to SU protein epitopes: high-resolution structural studies of the SU protein core have confirmed that N-linked oligosaccharides cover much of the protein's surface. Furthermore, the sugar chains are highly ordered, forming the outer surface of the Env spike. As predicted from this arrangement, N-linked glycosylation at specific sites blocks binding of monoclonal antibodies that recognize nearby sequences in the protein.

Several observations have led to the hypothesis that HIV-1 carries an evolving carbohydrate "shield" that enhances immune evasion. For example, the number of N-linked oligosaccharides added to SU tends to increase during the course of an HIV-1 infection, and the sites of N-linked glycosylation also change. Furthermore, broadly neutralizing antibodies that inhibit reproduction of multiple strains and clades of the virus recognize mannose-containing glycans, in some cases in conjunction with a protein epitope.

Chen B, Vogan EM, Gong H, Skehel JJ, Wiley DC, Harrison SC. 2005. Structure of an unliganded simian immunodeficiency virus gp120 core. *Nature* **433:**834–841.

Pejchal R, Doores KJ, Walker LM, Khayat R, Huang PS, Wang SK, Stanfield RL, Julien JP, Ramos A, Crispin M, Depetris R, Katpally U, Marozsan A, Cupo A, Maloveste S, Liu Y, McBride R, Ito Y, Sanders RW, Ogohara C, Paulson JC, Feizi T, Scanlan CN, Wong CH, Moore JP, Olson WC, Ward AB, Poignard P, Schief WR, Burton DR, Wilson IA. 2011. A potent and broad neutralizing antibody recognizes and penetrates the HIV glycan shield. *Science* **334:**1097–1103.

Scanlan CN, Offer J, Zitzmann N, Dwek RA. 2007. Exploiting the defensive sugars of HIV-1 for drug and vaccine design. *Nature* **446:**1038–1045.

Electron micrograph of HIV-1 particles, showing carbohydrates stained with ruthenium red (dark). Courtesy of Edwin P. Ewing, Jr., Centers for Disease Control and Prevention (CDC), Atlanta, GA (CDC Public Health Image Library).



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BOX 12.5

DISCUSSION

A viral thiol oxidoreductase system that operates in the cytoplasm

The intracellular mature virus particle of the poxvirus vaccinia virus is the first of two infectious particles assembled in infected cells. This particle carries an envelope containing viral membrane proteins surrounding an internal core in which the DNA genome is packaged. In 1999, it was reported that some viral core proteins synthesized in the cytoplasm, as well as the cytoplasmic domains of some membrane proteins, contain stable disulfide bonds. This property explained the previously reported sensitivity of vaccinia particles to disruption by reducing agents. In addition, it raised the intriguing question of how disulfide bonds could be introduced into viral proteins or domains that are **never** exposed to the major cellular site of thiol oxidation, the ER lumen. Within a few years, viral genes were shown to encode all the components necessary to catalyze formation of disulfide bonds. This viral thiol oxidoreductase system comprises three components, and the final substrates, which include the L1R and F9L proteins that are present in mature virus particles. The sequence in which the three viral enzymes act, summarized in the figure, was deduced from a variety of experimental observations.

The vaccinia virus E10R protein is a sulfhydryl oxidase that contains the motif CXXC common to proteins that participate in exchange of pairs of thiol groups for disulfide bonds. Such proteins include protein disulfide isomerase and other ER oxidoreductases that promote protein folding. The vaccinia virus enzyme belongs to a second class, which includes mitochondrial proteins that operate in the mitochondrial intermembrane space. As summarized in the figure, the viral enzyme system, like that of mitochondria, transfers electrons from substrates via intermediate oxidoreductases to an electron acceptor, typically O_2.

The proteins that comprise the viral thiol oxidoreductase pathway are conserved among all poxviruses. Sulfhydryl oxidases with low but readily discernible sequence identity were shown to be encoded in the genomes of other large DNA viruses, including African swine fever virus (an iridovirus) and mimivirus. An open reading frame exhibiting homology to the viral/mitochondrial family of sulfhydryl oxidases is also present in the genome of pandoraviruses. In all cases that have been examined, the viral enzymes are necessary for assembly of virus particles.

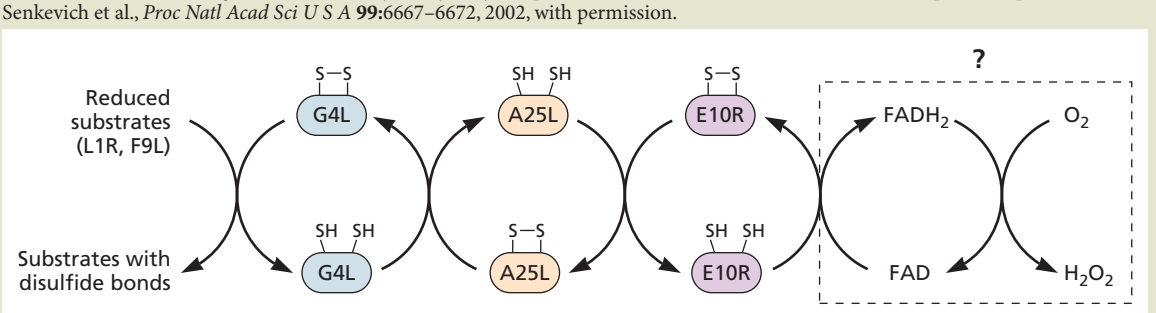
The abundance in the cytoplasm of compounds that reduce disulfide bonds, such as glutathione, indicates that viral proteins containing these bonds must be sequestered, for example, within the viral factories in which viral gene expression and protein synthesis take place.

Hakim M, Fass D. 2010. Cytosolic disulfide bond formation in cells infected with large nucleocytoplasmic DNA viruses. *Antioxid Redox Signal* **13:**1261–1271.

Locker JK, Griffiths G. 1999. An unconventional role for cytoplasmic disulfide bonds in vaccinia virus proteins. *J Cell Biol* **144:**267–279.

Senkevich TG, White CL, Koonin EV, Moss B. 2002. Complete pathway for disulfide bond formation encoded by poxviruses. *Proc Natl Acad Sci U S A* **99:**6667–6672.

The coupled oxidation-reduction (thiol-exchange) reactions among the proteins of the vaccinia virus disulfide bond formation are depicted in order (left to right). The transfer of electrons to oxygen via flavin adenine dinucleotide (FAD) (left) is based on homology of E10R with members of a family of FAD-containing sulfhydryl oxidases, and has not been demonstrated experimentally. These reactions are analogous to those catalyzed by enzymes present in the mitochondrial intermembrane space. Adapted from T. G. Senkevich et al., *Proc Natl Acad Sci U S A* **99:**6667–6672, 2002, with permission.



The ER chaperones, which include Grp78 and calnexin, are also crucial for the quality control processes that determine the fate of newly synthesized proteins translocated into the ER.

Grp78 is a member of the Hsp70 family of stress response proteins. It associates transiently with incompletely folded viral and cellular proteins. Binding of this chaperone, generally at multiple sites in a single nascent protein molecule, is thought to protect against misfolding and aggregation by sequestering sequences prone to nonspecific interaction, such as hydrophobic patches. The release of unfolded proteins from Grp78 is controlled by the hydrolysis of ATP bound to the chaperone. Multiple cycles of association with, and dissociation from, Grp78 probably take place as a protein folds. Once the sequences to which the chaperone binds are buried in the interior of the protein, such interactions cease. For example, molecules of vesicular stomatitis virus G, Semliki Forest virus E1, or influenza virus HA0 proteins that have acquired the full complement of correct disulfide bonds can no longer associate with Grp78. The ER contains many other folding catalysts and chaperones, some specific for particular proteins. Relatively little is known about the parameters that determine the chaperone(s) to which a newly synthesized protein binds,

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and the order in which chaperones operate. However, studies of specific viral glycoproteins in living cells indicate that the positions of oligosaccharides within the protein chain are one important determinant of chaperone selection (Box 12.6).

Calnexin is an integral membrane protein of the ER that also binds transiently to immature proteins. In contrast to Grp78, which recognizes protein sequences directly, calnexin distinguishes newly synthesized glycoproteins by binding to immature oligosaccharide chains. For example, the vesicular stomatitis virus G and influenza virus HA0 proteins bind to calnexin only when their oligosaccharide chains retain terminal glucose residues (Fig. 12.7B). In fact, formation of the mature oligosaccharide is intimately coupled with folding of glycoproteins and their retention within the ER (Fig. 12.8A). Proteins with sugars that include a single glucose residue are recognized by calnexin, but are released upon removal of the glucose by the enzyme glucosidase II. An enzyme that re-adds terminal glucose appears to be the "sensor" of the folded state of the glycoprotein: it recognizes incompletely folded proteins by virtue of exposed hydrophobic amino acids and specifically reglucosylates such proteins, controlling cycles of substrate binding and release from calnexin (Fig. 12.8A). This specificity ensures that only fully folded proteins can escape these chaperones and travel along the secretory pathway.

Proteins that are misfolded or not modified correctly cannot escape covalent or noncovalent associations with ER enzymes or molecular chaperones. For example, a temperature-sensitive vesicular stomatitis virus G protein remains bound to calnexin, and hence to the ER membrane, at a restrictive temperature. Consequently, egress of nonfunctional proteins from the ER to subsequent compartments in the secretory pathway is prevented. These interactions also target misfolded proteins for degradation. The mechanisms responsible for specific recognition of misfolded proteins, and induction of transport from the ER to the cytoplasm, are not fully understood. However, removal of multiple mannose residues, as well as

BOX 12.6

EXPERIMENTS

Selectivity of chaperones for viral glycoproteins entering the ER

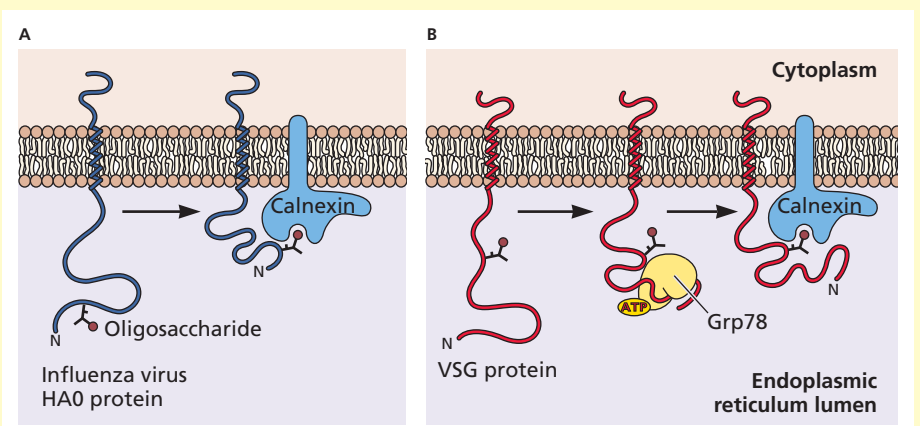
The parameters that determine which of the many ER chaperones operate on individual proteins are not fully understood. However, analysis of the folding and chaperone association of viral glycoproteins suggests that the position of glycosylation sites can determine chaperone selection.

The Semliki Forest virus E1 and p62 (pre-E2) glycoproteins enter the ER cotranslationally and are cleaved from a precursor by signal peptidase. However, E1, which folds via three intermediates differing in their disulfide bonding, initially associates with Grp78, whereas nascent p62 molecules bind to calnexin. One major difference between E1 and p62 is that the latter contains glycosylation sites close to the N terminus: addition of oligosaccharides at such sites could allow recognition of nascent p62 by calnexin and preclude association with Grp78.

This hypothesis was tested by elimination of N-linked glycosylation sites of the influenza virus HA0 protein, either close to the N terminus or in more C-terminal positions. The wild-type protein does not bind to Grp78, but prevention of glycosylation at N-terminal sites (positions 8, 22, and 38) led to association with this chaperone.

As summarized in the figure, these observations suggest that nascent proteins that carry N-linked glycosylation sites close to the N terminus (p62, HA0) enter the calnexin folding pathway directly **(A)**. Other proteins, such as the Semliki Forest virus E1 and the vesicular stomatitis virus G, associate initially with Grp78 and protein disulfide isomerase and are transferred to the calnexin pathway as they mature **(B)**.

Molinari M, Helenius A. 2000. Chaperone selection during glycoprotein translocation into the endoplasmic reticulum. *Science* **288:**331–333.



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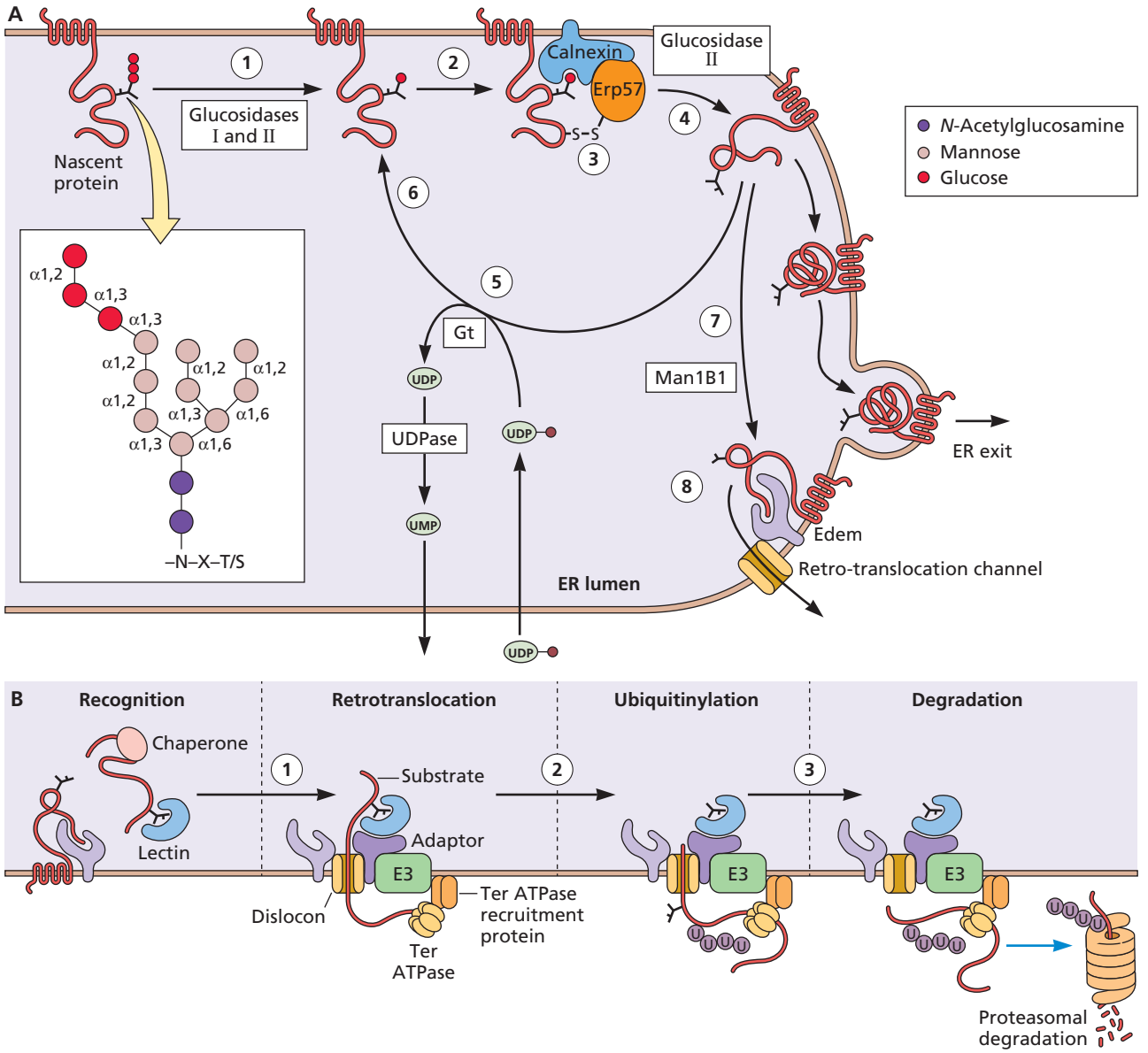


Figure 12.8 Integration of folding and glycosylation in the ER. The model illustrates the coordination of ER retention by calnexin with glycosylation and folding of a newly synthesized glycoprotein (red) containing an N-linked oligosaccharide, depicted as in Fig. 12.3. Trimming of terminal glucose residues by glucosidases I and II (1) yields a monoglucosylated chain, to which calnexin (or calreticulin) binds (2). Because the thiol oxidoreductase Erp57 associates with calnexin, the newly synthesized protein is brought into contact with Erp57, with which transient intermolecular disulfide bonds (-S-S-) can form. When the remaining glucose is removed by glucosidase II (3), the protein dissociates from the calnexin-Erp57 complex. If it has attained its native structure, the protein can leave the ER (4). However, if it is incompletely (or incorrectly) folded (5), the protein is specifically recognized by UDP-glucose glycoprotein transferase (Gt), which re-adds terminal glucose residues to the oligosaccharide (6) and therefore allows rebinding to calnexin. Cycles of binding and modification are repeated until the protein is either folded properly or targeted for degradation. Proteins that cannot escape this cycle by folding to the native conformation are subjected to progressive trimming of mannose residues by enzymes such as ER mannosidase 1 (Man1B1) and probably Edem1 and -2 (7). Removal of mannose residues prevents further reglucosylation and provides signals for recognition by one of several mannose-specific lectins, and direction to the ER membrane machinery for retrotranslocation and ubiquitinylation (the E3 complex) (8). Subsequently, nonglycosylated proteins that do not fold in the ER were found to be marked for retrotranslocation by addition of an O-linked mannose residue. Adapted from M. H. Smith et al., *Science* **334:**1086–1090, 2011, with permission. **(B)** Binding of a misfolded protein by a mannose-specific lectin (for example, amplified in osteosarcoma 9 [Os9] or ER lectin 1) is followed by association with adapter proteins, commonly Sel1L (suppressor/enhancer of lin-12-like proteins) (1). The adapter protein nucleates assembly of a large, membrane-associated complex that contains the protein destined for return to the cytoplasm and components required for retrotranslocation, such as derlin-1 or -2 and transitional ER-associated ATPase (Ter ATPase), which is thought to provide the necessary energy by hydrolysis of ATP (2). This complex also contains E3 ubiquitin ligases, such as synoviolin-1 (Sysn1, also known as Hrd1). These enzymes are thought to ubiquitinylate retrotranslocating protein chains upon entry into the cytoplasm (3) to target the proteins for degradation by the proteasome (4). Adapted from J. A. Olzmann et al., *Cold Spring Harb Perspect Biol* **5:**a013185, 2013, with permission.

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the participation of several ER proteins, has been implicated in diversion of misfolded glycoproteins for translocation to the cytoplasm. These proteins, such as the stress-induced ER-degradation-enhancing mannosidase-like proteins Edem1 and -2, promote association of misfolded proteins with ER membrane components for ubiquitinylation and transport to the cytoplasm via retrotranslocation (Fig. 12.8B). Initial identification of proteins required for the latter step came from studies of herpesviral proteins that induce translocation of major histocompatibility complex (MHC) class I molecules from the ER to the cytoplasm (Box 12.7). Once the proteins reenter the cytoplasm, they are degraded by the proteasome. The quality control functions of resident ER chaperones and other proteins therefore ensure that nonfunctional proteins are cleared from the secretory pathway at an early step.

Oligomerization. Most viral membrane proteins are oligomers that must assemble as their constituent protein chains are folded and covalently modified. Such assembly generally begins in the ER, as the surfaces that mediate interactions among protein subunits adopt the correct conformation. For many proteins, these reactions are also completed within the ER. For instance, influenza virus HA0 protein monomers are restricted to the ER lumen, whereas trimers are found in this and all subsequent compartments of the secretory pathway. Indeed, several viral and some cellular heteromeric membrane proteins must oligomerize to exit the ER, because folding of one subunit depends on association with the other(s). This requirement has been characterized in some detail for the glycoproteins of alphaviruses, such as Sindbis virus: the association of the two envelope proteins within the ER is essential for the productive folding, and exit, of both (Fig. 12.9). Similarly, the herpes simplex virus type 1 envelope glycoproteins gH and gL must interact with one another for the transport of either from the ER, and in the absence of gL, gH cannot fold correctly.

Assembly of other viral membrane proteins is completed following exit from the ER: disulfide-linked dimers of the hepatitis B virus surface antigen form higher-order complexes in the next compartment in the pathway, and oligomers of the human immunodeficiency virus type 1 Env protein can be detected only in the Golgi apparatus. At present, we can discern no simple rules describing the relationship of oligomer assembly and transport of membrane proteins from the ER. Nevertheless, oligomerization begins, and in some cases must be completed, within the ER, where it can be facilitated by the folding catalysts and chaperones characteristic of this compartment.

BOX 12.7

TRAILBLAZER

How a herpesviral glycoprotein led to identification of proteins required for ER retrotranslocation

Two human cytomegalovirus (a betaherpesvirus) membrane glycoproteins, US2 and US11, were known to insert into the ER membrane and induce rapid transfer of MHC class I heavy chains from the ER to the cytosol (retrotranslocation). These cellular proteins become polyubiquitinylated and degraded by the cytosolic proteasome. In the case of the viral US11 protein, a glutamine residue (Glu192) in the transmembrane domain is essential for retrotranslocation of MHC class I proteins.

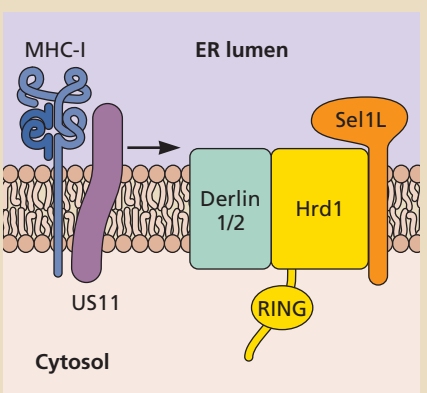
This property was exploited to purify human proteins that bound specifically to wild-type US11, but not to the viral protein carrying a Glu192 → Leu substitution. Several ER proteins bound to both US11 proteins, but only one associated specifically with wild-type US11. This protein, identified by mass spectrometry, showed some similarity to the yeast Der1p protein that is known to participate in degradation of misfolded ER proteins, and was named derlin-1. Overproduction of a dominant-negative derivative of derlin-1 inhibited US11-mediated retrotranslocation of MHC class I proteins.

In an alternative approach, components of a canine ER retrotranslocation channel were identified by virtue of their interaction with a cytoplasmic ATPase (ATPase p97) that was known to be essential for degradation of both misfolded ER proteins in yeast and MHC class I molecules in US11-producing human cells. The protein assembly identified in this way contained derlin-1 and a second ER membrane protein. These ER proteins were shown by immunoprecipitation to interact with both US11 and MHC class I proteins.

Subsequently, derlin-1 was shown to promote transport of other misfolded proteins from the ER to the cytoplasm, and many other proteins that participate in this process were identified (Fig. 12.8B). Nevertheless, the proteins that actually form the mammalian retrotranslocation channel have not been identified. The US11 protein acts as a virus-specific adapter, as shown in the figure.

Lilley BN, Ploegh HL. 2004. A membrane protein required for dislocation of misfolded proteins from the ER. *Nature* **429:**834–840.

Ye Y, Shibata Y, Yun C, Ron D, Rapoport TA. 2004. A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature* **429:**841–847.



Model for the function of the human cytomegalovirus US11 protein as a virus-specific adapter for ER-associated degradation. This viral proteins binds to MCH class I proteins to direct them to the E3 complex that contains the E3 ubiquitin ligase Hrd1 (HMG-CoA reductase degradation 1) and derlin-1 for return to the cytoplasm, ubiquitinylation, and degradation.

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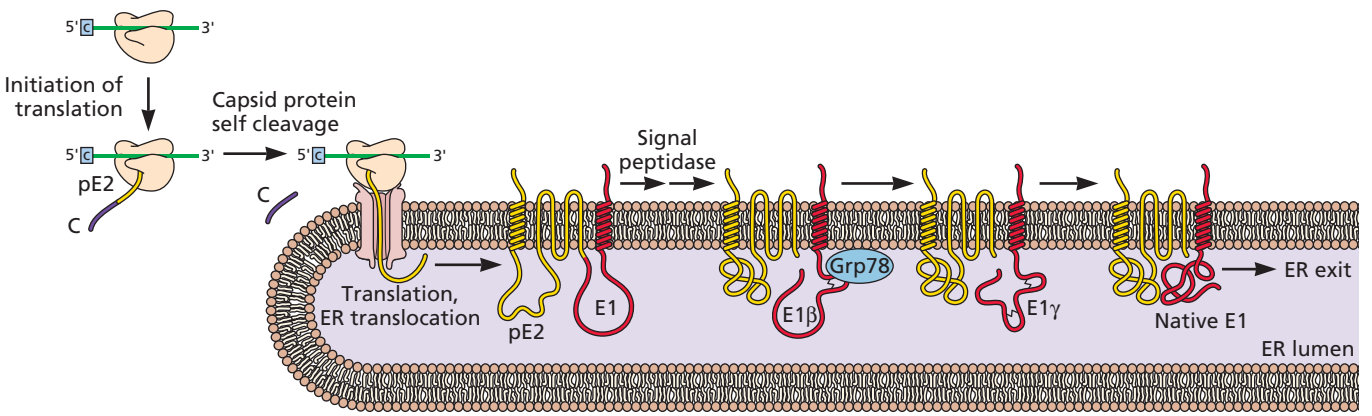
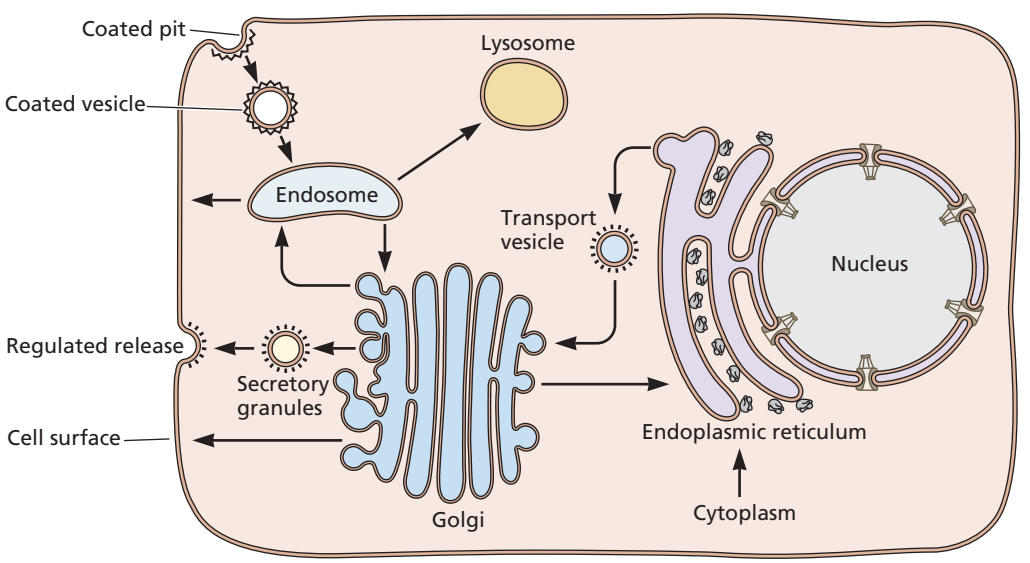


Figure 12.9 Folding of the two Sindbis virus envelope proteins depends on formation of heterodimers. The viral subgenomic mRNA encodes the precursor for the capsid (C) and envelope (pE2 and E1) proteins. Once the capsid protein emerges during translation by free cytoplasmic ribosomes, it is liberated by autoproteolysis. A hydrophobic sequence of pE2 exposed in this way directs association of the translating complex with the ER membrane, such that the envelope protein precursor is translocated across the ER membrane as its synthesis is completed. An unusual cleavage by signal peptidases releases the pE2 and E1 proteins. The nascent E1 protein becomes associated with Grp78, as well as thiol oxidoreductases, and folds via three intermediates (E1α [not shown], -β, and -γ) with different disulfide bonds. As shown, folding beyond E1β via E1γ to the native state depends on replacement of Grp78 by pE2, and folding of pE2 depends on interaction with E1: the pE2 protein misfolds when synthesized in the absence of E1 protein.

Vesicular Transport to the Cell Surface

The mechanism of vesicular transport. Viral membrane proteins, like their cellular counterparts, travel to the cell surface through a series of membrane-bound compartments and vesicles. The first step in this pathway, illustrated schematically in Fig. 12.10, is transport of the folded protein from the ER to the Golgi apparatus. Within the Golgi apparatus, proteins are sorted according to the addresses specified in their primary sequences or by their covalent modifications. **Transport vesicles**, and larger vesicular structures, which bud from one compartment and move to the next, carry cargo proteins between compartments of the secretory pathway (Box 12.8).

Figure 12.10 Compartments in the secretory pathway. The lumen of each membrane-bound compartment shown is topologically equivalent to the exterior of the cell. Proteins destined for secretion or for the plasma membrane travel from the ER to the cell surface via the Golgi apparatus. However, proteins can be diverted from this pathway to lysosomes or to secretory granules that carry proteins to the cell surface for regulated release. The return of proteins from the Golgi apparatus to the ER is indicated. The endocytic pathway discussed in Chapter 5 and the secretory pathway intersect in endosomes and the Golgi apparatus.



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BOX 12.8

EXPERIMENTS

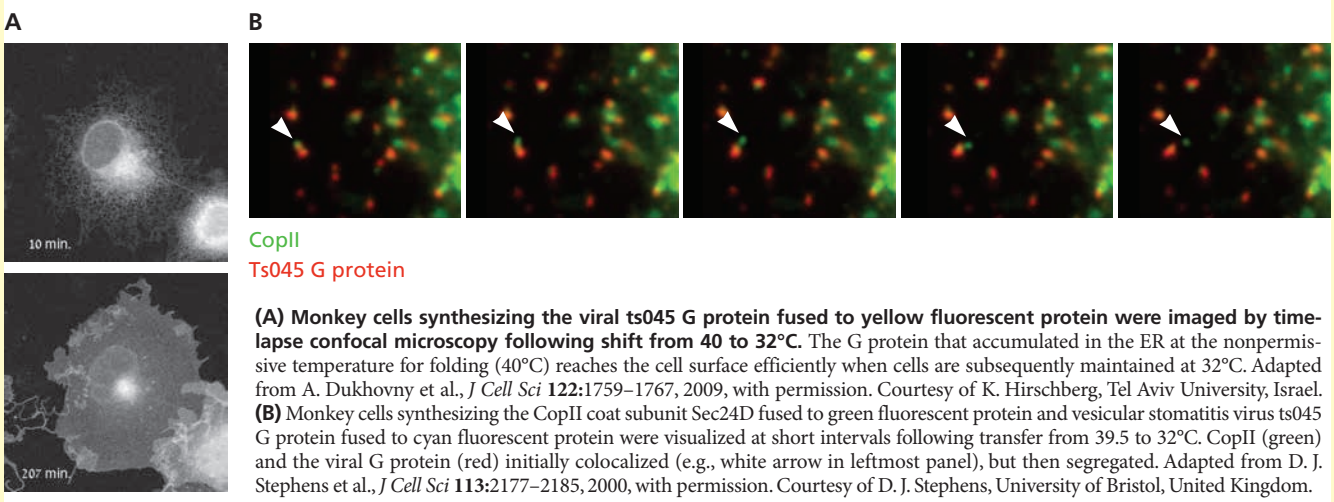
ER-to-Golgi transport in living cells

The vesicular stomatitis virus G protein made in cells infected with the mutant virus *ts*045 misfolds and is retained in the ER at high temperature (40°C). It refolds and is transported to the Golgi apparatus when the temperature is reduced to 32°C. This temperature-sensitive protein has therefore been used extensively to study transport through the secretory pathway. In initial studies of this process in living cells, the green fluorescent protein was attached to the cytoplasmic tail of the viral G protein. Control experiments established that this modification did not alter the temperature-sensitive folding or transport of the G protein. Time-lapse fluorescence microscopy of cells shifted from high to low temperature demonstrated that the chimeric G protein rapidly left the ER at multiple peripheral sites. The protein appeared in membranous structures, which were often larger than typical transport vesicles. These structures moved rapidly toward the Golgi in a stop-start manner, with maximal velocities of 1.4 μm/s. Such transport, but not formation of vesicles derived from the ER, was blocked when microtubules were depolymerized by treatment with nocodazole, or when the (−) end-directed microtubule motor dynein was inhibited. It was therefore concluded that vesicles and other membrane-bound structures that emerge from the ER at peripheral sites are actively transported along microtubules to the Golgi complex.

Extension of this approach to use fluorescent vesicular stomatitis virus as a model cargo in living cells that produce components of the secretory pathway fused to distinguishable fluorescent proteins has provided further insight into the mechanism of transport. For example, the viral G protein cargo was observed to leave the ER in vesicles with CopII coats, but the association between the cargo and this coat was reversed a short distance from the ER (panel B of the figure).

Presley JF, Cole NB, Schroer TA, Hirschberg K, Zaal KJ, Lippincott-Schwartz J. 1997. ER-to-Golgi transport visualized in living cells. *Nature* **389:**81–85.

Stephens DJ, Lin-Marq N, Pagano A, Pepperkok R, Paccaud JP. 2000. COPI-coated ER-to-Golgi transport complexes segregate from COPII in close proximity to ER exit sites. *J Cell Sci* **113:**2177–2185.



(A) Monkey cells synthesizing the viral ts045 G protein fused to yellow fluorescent protein were imaged by time-lapse confocal microscopy following shift from 40 to 32°C. The G protein that accumulated in the ER at the nonpermissive temperature for folding (40°C) reaches the cell surface efficiently when cells are subsequently maintained at 32°C. Adapted from A. Dukhovny et al., *J Cell Sci* **122:**1759–1767, 2009, with permission. Courtesy of K. Hirschberg, Tel Aviv University, Israel. **(B)** Monkey cells synthesizing the CopII coat subunit Sec24D fused to green fluorescent protein and vesicular stomatitis virus ts045 G protein fused to cyan fluorescent protein were visualized at short intervals following transfer from 39.5 to 32°C. CopII (green) and the viral G protein (red) initially colocalized (e.g., white arrow in leftmost panel), but then segregated. Adapted from D. J. Stephens et al., *J Cell Sci* **113:**2177–2185, 2000, with permission. Courtesy of D. J. Stephens, University of Bristol, United Kingdom.

Fusion of the vesicle membrane with that of the target compartment releases the cargo into the lumen of that compartment. Consequently, proteins that enter the secretory pathway upon translocation into the ER (and are correctly folded) are never again exposed to the cytoplasm of the cell. This strategy effectively sequesters proteins that might be detrimental, such as secreted or lysosomal proteases, and avoids exposure of disulfide-bonded proteins to a reducing environment.

Many soluble and membrane proteins that participate in vesicular transport have been identified and characterized by biochemical, molecular, and genetic methods. The properties of these proteins suggest that similar mechanisms control the budding and fusion of different types of transport vesicles. The general mechanism of vesicular transport is quite well understood. Budding of transport vesicles from the membranes of compartments of the secretory pathway requires proteins that form external coats of the vesicles, such as the protein complex called CopII, which initiates ER-to-Golgi transport, and small GTPases (Fig. 12.11A). The coat proteins induce membrane curvature and vesicle budding, and are subsequently removed by various mechanisms. The vesicle then moves to the next compartment, by either passive diffusion or active transport via microtubule-associated motor proteins over longer distances. When a transport vesicle encounters its target membrane, it docks as a result of specific interactions among Snare proteins present in the vesicle and target membranes. The coupled folding and assembly of Snare proteins on the two membranes lead to membrane fusion (Fig. 12.11B).

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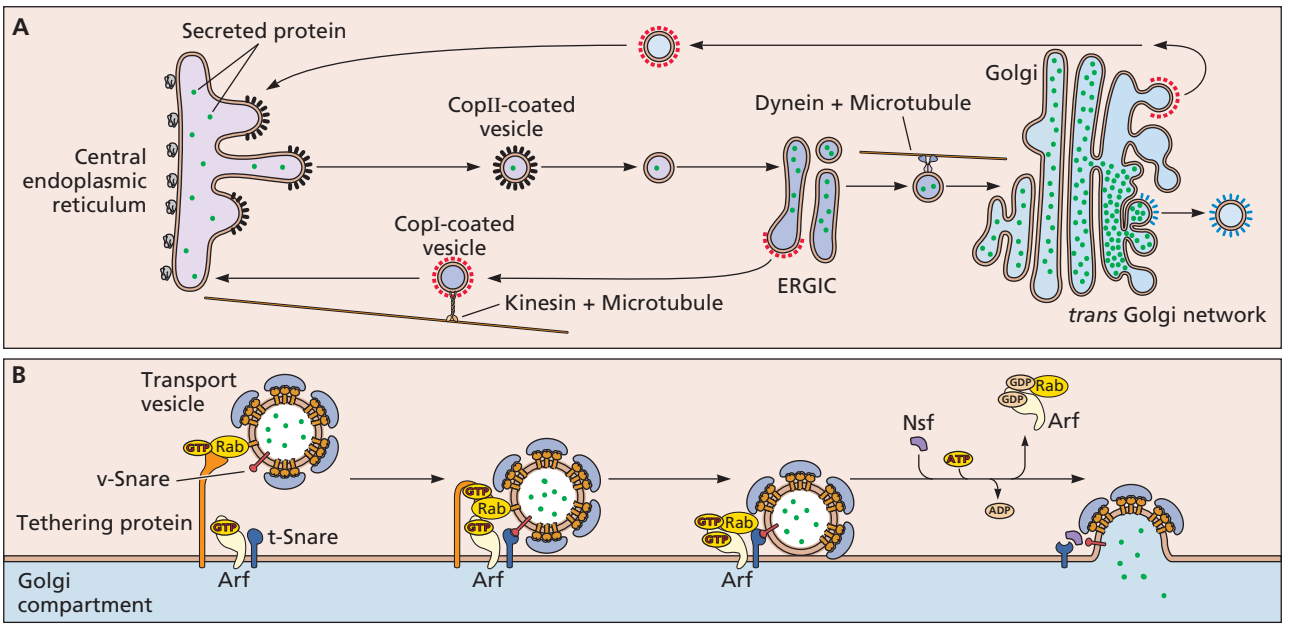


Figure 12.11 Protein transport from the ER to the Golgi apparatus. (A) Proteins leave the ER in transport vesicles at specialized ER exit sites, free of ribosomes. Vesicle formation is initiated by binding of cytoplasmic coat protein complex II (CopII), which contains a small GTPase (Sar1) and several other proteins, to the membrane. The vesicle membranes also carry proteins that direct them to appropriate destinations, such as particular v-Snares. Cargo is loaded by interactions between proteins of the CopII coat and either cytoplasmically exposed tails of cargo proteins or export receptors. The CopII coat induces budding and pinching off of vesicles, which move to the ER-Golgi intermediate compartment (ERGIC). Within this compartment, signals present in cargo proteins direct sorting for transport back to the ER, via CopI-coated vesicles, or for continued transport to the plasma membrane. The ERGIC matures into and/or fuses with the *cis*-Golgi. **(B)** Fusion of transport vesicle and target compartment membranes. Both vesicle (v-Snare) and target compartment (t-Snare) proteins govern the specificity of membrane fusion. The first step is thought to be tethering of a vesicle by interaction of a tethering protein with the GTP-bound Rab and/or components of the coat. Tethering proteins (e.g., Dsl1 and Cog proteins) contain multiple subunits, each built from several α-helical bundles to form extended structures. They interact with Snares, and may function as chaperones for the assembly of membrane-bridging complexes between the v- and t-Snares, a process known as docking. Membrane fusion takes places as v- and t-SNAREs finish zippering into highly stable helical bundles. Fusion is accompanied by ATP hydrolysis and disassembly of the fusion complex by the SNARE disassembly ATPase Nsf. The specificity of the v-Snare–t-Snare interaction contributes to the specificity of fusion, as do multisubunit tethering proteins. In addition, small GTP-binding proteins of the Rab family, each of which is associated with a specific organelle, provide, in the GTP-bound form, an "identity signal" recognized by proteins that participate in vesicle budding or fusion. Specific phosphoinositides (lipids) are also important determinants of the identity of some organelles, for example, of Golgi compartments.

A vesicle Snare and an appropriate Snare in the target membrane represent a minimal machinery for membrane fusion *in vitro*, but additional proteins perform essential regulatory functions in cells.

The high density of intracellular protein traffic requires considerable specificity during vesicle formation and fusion. For example, vesicles that transport proteins from the ER to the first compartment of the Golgi apparatus must take up only the appropriate proteins when budding from the ER, and must fuse only with the membrane of the *cis*-Golgi network. Specificity of cargo loading during formation of these (and other) transport vesicles is achieved by both direct association of cargo proteins with proteins of the vesicular coat and indirect interactions via transmembrane export receptors. Several types of protein establish the specificity of vesicular transport, including the Snare proteins resident in the vesicle and target compartment membranes.

Reactions within the Golgi apparatus. One of the most important staging posts in the secretory pathway is the Golgi apparatus, which is composed of a series of membrane-bound compartments. Proteins enter the Golgi apparatus from the ER via the *cis*-Golgi network, which is composed of connected tubules and sacs (Fig. 12.10). A similar structure, the *trans*-Golgi network, forms the exit face of this organelle. The *cis*- and *trans*-Golgi networks are separated by a variable number of cisternae termed the *cis*, medial, and *trans* compartments. Each of these compartments, which can comprise multiple cisternae, is the site of specific reactions, including those that form mature N-linked oligosaccharides (Fig. 12.12).

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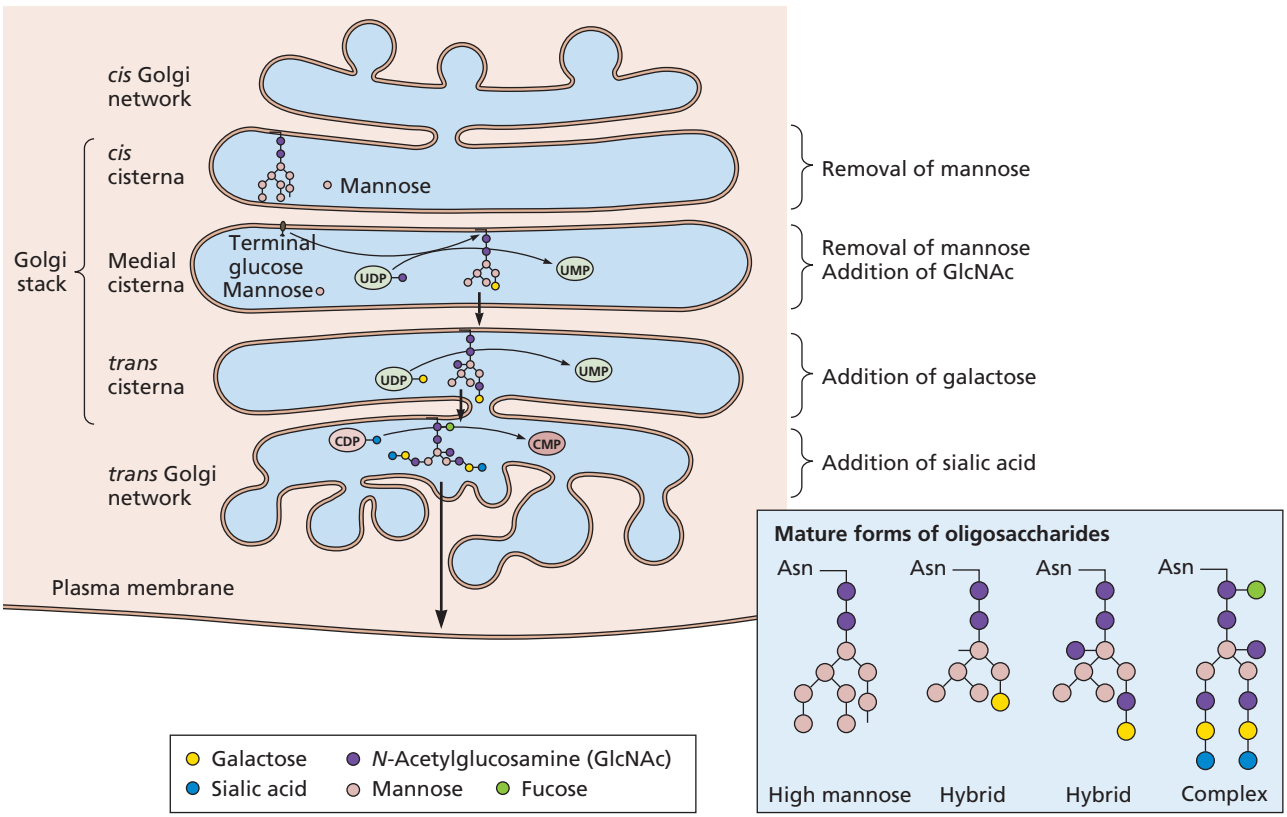


Figure 12.12 Compartmentalization of processing of N-linked oligosaccharides. The reactions by which mature N-linked oligosaccharide chains are produced from the high-mannose core precursor added in the ER (Fig. 12.7B) are shown in the Golgi compartment in which they take place. Trimming of terminal glucose and mannose residues of the common core precedes stepwise addition of the sugars found in the mature chains. The enzymes responsible for early reactions in maturation of oligosaccharides are located in *cis* cisternae, whereas those that carry out later reactions are present in the medial and *trans* compartments. Such spatial separation ensures that oligosaccharide processing follows a strict sequence as proteins pass through the compartments of the Golgi apparatus. Synthesis of O-linked oligosaccharides by glycosyltransferases, which add one sugar unit at a time to certain serine or threonine residues, also takes place in the Golgi apparatus.

A number of viral envelope glycoproteins are also processed proteolytically by cellular enzymes resident in late Golgi compartments. Retroviral Env glycoproteins are cleaved in the *trans*-Golgi network to produce the TM (transmembrane) and SU (surface unit) subunits from the Env polyprotein precursor (Fig. 12.13). Similarly, the HA0 protein of certain avian influenza A viruses is cleaved into the HA1 and HA2 chains (Fig. 12.4) in the same compartment. These and other viral membrane proteins (Table 12.1) are processed by members of a family of resident Golgi proteases that cleave after pairs of basic amino acids. The members of this family, which in mammalian cells include furins found in the *trans*-Golgi network, are serine proteases related to the bacterial enzyme subtilisin. Various furin family members have been shown by genetic and molecular methods to process viral glycoproteins; their normal function is to process cellular polyproteins, such as certain hormone precursors.

These proteolytic cleavages are not necessary for assembly but are essential for production of infectious particles. For example, proteolytic processing of envelope proteins of retroviruses and alphaviruses is necessary for infectivity, probably because sequences important for fusion and entry become accessible. Virulent strains of avian influenza A virus encode HA0 proteins that can be processed by the ubiquitous furin family proteases, such that virus particles carrying fusion-active HA protein are released (Volume II, Chapter 5). It seems likely that the common dependence on furin family proteases (Table 12.1), which act on proteins relatively late in the secretory pathway, helps minimize complications that would arise if viral glycoproteins were initially synthesized with their fusion peptides in an active conformation. Furthermore, exposure to the low-pH environment of *trans*-Golgi network compartments (pH ~6.0) can be a prerequisite for processing of viral envelope proteins.

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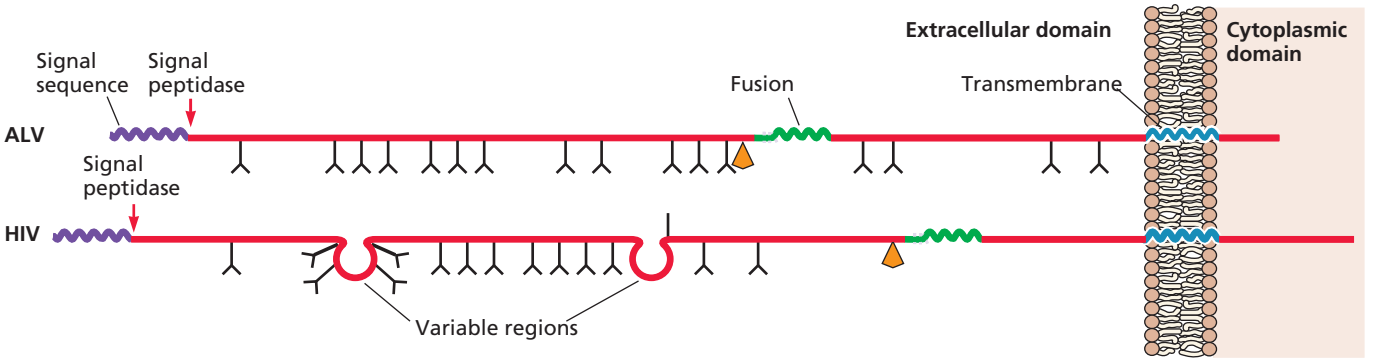


Figure 12.13 Modification and processing of retroviral Env polyproteins. Sequence features and modifications of the Env proteins of avian leukosis virus (ALV) and human immunodeficiency virus type 1 (HIV) are depicted as in Fig. 12.3. The variable regions of human immunodeficiency virus type 1 Env differ greatly in sequence among viral isolates. The translocation products shown here are cleaved by the ER signal peptidase (red arrows) and by furin family proteases in the *trans*-Golgi network (orange arrowheads). The latter liberates the transmembrane (TM) and surface unit (SU) subunits from the Env precursor.

This requirement is exemplified by the envelope proteins of flaviviruses, such as dengue virus (Fig. 12.14).

In the case of highly pathogenic influenza A viruses, the ion channel activity of the viral M2 protein helps to maintain HA in a fusion-incompetent conformation following cleavage. This HA protein switches to the fusion-competent conformation at a pH higher than that required by HA proteins of human influenza A viruses. The M2 protein, which forms a proton channel, is present at quite high concentrations in the membranes of secretory pathway compartments. By providing an exit channel for protons, and hence increasing the pH of normally acidic compartments, such as those of the *trans*-Golgi network, this protein prevents premature switching of proteolytically processed HA to the fusion-active conformation described in Chapter 5.

Although all the envelope proteins of viruses that assemble at the plasma membrane travel the cellular secretory pathway, there is considerable variation in the rate and efficiency of their transport. A champion is the influenza virus HA protein, which folds and assembles with a half time of only 7 min, with >90% of the newly synthesized molecules reaching the cell surface. Many other viral proteins do considerably less well. Parameters determining the rate and efficiency of transport may include the complexity of the protein and the inherent asynchrony of protein folding. With some exceptions (see "Inhibition of Transport of Cellular Proteins" below), cellular proteins continue to enter and traverse the secretory pathway as enveloped viruses assemble at the plasma membrane. Consequently, competition among viral and cellular proteins, which may vary with the nature and physiological state of the host cell, is also likely to affect the transport of viral proteins to the cell surface.

We have focused our discussion of viral envelope proteins on the well-understood maturation of their extracellular domains. However, the cytoplasmic portions of these proteins are also frequently modified. Many, including the influenza HA and human immunodeficiency virus Env proteins, are **acylated** by the covalent linkage of the fatty acid palmitate to cysteine residues in their cytoplasmic domains (Table 12.2). This modification can be necessary for optimal production of progeny virions. For example, inhibition of palmitoylation of the Sindbis virus E2 glycoprotein or of the human

Table 12.1 Some viral envelope glycoprotein precursors processed by secretory pathway proteases

Virus family	Precursor glycoprotein	Membrane-associated cleavage products
Signal peptidase		
Alphavirus	Envelope polyprotein precursor	E1, pE2
Bunyavirus	Translation product of M mRNA	Gn Gc
Flavivirus	Polyprotein	prM, E
Furin family proteases		
Alphavirus	pE2	E2
Flavivirus	PrM	M
Hepadnavirus	preC	C antigen[a,b]
Herpesvirus[b,c]	pre-gB	gB
Orthomyxovirus[d]	HA0	HA1, HA2
Paramyxovirus	F0	F1, F2
Retrovirus	Env	TM, SU

[a]This cleavage product is largely secreted into the extracellular medium, but is also associated with plasma membrane of infected cells.

[b]Cleavage is not necessary for production of infectious virus particles in cells in tissue culture.

[c]Some alphaherpesviruses (e.g., varicella-zoster virus), and all known betaherpesviruses.

[d]Virulent strains of avian influenza A virus.

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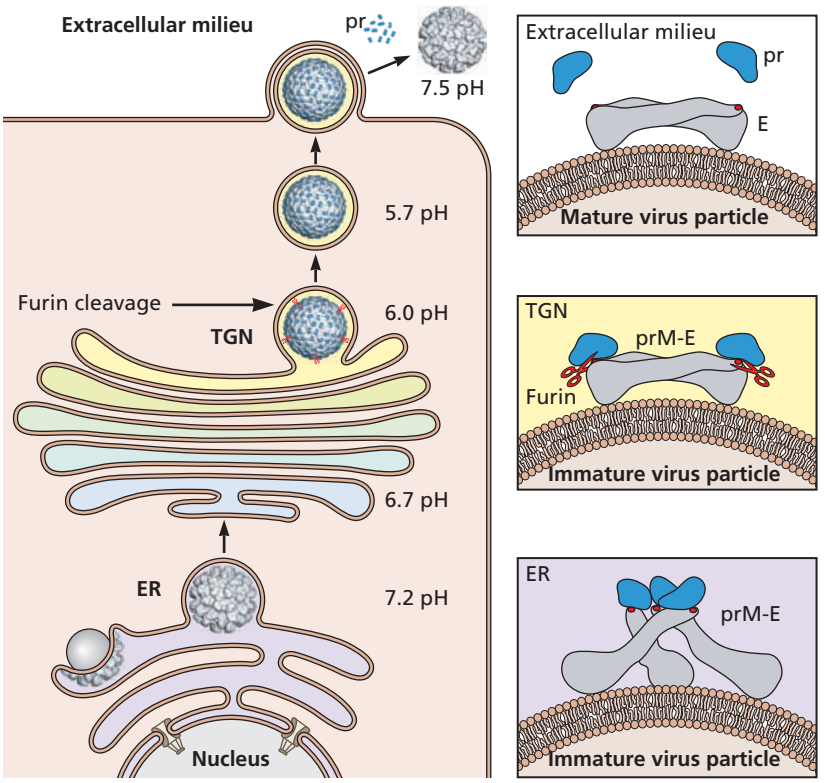


Figure 12.14 Low pH-induced conformational change and maturation of dengue virus particles. The envelope of mature particles of dengue virus (and other arthropod-borne flaviviruses) contains dimers of the envelope (E) protein that lie flat along the surface (see Fig. 4.23). However, this viral protein is initially inserted into membranes in association with the viral precursor membrane protein (prM) and forms heterotrimeric E-prM spikes on the surface of immature virus particles that bud into the ER lumen (step 1). The particles travel the secretory pathway, passing through compartments with decreasing internal pH, reaching pH 6.0 in the *trans*-Golgi network (TGN). The reduced pH induces a major reorganization of the surface proteins to form flat dimers (step 2) and conformational change that exposes a furin cleavage site in prM. Following cleavage, which is important for the infectivity of virus particles, a portion of prM (termed pr) remains associated with E proteins until its dissociation is triggered upon the release of particles into the neutral pH of the extracellular milieu (step 3). This model is based on comparison of the structures of mature virus particles and the immature particles containing uncleaved prM that are released when infected cells are exposed to compounds that increase the pH of the *trans*-Golgi network. Subsequent studies established that particles that carry uncleaved prM are quite prevalent in the populations released from animal cells, and in particular from mosquito cells. The impact of such heterogeneity on infectivity, antibody recognition, and pathogenesis is not yet clear. Adapted from I.-M. Yu et al., *Science* **319:**1834–1837, 2008, with permission. Reconstruction of virus particles courtesy of J. Chen, Purdue University.

immunodeficiency virus type 1 Env protein impairs virus assembly and budding. The bulky fatty acid chains attached to the short cytoplasmic tails may regulate envelope protein conformation or association with specific membrane domains.

Sorting of Viral Proteins in Polarized Cells

Proteins that are not specifically targeted to an intracellular address travel from the Golgi apparatus to the plasma membrane (Fig. 12.10). However, the plasma membrane is not uniform in all animal cells: differentiated cells often devote different parts of their surfaces to specialized functions, and the plasma membranes of such **polarized cells** are divided into correspondingly distinct regions. During infection by many enveloped viruses, the asymmetric surfaces of such cells are distinguished during entry and when components of virus particles are sorted to a specific plasma membrane region. In this section, we describe the final steps in the transport of proteins to specialized plasma membrane regions in two types

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Table 12.2 Examples of acylated or isoprenylated viral proteins

Virus	Protein	Lipid	Probable function
Envelope proteins			
Alphavirus			
Sindbis virus	E2	Palmitate	Efficient budding of virus particles
Coronavirus			
Severe acute respiratory syndrome virus	S	Palmitate	Fusion
Hepadnavirus			
Hepatitis B virus	L (pre-S1)	Myristate	Initiation of infection
Orthomyxovirus			
Influenza A virus	HA	Palmitate	Fusion and infectivity
Retrovirus			
Human immunodeficiency virus type 1, Moloney murine leukemia virus	Env (TM)	Palmitate	Budding of virus particles
Other viral proteins			
Hepatitis delta satellite virus	Large delta antigen	Geranylgeranol	Interaction with HBV L protein; assembly; inhibition of HDV RNA replication
Papovavirus			
Simian virus 40	VP2	Myristate	Assembly
Picornavirus			
Poliovirus	VP0, VP4	Myristate	Assembly; uncoating
Retrovirus			
Human immunodeficiency virus type 1, murine leukemia virus	Gag, MA	Myristate	Membrane association, assembly, and budding
Rous sarcoma virus	pp60^{src}	Myristate	Membrane association, transformation

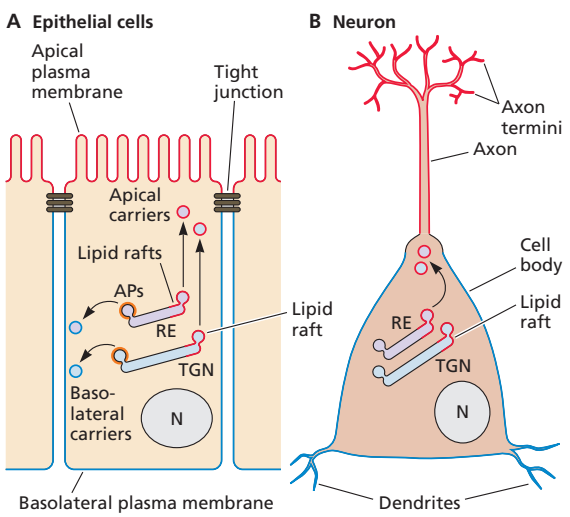


Figure 12.15 Polarized epithelial cells and neurons. (A) Tight junctions block the intercellular space between epithelial cells and delineate the apical and basolateral domains. Proteins destined for vesicular transport to these distinct domains are sorted, on the basis of the specific signals they carry, within the *trans*-Golgi network (TGN). The vesicles that carry cargo to both membrane domains can also arise from recycling endosomes (REs). AP, adapter protein. **(B)** The membrane of the axon of a neuron is equivalent to the apical domain of an epithelial cell, as indicated. It is demarcated from the remainder of the neuronal plasma membrane by the axon initial segment (gray membrane), which is not myelinated and contains bundled microtubules. The formation of axonal vesicular carriers is illustrated. Adapted from J. S. Bonifacino, *J Cell Biol* **204:**7–17, 2014, with permission.

of polarized cells in which animal viruses often reproduce, epithelial cells and neurons (Fig. 12.15).

Epithelial Cells

Epithelial cells, which cover the external surfaces of vertebrates and line all their internal cavities (such as the respiratory and gastrointestinal tracts), are primary targets of virus infection. The cells of an epithelium are organized into close-knit sheets, by both the tight contacts they make with one another and their interactions with the underlying basal lamina, a thin layer of extracellular matrix (Fig. 2.3). Within the best-characterized epithelia, such as those that line the intestine, each cell is divided into a highly folded **apical domain** exposed to the outside world and a **basolateral domain** (Fig. 12.15). The former performs more-specialized functions, whereas the latter is associated with cellular housekeeping. These two domains differ in their protein and lipid content, in part because they are separated by specialized cell-cell junctions (tight junctions), which prevent free diffusion and mixing of components in the outer leaflet of the lipid bilayer. However, such physical separation does not explain how the polarized distribution of plasma membrane proteins is established and maintained.

Viruses have been important tools in efforts to elucidate the molecular mechanisms responsible for the polarity of typical epithelial cells, because certain enveloped viruses bud asymmetrically. For example, in all epithelial cells studied,

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influenza A virus buds apically and vesicular stomatitis virus buds basolaterally. Such polarized assembly and release of virus particles can facilitate virus spread within or among host organisms (Volume II, Chapter 2). The polarity of virus budding is generally the result of accumulation of envelope proteins at the specific membrane regions, such as HA and G in the apical and basolateral domains, respectively. The most common mechanism for selective localization appears to be signal-dependent sorting of proteins in the *trans*-Golgi network, for packaging into appropriately targeted transport vesicles. Signals necessary for basolateral targeting comprise short amino acid sequences located in the cytoplasmic domains of membrane proteins (for example, YXXØ, where X is any and Ø is a bulky hydrophobic amino acid). These signals are recognized by components of coated vesicles that confer specificity during cargo selection and vesicle fusion, such as adapter protein 1, which interacts with clathrin or other scaffolding proteins. Indeed, many basolateral targeting signals overlap with those that direct proteins for clathrin-dependent endocytosis, and certain proteins are transferred through endosomes from one membrane domain to the other, a process termed transcytosis (see Volume II, Fig. 4.23). The sorting of viral glycoproteins to basolateral membrane domains can also be governed by additional viral proteins. When made in the absence of other viral proteins, the two envelope proteins of measles virus (F and H) are transported to the basolateral membrane. However, when the viral matrix protein binds to the cytoplasmic tails of F and H, these proteins are redirected from the default basolateral sorting pathway, and accumulate at the apical surface of epithelial cells.

A rather diverse set of determinants direct proteins to the apical membrane. They include certain sequences present in transmembrane domains (as are found in the influenza A virus HA and NA proteins), N- or O-linked oligosaccharides present in external or cytoplasmic domains, and a lipid anchor (glycosylphosphatidylinositol) that is added to some proteins made in the cytoplasm. It is thought that such signals confer affinity for specialized microdomains, termed **lipid rafts**. Such rafts, which are dynamic assemblies that can incorporate particular proteins selectively, were initially shown to mediate apical transport of glycosylphosphatidylinositol-anchored proteins. The influenza virus HA and NA proteins associate specifically with lipid rafts via their transmembrane domains, which determine apical sorting. Cellular proteins known to participate in apical trafficking of viral glycoproteins, such as caveolin-1 and myelin, are also associated with these membrane microdomains. Inhibition of the activity or synthesis of these proteins disrupts transport of the influenza virus HA protein (and other proteins) from the Golgi complex to the apical membrane. Lipid rafts seem likely to be more generally important in targeting of viral membrane proteins and assembly in nonpolarized cells: measles virus glycoproteins are selectively enriched in lipid rafts in nonpolarized cells, and association of the human immunodeficiency virus type 1 Gag polyprotein with these membrane domains promotes production of virus particles.

Neurons

Neurons are probably the most dramatically specialized of the many polarized cells of vertebrates. The axon is typically long and unbranched, whereas the dendrites form an extensive branched network of projections (Fig. 12.15). Axons are specialized for the transmission of electrical and chemical impulses, ultimately via the formation of synaptic vesicles and release of their contents. In contrast, dendrites provide a large surface area for the receipt of signals from other neurons. The nucleus, the rough ER, and the Golgi are also located in the dendritic region and the cell body of a neuron. Although axonal and dendritic surfaces are not separated by tight junctions, proteins must be distributed asymmetrically in neurons. Several mechanisms contribute to the establishment and maintenance of neuronal polarity, including transport of vesicles in specific directions along the highly organized microtubules of the axon (**axonal transport**), transport of particular mRNAs to specific regions of the neuron, and sorting and targeting of membrane proteins for delivery to axonal or dendritic surfaces.

The directional movement of vesicles and many cellular organelles in neurons is dependent on polarized microtubules and motor molecules that travel toward either their (−) or (+) ends. Such motors therefore mediate transport both toward the cell body from axons and dendrites and away from the cell body (Fig. 12.16). Infection, assembly, and egress of viruses that infect neurons depend on these mechanisms. An important example is provided by the neurotropic alphaherpesviruses, a group that includes the human pathogens herpes simplex virus type 1 and varicella-zoster virus. Following entry into sensory neurons, herpesvirus nucleocapsids and some tightly associated tegument proteins are transported along axons to the nucleus by microtubule-based transport, mediated by (−) end-directed motors such as dynein (Fig. 12.16). Later in the infectious cycle, virion components must be moved in the opposite direction (toward the synapse) upon association with proteins of the kinesin family. The spread of herpesviruses from neuron to neuron occurs at or near sites of synaptic contact, indicating that virus particles must be targeted to specific areas within neurons for egress. This attribute can be exploited to define neuronal connections in a living animal by using the virus as a tracer (Volume II, Chapter 1). Whether assembly is completed within the cell body of infected neurons or following transport of components of virus particles to sites of egress has been a subject of considerable debate (Box 12.9), but in the case of pseudorabies virus, there is compelling evidence for the former mechanism (Fig. 12.16).

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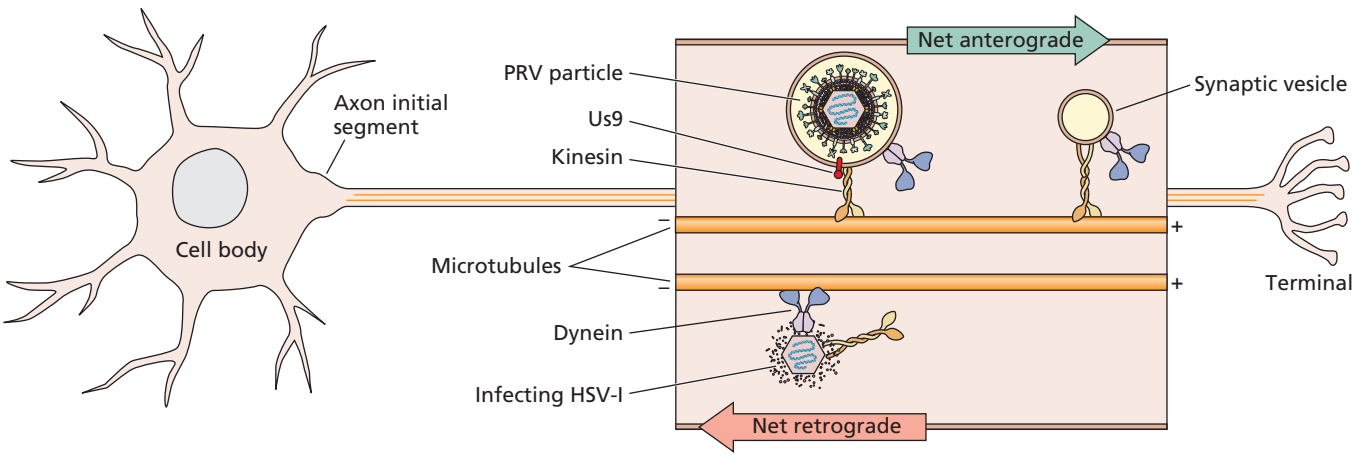


Figure 12.16 Axonal transport of herpesviral particles in neurons. At the beginning of an infectious cycle, nucleocapsids of alphaherpesviruses enter axon termini from epithelial cells (or other neurons) and are transported rapidly along microtubules in the retrograde direction upon association with the (−) end-directed motor dynein. Nucleocapsids associated with inner tegument proteins are transported efficiently, but which protein(s) carry dynein-binding sites is not yet clear. Later in the infectious cycle, the direction of transport must be reversed. There has been a long-standing debate about whether newly synthesized nucleocapsids are transported prior to or following secondary envelopment. In the case of pseuorabies virus (PRV), shown here, there is compelling evidence for the second mechanism. Efficient anterograde transport requires the glycoproteins gE and gI and the membrane protein Us9, which recruits the kinesin-3 motor Kif1A. It remains to be seen whether this model applies to human simplex virus type 1 (HSV-1).

Disruption of the Secretory Pathway in Virus-Infected Cells

Inhibition of Transport of Cellular Proteins

Some viral proteins interfere with transport to the plasma membrane of specific cellular proteins, notably MHC class I molecules. These proteins carry peptides derived from viruses (and other invaders) to the plasma membrane, where they alert cells of the adaptive immune response to infection. Prevention of transport of MHC class I proteins to the cell surface therefore helps prevent or delay the detection and destruction of infected cells (Volume II, Chapter 4). In the ER, the adenovirus E3 glycoprotein gp19 (Appendix, Fig. 1B) forms intramolecular disulfide bonds with these important components of the adaptive immune system and sequesters them within this organelle. Several herpesviral proteins also block transport of MHC class I molecules to the cell surface, including the human cytomegalovirus US11 and US2 gene products, discussed previously, which induce transport of cellular proteins from the ER to the cytosol for degradation by the cellular proteasome. The human immunodeficiency virus type 1 Vpu protein (Appendix, Fig. 29), a transmembrane phosphoprotein, also induces selective degradation of newly synthesized MHC class I proteins, and of CD4, by a similar mechanism. Such degradation of CD4, the major receptor for this virus, is important for assembly and release: tight binding of this cellular protein to the viral Env glycoprotein in the ER prevents transit of both proteins to the cell surface. Vpu also reduces the cell surface concentration of a third cellular protein, tetherin (also known as bone marrow stromal antigen 2 [Bst2]), which restricts release of human immunodeficiency virus from infected cells (Chapter 13). However, Vpu acts on tetherin not in the ER, but rather to induce its displacement from sites of virus budding in the plasma membrane, and to target the cellular protein for lysosomal degradation, thereby reducing recycling of tetherin from endosomes to the plasma membrane (Volume II, Chapter 7).

Drastic Effects on Compartments of the Secretory Pathway

Proteins encoded in the genomes of certain other viruses exert more-drastic effects on the cellular secretory pathway. For example, rotaviruses, which lack a permanent envelope but are transiently membrane enclosed during assembly, encode a protein that disrupts the ER membrane. This protein is thought to allow removal of the temporary envelope formed during assembly of virus particles. The replication of most (+) strand RNA viruses takes place in association with membranous structures derived from various cytoplasmic membranes of the host cell (Chapters 6 and 14). Such remodeling of cellular membranes can lead to dramatic reorganization of cytoplasmic compartments and inhibition of trafficking via the secretory pathway, effects well characterized in cells infected by poliovirus and other enteroviruses.

The Golgi complex is disrupted in poliovirus-infected cells, although such disassembly is not required for viral reproduction. Rather, it appears to be a consequence of diversion

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BOX 12.9

DISCUSSION

Which herpesviral components are targeted to axons for anterograde transport?

Alphaherpesviruses (e.g., herpes simplex virus and pseudorabies virus) reproduce within polarized neurons. Infection begins with entry at mucosal surfaces and spread of virus particles between cells of the mucosal epithelium. The peripheral nervous system is infected via axon termini innervating this region, and subsequent trafficking of nucleocapsids to the cell body. It is here that a reactivatable, latent infection that persists for the life of the host can be established. A well-known but poorly understood phenomenon is that, upon reactivation from latent infection, alphaherpesviruses rarely enter the central nervous system, despite having what seems to be two rather similar choices: cross one synapse and infect the central nervous system (rare) or traffic back down the axon and cross to the initial site of infection, mucosal epithelial cells (very common). Inherent in this choice is the fact that progeny viral particles or their components must be targeted to axons.

The mechanisms by which newly synthesized components of virus particles, such as nucleocapsids and envelope proteins, are sorted to axons for anterograde transport have been the subjects of considerable controversy. In fact, different processes have been proposed for herpes simplex virus and pseudorabies virus, separate transport of the nucleocapsid plus tegument and viral glycoprotein, and transport of enveloped virus particles, respectively. Similar methods have been used to examine anterograde transport of the two viruses in several laboratories. These methods include confocal microscopy, imaging of nucleocapsids and glycoproteins that carry distinguishable fluorescent labels in live cells, and immunoelectron microscopy. Nevertheless, the controversy has not been resolved. Although counterintuitive, it is possible that different processes operate in cells infected by these two viruses, which are the most distantly related among the alphaherpesviruses. This hypothesis implies that envelopment of naked herpes simplex virus nucleocapsids takes place at the membrane of axonal growth cones, whereas nonenveloped pseudorabies virus nucleocapsids can travel only in the retrograde direction.

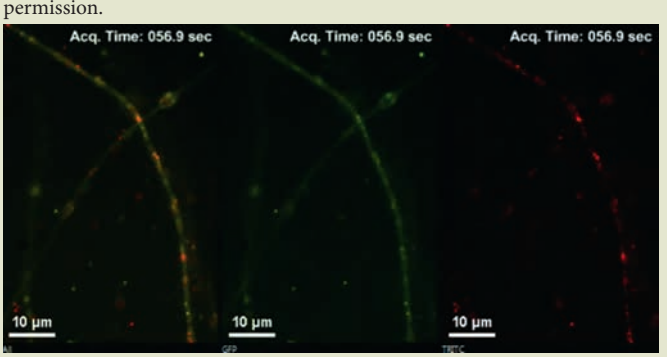
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The salivary glands of mice were infected with a virulent strain of dually fluorescent derivative of pseudorabies virus: the minor capsid protein VP26 was fused to mRFP (red) and the envelope protein Us9 was fused to GFP (green). Tissues including the salivary gland and submandibular ganglia were removed 24 h after infection, and the ganglia were exposed for time-lapse epifluorescence microscopy. Movie 12.1 (http://bit.ly/Virology_V1_Movie12-1) shows that all anterograde-moving red particles (nucleocapsids) also contain the envelope protein Us9 (green), consistent with sorting for axonal transport after formation of complete virus particles. Adapted from A. E. Granstedt et al., *mBio* **4:**e00358-13, 2013, with permission.



of membranes from earlier compartments in the secretory pathway. Poliovirus infection induces a transient increase in, but subsequent inhibition of, budding of CopII-coated vesicles from ER exit sites, where the viral 2B and 2BC proteins colocalize with cellular proteins that form this coat. The temporary acceleration in vesicular traffic from the ER may increase the supply of membranes and other components to the ER-Golgi intermediate compartment (ERGIC), the origin of the distinctive vesicles that serve as platforms for the replication of the viral RNA genome. These replication compartments are characterized by the presence of specific vesicle-associated proteins of the cell and the viral 3A protein, which recruits an enzyme that catalyzes synthesis of phosphoinositol 4-phosphate (PI4P)-containing lipids. The viral $3D^{pol}$ RNA polymerase localizes to the membranes of such replication compartments by virtue of its preferential binding to PI4P-containing lipids. The diversion of membranes from the ERGIC and disruption of Golgi compartments result in inhibition of protein traffic to the surface of infected cells, and may dampen antiviral responses mediated by MHC class I molecules and cytokines to facilitate survival of infected cells.

Later in infection, double-membrane vesicles 200 to 400 nm in diameter accumulate in the cytoplasm. These vesicles resemble autophagosomes, and can also be associated with replicating viral RNA genomes (Chapter 14). They may also play a role in nonlytic release of virus from cells (Chapters 13 and 14). The vesicles that serve as scaffolds for formation of replication complexes in cells infected by coronaviruses also

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exhibit properties of autophagosomes. The mechanisms by which infection by these viruses override the cellular circuits that normally prevent autophagy are not yet known. Nevertheless, it is clear that formation of autophagosomes facilitates virus reproduction: virus yield is reduced when synthesis of cellular proteins required for autophagy is prevented.

Induction or Inhibition of the Unfolded Protein Response

The quality control functions of the ER ensure that improperly folded proteins are retained in that organelle for degradation upon retrotranslocation to the cytoplasm. When the capacity of the ER protein folding and removal machinery is exceeded, three signaling pathways are activated by transmembrane receptors, because Grp78 is sequestered from their luminal domains by association with the large quantities of misfolded or unfolded proteins. These signal transduction cascades, collectively known as the unfolded protein response, lead to inhibition of translation and enhanced production of ER membranes and resident folding chaperones and catalysts, as well as ER proteins that clear improperly folded proteins (Fig. 12.17). However, when these measures fail to restore homeostasis, and the unfolded protein response is prolonged, apoptosis is induced via the Pkr-like ER kinase (Perk) and activating transcription factor 4 (Atf4).

Not surprisingly, the demands placed on the biosynthetic capacity of virus-infected cells can induce the unfolded protein response. For example, synthesis of the ER chaperone Grp78 is stimulated in cells infected by a variety of enveloped viruses, including bunyaviruses, flaviviruses, herpesviruses, influenza A virus, and paramyxoviruses. In most cases, accumulation of improperly folded viral proteins is likely to trigger activation of Atf6 (Fig. 12.17). However, during the early stages of infection, the major immediate early protein of the betaherpesvirus human cytomegalovirus directly activates transcription from the promoter of the Grp78 gene, and translation of Grp78 mRNA is also stimulated. A second viral protein, UL50, binds to inositol-requiring enzyme 1 (Ire1) to block signaling from this protein and increase synthesis of others that mediate ER-associated degradation late in infection.

From the point of view of successful virus reproduction, activation of the unfolded protein response is a mixed blessing: increased ER capacity would facilitate the production of large quantities of viral proteins, but attenuation of translation, induction of apoptosis, and increased ER-associated degradation (Fig. 12.17) could limit virus reproduction. This property may account for the differential impact of certain viruses on the various arms of the unfolded protein response. For example, in cells infected by the flaviviruses West Nile virus and dengue virus, signaling via Atf6 and Ire1 is increased, but Perk-mediated inhibition of translation and induction of apoptosis is blocked. The mechanisms that allow such discrimination among the three signaling pathways are not yet known, but clearly facilitate virus reproduction: the yield of infectious dengue virus particles is reduced by an order of magnitude in cells that lack Atf6, but increased to the same or a greater degree in *Perk*$^{-/-}$ cells.

Signal Sequence-Independent Transport of Viral Proteins to the Plasma Membrane

Many enveloped viruses contain matrix or tegument proteins lying between, and making contact with, the inner surface of the membrane of the particle and the capsid or nucleocapsid (Chapter 4). In contrast to the integral membrane proteins of enveloped viruses, such internal proteins of virus particles do not enter the secretory pathway, but are synthesized in the cytoplasm of an infected cell and directed to membrane assembly sites by specific signals.

Lipid-plus-Protein Signals

It has been known for many years that cytoplasmic proteins can be modified by the covalent addition of lipid chains (Table 12.2). Best characterized are the addition of the 14-carbon saturated fatty acid myristate to N-terminal glycine residues, and of unsaturated polyisoprenes, such as farnesol (C_{15}) or geranylgeranol (C_{20}), to a specific C-terminal sequence (Fig. 12.18). Palmitate is also added to some viral proteins that do not enter the secretory pathway. The discovery that transforming proteins of oncogenic retroviruses, the Src and Ras proteins, are myristoylated and isoprenylated, respectively, led to a resurgence of interest in these modifications. In this section, we focus on myristoylation and isoprenylation of viral structural proteins.

Myristoylation of the cytoplasmic Gag proteins of retroviruses and its consequences have been examined in detail. The internal structural proteins of these viruses, MA (matrix), CA (capsid), and NC (nucleocapsid), are produced by proteolytic cleavage of the Gag polyprotein following virus assembly. The Gag proteins of the majority of retroviruses are myristoylated at their N-terminal glycines. Mutations that prevent such acylation of murine leukemia virus or human immunodeficiency virus type 1 Gag proteins block interaction of the protein with the plasma membrane, induce cytoplasmic accumulation of Gag, and inhibit virus assembly and budding. In the case of the human immunodeficiency virus type 1 Gag protein, the myristoylated N-terminal segment and a highly basic sequence located a short distance downstream form a bipartite signal, which allows membrane binding *in vitro* and virus assembly and budding *in vivo* (Fig. 12.19). The MA domain of the Gag protein of this protein binds to phosphatidylinositol (4,5)-bisphosphate, and the acyl chains of other lipids enriched in the inner leaflet of the plasma membrane. This interaction accounts for the preferential association of

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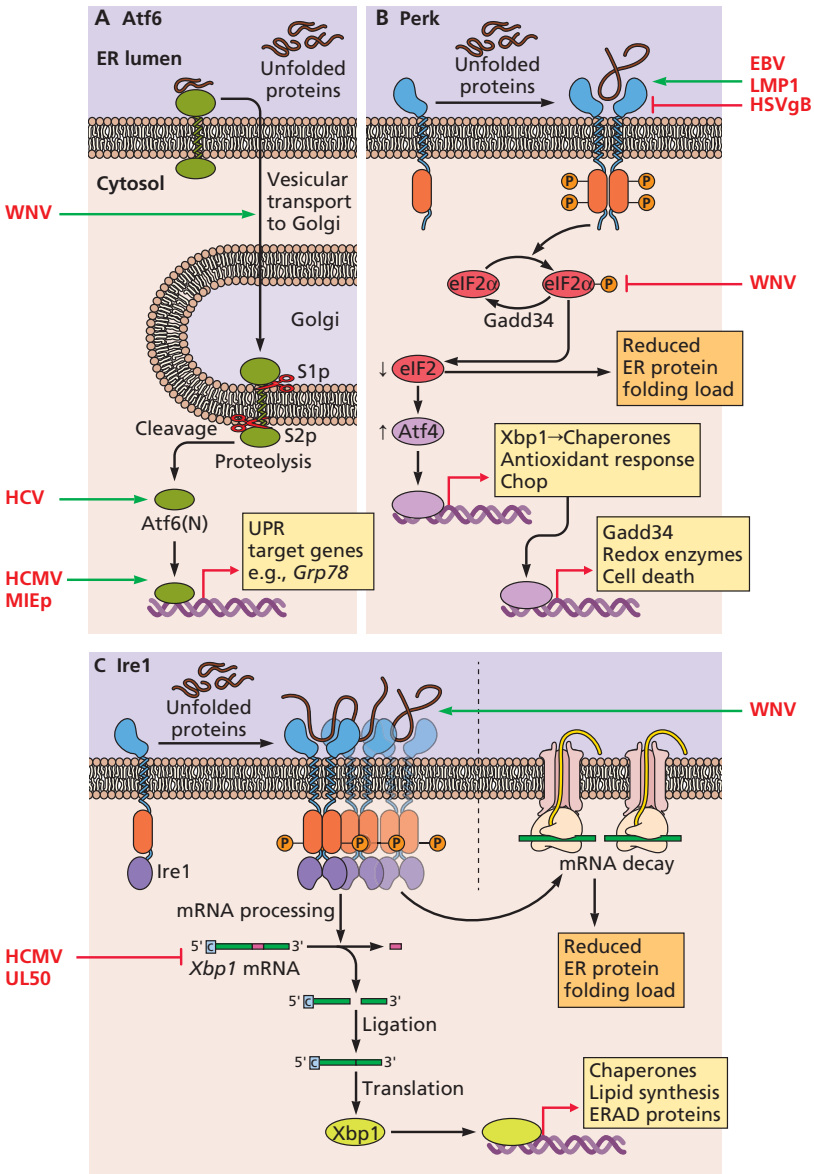


Figure 12.17 Modulation of the unfolded protein response in cells infected by flaviviruses and herpesviruses. Within the ER, Grp78 associates with the luminal domains of members of three families of signal transducers, Atf6 (activating transcription factor 6), Perk (double-stranded RNA activated protein kinase [Pkr]-like ER kinase), and Ire1 (inositol-requiring enzyme 1). Sequestration of the chaperone when concentrations of incompletely or improperly folded proteins in the ER lumen are high leads to activation of these signaling molecules, by different mechanisms. **(A)** Atf6 is released for transport to Golgi compartments, where it is cleaved by the site 1 (S1p) and site 2 (S2p) proteases. The N-terminal segment liberated into the cytoplasm enters the nucleus, where it activates transcription of specific genes. The majority of these genes encode ER proteins, including chaperones such as Grp78 and protein folding catalysts like Pdi, to increase the capacity of the ER to handle protein folding. **(B)** Signaling from Perk is initiated by dimerization and autophosphorylation of its cytoplasmic domain. The active kinase then phosphorylates the α subunit of the translation initiation protein eIF2 to inhibit translation (see Chapter 11). This response decreases the flow of newly synthesized proteins into the ER. However, some mRNAs, including Atf4 mRNA, are translated preferentially when eIF2 concentrations are limiting. Genes increased in expression in response to this regulator include those for X box-binding protein 1 (Xbp1) and transcriptional activator c/Ebp homologous protein (Chop). The latter protein in turn stimulates transcription of genes that encode proapoptotic proteins such as growth arrest and DNA damage-inducible 34 (Gadd34). Gadd34 also functions as a regulatory subunit of protein phosphatase to reverse phosphorylation and inhibition of eIF2. Consequently, prolonged signaling from Perk can induce cell death. **(C)** Ire1, the only unfolded protein signal transducer present in yeast, contains cytoplasmic kinase and RNase domains. Binding of unfolded proteins to the luminal domain is thought to induce oligomerization, autophosphorylation, and activation of the RNase. This enzyme initiates a very unusual splicing reaction by excision of the intron of Xbp1 mRNA, for subsequent ligation of its exons (by tRNA ligase in yeast and an as yet unidentified enzyme in mammalian cells). Subsequent synthesis of active Xbp1 leads to induction of transcription of genes that code for enzymes that catalyze lipid synthesis and proteins that facilitate removal of misfolded proteins from the ER. In addition to the exquisitely specific cleavages of Xbp1 mRNA, Ire1 initiates degradation of mRNAs associated with the ER by low-specificity endonucleolytic cleavage. As indicated, virus infection can lead to activation or inhibition of the three arms of the unfolded protein response, although in most cases the mechanisms of such modulation remain to be established. HCMV, human cytomegalovirus; EBV, Epstein Barr virus, HCV, hepatitis C virus; HSV, herpes simplex virus type 1; WNV, West Nile virus; MIEp, major immediate early protein; LMP1, latent membrane protein 1; ERAD, ER-associated degradation; UPR, unfolded protein response. Adapted from P. Walter and D. Ron, *Science* **334:**1081–1086, 2011, with permission.

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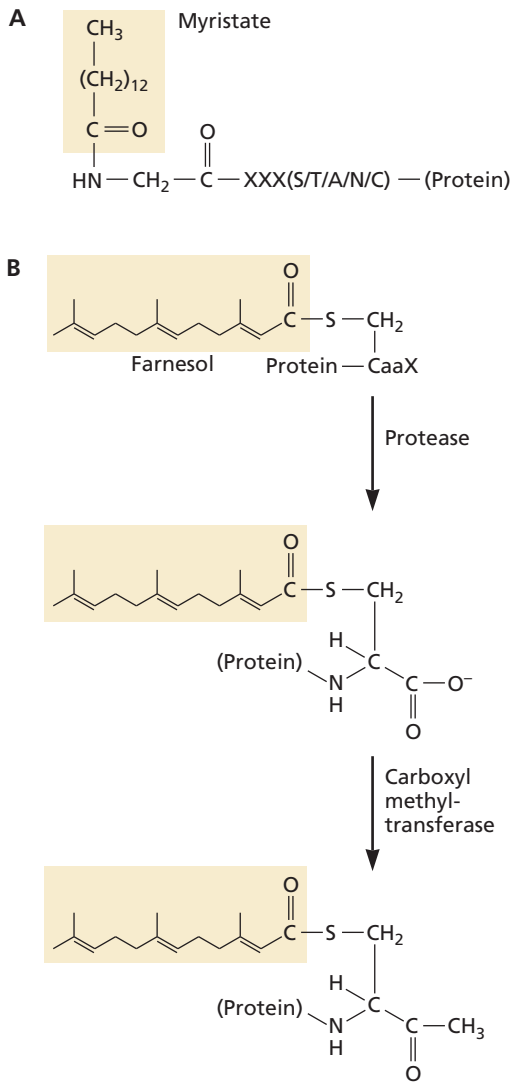


Figure 12.18 Addition of lipids to cytoplasmic proteins. **(A)** N-terminal myristoylation. An amide bond links the saturated fatty acid myristate to an N-terminal glycine present in the myristoylation site consensus sequence (X is any amino acid except proline). The initiating methionine must be removed, a reaction that is facilitated by uncharged amino acids in the positions denoted X. **(B)** C-terminal isoprenylation. A thioether bond links the unsaturated lipid farnesol to a cysteine in the isoprenylation consensus sequence ("a" is an aliphatic amino acid). In many proteins, isoprenylation is followed by proteolytic cleavage to expose the C-terminal cysteine, which is then methylated.

Gag with the plasma membrane. It also induces a conformational change that leads to exposure of the N-terminal myristate, and presumably tighter association of Gag with the membrane.

The hepatitis B virus large surface (L) protein is also myristoylated at its N terminus. However, in contrast to retroviral Gag, the L protein is present in the envelope of virus particles. Modification of its N terminus must therefore occur while it traverses the secretory pathway. In this case, myristoylation is not necessary for assembly or release of virus particles, but is required for infection of primary hepatocytes, presumably because it contributes to the initial interaction of the virus with, or its entry into, the host cell. More surprising is the myristoylation of structural proteins of poliovirus (VP4) and polyomavirus (VP2): although these virus particles do not contain an envelope, this modification is necessary for efficient assembly. In mature poliovirus particles, the myristate chain at the N terminus of VP4 interacts with the VP3 protein on the inside of the capsid (Fig. 4.12B). The hydrophobic lipid chain must therefore facilitate protein-protein interactions necessary for the assembly. The fatty acid is also important during entry into cells of poliovirus particles and their uncoating at the beginning of an infectious cycle (Chapter 5).

Among viral structural proteins, only the large delta protein of the hepatitis delta satellite virus has been found to be isoprenylated. Formation of the particles of this satellite virus depends on structural proteins provided by the helper virus, hepatitis B virus. The isoprenylation of large delta protein is necessary, but not sufficient, for its binding to the hepatitis B virus S protein during assembly of the satellite virus. This hydrophobic tail of large delta protein seems likely to facilitate interaction with the plasma membrane adjacent to regions that contain helper virus S protein in cells infected by the two viruses.

Protein Sequence Signals

The matrix proteins of members of several families of (−) strand RNA viruses are essential for correct localization and packaging of RNA genomes. During assembly, matrix proteins, such as M of vesicular stomatitis virus and M1 of influenza A virus, must bind to the inner surface of the plasma membrane of infected cells. These proteins are produced in the cytoplasm, but receive no lipid after translation. When the influenza virus M1 protein is synthesized in host cells in the absence of other viral proteins, it associates tightly with cellular membranes. Both this protein and the vesicular stomatitis virus M protein contain specific sequences that are necessary for their interaction with the plasma membrane *in vivo* or with lipid vesicles *in vitro*. This region of the influenza A virus M1 protein contains two hydrophobic sequences (Fig. 12.20A), which might form a hydrophobic surface in the folded protein. In addition to hydrophobic segments, membrane association of the vesicular stomatitis virus M protein requires a basic N-terminal sequence (Fig. 12.20B). This latter segment might participate directly in membrane binding, like the basic sequence of the human immunodeficiency virus type 1 Gag membrane-targeting signal, or it might stabilize a conformation of the internal sequence favorable for interaction with the membrane.

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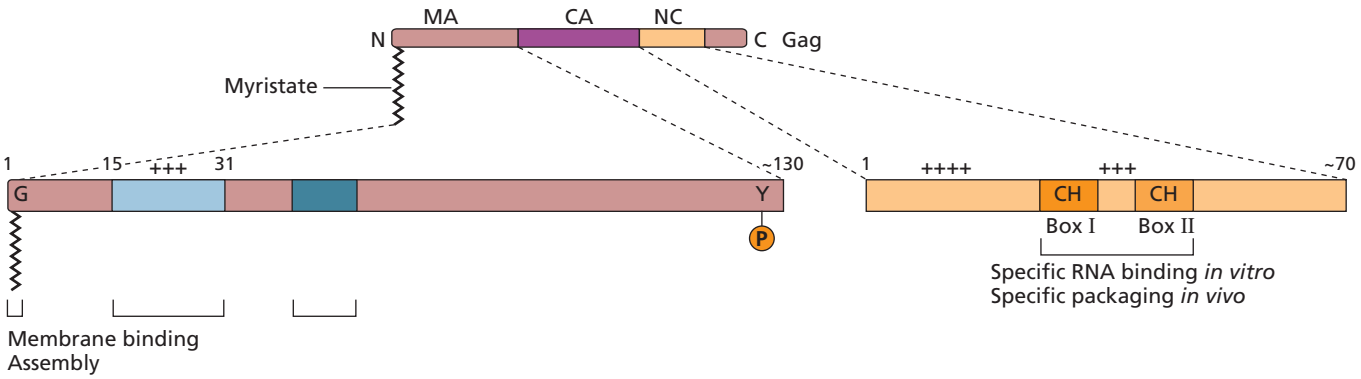


Figure 12.19 Human immunodeficiency virus type 1 Gag proteins and their targeting signals. The locations of the internal structural proteins MA (matrix), CA (capsid), and NC (nucleocapsid) in the Gag polyprotein are shown at the top. Sequence features, localization signals (MA), and the RNA-binding domain (NC) are shown below. The lengths of the MA and NC proteins are listed as approximate because of the variation among virus isolates. Specific amino acids are given in the single-letter code in the boxes, and a plus sign indicates a basic amino acid. The basic region of MA of retroviruses with simple genomes, such as avian sarcoma virus, is not required for membrane binding. The CH boxes of NC contain three cysteines and one histidine, and each coordinates one Zn^{2+} ion. CH box I is conserved among retroviruses, but CH box II is not.

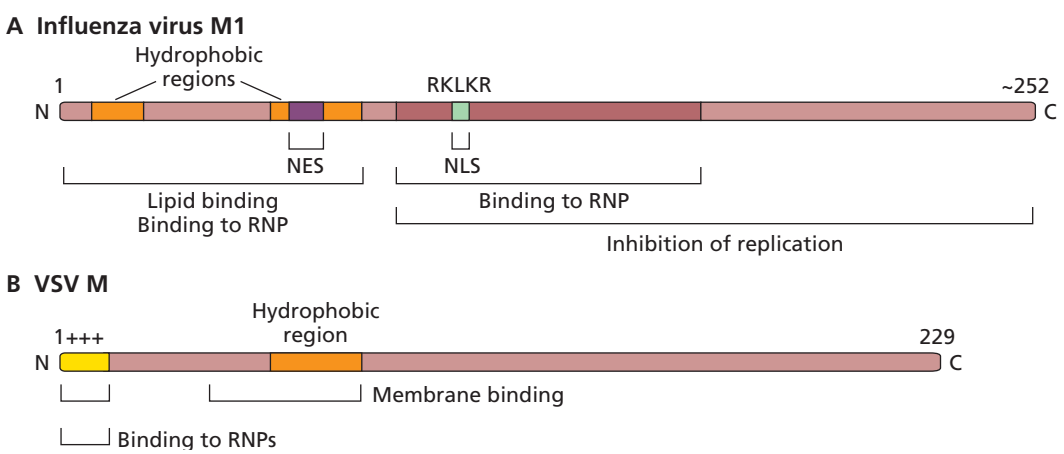
In some cases, such as the vesicular stomatitis virus M protein, specificity for the plasma membrane is an intrinsic property, suggesting that these proteins might recognize phospholipids enriched in the inner leaflet of the plasma membrane, such as phosphatidylserine and phosphatidylinositol. However, binding of matrix proteins to the cytoplasmic tails of viral envelope glycoproteins can also be an important determinant of membrane association. The cytoplasmic domains of both the NA and HA proteins of influenza A virus stimulate membrane binding by M1 protein. Similarly, membrane binding by the matrix protein of Sendai virus (a paramyxovirus) is independently stimulated by the presence of either of the two viral glycoproteins (F or HN) in the membrane.

Interactions with Internal Cellular Membranes

The envelopes of a variety of viruses are acquired from internal membranes of the infected cell, rather than from the plasma membrane. The majority of these viruses assemble at the cytoplasmic faces of compartments of the secretory pathway (Table 12.3). Although a single budding reaction is typical, the more complex herpesviruses and poxviruses interact with multiple internal membranes during assembly and exocytosis (Chapter 13).

The diversity of the internal membranes with which these viruses associate during envelope acquisition and exocytosis is the result of variations on a single mechanistic theme: the site

Figure 12.20 Targeting signals of matrix proteins of influenza virus (A) and vesicular stomatitis virus (B). Sequence features of specific segments of the proteins and the boundaries of targeting and RNP-binding domains are shown. Amino acids are written in the one-letter code, and a plus sign indicates a basic amino acid. NES, nuclear export signal; NLS, nuclear localization signal; VSV, vesicular stomatitis virus.



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Table 12.3 Interactions of viruses with internal cellular membranes

Virus family	Example	Integral membrane protein(s)	Intracellular membrane(s)	Mechanism of envelopment
Bunyaviruses	Uukuniemi virus Hantaan virus	Gn Gc	*cis*-medial Golgi cisternae	Budding into Golgi cisternae
Coronavirus	Mouse hepatitis virus Severe acute respiratory syndrome coronavirus	M, S	ERGIC and *cis*-Golgi network	Budding into ERGIC and *cis*-Golgi network
Flavivirus	Dengue virus West Nile virus	E, prM	ER	Budding into ER
Hepadnavirus	Hepatitis B virus	L, M, S	ER and other compartments	Budding into multivesicular body
Herpesvirus	Herpes simplex virus type 1	gB, gH, UL34 gE-gI	Nuclear membrane *trans*-Golgi cisternae	Primary envelopment; budding of capsids from inner nuclear membrane Budding at *trans*-Golgi membrane
Poxvirus	Vaccinia virus, immature Vaccinia virus, intracellular mature virus	A14L, A13L, A17L A56R (HA), F13L, B5R	ER Late *trans*-Golgi cisternae and post-Golgi vesicles	Formation of mature virion Wrapping of mature virion
Rotavirus	Simian rotavirus	VP7, NS28	ER	Budding into ER

of assembly is determined by the intracellular location of viral envelope proteins (Fig. 12.21), just as assembly at the plasma membrane is the result of transport of such proteins to that site. Assembly of viruses at internal membranes therefore requires transport of envelope proteins to, and their retention within, appropriate intracellular compartments.

Localization of Viral Proteins to Compartments of the Secretory Pathway

The bunyaviruses, a family that includes Uukuniemi and Hantaan viruses, are among the best-studied viruses that assemble by budding into compartments of the secretory pathway. Bunyavirus particles contain two integral membrane glycoproteins, called Gn and Gc, which are encoded within a single open reading frame of the M genomic RNA segment. Like alphaviral envelope proteins, the bunyaviral polyprotein containing Gn and Gc is processed cotranslationally by signal peptidase as the precursor enters the lumen of the ER (Table 12.1). However, association of the glycoproteins with one another is required for transport of Gc to Golgi compartments: when synthesized alone, Gn accumulates in the Golgi complex as it does in infected cells, but Gc fails to leave the ER. The signals necessary for Golgi residence of Gn and associated Gc lie in the transmembrane domain of Gn.

Golgi cisternae are by no means the only compartments of the secretory pathway at which virus budding can occur. For example, rotaviruses transiently acquire an envelope by budding into and out of the ER, whereas coronaviruses bud into the ERGIC and the Golgi apparatus. In these and other cases, it is the presence of viral glycoproteins in specific cellular membranes (Table 12.3) that determines the site of assembly and budding.

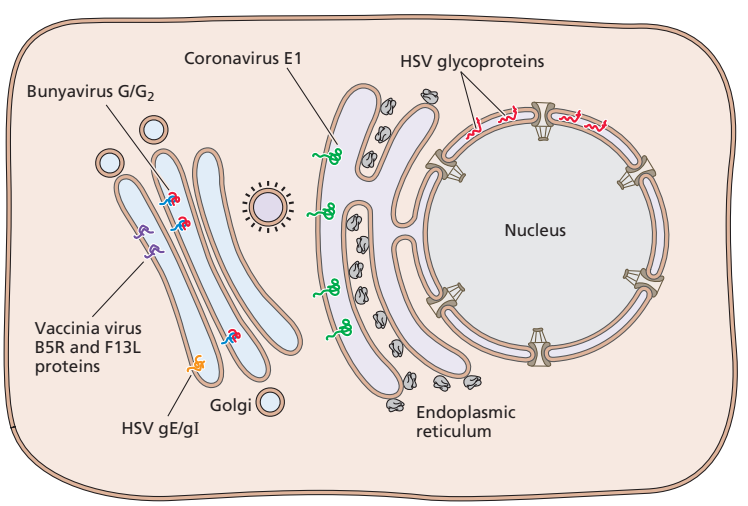


Figure 12.21 Sorting of viral glycoproteins to internal cell membranes. The destinations of membrane glycoproteins of viruses that bud into compartments of the secretory pathway (bunyaviruses and coronaviruses) or from the inner nuclear membrane and compartments of the *trans*-Golgi network (herpesviruses [HSV]) or are wrapped by cellular membranes during assembly (poxviruses) are indicated.

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Localization of Viral Proteins to the Nuclear Membrane

Herpesviruses such as herpes simplex virus type 1 are the only enveloped viruses that are known to assemble initially within, and bud from, the nucleus. The first association of an assembling herpesvirus with a cellular membrane is therefore budding of the nucleocapsid through the inner membrane of the nuclear envelope. This process, which is described in Chapter 13, depends on association of particular viral proteins with the inner nuclear membrane (Fig. 12.21; Table 12.3).

Transport of Viral Genomes to Assembly Sites

Like the structural proteins and enzymes of virus particles, progeny genomes must be available, or concentrated, at the intracellular site at which assembly takes place. In several cases, this requirement is met by genome replication within the same cellular organelle or structure as assembly of new virus particles. The genomes of DNA viruses that are synthesized in infected cell nuclei are encapsidated within that organelle. Similarly, the replication of the genomes of many (+) strand viral RNAs (including those of picornaviruses and flaviviruses) and of large DNA viruses such as poxviruses, and assembly of virus particles, are restricted to specialized cytoplasmic structures derived from host cell membranes (Chapters 13 and 14). In contrast, the genomes of many (−) strand RNA viruses must be transported to the cytoplasmic faces of the appropriate membrane, most commonly the plasma membrane. Yet other RNA genomes must travel even farther: both influenza virus and retroviral genomic RNAs are synthesized within the infected cell nucleus, but progeny virus particles bud from the plasma membrane.

Transport of Genomic and Pregenomic RNA from the Nucleus to the Cytoplasm

Retroviral genomes are unspliced RNA transcripts synthesized in infected cell nuclei by host cell RNA polymerase II, as is hepadnaviral pregenomic RNA. These RNAs must be exported to the cytoplasm for assembly, a process that requires that the inefficient export of unspliced mRNAs characteristic of host cells be circumvented. Viral RNA-binding proteins promote export of unspliced RNA of retroviruses with complex genomes, such as human immunodeficiency virus type 1, whereas specific sequences that are recognized by cellular proteins direct export of genomic RNAs of other retroviruses and hepadnaviral pregenomic RNA (Chapter 10).

Perhaps the most elaborate requirements for transport of viral RNA species between nucleus and cytoplasm are found in influenza A virus-infected cells: both the direction of transport of genomic RNA and the nature of the viral RNA exported from the nucleus change as the infectious cycle progresses. When the cycle is initiated, viral genomic ribonucleoproteins (RNPs) enter the nucleus under the direction of the nuclear localization signal of the NP protein (Chapter 5). The mechanisms that ensure export of viral (+) strand mRNAs for translation are not well understood. With the switch to replication, genomic (−) strand RNA segments are synthesized in infected cell nuclei, where they accumulate as viral RNPs containing the NP protein and the three P proteins. These RNPs must be exported to allow virus assembly and completion of the infectious cycle, a reaction that requires the viral M1 and NEP proteins (Fig. 12.22). The M1 protein binds to both viral RNPs and NEP. Efficient transport of this complex to the cytoplasm requires two leucine-rich nuclear export signals present in NEP and one in M1. This requirement may restrict export of genome segments to the late phase of infection, when NEP accumulates, and ensure association of RNPs with the protein (M1) necessary for guiding them to the plasma membrane.

Transport of Genomes from the Cytoplasm to the Plasma Membrane

Accumulation of the RNA genomes of enveloped viruses at the appropriate cellular membrane depends on signals present in viral proteins bound to the RNA. The membrane-binding domain of influenza virus M1, described previously, allows association of the genomic RNPs with the plasma membrane following transport from the juxtanuclear region, at which they accumulate upon export from the nucleus. This region contains the microtubule organizing center, and the RNPs become associated with recycling endosomes and are transported along microtubules to the plasma membrane (Fig. 12.22). The concentration of RNPs on the membranes of recycling endosomes may also facilitate association and packaging of the full complement of genome segments during assembly (Chapter 13).

Although more limited in its RNA transport functions, the M protein of vesicular stomatitis virus shares several properties with the influenza virus M1 protein. Newly synthesized genomic RNA molecules assemble with the N, L, and NS proteins to form helical RNPs. Genomic RNA molecules within RNPs can serve as templates for additional cycles of replication or for mRNA synthesis. However, these RNPs eventually must travel to the plasma membrane for association with the G protein and incorporation into virus particles. Entry into the latter pathway is determined by the viral M protein, which associates with RNPs containing genomic RNA to induce formation of a tightly coiled RNA-protein "skeleton" (Fig. 12.23). Formation of this structure precludes replication and mRNA synthesis and allows transport of nucleocapsids to the plasma membrane via microtubules (Box 12.10). The membrane-binding domains of the M protein described above then direct association with the plasma membrane.

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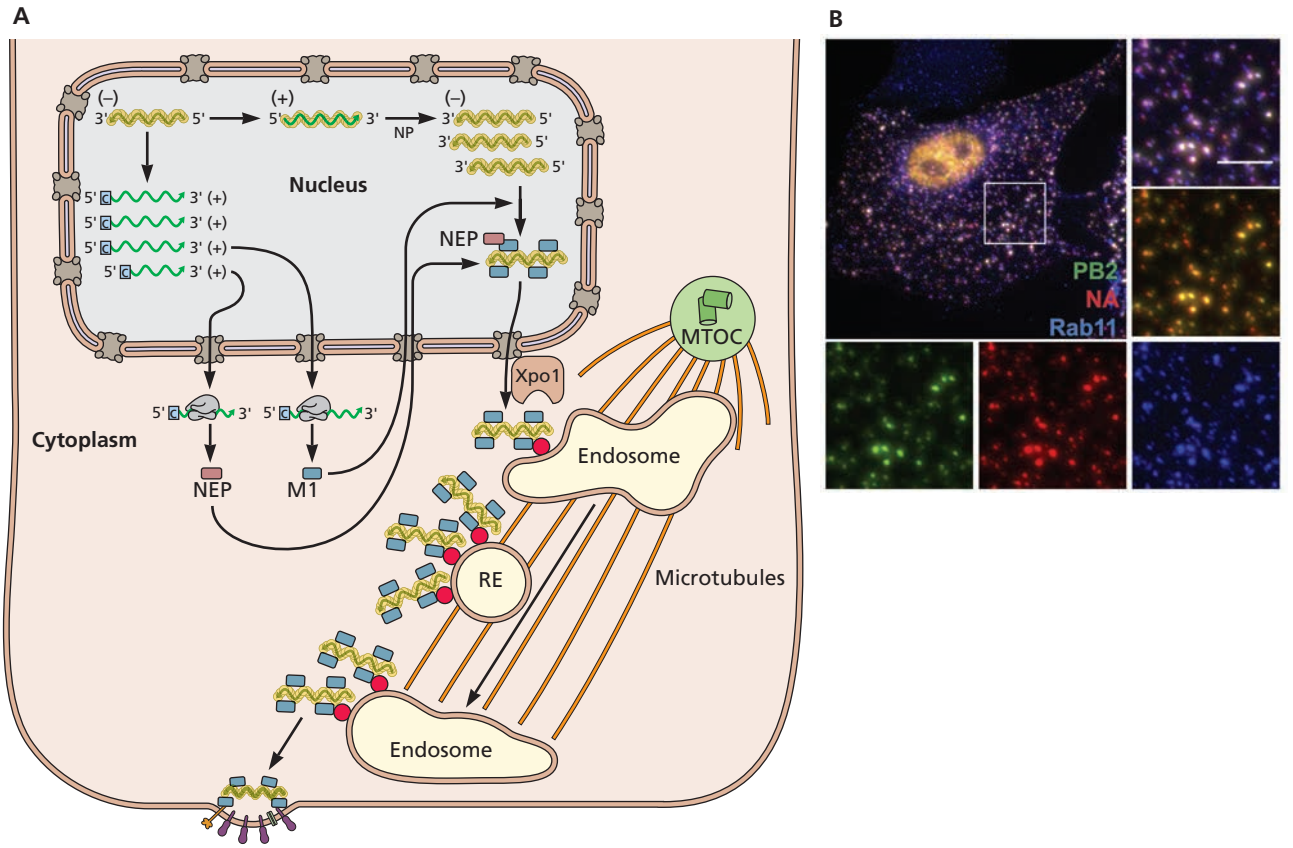


Figure 12.22 Transport of influenza A virus genomic RNA segments from the nucleus to the plasma membrane. (A) Genomic RNA segments are bound by the NP protein as they are synthesized (see Chapter 6) and subsequently by the M1 protein. M1 is the most abundant protein of the virus particle and enters the nucleus by means of a typical nuclear localization signal (Fig. 5.22). Binding of M1 to genomic RNPs (Fig. 12.20A) both inhibits RNA synthesis and promotes genomic RNP export. M1-containing RNPs are directed to the cellular Xpo1 export pathway upon binding of NEP, which contains two nuclear export signals. NEP possesses no intrinsic RNA-binding activity, but includes a C-terminal M1-binding domain. This domain is thought to allow recognition of RNPs to which the M1 protein is bound. Following export from the nucleus, RNPs accumulate in the region that contains the microtubule organizing center (MTOC) and, via interaction of the viral polymerase with the GTP-bound form of Rab11, become associated with recycling endosomes (REs), which are transported to the plasma membrane along microtubules. **(B)** It has been proposed that Rab11-containing organelles, such as REs, serve as platforms for association of the individual vRNA segments that comprise a genome with one another, based in part on colocalization. In the experiment shown here, human cells were infected with influenza virus for 10 h (later in the infectious cycle), and subjected to two-color, high-sensitivity fluorescence *in situ* hybridization (FISH) with oligonucleotides complementary to the PB2 and NA vRNA segments labeled with different fluorophores, Cy3 (green) and Cy5 (red), respectively, and then to immunostaining for Rab11. A high degree of colocalization of these vRNAs with one another (yellow puncta in the two-color FISH image, top, lower panel on right) and with Rab11 (white puncta in the merged FISH and immunostaining image, top, upper panel on right) was observed. Quantification of the degree of colocalization of the Rab11-associated vRNAs and the vRNAs that were not so associated suggested that interaction with Rab11 promoted vRNA colocalization. Consistent with a model in which Rab11-bound organelles provide a niche for assembly of individual vRNA segments in transit to the plasma membrane for assembly, synthesis of a dominant-negative derivative of Rab11 in infected cells reduced the efficiency of colocalization of the PB2 and NA vRNAs. Adapted from Y.-Y. Chou et al., *PloS Pathog* **9:**e1003358, 2013, with permission.

The retroviral proteins that mediate membrane association of genomic RNA are similar to the matrix proteins of these (−) strand RNA viruses in several respects. Once within the cytoplasm, unspliced retroviral RNA is translated on polyribosomes into the Gag and, at low frequency, Gag-Pol polyproteins. The functions of Gag include transport of unspliced RNA molecules to membrane assembly sites and packaging of the RNA into assembling capsids. Consequently, whether unspliced genomic RNA molecules continue to be translated or are redirected for assembly is controlled by the cytoplasmic concentration of Gag.

The NC segment of Gag contains an RNA-binding domain required for specific recognition of the RNA-packaging sequences described in Chapter 13. NC functions as an

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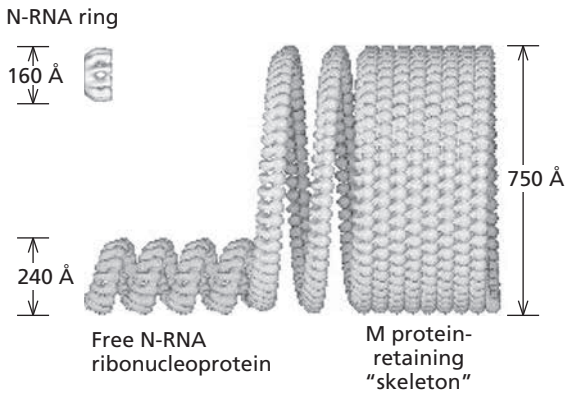


Figure 12.23 Models of the rabies virus nucleocapsid, showing the free nucleocapsid and the nucleocapsid present in virus particles. The models are based on cryo-electron microscopy and image reconstruction of the two forms of the nucleocapsid, as well as of rings of 9 or 10 molecules of the viral N protein and RNA assembled when the protein is produced in insect cells (160-Å N-RNA ring). The free nucleocapsid, which is the template for viral RNA synthesis, is a loosely coiled helix with a variable pitch and diameter of 240 Å. In contrast, the nucleocapsid helix incorporated into virus particles is tightly wound, with a small pitch and a much larger diameter (750-Å M protein-retaining "skeleton"). These structural transitions are induced by binding of the M protein to the free nucleocapsid. Adapted from G. Schoehn et al., *J Virol* **75:**490–498, 2001, with permission.

independent protein in mature virus particles, is very basic, and contains at least one copy of a zinc-binding motif (Fig. 12.19). This domain makes a major contribution to the specificity with which Gag or NC proteins bind to unspliced retroviral RNA and, in conjunction with basic amino acids located nearby, is responsible for the RNA-packaging activity of Gag. The N-terminal MA portion contains the signals described above that target the polyprotein to the plasma membrane. Binding of Gag to unspliced retroviral RNA therefore allows delivery of the genome to assembly sites at the plasma membrane. The interaction probably takes place at the juxtanuclear sites where newly synthesized Gag accumulates. Movement of Gag (and presumably associated viral RNA genomes) to the plasma membrane requires the kinesin Kif4, a motor that moves toward the (+) (plasma membrane) end of microtubules and binds to the viral protein. A variety of other cellular proteins, including the RNA-binding protein Staufen1 and proteins that interact with it, have been implicated in transport of retroviral genomes to the plasma membrane. However, whether transport is direct, or via association with membrane-bound structures such as multivesicular bodies, or whether these different mechanisms dominate in different cell types is not yet clear.

The influenza virus M1, vesicular stomatitis virus M, and retroviral Gag proteins each possess the ability to bind directly or indirectly to RNPs containing genomic RNA and to membranes. Such interactions commit genomic RNA to the assembly pathway, direct the RNA to the plasma membrane, and promote interactions among internal and envelope components of virus particles. These properties are essential at the end of an infectious cycle, when the primary task is assembly of progeny. On the other hand, they would be disastrous if the interactions could not be reversed before or at the beginning of a new cycle, when the infecting genome must reach nuclear (influenza virus) or cytoplasmic (vesicular stomatitis virus and retroviruses) sites distant from the plasma membrane. In the case of (−) strand viruses, matrix proteins are removed during virus entry. The retroviral mechanism is more elegant: following virus assembly and budding, Gag (and Gag-Pol) polyproteins are processed by the viral protease to the individual structural proteins shown in Fig. 12.19. Such cleavages place the RNA-binding domain of NC in protein molecules separate from membrane-binding signals of MA, so that matrix-free core RNPs can be released into the cell to initiate a new infectious cycle.

Perspectives

The cellular trafficking systems described in this chapter are just as crucial for virus reproduction as the host cell's biosynthetic capabilities. The trafficking requirements during the infectious cycle can be quite intricate, with transport of viral macromolecules (or structures built from them) over large distances, or in opposite directions during different periods of the infectious cycle. Assembly of progeny particles of all viruses depends on the prior sorting of their components by at least one cellular trafficking system.

The intracellular sorting of viral proteins or nucleic acids synthesized in large quantities in infected cells has provided important tools with which to study these processes, which are also essential to cellular physiology. Indeed, the fundamental principle of protein sorting, that a protein's final destination is dictated by specific signals within its amino acid sequence and/or covalently attached sugars or lipids, was established by analyses of viral proteins. Furthermore, the study of viral proteins that enter the secretory pathway has provided much of what we know of the reactions by which proteins are folded and processed within the ER, as well as those that clear misfolded proteins from the pathway. It therefore seems certain that viral systems will provide equally important insights into signals and sorting mechanisms that are presently less well characterized, such as those responsible for the direction of proteins to the specialized membrane regions of polarized cells.

One of the greatest current challenges in this field remains the elucidation of the mechanics of the movement of proteins, nucleic acids, nucleoproteins, or transport vesicles from one cellular compartment or site to another. The development and application of techniques that exploit fluorescent proteins to visualize transport in living cells is providing important new insights into these processes. Transport of components of virus particles to sites of assembly results in formation of

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BOX 12.10

EXPERIMENTS

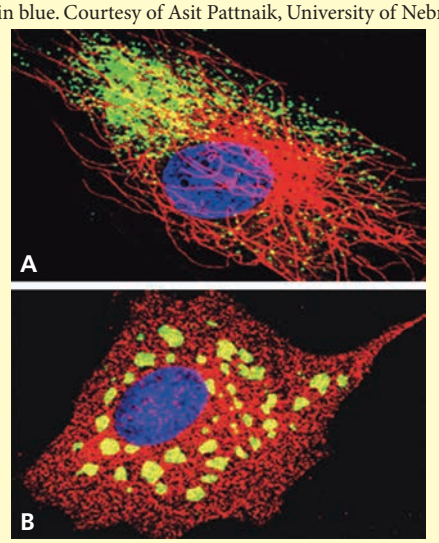
Movement of vesicular stomatitis virus nucleocapsids within the cytoplasm requires microtubules

Vesicular stomatitis virus (VSV) nucleocapsids that must be transported to the plasma membrane for assembly and budding of virus particles contain the (−) strand RNA genome and several viral proteins, including the P protein. To examine intracellular trafficking of nucleocapsids, a sequence coding for a green fluorescent protein (eGFP) was inserted into that for the hinge region of the P protein. Control experiments established that the P-eGFP fusion protein catalyzed both viral mRNA synthesis and genome replication, although it exhibited somewhat reduced activity. Furthermore, mutant particles containing P-eGFP in place of the P protein were infectious.

In infected cells, P-eGFP colocalized with newly synthesized viral RNA, as well as with the N and L proteins, in cytoplasmic structures of the size predicted for nucleocapsids. Time-lapse imaging of these P-eGFP-containing structures indicated that nucleocapsids move toward the cell periphery. Indeed, nucleocapsids were observed to be distributed throughout the cytoplasm in close association with microtubules (panel A of the figure). Treatment of infected cells with drugs that disrupt microtubules, such as nocodazole, dramatically altered this pattern: nucleocapsids became clustered in the absence of microtubules in large aggregates and did not reach the plasma membrane (panel B). Such drugs also reduced virus yield significantly, confirming the importance of microtubules in the transport of vesicular stomatitis virus nucleocapsids to sites of assembly.

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Localization of P-eGFP-containing nucleocapsids (green) and microtubules (red) in cells infected by the mutant virus VSV-PeGFP and untreated (A) or treated with nocodazole prior to infection (B). Nuclei are in blue. Courtesy of Asit Pattnaik, University of Nebraska-Lincoln.



microenvironments containing high concentrations of viral structural proteins and the nucleic acid genome. Such microenvironments are ideal niches for the assembly of progeny particles from their multiple parts.

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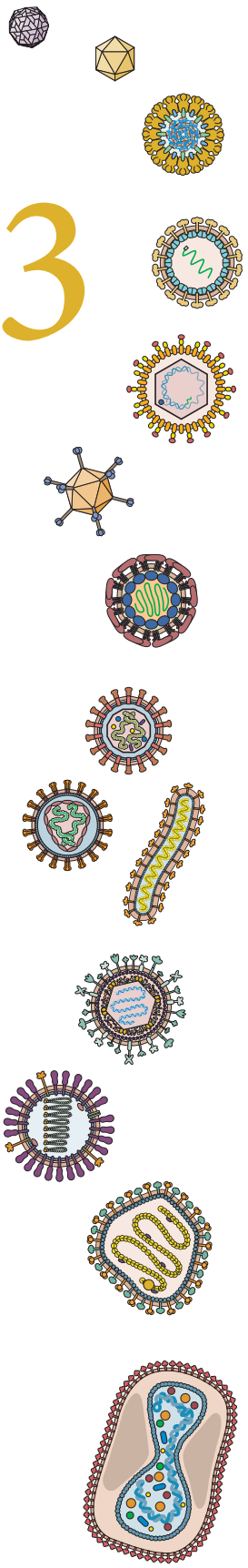
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13 Assembly, Exit, and Maturation

Introduction

Methods of Studying Virus Assembly and Egress
- Structural Studies of Virus Particles
- Visualization of Assembly and Exit by Microscopy
- Biochemical and Genetic Analyses of Assembly Intermediates
- Methods Based on Recombinant DNA Technology

Assembly of Protein Shells
- Formation of Structural Units
- Capsid and Nucleocapsid Assembly
- Self-Assembly and Assisted Assembly Reactions

Selective Packaging of the Viral Genome and Other Components of Virus Particles
- Concerted or Sequential Assembly
- Recognition and Packaging of the Nucleic Acid Genome
- Incorporation of Enzymes and Other Nonstructural Proteins

Acquisition of an Envelope
- Sequential Assembly of Internal Components and Budding from a Cellular Membrane
- Coordination of the Assembly of Internal Structures with Acquisition of the Envelope

Release of Virus Particles
- Assembly and Budding at the Plasma Membrane
- Assembly at Internal Membranes: the Problem of Exocytosis
- Release of Nonenveloped Viruses

Maturation of Progeny Virus Particles
- Proteolytic Processing of Structural Proteins
- Other Maturation Reactions

Cell-to-Cell Spread

Perspectives

References

LINKS FOR CHAPTER 13

- ⏭ *Video: Interview with Dr. Wesley Sundquist*
  http://bit.ly/Virology_Sundquist
- ⏭ *Movie 13.1: Active repulsion of vaccinia virus particles from infected cells*
  http://bit.ly/Virology_V1_Movie13-1
- ⏭ *Covering up a naked virus*
  http://bit.ly/Virology_3-19-15
- ⏭ *Cutting through mucus with the influenza virus neuraminidase*
  http://bit.ly/Virology_1-8-14

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The probability of formation of a highly complex structure from its elements is increased, or the number of possible ways of doing it diminished, if the structure in question can be broken down in a finite series of successively smaller substrates!

J. D. Bernal
in A. I. Oparin (ed.), *The Origins of Life on Earth*
(Pergamon, Oxford, United Kingdom, 1959)

Introduction

Virus particles exhibit considerable diversity in size, composition, and structural sophistication, ranging from those comprising a single nucleic acid molecule and one structural protein to complex structures built from many different proteins and other components. Nevertheless, successful reproduction of all viruses requires execution of a common set of *de novo* assembly reactions. These processes include formation of the structural units of the protective protein coat from individual protein molecules, assembly of the coat by interaction among the structural units, and incorporation of the nucleic acid genome (Fig. 13.1). In many cases, formation of internal virion structures must be coordinated with acquisition of a cellular membrane into which viral proteins have been inserted, or additional maturation steps must be completed to produce infectious particles. Assembly of even the simplest viruses is therefore a remarkable process that requires considerable specificity in, and coordination among, each of multiple reactions. In the extreme case of giant viruses, such as mimivirus and Pandoravirus, hundreds of proteins must interact appropriately with one another, with host cell membranes, and with the viral genome. Furthermore, virus reproduction is successful only if each of the assembly reactions proceeds with reasonable efficiency and if the overall pathway is irreversible. The diverse mechanisms by which viruses assemble represent powerful solutions to these problems associated with *de novo* assembly. Indeed, infectious virus particles are produced in prodigious numbers with great specificity and efficiency.

The architecture of a virus particle determines the nature of the reactions by which it is formed (Fig. 13.1). Despite variations in structure and biological properties, all virus particles must be well suited for protection of the nucleic acid genome in extracellular environments. They must also be metastable structures, that is, built in a way that allows their ready disassembly during entry into a new host cell. A number of elegant mechanisms resolve the apparently paradoxical requirements for very stable associations among virion components during assembly and transmission but the ready reversal of these interactions when appropriate signals are encountered upon infection of a host cell.

Like synthesis of viral nucleic acids and proteins, assembly of virus particles depends on host cell components, such as the cellular proteins that catalyze or assist the folding of individual protein molecules. Furthermore, the building blocks of virus particles are transported to the appropriate assembly site by cellular pathways (Chapter 12). Concentration of components of virus particles to a specific intracellular compartment or region undoubtedly facilitates virus production by increasing the rates of intermolecular assembly reactions. It is also likely to restrict the number of interactions in which particular components can engage, thereby increasing the specificities of these reactions.

The survival and propagation of a virus in a host population generally require dissemination of the virus beyond the cells initially infected. Progeny virus particles must therefore escape from the infected cell for transmission to new cells within the same host or to new hosts. The majority of viruses leave an infected cell by one of two general mechanisms: they are released into the external environment in various ways, or they are transferred directly from one cell to another.

PRINCIPLES *Assembly, exit, and maturation*

- Some structural units are formed from individual protein subunits; an alternative mechanism is assembly while individual protein coding sequences are covalently linked in a polyprotein precursor.
- The structural units of some protein shells assemble from individual units.
- An assembly line mechanism is well-suited for orderly formation of some virus particles.
- Accurate assembly of some large icosahedral protein shells requires scaffolding or chaperone proteins.
- Structural proteins contain the information necessary to specify assembly, but this process may be improved by the participation of cellular or viral chaperones.
- During encapsidation, viral genomes must be distinguished from cellular RNA or DNA and therefore often contain specific packaging signals.
- All viral genomes are packaged by one of two mechanisms, in conjunction with or following assembly of a protein shell (concerted or sequential encapsidation).
- Acquisition of an envelope by budding from the plasma membrane or internal membranes may be coordinated with, or follow, assembly of internal structures.
- Viral protein L domain sequences promote budding of enveloped viruses by recruitment of cellular proteins that participate in vesicular trafficking.
- When viral particles are assembled from polyproteins or precursor proteins, proteolytic cleavage by viral proteases is essential to produce infectious viral particles.
- Viruses may be released as free particles or spread from cell to cell without exposure to the extracellular milieu.

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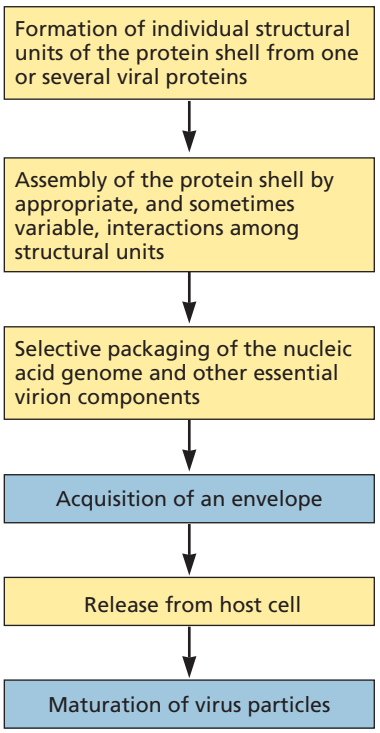


Figure 13.1 Hypothetical pathway of virus particle assembly and release. Reactions common to all viruses are shown in yellow, and those common to many viruses are shown in blue. The structural units that are often the first assembly intermediates are the homo- or hetero-oligomers of viral structural proteins from which virus particles are built (see Table 4.1). The arrows indicate a general sequence that applies to only some viruses. Packaging of the genome can be coordinated with assembly of the capsid or nucleocapsid, and for enveloped viruses, the assembly of internal components can be coordinated with acquisition of the envelope.

Methods of Studying Virus Assembly and Egress

Mechanisms of virus assembly and release can be understood only with the integration of information obtained by structural, biochemical, genetic, and imaging approaches. These methods are introduced briefly in this section.

Structural Studies of Virus Particles

The mechanisms by which virus particles form within, and leave, their host cells are intimately related to their structural properties. Our understanding of these processes therefore improves dramatically whenever the structure of a virus particle is determined. An atomic-level description of the contacts among the structural units that maintain the integrity of the particle identifies the interactions that mediate assembly and the ways in which these interactions must be regulated. For example, the X-ray crystal structure of the polyomavirus simian virus 40 described in Chapter 4 solved the enigma of how VP1 pentamers could be packed in hexameric arrays and identified three distinct modes of interpentamer contact. Assembly of the simian virus 40 capsid therefore must require specific variations in the ways in which pentamers associate, depending on their position in the capsid shell. Such subtle, yet sophisticated, regulation of the association of structural units was not anticipated and could be revealed only by high-resolution structural information.

Visualization of Assembly and Exit by Microscopy

While high-resolution structural studies of purified virus particles or individual proteins provide a molecular foundation for describing virus assembly, they offer no clues about how assembly (or exit) actually proceeds in an infected cell. Electron microscopy can be applied to investigation of these processes. Examination of thin sections of cells infected by a wide variety of viruses has provided important information about intracellular sites of assembly, the nature of assembly intermediates, and mechanisms of envelope acquisition and release of particles. This approach can be particularly useful when combined with immunocytochemical methods for identification of individual viral proteins or of the structures that they form via binding of specific antibodies attached to electron-dense particles of gold (Fig. 13.2A). More recently, intracellular viral structures and sites of assembly have been visualized by scanning electron and cryo-electron tomography (Chapter 4), which can capture three-dimensional information (Fig. 13.2B and C).

The labeling of viral proteins by fusion with green fluorescent protein or its derivatives (Chapter 2) (or of membranes with fluorescent lipophilic dyes) allows direct visualization of assembly and egress, an approach inconceivable even a few years ago. Such chimeric proteins and virus particles containing them can be observed in living cells, and their associations and movements can be recorded by video microscopy (see, for example, Chapter 12, Boxes 8 and 9). Consequently, these techniques overcome the limitations associated with traditional methods of microscopy, which provide only static views of populations of proteins or virus particles. On the other hand, the resolution that can be achieved by conventional fluorescence microscopy ($<$200 nm) is very low. Superresolution methods of fluorescence microscopy offer sufficiently improved resolution to visualize structural features of virus particle or assembly intermediates but have not yet been developed for routine live-cell imaging.

Biochemical and Genetic Analyses of Assembly Intermediates

Although of great value, the information provided by X-ray crystallography or microscopy is not sufficient to describe the dynamic processes of virus assembly and release; such understanding requires identification of the intermediates

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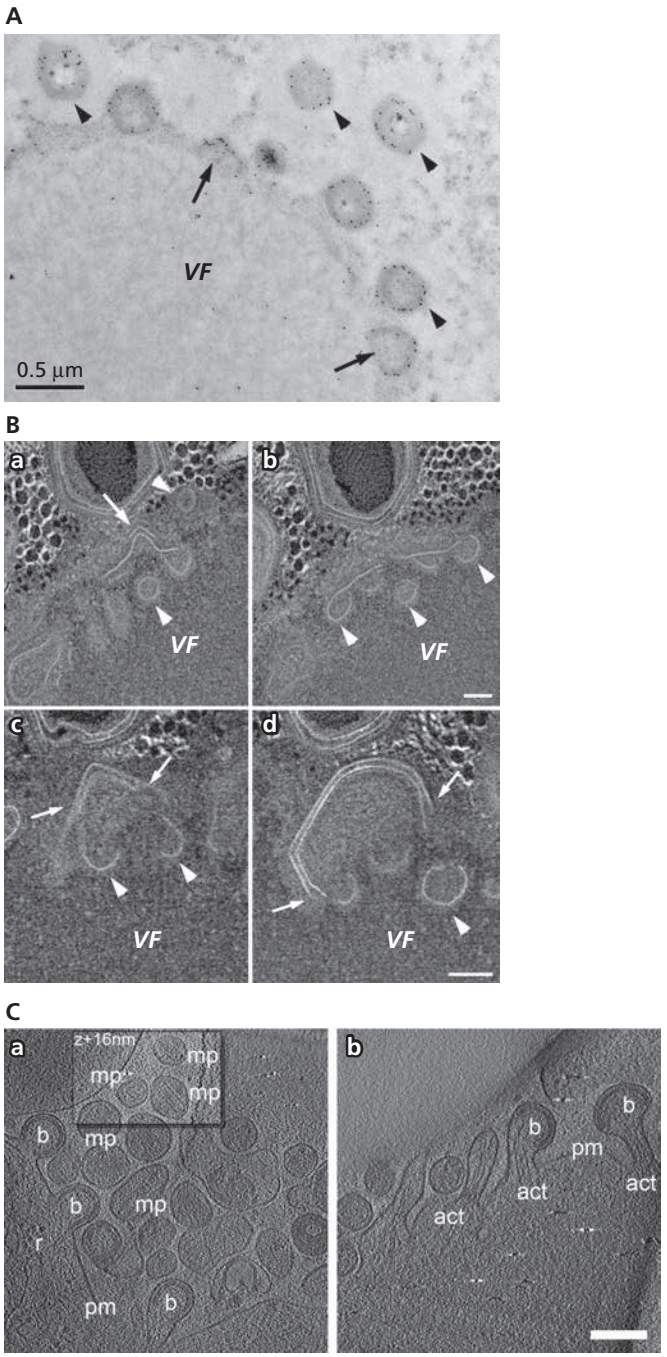


Figure 13.2 Examination of virus assembly by electron microscopy. Increasingly powerful methods of electron microscopy have been developed and applied to virus-infected cells. These approaches are illustrated using cells infected by mimivirus and human immunodeficiency virus type 1. **(A)** In immunoelectron microscopy, samples of infected cells suspended in a solid (but transparent) resin are sliced into sections, prior to reaction with gold-labeled antibodies against proteins of interest. This example shows 100- to 120-nm-thick sections of mimivirus-infected acanthamoeba and structures detected with antibodies that recognize the scaffolding protein of mimivirus (L425). This protein can be seen both in assembling capsids that form at the periphery of viral factories (VF), indicated by the arrows, and in closed capsids that are more distant from factors (arrowheads). **(B)** In scanning electron tomography, thick sections (250 to 400 nm in this example) of embedded samples are examined by electron microscopy at multiple tilt angles, and three-dimensional reconstructions are then computed from the images collected. Shown are 10-nm digital slices 40 nm apart derived from a tomogram of an amoeba infected with mimivirus for 8 h (a, b), in which can be seen viral factories and angular structures (arrows) forming on top of an open membrane sheet and surrounded by vesicles (arrowheads). By a later stage in assembly (c, d), angular structures (arrows) with truncated icosahedral symmetry (arrowheads) can be observed. These structures correspond to the scaffold protein-containing open structure shown in panel A. Adapted from Y. Mutsafi et al., *PLoS Pathog.* **9**(5)**:**e1003367, 2013, doi:10.1371/journal.Ppat.1003367, with permission. Courtesy of A. Minsky, The Weitzmann Institute of Science, Israel. **(C)** In cryo-electron tomography, vitrified samples of infected cells are examined at a series of tilt angles and three-dimensional images are reconstructed (see Chapter 4). In this example, human cells with thin peripheral areas amenable to visualization by this method (≤500 nm) were infected with an adenovirus vector for expression of the human immunodeficiency virus type 1 Gag-Pol coding sequence. Shown are computational slices 1.6 nm in thickness through a cryo-electron tomogram with budding particles (b), mature particles (mp), the plasma membrane (pm), actin filaments (act), and ribosomes (r) indicated. The inset in the left panel is offset by 16 nm perpendicular to the image plane to show the morphology of mature particles with a discrete cone-shaped internal core. Adapted from L.-A. Carlson et al., *PLoS Pathog.* **6**(11)**:**e1001173, 2010, doi:10.1371/journal.ppat.1001173, with permission. Courtesy of K. Grünewald, University of Oxford, United Kingdom.

and reactions in the pathway by which individual viral proteins and other components of virus particles are converted to mature infectious virus particles.

When extracts are prepared from the appropriate compartment of infected cells under conditions that preserve protein-protein interactions, a variety of viral assemblies can often be detected by techniques that separate them on the basis of mass and conformation (velocity sedimentation in sucrose gradients or gel filtration) or of density (equilibrium centrifugation). These assemblies range from the simplest structural units (see Table 4.1 for the definitions) to empty capsids and mature virus particles. Similar methods have identified various complexes formed by viral structural proteins in *in vitro* reactions. Furthermore, such structures can be organized into a sequence logical for assembly, from the least to the most complete. On the other hand, it is often quite difficult to **prove** that structures identified by these approaches, such as empty capsids, are true intermediates in the pathway.

By definition, the intermediates in any pathway do not accumulate unless the next reaction is rate limiting. For this reason, assembly intermediates are generally present within infected cells at low concentrations against a high background of the starting material (mono- or oligomeric structural proteins) and the final product (virus particles). This property makes it difficult to establish precursor-product relationships

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by pulse-chase experiments; the large pools of structural proteins initially labeled are converted only slowly and inefficiently into subsequent intermediates in the pathway. Genetic methods of analysis provide one powerful solution to this problem. Mutations that confer temperature sensitivity or other phenotypes that block a specific reaction have been invaluable in the elucidation of assembly pathways. A specific intermediate may accumulate in mutant-virus-infected cells and can often be purified and characterized more readily. Temperature-sensitive mutants can allow the reactions in a pathway to be ordered, and second-site suppressors of such mutations can identify viral proteins that interact with one another. Of even greater value is the combination of genetics with biochemistry, an elegant approach pioneered more than 35 years ago with the development of *in vitro* complementation for studies of the assembly of bacteriophage T4 (Box 13.1).

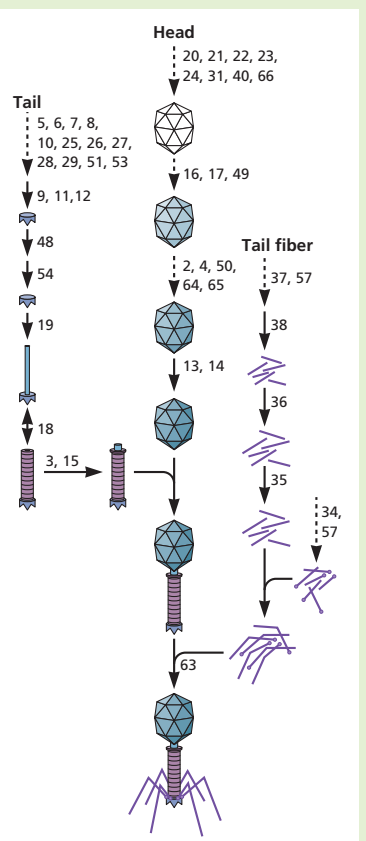
The difficulties inherent in kinetic analyses are compounded by the potential for formation of dead-end products and the unstable nature of some assembly intermediates. Dead-end assembly products are those that form by off-pathway (side) reactions. Because they are not true intermediates, they may accumulate in infected cells and be identified incorrectly as components in the pathway. Authentic intermediates by definition exist only transiently, and some may be fragile structures because they lack the complete set of intermolecular interactions that stabilize the virus particle. Less obvious is the conformational instability of some intermediates; such assemblies do not fall apart during isolation and purification but, rather, undergo irreversible conformational changes so that the structures studied experimentally do not correspond to **any** present in the infected cell. Such conformational change may well escape notice, as was initially the case for poliovirus empty capsids.

BOX 13.1

BACKGROUND

Late steps in T4 assembly

As illustrated, the head, tail, and tail fibers of this morphologically elaborate bacteriophage first form separately and then assemble with one another. The many genes encoding products that participate in building the T4 particle are listed by the reaction for which they are required. These gene products, and the order in which they act, were identified by genetic methods that included mapping of second-site suppressors of specific mutations (Chapter 3). The development of *in vitro* systems in which specific reactions were reconstituted was also of the greatest importance, allowing biochemical complementation. For example, noninfectious T4 particles lacking tail fibers accumulate in infected cells when the tail fiber pathway (right part of figure) is blocked by mutation. These incomplete particles can be converted to infectious phage when mixed *in vitro* with extracts prepared from cells infected with T4 mutated in the gene encoding the major head protein. The fact that the bacteriophages formed in this way were infectious established that assembly was accurate. This type of system was used to identify the genes encoding proteins that are required for assembly of heads or tails, as well as scaffolding proteins that are essential for assembly of the head, but not are present in the virus particle. Adapted from W. B. Wood, *Harvey Lect.* **73:**203–223, 1978, and W. B. Wood et al., *Fed. Proc.* **27:**1160–1166, 1968, with permission.



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Methods Based on Recombinant DNA Technology

Modern methods of molecular biology and the application of recombinant DNA technology have greatly facilitated the study of virus assembly. Especially valuable is the simplification of this complex process, that can be achieved by the synthesis of an individual viral protein or small sets of proteins in the absence of other viral components (Box 13.2).

Assembly of Protein Shells

Although virus particles are far simpler in structure than any cell, they are built from multiple components, such as a capsid, a nucleoprotein core containing the genome, and a lipid envelope carrying viral glycoproteins. The first steps in assembly are therefore the formation of the various components of virus particles from their parts. To complete the construction of the virus particle, these intermediates must then associate in ordered fashion, in some cases after transport to the appropriate intracellular site. Application of the techniques described in the previous section has allowed us to delineate the pathways by which many viruses are assembled and to describe some specific reactions in exquisite detail. In this section, we draw on this large body of information to illustrate mechanisms for the efficient assembly of protective protein coats for genomes, the first reaction listed in Fig. 13.1.

Formation of Structural Units

In some cases, notably assembly of (−) strand RNA viruses, structures built entirely from proteins do not accumulate because fabrication of a protein shell is coordinated with binding of structural proteins to the viral genome. In other cases, the first assembly reaction is the formation of the structural units from which the capsid is constructed (Fig. 13.1). This process is relatively simple: individual structural units contain a small number of protein molecules, typically two to six, that must associate appropriately following (or during) their synthesis. Nevertheless, structural units are formed by several different mechanisms, and in some cases additional proteins are required to assist the reactions (Fig. 13.3).

Assembly from Individual Proteins

The structural units of some protein shells, including the VP1 pentamers of simian virus 40, assemble from their individual protein components (Fig. 13.3A). This straightforward mechanism is analogous to the formation of cellular structures containing multiple proteins, such as nucleosomes. In this kind of reaction, the surfaces of individual protein molecules that contact other molecules of either the same protein or a different protein are formed prior to assembly of the structural unit. This mechanism facilitates specific binding when appropriate protein molecules encounter one another; no energetically costly conformational change is required, and subunits that come into contact can simply interlock. Production of these structural units generally can be reconstituted *in vitro* or in cells that synthesize the component proteins. Such experiments confirm that all information necessary for accurate assembly is contained within the primary sequence and, hence, the folded structure of the protein subunits. On the other hand, the individual protein subunits must find one another in a dense intracellular environment in which the concentration of irrelevant (cellular) proteins is very high (20 to 40 mg/ml). Such a milieu offers opportunities for nonspecific binding of viral proteins to unrelated cellular proteins. This problem can

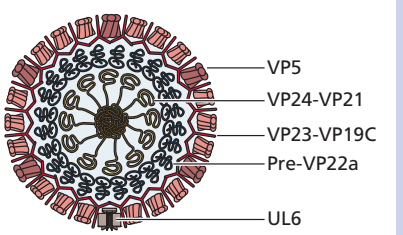
BOX 13.2

METHODS

Assembly of herpes simplex virus 1 nucleocapsids in a simplified system

The assembly and egress of herpesviruses from infected cells are complicated processes that comprise multiple steps (Fig. 13.8 and 13.23). To facilitate analysis of the initial reactions that lead to assembly of the protein shell, the viral genes that encode the proteins of the nucleocapsid were introduced into baculovirus vectors. Formation of the nucleocapsid was examined by electron microscopy of insect cells infected with various combinations of the recombinant baculoviruses. Empty capsids indistinguishable from those formed in herpes simplex virus 1-infected cells were observed when six viral genes were expressed together. Four of these encode the structural proteins VP5 (hexons and pentons), VP19C and VP23 (triplexes that link VP5 structural units), and VP26 (which caps hexons of VP5). By omission of individual recombinant baculoviruses, it was shown that VP26 is not necessary for nucleocapsid assembly. Furthermore, only partial or deformed structures assemble in the absence of VP24, VP21, and VP22a, the protease and scaffolding proteins (see "Viral Scaffolding Proteins: Chaperones for Assembly").

Tatman JD, Preston VG, Nicholson P, Elliot RM, Rixon FJ. 1994. Assembly of herpes simplex virus 1 capsids using a panel of recombinant baculoviruses. *J Gen Virol* **75:**1101–1113.



Herpes simplex type 1 procapsid showing the capsid proteins (VP5, VP23, VP19C), the portal (UL216), and the proteins that facilitate assembly and subsequent maturation (VP24, VP21 and VP22a).

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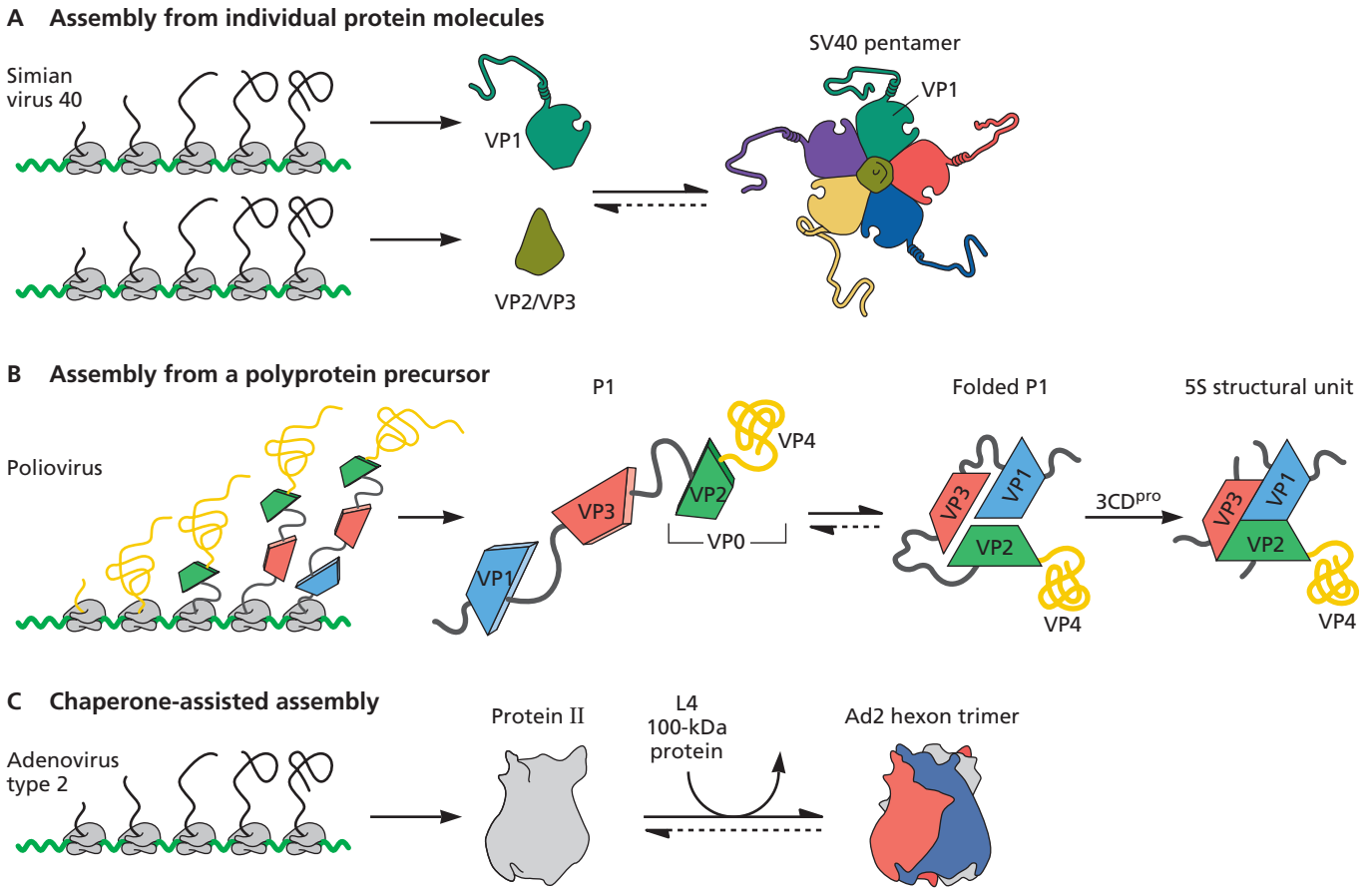


Figure 13.3 Mechanisms of assembly of viral structural units. (A) Assembly from folded protein monomers, illustrated with simian virus 40 (SV40) VP1 pentamers. The assembly reaction is the result of specific interactions among the proteins that form structural units. In many cases, the interactions have been described at atomic resolution (Chapter 4). These assembly reactions are driven in a forward direction by the high concentrations of protein subunits synthesized in infected cells, as indicated by the solid arrows. Other structural units that assemble in this way are the adenoviral fiber (trimer of protein IV) and penton (pentamer of protein III), the hepatitis B virus capsid **(C)** protein dimer, and the s3-μ1c hetero-oligomers of the outer capsid shell of reovirus. **(B)** Assembly from a polyprotein precursor, illustrated with the poliovirus polyprotein that contains the four proteins that form the heteromeric structural unit. The latter proteins are synthesized as part of the single polyprotein precursor from which all viral proteins are produced by proteolytic processing. For simplicity, only the P1 capsid protein precursor and its cleavage by the viral 3CD protease following the folding and assembly of the immature structural unit (VP0, VP3, and VP1) are shown. The flexible covalent connections between VP1, VP3, and VP0 in the P1 precursor, which are exaggerated for clarity, are severed by the protease to form the 5S structural unit. However, VP4 remains covalently linked to VP2 in VP0 until assembly is completed (see the text). **(C)** Assisted assembly. Some structural units are assembled only with the assistance of viral chaperones, such as the adenoviral L4 100-kDa protein, which is required for formation of the hexon trimer from the protein II monomer. Similarly, the herpes simplex virus 1 VP22a protein assists in the assembly of VP5 pentamers and hexamers.

be circumvented by both the synthesis of viral structural proteins in quantities far in excess of those incorporated in virus particles and their accumulation at specialized assembly sites, common features of virus-infected cells. Such high concentrations not only increase the probability that viral proteins will encounter one another by random diffusion but also provide a sufficient reservoir to compensate for any loss by nonspecific binding to cellular components. Another benefit of high protein concentration is that the formation of structural units proceeds efficiently (Fig. 13.3A), driving the assembly pathway in the productive direction.

Assembly from Polyproteins

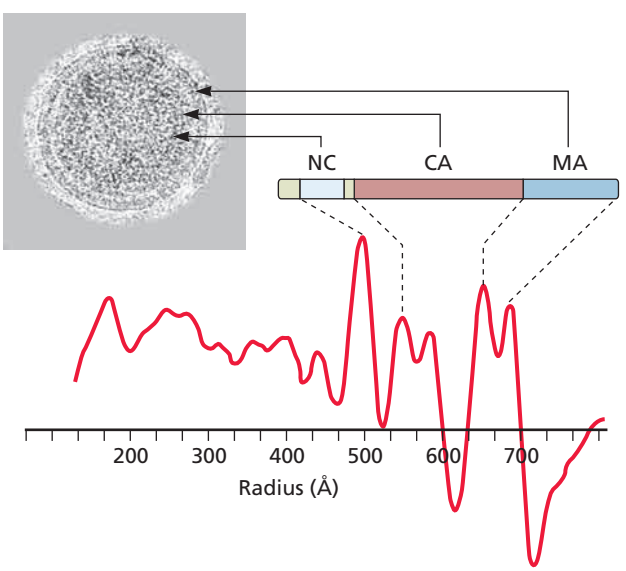
An alternative mechanism for the production of structural units is assembly while covalently linked in a polyprotein precursor. This mechanism, exemplified by formation

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of picornaviral capsids, circumvents the need for protein subunits to meet by random diffusion and avoids competition from nonspecific binding reactions. The first poliovirus intermediate, which sediments as a 5S particle, is the immature structural unit that contains one copy each of VP0, VP3, and VP1 (Fig. 13.3B). It is thought that folding of their central β-barrel domains (Fig. 4.11) takes place during synthesis of their precursor (P1). The poliovirus structural unit can then form by intramolecular interactions among the surfaces of these β-barrel domains, before the covalent connections that link the proteins are severed by the viral $3CD^{pro}$ protease.

Retrovirus assembly illustrates an elegant and effective variation on the polyprotein theme. Mature retrovirus particles contain three protein layers. An inner coat of NC protein, which packages the dimeric RNA genome, is enclosed within the capsid built from the CA protein. The capsid is in turn surrounded by the MA protein, which lies beneath the inner surface of the viral envelope (see Appendix Fig. 30A). These three structural proteins are synthesized as the Gag polyprotein precursor, which contains their sequences in the order of the protein layers that they form in virus particles, with MA at the N terminus (Fig. 13.4). Retrovirus particles assemble from such Gag polyprotein molecules by a unique mechanism that allows orderly construction of the three protein layers and, as we shall see, coordination of this reaction with encapsidation of the genome and acquisition of the envelope.

Figure 13.4 Radial organization of the Gag polyprotein in immature human immunodeficiency virus type 1 particles. The model for the arrangement of the Gag polyprotein shown to the right of the cryo-electron micrograph of a virus-like particle assembled from Gag was deduced from radial density measurements of digitized images of the particles. The plot indicates density as a function of distance from the particle center, in angstroms. Courtesy of T. Wilk, European Molecular Biology Laboratory.



Participation of Cellular and Viral Chaperones

Chaperones are specialized proteins that facilitate the folding of other proteins by preventing improper, nonspecific associations among sticky patches exposed on nascent and newly synthesized proteins. The first chaperone to be identified, the product of the *Escherichia coli groEL* gene, was discovered because it is essential for reproduction of bacteriophages T4 and lambda (Fig. 13.5A). The participation of chaperones resident in the lumen of the endoplasmic reticulum (ER) in folding and assembly of oligomeric viral glycoproteins is well established (Chapter 12). Cytoplasmic and nuclear chaperones are probably equally important for the formation of structural units or later reactions in virus assembly. A number of viral structural proteins have been shown to interact with one or more cellular chaperones, but in most cases, a role for these proteins in viral assembly is based on "guilt by association." However, some cellular chaperones have been directly implicated in assembly reactions (Fig. 13.5). For example, association of molecules of the Gag protein of the betaretrovirus Mason-Pfizer monkey virus with one another and accumulation of capsids depend upon interaction of Gag with the cytoplasmic chaperone TriC, which facilitates proper folding of the polyprotein.

Chaperones are abundant in all cells, and some accumulate to concentrations even greater than those of the very numerous ribosomes. Nevertheless, the genomes of several viruses encode proteins with chaperone activity, some with sequences and functions homologous to those of cellular proteins (Table 13.1). Some viral chaperones are essential participants in the reactions by which structural units are formed. For example, assembly of adenoviral hexon trimers, which form the faces of the icosahedral capsid, depends on such an accessory protein, the viral L4 100-kDa protein (Fig. 13.3C).

Capsid and Nucleocapsid Assembly

The accumulation of viral structural units within the appropriate compartment of an infected cell sets the stage for the assembly of more-elaborate capsids or nucleocapsids (see Table 4.1 for nomenclature). For reasons discussed previously, the reactions by which these structures are formed are often not understood in detail. Nevertheless, several different mechanisms for their assembly can be distinguished.

Intermediates in Assembly

A striking feature of well-characterized pathways of bacteriophage assembly (Box 13.1) is the sequential formation of progressively more elaborate structures; heads, tails, and tail fibers are each assembled in stepwise fashion via defined intermediates. Such an assembly line process appears ideally suited for orderly formation of virus particles, which can be large and architecturally intricate. Discrete intermediates also form during the assembly of some icosahedral animal viruses.

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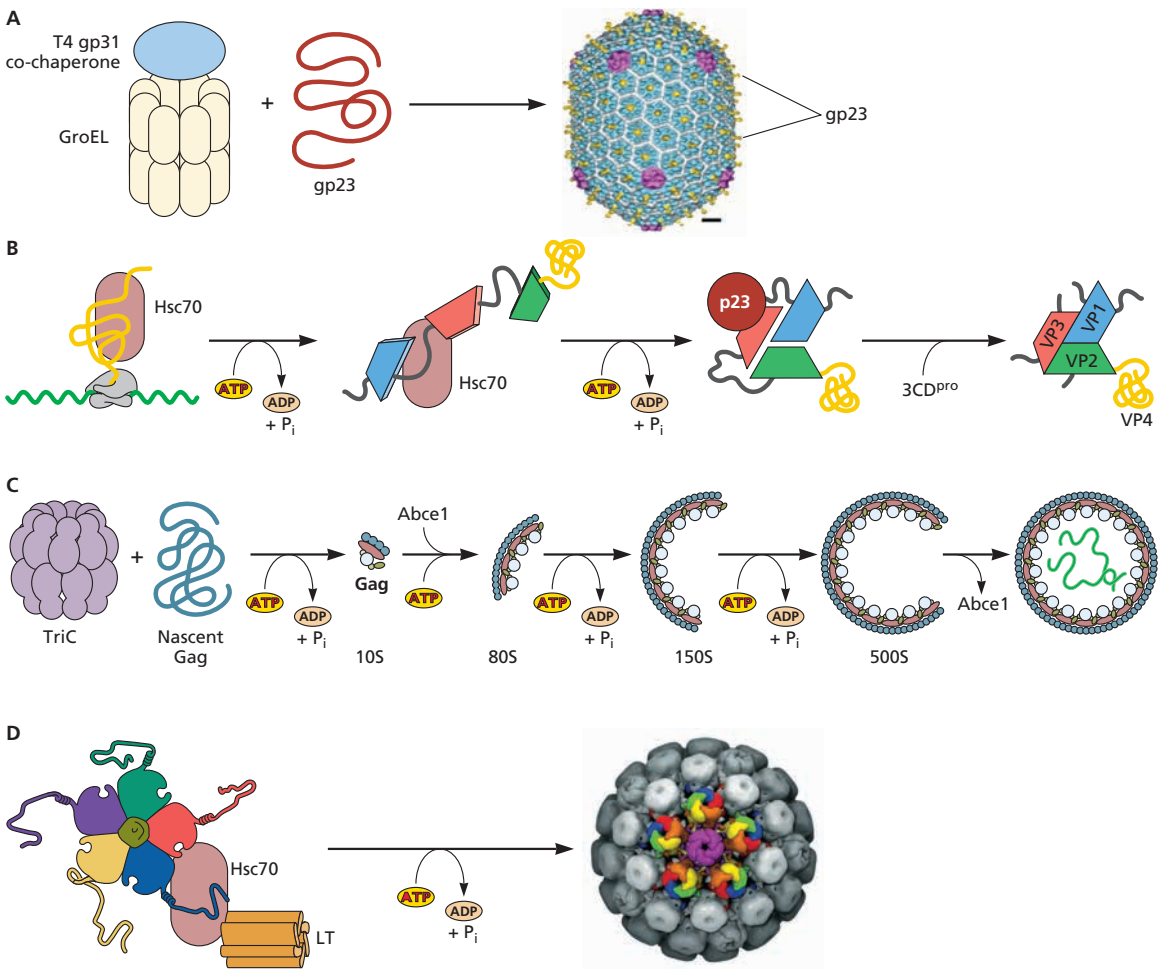


Figure 13.5 Some assembly reactions assisted by cellular chaperones. (A) The *E. coli* chaperone GroEL, which comprises two rings of eight identical subunits, promotes folding of the bacteriophage T4 major coat protein (gp23) and its assembly to form the prohead (Box 13.1). GroEL normally functions in concert with the co-chaperone GroES, but the bacteriophage protein gp31 replaces GroES for the folding of gp23. GroEL is also necessary for reproduction of bacteriophage λ. Adapted from A. Fokine et al., *Proc. Natl. Acad. Sci. U. S. A.* **101:**6003–6008, 2004, with permission. Courtesy of M. Rossmann, Purdue University. **(B)** The P1 polyprotein precursor of poliovirus (and other picornaviruses) associates with the host cell cytoplasmic chaperone Hsc70. This chaperone promotes productive folding in cycles of binding to, and release from, hydrophobic patches in the nascent protein, governed by ATP binding and hydrolysis. The interaction of P1 with a second chaperone, Hsp90, and its co-chaperone p23 is required for cleavage of P1 by the viral $3C^{pro}$ protease. It is thought that Hsp90 promotes the folding of P1 to a conformation that allows recognition of the cleavage sites for the viral enzyme. **(C)** The contribution to the folding of Gag of the cytoplasmic chaperone TriC, a ring-like structure built from eight different subunits, is based on studies of Mason-Pfizer monkey virus (see the text). Whether TriC also facilitates folding of Gag proteins of other retroviruses is not known. However, Gag proteins of several primate retroviruses, including human immunodeficiency virus type 1, associate with the cellular protein ATP-binding cassette sub-family E member 1 (Abce1) *in vitro* and in cells in which the viral protein is produced. Abce1 interacts with several intermediates (80S, 150S, and 500S) in the assembly of virus-like particles but not with this final product of assembly. The Abce1 protein contains ATP-binding domains, and depletion of ATP from Gag-producing cells leads to the accumulation of the 80S and 150S assembly intermediates. These observations suggest that Abce1 serves as a chaperone or scaffolding protein for assembly of retrovirus particles. **(D)** The VP1 and VP3 proteins of the polyomavirus simian virus 40 form pentamers efficiently *in vitro* or when made in *E. coli*. Such pentamers assemble into capsid-like particles but only when incubated with ATP, the cellular chaperone Hsc70, and the viral large T antigen (LT) (or with the bacterial chaperones GroEL and -ES). The viral protein contains a J domain, which is similar in sequence to a specific domain in cellular chaperones of the DnaJ family, and like these cellular proteins, LT stimulates the ATPase activity of Hsp70 chaperones. This N-terminal domain of LT is also present in the small T antigen (sT). LT and sT are associated with VP1 and the cellular chaperone during the late phase of infection, but the folding or assembly reactions that they assist have not been identified. The image of the simian virus particle was created by Jason Roberts, Doherty Institute, Melbourne, Australia.

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Table 13.1 Some viral chaperones and scaffolding proteins

Protein	Properties and/or function(s)
Chaperones	
Adenovirus type 2 L4 100-kDa protein	Formation of hexon trimmers
African swine fever virus CAP80	Productive folding of the major capsid protein p73
Herpes simplex virus 1 VP22a	Formation of VP5 pentamers
Simian virus 40 LT antigen	N-terminal J domain necessary for assembly of virus particles; binds to and stimulates the activities of cellular Hsc70 proteins
Scaffolding proteins	
Adenovirus type 5 L1 52/55-kDa proteins	Necessary for formation of capsids; present in immature, but not mature, particles; may be required for encapsidation
Adenovirus-associated virus type 2 assembly-activating protein (AAP)	Interacts with common C terminus VP1, VP2, and VP3; promotes capsid assembly; forms high-molecular-weight oligomers
Herpes simplex virus 1 VP22a	Forms a scaffold-like structure that organizes assembly of the empty nucleocapsid

A stepwise assembly mechanism has been well characterized for poliovirus; the 5S structural unit described in the previous section is the immediate precursor of a 14S pentamer, which in turn is incorporated into virus particles in a two-step process (Fig. 13.6). The pentamer is stabilized by extensive protein-protein contacts and by interactions mediated by the myristate chains present on the five VP0 N termini (Fig. 4.12C). The contribution of the lipids to pentamer stability is so great that this structure does not form at all when myristoylation of VP0 is prevented. Formation of the very stable 14S assembly intermediate is irreversible under normal conditions, a property that imposes the appropriate directionality on the entire assembly pathway (Fig. 13.6).

Discrete assembly intermediates like the poliovirus pentamer have been difficult to identify in cells infected by many viruses. In some cases, the absence of intermediates can be attributed to coordination of assembly of protein shells with binding of the structural proteins to the nucleic acid genome. This mode of assembly is exemplified by the ribonucleoproteins of (−) strand RNA viruses, which assemble as genomic RNA is synthesized. Nucleocapsid formation depends on interactions of the protein components with both the nascent RNA and other protein molecules previously bound to the RNA.

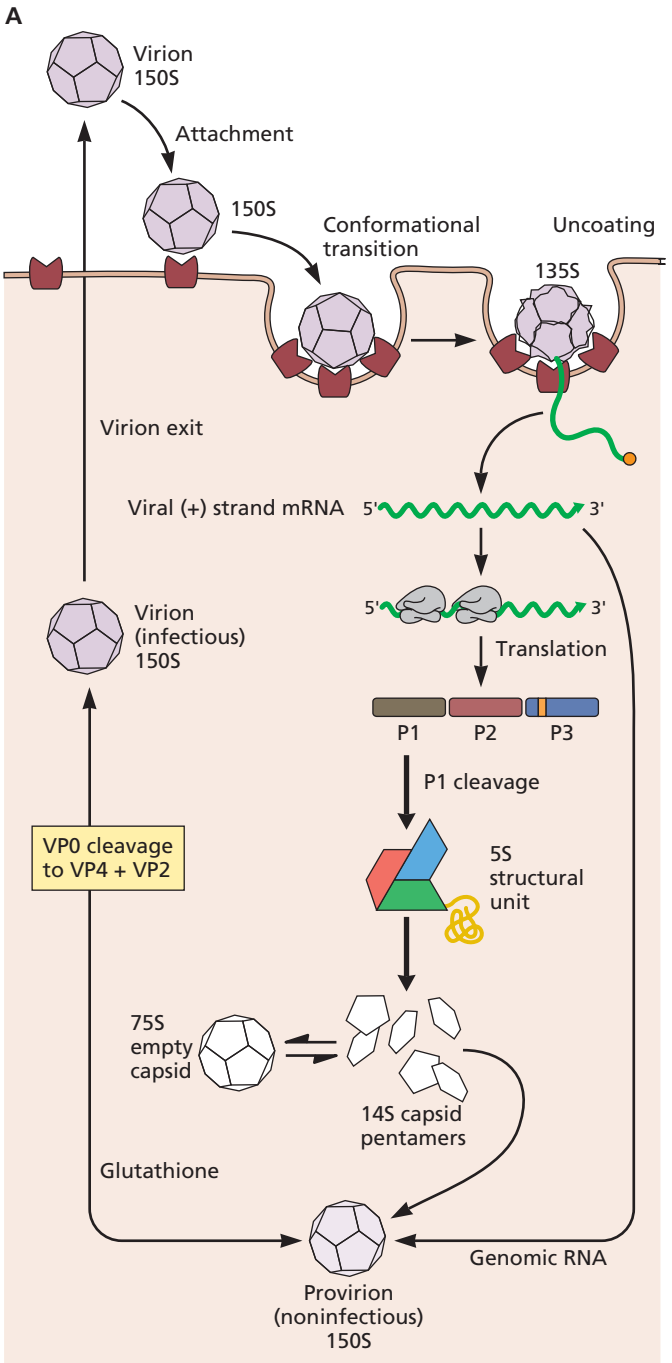
Methods that permit the synthesis of subsets of structural proteins have begun to provide insights into how such ribonucleoproteins assemble. The vesicular stomatitis virus N protein, which is a dimer in the helical nucleocapsid, aggregates when synthesized alone in *E. coli*. However, when the viral P protein is also made, aggregation does not occur, and discrete, disk-like oligomers assemble. The assembly contains 10 molecules of the N protein, 5 molecules of the P protein, and an RNA molecule (of bacterial origin) of some 90 nucleotides (Fig. 4.6). The disk-like oligomer is equivalent to one turn of the ribonucleoprotein helix formed in vesicular stomatitis virus-infected cells. No further assembly takes place in bacterial cells, perhaps because the viral proteins cannot be posttranslationally modified in the appropriate fashion. However, N-RNA complexes purified from virus particles or insect cells synthesizing N are competent to form bullet-shaped structures with the morphology of the virion ribonucleoprotein (Fig. 13.7; compare to Fig. 4.6). This property indicates that the interactions of N protein molecules with one another and with RNA are sufficient to specify assembly of the helical ribonucleoprotein.

Assembly of the protein shells of many enveloped viruses, including retroviruses, is coordinated with binding of structural proteins to a cellular membrane. This property makes isolation of intermediates a technically demanding task. Nevertheless, new methods for separation of intermediates make it possible to examine assembly reactions of these viruses. Some assembly reactions can also be studied by using simplified experimental systems. When synthesized in a cell-free transcription-translation system, the human immunodeficiency virus type 1 Gag protein multimerizes through a series of discrete intermediates to form 750S particles (Fig. 13.5C) that resemble virus-like particles released when Gag is the only viral gene expressed in mammalian cells. These observations illustrate the power of simplified approaches to the study of virus assembly. An important caveat is that such experimental systems must faithfully reproduce reactions that take place within infected cells. There is good reason to conclude that the *in vitro* assembly of Gag particles meets this crucial criterion: the assembly phenotypes exhibited by altered Gag proteins *in vitro* correspond closely to those observed in infected cells, and binding of Gag to the cellular chaperone Abcel is required for the assembly of later intermediates in both cases.

Self-Assembly and Assisted Assembly Reactions

The primary sequences of viral structural proteins contain all the information necessary to specify assembly, including intricate reactions like the alternative 5- and 6-fold packing of VP1 pentamers in the simian virus 40 capsid; when synthesized in *E. coli*, VP1 is isolated as pentamers that assemble into capsid-like structures *in vitro*. Such self-assembly of structural proteins is the primary mechanism for formation of protein shells, but other viral components or cellular proteins can assist the process.

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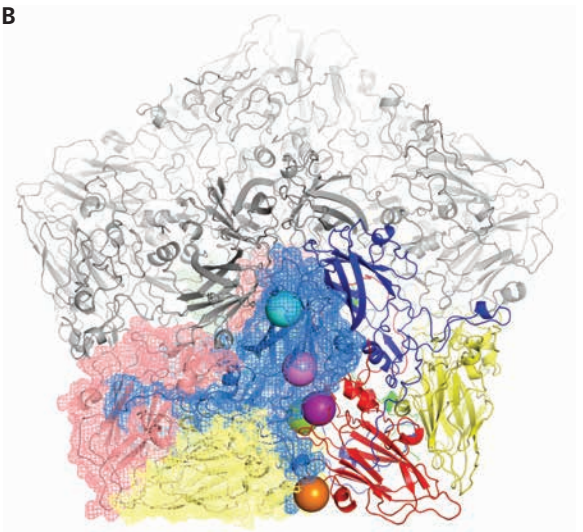


Figure 13.6 Assembly of poliovirus in the cytoplasm of an infected cell. (A) Most of the assembly reactions are essentially irreversible, because of proteolytic cleavage (formation of 5S structural units and mature virus particles) or extensive stabilizing interactions in the assembled structure (formation of 14S pentamers and of provirions). Stable, empty capsids, originally considered the precursors of provirions, do not possess the same conformation as the mature virus particle, as symbolized by the white color, and are dead-end products. Formation of the capsid shell from 14S pentamers is coordinated with genome encapsidation and requires replication of genomic RNA. The conformational transition upon attachment to the poliovirus receptor, for which the virus particle is primed by cleavage of VP0 to VP2 and VP4, is also illustrated. Some evidence for this mechanism is summarized in the text. In addition, in a cell-free system for the synthesis of infectious poliovirus particles, exogenously added 14S pentamers assemble with newly synthesized viral (+) strand RNA to form virus particles with antigenic sites characteristic of those produced in infected cells. In contrast, exogenously added empty capsids undergo no further assembly, even when genomic RNA is synthesized, confirming that they are dead-end products. **(B)** The sites of substitutions that render the reproduction of poliovirus resistant to depletion of glutathione are shown as colored spheres on one structural unit of a pentamer, in which VP1, VP2, and VP3 are colored blue, green, and red, respectively. The other structural units are shown in gray. Most of these substitutions lie at the interface between adjacent structural units. Adapted from H.-C. Ma et al., *PLoS Pathog.* **10:**e1004052, doi:10.1371/journal.ppat.1004052, 2014, with permission. Courtesy of P. Jiang and E. Wimmer, State University of New York, Stonybrook, MY.

Viral and Cellular Components That Regulate Self-Assembly

Interactions among viral structural proteins may be the mortar for the construction of virus particles, but other components of the particle often provide an essential foundation or the blueprint for correct assembly. As we have seen, assembly of the nucleocapsids of (−) strand RNA viruses is both coordinated with and dependent on synthesis of genomic RNA. The RNA serves as a template for productive and repetitive binding of nucleocapsid proteins to one another. Interactions of retroviral Gag proteins with RNA mediated by the NC RNA-binding domain also appear to be essential to initiate assembly of the Gag protein shell (Box 13.3). In other cases, the viral genome plays a more subtle yet equally important

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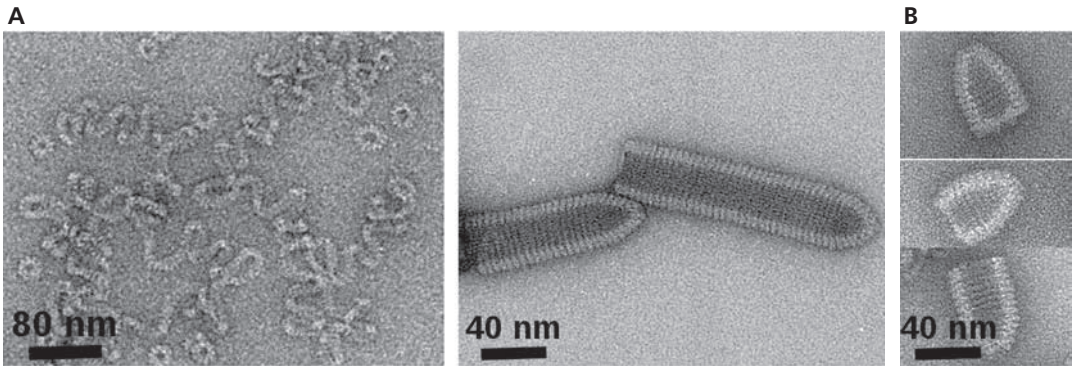


Figure 13.7 Formation of bullet-shaped particles by the vesicular stomatitis virus N protein. (A) At neutral pH and physiological ionic strength, N-RNA complexes purified from vesicular stomatitis virus particles form loosely coiled ribbons (left), but at lower pH and ionic strength, they assemble as bullet-shaped particles (right). These particles exhibit the morphology of the ribonucleoprotein in virus particles but vary in diameter. **(B)** The formation of bullet-shaped particles by the N protein synthesized in insect cells in the absence of other viral proteins and bound to cellular RNA indicates that this viral protein contains the information to specify assembly of these nonuniform structures. Adapted from A. Derfosses et al., *Nat. Commun.* **4:**1429, doi:10.1038/ncomms2435, 2013, with permission. Courtesy of I. Gutsche, UJF-EMBL-CNRS, Grenoble, France.

BOX 13.3

DISCUSSION

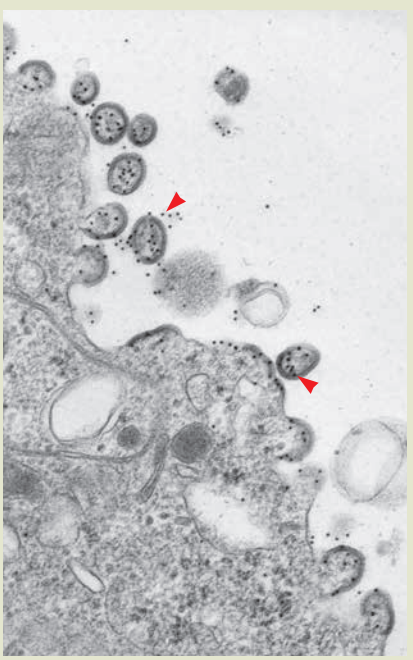
A scaffolding function for RNA

When synthesized in the absence of any other viral component, retroviral Gag polyproteins direct assembly and release of the virus-like particles shown in the figure. It was therefore assumed for many years that this protein contains all the information necessary and sufficient for assembly of particles. However, the results of subsequent experiments indicate that RNA acts as a scaffold during Gag assembly.

In vitro studies of the ability of truncated Gag proteins to multimerize with the full-length protein initially underscored the importance of the nucleocapsid (NC) RNA-binding domain for efficient assembly. The association of Gag with RNA is also required for multimerization in this system. The apparent contradiction between these findings and efficient assembly of Gag in mammalian cells in the absence of genomic RNA was subsequently resolved; virus-like particles contain cellular RNAs when they form in cells infected by a Moloney murine leukemia virus mutant with a deletion in the signal that directs packaging of the RNA genome. Furthermore, RNase digestion of cores assembled from Gag in wild-type Moloney murine leukemia virus-infected cells was shown to dissociate these structures. These observations indicate that interactions of Gag molecules with RNA, as well as with one another, are required for assembly and to maintain particle stability.

Campbell S, Vogt VM. 1995. Self assembly in vitro of purified CA-NC proteins from Rous sarcoma virus and human immunodeficiency virus type 1. *J Virol* **69:**6487–6497.

Muriaux D, Mirro J, Harvin D, Rein A. 2001. RNA is a structural element in retrovirus particles. *Proc Natl Acad Sci U S A* **98:**5246–5251.



Electron micrograph showing a thin section (fixed and stained) of a human T cell synthesizing the viral Gag polyproteins. Prior to electron microscopy, viral particles (red arrowheads) were labeled with polyclonal antibodies (attached to gold beads) recognizing the CA protein. Bar, 1.0 μm. N, nucleus; M, mitochondrion. Courtesy of J. J. Wang, Institute of Biomedical Sciences, Academica Sinica, Taipei, Taiwan, and B. Horton and L. Ratner, Washington University School of Medicine, St. Louis, MO.

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role, ensuring that the interactions among structural units are those necessary for infectivity. For example, poliovirus empty capsids lack internal structural features characteristic of the mature virus particle, because VP0 is not cleaved to form VP4 and VP2. The RNA genome is thought to participate in the autocatalytic cleavage of this precursor, which is essential for the production of infectious particles. Association of structural proteins with a cellular membrane is essential for the assembly of some virus particles, a situation exemplified by many retroviruses; the sequences of MA that specify Gag myristoylation and binding to the cytoplasmic surface of the plasma membrane (described in Chapter 12) are also required for assembly.

Binding of structural proteins to the genome or to a cellular membrane might simply raise their local concentrations sufficiently to drive self-assembly, might organize the proteins in such a way that their interactions become cooperative, or might induce conformational changes necessary for the productive association of structural units. These mechanisms, which are not mutually exclusive, have not been distinguished experimentally, but there is evidence for induction of conformational transitions in specific cases. We do not understand adequately the molecular mechanisms by which binding of structural proteins to other components directs or regulates particle assembly. However, such a requirement offers the important advantage of integration of the formation of protein shells with the acquisition of other essential virion components.

Cellular components can also modulate the fidelity with which viral structural proteins bind to one another. The capsid-like structures assembled when simian virus 40 VP1 is made in insect or mammalian cells are much more regular in appearance than those formed *in vitro* by bacterially synthesized VP1. Modification of VP1 (by acetylation and phosphorylation) or the participation of chaperones, such as Hsc70 and the J domain of the viral large T antigen (LT), must therefore improve the accuracy with which VP1 pentamers associate to form capsids (Fig. 13.5D). Similarly, *in vitro* self-assembly of poliovirus structural proteins is very slow, proceeding at least 2 orders of magnitude more slowly than that observed in infected cells. Furthermore, the empty capsids that form have the altered conformation described previously, unless the reaction is seeded by 14S pentamers isolated from infected cells. This property indicates that the appropriate folding, modification, and/or interactions of the viral structural proteins are critical for subsequent assembly reactions to proceed productively. Within infected cells, these crucial reactions are likely to be modulated by cellular chaperones, such as Hsc70, which is associated with the polyprotein during its folding to form 5S structural units. Remarkably, a small host cell molecule, glutathione, also facilities assembly of poliovirus (Fig. 13.6); depletion of glutathione reduces both virus yield and the accumulation of pentamers. This molecule binds to VP1 and VP3, and mutations that confer resistance to glutathione depletion carry amino acid substitutions at the interfaces between adjacent structural units in the pentamer, consistent with stabilization of this intermediate upon binding of glutathione. It is clear from these examples that host cells provide a hospitable environment for productive virus assembly, one that is not necessarily reproduced when viral structural proteins are assembled *in vitro*.

Viral Scaffolding Proteins: Chaperones for Assembly

Accurate assembly of some large icosahedral protein shells, such as those of adenoviruses and herpesviruses, is mediated by proteins that are not components of mature virus particles (Table 13.1). Because these proteins participate in reactions by which the capsid or nucleocapsid is constructed but are then removed, they are termed **scaffolding proteins**. Among the best characterized is the precursor of the herpes simplex virus 1 VP22a protein.

This protein is the major component of an interior core present in assembling nucleocapsids (Fig. 13.8A). In the absence of other viral proteins, it forms specific scaffold-like structures and appears as an ordered sphere in immature nucleocapsids isolated from infected cells. Self-association of pre-VP22a stimulates binding to VP5, the protein that forms the hexameric and pentameric structural units of the nucleocapsid. The interactions of VP5 with the scaffolding protein guide and regulate the intrinsic capacity of VP5 hexamers (and other nucleocapsid proteins) for self-assembly; omission of the scaffolding protein from a simplified assembly system (Box 13.2) leads to the production of partial and deformed nucleocapsid shells.

One of the 12 vertices of the herpesviral nucleocapsid comprises not a VP5 pentamer but rather the portal through which the DNA enters (Fig. 13.8A; see also Fig. 4.29). This unique structural unit, a dodecamer of the UL6 protein, must be incorporated at just one vertex during assembly. The reaction requires interaction of the portal with the major scaffolding protein; a small molecule that blocks the interaction prevents assembly of portal-containing nucleocapsids in infected cells. Although the portal is dispensable for the formation of **procapsids** or nucleocapsids, the results of *in vitro* studies indicate that it can be incorporated only during the initial stages of assembly. The mechanism that ensures that each nucleocapsid contains only one portal remains an enigma.

Once nucleocapsids have assembled, scaffolding proteins must be discarded, so that viral genomes can be accommodated (Fig. 13.8A). The virion protease VP24 is essential for such DNA encapsidation. This protein is incorporated into the assembling nucleocapsid as a precursor (Fig. 13.8B).

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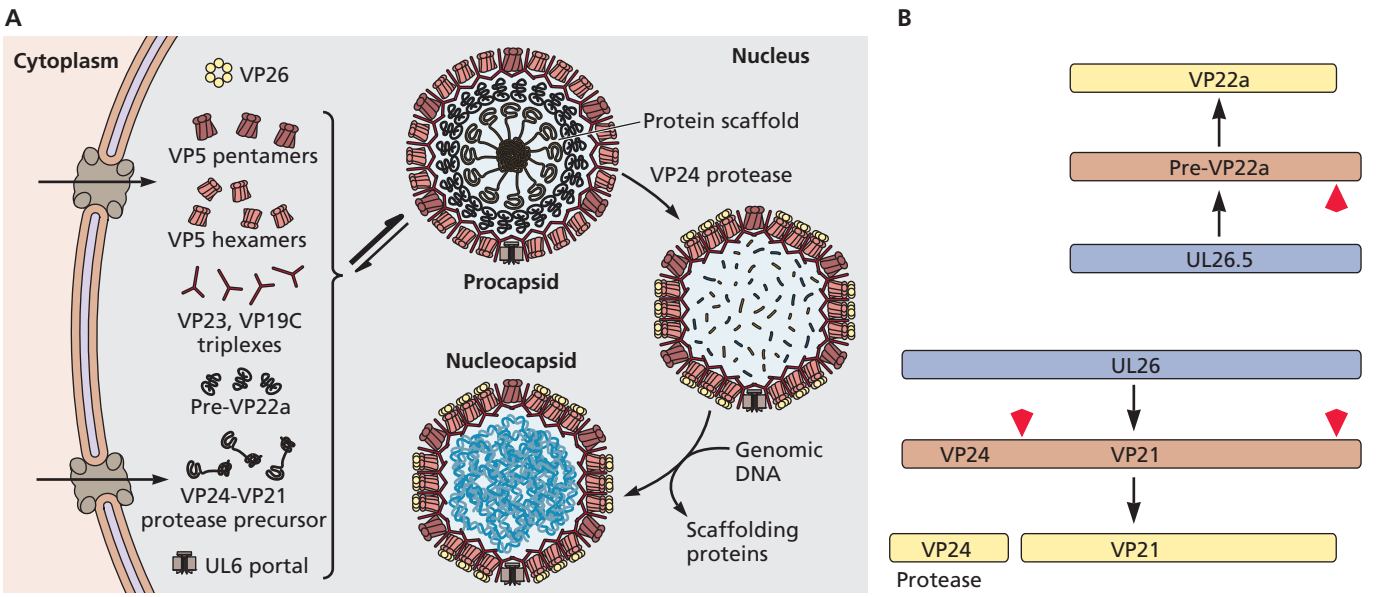


Figure 13.8 Assembly of herpes simplex virus 1 nucleocapsids. (A) Assembly begins as soon as nucleocapsid proteins accumulate to sufficient concentrations in the infected cell nucleus. Intermediates include pentamers and hexamers of the major capsid protein VP5, which form pentons and hexons in the capsid, and triplexes of the minor proteins VP23 and VP19C. Whether structural units assemble prior to transport into the nucleus is not clear. Viral proteins essential for assembly of the nucleocapsid but not present in mature virus particles, namely, the scaffolding protein (pre-VP22a) and the viral protease precursor (VP24-VP21), must also enter the nucleus. Assembly of nucleocapsids depends on the formation of an internal scaffold around which the protein shell assembles. The VP5 and pre-VP22a proteins form a core via hydrophobic interactions, to which additional VP5 hexamers and the triplexes of VP19 and VP23 are added. These structures are required for further assembly, which occurs by sequential formation of partial dome-like structures and the spherical immature nucleocapsid. Subsequent reactions require the viral protease to remove the scaffolding protein, allowing entry of the DNA genome and morphological transitions. As discussed in the text, encapsidation is concurrent with cleavage of the concatemeric products of herpesviral DNA replication. **(B)** Overlapping sequences of scaffolding proteins. The UL26 and UL26.5 reading frames are shown in purple, and their primary translation products are shown in light brown. The initiating methionine of the VP22a protein is within the larger reading frame that encodes the VP24-VP21 polyprotein. Consequently, VP21 and VP22a are identical in sequence, except that the former contains a unique N-terminal segment. All proteolytic cleavages at sites indicated by the red arrowheads, including those that liberate the protease itself from the VP24-VP21 precursor, are carried out by the VP24 protease. The cleavage at the C-terminal site in VP22a disengages the scaffolding from the capsid proteins.

The protease precursor possesses some activity and initiates cleavage to produce VP24, which then cleaves the scaffolding protein to remove a short C-terminal sequence that is required for binding to VP5. Such processing presumably disengages scaffolding from structural proteins, once assembly of the nucleocapsid is complete. The protease also degrades the scaffolding protein so that encapsidation of the genome can begin.

The proteolytic cleavages that liberate the VP5 structural units from their association with the scaffold also induce major changes in the organization and stability of the nucleocapsid shell. Studies of nucleocapsid assembly *in vitro* and in cells infected by a mutant virus encoding a temperature-dependent viral protease suggest that the uncleaved spherical precursor is analogous to the well-characterized **procapsids** that are formed during the assembly of certain DNA-containing bacteriophages (Box 13.4).

Assembly of simpler protein shells can also depend critically on a viral protein. In addition to its many other functions, simian virus 40 LT participates in assembly of virus particles (Fig. 13.5D). This protein does not form a scaffold, but an N-terminal domain of LT appears to be essential for organization of the capsid; alterations within this domain block production of particles and induce the accumulation of an incomplete structure that contains the viral chromatin and VP1. The N-terminal segment of LT possesses chaperone activity, which may ensure the productive binding of VP1 pentamers to one another and to other components of the particle during assembly.

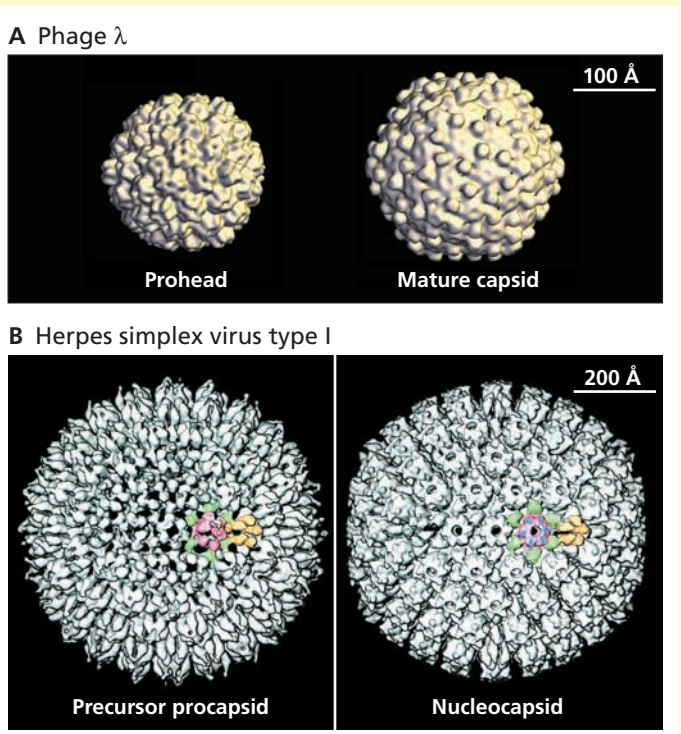
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BOX 13.4

EXPERIMENTS

Visualization of structural transitions during assembly of DNA viruses

The assembly of viruses that package double-stranded DNA genomes into a preformed protein shell exhibits several common features, regardless of the host organism. These include the presence of a portal for DNA entry in the capsid or nucleocapsid precursor and probably the mechanism of DNA packaging (see the text). In addition, as illustrated for bacteriophage λ and herpes simplex virus 1, formation of DNA-containing structures is accompanied by major reorganizations of the protein shell. **(A) Cryo-electron micrographs of the bacteriophage λ prohead and the DNA-containing mature capsid.** The former comprises hexamers and pentamers of the capsid protein gpE organized with $T = 7$ icosahedral symmetry and is assembled prior to encapsidation of the DNA genome. It is smaller than the mature capsid (270 and 315 Å in diameter, respectively), but its protein shell is considerably thicker. Packaging of the DNA genome leads to an expansion of the capsid as a result of reorganization of gpE hexamers. This change is accompanied by binding of the gpD protein, which contributes to capsid stabilization. Adapted from T. Dokland and H. Murialdo, *J. Mol. Biol.* **233:**682–694, 1993, with permission. **(B) Cryo-electron micrographs of herpes simplex virus 1 nucleocapsid precursor and mature nucleocapsid, viewed along a 2-fold axis of icosahedral symmetry.** Some copies of the proteins that form the particles' surfaces are colored as follows: VP5 hexons, red; VP5 pentons, yellow; and triplexes containing one molecule of VP19C and two of VP23, green. The precursor nucleocapsid is spherical (rather than icosahedral), and its protein shell is thicker. Furthermore, the VP5 hexamers are not organized in a highly regular, symmetric manner in the precursor, resulting in a more open protein shell. The precursor nucleocapsid also lacks the VP26 protein, which binds to the external surfaces of VP5 hexamers, but not pentamers, in the mature nucleocapsid. Adapted from A. C. Steven et al., *FASEB J.* **10:**733–742, 1997, with permission.



Selective Packaging of the Viral Genome and Other Components of Virus Particles

Concerted or Sequential Assembly

Incorporation of the viral genome into assembling particles, often called **packaging**, requires specific recognition of genomic RNA or DNA molecules. All viral genomes are packaged by one of two mechanisms, concerted or sequential assembly.

In concerted assembly, the structural units of the protective protein shell assemble productively only in association with the genomic nucleic acid. The nucleocapsids of (−) strand RNA viruses form by a concerted mechanism (Fig. 13.9), as do retrovirus particles (Fig. 13.10) and those of other (+) strand RNA viruses. In many cases, these assembly reactions are coordinated with synthesis of the viral genome. In the alternative mechanism, sequential assembly, the genome is inserted into a preformed protein shell. The formation of herpesviral nucleocapsids provides a clear example of this packaging mechanism (Fig. 13.8). Mutations that inhibit viral DNA synthesis or that prevent DNA packaging do not block assembly of capsid-like structures that lack DNA. These phenotypes establish unequivocally that the DNA genome must enter preformed nucleocapsids. In contrast to concerted assembly, encapsidation of the genome in a preformed structure requires specialized mechanisms to maintain or open a portal for entry of the nucleic acid to pull or push the genome

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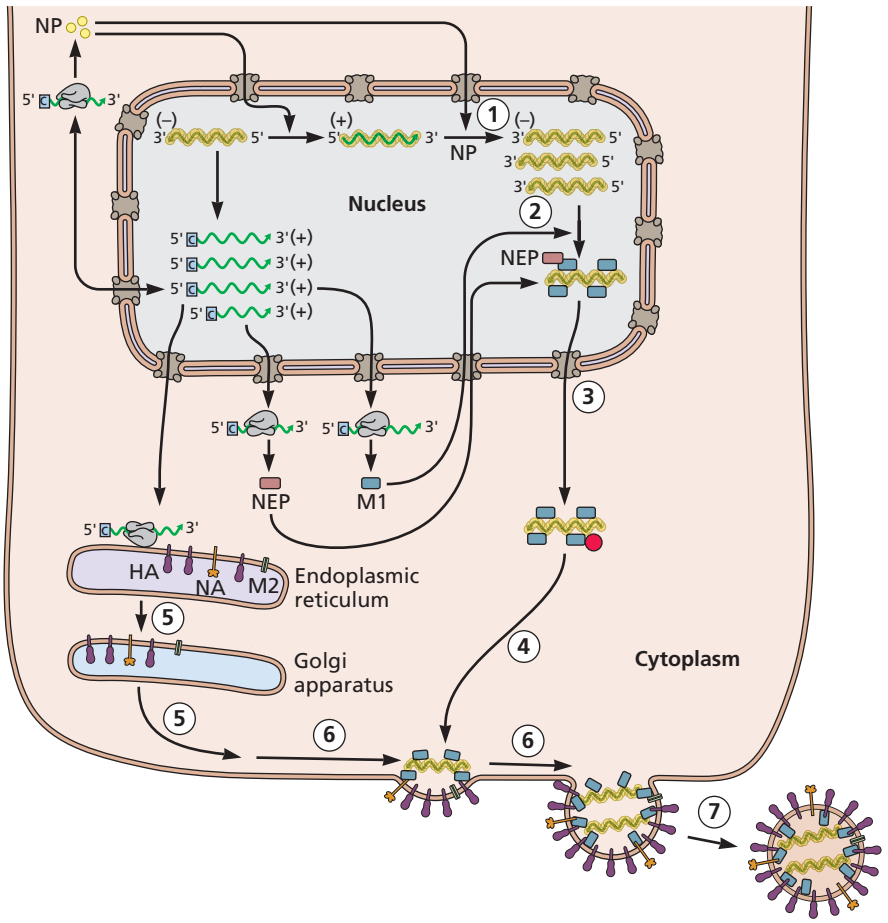


Figure 13.9 Assembly of influenza A virus. Assembly proceeds in stepwise fashion within different compartments of an infected cell. As (−) strand genomic RNA is synthesized in the nucleus, it is packaged by the NP RNA-binding protein (step 1). These ribonucleoproteins may serve as templates for mRNA synthesis, participate in further cycles of replication, or bind the M1 protein (step 2). The latter interaction prevents further RNA synthesis and allows binding of the viral nuclear export protein (NEP) and export of the nucleocapsid to the cytoplasm (step 3). The M1 protein also binds to the cytoplasmic face of the plasma membrane via specific sequences and directs the nucleocapsid to the plasma membrane (step 4). The plasma membrane carries the viral HA, NA, and M2 proteins, which reach this site via the cellular secretory pathway (step 5). The M1 protein probably controls budding (step 6) via recruitment of cellular components (see the text). Fusion of the membrane bud releases the enveloped particle (step 7). Only two of the eight genome segments are illustrated for clarity.

into the capsid (see the next section). The herpesviral portal UL6, which is present at only 1 of the 12 vertices of the nucleocapsid (Fig. 13.8; see also Fig. 4.29), fulfills the latter function. The DNA genomes of adenovirus (Fig. 13.11) and adenovirus-associated virus also appear to be packaged into preformed capsids.

Recognition and Packaging of the Nucleic Acid Genome

During encapsidation, viral nucleic acid genomes must be distinguished from the cellular DNA or RNA molecules present in the compartment in which assembly takes place. This process requires a high degree of discrimination among similar nucleic acid molecules. For example, retroviral genomic RNA constitutes much less than 1% of an infected cell's cytoplasmic mRNA population and bears all the hallmarks of cellular messenger RNAs (mRNAs), yet it is **the** RNA packaged in the great majority of retrovirus particles. Such discrimination is the result of specific recognition of sequences or structures unique to the viral genome, termed **packaging signals**. These can be defined by genetic analyses as the sequences that are necessary for incorporation of the nucleic acid into the assembling virus particle or sufficient to direct incorporation of foreign nucleic acid. The organization

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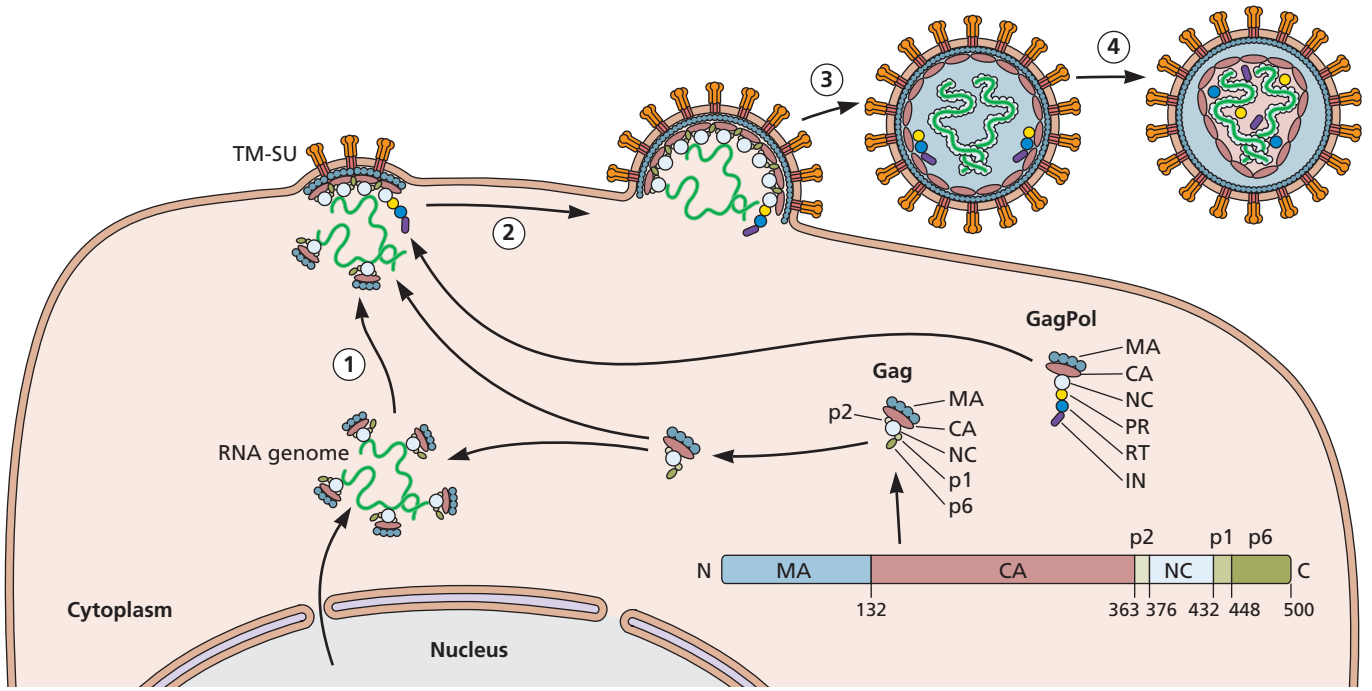


Figure 13.10 Assembly of a retrovirus from polyprotein precursors. The Gag polyprotein of all retroviruses contains the MA, CA, and NC proteins linked by spacer peptides that vary in length and position. The proteins are in the order (from the N to the C terminus) of the protein shells of the virus particle, from the outer to the inner. The organization of human immunodeficiency virus type 1 Gag is illustrated on the right. A minor fraction, about 1 in 10, of Gag translation products carry the retroviral enzymes, denoted by PR, RT, and IN, at their C termini. The association of Gag molecules with the plasma membrane, with one another, and with the RNA genome via binding of NC segments initiates assembly at the inner surface of the plasma membrane (step 1). In some cases, such as human immunodeficiency virus type 1, the MA segment also binds specifically to the internal cytoplasmic domain of the TM-SU glycoprotein. Assembly of the particle continues by incorporation of additional molecules of Gag (step 2). This pathway is typical of many retroviruses, but some (e.g., betaretroviruses) complete assembly of the core in the interior of the cell prior to its association with the plasma membrane. The dimensions of the assembling particle are determined by interactions among Gag polyproteins. Eventually, fusion of the membrane around the budding particle (step 3) releases the immature noninfectious particle. Cleavage of Gag and Gag-Pol polyproteins by the viral protease (PR) produces infectious particles (step 4) with a morphologically distinct core (see Fig. 13.25).

of the packaging signals of several viruses is therefore quite well understood.

Nucleic Acid Packaging Signals

DNA signals. The products of adenoviral or polyomaviral DNA synthesis are genomic DNA molecules that can be incorporated into assembling virus particles without further modification. These DNA genomes contain discrete packaging signals with several common properties (Fig. 13.12). The signals comprise repeats of short sequences, some of which are also part of viral promoters or enhancers; they are positioned close to an origin of replication, and their ability to direct DNA encapsidation depends on this location. They differ in whether they are recognized directly or indirectly by viral proteins.

The encapsidation signal of the adenoviral genome, which is located close to the left inverted repeat sequence and origin, comprises a set of repeated sequences. The sequences are recognized by the viral late proteins IVa_2 and L4 22-kDa, while the L1 52/55-kDa protein is recruited by interaction with the IVa_2 protein. Cooperative binding of these proteins to the repeated sequence is thought to form a higher-order nucleoprotein structure that promotes packaging of the genome. The results of genetic experiments have established the importance of these proteins in assembly: mutations that prevent production of the proteins block the formation of mature virus particles generally with accumulation of empty, immature capsids, consistent with a sequential encapsidation mechanism (Fig. 13.11)

The simian virus 40 DNA-packaging signal is located in the regulatory region of the genome that contains the origin of replication, the enhancer, and early and late promoters. Several sequences within this region contribute to the encapsidation signal, which includes multiple binding sites for the

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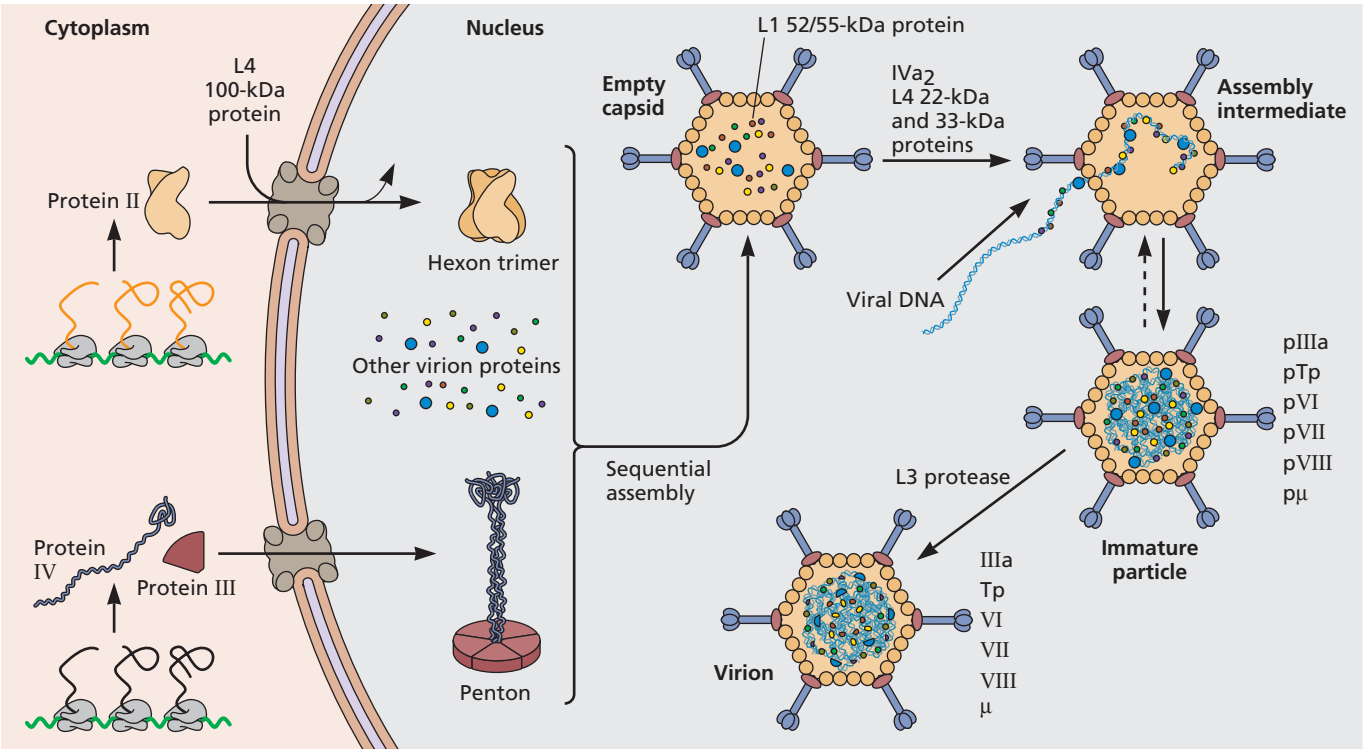
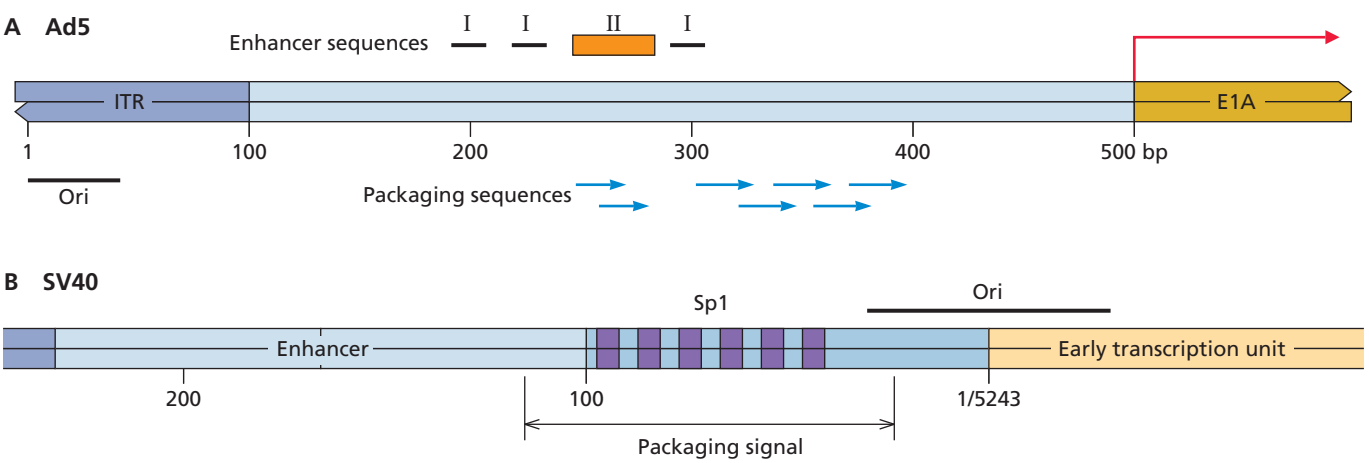


Figure 13.11 Adenovirus assembly. Synthesis and assembly of hexons and pentons and their transport into the nucleus set the stage for assembly. The L4 100-kDa protein is required for formation of hexons, but its molecular function is not known. These structural units together with the proteins that stabilize the capsid assemble into empty capsids. The L1 52-/ 55-kDa proteins are necessary for the formation of structures that can complete assembly and decrease in concentration as assembly proceeds. The DNA is then inserted into this structure via the packaging signal located near the left end of the genome. The viral IVa_2 and L4 22-kDa proteins bind specifically to this sequence *in vitro* and are required for assembly in infected cells. Premature breakage of DNA in the process of insertion would yield the structure designated "Assembly intermediate," in which an immature capsid is associated with a DNA fragment derived from the left end of the viral genome. Core proteins are encapsidated with the viral genome to yield noninfectious young virus particles. Mature particles are produced upon cleavage of the precursor proteins listed to the right of the young particle.

Figure 13.12 Viral DNA-packaging signals. (A) Human adenovirus type 5 (Ad5). The locations of the repeated sequences (blue arrows) of the packaging signal relative to the left inverted terminal repeat (ITR), the origin of replication (Ori), and the E1A transcription unit are indicated. The repeated sequences are AT rich and functionally redundant, and several overlap enhancers that stimulate transcription of viral genes. The viral IVa_2 protein binds directly to the 3′ portion of the sequence that is conserved in each of the repeats. Once the IVa_2 protein is associated, the L4 22-kDa protein interacts with the 5′ segment of the conserved sequences. The positions of transcriptional enhancers within this region are also shown. Enhancer 1 stimulates transcription of the immediate early E1A gene, whereas enhancer II increases the efficiency of transcription of all viral genes. **(B)** Simian virus 40 (SV40). The region of the genome containing the enhancer, origin of replication (Ori), and packaging signal is shown, with positions (base pairs) in the circular genome indicated below. The Sp1-binding sites within the packaging sequence are required for genome packaging.



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cellular transcriptional regulator Sp1. Although the cellular genome contains many such binding sites, the particular arrangement of sequences recognized by Sp1 in the viral packaging signal is unique. The internal proteins of the simian virus capsid (VP2 and -3) bind to the packaging signal with high affinity and specificity only in the presence of Sp1. The observation that this cellular protein stimulates the *in vitro* assembly of infectious virus particles by an order of magnitude is consistent with a role in mediating indirect recognition of the packaging signal by capsid proteins. Subsequently, highly cooperative interactions among the structural units appear to drive the concerted assembly of the capsid, concomitantly with displacement of Sp1 and nonspecific binding of capsid proteins to viral minichromosomes.

The products of herpesviral DNA synthesis are not genomic DNA molecules but, rather, concatemers containing many head-to-tail copies of the genome. Individual genomes must therefore be liberated from these long molecules. The herpes simplex virus 1 packaging signals *pac1* and *pac2*, which lie within the terminal *a* repeats of the genome, are necessary for both recognition of the viral DNA and its cleavage within the adjacent direct repeats (Fig. 13.13A). It is generally thought that cleavage is concomitant with genome encapsidation. One model (Fig. 13.13B) proposes that a protein complex formed on the unique short *pac* sequence interacts with the portal in the nucleocapsid. Following the first DNA cleavage, a unit-length genome is reeled into the nucleocapsid from the concatemer prior to the second DNA cleavage. This mechanism is analogous to that by which concatemeric DNA products of bacteriophage T4 replication are cleaved and packaged by a multisubunit terminase, which hydrolyzes ATP and associates transiently with the portal protein of the preformed capsid. The products of at least seven herpes simplex virus 1 genes are dedicated to encapsidation of the viral genome. The UL15, UL28, and UL33 proteins, which interact with one another and with the portal protein, exhibit the properties predicted for the terminase. For example, the UL15 protein contains a sequence motif characteristic of ATPases that is essential for encapsidation of viral genomes, while the UL28 gene product binds to *pac* sequences required for DNA cleavage.

RNA signals. Because it is also an mRNA, the retroviral genome must be distinguished during encapsidation from both cellular and subgenomic viral mRNAs. In addition, two genomic RNA molecules must interact with one another, for the retroviral genome is packaged as a dimer. This unusual property is thought to help retroviruses survive extensive damage to their genomes (Chapter 7). In virus particles, the dimeric genome is in the form of a 70S complex held together by many noncovalent interactions between the RNA molecules. However, most attention has focused on sequences that allow the formation of stable dimers, termed the **dimer**

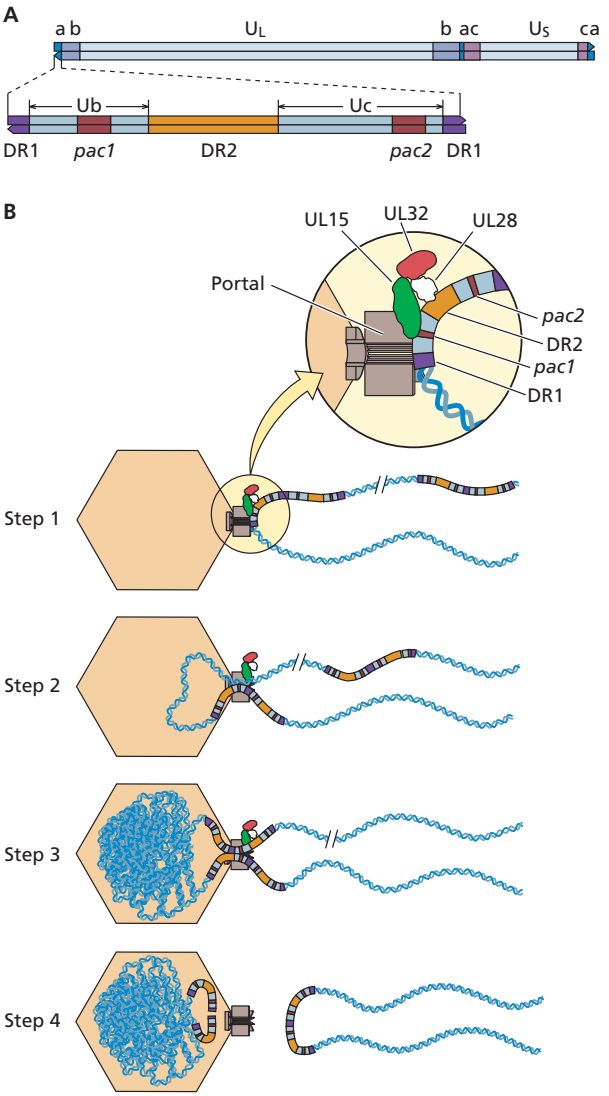


Figure 13.13 Packaging of herpes simplex virus 1 DNA. **(A)** Organization of the *a* repeats of the viral genome, showing the locations of the *pac1* and *pac2* sequences within the nonrepeated sequences Ub and Uc and relative to the flanking direct repeats 1 and 2 (DR1 and DR2). One to several copies of the *a* sequence are present at the end of the unique long (U_L) segment and at the internal L-S junction, but only one copy lies at the end of the unique short (U_S) region. **(B)** Model of herpes simplex virus 1 DNA packaging, in which encapsidation is initiated by formation of a terminase, which includes the proteins indicated, bound to the packaging sequence. This protein-DNA complex is oriented to interact with the portal of the nucleocapsid (step 1). The DNA is then reeled into the capsid (steps 2 and 3) until a headful threshold is reached and an *a* sequence in the same orientation (i.e., 1 genome equivalent) is encountered (step 3), when cleavage in DR1 sequences takes place (step 4). In addition to the proteins shown, the UL17 protein is essential for DNA cleavage, but its function in this process is not known. It is also necessary for recruitment of the UL15 protein subunit of the terminase. The UL25 protein is also necessary for efficient packaging of the genome. In its absence, DNA cleavage does occur, but fewer nucleocapsids are formed. It has therefore been suggested that one function of this protein is to stabilize the protein shell so that it can withstand the pressure exerted by the encapsidated genome.

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linkage sequence. *In vitro* experiments with human immunodeficiency virus type 1 RNA have provided evidence for base pairing between loop sequences of a specific hairpin (SL1) within the dimer linkage sequence (Fig. 13.14A) and the formation of an intermolecular, four-stranded helical structure (known as a G tetrad or G quartet). The effects of mutations in or duplication of this sequence indicate that it nucleates formation of genome RNA dimers *in vivo* and that dimerization is required for efficient genome packaging. Indeed, the dimer linkage sequence lies within the series of hairpin loops that comprise the RNA-packaging signal (Fig. 13.14A).

Sequences necessary for packaging of retroviral genomes, termed psi (ψ), vary considerably in complexity and location. In some cases, exemplified by Moloney murine leukemia virus, a ψ sequence of about 350 contiguous nucleotides (Fig. 13.14B) is both necessary and sufficient for RNA encapsidation. As this sequence lies downstream of the 5′ splice site, only unspliced genomic RNA molecules are recognized for packaging. The human immunodeficiency virus type 1 genome also contains a primary packaging sequence (Fig. 13.14A) that distinguishes the full-length genome from spliced viral RNA molecules. However, this sequence fails to direct packaging when it is incorporated into heterologous RNA species, indicating that it is not sufficient. Additional sequences required for genomic RNA encapsidation lie within tar and adjacent sequences and at more-distant locations. One function of sequences upstream of ψ is to participate in a structural switch that governs the accessibility of the dimer initiation sequence and the initiation codon of the Gag coding sequence and hence determine whether full-length (+) strand RNA is dimerized and packaged or translated (Box 13.5). Sequestration of the complete ψ sequence present in the subgenomic mRNA of avian retroviruses (Fig. 13.14B) in a folded structure may also account for the inefficient encapsidation of the mRNA.

The NC domain of Gag mediates selective and efficient encapsidation of genomic RNA during retroviral assembly and can facilitate annealing of RNA molecules. The central region of NC containing the zinc-binding motif(s) and adjacent basic sequences binds specifically to RNAs that carry ψ sequences *in vitro* and is necessary for selective packaging of the genome in infected cells. Structural studies of NC proteins bound to RNA-packaging signals indicate that NC binds specifically to short RNA sequences. The zinc-binding motifs form a compact structure that makes specific contacts with bases and is complementary in charge and shape to the bound RNA. The Moloney murine leukemia virus ψ signal region contains 13 copies of the sequence recognized specifically by its NC protein, a higher frequency than elsewhere in the genome. However, this high-affinity NC-binding signal is exposed only after dimerization of the RNA, a property that promotes selective packaging of genome dimers.

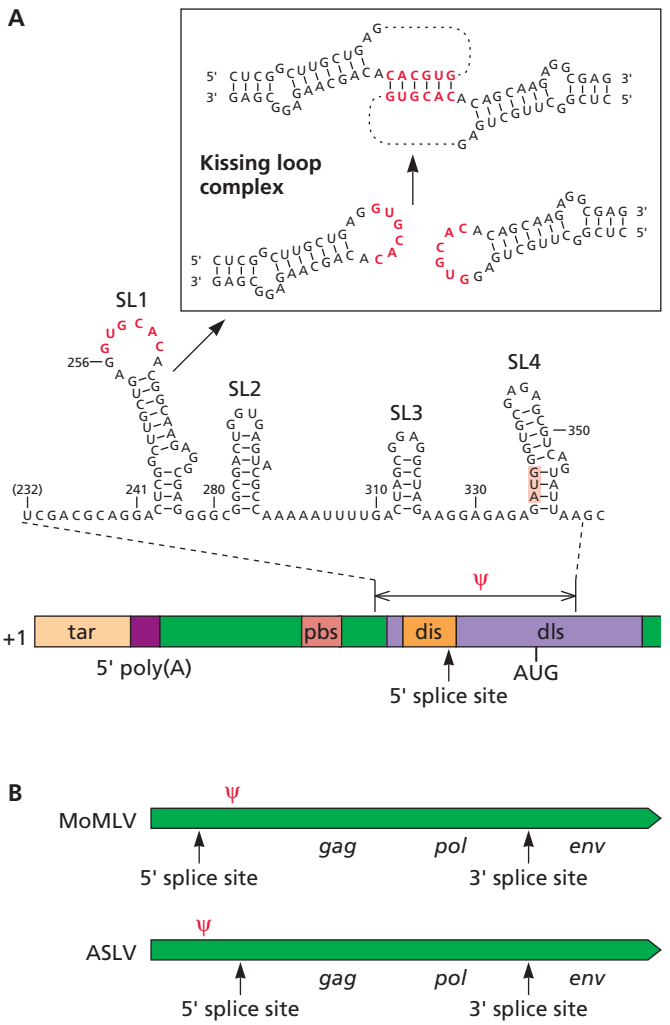


Figure 13.14 Sequences important for the packaging of retroviral genomes. (A) The 5′ end of the human immunodeficiency virus type 1 genome is shown to scale at the bottom, indicating the positions of tar, the 5′ polyadenylation signal [5′ poly (A)], the tRNA primer-binding site (pbs), the 5′ splice site, a packaging signal designated ψ, the sequence that forms the dimer linkage structure (dls), and the dimerization initiation site (dis), which can initiate dimerization *in vitro*. The four hairpins (SL1 to SL4) formed by the ψ sequence are shown above. The SL1 hairpin is the dimer initiation sequence. The loop-loop "kissing" complex proposed to form when two genomic RNA molecules dimerize via the self-complementary sequence shown in red is depicted at the top. The ψ sequence, which includes intronic sequences and therefore is present only in unspliced RNA, appears to be necessary but not sufficient for encapsidation of genomic RNA. **(B)** Locations within the RNA genomes of sequences necessary for the encapsidation of Moloney murine leukemia virus (MoMLV) and avian sarcoma/leukosis virus (ASLV) RNAs, designated ψ. The ψ signal resides only upstream of the 5′ splice site. Even though both genomic and subgenomic RNAs contain this sequence, spliced mRNA molecules are not encapsidated efficiently.

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BOX 13.5

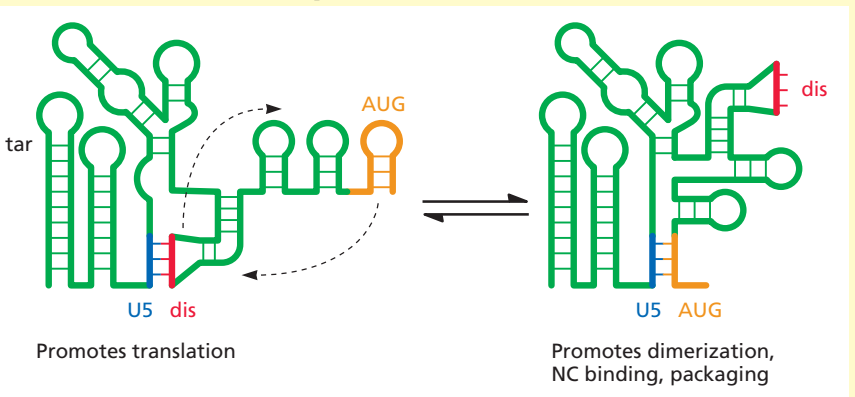
EXPERIMENTS

Dimerization-induced conformational change and encapsidation of the human immunodeficiency virus type 1 genome

The figure shows models of the secondary structure of the 5′ leader RNA determined using nuclear magnetic resonance (NMR) methods for analysis of RNAs of >50 nucleotides. At low ionic strength, the RNA was predominantly monomeric, and the NMR signals indicated that the AUG codon for initiation of translation of Gag was present in a hairpin (left). With increasing ionic strength, RNA dimers were formed and the NMR signals of the AUG-containing region were consistent with base pairing to the U5 sequence, displacing the dimerization initiation site (dis; right). These models were confirmed by various observations, including the results of site-directed mutagenesis. For example, mutations in the AUG region designed to disrupt base pairing promoted dimer formation. Furthermore, the AUG-U5 base-pairing interaction increased the affinity with which NC bound to RNA, and mutations that precluded such base pairing severely impaired RNA packaging. It has therefore been proposed that U5:AUG base pairing serves as a regulatory switch that governs the accessibility of both dis and the Gag AUG and hence dimerization and packaging versus translation of full-length (+) strand viral RNA. How this switch might be induced in infected cells is not known.

Lu K, Heng X, Garyu L, Monti S, Garcia EL, Karytonchy S, Dorjseuren B, Kulandaivel G, Jones S, Hirimath A, Divakaruni SS, LaColti C, Barton S, Tummillo D, Hosic A, Edme K, Albrecht S, Telesnitsky A, Summers MF. 2011. NMR detection of structures in the 5′-leader RNA that regulate genome packaging. *Science* **334:**242–245.

Shown are models of the secondary structures of an RNA comprising residues 1 to 356 of human immunodeficiency virus type 1 RNA determined by NMR methods. Those on the left and right predominate when the RNA is monomeric and dimeric, respectively. Adapted from K. Lu et al., *Science* **334:**242–245, 2011, with permission.



Other parameters that govern genome encapsidation. Specific signals may be required to mark a viral genome for encapsidation, but their presence does not guarantee packaging. The fixed dimensions of the closed icosahedral capsids or nucleocapsids of many viruses impose an upper limit on the size of viral nucleic acid that can be accommodated. Consequently, even when they contain appropriate packaging signals, nucleic acids that are more than 5 to 10% larger than the wild-type genome cannot be encapsidated. This property has important implications for the development of viral vectors. In some cases, the length of the DNA that can be accommodated in the particle (a "headful") is a critical parameter. This size limitation is exemplified by the coupled cleavage and encapsidation of genomic herpesviral DNA molecules from the concatemeric products of replication, as both specific sequences and a headful of DNA are recognized. Indeed, the packaging of some viral DNA genomes, such as T4 DNA, depends solely on the latter parameter (Box 13.6).

The specificity with which the viral genome is incorporated into assembling structures may also be the result of the coupling of encapsidation with its synthesis. As mentioned previously, such coordination is typical of the assembly of (−) strand RNA viruses (see, e.g., Fig. 13.9). Coordination of replication and encapsidation may contribute to the great specificity with which picornaviral genomes are packaged; not only abundant cytoplasmic cellular RNA species, but also (−) strand viral RNA and viral mRNA lacking VPg are excluded from virus particles. However, no packaging signal has yet been identified in the poliovirus genome. Encapsidation of the (+) strand, Vpg-linked RNA genome, which initiates assembly of virus particles (Fig. 13.6), is coordinated with genome replication, and both processes take place in association with cytoplasmic vesicles unique to poliovirus-infected cells (Chapter 14). Such sequestration of genomic RNA molecules with the viral proteins that must bind to them could promote specific packaging by reducing competition from cytoplasmic cellular RNAs. However, selective encapsidation of newly synthesized genomes is also facilitated by interaction of an essential component of the viral replication machinery (the viral 2C protein ATPase) with the capsid protein VP3. As packaging of flavivirus RNA also depends on replication of genomic RNA, coincident genome synthesis and assembly may be a general feature of (+) strand RNA viruses.

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BOX 13.6

BACKGROUND

Packaging a headful of viral DNA

During assembly of herpesvirus and several bacteriophages with large, double-stranded DNA genomes, including bacteriophage T4, the linear genome is cleaved from concatemeric products of viral genome replication as it is inserted into a preformed protein shell. While encapsidation of T4 DNA is coordinated with cleavage of concatemers, the T4 genome exhibits several unusual features:

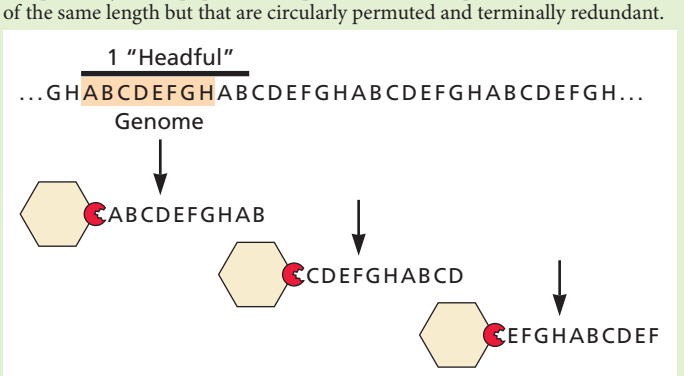
- the linear genomes do not have unique terminal sequences;
- the genetic map is circular, even though the genome is linear;
- the terminal sequences, which are different in each DNA molecule, are repeated at each end of DNA; and
- each virus particle contains more than a genome's length of DNA.

It was deduced from these properties that the T4 genome is circularly permuted and terminally redundant. These properties can be accounted for by essentially random cleavage of head-to-tail concatemers (the preferred substrate for DNA packaging) resulting in encapsidation of DNA molecules that are **longer** than the unique sequence in the genome (see the figure). No specific DNA sequence dictates the cleavages that liberate linear DNA during encapsidation. Rather, the first cleavage occurs randomly, and the second takes place once the phage T4 head has been filled with DNA. As predicted by this "headful" packaging mechanism, when head size is increased or decreased by mutation in specific genes (or other manipulations), longer and shorter DNA molecules, respectively, are encapsidated. Furthermore, when sequences are deleted from, or inserted into, the genome, the length of the terminal repeats increases or decreases to the corresponding degree. These properties demonstrate directly that a fixed length of DNA, a headful, is incorporated during assembly.

A headful of DNA is packaged during assembly of other bacteriophage and animal viruses with double-stranded DNA genomes, including herpesviruses. Structural studies of bacteriophage P22 virus particles revealed that tight spooling of DNA in the nucleocapsid induces major conformational change in the portal, through which DNA enters. It has therefore been proposed that the change in portal structure provides the signal that the nucleocapsid is full to activate termination of DNA encapsidation.

Lander GC, Tang L, Casjens SR, Gilcrease EB, Prevelige P, Poliakov A, Potter CS, Carragher B, Johnson JE. 2006. The structure of infectious P22 virion shows the signal for headful DNA packaging. *Science* **312:**1791–1795.

A head-to-tail concatemer, in which the unique genome sequence is represented by ABCDEFGH, is shown at the top. Initial cleavage between H and A is followed by packaging of a headful length that is longer than the length of the unique genome sequence, and the second cleavage. Repetition of this process yields a population of particles with encapsidated DNA molecules of the same length but that are circularly permuted and terminally redundant.



Packaging of Segmented Genomes

Segmented genomes pose an intriguing packaging problem. The best-studied example among animal viruses is the influenza A virus genome, which comprises eight molecules of RNA. It has been appreciated for many years that formation of an infectious virus particle requires incorporation of at least one copy of each of the eight genomic segments. However, it proved difficult to distinguish random packaging from a selective mechanism for inclusion of a full complement of genomic RNAs.

Packaging of the bacteriophage ϕ6 genome provides clear precedent for a selective mechanism. The genome of this bacteriophage comprises one copy of each of three double-stranded RNA segments designated S, M, and L. The (+) strand of each segment is packaged prior to synthesis of the double-stranded RNA segments, as with the packaging and synthesis of the reovirus genome (Chapter 6). The particle-to-PFU ratio of ϕ6 is close to 1, indicating that essentially all particles contain a complete complement of genome segments. Such precise packaging appears to be the result of the serial dependence of packaging of the (+) strand RNA segments. In *in vitro* reactions, the S segment packages alone, but entry of M RNA requires the presence of S RNA within particles, and packaging of the L segments is dependent on the prior entry of both S and M RNAs.

A random-packaging mechanism in which any eight RNA segments of the influenza virus genome were incorporated into virus particles would yield a maximum of 1 infectious particle for every 400 or so assembled ($8!/8^8$). This ratio might seem impossibly low, but it is within the range of ratios of noninfectious to infectious particles found in virus preparations. Furthermore, if packaging of more than eight RNA segments

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were possible, the proportion of infectious particles would increase significantly. For example, with 12 RNA molecules per particle, 10% would contain the complete viral genome. Particles containing more than eight RNA segments have been isolated, consistent with random packaging. Nevertheless, it has become clear in the last decade that the packaging of influenza virus genome segments **is** selective. For example, eight RNA segments were observed in all particles by electron tomography (Fig. 13.15). Furthermore, estimation of the copy number of each viral RNA segment by single-molecule fluorescent *in situ* hybridization established that each particle contains one copy of each of the eight segments of the genome (Box 13.7).

A selective mechanism implies that each of the eight (−) strand genome RNAs (vRNAs) carries a unique signal that ensures its packaging. These sequences comprise the short 5′ and 3′ noncoding regions of each segment but extend short distances into adjacent coding regions. The extreme 5′- and 3′-terminal sequences are highly conserved among vRNA segments of influenza A virus isolates and might distinguish vRNAs from viral and cellular mRNAs. Although the mechanisms by which the full complement of the eight vRNA segments that comprise the viral genome is selected for packaging is not fully understood, the available evidence implicates direct interactions among RNA segments. This mechanism was first suggested by the observation that deletion of packaging signals from the vRNA segments for the polymerase proteins impaired the encapsidation not only of these vRNAs but also of others. The greater importance of some vRNA packaging signals (those of the PA, PB2, M, and NP RNA segments) is consistent with a hierarchical mechanism of packaging. Each vRNA segment associates with at least one other *in vitro*, and specific base pairing between two segments (vRNA2 and vRNA8) has been shown to be necessary for the packaging of both into virus particles in infected cells and for optimal virus reproduction. It therefore appears that base-pairing interactions drive formation of an organized assembly containing one molecule of each vRNA segment prior to encapsidation. Such interactions may occur during transport of vRNAs to the plasma membrane (Chapter 12) and are consistent with the nonrandom organization of vRNAs observed in budding virus particles (Fig. 13.15).

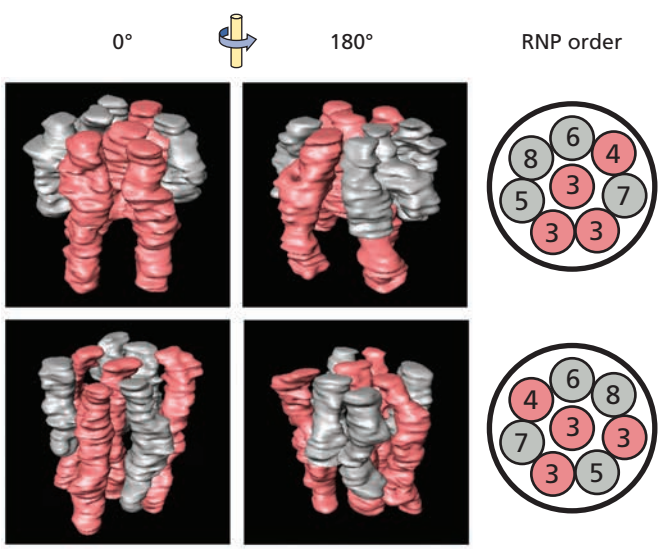


Figure 13.15 Organization of ribonucleoproteins in influenza A virus particles. Purified influenza A virus particles were examined by scanning transmission electron tomography. Shown are three-dimensional models of the viral ribonucleoproteins (vRNPs) observed in the particles. All particles examined contained 8 vRNPs, most of which could be distinguished by their lengths and are designated 3, 3, 3, 4, 5, 6, 7, and 8 in terms of decreasing length. Longer (3, 4) and shorter RNPs are shown in pink and gray, respectively. The RNPs are packaged in the 7+1 arrangements illustrated at the right and associated with one another in all particles examined. Adapted from T. Noda et al., *Nat. Commun.*, 2012, doi:10.1038/ncomms1647. Courtesy of Y. Kawaoka, University of Tokyo, Tokyo, Japan.

Incorporation of Enzymes and Other Nonstructural Proteins

In many cases, the production of infectious particles requires essential viral enzymes or other proteins that are important in establishing an efficient infectious cycle. Some of these proteins are also structural proteins. For example, the herpes simplex virus 1 VP16 protein is both a major component of the tegument and the activator of transcription of viral immediate early genes.

A simple, yet elegant, mechanism ensures entry of retroviral enzymes (protease [PR], reverse transcriptase [RT], and integrase [IN]) into the assembling core. In most cases, the precursors to these enzymes are synthesized as C-terminal extensions of the Gag polyprotein. The organizations and complements of these translation products, designated Gag-Pol, vary among retroviruses, but the important point is that they contain not only Pol but also the sequences specifying Gag-Gag interactions, which can direct incorporation of Gag-Pol molecules into assembling particles (Fig. 13.10). The low efficiency with which Gag-Pol polyproteins are translated determines their concentrations relative to Gag in the cell and in virus particles (1:9). The enzymes present in other virus particles, such as the RNA-dependent RNA polymerases of (−) strand RNA viruses (see Table 4.3), are synthesized as individual molecules and therefore must enter assembling particles by noncovalent binding to the genome or to structural proteins.

All retroviral capsids also contain the cellular tRNA primer for reverse transcription, brought into particles by its base pairing with a specific sequence in the RNA genome and by specific binding to RT. In some cases, including human immunodeficiency virus type 1, the host amino acyl tRNA

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BOX 13.7

EXPERIMENTS

Counting the number of unique viral RNA segments in influenza virus particles

To provide single-molecule sensitivity, "counting" experiments exploited fluorescent *in situ* hybridization (FISH), with individual viral RNA segments detected by hybridization to multiple oligonucleotides complementary to different sequences of the RNA.

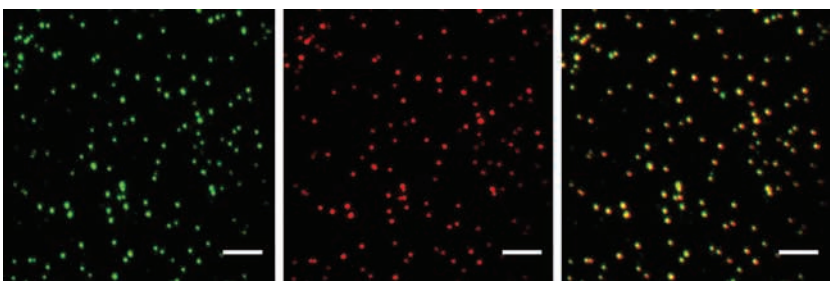
Purified influenza A virus particles were immobilized at low density on glass slides by binding to surface-bound antibodies against the viral HA protein. Particles were fixed and permeabilized prior to hybridization. The specificity of the method was first established by showing that fluorophore (Cy3 or Cy5)-labeled probes for two viral RNA segments each detected >50 spots, but a probe against a cellular RNA yielded few, if any.

Hybridization with a mixture of Cy3- and Cy5-labeled probes against a single segment established that the efficiency of colocalization was high (see the figure). When Cy3-labeled oligonucleotides complementary to the PB2 RNA segment and Cy5-labeled probes for each of the other 7 segments were applied, efficient colocalization of PB2 with each of the other RNAs was observed. Calculations based on the colocalization efficiencies indicated that over 50% of the virus particles examined contained all 8 genomic RNA segments.

The copy number of each segment in viral particles was examined by using photobleaching. This approach relied on the stepwise loss of fluorescent intensity as a function of time when multiple probes were hybridized to an RNA segment. Control experiments with a recombinant virus containing 2 copies of the HA RNA segment established that this approach can distinguish clearly 1 from 2 copies of an RNA segment per particle. The results obtained when the other RNA segments were "counted" in this way indicted that a single copy of each of the eight genomic segments was packaged in >90% of the hundreds of virus particles examined. These experiments provided compelling evidence for selective and efficient packaging of all the parts of this segmented genome in each virus particle.

Chou YY, Vufabakhsh R, Doganay S, Gao Q, Ha T, Palese P. 2012. One influenza virus particle packages eight unique viral RNAs as shown by FISH analysis. *Proc Natl Acad Sci U S A* **109:**9101–9106.

Images of influenza A virus particles labeled by FISH with 23 Cy3-labeled and 25 Cy5-labeled oligonucleotides complementary to the NA RNA segment. As illustrated in the merged image, the great majority (90%) of particles are labeled with both probes (right). Scale bar = 5 μm. Adapted from Y.-Y. Chou et al., *Proc. Natl. Acad. Sci. U. S. A.* **109:**9101–9106, 2012, with permission. Courtesy of P. Palese, Mount Sinai School of Medicine, New York, NY.



synthetase that aminoacylates the particular tRNA used as primer is also encapsidated (Chapter 7). The absence from virus particles of other amino acid tRNA synthetases and the similar concentrations of the enzyme and its tRNA substrate in human immunodeficiency virus type 1 particles suggest that the synthetase may be recognized by viral components during packaging (Box 7.3).

Acquisition of an Envelope

The formation of many types of virus particle requires envelopment of capsids or nucleocapsids by a lipid membrane carrying viral proteins. Most such enveloped viruses assemble by virtue of specific interactions among their components at a cellular membrane before budding and pinching off of a new virus particle. There is considerable variety in the interactions of viral proteins with membranes (and with one another) that induce membrane curvature (bud formation) (Fig. 13.16). Whether particles assemble at the plasma or an internal membrane is determined by the destination of viral proteins that enter the cellular secretory pathway (Chapter 12). Enveloped viruses assemble by one of two mechanisms, distinguished by whether acquisition of the envelope follows assembly of internal structures or whether these processes take place simultaneously.

Sequential Assembly of Internal Components and Budding from a Cellular Membrane

The assembly of the internal structures of most enveloped virus particles and their interaction with a cellular membrane modified by insertion of viral proteins are spatially and temporally separated. This class of assembly pathways is exemplified by (−) strand RNA viruses, such as influenza A virus (Fig. 13.9) and vesicular stomatitis virus. Influenza A virus ribonucleoproteins containing individual genomic RNA segments, NP protein, and the polymerase proteins are assembled in the infected cell nucleus as genomic RNA segments are synthesized and are then transported to the cytoplasm (Chapter 12). The viral glycoproteins HA and NA and the M2 membrane protein travel separately to specialized regions in

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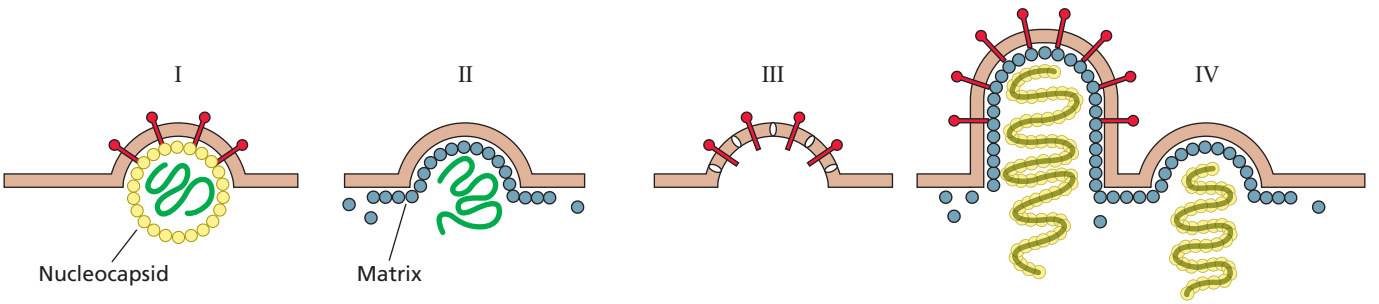


Figure 13.16 Interaction of viral proteins responsible for budding at the plasma membrane. Four distinct budding strategies have been identified. In type I budding, exemplified by alphaviruses, such as Sindbis virus, both the envelope glycoproteins and the internal capsid are essential. Quite detailed structural pictures of alphaviruses are now available (Chapter 4). Certain altered or chimeric envelope proteins that reach the membrane normally do not support budding. These observations indicate that lateral interactions among the envelope heterodimers, as well as those of the heterodimers with the capsid, cooperate to drive budding. Type II budding, such as Gag-dependent budding of many retroviruses, requires only the internal Gag polyprotein. For other viruses, type II budding requires only capsid proteins. Conversely, budding can be driven solely by envelope proteins (type III), a mechanism exemplified by the envelope proteins of the coronavirus mouse hepatitis virus. Type IV budding is driven by matrix proteins, but its proper functioning depends on additional components. For example, in the case of rhabdoviruses and orthomyxoviruses, internal matrix proteins alone can drive budding. However, this process is inefficient or results in deformed or incomplete particles in the absence of envelope glycoproteins or the internal ribonucleoprotein. Adapted from H. Garoff et al., *Mol. Microbiol. Rev.* **62:**1171–1190, 1998, with permission.

the plasma membrane (lipid rafts) via the cellular secretory pathway (Fig. 13.9; Chapter 12). The M1 protein interacts with both viral nucleocapsids and the inner surface of the plasma membrane to direct the assembly of progeny particles at that membrane. Vesicular stomatitis virus assembles in a similar fashion. The matrix proteins of these (−) strand RNA viruses therefore provide the links between ribonucleoproteins and the modified cellular membrane necessary for assembly and budding.

The cellular membranes destined to form the envelopes of virus particles contain viral integral membrane proteins that play essential roles in the attachment of virus particles to, and their entry into, host cells. In simple enveloped alphaviruses, direct binding of the cytoplasmic portions of the viral glycoproteins to the single nucleocapsid protein (see Fig. 4.24) is necessary for acquisition of the envelope during budding from the plasma membrane. The crucial role and specificity of these interactions in the final steps in assembly are illustrated by the failure of a chimeric Sindbis virus containing the coding sequence for the E1 glycoprotein of a different togavirus to bud efficiently. The heterodimeric glycoproteins (E1 plus E2) are formed and transported to the plasma membrane. However, these chimeras exhibit an altered conformation and fail to bind to nucleocapsids at the plasma membrane. Binding of viral glycoproteins to internal components also appears to be important for the production of structurally more complicated enveloped viruses. For example, interactions between the influenza virus M1 protein and the cytoplasmic tails of the HA and NA glycoproteins are necessary for formation of virus particles with normal size and morphology.

Coordination of the Assembly of Internal Structures with Acquisition of the Envelope

The alternative pathway of acquiring an envelope, in which assembly of internal structures and budding from a cellular membrane are largely coincident in space and time, is exemplified by many retroviruses. Assembling cores of the majority first appear as crescent-shaped patches at the inner surface of the plasma membrane. These structures extend to form a closed sphere as the plasma membrane wraps around and the assembling particle is eventually pinched off (Fig. 13.10). Formation of the assembling particles depends on the interaction of Gag polyprotein molecules with one another to form the protein core, with the RNA genome via the NC portion, and with the plasma membrane via the MA segment.

Specific segments of Gag mediate the orderly association of polyprotein molecules with one another and are required for proper assembly. These sequences include an essential C-terminal multimerization domain of the CA segment; substitutions that disrupt the CA dimer interface block assembly of the CA protein *in vitro* and severely inhibit Gag assembly and formation of virus particles in infected cells. The capsids of retroviruses can be spherical, conical, or cylindrical (Fig. 4.17), and specific CA sequences that determine the morphology of mature particles have also been identified. Certain sequences present only in the Gag polyprotein also govern morphology, for their removal results in the assembly of misshapen particles.

As discussed previously, Gag multimerization during particle assembly of human immunodeficiency virus type 1 (and many other retroviruses) is regulated by binding of the NC

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domain to the RNA genome. This process is also promoted by interaction of Gag with the plasma membrane via the MA membrane-binding signals (Fig. 12.18). Elimination of the signal for myristoylation prevents assembly, as does alteration of the sequence predicted to lie at the interfaces of the MA trimers formed in crystals (Fig. 4.26). It has been suggested that MA trimerization increases the accessibility of the myristate chain. Conversely, efficient membrane binding of Gag depends on sequences other than the membrane-binding region of MA, such as a sequence in the N-terminal portion of NC. Because this sequence is not required for production of stable Gag or its transport to the plasma membrane, it may promote Gag-Gag or Gag-RNA interactions that lead to cooperative and stable binding of Gag molecules to the membrane. In this context, it is noteworthy that Gag-Gag interactions have been observed in living cells only at the plasma membrane.

In some cases, the MA segment of Gag also binds to the cytoplasmic tail of the viral envelope glycoprotein. For example, association of the assembling human immunodeficiency virus type 1 core with the TM-SU glycoprotein requires the N-terminal 100 amino acids of MA. Such Gag-Env interactions ensure specific incorporation of viral glycoproteins into virus particles. Nonetheless, they do not appear to be universal; glycoprotein-containing virus particles are produced even when the C-terminal tails of TM of other retroviruses (e.g., avian sarcoma virus) are deleted. Nor can a model based solely on Gag-Env interactions account for the ease with which "foreign" viral and cellular glycoproteins are included in the envelopes of all retroviruses. The final reaction, fusion of membrane regions juxtaposed as the particle assembles (Fig. 13.10), is shared with other viruses that assemble at the plasma membrane. This process is considered in the next section.

Release of Virus Particles

Many enveloped viruses assemble at, and bud from, the plasma membrane. Consequently, the final assembly reaction, fusion of the membrane around the internal viral components, releases the newly formed virus particle into the extracellular environment. When the envelope is derived from an intracellular membrane, the final step in assembly, budding, is also the first step in egress, which must be followed by transport of the particles to the cell surface. The assembly of enveloped viruses is therefore both mechanistically coupled and coincident with (or at least shortly followed by) their exit from the host cell. In some cases, nondestructive budding permits a long-lasting relationship with the host cell. The progeny of many retroviruses are released throughout the lifetime of an infected cell, which is not harmed (but may be permanently altered [see Volume II, Chapter 6]). The egress of some viruses without envelopes from certain cell types also occurs by specific mechanisms. However, reproduction of such viruses more commonly results in destruction (lysis) of the host cell. Large quantities of assembled virus particles may accumulate within infected cells for hours, or even days, prior to their release.

Assembly and Budding at the Plasma Membrane

The release of enveloped virus particles from the plasma membrane is an intricate process that comprises induction of membrane curvature by viral components (bud formation), bud growth, and fusion of the membrane (scission) to liberate virus particles. As discussed in the previous section, interactions among internal viral proteins and the membrane (and/or viral glycoproteins within it) induce membrane curvature and bud formation. However, with some exceptions (see the next section), viral proteins are not sufficient for membrane scission. In fact, it is now clear that the cellular endosomal complex required for transport (Escrt) mediates the release of many viruses.

Escrt-Dependent Budding

Common sequence motifs are required for budding. A major breakthrough in our understanding of how particles of some viruses bud from the plasma membrane came with the identification of mutants of human immunodeficiency virus type 1 with an unusual assembly phenotype; amino acid substitutions in the p6 region unique to the Gag polyprotein did not impair assembly of immature particles, but the particles remained attached to the host cell by a thin membrane stalk (Fig. 13.17A). It was therefore concluded that these Gag sequences are required for the fusion reaction that separates the viral envelope from the plasma membrane. Subsequently, functionally analogous sequences, termed late-assembly (L) domains, were identified in Gag proteins of several other retroviruses. These L domains are not conserved in their location within Gag or in amino acid sequence but nevertheless can substitute for one another to promote budding.

Retroviral L domains contain a small number of short, core sequence motifs, such as PTAP and PPXY. The recognition of such motifs, and their ability to function independently of position or sequence context, led to identification of L domains in the proteins required for the budding of viruses of several different families (Table 13.2). These **L domain sequences** promote budding by recruitment of cellular proteins that participate in specific steps in vesicular trafficking.

The activity of viral L domains depends on vesicular sorting proteins. The autonomous activity (and in some cases the sequence) of L domains suggested that these sequences mediate protein-protein interactions. Cellular proteins that bind to each of the prototype sequences have now been identified (Fig. 13.17B). The PTAP motif was first shown to

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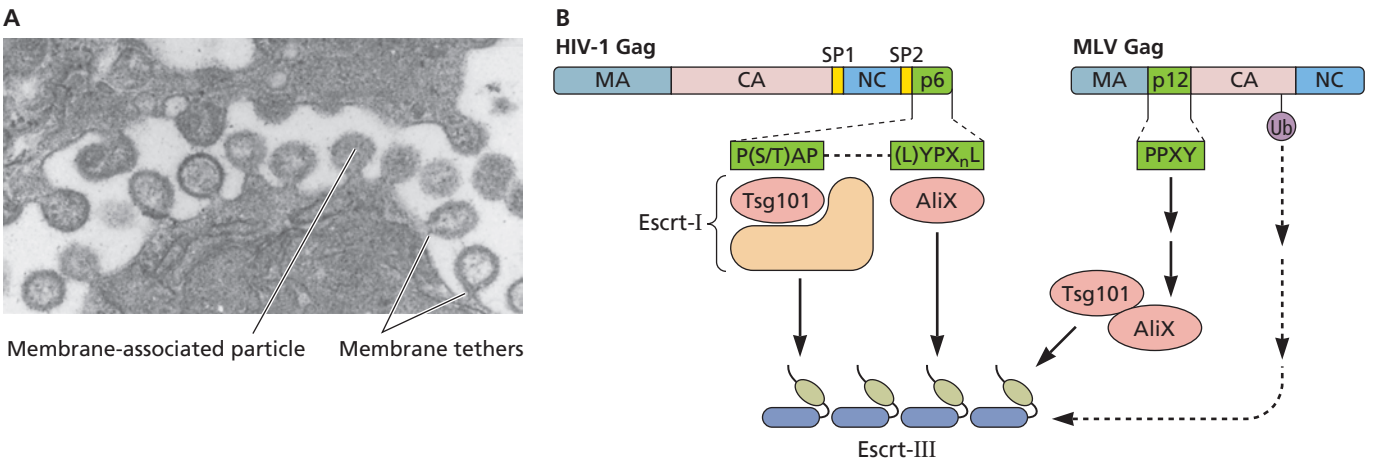


Figure 13.17 L domains and release of retroviral particles. (A) Electron micrograph of monkey Cos-7 cells containing a human immunodeficiency virus type 1 mutant provirus from which Gag p6 cannot be expressed. The plasma membrane-associated particles exhibit normal morphology but remain tethered to the membrane. Adapted from H. G. Göttlinger et al., *Proc. Natl. Acad. Sci. U. S. A.* **88:**3195–3199, 1991, with permission. Courtesy of H. Göttlinger, University of Massachusetts Medical Center. **(B)** Summary of the association of cellular trafficking proteins with core sequence motifs of L domains present in retroviral Gag proteins (and other proteins) required for release of viral particles. Interactions are shown by direct contact between motifs and proteins and by double-headed arrows. The various adapter proteins, such as Escrt-I, Alix, and likely Nedd4 family ubiquitin ligases, recruit Escrt-III to sites of budding. For example, interaction of Alix with the Escrt-III protein Chmp4 (charged multivesicular body protein 4) is required for budding of human immunodeficiency virus type 1. Mammalian cells contain 12 different Escrt-III-like proteins, of which 2 (Chmp2 and Chmp4) are essential for release of human immunodeficiency virus type 1. These proteins are auto-inhibited by interaction of C-terminal segments with a long α-helical core domain. They form homo- and heteromeric filaments upon relief of auto-inhibition, an activity that is thought to drive membrane constriction.

recruit the product of tumor susceptibility gene 101 (Tsg101), an interaction that is essential for budding of human immunodeficiency virus type 1. Mammalian Tsg101 participates in sorting and trafficking of cellular proteins from late endosomes to structures called multivesicular bodies, which fuse with lysosomes. As their name implies, multivesicular bodies contain vesicles within vesicles. The formation of these structures and budding of virus particles are topologically equivalent processes; in both cases, membranes invaginate away from the cytoplasm and fusion releases vesicles with cytoplasmic contents into a lumen or the extracellular space. Recruitment of Tsg101 by the PTAP L domain therefore suggested that the cellular machinery that mediates sorting and trafficking of endocytic vesicles is diverted to promote budding and release of virus particles. In fact, Tsg101 proved to be the human homolog of one subunit of the heteromeric protein Escrt-I, first identified because it is required for sorting of yeast proteins to the vacuole/lysosome. The other subunits of human Escrt-I are also required for release of human immunodeficiency virus type 1. Escrt-I is but one of several multiprotein assemblies that participate in trafficking by way of multivesicular bodies and are necessary for release of retroviruses and other enveloped viruses, including arenaviruses, filoviruses, paramyxoviruses, and rhabdoviruses. Of these, the filamentous protein Escrt-III and the ATPases that associate with it (Vsp4A or -B) act late in budding to drive membrane constriction and fission. The subunits of Escrt-III form a filamentous spiral in the bud neck that is thought to constrict this structure and juxtapose the membranes to promote scission (Fig. 13.18). It therefore appears that formation and release of virus particles with very different structures, genomes, and composition are driven by the same cellular components and mechanism.

Table 13.2 Common sequence motifs required for budding of enveloped virus particles

L domain motif	Escrt component	Viral protein
P (T/S)AP	Tsg101	Human immunodeficiency virus type 1 Gag Murine leukemia virus Gag3 Ebola virus GP40 Bluetongue virus NS
YPXnL	Alix	Human immunodeficiency virus type 1 Gag Rous sarcoma virus Gag Sendai virus M Yellow fever virus NS3
PPXY	Nedd4	Rous sarcoma virus Gag Ebolavirus GP40 Vesicular stomatis virus M

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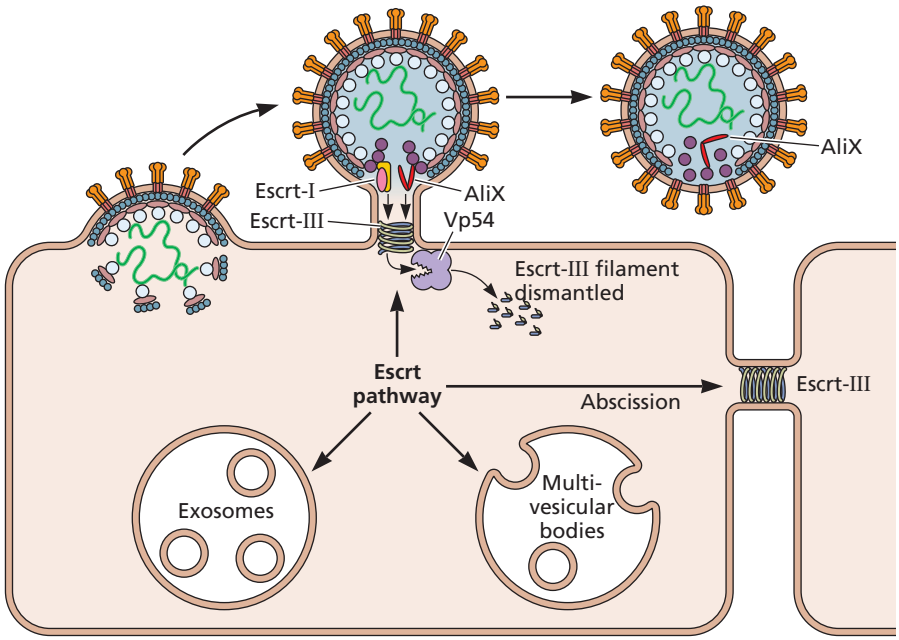


Figure 13.18 Functions of the Escrt pathway in uninfected and virus-infected cells. The Escrt machinery (>30 proteins) catalyzes the membrane fission reactions required for several important cellular processes, including formation of multivesicular bodies and exosomes, as well as the scission reaction that severs the connection between daughter cells in the final step of cell division (abscission). The Escrt pathway is also necessary for the budding and release of retroviruses and a variety of other enveloped viruses; structural proteins of these viruses recruit the core Escrt-III proteins via different adapter proteins (Table 13.2). A model for how cooperation among the various Escrt pathway proteins that interact with L domains present in the human immunodeficiency virus type 1 Gag protein might promote the release of virus particles is illustrated. This model is based on the observations that retrovirus particles contain ubiquitin, which, when attached to Gag, can function as an L domain and that the adapter proteins Alix and Escrt-I can bind to both Gag and ubiquitin and to each other. They may therefore cooperate to recruit Escrt-III for membrane scission at the bud neck. This protein is shown as a filament at this location (middle) and as depolymerized and auto-inhibited individual subunits after release of the virus particle (right). The Vsp4A and -B ATPases can depolymerize Esct-III, presumably to allow its participation in multiple cycles of membrane budding. Vsp4 is required for the release of retrovirus particles, but its molecular role in this process is not yet clear. Adapted from J. Votteler and W. I. Sundquist, *Cell Host Microbe* **14:**2320241, 2013, with permission.

A second L domain (the YPXL domain) that is present in Gag proteins of several retroviruses and facilitates the release of multiple viruses (Table 13.2) also recruits components of the Escrt pathway, in this case via the adapter protein Alix (Fig. 13.17B). A small fraction of retroviral Gag is ubiquitinylated, and a third type of L domain (PPXY) recruits specific ubiquitin ligases (Fig. 13.17B). A catalytically active ubiquitin ligase is necessary for release of retroviruses with this Gag L domain sequence. Furthermore, ubiquitinylation of human immunodeficiency virus type 1 Gag at sites C terminal to the CA domain is necessary for efficient release; substitutions that prevent modification at these sites lower the rate of release and induce the accumulation of virus particles tethered to the plasma membrane. As ubiquitin is recognized by several of the endocytic trafficking proteins, this modification might promote the assembly of the machine that mediates budding and release of retroviruses (Fig. 13.18).

After the identification of its role in release of enveloped virus particles, the Escrt pathway was shown to mediate an analogous reaction in uninfected cells, scission of the thin intercellular bridges between daughter cells during the final step in division (Fig. 13.18) (See the interview with Dr. Wesley Sundquist: http://bit.ly/Virology_Sundquist). Escrt proteins were first identified in budding yeast, and Escrt-III components are also required for cell division of a subset of archaea. These ancient and conserved proteins are thus available in many different species and types of cell in which viruses reproduce.

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Escrt-Independent Budding

Although Escrt-dependent budding is a common mechanism for release of enveloped particles from the plasma membrane, it is not the only one; the structural proteins of other enveloped viruses, including influenza viruses and togaviruses, contain no L domains, and budding of these particles is not inhibited by dominant negative derivatives of Escrt pathway proteins.

Budding of togaviruses, such as Ross River and Sindbis viruses, is driven by interactions between capsid (C) protein and envelope glycoproteins (E1 and E2) in the plasma membrane. It is thought that formation of the highly ordered, external glycoprotein shell (Fig. 4.24) facilitates membrane constriction at the neck of budding particles and, hence, scission and release of particles. Interactions among viral glycoproteins (HA, NA) and the internal protein M1 also mediate the assembly of influenza virus particles and induce membrane curvature. However, the viral M2 protein is required for final membrane scission. This protein is recruited to budding particles by interaction with M1 and localizes to the bud neck. It can alter membrane curvature at this site and may do so by membrane insertion of an amphipathic α-helix.

Nonstructural Proteins Can Facilitate Release

Release from the plasma membrane can also depend on viral proteins other than major structural proteins. For example, in some cell types, efficient release of human immunodeficiency virus type 1 requires the viral Vpu protein. In the absence of Vpu, particles accumulate in intracellular vacuoles or are attached to the infected cell surface. This viral protein was shown to counteract the action of an antiviral protein that tethers virus particles to the cell surface and is produced when cells are exposed to interferon α. The organization of this protein, termed tetherin (or bone marrow stromal antigen 2 [Bst2]), suggests that interactions between tetherin molecules inserted in the plasma membrane and the viral envelope are responsible for retaining virus particles at the cell surface. Vpu associates with tetherin in the *trans*-Golgi network to reduce transport of the cellular protein to the plasma membrane. In some cell types, this association leads to ubiquitinylation of tetherin, which is then sorted in an Escrt-dependent manner for lysosomal degradation.

Tetherin is now known to limit the release of many other retroviruses, as well as filoviruses, rhabdoviruses, and herpesviruses, even though the latter buds at an internal membrane (see below). This interferon-inducible protein is an important component of antiviral defense and may also serve as a sensor of viral infection (Volume II, Chapter 3).

Assembly at Internal Membranes: the Problem of Exocytosis

Cytoplasmic Compartments of the Secretory Pathway

Several enveloped viruses are assembled at the cytoplasmic surfaces of compartments of the secretory pathway under the direction of specifically located viral glycoproteins and form by budding of the particle into the lumen of one of these compartments (Table 12.3). These particles therefore lie within membrane-bound organelles. It is generally assumed that such virus particles must be packaged within cellular transport vesicles for travel along the secretory pathway to the cell surface, but few details have been reported. On the other hand, there is accumulating evidence for the participation of other vesicular transport pathways. For example, release but not the intracellular accumulation of infectious hepatitis C virus particles, which bud into the ER, depends on components of the Escrt machinery. This observation suggests that these virus particles reach the cell surface via recycling endosomes. The endocytic pathway has also been implicated in transport to the plasma membrane of immature capsids of the betaretrovirus Mason-Pfizer monkey virus, which assembles at internal cytoplasmic sites near the centrioles. Proteins that function late in the Escrt pathway are also required for budding and release of hepatitis B virus, although the site of particle formation and route of travel to the cell surface are not yet clear.

The budding of virus particles into internal compartments of the secretory pathway is initiated by interactions among the cytoplasmic domains of viral membrane proteins and internal components of the particle. Consequently, this process generally begins as soon as the integral membrane and cytoplasmic viral proteins attain sufficient concentrations in the infected cell. For example, the concentration of viral membrane proteins (surface proteins) determines the fate of hepadnaviral cores, which contain the capsid (C) protein, a DNA copy of the pregenomic RNA, and the viral polymerase (Appendix; Fig. 11). Early in infection, the concentration of the large surface protein (L) in membranes is too low for efficient envelopment of cores, and these structures enter the nucleus, where they contribute to the pool of viral DNA templates for transcription (Fig. 13.19). As the concentration of the L protein increases, it interacts with cores, and enveloped particles form. The ability of hepadnaviral cores to bind to this viral glycoprotein is also regulated by the nature of the nucleic acid that they contain; the synthesis of DNA from the pregenomic RNA induces significant conformational changes in the exterior surfaces of the C protein, notably a more open geometry of a hydrophobic pocket that is lined with residues required for envelopment and thought to make contact with viral envelope proteins.

Although budding into internal compartments imposes the need for subsequent transport and release of virus particles, this mechanism may confer some advantages. Intracellular budding may reduce the concentration of viral glycoproteins exposed on the surface of the infected cell. This property would decrease the likelihood that an infected cell would be recognized by components of the immune system

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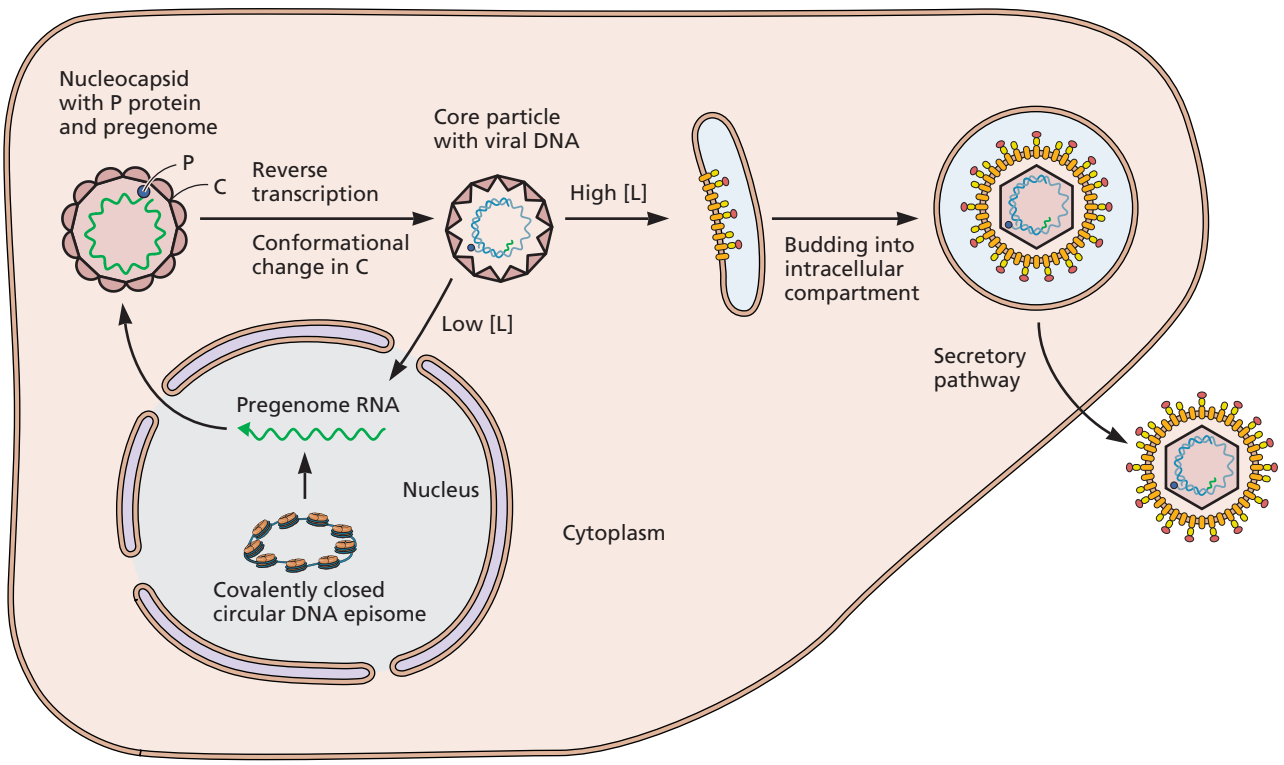


Figure 13.19 Model of hepatitis B virus envelopment. The pregenome RNA synthesized in infected cell nuclei (see Chapter 7) is exported to the cytoplasm, where it is incorporated into particles built from the capsid (C) protein. Reverse transcription to produce the DNA genome induces a conformational change in the C protein that allows interaction of capsid with the large surface protein (L) inserted into internal membranes. Whether core particles containing DNA enter the nucleus or become enveloped by budding into compartments of the secretory pathway is determined by the concentration of the L protein. The L, middle (M), and small (S) envelope glycoproteins accumulate in membranes of the ER-Golgi intermediate compartment, into which subviral particles that contain only lipid and envelope proteins (primarily S) also appear to bud. As the S, as well as the L, protein is required for envelopment, it is generally accepted that virus particles are also formed by budding into this same compartment of the secretory pathway. However, the results of recent experiments indicate that cellular proteins that participate in endocytic trafficking (see the text) participate in hepatitis B virus budding and release.

before the maximal number of progeny particles was assembled and released. Alternatively, the simpler cytoplasmic surfaces of internal membranes, which are not burdened with cytoskeletal structures and the proteins that attach them to the extracellular matrix, may make for more-facile assembly or budding reactions, or the distinctive lipid composition of internal membranes may confer some (as-yet-unknown) special property that is advantageous to these viruses.

Envelopment by a Virus-Specific Mechanism

The interaction of components of the poxvirus vaccinia virus with internal cellular membranes during assembly is most unusual. One remarkable feature is the assembly of two **different** infectious particles, which have been termed the intracellular mature and the extracellular enveloped virions, that differ in the number and origin of their lipid membranes. Furthermore, the initial acquisition of a membrane early in assembly occurs by a virus-specific mechanism that appears to be shared with other large DNA viruses that assemble in the cytoplasm, such as mimivirus. Finally, infectious particles leave the host cell by at least three distinct routes.

Vaccinia virus assembly includes the formation of several intermediates, such as crescents (see below) and immature particles, and major morphological rearrangements as infectious particles are formed (Fig. 13.20). The assembly pathway was elucidated initially by electron microscopy in some of the earliest studies of vaccinia virus. Numerous viral proteins that participate in the various assembly reactions have been identified by genetic experiments (Table 13.3). Synthesis of viral DNA genomes and structural proteins takes place in discrete cytoplasmic domains termed viral factories. The first morphological sign of assembly is the appearance within viral factories of rigid, curved structures 10 to 15 nm

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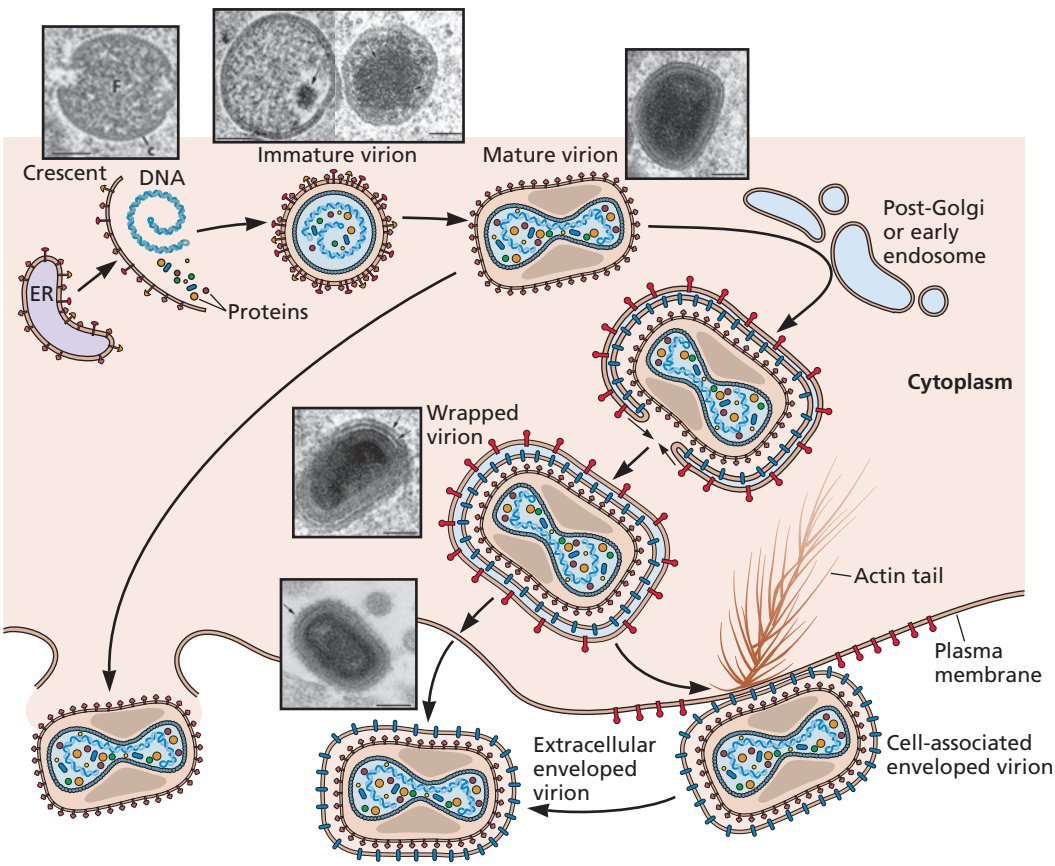


Figure 13.20 Vaccinia virus assembly and exocytosis. Viral structures observed when HeLa cells infected with vaccinia virus for 10 or 24 h were prepared for electron microscopy by quick freezing and negative staining while frozen are shown in a schematic model of assembly and exocytosis. Assembly begins with the formation of crescents by diversion of membrane from the ER. That shown in the electron micrograph (c) is present in a viral factory (F) and encircles a dense focus of viral material (viroplasm). The viral D13 protein, which is associated with the outer leaflets of crescents, maintains the curvature and rigidity of the crescent membrane as it enlarges and eventually closes with the incorporation of viral DNA and proteins from viral factories to form immature virus particles, two examples of which are shown. As the D13 protein is lost during the morphological transitions that form the brick-shaped mature virus particle, it is considered a scaffolding protein. The mature particle is released from infected cells only upon lysis. However, a significant proportion of these structures acquire additional membranes by wrapping in membranes derived from a late or post-Golgi compartment to form the intracellular enveloped virus particle. The additional double membrane is indicated by the arrows in the electron micrograph. This particle is transported to the plasma membrane, where fusion with this membrane forms the cell-associated enveloped virus, which has lost one outer membrane layer. This particle induces formation of actin tails. Adapted from B. Sodeik and J. Krijnse-Locker, *Trends Microbiol.* **10:**15–24, 2002, with permission. Electron micrographs are adapted from C. Risco et al., *J. Virol.* **76:**1839–1855, 2000, with permission. Bars, 100 nm.

thick (Fig. 13.20). There has never been any doubt that these structures, termed crescents, contain at least one lipid bilayer. In contrast, the origin of this membrane has been a subject of much debate. The current consensus is that crescents contain a single lipid membrane that is derived from the ER membrane but by a virus-specific mechanism (Box 13.8). As the crescents enlarge, they retain their original curvature and therefore eventually form spheres surrounding viral macromolecules present in viral factories, including the DNA genome (Fig. 13.2). Such immature particles then undergo major morphological transitions to form brick-shaped mature virions (Fig. 13.20). This maturation process requires several distinct reactions, including proteolytic cleavage of several structural proteins by a viral protease(s) (Table 13.3), the action of the viral redox system (Chapter 12), and removal of at least one crescent-associated protein. These changes resemble those that occur during assembly of herpesviruses and adenoviruses.

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Table 13.3 Some proteins implicated in vaccinia virus assembly[a]

Assembly reaction	Protein(s)	Function(s)/properties
Crescent formation	AG	Localization of major viral membrane proteins to viral factories
	A11	Colocalizes with ER at viral membranes in viral factories
	A14, A17	Essential for this step; integral membrane proteins; phosphorylated by F10; form a disulfide bond-stabilized lattice
	D13	Imparts crescent curvature and rigidity; interacts with A17
	F10	Essential for ER membrane remodeling and appearance of crescents; dual-specificity protein kinase
Assembly of IV	Complex of 7 proteins, including F10	Association of viroplasm with crescent membranes
	A32	Genome encapsidation; required for packaging DNA and I6
	I6	Genome encapsidation; binds specifically to terminal hairpins in DNA
Formation of MV	A4	Core assembly during morphogenesis; present in outer palisade layer of core wall
	A3	Formation of morphologically normal and transcriptionally active cores; proteolytically processed during morphogenesis
	A19	Efficient processing of core proteins
	G1	IV-to-MV transition; metalloprotease
	I7	IV-to-MV transition; cysteine protease, required for processing A3 and other proteins
	E10, A2.5, G4	IV-to-MV transition; thiol redox proteins (see Chapter 12)
Formation of wrapped virus particles	A27	Essential for this step; disulfide-bonded trimer bound to MV membrane
	B5, F13	Required for efficient wrapping; transmembrane proteins sorted to intracellular wrapping site(s)

[a]IV, immature virus particles; MV, mature virus particles.

The mature virus is released only upon lysis of the infected cell. However, some of these particles become engulfed by the membranes of a second intracellular compartment, probably a *trans*-Golgi or early endocytic compartment, to form the wrapped virus particle (Fig. 13.20). The mature particle is transported to the site(s) of wrapping via microtubules. The remodeling of organelle membranes to form the wrapped particle depends on a number of viral proteins that are present only in this type of particle (Table 13.3) and that appear to be sorted to wrapping sites via the secretory pathway. The wrapped particle can be released from the cell as the two-membrane-containing extracellular enveloped virus particle, following transport to the cell surface and fusion of its outer membrane with the plasma membrane (Fig. 13.20). As the mature and the extracellular enveloped virions bind to different cell surface receptors, the release of two types of infectious particle may increase the range of cell types that can be infected. A significant proportion of enveloped virus particles are not released following membrane fusion but, rather, remain attached to the host cell surface as cell-associated enveloped virions. The mechanisms of transport and egress that produce these cell-associated particles are amazing processes that depend on major reorganization of components of the host cell cytoskeleton.

Wrapped virus particles initially travel from sites of assembly to the plasma membrane on microtubules, carried by a cellular motor protein of the kinesin family. The interaction of these particles with the motor depends on the viral A36 protein present in their outer membrane, which binds to the light chain of the kinesin motor. Such active transport allows movement of the large wrapped virus particles to the cell periphery in less than 1 min (compared to an estimated 10 h that would be required by passive diffusion!). Remodeling of the dense layer of cortical actin that lies beneath the plasma membrane (Fig. 2.4) is also required to deliver these particles to the cell surface. This phenomenon is induced by a viral protein that modulates the cellular signaling pathway that regulates the dynamics of cortical actin.

The particles formed by fusion of wrapped virus particles with the plasma membrane remain cell associated because of a remarkable activity: they induce a further dramatic reorganization of the actin cytoskeleton just below the site of fusion. The number of typical actin stress fibers is significantly decreased, as the virus induces the formation of

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BOX 13.8

DISCUSSION

The enigma of how the vaccinia virus crescent membrane formed

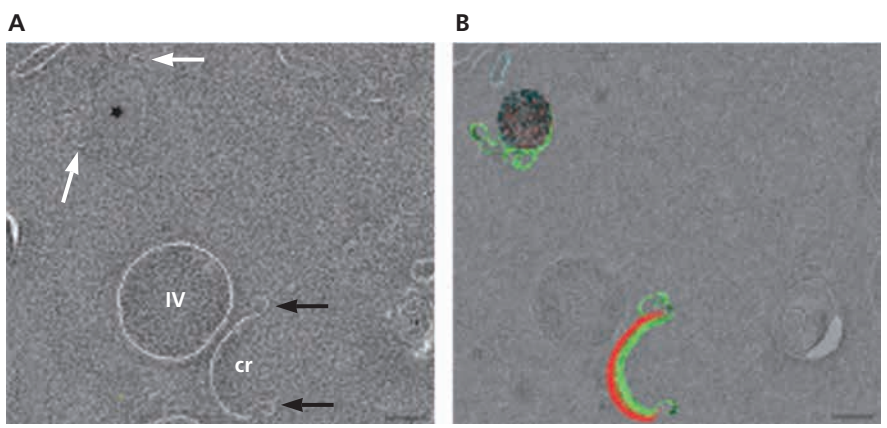
It is simple to visualize how reorganization and fusion of internal cellular membranes can "wrap" structures in a double membrane, as during formation of wrapped particles of vaccinia virus (Fig. 13.20). In contrast, it is not at all obvious how viral structures containing a **single** lipid bilayer, namely, the crescent that is the first structure built during vaccinia virus assembly (see the figure), can arise by a nonbudding mechanism. This conundrum led to the early proposal that the crescent membrane is synthesized *de novo* from cellular lipids. No mechanism for such *de novo* assembly has been identified, and it is generally agreed that crescents are derived from preexisting cellular membranes. There is accumulating evidence that the ER is the source of the crescent membrane.

- Several of the major viral membrane proteins are inserted into the ER membrane in infected cells, and one (A9) is present, near sites of assembly, in tubular structures that contain the ER luminal enzyme protein disulfide isomerase. Furthermore, when a heterologous signal sequence was added to the N terminus of A9, the signal sequence was cleaved off and only the truncated protein was detected in immature and mature virus particles. As signal peptidase, which removes signal sequences, resides in the ER (Chapter 12), this observation provides compelling support for the view that viral membrane proteins travel from the ER to the crescent membrane.
- Inhibition of transport from the ER to the Golgi compartments via the secretory pathway did not impair assembly of immature or mature virus particles (although the subsequent wrapping step was blocked).
- Repression of synthesis in infected cells of the major components of the crescent membrane, namely, the transmembrane proteins A14 and A17 and the scaffold protein D13, led to formation of only irregular membranes or small vesicles and tubules and the accumulation of dense, virus-specific inclusions.
- Several other viral proteins are required for assembly of the crescent membrane and immature virus particles. Of these, the L2 protein is synthesized in infected cells before viral factories appear and is associated with the ER at sites at which crescent membranes will form. Repression of L2 synthesis resulted in complete inhibition of vaccinia virus reproduction and assembly of mature virus particles. Some immature virus-like particles did assemble but contained greatly reduced quantities of several viral membrane proteins. Very short crescents associated with the major crescent proteins and the ER chaperone calnexin were also observed.

These observations indicate that the crescent membrane is constructed from the ER under the direction of viral proteins, but the mechanism remains an enigma.

Hussain M, Weisberg AS, Moss B. 2006. Existence of an operative pathway from the endoplasmic reticulum to the immature poxvirus membrane. *Proc Natl Acad Sci U S A* **103:**19506–19511.

Maruri-Avidal L, Weisberg AS, Bisht H, Moss B. 2013. Analysis of viral membranes formed in cells infected by a vaccinia virus L2 deletion mutant suggests their origin from the endoplasmic reticulum. *J Virol* **87:**1861–1871



Cryo-electron tomography of vaccine virus-infected HeLa cells showing a 0.9-nm slice taken from a 200-nm-thick cryo-section, with an immature virus particle (IV), a cresent (cr), and a small patch of scaffold protein (star) indicated. The black arrows indicate the ends of the crescent membrane that typically curl away from the coated scaffold region, and the white arrows the small membrane curls that are seen close to patches of the scaffold protein. In the right panel, the crescent membrane and scaffold have been rendered in green and red, respectively. Adapted from C. Suarez et al., *Cell. Microbiol.* **15:**1883–1895, 2010. Courtesy of J. Krijnse Locker, Heidelberg University, Heidelberg, Germany.

new, filamentous, actin-containing structures. Each of these, which are termed actin tails, is in contact with a single virus particle (Fig. 13.21A and B). Viral particles attached to the tips of actin tails are propelled by the polymerization of actin at the front end of the tail and its depolymerization at the back end. As the infection progresses, they can be seen on large microvilli induced by the actin tails (Fig. 13.21B). Formation of actin tails in vaccinia virus-infected cells requires the same viral protein (A36) that allows transport of wrapped virions along microtubules. This protein is phosphorylated at specific positions by the cellular tyrosine kinase Src, which plays an important role in the regulation of actin dynamics in uninfected cells. Phosphorylation of A36 triggers its dissociation from kinesin and allows binding of cellular proteins that promote actin polymerization (Fig. 13.22).

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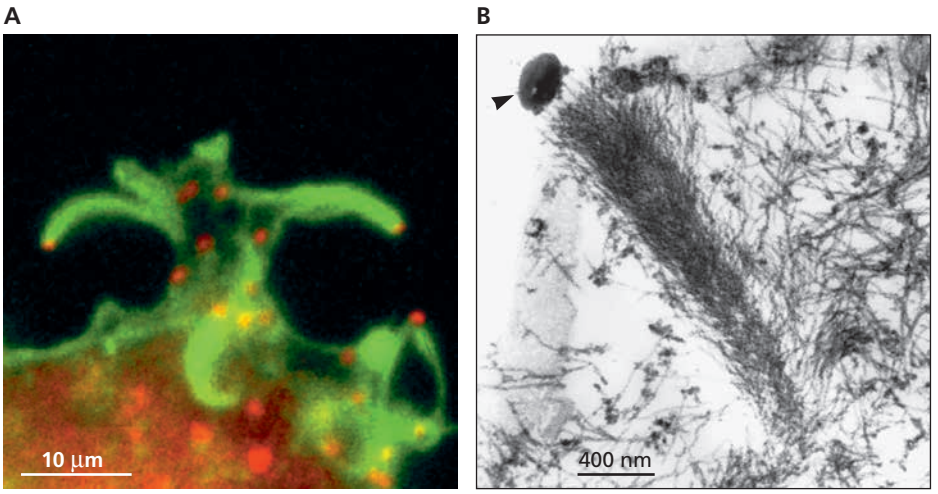


Figure 13.21 Movement of vaccinia virus on actin tails. (A) Immunofluorescence micrograph of virus particles (red) at the ends of the cell surface projections containing actin tails (green). The coincidence of the tips of the projecting actin and viral particles gives yellow-orange signals, indicating that the particles are projected from the cell surfaces on the tips of actin tails. When infected cells are plated with uninfected cells, such actin-containing structures to which virus particles are attached can be seen extending from the former into the latter. **(B)** Electron micrograph of a virus particle attached (arrowhead) to an actin tail. From S. Cudmore et al., *Nature* **378:**636–638, 1995, with permission. Courtesy of S. Cudmore and M. Way, European Molecular Biology Laboratory.

The formation of vaccinia-actin tails is necessary for efficient spread of the virus; mutants that cannot induce these structures form only small plaques on cells in culture. Cellular projections containing actin tails with virus particles at their tips can extend from infected cells toward neighboring uninfected cells, suggesting that they may facilitate direct cell-to-cell spread of infectious particles. More importantly for rapid spread of vaccinia virus, they mediate a remarkable mechanism of repulsion of virus particles from infected cells (Box 13.9).

Intranuclear Assembly

The problem of egress is especially acute for the enveloped herpesviruses, because the nucleocapsids assemble in the nucleus. The pathway by which the virus leaves the cell has been a topic of fierce controversy, centered on where and when the viral envelope is acquired. A large body of evidence now favors the less intuitive double-envelopment model summarized in Fig. 13.23.

The first step in egress is exit of nucleocapsids from the nucleus, which is achieved not by transport through nuclear pore complexes but, rather, by an unusual budding mechanism (Fig. 13.23), which was shown subsequently to export large ribonucleoproteins containing certain cellular mRNAs (Box 13.10). In the case of herpes simplex virus 1, a subset of the tegument proteins, including VP16, associates with the nucleocapsid prior to budding. Late in infection, two viral proteins act in concert with the cellular protein kinase C to induce disruption of the nuclear lamina (Fig. 13.24) and subsequently drive budding of the nucleocapsid through the inner nuclear membrane. The de-envelopment reaction that subsequently releases nucleocapsids into the cytoplasm (Fig. 13.23) requires either the gB or the gH glycoprotein and the cellular ATPase torsinA.

The second envelopment, in which particles acquire their envelopes, takes place at the cytoplasmic surfaces of compartments of the *trans*-Golgi network. Viral membrane proteins, including those necessary for secondary envelopment (e.g., gD, gE/gI, gM, and the UL20 protein), are sorted to these cellular compartments via the secretory pathway (Chapter 12). Some tegument proteins accumulate at the sites of secondary envelopment and are required for this step. Others associate with the nucleocapsid in the cytoplasm. The latter proteins include the US3 required for nuclear exit and the UL36 and UL37 proteins, which are required for transport of nucleocapsids through the cytoplasm. Once the nucleocapsid reaches the *trans*-Golgi network, interactions between these two classes of tegument protein must take place prior to envelopment. Nevertheless, the proteins that mediate such final assembly of the tegument have not yet been identified, nor have those that induce membrane budding and fusion. Some recent observations hint that such viral proteins may function via some components of the cellular Escrt machinery that mediates the release of simpler enveloped viruses.

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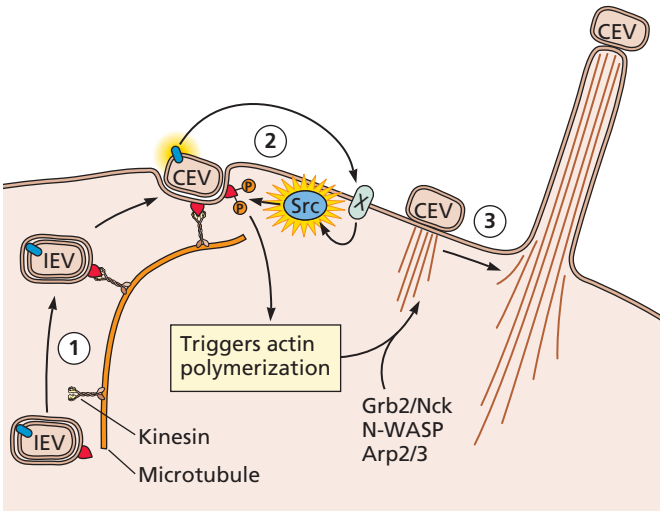


Figure 13.22 Model for the switch from microtubule- to actin-dependent transport of vaccinia virus particles. The A36R protein (red) present in the outer membranes of wrapped virus particles binds to the light chain of the kinesin motor, which then transports the particles to the cell periphery. Remodeling of cortical actin by viral proteins allows close approach of the particles to the plasma membrane. Fusion of the outer membrane of wrapped virus particles with the plasma membrane releases cell-associated virus particles, which carry the B5R glycoprotein (blue) in their new outer membrane. This viral protein activates the cellular Src tyrosine kinase, presumably via interaction with one or more cellular membrane proteins (X). Src then phosphorylates the membrane-associated A36R protein, a modification shown by genetic experiments to be essential for formation of actin tails. Furthermore, A36 remains bound to kinesin in vaccinia virus-infected cells that lack Src or that are treated with inhibitors of this kinase. Phosphorylated A36R binds via adapter (Grb and Nck) and scaffolding (N-Wasp) proteins to proteins that induce actin polymerization. Such polymerization drives the formation of actin tail-containing protrusions that project cell-associated virus particles away from the host cell. The viral F11 protein-induced inhibition of signaling via the small G protein RhoA leads to increased microtubule dynamics and facilitates transport of progeny virus particles to the plasma membrane. Such inhibition also stimulates the migration of vaccinia virus-infected cells, a property that promotes spread of progeny virus particles. CEV, Cell-associated enveloped virions; IEV, Intracelleular enveloped virion. Adapted from A. Hall, *Science* **306:**65–67, 2006, with permission.

Release of Nonenveloped Viruses

The most usual fate of host cells permissive for reproduction of nonenveloped viruses is death (but see Volume II, Chapter 5). In natural infections, the host defenses are an important cause of infected-cell destruction. However, infection by these viruses destroys host cells more directly; they are cytopathic to cells in culture. Although the mechanisms by which reproduction of nonenveloped viruses induces death and lysis of host cells are not well understood, some viral proteins that induce rupture of particular membranes and/or cell lysis have been identified.

The VP4 protein of the polyomavirus simian virus 40, which accumulates late in infection, perforates membranes *in vitro* by forming pores in them. It is considered a **viroporin**, a class of small, hydrophobic proteins that are encoded in the genomes of a variety of viruses; examples include the influenza A virus M2 protein and Vpu of human immunodeficiency virus type I (see Volume II, Chapter 7). Simian virus 40 VP4 associates with the nuclear envelope, where it induces release of nuclear contents into the cytoplasm. Such activity assuredly contributes to the escape of newly assembled virus particles from the nucleus and might contribute to lysis of the host cell. However, the viral agnoprotein is also likely to be important; the analogous protein of the human polyomavirus JC virus has been reported to form pores in the plasma membrane to facilitate release of progeny virus particles. A small viral protein is also necessary for efficient nuclear disruption and lysis of cells infected by human adenovirus. This adenovirus death protein (ADP) accumulates in the nuclear envelope late in infection and stimulates release of virus particles, but its mechanism of action is not clear. The severe inhibition of cellular protein synthesis toward the end of the infectious cycle and disruption of cytoplasmic intermediate filaments upon cleavage of their components by the viral L3 protease are likely to facilitate release of adenovirus particles by compromising the structural integrity of the infected cell.

While cell lysis is the most common means of escape of naked viruses, there is evidence that some are released in the absence of any cytopathic effect. When poliovirus replicates in polarized epithelial cells resembling those lining the gastrointestinal tract (a natural site of infection), progeny virus particles are released exclusively from the apical surface by a nondestructive mechanism. The viral 2BC and 3A proteins induce the formation of infected-cell-specific vesicles that closely resemble autophagosomes (see Chapter 14). Coxsackie B virus particles are also released in autophagosomes. It has been proposed that these vesicles, which contain two membranes and virus particles late in infection provide a route for nonlytic release of particles assembled to the cytoplasm (Fig. 13.25A). Another pathogenic picornavirus, hepatitis A virus, leaves liver cells in culture in enveloped particles that resemble exosomes (Fig. 13.25B). This "wolf-in-sheep's-clothing" strategy for release prevents recognition of virus particles by neutralizing antibodies *in vitro* and presumably aids spread of the virus in the liver.

Maturation of Progeny Virus Particles

Proteolytic Processing of Structural Proteins

The products of assembly of several viruses are noninfectious particles. In all cases, proteolytic processing of specific proteins with which the particles are initially built converts them to infectious virions. The maturation reactions are carried out

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BOX 13.9

EXPERIMENTS

Repulsion of virus particles from infected cells accelerates vaccinia virus spread

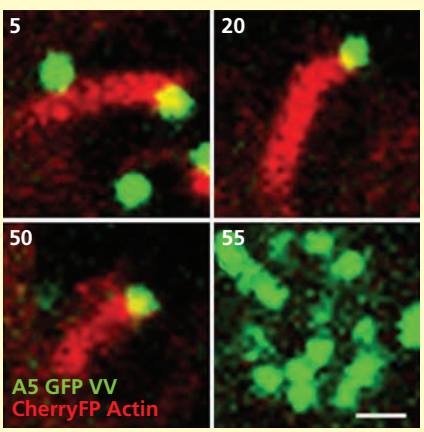
Vaccinia virus particles are spread by mechanisms that include increased migration of cells induced by infection and propulsion toward neighboring cells on actin projections (Fig. 13.21). Measurement of the rate of increase in the size of vaccinia virus plaques in various cell lines indicated that the virus crossed one cell every 1.2 h. This rate of spread is considerably higher than can be explained by either the assembly of progeny virus particles or the induction of infected-cell motility, both of which require 5 to 6 h after the initial infection. Mutant viruses defective for formation of actin tails infected new cells only every 5 to 6 h, consistent with the kinetics of the infectious cycle. This finding implicated actin tail formation in the rapid spread of vaccinia virus.

Virus particles containing a structural protein fused to enhanced green fluorescent protein (eGFP) and cells producing actin fused to Mcherry fluorescent protein were used to investigate the mechanism of rapid spread. Green virus particles were detected on red actin tails in cells that contained no viral factories or progeny virus particles. These structures appeared before viral factories, and particles on a red actin tail induced the formation of a new actin tail upon recontact with the same cells (see the figure and Movie 13.1: http://bit.ly/Virology_V1_Movie13-1), suggesting a mechanism of active repulsion of virus particles from infected cells.

The viral A33 and A36 proteins, which are required for formation of actin tails, are made early in the infectious cycle and accumulate in the plasma membrane at the edges of plaques. Mutant viruses that direct the synthesis of these proteins late rather than early in infection produce only small plaques. Furthermore, the synthesis of just these two proteins in uninfected cells allowed the formation of actin tails within 15 to 30 min after exposure to extracellular enveloped virus particles.

These observations identified a previously unrecognized mechanism of spread of vaccinia virus particles, repulsion from infected cells on actin tails toward neighboring cells. This process prevents superinfection and hence accelerates the rate of spread of the virus.

Doceul V, Hollinshead M, van der Linden L, Smith GL. 2010. Repulsion of superinfecting virions: a mechanism for rapid virus spread. *Science* **327:**873–876.



Simian BSC-1 cells synthesizing actin fused to cherry fluorescent protein (red) were infected at a low multiplicity of infection with vaccinia virus with a structural protein (A5L) fused to eGFP (green). Shown are actin tails formed at the times indicated (min) after infection, and before the appearance of large green viral factories at 55 min. Scale bar = 5 μm. The time-lapse movie shows such a cell and induction of a new actin tail when a virus particle at the tip of a red actin tail recontacts the cell surface. Adapted from C. Doceul et al., *Science* **327:**878–867, with permission. Courtesy of G. L. Smith, Imperial College, London, United Kingdom.

by virus-encoded enzymes and take place late in assembly of particles or following their release from the host cell. Proteolytic cleavage of structural proteins introduces an irreversible reaction into the assembly pathway, driving it in a forward direction. This modification can also make an important contribution to resolving the contradictory requirements of assembly and virus entry. One consequence of proteolytic processing is the exchange of covalent linkages between specific protein sequences for much weaker noncovalent interactions, which can be disrupted in a subsequent infection. A second is the liberation of new N and C termini at each cleavage site and, hence, opportunities for additional protein-protein contacts. Such changes in chemical bonding among structural proteins clearly facilitate virus entry, for the proteolytic cleavages that introduce them are necessary for infectivity. Accordingly, viral proteases and the structural consequences of their actions are of considerable interest. Moreover, these enzymes are excellent targets for antiviral drugs, as exemplified by the success of therapeutic agents that inhibit the human immunodeficiency virus type 1 protease.

Cleavage of Polyproteins

The alterations in the structure of the virus particle following proteolytic processing and their functional correlates are best understood for small RNA viruses, such as the picornavirus poliovirus. A single cleavage to liberate VP4 and VP2 from VP0 converts noninfectious provirions to mature virus particles (Fig. 13.5). As the viral proteases are not incorporated into particles, VP0 cleavage may be catalyzed by a specific feature of the capsid itself, with internal genomic RNA participating in the reaction. The structural changes induced by such maturation cleavage can be described in great detail, for the structures of mature and empty particles in which VP0 has not been cleaved have been determined at high resolution. Cleavage of VP0 allows the extensive internal structures of the particle (Fig. 4.12C) to be established and consequently is important for the stability of the virion.

Cleavage of VP0 to VP4 and VP2 is also necessary for the release of the RNA genome into a new host cell. The conformational transitions that mediate entry of the genome

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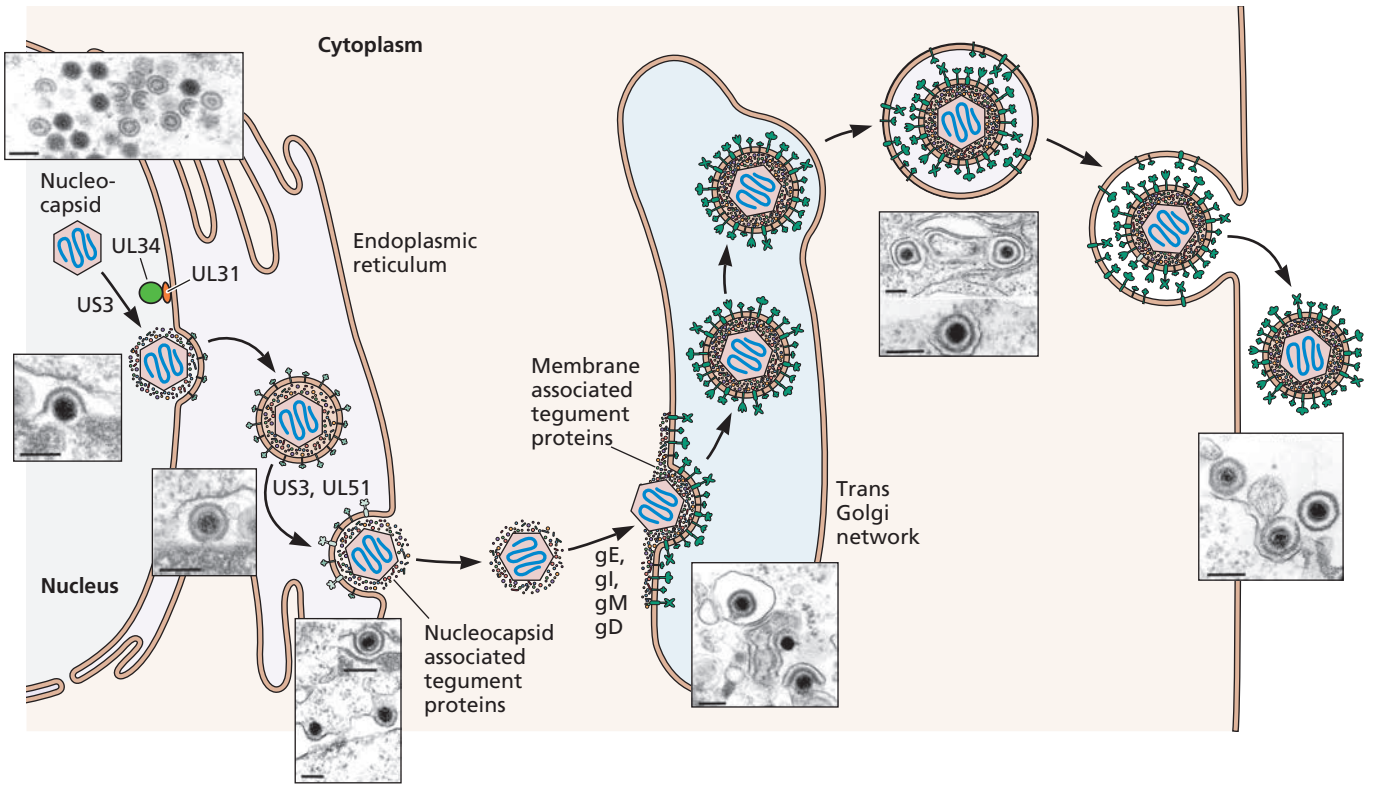


Figure 13.23 Pathway of herpesvirus egress. The mature nucleocapsid assembled within the nucleus (Fig. 13.8) initially acquires an envelope by budding through the inner nuclear membrane. The dense meshwork of protein filaments that abuts the inner nuclear membrane (the nuclear lamina) is dramatically reorganized and perforated (Fig. 13.24), presumably to allow juxtaposition of the nucleocapsid and membrane. Such disruption of the nuclear lamina requires the viral UL31 phosphoprotein and the UL34 transmembrane protein. These proteins, often called the nuclear export complex, associate with one another at the inner surface of the inner nuclear membrane and bind the proteins that form the lamina (lamins A/C and B) and cellular protein kinase C. This enzyme phosphorylates the lamins, while the viral US3 kinase phosphorylates the nuclear membrane protein emerin, which binds to lamins and has been implicated in the maintenance of nuclear integrity. These modifications are thought to disrupt the interactions that form the nuclear lamina. The UL31 protein interacts with nucleocapsids, and the UL31-UL34 assembly is sufficient to induce deformation and scission of membranes *in vitro*. These activities are inhibited by substitutions in the UL34 protein that block nuclear exit of nucleocapsids but not their assembly with UL31 at the inner nuclear membrane, suggesting that the two viral proteins drive budding through this nuclear membrane. Upon fusion with the outer nuclear membrane, this membrane is lost as unenveloped nucleocapsids are released into the cytoplasm. Some tegument proteins interact with the nucleocapsid in the cytoplasm, whereas others, including the UL11, UL46, and UL49 proteins, concentrate at sites of secondary envelopment. The latter are presumably localized at membranes of *trans*-Golgi compartments by interactions with the cytoplasmic domains of viral glycoproteins, such as the binding of the UL11 and UL49 proteins to the cytoplasmic domains of gE and gD. The myristoylated UL11 protein accumulates at the membranes of *trans*-Golgi compartments and directs other tegument proteins to sites of secondary envelopment. The viral envelope is acquired upon budding of tegument-containing structures into compartments of the *trans*-Golgi network. Virus particles formed in this way are thought to be transported to the plasma membrane in secretory transport vesicles and released upon membrane fusion, as illustrated. Viral gene products implicated in specific reactions are indicated. The reactions are illustrated in the electron micrographs of cells infected by the alphaherpesvirus pseudorabies virus. Bar, 150 nm. Adapted from T. C. Mettenleiter, *J. Virol.* **76:**1537–1547, 2002, with permission. Courtesy of T. C. Mettenleiter, Federal Research Center for Virus Diseases of Animals, Insel Riems, Germany.

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BOX 13.10

TRAILBLAZER

Budding as a mechanism for export of cellular mRNAs from the nucleus

Until quite recently, nuclear pore complexes were thought to provide the only routes for transport of molecules and macromolecules between the nucleus and cytoplasm. These large and architecturally elaborate structures (Fig. 5.23) allow free passage of molecules of less than 20 kDa and bidirectional transport up concentration gradients of larger proteins and RNAs (almost always packaged as ribonucleoproteins [RNPs]). Many viral genomes reach the nucleus by way of nuclear pore complexes (Chapter 5). Toward the end of infectious cycles, progeny genomes of several viruses, including influenza viruses and retroviruses, are exported to the cytoplasm via these structures, whereas particles that complete assembly in the nucleus (for example, adenoviruses and polyomaviruses) escape that organelle upon destruction and lysis of the host cell.

When initially discovered, nuclear budding of newly assembled nucleocapsids of alphaherpesviruses was a virus-specific mechanism with no counterpart in uninfected cells. Subsequently, however, budding of cellular mRNAs was discovered in studies of the development of neuromuscular junction synapses in the body wall muscles of *Drosophila* larvae. This process depends on the secreted signaling protein wingless (Wnt). Binding of Wnt to its receptor, *Drosophila* frizzled-2 (Dfz2), on a postsynaptic cell induces endocytosis of the receptor, its cleavage, and import of the C-terminal segment (Dfz2C) into the nucleus. Application of various imaging techniques established that the latter protein is associated with large, RNA-containing granules in the space between the inner and outer nuclear membranes. These granules could be seen leaving the nucleus. They contained multiple mRNAs coding for postsynaptic proteins, and mutations that impaired the formation of the granules prevented proper synaptic differentiation. Like budding of herpesviral nucleocapsids, formation of Dfz2C-containing granules and invaginations of the inner nuclear membrane require protein kinase C and phosphorylation of lamins. Furthermore, exit of both the mRNA-containing granules in *Drosophila* larvae and herpesviral nucleocapsids in mammalian cells to the cytoplasm depends on torsinA.

A budding mechanism might be necessary for export of very large structures, be they ribonucleoprotein granules or viral assemblies. Such a mechanism might also ensure simultaneous export of multiple mRNAs coding for proteins that operate together for subsequent cotransport and cotranslation. Despite previous reports of perinuclear granules and inner nuclear membrane invaginations in both animal and plant cells, budding from the nucleus of other cellular mRNAs has not yet been demonstrated.

Speese SD, Ashly J, Joki V, Nunnari J, Barniw R, Alaman B, Koon A, Chang Y-T, Li Q, Moore MJ, Budnik V. 2012. Nuclear envelope budding enables large ribonucleoprotein particle export during synaptic Wnt signaling. *Cell* **149:**832–846.

Nuclei from Drosophila larval body wall muscle were examined by live-cell imaging after incubation with an RNA-specific dye (E46) (green; left panel) and then visualized after being fixed and stained with antibodies to lamin C (red; middle panel). These images are shown superimposed on the right. The arrow indicates an RNA-containing granule. Such granules were observed moving away from the nucleus during time-lapse imaging. Adapted from S. Speese et al., *Cell* **149:**832–836, 2012, with permission. Courtesy of V. Budnik, University of Massachusetts Medical School.



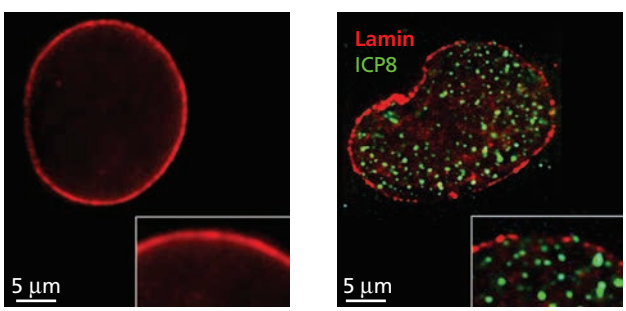


Figure 13.24 Disruption of the nuclear lamina in herpes simplex virus 1-infected cells. Human cells mock infected or infected with herpes simplex virus 1 for 16 h were examined by indirect immunofluorescence. The cellular lamin A/C and viral 1CP8 proteins are in red and green, respectively. The insets show magnified regions of equal sizes. Adapted from M. Simpson-Holley et al., *J. Virol.* **79:**12840–12851, with permission. Courtesy of D. Knipe, Harvard University Medical School.

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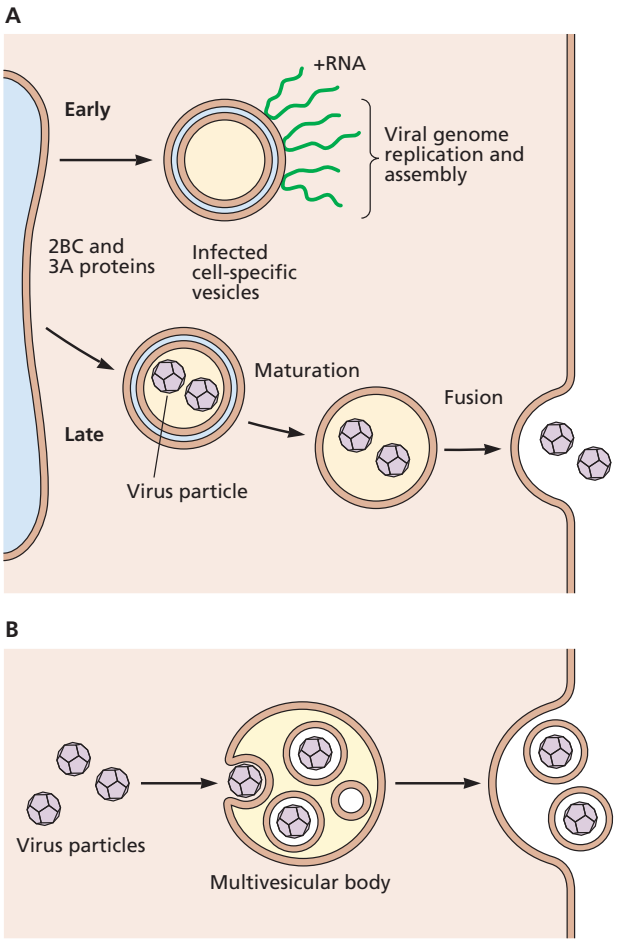
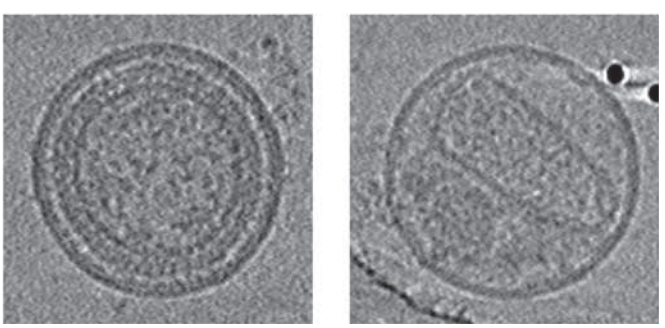


Figure 13.25 Models for nonlytic release of picornavirus particles. (A) Synthesis of the poliovirus 2BC and 3A proteins leads to formation of infected-cell-specific vesicles that resemble autophagosomes. The surfaces of these vesicles are sites of genome replication and assembly (top). It has been proposed that as autophagosome-like vesicles are formed from these membranes later in infection, they enclose virus particles. Maturation of such particle-containing vesicles in a manner analogous to the maturation of autophagosomes would result in complete or partial degradation of the inner membrane. Subsequent fusion of the mature vesicle with the plasma membrane would release virus particles. This model is based on the observation that RNA interference-mediated knockdown of proteins required for the formation of autophagosome-like vesicles reduced the yield of extracellular virus particles to a greater degree than the yield of intracellular particles. **(B)** Hepatitis A virus, also a member of the family *Picornaviridae*, is a common cause of hepatitis and is transmitted by an enteric route. Virus particles released from hepatocytes infected in culture were found to be enclosed within membrane vesicles that carried 1 to 4 particles. Such membrane-enclosed particles were also observed in the blood of humans suffering from hepatitis A virus infection. These particles are infectious and resistant to inhibition by neutralizing antibodies. The enveloped virus particles resemble exosomes in size, and their formation requires cellular proteins that participate in the formation of multivesicular bodies and exosomes, such as Alix and Escrt-III (Fig. 13.17). It has therefore been proposed that hepatitis A virus particles bud into multivesicular bodies upon interaction of the capsid with such proteins. Fusion of the multivesicular body with the plasma membrane would result in release of virus particle enclosed within cellular membrane that is not modified by insertion of viral proteins.

following attachment of the virus to its receptor are not fully understood. However, many alterations that impair receptor binding and entry map to those regions of the capsid proteins that adopt their final organization only upon VP0 cleavage. Cleavage of VP0 therefore not only stabilizes the virus particle but also "spring-loads" it for the conformational transitions that take place during the entry and release of the genome.

Following or during release of most retrovirus particles, the Gag polyprotein is processed by the viral protease, concomitantly with substantial morphological and conformational rearrangements (Fig. 13.26; Box 13.11). Such processing plays an essential part in the mechanisms by which most infectious retroviruses are assembled and released. As we have seen, interactions among Gag polyproteins and the viral RNA and between their NC and MA domains and the plasma membrane build and organize an assembling retrovirus particle. Efficient and orderly assembly also depends on "spacer" peptides that are removed during proteolysis. Furthermore, the membrane-binding signal of MA of human immunodeficiency virus type 1 is exposed when MA is part of Gag but is blocked by a C-terminal α-helix of MA in the mature protein. It is therefore very unlikely that retrovirus particles could be constructed correctly from mature Gag proteins. Indeed, alterations that increase the catalytic activity of the viral protease inhibit budding and production of infectious particles, indicating that premature processing of the polyproteins is detrimental to assembly. On the other hand, the covalent connection of the structural proteins that is so necessary during assembly is incompatible with the release of the internal core following fusion of the viral envelope with the membrane of a new host cell. Such covalent linkage also precludes efficient activity of virion enzymes, which are incorporated as Gag-Pol polyproteins. In some particles, including those of

Figure 13.26 Morphological rearrangement of retrovirus particles upon proteolytic processing of the Gag polyprotein. These two cryo-electron micrographs show the maturation of human immunodeficiency virus type 1 virus particles. **(Left)** The immature particles contain a Gag polyprotein layer below the viral membrane and its external spikes. **(Right)** Processing of Gag converts such particles to mature virus particles with elongated cone-shaped internal capsids. Courtesy of G. Jensen and W. Sundquist, University of Utah School of Medicine.



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BOX 13.11

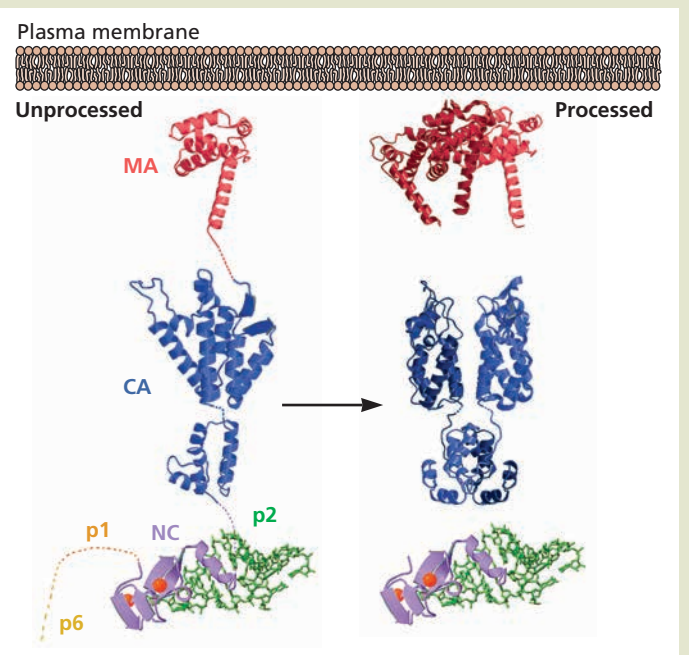
DISCUSSION

Model for refolding of the human immunodeficiency virus type 1 CA protein on proteolytic processing of Gag

The model for the radial organization of the human immunodeficiency virus type 1 Gag polyprotein (left), which contains the spacer peptides p1, p2 and p6, is based on cryo-electron micrographs like those shown in Fig. 13.4. The three-dimensional structures of the processed proteins, the MA trimer (red), the CA dimer (blue), and monomeric NC (violet) bound to the SL3 packaging signal (green), shown on the right, are derived from high-resolution structures discussed in this and preceding chapters.

In the X-ray crystal structure of the N-terminal portion of mature CA (right), the charged N terminus is folded back into the protein by a β hairpin formed by amino acids 1 to 13 and forms a buried salt bridge with the carboxylate of Asp51. The lack of a charged N terminus prior to cleavage of CA from MA and the steric difficulties of burying the N terminus of CA attached to an MA extension (left) indicate that the β hairpin and buried salt bridge can form only after proteolytic cleavage. Furthermore, the viral protease recognizes the cleavage site between MA and CA in an extended conformation. As the N-terminal β hairpin of mature CA forms a CA-CA interface, it has been proposed that proteolytic cleavage and the consequent refolding of the N terminus of CA facilitate the rearrangements to form the conical core during maturation of virus particles. Alteration of amino acids in this interface inhibits core assembly and formation of infectious viral particles in infected cells, consistent with this model. Courtesy of T. L. Stemmler and W. Sundquist, University of Utah.

von Schwedler UK, Stemmler TL, Klishko VY, Li S, Albertine KH, Davis DR, Sundquist WI. 1998. Proteolytic refolding of the HIV-1 capsid protein amino-terminus to facilitate viral core assembly. *EMBO J* **17:**1555–1568.



Moloney murine leukemia virus, the protease also removes a short C-terminal segment of the cytoplasmic tail of the TM envelope protein to activate the fusionogenic activity of TM. The retroviral proteases that sever such connections therefore are absolutely necessary for the production of virions, even though they are dispensable for assembly.

The retroviral proteases belong to a large family of enzymes with two aspartic acid residues at the active site (aspartic proteases). The viral and cellular members of this family are similar in sequence, particularly around the active site, and are also similar in three-dimensional structure. All aspartic proteases contain an active site formed between two lobes of the protein, each of which contributes a catalytic aspartic acid. The retroviral proteases are homodimers in which each monomer corresponds to a single lobe of their cellular cousins. Consequently, the active site is formed only upon dimerization of two identical subunits. This property undoubtedly helps avoid premature activity of the protease within infected cells, in which the low concentration of the polyprotein precursors mitigates against dimerization. Indeed, dimerization of the protease appears to be rate limiting for maturation of virus particles. Fusion of the protease to the NC domain of Gag also inhibits dimerization. Consequently, synthesis of the protease as part of a polyprotein precursor not only allows incorporation of the enzyme into assembling particles but also contributes to regulation of its activity. These properties raise the question of how the protease is activated, a step that requires its cleavage from the polyprotein. Polyproteins containing the protease (e.g., made in bacteria) possess some activity, sufficient to liberate fully active enzyme at a very low rate *in vitro*. It is therefore thought that such activity of the polyproteins initially releases protease molecules within the particle. Furthermore, it has been shown, using Gag-Pol proteins yielding distinguishable cleavage products, that the initial proteolytic cleavages are intramolecular. The high local concentrations of protease molecules within the assembling

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particle would facilitate subsequent dimerization of protease molecules to form the fully active enzyme.

Cleavage of Precursor Proteins

Like its retroviral counterpart, the adenoviral protease converts noninfectious particles to infectious particles, in this case by cleavage at multiple sites within six structural proteins (Fig. 13.10). Although the adenoviral enzyme does not process polyprotein precursors, the cleavage of so many proteins alters protein-protein interactions necessary for assembly in preparation for early steps in the next infectious cycle; particles that lack the protease are not infectious. This enzyme is a cysteine protease containing an active-site cysteine and two additional cysteines, all highly conserved. One mechanism by which its activity is regulated is by interaction with a small peptide, a product of cleavage of the structural protein pVI, or with pVI itself. The pVI peptide binds covalently via a disulfide bond to the proteases both *in vitro* and in virus particles to increase the catalytic efficiency of the enzyme over 1,000-fold. A second cofactor is the viral DNA genome, along which the protease-pVI assembly moves rapidly by one-dimensional diffusion. Several lines of evidence indicate that this movement facilitates the association of the activated protease with its far more numerous substrates and their cleavage within virus particles.

Other Maturation Reactions

Newly assembled virus particles appear to undergo few maturation reactions other than proteolytic processing. However, the trimming of certain oligosaccharides, or formation of disulfide bonds, is known to be required for the infectivity in some cases. Moreover, a surprising extracellular assembly process has been identified recently (Box 13.12).

Terminal sialic acid residues are removed from the complex oligosaccharides added to the envelope HA and NA glycoproteins of influenza A virus during their transit to the plasma membrane. The influenza A virus receptor is sialic acid, which is specifically recognized by the HA protein. Consequently, newly synthesized virus particles have the potential to aggregate with one another and with the surface

BOX 13.12

EXPERIMENTS

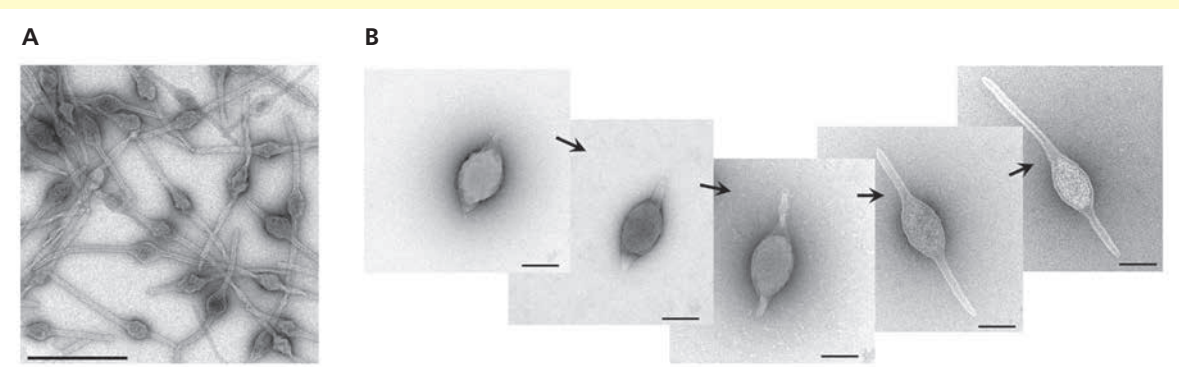
A notable example of virus maturation: extracellular assembly of specific structures

Acidianus two-tailed virus was discovered in an acidic hot spring (pH 1.5, 85 to 93°C) at Pozzuoli, Italy, where it reproduces in the thermophilic archaeon *Acidianus convivator*. **(A)** The virus particles isolated from this source have a lemon-shaped body with filamentous tails of different lengths protruding from each end. **(B)** However, when the virus was propagated in host cells grown in culture at 75°C, the released particles lacked such tails. Remarkably, tails formed over 1 week when such particles were incubated at 75°C in the **absence** of host cells (left to right). Moreover, this extracellular assembly reaction was complete in less than 1 h when particles were incubated at the temperatures optimal for host cell growth, 85 to 90°C.

Although the morphological changes that accompany maturation of virus particles are well documented (see the text), *Acidianus* two-tailed virus represents the first example of extracellular assembly. This capacity implies that the tailless particles released from host cells contain all the components and information necessary for tail assembly. The tails are presumed to facilitate attachment of virus particles to host cells.

Häring M, Vestergaard G, Rachel R, Chen L, Garrett RA, Prangishvili D. 2005. Independent virus development outside a host. *Nature* **436:**1101–1102.

Electron micrographs of *Acidianus* two-tailed virus particles isolated from a hot spring (A) or released from host cells infected in culture at 75°C and maintained in cell-free medium at 75°C for 0, 2, 5, 6, and 7 days (B, left to right). Scale bars, 0.5 μm **(A)** and 0.1 μm **(B)**. From M. Häring et al., *Nature* **436:**1101–1102, 2005, with permission. Courtesy of David Prangishvili, Institut Pasteur, Paris, France.



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of the host cell by binding of an HA molecule on one particle to a sialic acid present in an envelope protein of another particle or in cell surface proteins. Such aggregation is observed when the viral neuraminidase is inactivated. The neuraminidase eliminates such binding of newly synthesized virus particles to one another and to cell surface proteins. The activity of this enzyme, which removes terminal sialic acid residues from oligosaccharide chains, is essential for effective release of progeny virus particles from the surface of a host cell. This requirement has been exploited to develop new drugs (e.g., oseltamivir phosphate [Tamiflu]) designed specifically to inhibit the viral neuraminidase.

The capsids of nonenveloped papillomaviruses, which are built from 72 pentamers of the major structural protein L1, are stabilized by intermolecular disulfide bonds between specific L1 cysteine residues. This protein does not travel the secretory pathway, raising the question of how such cysteines become oxidized. When the human papillomavirus type 16 (or 18) L1 and the minor capsid (L2) proteins are made in mammalian cells, they assemble to form particles that lack disulfide bonds and are less stable and less infectious than mature capsids. Disulfide bonds form spontaneously at a low rate when immature particles are incubated 37°C and more quickly in the presence of oxidizing agents. This process is accompanied by increased stability and infectivity and the appearance of more regularly structured particles. Papillomaviruses are thought to be released slowly during natural infections as the outer layers of the epithelia in which they replicate are shed. It is therefore likely that newly assembled capsids are exposed to an oxidizing environment for a considerable period (several days) prior to release.

Cell-to-Cell Spread

All progeny virions must infect a new host cell in which the infectious cycle can be repeated. Many viruses are released as free particles by the mechanisms described in preceding sections and must travel within the host until they encounter a susceptible cell. The new host cell may be an immediate neighbor of that originally infected or a distant cell reached via the circulatory or nervous systems of the host. Virus particles are designed to withstand such intercellular passage, but they are susceptible to several host defense mechanisms that can destroy them (Volume II, Chapters 2 to 4). Localized release of virus particles only at points of contact between an infected cell and its uninfected neighbor(s) can minimize exposure to these host defense mechanisms. Furthermore, some viruses can spread from one cell to another by mechanisms that circumvent the need for release of progeny virus particles into the extracellular environment.

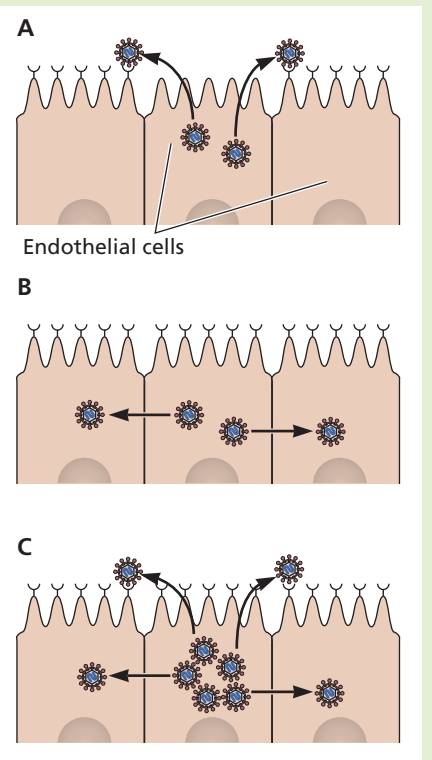
In some cases, virus particles can be transferred directly from an infected cell to its neighbors (Box 13.13), a strategy that avoids exposure to host defense mechanisms targeted against extracellular virus. Such cell-to-cell spread, which depends on the viral fusion machinery, is defined operationally as infection that still occurs when released virus particles are neutralized by addition of antibodies. In the case of herpes simplex virus 1, the glycoproteins that promote fusion during entry (gB, gH, and gL) and another glycoprotein (gD) are required. The latter protein binds to the cell surface protein nectin-1, which is localized to cell-cell junctions. Two additional glycoproteins (as well as other proteins, including UL34) are also necessary for efficient cell-to-cell spread but have no known role in entry of extracellular particles. Mutant viruses that lack the gE or gI gene form only small plaques when transfer of free virus particles from one cell to another is prevented. They are also defective for both lateral spread of infection in polarized epithelial cells and the spread of infection from an axon terminal to an uninfected neuron in animals. Such cell-to-cell spread of herpesviruses

BOX 13.13

BACKGROUND

Extracellular and cell-to-cell spread

(A) Many viruses spread from one host cell to another as extracellular virus particles released from an infected cell. Such extracellular dissemination is necessary to infect another naive host. Some viruses, notably alphaherpesviruses, paramyxoviruses, and some retroviruses, can also spread from cell to cell without passage through the extracellular environment **(B)** and can therefore be disseminated by both mechanisms **(C)**.



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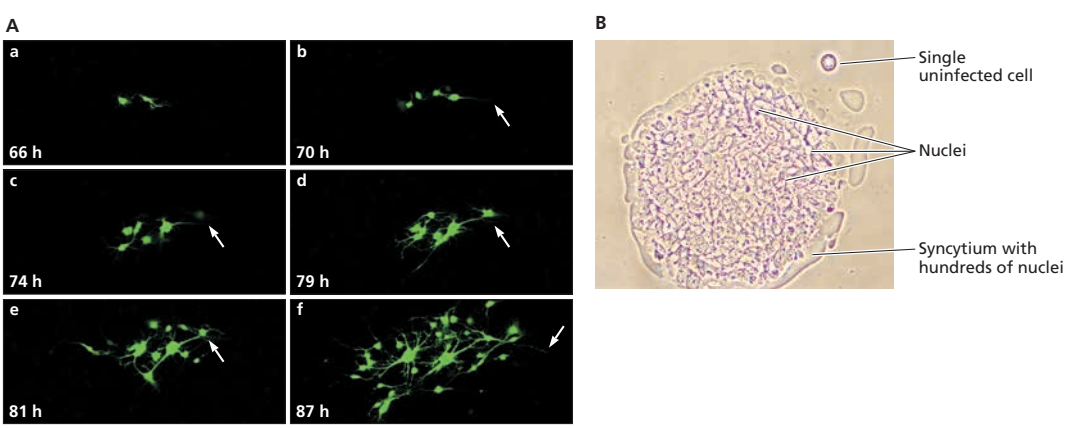
is thought to occur at specialized sites, such as tight junctions of epithelial cells, and by synaptic contacts between individual neurons, but the molecular mechanism is not clear.

Direct cell-to-cell spread is the predominant mechanism for transmission of human immunodeficiency virus type 1 and other retroviruses. Specialized sites of close intercellular contact called **virological synapses** assemble when an infected cell contacts an uninfected neighbor. Virological synapses form at lipid raft regions of the plasma membrane that are enriched in cholesterol and sphingomyelin (see Chapter 12) and also the sites of release of viral particles by budding into the extracellular space. In virological synapses, the viral Gag and Env proteins accumulate in the donor cell membrane, and the CD4 and CxCr4 coreceptors in those of acceptor cells. Env-CD4 interactions are required for intercellular transfer of human immunodeficiency virus type 1 (Volume II, Fig. 7.14). This mode of transmission is some 2 to 3 orders of magnitude more efficient than infection via entry of extracellular virions. Formation of stable filopodia or nanotube contacts between uninfected and infected cells as a result of strong association of the viral Env protein of released virus particles with its receptor on uninfected cells has also been observed. Virus particles then travel toward the uninfected cell along the outer surfaces of such bridges.

Persistent measles virus (a paramyxovirus) infection of the brain is associated with subacute sclerosing panencephalitis (Volume II, Appendix, Fig. 15). Little infectious virus can be recovered from brain tissue of patients with this disease, although the genomic RNA and viral proteins are present. Indeed, budding of virus particles does not take place from the surfaces of infected mouse or human neurons in culture, which contain nucleocapsids accumulating at presynaptic membranes, nor does spread of measles virus between cultured neurons require the viral receptors (cell-cell contact and the fusion protein are necessary). In neurons, measles virus therefore spreads without the release and attachment to infected cells of free virus particles; rather, it spreads from cell to cell, most likely through synapses. Measles virus may also spread directly between other cell types as virological synapses form between infected dendritic cells and uninfected T cells.

There are other examples of more-radical mechanisms of transfer. In astrocytes (supporting cells of the central nervous system), measles virus spreads by inducing the formation of syncytia, sheets of neighboring cells fused to one another (Fig. 13.27A). Certain cell types infected by human immunodeficiency virus type 1 also form syncytia when they would not normally do so (Fig. 13.27B). Even more remarkable are the mechanism by which formation of actin tails repels vaccinia virus particles from infected cells to accelerate their spread to uninfected neighbors (Box 13.9) and the actions of the movement proteins encoded in the genomes of all plant viruses (Box 13.14).

Figure 13.27 Formation of syncytia. (A) Cell-to-cell spread by measles virus. Human astrocytoma cells were infected at a low multiplicity of infection with a recombinant measles virus encoding a green fluorescent protein. The autofluorescence of this protein identifies infected cells (a) and allows the spread of the virus to be monitored in living cells. With increasing time, the virus spreads to cells neighboring those initially infected and can be clearly seen in the processes connecting the cells that become infected (b to f). The arrows point to an extended astrocyte process of a newly infected cell (b), the weak autofluorescence of the nucleus of a cell in a very early phase of infection (c), the nucleus from the same cell 5 and 7 h later (d and e, respectively), and an extended astrocytic process issuing from the cell shown in panels d and e (f). From W. P. Duprex et al., *J. Virol.* **73:**9568–9575, 1999. Courtesy of W. D. Duprex, Queen's University, Belfast, United Kingdom. **(B)** Syncytia formed by human immunodeficiency virus type 1 in a T cell line. The photograph shows a large syncytium of SupT1 cells (a $CD4^+$ T-lymphotropic cell line) that had been infected with a viral vector that expresses the *env* gene. Large quantities of the Env protein accumulate at the cell surfaces, mediating fusion. A single cell is indicated for comparison. Courtesy of Matthias Schnell, Philip McKenna, and Joseph Kulkosky, Thomas Jefferson University School of Medicine, Philadelphia, PA.



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BOX 13.14

DISCUSSION

Intercellular transport by plant virus movement proteins

Plant cells are encased in a thick and rigid cell wall. The possible impact of this feature on the architecture of plant viruses is discussed in Box 12.2. The cell wall hampers release of progeny virus particles and cell-to-cell spread by the mechanisms described in the text. In fact, the spread of viruses within an infected plant relies on a second characteristic feature of plant cells, the specialized structures (plasmodesmata) that connect neighboring cells to one another. Each cell has many (up to 10^5) plasmodesmata, and these structures can be formed by all plant cells. Consequently, a plant is, at least in principle, a monstrous syncytium ideal for the local and systemic spread of a virus.

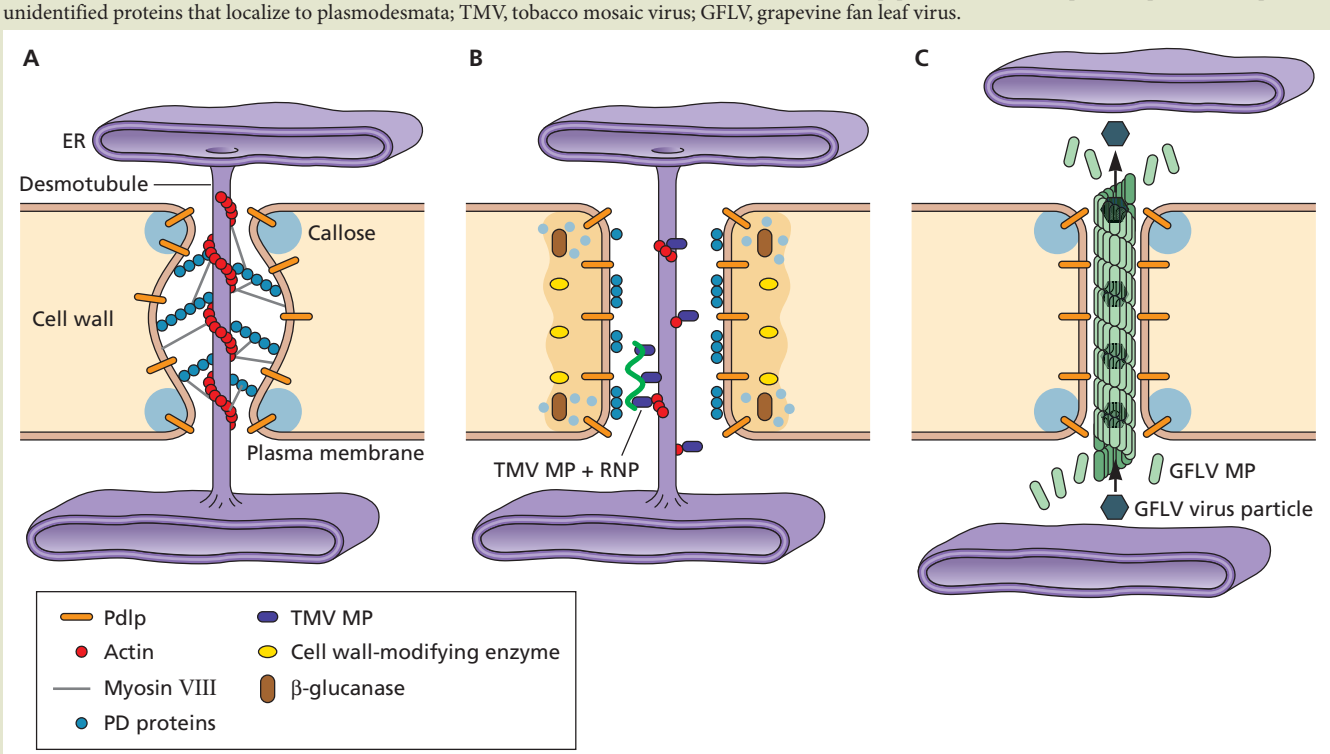
Plasmodesmata comprise the central desmotubule derived from the endoplasmic reticulum within a second membrane originating from the plasma membrane. The intervening space, termed the cytoplasmic sleeve, which contains many proteins, including actin and myosin, is the major conduit for intercellular transport. The inherent size exclusion limits of plasmodesmata for passive diffusion vary with cell type and physiological state but are very low (some 1 to 7 kDa). However, plasmodesmata are dynamic, and proteins of up to 50 kDa can travel through them under appropriate conditions. This "normal" expanded capacity is still insufficient for the passage of even the smallest plant virus particles (members of the *Nanoviridae*, such as fava bean necrotic yellow virus, with a molecular mass of $\sim 1.6 \times 10^3$ kDa) or of viral genomes in the form of ribonucleoproteins. This potential barrier to virus spread and propagation is circumvented by the movement proteins encoded in the genomes of all plant viruses.

The first such protein to be identified was the 30-kDa movement protein of tobacco mosaic virus; progeny virus particles of temperature-sensitive strains of the virus with substitutions in the 30-kDa protein coding sequence are unable to move from the infected cell. The plant virus movement proteins can be compared and grouped on the basis of various properties, including sequence and interactions with other viral components. However, they fall into two broad functional classes (see the figure). A large group, including the movement protein of tobacco mosaic virus, induces transient increases in the size exclusion limit of plasmodesmata indirectly by a variety of mechanisms. Depending on the virus, such proteins allow transport from cell to cell of either progeny virus particles or ribonucleoproteins containing the viral genome. Members of the second class of movement proteins actually form tubules within plasmodesmata, displacing desmotubules. These structures provide tracks for cell-to-cell spread, but what viral components are transported and how are not yet clear.

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The organization of a plasmodesma in an uninfected cell (left) and the impact of plant virus movement proteins that increase the size exclusion limit indirectly (middle) or form transport tubules (right) are illustrated schematically. Pdlp, plasmodesmata receptor-like proteins; PD proteins, unidentified proteins that localize to plasmodesmata; TMV, tobacco mosaic virus; GFLV, grapevine fan leaf virus.



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The production of "decoys," noninfectious particles released in large quantities, is one alternative strategy to avoid host defense mechanisms during transmission. The vast majority of particles detected in hepatitis B virus-infected humans are empty. Another strategy would be to disguise virus particles with normal products of a host cell, as during releases of hepatitis A virus in exosomes. Some viral envelopes retain cellular proteins, such as major histocompatibility complex class II proteins and the adhesion receptor Icam-1, in their membranes. The latter protein substantially increases the infectivity of human immunodeficiency virus type 1 particles. However, the importance of such a masking strategy for the spread of a virus from one cell to another in the host has yet to be documented.

Perspectives

The assembly of even the simplest virus is a complicated process in which multiple reactions must be completed in the correct sequence and coordinated in such a way that the overall pathway is irreversible. These requirements for efficient production and release of stable structures must be balanced with the fabrication of virus particles primed for ready disassembly at the start of a new infectious cycle. The integration of information collected by the application of structural, imaging, biochemical, and genetic methods of analysis has allowed an outline of the dynamic processes of assembly, release, and maturation for many viruses. Despite the considerable structural diversity of virions, the repertoire of mechanisms for successful completion of the individual reactions is limited. Furthermore, we can identify common mechanisms that ensure that assembly proceeds efficiently and irreversibly or that resolve the apparent paradox of great particle stability during assembly and release but facile disassembly at the start of the next infectious cycle. These mechanisms include high concentrations of virion components at specific sites within the infected cell and proteolytic cleavage of viral proteins at one or more steps in the production of infectious particles. Indeed, for some smaller viruses, the structural changes that accompany the production of infectious virions from noninfectious precursor particles can be described in atomic detail. Such information has revealed unanticipated relationships between structures that stabilize virus particles and interactions that prime them for conformational rearrangements during entry.

On the other hand, the pathways for assembly, production, and release of even the simplest virus particles cannot be described fully. These reactions are difficult to study in infected cells, and even the simplest proved more difficult to reconstitute *in vitro* than originally anticipated. The latter observation emphasizes the crucial contributions to virus assembly that can be made by cellular proteins that assist protein folding and oligomerization (chaperones) or that covalently modify virion proteins. Historically, assembly reactions have received less attention than mechanisms of viral gene expression or replication of viral genomes. However, the development of new structural and imaging methods, coupled with the experimental power and flexibility provided by modern molecular biology, has revitalized investigation of the essential processes of assembly, release, and maturation of virus particles. This renaissance has been further stimulated by the success of therapeutic agents designed to inhibit virus-specific reactions crucial for the production of infectious particles.

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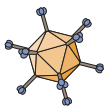
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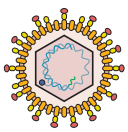
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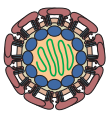
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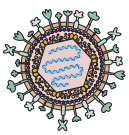




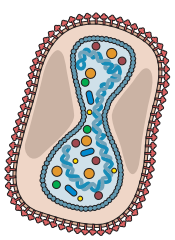
14 The Infected Cell











Introduction

Signal Transduction
- Signaling Pathways
- Signaling in Virus-Infected Cells

Gene Expression
- Inhibition of Cellular Gene Expression
- Differential Regulation of Cellular Gene Expression

Metabolism
- Methods To Study Metabolism
- Glucose Metabolism
- The Citric Acid Cycle
- Electron Transport and Oxidative Phosphorylation
- Lipid Metabolism

Remodeling of Cellular Organelles
- The Nucleus
- The Cytoplasm

Perspectives

References

LINKS FOR CHAPTER 14

⏭ *Video: Interview with Dr. Thomas Shenk*
http://bit.ly/Virology_Shenk

⏭ *Herpes and the sashimi plot*
http://bit.ly/Virology_Twiv339

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He hath eaten me out of house and home.

WILLIAM SHAKESPEARE
King Henry IV, Part II

Introduction

In previous chapters, we have described the reactions that comprise viral infectious cycles, from initial attachment to a receptor on the surface of a susceptible cell to assembly and release of progeny particles. The focus has been on the mechanisms that ensure successful viral gene expression, replication of viral genomes, and production of virus particles. These processes depend to a greater or lesser degree on the host cell's metabolic and biosynthetic capabilities, signal transduction pathways, and trafficking systems. Consequently, productive virus infection inevitably redirects, and frequently compromises, normal cellular physiology, and indeed can result in lysis and death of the infected cell within a matter of hours to days. Some of the mechanisms by which viral gene products fashion cellular systems to virus-specific ends have been touched on in previous chapters. Here, we present an integrated description of cellular responses to illustrate the marked, and generally irreversible, impact of virus infection on the host cell.

The initial responses of a host cell to virus infection are rapid, initiated upon contact of a virus particle with a receptor or immediately following entry of virus particles (or components thereof) into the cell. A major consequence of entry is the recognition of viral components by cellular proteins specialized for detection of microbial invaders (pattern recognition receptors). Such recognition initiates signal transduction cascades that mobilize host defenses, such as those mediated by interferons. These defensive responses, which can include alterations in expression of large sets of cellular genes, for example, of up to 1,000 or so interferon-inducible genes, are described in Volume II, Chapter 3. Virus infection also elicits alterations in host cell processes that facilitate production and release of progeny virus particles. Infection may modify expression of cellular genes, redirect metabolic pathways, disrupt trafficking of cellular macromolecules, or remodel cellular components and organelles to promote specific reactions in an infectious cycle. The extent and magnitude of such alterations depend on properties of the host cell, such as whether it is normal or transformed, quiescent or proliferating, as well as whether an infection is productive: when an infection is latent or persistent, only a subset of viral genes is expressed and their products promote survival of infected cells, rather than the widespread reprogramming of cellular gene expression observed in cells productively infected by many viruses.

Our understanding of the cellular response to viral infection has deepened enormously since the development of the techniques of systems biology as well as improved imaging methods. Indeed, application of these approaches has revealed just how different an infected cell that is supporting virus reproduction can be from its uninfected cell counterpart.

Signal Transduction

Signaling Pathways

All cells, be they individual organisms (e.g., bacteria, archaea, and protozoa) or but one of millions in a multicellular animal or plant, must be capable of sensing their environment and responding in an appropriate manner. They must also possess mechanisms to perceive internal cues that provide information about the need for particular metabolites, the integrity of the genome, or the presence of microbes. In multicellular organisms, the coordination of the properties and behaviors of individual cells with those of local neighbors, or more distant cells, is critical for successful differentiation and development, and for maintaining homeostasis among functionally specialized organs and tissues. Cells therefore possess elaborate sensing mechanisms that monitor, and when appropriate, initiate a response to, information about the external and internal milieus. These **signal transduction pathways** govern and integrate every aspect of cell physiology and conduct, from the rate of metabolic reactions to the decisions to move in a particular direction, to divide, or to differentiate.

PRINCIPLES *The infected cell*

- A single signal transduction pathway can be modified in cells infected by many different viruses.
- Infection of cells with a single virus can result in modification of multiple signaling pathways.
- Inhibition of cellular gene expression is a common outcome of viral infection.
- Cellular gene expression can be inhibited in virus infected cells by blocking cellular mRNA production, inhibiting translation, or inducing increased degradation of cellular mRNAs.
- A second common pattern is differential regulation, in which expression of some genes is increased and that of others decreased.
- The rates of glucose uptake and metabolism are increased in cells infected by a wide variety of viruses, but the products of this pathway have virus-specific uses.
- Virus-induced changes in lipid metabolism are required for energy production, formation of replication centers, generation of viral envelopes with unusual characteristics, or maturation of virus particles.
- Remodeling of the nucleus or cytoplasm during virus infection can facilitate genome replication, assembly of progeny virus particles, or both.

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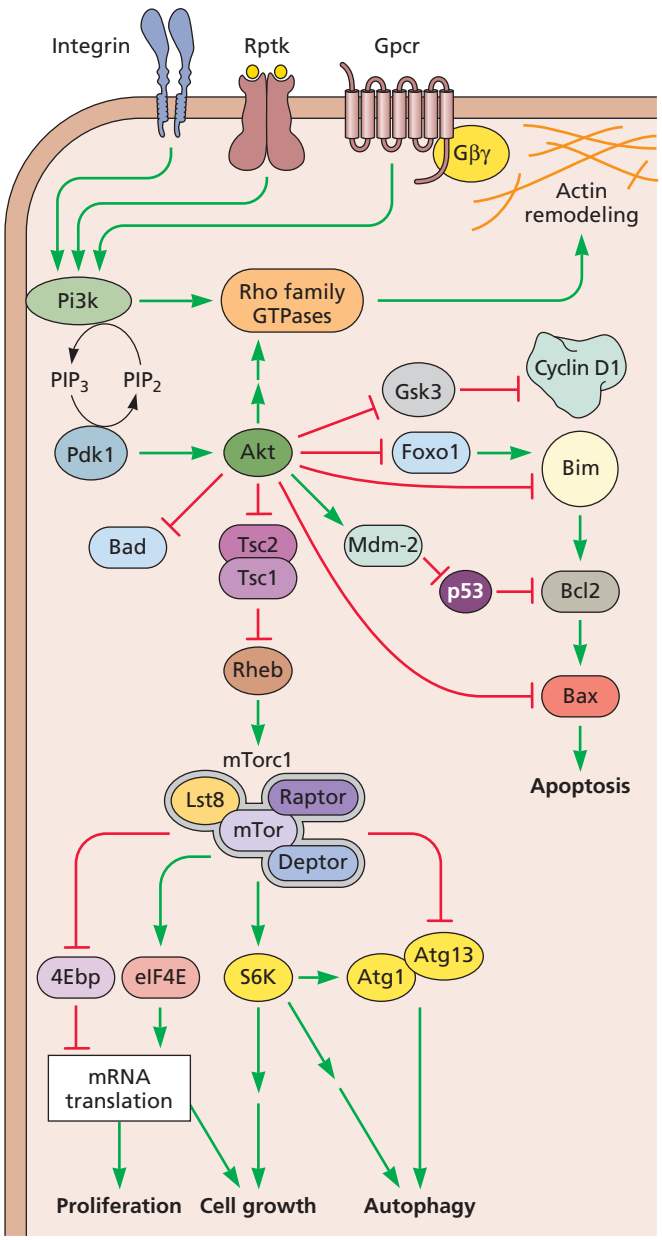


Figure 14.1 The mammalian Pi3k-Akt-mTor signaling route. The core features of this signaling transduction system are illustrated. Binding of ligand to any one of several types of plasma membrane receptors initiates signaling to Pi3k, which is associated with the inner surface of the plasma membrane, and activation of this kinase via phosphorylation. Mammalian cells contain three classes of Pi3ks, distinguished by their intracellular distributions, mechanisms of activation, and substrate specificity. Shown is the most common, class I Pi3ks, which comprise a regulatory (p85) and a catalytic (p110) subunit. Once activated, these kinases phosphorylate phosphoinositol present on membrane lipids to produce phosphoinositol 3,4,5-triphosphate (PIP_3). These modified lipids are bound by particular domains of other proteins, such as phophoinositide-dependent kinase 1 (Pdk1), which then transmit the signal to Akt. Synthesis of PIP_3 also leads to activation of small G proteins of the Rho (Ras homology) family that control actin polymerization and depolymerization, such as Rac (Ras-related C3 botulism toxin substrate 1) and Cdc42 (cell division control protein 42 homolog). Activation of Akt modulates numerous substrates and several processes. Shown are consequences that promote cell growth and proliferation via activation of the mTor kinase present in mTorC1. Activated mTor facilitates translation by multiple mechanisms and also induces **autophagy**, a process that helps cells survive extreme forms of stress, such as amino acid starvation. The signaling hubs Pi3k, Akt, and mTor are connected to, and regulated by, other signaling systems and to one another by various feedback circuits. Atg, autophagy-related protein; Bad, Bcl2-associated death protein; Bax, apoptosis-regulator Bcl2-associated protein; Bcl2, apoptosis-regulator Bcl2 (B cell CLL/lymphoma 2); Bim, Bcl2-interacting mediator of cell death; Deptor, Dep domain-containing mTor-interacting protein; 4Ebp, eukaryotic initiation factor 4E-binding protein; eIF4G, eukaryotic translation initiation factor 4G; Foxo1, forkhead box protein 1; Gsk3, glycogen synthase kinase 3; Lst8, target of rapamycin complex subunit Lst8 homology; Mdm2, E3 ubiquitin ligase Mdm2 (double minute protein 2); Raptor, regulatory-associated protein of mTor complex 1; Rheb, Ras-homology enriched in protein; S6k, ribosomal protein S6 kinase; Tsc, tuberous sclerosis protein.

Consequently, a considerable fraction of a cell's coding capacity is devoted to genes that encode signaling proteins: of the some 30,000 human genes, 918 (~1.7%) specify protein kinases, just one of the several classes of signal transduction protein. Extensive alteration in cellular signaling is an inevitable consequence of virus infection.

In signal transduction pathways, detection of an informational molecule, such as a metabolite (e.g., glucose), hormone, or growth factor, by a receptor initiates amplification of the signal as it is transmitted to effectors. Amplification is achieved by the actions of protein kinases that catalyze sequential phosphorylation and activation of additional kinases or other substrates, and the synthesis of small, diffusible molecules that act as messengers, for example, cyclic AMP and phosphoinositol 3-phosphate (PI3P). Proteins that operate in any cellular process may be effectors, but those that regulate gene expression are common targets. Many of the numerous signaling pathways of mammalian cells respond to more than a single input, regulate multiple molecular processes, and communicate with one another. The phosphoinositol 3-kinase (Pi3k)-Akt pathway exemplifies these properties: it receives input from multiple membrane receptors and regulates many aspects of cell metabolism, proliferation, and survival both directly and via connections to other pathways, such as that centered on the serine/threonine protein kinase mammalian target of rapamycin (mTor) (Fig. 14.1). The kinases Pi3k and Akt are focal points or hubs in the signaling network, with multiple inputs and outputs.

Signaling in Virus-Infected Cells

Much of our understanding of the impact of virus infection on host cell signal transduction cascades comes from investigation of the functions of viral gene products in cells in culture. In such infected cells, alterations in signaling are both rapid and substantial. For example, quantitative analysis

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of protein phosphorylation by mass spectrometry revealed changes in the frequency of phosphorylation at specific sites on 175 cellular proteins within a minute of exposure of host $CD4^+$ T cells to human immunodeficiency virus type 1. Furthermore, it is clear that viral infection can effect changes in signaling that promote every reaction in the infectious cycle. Many viral gene products intervene to block defensive responses of the host that would inhibit virus reproduction. In fact, every virus that has been examined has been found to direct synthesis of at least one viral gene product that impairs detection of infection or blocks the initial antiviral responses (Volume II, Chapter 3). Although most alterations are transient (because infected cells generally do not survive), some viral proteins can induce permanent changes in cellular signaling systems that allow cells to proliferate indefinitely. This process, termed transformation, is essential for **oncogenesis** and is described in Volume II, Chapter 6.

In this section, we focus on modulations of signaling pathways that facilitate virus reproduction, and use specific examples to illustrate two general principles: the same signal transduction pathway can be modified in cells infected by many different viruses, and individual viruses can modulate multiple signaling pathways.

Activation of Common Signaling Pathways

A core set of processes, including entry into a host cell, translation of viral mRNAs, and synthesis of viral nucleic acids, are common to all viral infectious cycles. Consequently, it is not unexpected that the same signal transduction pathway can be modulated in cells infected by viruses belonging to different families. One example of this phenomenon is activation of the transcriptional regulator Nf-κb in cells infected by several viruses with DNA genomes and some retroviruses to facilitate transcription of viral DNA templates (Fig. 8.11). However, viral gene products also frequently block this pathway, because Nf-κb is critical for activation of innate immune defenses (Volume II, Chapter 3). Signaling via Pi3k and Akt regulates a broad range of cellular processes (Fig. 14.1) and is modulated following infection by a large number of viruses. We therefore illustrate the varied impact of infection on one signal transduction cascade using this pathway.

Among many other aspects of cell physiology, this signaling pathway regulates remodeling of the cytoskeleton by polymerization and depolymerization of actin fibers (Fig. 14.1). Such resculpting of these structural components of the cell is essential for movement of cells; formation of extensions, such as lamellipodia; and other processes that require reorganization of the external surface of the cell, including virus entry. Attachment of viruses belonging to numerous families to their cognate cell surface receptors induces rapid activation (phosphorylation) of Pi3k. This response is required for efficient virus entry, as inhibition of Pi3k or of downstream effectors (see Fig. 14.2) impairs this process. Although Pi3k is activated in all cases, the downstream pathways are virus specific, because the mechanisms of entry differ from virus to virus. Attachment of human adenovirus to its integrin receptor leads to signaling from Pi3k via small G proteins to induce actin reorganization and facilitate endocytosis of virus particles. In other cases, it is signaling from Pi3k to Akt that has been implicated in promoting entry of virus particles by endocytosis (Fig. 14.2). Attachment of influenza A virus particles, which leads to clustering of lipid rafts and associated receptor protein tyrosine kinases and subsequent activation of Pi3k, stimulates not only actin remodeling, but also the acidification of endosomes necessary for disassembly of virus particles. In contrast, activation of the Pi3k-Akt pathway by binding of Zaire ebolavirus to its receptor does not promote virus entry directly, but rather prevents the diversion of endosomal virus particles to cytoplasmic vesicles in which fusion of viral and cellular membranes cannot occur. Presumably, these distinct outputs of Pi3k and Akt signaling are determined by the virus-specific mechanisms of activation of the kinases. Entry of all viruses that reproduce in mammalian cells depends on some degree of refashioning of the plasma membrane and associated cytoskeleton. It therefore seems likely that subversion of the normal function of Pi3k, Akt, or both in regulating membrane transactions will prove to be a more general response to the encounter of host cells with virus particles.

Signaling initiated by activation of Pi3k also facilitates later steps in virus reproduction. This kinase signals to not only Akt but also a second kinase, mTor, present in mTor complex 1 (mTorC1). Outputs from these downstream hubs in the cascade increase the rate of translation (and hence support cell growth and proliferation), modulate metabolic pathways, and promote cell survival (Fig. 14.1). All these responses would be expected to be beneficial for completion of viral infectious cycles. In fact, in every case that has been examined, virus infection has been observed to activate signaling via Pi3k to Akt, and in many cases, mTorC1. Modulation of such kinases can also contribute to pathogenesis (Box 14.1).

The genomes of a number of DNA viruses and retroviruses include oncogenes. The products of such genes can induce permanent activation of cell proliferation, a process termed transformation, and sometimes acquisition of the ability to form tumors in animals. These viral proteins stimulate cell proliferation by a variety of mechanisms, and typically also activate the Pi3k-Akt-mTorC1 signaling cascade to support cell growth and promote cell survival (Volume II, Chapter 6). Infection by many other viruses with both RNA and DNA genomes also circumvents the normal mechanisms of regulation of Pi3k (or downstream signaling molecules) to facilitate translation of viral mRNAs and/or to block apoptosis, a defense to virus infection of last resort. The genomes of several viruses, including human adenovirus type 5, hepatitis C

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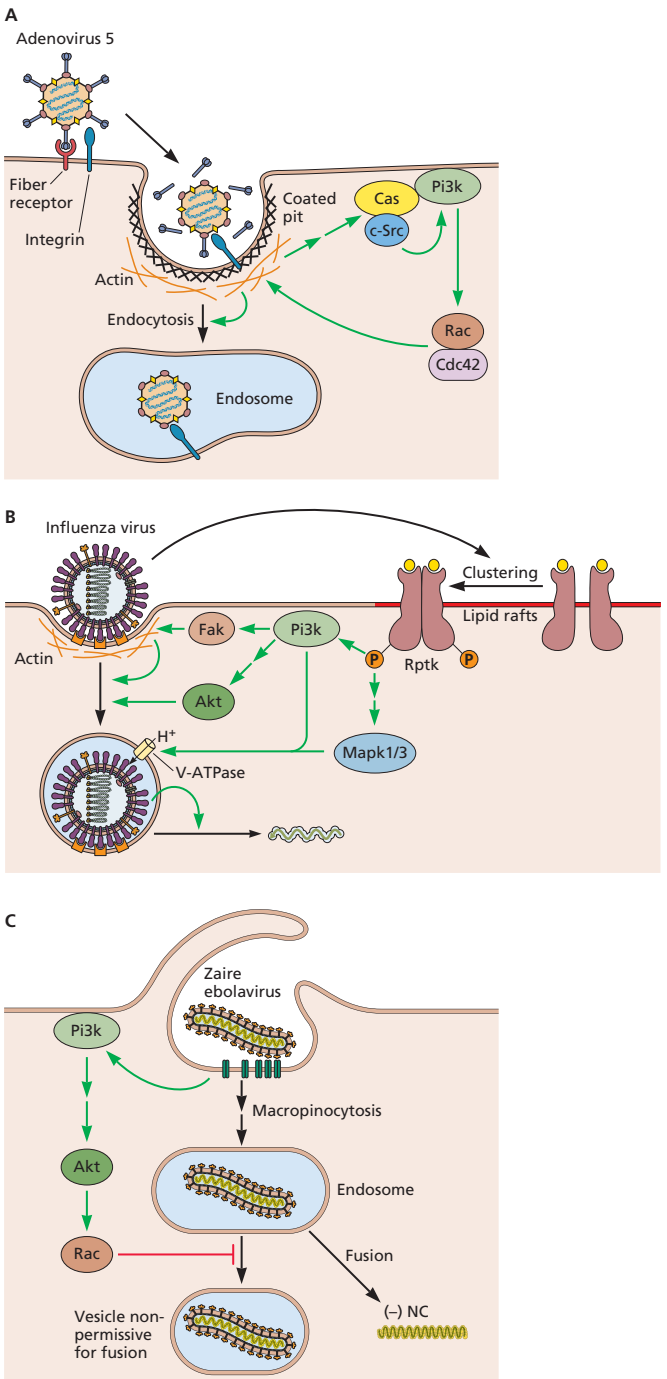


Figure 14.2 Signaling via Pi3k facilitates virus entry by a variety of mechanisms. Pi3k is activated following attachment to cellular receptors of adenoviruses, filoviruses, flaviviruses, influenza viruses, herpesvirus, and poxviruses, among others. Shown are three examples of the consequences of activated signaling from Pi3k. **(A)** Binding of a nonenveloped adenovirus type 5 particle to the αv integrin receptor leads to activation of Pi3k upon association of its p85 regulatory subunit with phosphorylated Crk-associated subunit (Cas), which is a substrate of the c-Src tyrosine kinase. Signaling initiated by the action of Pi3k results in actin remodeling via activation of the small G proteins Rac and Cdc42. Inhibition of phosphorylation of Cas or production of a dominant-negative derivative of Rac inhibits adenovirus entry, emphasizing the importance of this cellular signaling pathway for efficient internalization. **(B)** Attachment of an influenza A virus particle to its sialic acid receptor also induces activation of Pi3k to promote actin remodeling and endocytosis. In this case, these processes depend on signaling via Akt and focal adhesion kinase (Fak), and Pi3k is activated following clustering of lipid rafts and their associated receptor protein tyrosine kinases (Rptks) in the plasma membrane. Such concentration of receptors facilitates their activation by cross-phosphorylation, and also activates Mapk1 and -3. These kinases, in conjunction with signals transmitted from Pi3k, increase the activity of the vacuolar ATPase (V-ATPase) present in the membrane of endosomes, which pumps protons into the vesicles. The increased flux of protons reduces pH in the endosomal lumen and facilitates disassembly of virus particles for release of genome RNA segments into the cytoplasm. **(C)** Binding of the filovirus Zaire ebolavirus to its receptor induces entry via macropinocytosis and also activates signaling via Pi3k, Akt, and Rac by a mechanism that is not yet known. Such signal transduction indirectly facilitates release of the viral genomes into the cytoplasm by blocking diversion of endosomes containing virus particles to a nonproductive pathway.

virus, and rotavirus, encode the proteins that bind directly to the regulatory subunit of Pi3k to activate the kinase (Fig. 14.3A). Infection by human enterovirus 71, a pathogenic picornavirus, also leads to activation of Pi3k via its regulatory subunit, but in this case the interaction is with a cellular protein (Sam68) that is induced to relocalize from the nucleus to the cytoplasm. Other viral proteins act upstream of Pi3k or by mechanisms that are not yet known (Fig. 14.3B).

While various responses have been ascribed to activation of the Pi3k pathway by specific viral proteins, or in cells infected by different viruses (Fig. 14.3), only certain outputs were examined in each case, and it is therefore possible that the consequences of the increased activity of this cascade are more far-reaching. Furthermore, the genomes of a variety of viruses encode proteins that intervene downstream of Pi3k to maintain mTor activity and consequently efficient translation (Chapter 11).

Infection with a Particular Virus Modulates Multiple Signal Transduction Pathways

Virus reproduction is invariably accompanied by alterations in more than a single signaling relay, typically with one or more pathways blocked and others stimulated. Prominent among those that are inhibited in infected cells are pathways that detect microbes and mediate cellular defenses (Volume II, Chapter 3). Concurrently, signaling cascades that govern other processes are modulated to support the reactions necessary for expression and replication of viral genomes and assembly of progeny virus particles.

Infection by viruses with even relatively simple genomes and mechanisms of reproduction that depend on a minimal set of cellular systems and components leads to modification of several signaling pathways. For example, infection with Coxsackie B

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BOX 14.1

EXPERIMENTS

A viral pathogenic RNA blocks Akt activation to induce apoptosis

In cells infected by arthropod-transmitted flaviviruses such as West Nile and dengue viruses, degradation of the viral (+) strand RNA genome by the cellular 5′ → 3′ exonuclease Xrn1 produces small, noncoding RNAs derived from the 3′ untranslated region of the genome (Box 10.9). Initial characterization of this subgenomic (sg) RNA of West Nile virus established that it is necessary for formation of plaques and killing of infected cells in culture, and for pathogenicity in mice.

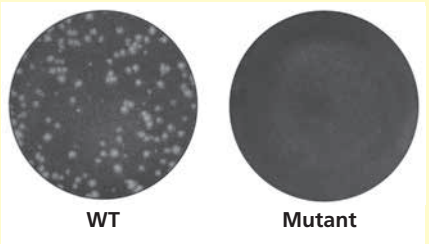
Production of dengue virus sgRNA (of some 400 nucleotides) is prevented by a deletion that removes a segment predicted to form two hairpin stem-loop structures near the end of the 3′ untranslated region. This mutation does not have much effect on synthesis of viral proteins, replication of the genome, or the yield of infectious virus particles following infection of mammalian cells. However, the ability of the mutant virus to form plaques on these cells was impaired (see the figure), as was induction of apoptosis when assessed by three different assays. At the time of peak genome RNA concentration in cells infected by the wild-type virus, Akt was inactivated (loss of phosphorylation at a specific residue) and the concentration of the antiapoptotic protein Bcl-2 was reduced. Neither of these alterations was observed in cells infected by the mutant virus. Introduction of a plasmid that directed synthesis of sgRNA into cells infected by the mutant led to a partial restoration of plaque formation and increases in cell death and the number of apoptotic cells.

These observations indicate that the dengue virus sgRNA inhibits signaling from Akt late in infection, and are consistent with a model in which this function of the sgRNA promotes apoptosis as a result of reduced concentrations of Bcl-2 (see Fig. 14.1). How a small RNA blocks activation of Akt remains to be established.

Liu Y, Liu H, Zou J, Zhang B, Yuan Z. 2014. Dengue virus subgenomic RNA induces apoptosis through the Bcl-2-mediated PI3k/Akt signaling pathway. *Virology* **448:**15–25.

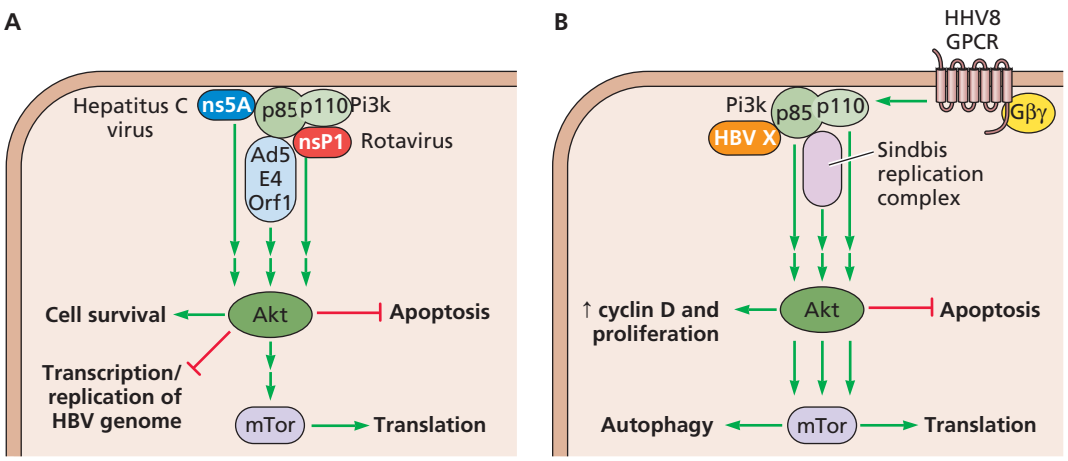
Pijlman GP, Funk A, Kondratieva N, Leung J, Torres S, van der Aa L, Liu WJ, Palmenberg AC, Shi PY, Hall RA, Khromykh AA. 2008. A highly structured, nuclease-resistant, noncoding RNA produced by flaviviruses is required for pathogenicity. *Cell Host Microbe* **4:**579–591.

Rubyh JA, Pijlman GP, Wilusz J, Khromykh AA. 2014. Noncoding subgenomic flavivirus RNA: multiple functions in West Nile virus pathogenesis and modulation of host responses. *Viruses* **6:**404–427.



Plaques formed on BHK-21 cells by wild-type dengue virus (WT) and a mutant with a deletion near the 3′ end of the 3′ untranslated RNA that prevents production of sgRNA. Cells were fixed and stained with crystal violet. Adapted from Y. Liu et al., *Virology* **448:**15–25, 2014, with permission. Courtesy of Z. Yuan, Chinese Academy of Sciences, Wuhan, China.

Figure 14.3 Common activation of the Pi3k-Akt-mTor relay in virus-infected cells. (A) Direct association (double-headed arrows) of several viral proteins with the p85 regulatory subunit activates Pi3k and hence Akt to promote cell survival and block apoptosis, and in the case of adenovirus 5 (Ad5) E4 Orf1 protein, to activate mTor and stimulate translation. **(B)** The hepatitis B virus (HBV) X protein activates Pi3k in the same way, but subsequent signal transduction induces increased production of cyclin D1, which promotes cell proliferation and autophagy. The human herpesvirus 8 (HHV8)-encoded G protein-coupled receptor (GPCR) and formation of replication complexes of the alphavirus Sindbis virus also stimulate Pi3k-dependent signaling, by unknown mechanisms.



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virus, a pathogenic picornavirus with an RNA genome of some 7.5 kb that is replicated by viral proteins in the cytoplasm, leads to signaling via not only Pi3k and mTor, but also the mitogen-activated protein kinase (Mapk) and the Nf-κb pathways and the tyrosine kinase Src. As might be anticipated from the additional participation of nuclear components such as the primers for synthesis of viral mRNA from the (−) strand genomic RNA segments and splicing proteins in influenza A virus reproduction, infection by this virus leads to activation of a larger number of signaling pathways than does picornavirus infection.

In general, it appears that the more elaborate the strategy for viral reproduction, the greater the impact of infection on signaling pathways. However, direct comparisons of the responses of signal transduction cascades in a particular cell type to infection with different viruses have not been reported. Furthermore, how radically cellular signaling systems are altered will also be determined by the origin and proliferation state of the host cell. Many human cells in routine use in the laboratory are derived from tumors (Chapter 2), and consequently are abnormal in many respects, including unrestrained proliferation and permanent activation of signaling circuits that promote cell growth and progression through the cell cycle. In contrast, in natural infections, many host cells proliferate only slowly or are quiescent (withdrawn from the cell cycle). Successful virus reproduction in such cells is therefore likely to depend to a greater degree on activation of signaling pathways that control these processes than does reproduction in tumor-derived cell lines.

The most common bit of information that is transmitted during biological signaling is the presence (or absence) of a phosphate group on specific amino acids in a protein. Application of methods of mass spectrometry that allow detection and very accurate quantification of differences in concentration among samples of thousands of phosphopeptides can therefore provide global, unbiased views of changes in signal transduction pathways under specific conditions. The results of recent applications of these methods to comparison of uninfected and virus-infected cells suggest that the impact of particular viruses on host cell signaling is even broader than previously appreciated. For example, comparison of the concentrations of phosphopeptides in uninfected, quiescent mouse fibroblasts and 18 h after infection with murine herpesvirus 68 (a gammaherpesvirus) identified changes in 86% of the nearly 2,500 unique peptides examined. This infection-induced difference is far larger than that observed following exposure of cells to growth factors (<13%) or assaults, such as damage to the genome or exposure of human cells to *Salmonella* (~24%).

Large-scale analyses of phosphoproteins in infected cells can also identify cellular substrates of signaling pathways that are important for virus reproduction. The abundance of phosphorylation sites in 175 host proteins was observed to increase or decrease within 1 min of exposure of unstimulated $CD4^+$ T cells to human immunodeficiency virus type 1. Bioinformatics analyses of the amino acid sequences spanning these phosphorylation sites indicated activation of signaling via Mapk and calmodulin-dependent kinase II (CamkII), in agreement with previous studies, but inhibition of signaling from protein kinase A. Subsequent functional analysis of production of proteins with increased phosphorylation in infected cells established the important contribution of host proteins not previously implicated in reproduction of human immunodeficiency virus type 1, notably a specific set of splicing proteins.

Gene Expression

Altered host cell gene expression is a universal consequence of virus infection. The altered patterns range from inhibition of the synthesis or translation of the majority if not all cellular mRNAs, to differential increases or decreases in expression of particular sets of cellular genes as an infection proceeds. These changes may be the result of modulation of any of the several reactions by which mammalian pre-mRNAs are produced, used as template for protein synthesis, and turned over.

The impact of virus infection on cellular gene expression and the mechanism(s) by which this process is altered vary with the strategies by which viral genes are expressed. For example, transcription of viral genes from DNA templates by the cellular transcriptional machinery and processing of the transcripts in the same manner as cellular pre-mRNAs precludes inhibition of these reactions (although their selectivity may be redirected). Furthermore, the genomes of such viruses often encode powerful activators of transcription that promote viral gene expression, but can also exert broad effects on cellular mRNA synthesis.

Inhibition of Cellular Gene Expression

Viral genomes typically encode one or more proteins (or RNAs) that inhibit cellular gene expression indirectly, by blocking mechanisms that expedite antiviral responses (Volume II, Chapter 3) or modulating signal transduction pathways of the host cell (see previous section). However, reactions in the pathways by which cellular mRNAs are produced, translated, or degraded are inhibited directly by proteins of viruses with diverse reproduction strategies. Many of these proteins are described in previous chapters (Chapters 8, 10, and 11). Their impact on cellular gene expression emphasizes the fact that such proteins operate by a considerable variety of mechanisms (Fig. 14.4).

Host cell mRNA production can be inhibited following infection of permissive cells by viruses with (+) or (−) strand RNA genomes because the viral genomes are expressed and replicated with minimal dependence on cellular systems. Such inhibition facilitates selective and efficient synthesis of

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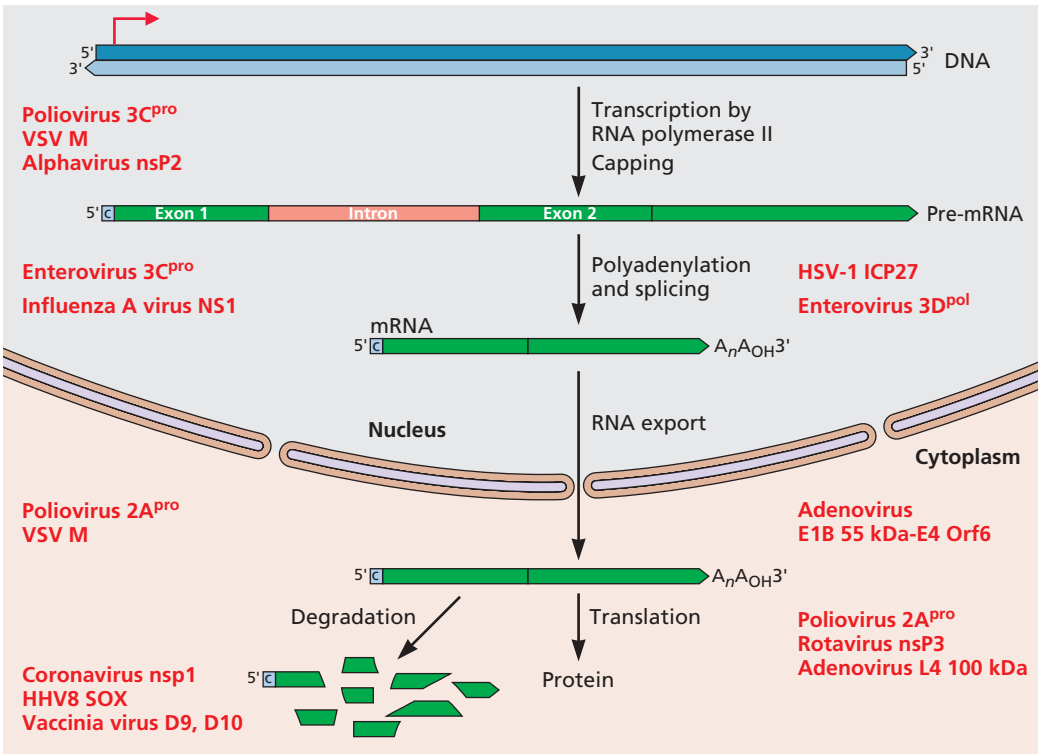


Figure 14.4 Inhibition of cellular gene expression by viral proteins. Viral proteins can inhibit the reactions by which mammalian mRNAs are made in the nucleus or exported to the cytoplasm and translated, or can induce accelerated mRNA degradation. (Transcription) The poliovirus $3C^{pro}$, nsP2 of Old World alphavirus, and the vesicular stomatitis virus (VSV) M protein all target components of the basal transcriptional machinery, Tbp, RNA polymerase II, and transcription initiation protein IID (TfIId), respectively. The first two are degraded in infected cells, but the mechanism by which the VSV M protein inactivates TfIId has not been established. Cleavage of Tbp by poliovirus $3C^{pro}$ also inhibits transcription by RNA polymerases I and III. (Polyadenylation) Both $3C^{pro}$ of enterovirus 71 (a picornavirus) and influenza A virus NS1 protein block polyadenylation of cellular pre-mRNAs, by inducing degradation of a subunit of cleavage stimulatory protein (Cstf) and sequestering cleavage and polyadenylation specificity protein (Cpsf), respectively. In both cases, the 3′ poly(A) sequences of viral mRNAs are synthesized by the viral RNA polymerase (Chapter 6). (Splicing) Splicing, essential for the production of the majority of cellular mRNAs, is perturbed by herpes simplex virus 1 (HSV-1) ICP27, which inhibits an early reaction in splicing and disrupts the nuclear foci in which splicing proteins are concentrated. This process is blocked in cells infected by enterovirus 71 as a result of interaction of the viral $3D^{pol}$ protein with a core component of the cellular splicing machinery, Prp8 (pre-mRNa processing protein 8). (Export) Export of RNAs from the nucleus is disrupted by a second poliovirus protease, $2A^{pro}$, and the VSV M protein, while export of cellular mRNAs is selectively blocked by the virus-specific E3 ubiquitin ligase containing the adenoviral E1B 55 kDa and E4 Orf6 proteins and several proteins co-opted from cellular enzymes of this type. (Translation) Translation of cellular mRNAs is also selectively inhibited in cells infected by a variety of viruses. The viral proteins shown block the function of proteins critical for initiation of translation of cellular mRNAs, because the viral mRNAs carry distinctive features that reduce or eliminate the dependence of their translation on these proteins (Chapter 11). (Degradation) The genomes of larger DNA and RNA viruses encode proteins that initiate mRNA degradation by removal of the 5′ cap (vaccinia virus D9 and D10 proteins) or endonucleolytic cleavage (coronavirus nsp1 and human herpesvirus 8 SOX protein). In some cases, cellular but not viral RNAs are degraded (see text).

viral proteins by reducing competition of cellular with viral mRNAs for components of the translation machinery, and can also mitigate host responses that impair virus reproduction. Although the specific reactions that are disrupted by viral proteins vary, the targets are cellular proteins necessary for production of cellular mRNAs in the nucleus or their export to the cytoplasm. Such proteins include essential components of the transcriptional initiation machinery, such as TATA-binding protein (Tbp), which is cleaved and inactivated by the poliovirus protease $3C^{pro}$; and a catalytic subunit of RNA polymerase II targeted for degradation by alphavirus nsP2. Proteins necessary for splicing or export of viral mRNAs to the cytoplasm can also be destroyed or inhibited by viral proteins (Fig. 14.4). The impact of such inhibition can be both substantial and widespread. For instance, only some 7% of the >5,000 cellular cytoplasmic poly(A)-containing mRNAs examined by microarray hybridization could be detected by 18 h after infection with the alphavirus Sindbis virus (Fig. 14.5A).

In some cases, viral proteins also block translation. This property is illustrated by poliovirus proteins that inhibit not only production of cellular mRNAs but also their translation.

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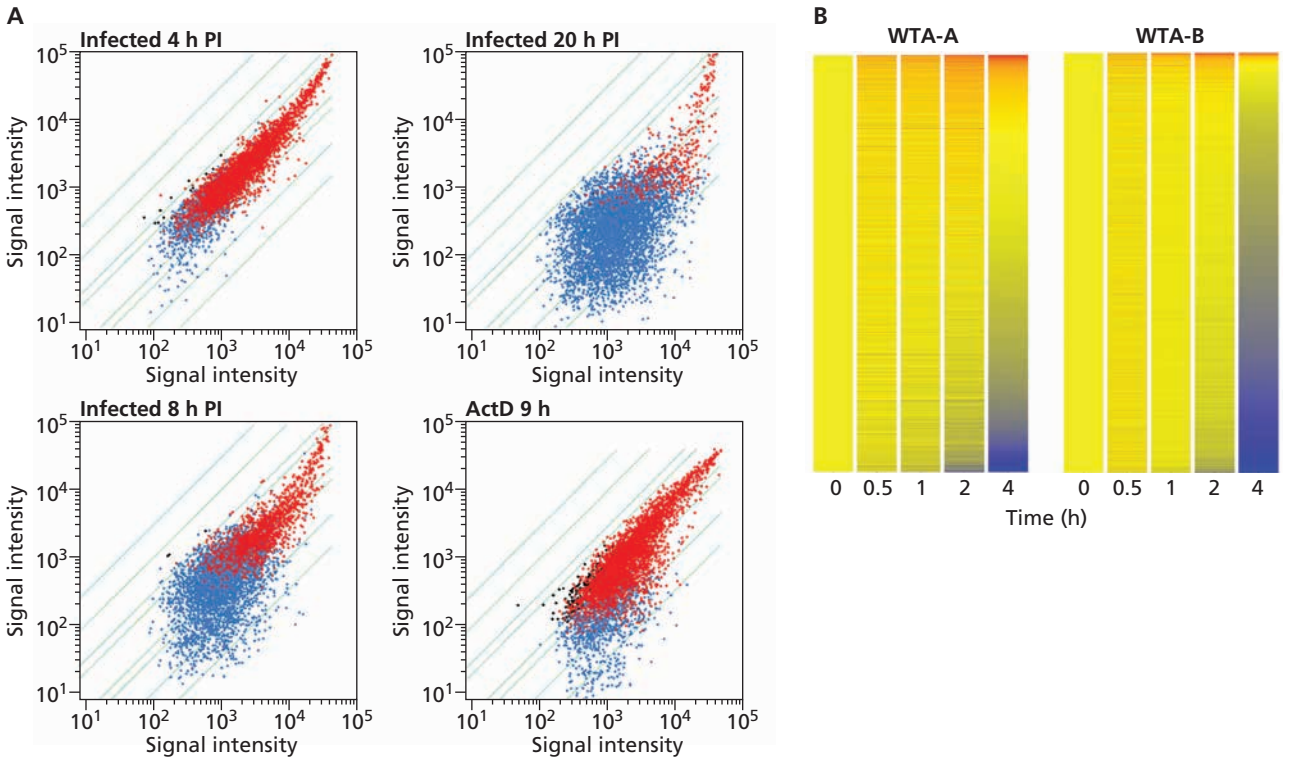


Figure 14.5 Decreases in cellular mRNA concentration in virus-infected cells. (A) Murine NIH 353 cells were infected with the alphavirus Sindbis virus (20 PFU/cell) (Infected). Total cell RNA was isolated after the periods of infection (PI) indicated, and from uninfected cells exposed to the transcriptional inhibitor actinomycin D (ActD) for 9 h. The concentrations of mRNAs were examined by hybridization to microarrays, using bacterial RNA added to each sample to provide an internal control. The mRNA signals are shown with those from infected or actinomycin D-treated cells plotted against the signals from uninfected cells. The mRNAs that changed little in concentration and decreased are shown in red and blue, respectively. Note that Sindbis virus infection leads to reductions in the concentration of far more cellular mRNAs than does exposure to actinomycin D. Adapted from R. Gorchakov et al., *J Virol* **79:**9397–9409, 2005. Courtesy of I. Frolov, University of Texas Medical Branch, with permission. **(B)** Human HeLa cells were infected with purified vaccinia virus under conditions that ensure infection of all cells, and total polyadenylated RNA was isolated at the times indicated. cDNAs were prepared and subjected to high-throughput sequencing. The number of read counts for each cellular mRNA (individual horizontal lines) is shown as the fold change from time zero, after normalization to the total number of reads for two independent infections (A and B). Colors from yellow to blue and to red indicate decreases and increases, respectively. Adapted from Z. Yang et al., *Proc Natl Acad Sci U S A* **107:**11513–11518, 2010, with permission. Courtesy of B. Moss, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Such seeming redundancy ensures efficient synthesis of viral proteins: inhibition of transcription and export of RNAs from the nucleus blocks the flow of newly synthesized cellular mRNAs into the cytoplasm, but those mRNAs made before infection, many of which are quite stable, are still present and potential templates for translation. The unusual mechanism of initiation of translation of viral mRNAs and concomitant cleavage of the initiation protein eIF4G by the viral protease $2A^{pro}$ eliminates such potential competition from cellular mRNAs (Chapter 11). The very short infectious cycle of poliovirus (and other picornaviruses; some 8 h) may necessitate particularly effective measures to prevent synthesis of cellular proteins.

The genomes of poxviruses and other large DNA viruses that reproduce in the cytoplasm are expressed by virally encoded transcription and RNA-processing systems that synthesize viral mRNAs with 5′ caps and 3′ poly(A) tails (Chapters 8 and 10). As might therefore be anticipated, loss of cellular mRNAs has been observed using high-throughput methods following infection by such viruses. Only ~10% of the RNA sequences in amoebae infected by mimivirus for 6 h were cellular in origin. Similarly, the concentrations of the majority of cellular mRNAs decreased by 4 h after vaccinia virus infection (Fig. 14.5B). The effect of infection on host cell transcription is not clear. However, the viral enzymes that remove 5′ caps from mRNA to initiate exonucleolytic degradation by the cellular nuclease Xrn1 are thought to make a major contribution to decreasing cellular mRNA concentrations.

Cellular gene expression can also be impaired in several different ways when viral mRNA synthesis depends on host cell components. Such selective inhibition targets reactions that are less critical for production of viral mRNAs than for those of the

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host cell, for example, pre-mRNA splicing in cells infected by herpesviruses or influenza viruses: in both cases, the majority of viral mRNAs comprise a single exon and cannot be spliced (Chapter 10). Similarly, the adenovirus L4 100-kDa protein induces selective translation of viral major late mRNAs. These mRNAs share a common 5′ untranslated region that bypasses the requirement for specific translation initiation proteins reduced in activity in infected cells (Chapter 11). Infection by gammaherpesviruses, such as Epstein-Barr virus and human herpesvirus 8, and severe acute respiratory syndrome coronavirus induces selective degradation of cellular mRNAs, a very effective mechanism for favoring synthesis of viral proteins. In the case of human herpesvirus 8, such degradation is initiated by a viral endonuclease (the SOX protein) that acts in conjunction with Xrn1. How cellular and viral mRNAs are distinguished is not known. However, destruction of cellular mRNAs triggers changes in cellular proteins that participate in production of host cell mRNAs to further restrict expression of cellular genes (Box 14.2).

Despite favoring translation of viral mRNAs, decreases in the production or acceleration of turnover of cellular mRNAs in virus-infected cells would not be expected to change the population of host cell proteins radically: many cellular mRNAs are quite stable, with half-lives longer than the time required for production of progeny particles of some viruses, such as picornaviruses or poxviruses (6 to 8 h). Indeed, when the concentrations of cellular proteins were assessed early and late after vaccinia virus infection of human cells, <10% were observed to change significantly.

Virus reproduction depends on many stable cellular proteins, including ribosomal proteins and structural proteins of the cytoskeleton. However, it may also be necessary to maintain the production of much less stable host proteins for optimal reproduction of particular viruses, even in the face of widespread inhibition of cellular gene expression. The mechanisms that allow such selective expression of specific sets of cellular genes in virus-infected cells are not well understood, but synthesis of particular proteins can be maintained

BOX 14.2

DISCUSSION

Domino effects of the human herpesvirus 8 SOX protein on cellular and viral gene expression

Synthesis of an early human herpesvirus 8 gene product, the SOX protein, in infected cells leads to degradation of the majority of cellular mRNAs. One consequence of such degradation is release of the poly(A)-binding protein PabpC1, which is normally bound to the 3′ poly(A) tails of cytoplasmic mRNAs to regulate their stability. This cellular protein then becomes relocalized to the nucleus (see the figure), where it leads to accumulation of poly(A), hyperadenylation of mRNAs, and impaired export of mRNAs from the nucleus.

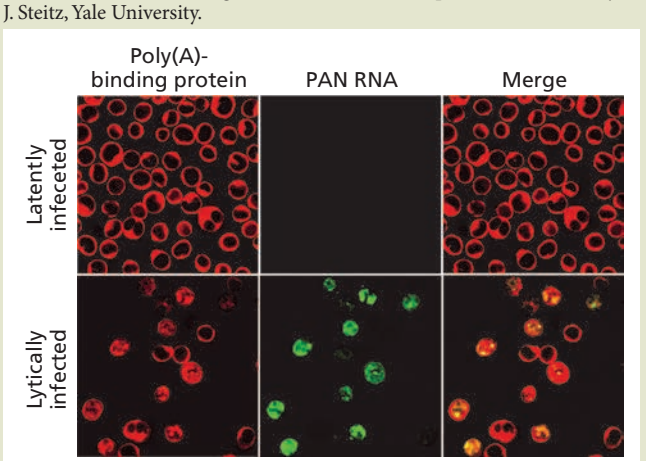
In the nucleus, PabpC1 associates with a viral noncoding RNA, the polyadenylated nuclear (PAN) RNA (see the figure). This viral RNA accumulates to very high concentrations in infected cell nuclei (~500,000 copies per nucleus) and is not transported to the cytoplasm. The interaction of PabpC1 with PAN RNA may contribute to such accumulation by stabilizing PAN RNA: the RNA-degradation function of SOX and nuclear PabpC1 increase the nuclear concentrations of PAN RNA. This viral RNA does not contribute to inhibition of cellular gene expression. Rather, PAN RNA promotes synthesis of viral late proteins and release of DNA-containing viral particles: both were impaired when PAN RNA concentrations were reduced by introduction of complementary oligonucleotides and RNAse H.

The direct action of SOX and subsequent indirect consequences of release of PabpC1 therefore ensure efficient synthesis of viral late proteins.

Borah S, Darricarrère N, Darnell A, Myoung J, Steitz JA. 2011. A viral nuclear noncoding RNA binds re-localized poly(A) binding protein and is required for late KSHV gene expression. *PLoS Pathog* **7:**e1002300. doi:10/1371/journal.ppat.10023000.

Kumar GR, Glaunsinger BA. 2010. Nuclear import of cytoplasmic poly(A) binding protein restricts gene expression via hyperadenylation and nuclear retention of mRNA. *Mol Cell Biol* **30:**4996–5008.

The localization of PabpC1 (red) and PAN RNA (green) determined by immunofluorescence and *in situ* hybridization, respectively, in human cells latently infected with human herpesvirus 8 (top) or following entry into the lytic replication cycle by synthesis of the viral regulator (RTA) that is necessary and sufficient to induce this switch (bottom). Adapted from S. Borah et al., *PLoS Pathog* **7:**e1002300, 2011, with permission. Courtesy of J. Steitz, Yale University.



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in various ways. For example, a small number of cellular mRNAs enriched in those for proteins that participate in signal transduction or regulation of apoptosis initially escape destruction in vaccinia virus-infected cells (Fig. 14.5B), and translation of specific host cell mRNAs is stimulated following hepatitis C virus infection.

Differential Regulation of Cellular Gene Expression

Although widespread inhibition of cellular gene expression is a common outcome of infection, more-subtle alterations also occur, particularly when viral gene expression depends on the host cell transcription and RNA-processing machineries. The scale and complexity of such modulation of host cell gene expression have become apparent only since the development of genome-wide methods for measurement of mRNA concentration, initially microarray hybridization and more recently high-throughput RNA sequencing. The latter method does not require hybridization to DNA and hence selection of DNA probes. It can therefore also provide information about noncoding RNAs. Alterations in the concentrations of micro-RNAs, long intergenic noncoding RNAs, small nucleolar RNAs, antisense RNAs, and transcripts of pseudogenes have been observed in cells infected by several viruses, but the significance of such broad impacts on the host cell RNA population is not yet clear.

These methods of RNA profiling are typically applied to total cell RNA populations or those enriched in mRNA by selection for the presence of a 3′ poly(A) tail. Consequently, they measure steady-state concentrations of mRNA (or other RNAs). Changes in this parameter are generally interpreted in terms of increases or decreases in transcription of individual genes, although they could be the result of alterations in any of the reactions by which an mRNA is produced, or in its rate of turnover (Box 14.3). More-precise information about the mechanisms that result in modulation of RNA accumulation in virus-infected cells can be collected by isolation of specific populations of RNA, for example, mRNAs that are serving as translational templates, prior to application of high-throughput quantification methods (Box 14.4).

The impact of infection on patterns of cellular gene expression varies with virus. Expression of some 800 host genes is altered in activated $CD4^+$ T cells infected by human immunodeficiency virus type 1. In contrast, the concentrations of ~10,000 cellular mRNAs increased or decreased by at least a factor of 2 in primary mouse fibroblasts infected by the herpesvirus murine cytomegalovirus, which possesses a large genome that encodes several transcriptional or posttranscriptional regulators. Furthermore, cellular mRNAs can even accumulate when viral gene products block production or increase turnover of the majority. This phenomenon is illustrated by the detection of increased concentrations of 400 or so cellular mRNAs by 7 h after herpes simplex virus 1 infection of normal human cells, despite the action of the viral Vhs endonuclease that leads to mRNA degradation (Chapter 10). When combined with various types of bioinformatics analysis, such as classification of differentially expressed genes by their functional annotations (gene ontology analysis), the results of these descriptive studies can help identify cellular gene products and pathways that promote or counter virus reproduction, or that correlate with virus pathogenicity or the responses of individual hosts to infection (Box 14.5).

In most cases, indirect effects of infection on cellular RNA populations, for example, as a result of modulation of signal transduction pathways, have not been distinguished from the direct actions of viral gene products. One exception is provided by cells infected by adenovirus. Infection of quiescent, normal human fibroblasts by human adenoviruses is followed by increases or decreases of at least 2-fold in expression of 10% of cellular genes. Many of these changes are associated with reentry of quiescent cells into the cell cycle, or support genome replication and expression. Transcription of a subset of genes repressed in infected cells, particularly interferon-sensitive and other genes associated with antiviral defenses, is inhibited by the viral E1B 55-kDa protein. How-

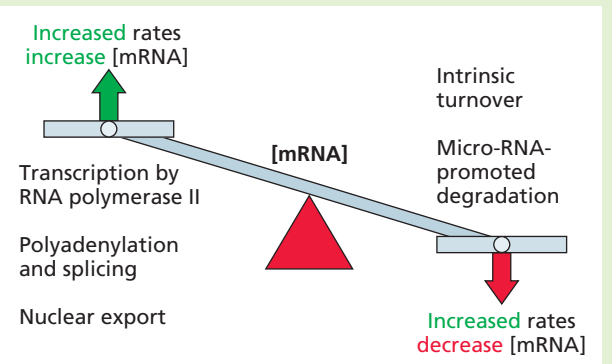
BOX 14.3

BACKGROUND

Multiple parameters govern the steady-state concentration of a cellular mRNA

The steady-state concentration of a cellular mRNA is determined by the balance between the overall rate of production of the mRNA and the rate at which it is degraded. Consequently, changes in concentration measured by microarray hybridization or high-throughput sequencing can be the result of alterations in the rate of either synthesis of the mRNA or its degradation, or changes in both parameters.

As summarized in the figure, the appearance of a functional mRNA available for translation in the cytoplasm is the end result of several processes. The rate of turnover of the mRNA can also be influenced by various parameters. Consequently, it is not correct to ascribe alterations in mRNA concentration measured by these techniques to increased or decreased transcription.



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BOX 14.4

METHODS

Increasing the specificity of RNA profiling

Microarray hybridization or high-throughput sequencing of total cell RNA or mRNA populations does not yield direct information about the mechanism(s) responsible for changes in RNA concentration or their consequences for the protein repertoire of a cell. The first of these limitations can be circumvented by isolation of specific classes of RNA prior to analysis.

The impact of a particular condition, such as virus infection, on translation can be assessed by purification of mRNAs present in polyribosomes and therefore serving as templates for protein synthesis. Methods that fractionate macromolecules on the basis of size and shape, such as density gradient centrifugation, readily separate larger polyribosomes from free mRNAs and ribosomal subunits (panel A of the figure). When combined with parallel analysis of total mRNA populations, this approach can establish whether cellular gene expression is subject to translational regulation, as has been observed in permissive cells infected by hepatitis C virus or human cytomegalovirus.

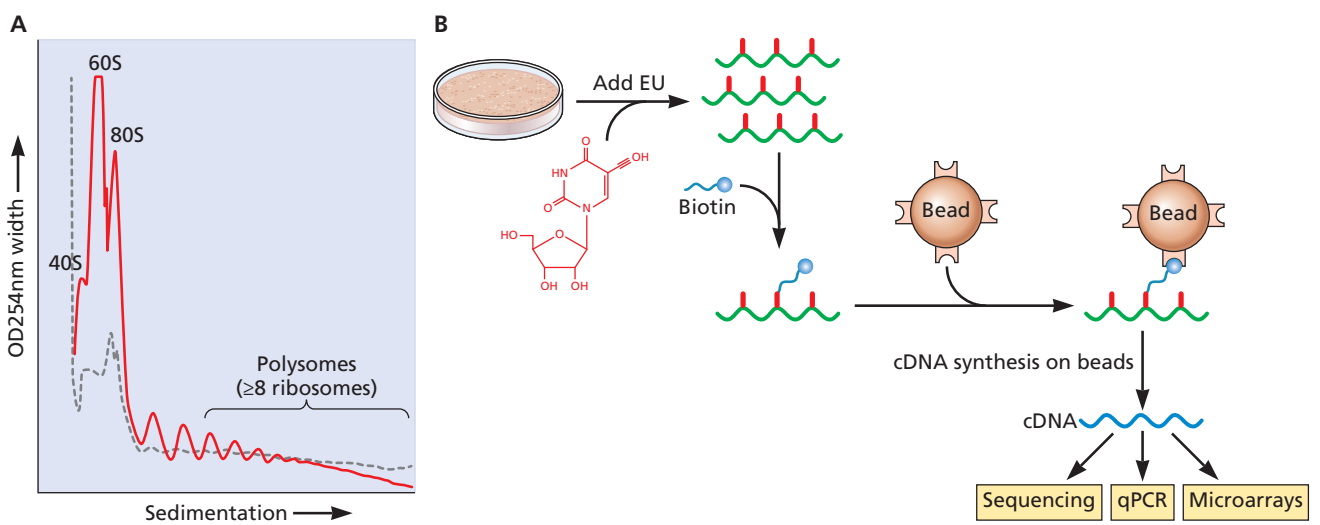
To distinguish transcriptional from post-transcriptional regulation, it is necessary to examine newly synthesized RNA (a small fraction of the total population in a cell). Selection of this population can be achieved by incorporation of modified derivatives of uridine, such as 4-thiouridine or 5-ethynyluridine (EU). RNA containing these modified bases, at the relatively low frequency of 1 per 50 to 100 nucleotides, is biotinylated after purification and then separated from the bulk RNA pool by binding to streptavidin attached to beads (panel B). When the uridine derivative is supplied to uninfected and infected cells for short periods (≤1 h), newly transcribed RNAs can be isolated and compared to identify changes in transcription. Application of this approach to normal murine cells infected by murine cytomegalovirus has established an almost perfect concordance between changes in the total RNA and the newly synthesized RNA pools during the early phase of infection, indicating that the rates of turnover of cellular mRNAs were not modulated at this time. Particles of murine cytomegalovirus and other herpesviruses contain substantial quantities of viral RNA species that are incorporated nonspecifically and transferred into new host cells. Tagging and isolation of newly synthesized RNA therefore facilitates elucidation of the pattern of viral gene expression early after infection, because the viral RNAs introduced from virus particles are not labeled.

This approach can also be used to measure the rates of turnover of mRNAs, by using a pulse-chase method in which the uridine derivative is supplied for a period sufficient for incorporation into mature mRNA, and the cells are then exposed to medium containing a vast excess of unmodified uridine. Measurement of the concentrations of the tagged mRNAs as a function of time after this switch (the "chase") allows rates of decay to be determined.

Colman H, Le Berre-Scoul C, Hernandez C, Pierredon S, Bihouée A, Houlgatte R, Vagner S, Rosenberg AR, Féray C. 2013. Genome-wide analysis of host mRNA translation during hepatitis C virus infection. *J Virol* **87:**6668–6677.

Marcinowski L, Lidschreiber M, Windhager L, Rieder M, Bosse JB, Rädle B, Bonfert T, György I, de Graaf M, Prazeres da Costa O, Rosenstiel P, Friedel CC, Zimmer R, Ruzsics Z, Dölken L. 2012. Real-time transcriptional profiling of cellular and viral gene expression during lytic cytomegalovirus infection. *PLoS Pathog* **8:**e1002908. doi:10.1371/journal.ppat.1002908.

McKinney C, Zavadil J, Bianco C, Shiflett L, Brown S, Mohr I. 2014. Global reprogramming of the cellular translational landscape facilitates cytomegalovirus replication. *Cell Rep* **16:**9–17.



(A) Comparison of the polyribosome profiles of normal human fibroblasts infected by human cytomegalovirus for 48 h (red) and uninfected cells (gray). Cell extracts were prepared under conditions that do not disrupt polyribosomes and sedimented through 15 to 50% linear sucrose gradients. The absorbance at 254 nm (shown) was monitored during collection of gradient fractions. Adapted from C. McKinney et al., *Cell Rep* **16:**9–17, 2014, with permission. **(B)** Isolation of newly synthesized RNA is achieved by incorporation of 5-ethynyluridine (EU; structure shown above the first step) for a short period followed by *in vitro* biotinylation, and subsequent separation from the bulk RNA population by binding to bead-bound streptavidin. The bead-bound RNA is then converted to cDNA for analysis by microarray hybridization, high-throughput sequencing, or quantitative PCR (qPCR).

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BOX 14.5

DISCUSSION

Insights into virus-host interactions from RNA profiling studies

It is well established that gene expression profiling can make quite fine distinctions among multiple subtypes of particular cancers, for example, breast cancers, and provide important information about prognosis. When combined with genome-wide analysis of mutations and changes in copy number, this approach has the potential to inform rationally based personalized treatment. As the two examples below illustrate, RNA profiling is also beginning to yield new insights into the interactions of viruses with their hosts.

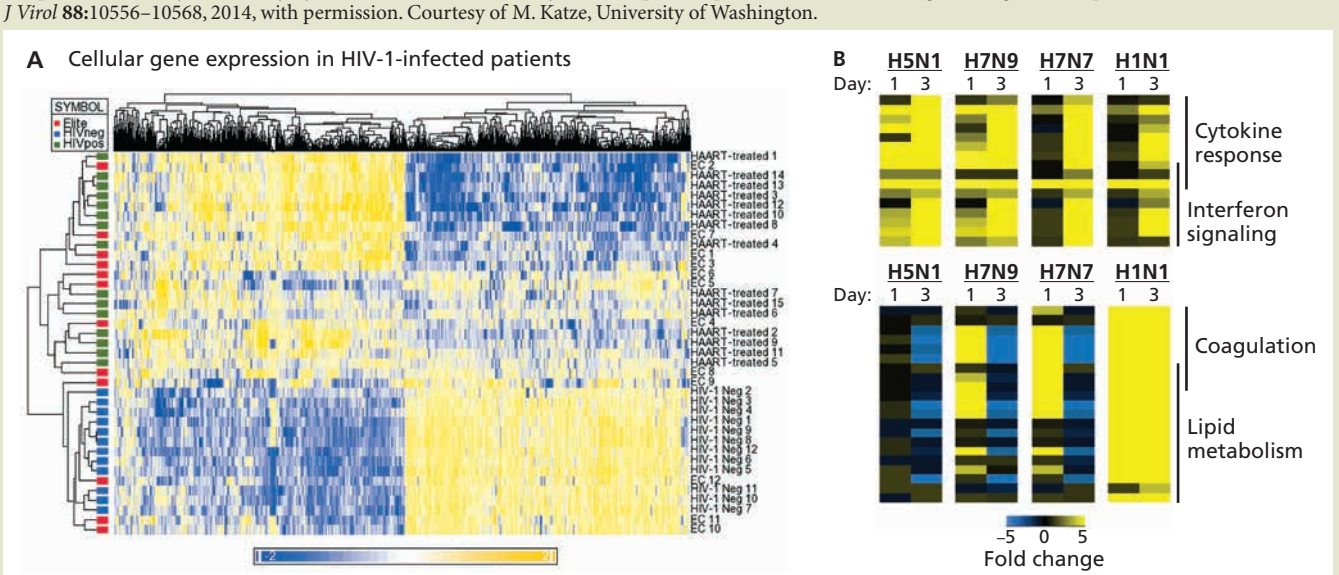
In a very rare subset of individuals infected with human immunodeficiency virus type 1, reproduction of the virus (that is, synthesis of genomes) is undetectable by all but extremely sensitive assays. Studies of such "elite controllers" have identified various features of the immune response that correlate with restriction of virus reproduction (Volume II, Chapter 7). Unexpectedly, two groups of elite controllers were distinguished by the results of microarray hybridization of RNA isolated from $CD4^+$ T cells of uninfected individuals, human immunodeficiency virus type 1-infected patients undergoing highly active antiretroviral therapy (HAART), and elite controllers. Members of one group of elite controllers exhibited gene expression patterns closely resembling those of virus-negative individuals, while the expression patterns of the second group clustered with those of patients receiving HAART (panel A of the figure). Subsequently, the elite controllers in the first group were shown to have higher $CD4^+$ T cell counts and reduced $CD8^+$ T cell responses compared with the members of the second group. The number of elite controllers with gene expression profiles like those of uninfected persons was small (4 of the 12 elite controllers examined), but further studies of the properties of their T cells may provide clues in the search for a cure for human immunodeficiency virus type 1.

Infection of humans by highly pathogenic avian influenza virus is occurring more frequently since the first case of H5N1 avian virus in humans was reported in 1997 (Volume II, Chapter 11). So far, such outbreaks, which are associated with high case-fatality ratios, have been sporadic, because the avian viruses are not transmitted from human to human. The pathogenicity of these avian viruses in mammals cannot be predicted a priori from their genome sequences, and is traditionally assessed in such model animals as mice and ferrets. In one complementary approach to such traditional methods, the gene expression profiles of lung tissue of mice infected with influenza virus strains that differ in pathogenicity were compared. These viruses were

- an H5N1 avian influenza virus strain that is highly pathogenic in laboratory animals but poorly transmissible
- an H7N9 avian influenza virus that is somewhat less pathogenic but more transmissible
- an H7N7 avian influenza virus strain with lethality in animals similar to that of the H7N9 virus
- a strain from the 2009 H1N1 pandemic in humans, which was observed to be much less pathogenic in mice than any of the avian virus isolates

Gene expression differences in lung tissue in mice infected by these four influenza virus strains were observed. However, the

(A) Clustering of genes differentially expressed in uninfected individuals, human immunodeficiency virus type 1 (HIV-1)-infected patients receiving HAART, and elite controllers, indicated by the green, blue, and red squares, respectively, at the left. These differences are based on the results of microarray hybridization of duplicate samples of RNA isolated from $CD4^+$ T cells. Note the clear separation of the profiles from uninfected persons and infected patients, and the dispersal of those from elite controllers in both clusters. Adapted from F. Vigneault et al., *J Virol* **85:**3015–3019, 2011, with permission. Courtesy of M. Lichterfeld, Massachusetts General Hospital. **(B)** Mice were infected with the types of influenza virus strain indicated at the top, and RNA was isolated from lung tissue after 1 or 3 days of infection and from mock-infected animals. Differences between the infected and mock-infected samples identified by microarray hybridization are shown for cytokine response, lipid metabolism, and coagulation genes. Adapted from J. Morrison et al., *J Virol* **88:**10556–10568, 2014, with permission. Courtesy of M. Katze, University of Washington.



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most striking was the correlation of high pathogenicity with the increased expression of genes that encode cytokines, interferons, and proteins that mediate the interferon response and the decreased expression of genes associated with lipid metabolism and coagulation (panel B). The latter changes were also observed in animals infected by the highly pathogenic 1918 H1N1 influenza virus, although their significance is not yet known. It has been suggested that such a gene expression signature of highly pathogenic influenza viruses could help identify new therapeutics that target specific host proteins or pathways.

Morrison J, Josset L, Tchitchek N, Chang J, Belser JA, Swayne DE, Pantin-Jackwood MJ, Tumpey TM, Katze MG. 2014. H7N9 and other pathogenic avian influenza viruses elicit a three-pronged transcriptomic signature that is reminiscent of 1918 influenza virus and is associated with a lethal outcome in mice. *J Virol* **88:**10556–10568.

Vigneault F, Woods M, Buzon MJ, Li C, Pereyra F, Crosby SD, Rychert J, Church G, Martinez-Picado J, Rosenberg ES, Telenti A, Yu XG, Lichterfeld M. 2011. Transcriptional profiling of CD4 T cells identifies distinct subgroups of HIV-1 elite controllers. *J Virol* **85:**3015–3019.

ever, the majority of the changes can be attributed to the viral 243R E1A protein (Fig. 8.18), as they also occur when cells are infected by a mutant virus that directs efficient synthesis of only this protein. The E1A protein associates with the promoters of host cell genes altered in expression in infected cells, where it modulates the recruitment to promoters of the cellular repressor Rb and acetylation of histones at specific residues (Fig. 14.6).

Metabolism

Host cells supply not only the molecular machinery needed for synthesis of viral nucleic acids and proteins (at a minimum the translational machinery), but also the essential building blocks, nucleotides and amino acids. Assembly of enveloped viruses also requires cellular membranes and the lipids from which these structures are constructed. The production of large quantities of viral macromolecules and virus particles, often within a short period (a day or less), imposes heavy demands on the host cell's biosynthetic systems that manufacture nucleotides, amino acids, and, in many cases, fatty acids. Synthesis of these molecules consumes energy, typically supplied by the hydrolysis of ATP, as does production of viral macromolecules: synthesis of a single peptide bond, for example, consumes the equivalent of 4 molecules of ATP, and energy is also expended during the folding of viral proteins and intracellular transport of viral nucleic acids and proteins during the infectious cycle. Consequently, virus infection can lead to alterations in the pathways by which cells generate energy from molecular fuels (**catabolism**), as well as those that make the precursors of nucleic acids, proteins, and membranes (**anabolism**). Perhaps not surprisingly, the impact of infection on host metabolism is virus specific, ranging from relatively simple alterations in the rates of particular reactions to extensive redirection of multiple pathways to virus-specific ends. Indeed, infection by some viruses has been associated with development of metabolic diseases.

Methods To Study Metabolism

Some of the earliest studies of host cell responses to virus infection examined rates of catabolism by measuring the uptake of molecular oxygen or release of lactic acid (Fig. 14.7), the end product of anaerobic glycolysis. It has therefore been appreciated for decades that virus infection modulates cellular energy metabolism, often increasing the rate of glycolysis. However, this aspect of virus-host cell interactions was difficult to study in detail until the development of methods for simultaneous and comprehensive measurement of the concentrations of large numbers of metabolites and of changes in flux through individual pathways and reactions, so-called metabolomics. In the past decade or so, application of these methods to virus-infected cells (and their mock-infected counterparts) has revealed just how extensive the modulation of catabolism or anabolism can be, and unexpected ways in which virus infection can redirect metabolic networks.

Substrates and intermediates of metabolic pathways turn over as they are converted to other compounds, and many do so at high rates. Accurate measurement of the concentrations of metabolites under a particular condition therefore requires that metabolic pathways be halted quickly as samples are collected. This imperative is typically met by rapidly transferring cells to ice-cold organic solvents, a process that also contributes to extraction of metabolites. These compounds are then separated and identified by a variety of analytic techniques, most commonly liquid or gas chromatography followed by one- or two-dimensional mass spectrometry. The ions separated in this way are identified by comparison of their properties to the contents of reference libraries of metabolites, and can be quantified. Accurate comparison among samples is facilitated by examination of multiple experimental replicates and the addition of internal standards.

This approach can identify changes in the concentration of many metabolites, and hence indicates that the rates of particular pathways may be altered following virus infection. More-precise information can be obtained by supplying infected cells with a metabolic precursor labeled with a heavy atom (such as ^{13}C or ^{15}N). Because mass spectrometry separates compounds on the basis of mass and charge, the flow of the heavy atom to other metabolites can be then traced as a function of time. This method allows measurement of the

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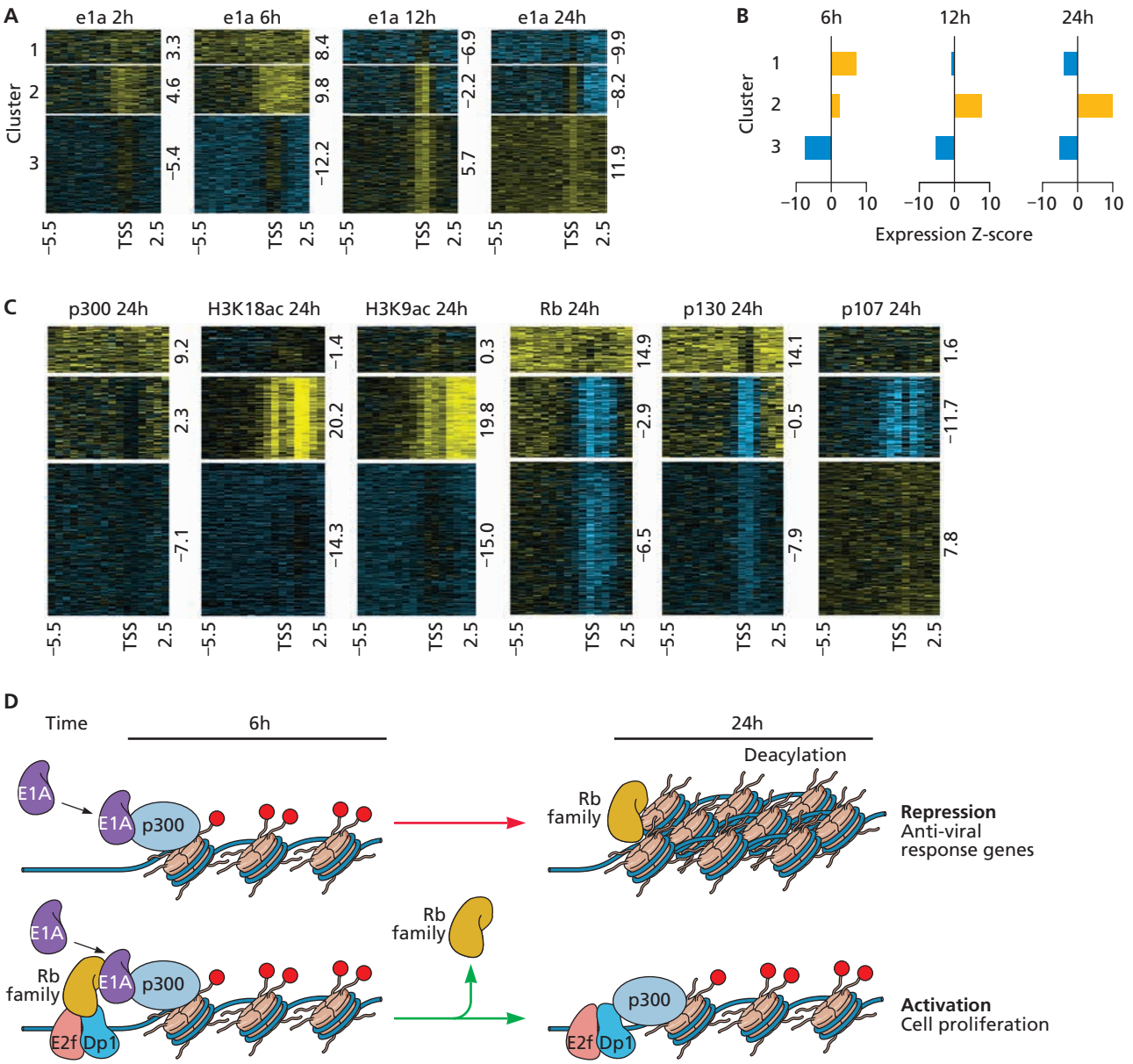


Figure 14.6 Reprogramming of promoter-associated transcriptional regulators by an adenovirus protein. **(A)** Contact-inhibited (quiescent) normal human fibroblasts were infected by a mutant of human adenovirus type 2 that directs synthesis in infected cells of only the smaller (243R) E1A protein. At the times after infection indicated, DNA bound to the E1A protein was isolated by chromatin immunoprecipitation (Box 8.1) and hybridized to microarrays containing probes that span from −5.5 kb to +2.5 kb (relative to the transcriptional start site [TSS]) of some 17,000 human promoters (so-called tiling arrays). The patterns of enrichment or loss of E1A across the promoters for 70% of the promoters to which E1A bound defined three clusters as indicated. **(B)** Comparison of the RNA profiles of infected and mock-infected cells showed that the expression of the genes present in each of the three clusters defined by kinetic patterns of E1A protein association exhibited different responses to synthesis of the E1A protein (note the change of scale at 24 h postinfection). **(C)** Chromatin immunoprecipitation was performed with antibodies that recognize cellular proteins that bind to the E1A protein (p300/Cbp, Rb, and the related proteins p130 and p107) or histone H3-bearing acetyl groups at specific lysine residues. The recovered DNA was hybridized to the same arrays. **(D)** These data indicated mechanisms by which the E1A protein alters expression of cellular genes. For example, the large increase in expression of genes in cluster 2 by 24 h postinfection correlated with loss of the transcriptional repressor Rb (and its relatives) and concomitant large increases in the association of histone H3 acetylated at Lys9 or Lys18, posttranslational modifications associated with activation of transcription. This cluster was enriched in genes associated with cell proliferation (growth and progression through the cell cycle) and DNA synthesis, and with promoters that contain binding sites for transcriptional activators of the E2F family, the primary targets of repression of gene expression by Rb proteins (Chapter 8). These E1A-induced alterations in proteins associated with the promoters of cluster 2 genes are consistent with ability of the E1A protein to overcome Rb-mediated inhibition of transcription of E2F-dependent genes observed in simplified experimental systems. Adapted from R. Ferrari et al., *Science* **321:**1086–1088, 2008, with permission. Panels A to C courtesy of S. Kurdistani, University of California, Los Angeles.

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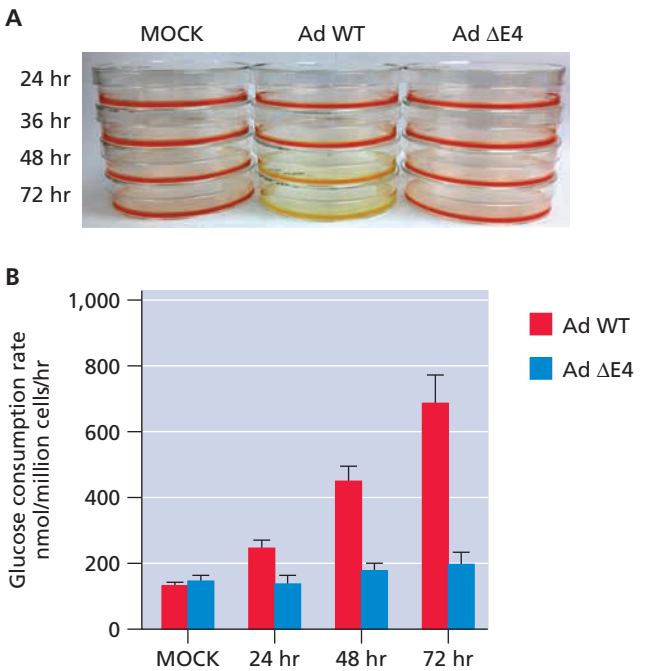


Figure 14.7 Increased glycolysis in virus-infected cells. **(A)** Infection by a variety of viruses (including adenoviruses, hepdnaviruses, herpesviruses, orthomyxoviruses, papillomaviruses, polyomavirus, and retroviruses) increases the rate of glycolysis, as illustrated for adenovirus. Plates of human breast epithelial cells infected with human adenovirus type 5 (Ad5) or a mutant that lacks the E4 gene (Ad ΔE4) or mock-infected (mock) were incubated for the periods indicated at the left. The pH of the medium is indicated by the color of the indicator it contains, where red and yellow indicate neutral and acidic pH, respectively. The increasing acidity of the medium of Ad5-infected cells is the result of the increased production of lactic acid, the product of glycolysis under anaerobic (or hypoxic) conditions. **(B)** This change was accompanied by increased rates of glucose consumption (but decreased O_2 uptake). Adapted from M. Thai et al., *Cell Metab* **19:**694–701, 2014, with permission. Courtesy of H. Christofk, University of California, Los Angeles.

rates of metabolic reactions of interest. It has been particularly valuable in tracing some unusual fates of common metabolites in virus-infected cells.

Comparison of the concentrations of metabolic enzymes (or of the mRNAs that encode them) in uninfected and infected cells can also indicate virus-induced perturbations of particular metabolic pathways, or mechanisms by which the production or consumption of metabolites is modulated. Application of these approaches, in conjunction with examination of the effects of inhibition of individual enzymes on viral reproduction, has illuminated various ways in which virus infection deranges the metabolic homeostasis of the host cell.

Glucose Metabolism

During glycolysis, the 6-carbon sugar glucose, the major product of breakdown of dietary carbohydrate, is converted to 2 molecules of the 3-carbon compound pyruvate (Fig. 14.8). From 1 molecule of glucose, this series of 10 reactions generates energy in the form of 2 molecules of ATP, which can be used directly in numerous reactions and processes, and 2 molecules of NADH (reduced nicotinamide adenine dinucleotide). The latter compound can be used for production of

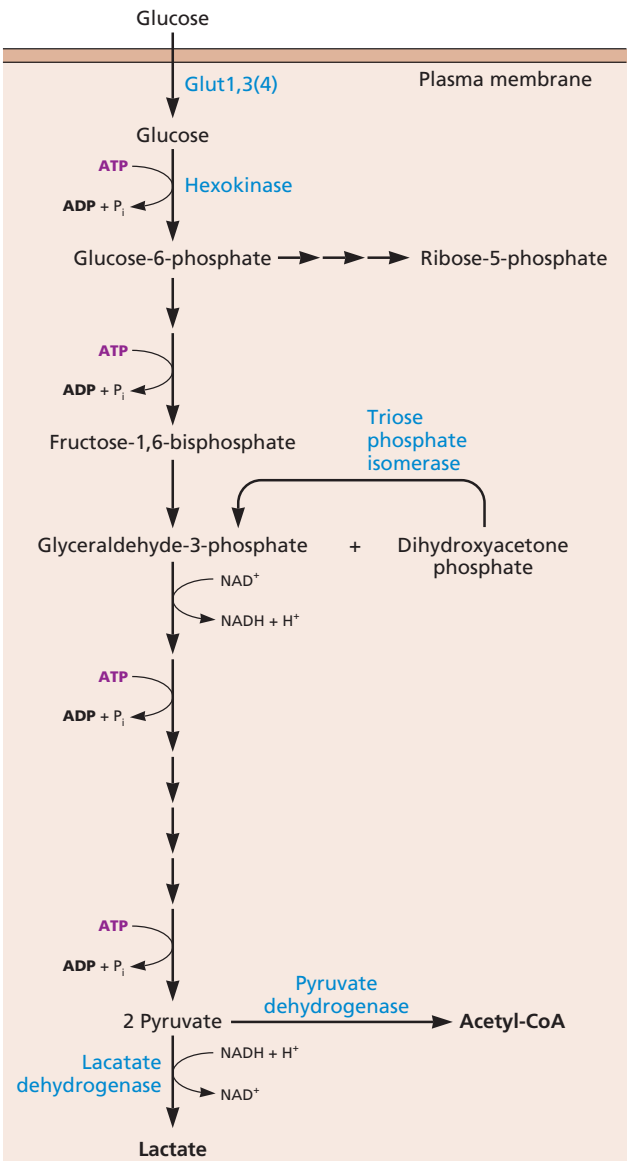


Figure 14.8 Glucose metabolism. Following transport into cells via glucose transporters (Gluts) and phosphorylation by hexokinase, glucose can enter glycolysis or, in rapidly growing cells in which nucleotide biosynthesis is required, the pentose phosphate pathway. The numbers of reactions in these pathways are indicated by the arrows, and the products, some intermediates, and some enzymes are listed. The 6-carbon molecule fructose-1,6-bisphosphate is converted to two 3-carbon compounds, both of which can be converted to pyruvate. Consequently, glycolysis produces 2 molecules of pyruvate from 1 of glucose, with a net energy yield of 2 ATP and 2 NADH.

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additional ATP by the electron transport chain and oxidative phosphorylation in mitochondria, or be consumed in other metabolic reactions. One important function of glycolysis is to allow synthesis of ATP, and in some cells glucose is the only (red blood cells) or preferred (neurons) source of energy. However, this pathway also yields intermediates and products that allow synthesis of much larger quantities of ATP, or provide carbon skeletons for biosynthetic reactions. For example, glucose-6-phosphate is not only the substrate of the second glycolytic reaction, but also the precursor for synthesis of ribose, and hence nucleotides, RNA, and DNA. Similarly, pyruvate, the final product of glycolysis, is often converted to acetyl coenzyme A (acetyl-CoA), which serves as a precursor for synthesis of fatty acids and sterols or enters the citric acid cycle. This cycle generates energy and precursors to amino acids and bases (purines and pyrimidines).

Our understanding of the impact of virus infection on glycolysis, particularly the mechanisms by which viral gene products regulate this important pathway, is far from complete. Nevertheless, it is clear that infection of mammalian cells by a variety of viruses increases the rate of glycolysis by altering the concentration, activity, or other properties of cellular enzymes and other proteins that execute the initial metabolism of glucose. An increased rate of flux from glucose to pyruvate (Fig. 14.8) is a common response to virus infection, but is not inevitable, a perhaps counterintuitive fact emphasized by the opposite effects on this pathway of infection by two human herpesviruses (Box 14.6). In this section, we use some specific examples to illustrate the ways in which glucose metabolism can be perturbed in virus-infected cells and the association of such responses to virus infection with human disease.

Virus Infection Can Alter the Rate of Glycolysis by Several Mechanisms

Regardless of its final fate, glucose must enter cells before it can be metabolized. This hydrophilic molecule is transported across the plasma membrane by any one of a number of glucose transporters (12 are encoded in the human genome). These include the ubiquitous protein glucose transporter 1 (Glut1); tissue-specific transporters such as Glut3, present in neurons of the central nervous system; and the insulin-regulated transporter Glut4, which is present in skeletal muscle and adipose tissue. Localization of Glut4 to the plasma membrane depends on the presence of the hormone insulin, which is synthesized in and released from specialized cells (β cells) in the pancreas in response to high concentrations of blood glucose. Glucose is phosphorylated to glucose-6-phosphate upon entry into cells, a reaction that is irreversible under normal physiological conditions. This modification ensures retention of glucose within cells, and activates it for subsequent glycolysis or entry into the pentose phosphate pathway (Fig. 14.8). One parameter contributing to the increased rate of glycolysis observed in cells infected by hepatitis C virus, herpesviruses, and human immunodeficiency virus type 1 is an increased rate of glucose uptake.

In several cases, such accelerated transport of glucose into virus-infected cells can be attributed to elevated concentrations of Glut1 or Glut3, as a result of alterations in signal transduction pathways that regulate transcription. For example, in cells infected by the gammaherpesvirus Epstein-Barr virus or human papillomaviruses, the concentration of hypoxia-inducible factor 1α (Hif-1α) is increased. This transcriptional regulator activates expression of the genes encoding Glut1 and Glut3 (as well as several that encode glycolytic enzymes). This mechanism resembles the normal responses of uninfected cells to low availability of oxygen (hypoxia) and nutrients. In contrast to modulation of Glut1 or Glut3, the increased uptake of glucose into human cytomegalovirus-infected fibroblasts is mediated by the insulin-regulated transporter Glut4. This protein is not made in uninfected fibroblasts, but transcription of the gene that encodes it is turned on in infected cells as a result of increased production of the transcriptional regulator Chrebp (carbohydrate response element-binding protein): human cytomegalovirus infection therefore overrides the mechanisms that normally control production of Glut4. The restriction of the transporter to intracellular vesicles unless insulin stimulates signaling via Akt is also circumvented, because infection activates this signal transduction pathway. Inhibition of synthesis of Chrebp by RNA interference reduced glucose uptake by infected cells, prevented production of Glut4 mRNA, and reduced the yield of progeny virus particles. These observations emphasize the importance of the switch from Glut1 to Glut4 for efficient human cytomegalovirus reproduction, a necessity that would not have been predicted from the relatively modest increase (some 3-fold) in the affinity of Glut4 for glucose.

In principle (the law of mass action), increased intracellular concentrations of glucose as a result of more efficient transport across the plasma membrane could account for increased rates of glycolysis in virus-infected cells. Nevertheless, in several cases, the rate of flux through this pathway is also accelerated by increases in the intracellular concentration or activity of one or more glycolytic enzymes. For example, the activity of phosphofructokinase 1, which catalyzes the committed reaction in this pathway (Fig. 14.8), is increased in cells infected by human cytomegalovirus. The concentrations of several glycolytic enzymes (or their mRNAs) are also elevated following infection by this virus, and early during acute infection of hepatocytes in culture with hepatitis C virus.

Virus Infection Can Redirect the Utilization of Glycolytic Intermediates and Products

Acceleration of glycolysis in virus-infected cells can help provide the additional energy required for production and transport of viral macromolecules. However, this response to

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BOX 14.6

EXPERIMENTS

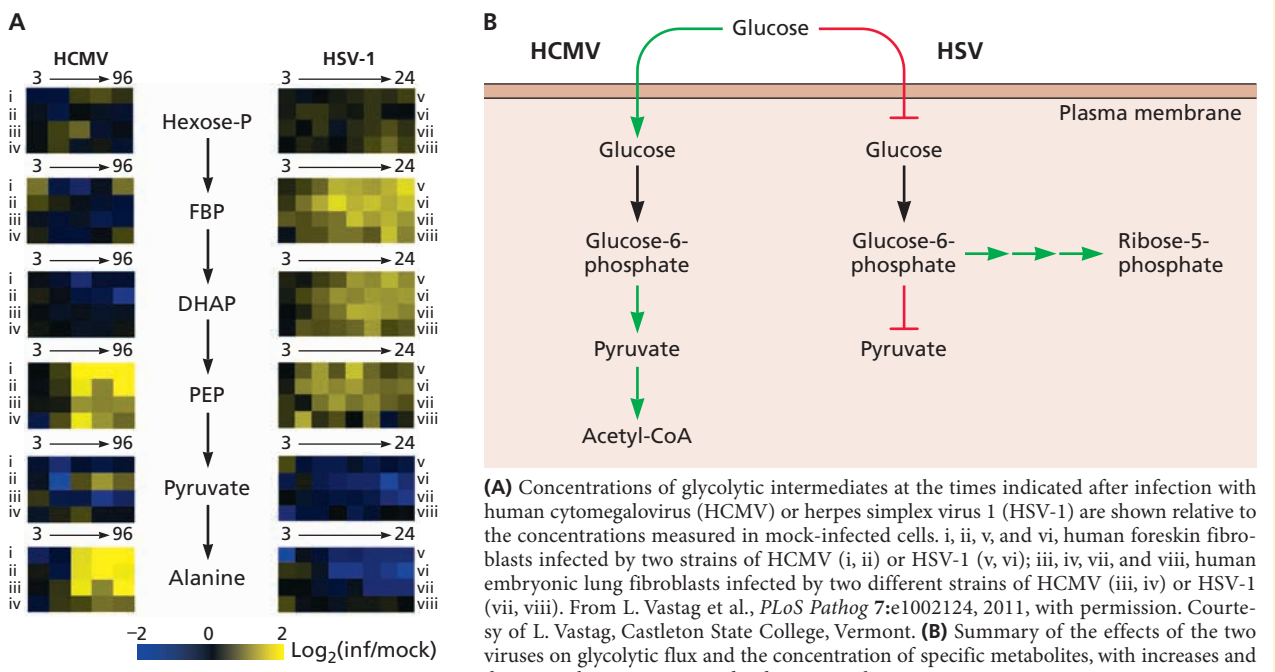
Members of the same virus family can exert different effects on metabolism: glycolysis in cells infected by two human herpesviruses

The impact of infection with the alpha- and betaherpesvirus herpes simplex virus 1 and human cytomegalovirus, respectively, on carbon metabolism in normal human fibroblasts or epithelial cells was compared initially by measuring the concentration of >80 metabolites as a function of time after infection. Analysis of these data identified some host-cell-type-specific responses and some changes common to infection by the two viruses, such as increased concentrations of dTTP in infected cells. However, major differences were also detected, notably increased accumulation of glycolytic intermediates in cells infected by herpes simplex virus 1, but decreased concentrations in human cytomegalovirus-infected cells (panel A of the figure).

The reasons for this difference were investigated further, for example, by supplying infected cells with ^{13}C-labeled glucose and monitoring its incorporation into downstream metabolites as a function of time thereafter. In human cytomegalovirus-infected cells, the uptake of glucose and the labeling of glycolytic intermediates such as fructose-1,6-bisphosphate were increased, indicating stimulation of glycolytic flux (panel B). However, these parameters were decreased in herpes simplex virus-infected cells, accounting for the buildup of glycolytic intermediates (panel A). This response was accompanied by increased concentrations of intermediates in the pentose phosphate pathway and of its product, ribose-5-phosphate (panel B). Synthesis of pyrimidines is also increased in herpes simplex virus 1-infected cells (see text). The increased production of pyruvate in human cytomegalovirus-infected cells because of the acceleration of glycolytic flux supports increased production of fatty acids, following synthesis of the precursor, acetyl-CoA (see the text).

It has been proposed that the quite different fates of carbon from glucose in cells infected by these two human herpesviruses is the result of the much shorter replication cycle of herpes simplex virus 1 than of human cytomegalovirus, some 24 and 96 h, respectively, to attain the maximal yield of progeny virus particles. The relatively rapid reproduction of herpes simplex virus and some 10-fold-higher yield of virus particles require synthesis of a large number of viral DNA genomes (and large quantities of viral RNAs) in a short period, and hence impose a greater demand for nucleotide precursors from the host cell.

Vastag L, Koyuncu E, Grady SL, Shenk TE, Rabinowitz JD. 2011. Divergent effects of human cytomegalovirus and herpes simplex virus-1 on cellular metabolism. *PLoS Pathog* **7:**e1002124. doi:10.1371/journal.ppat.1002124.



(A) Concentrations of glycolytic intermediates at the times indicated after infection with human cytomegalovirus (HCMV) or herpes simplex virus 1 (HSV-1) are shown relative to the concentrations measured in mock-infected cells. i, ii, v, and vi, human foreskin fibroblasts infected by two strains of HCMV (i, ii) or HSV-1 (v, vi); iii, iv, vii, and viii, human embryonic lung fibroblasts infected by two different strains of HCMV (iii, iv) or HSV-1 (vii, viii). From L. Vastag et al., *PLoS Pathog* **7:**e1002124, 2011, with permission. Courtesy of L. Vastag, Castleton State College, Vermont. **(B)** Summary of the effects of the two viruses on glycolytic flux and the concentration of specific metabolites, with increases and decreases shown in green and red, respectively.

infection can also foster virus reproduction by increasing the supply of precursors for biosynthesis of lipids and nucleotides.

Under aerobic conditions in mammalian cells, pyruvate (the final product of glycolysis) enters mitochondria and is converted to acetyl-CoA. This compound is an activated carrier of units of 2 carbon atoms that is a major source of energy following its entry into the citric acid cycle (see below), and is also the precursor for synthesis of fatty acids and sterols. In cells infected by human cytomegalovirus, most of the acetyl-CoA produced from pyruvate is rerouted

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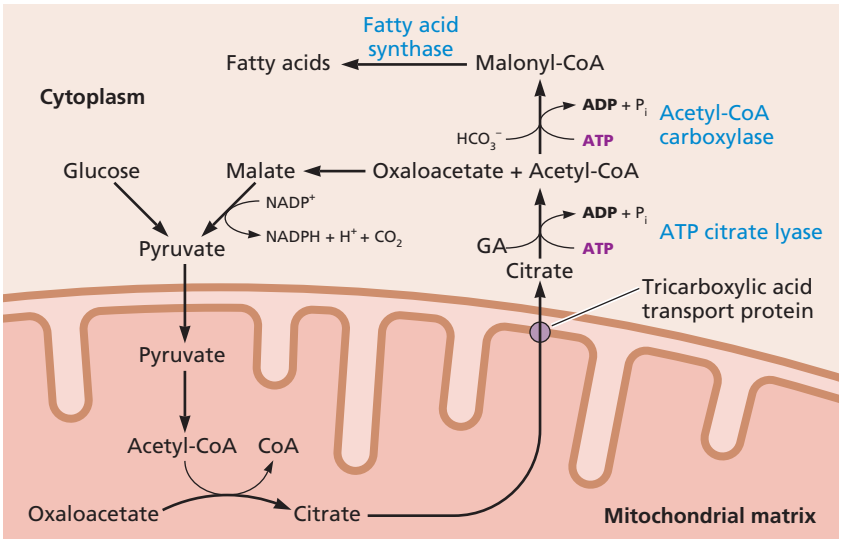


Figure 14.9 Diversion of acetyl-CoA for fatty acid synthesis in human cytomegalovirus-infected cells. Acetyl-CoA is the precursor for synthesis of fatty acids. However, this process takes place in the cytoplasm, whereas acetyl-CoA is produced within the mitochondrial matrix, as indicated. As this metabolite cannot be transported across the mitochondrial membrane, it is first converted to citrate via the first reaction in the citric acid cycle. Citrate then enters the cytoplasm via the tricarboxylate transport protein, where it is reconverted to acetyl-CoA and oxaloacetate by ATP citrate lyase. Formation of malonyl-CoA by acetyl-CoA carboxylase initiates fatty acid synthesis, which is catalyzed by the multi-active-site enzyme fatty acid synthase. As shown, pyruvate can be regenerated from oxaloacetate, with production of NAPDH, which is consumed during fatty acid synthesis.

from mitochondria to the cytoplasm by the shuttle shown in Fig. 14.9 to promote biosynthesis of fatty acids. In contrast, an important function of glycolysis during the early phase of herpes simplex virus 1 infection is to allow increased synthesis of ribose and deoxyribose (and hence nucleotides and nucleic acids): the concentrations of intermediates and enzymes of the pentose phosphate pathway are elevated in fibroblasts infected by this human herpesvirus. Furthermore, the concentrations of an enzyme (and its mRNA) that consumes aspartate, the critical donor of amino groups during purine biosynthesis, are decreased in infected cells, and inhibition of production of the enzyme stimulates viral genome replication and the yield of virions. It has been proposed that the different fates of glycolytic intermediates or products in cells infected by these two human herpesviruses are a consequence of the much more rapid reproduction of herpes simplex virus 1 than of human cytomegalovirus (Box 14.6). Human cells infected by the poxvirus vaccinia virus are also characterized by elevated concentrations of several nucleotides, including TMP, dATP, and dGTP, but whether flux through the pentose phosphate pathway is increased is not clear.

Human Disease Associated with Virus-Induced Alterations in Glucose Metabolism

In humans (and other mammals), the liver makes a critical contribution to glucose homeostasis: it is a major site of both the synthesis of glycogen, the polymer in which excess glucose is stored, and *de novo* synthesis of the sugar from 2-carbon compounds. The latter activity is essential to maintain blood glucose concentrations when food intake is low or during prolonged exercise or stress. Among the detrimental effects of infection by some hepatotropic viruses are major perturbations of glucose homeostasis that can lead to development of disease.

Infection by the hepadnavirus hepatitis B virus is strongly associated with the development of type 2 diabetes (insulin-independent) in certain populations (for example, Asians) and with increased blood glucose levels (hyperglycemia) in patients with acute infection. Such a systemic perturbation in glucose concentrations arises because a high proportion of liver cells can be infected by this virus. In both human hepatocytes in culture and the livers of transgenic mice, expression of the viral X gene is sufficient to stimulate expression of several cellular genes that encode enzymes required for synthesis of glucose from 2-carbon precursors (gluconeogenesis), notably phosphoenolpyruvate carboxykinase, which catalyzes the first (and committed) reaction in the synthesis of glucose from pyruvate. This response is likely to be of physiological significance, because mice transgenic for the viral X gene exhibit increased synthesis of glucose compared to control mice. They are also glucose intolerant; that is, glucose is not removed effectively from the blood when the animals are either fed or starved. Both activation of glucogenesis and glucose intolerance are symptoms of type 2 diabetes.

Insulin resistance, another diagnostic marker for development of type 2 diabetes, is exhibited by some 25% of patients with chronic hepatitis C virus infection. Insulin-dependent signaling is disrupted by the viral C (capsid) protein, which induces altered phosphorylation and increased degradation of a critical signal transducer in this pathway. However, hepatitis C virus infection of human hepatocytes in culture also results in increased concentrations of phosphoenolpyruvate carboxykinase (and its mRNA) and reduced quantities of Glut4 and cell-surface Glut2. These observations suggest that increased glucose synthesis and reduced uptake of glucose by infected hepatocytes may also promote development

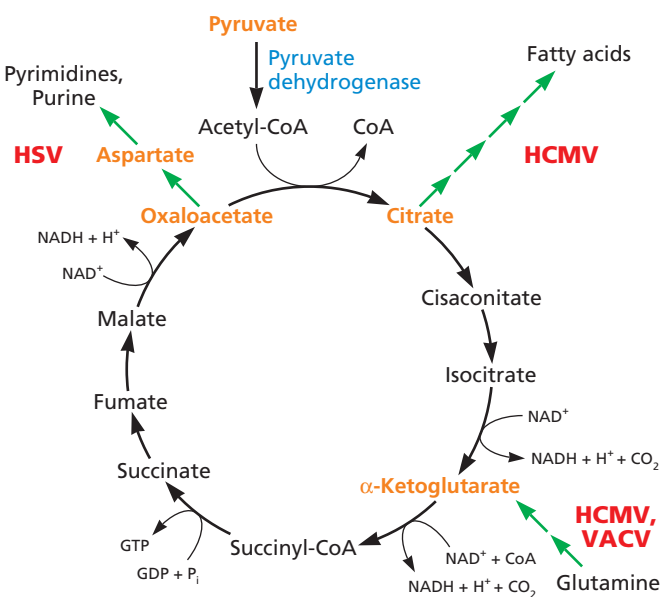
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of type 2 diabetes in patients with chronic hepatitis C virus infection.

The Citric Acid Cycle

The citric acid cycle (also called the tricarboxylic acid [TCA] or Krebs cycle) is an important central hub of carbon metabolism, serving as the final common pathway for oxidation of carbon from glucose and other fuels (such as fatty acids and the carbon skeletons of amino acids). Under normal conditions, 1 acetyl group, which enters the cycle as acetyl-CoA, is oxidized to CO_2 in 1 turn of the cycle with generation of energy in the form of the reduced electron carriers NADH and $FADH_2$ (reduced flavin adenine dinucleotide), and GTP. However, the 8 reactions that comprise the citric acid cycle are amphibiotic; that is, they also yield precursors for biosynthesis of a great variety of compounds (e.g., Fig. 14.10). Although such biosynthetic reactions remove intermediates from the cycle, their concentrations are normally almost constant, because the citric acid cycle is replenished by several reactions. Despite its importance in both catabolism and anabolism, the citric acid cycle in virus-infected cells has received relatively little attention: only the impacts of infection by some of the larger DNA viruses have been examined in detail.

Figure 14.10 The citric acid cycle and some alterations induced in virus-infected cells. Acetyl-CoA produced by oxidation of pyruvate enters the citric acid cycle when its 2 carbon atoms are transferred to oxaloacetate to form citrate. The subsequent 8 reactions of the cycle accomplish complete oxidation of the acetate group to 2 molecules of CO_2, with production of energy in the form of GTP and the reduced electron carriers NADH and $FADH_2$, as shown. Reactions consuming intermediates in the cycle or replenishing them that are stimulated in virus-infected cells are indicated. HSV, herpes simplex virus 1; HCMV, human cytomegalovirus; VACV, vaccinia virus.



Alternative Mechanisms of Entry of Carbon Atoms from Glucose into the Citric Acid Cycle in Cells Infected by Alpha- and Betaherpesviruses

When infected cells are supplied with ^{13}C-labeled glucose, it is possible to trace the flow of the heavy carbon atoms from this metabolite into intermediates in the citric acid cycle (or in other pathways). Such studies established that 2 carbon atoms are incorporated into citrate (6 carbon atoms) from glucose in cells infected by herpes simplex virus 1, but that 3 carbon atoms of citrate become labeled in human cytomegalovirus-infected cells. This difference indicates that pyruvate (the product of glycolysis) is first converted to acetyl-CoA or oxaloacetate, respectively (Fig. 14.11). The choice between these fates of pyruvate is strongly regulated by the concentration of acetyl-CoA. This important intermediate in metabolism is produced by catabolism of a variety of fuels, and its concentration is therefore an indicator of how well a cell is supplied with a source of energy. It is a positive regulator of pyruvate carboxylase, but an allosteric inhibitor of pyruvate dehydrogenase (Fig. 14.11). In human cytomegalovirus-infected cells, much acetyl-CoA is consumed for synthesis of fatty acids (see "Lipid Metabolism" below). Consequently, the mitochondrial concentration of this compound would be low, favoring activation of pyruvate dehydrogenase. However, the mechanisms responsible for the differential regulation of these enzymes in cells infected by these two herpesviruses have not been examined.

Even though synthesis of oxaloacetate catalyzed by pyruvate carboxylase is a reaction that replenishes the citric acid cycle, the concentration of intermediates in this cycle decreases as the herpes simplex virus infectious cycle proceeds. This reduction is the result of diversion of aspartate from participation in the cycle to synthesis of pyrimidine nucleotides, such as UTP (Fig. 14.10). Inhibition of pyruvate carboxylase or the first enzyme in the pathway by which pyrimidines are made from aspartate impaired production of infectious particles of herpes simplex virus, but not of human cytomegalovirus, emphasizing the very different needs for cellular metabolic pathways for efficient reproduction of these human herpesviruses.

Enhanced Replenishment of the Citric Acid Cycle by Metabolism of Glutamine

Analysis of the flux of labeled carbon atoms from glucose in cells infected by human cytomegalovirus indicated that most of the citrate produced from acetyl-CoA and pyruvate leaves the mitochondria for conversion to oxaloacetate and acetyl-CoA in the cytoplasm (Fig. 14.9). Halting of the citric acid cycle as a result of such withdrawal of citrate is prevented by the enhanced uptake of glutamine and its conversion to the citric acid cycle intermediate α-ketoglutarate, because of increased production and activity of the enzymes that catalyze this process in infected cells.

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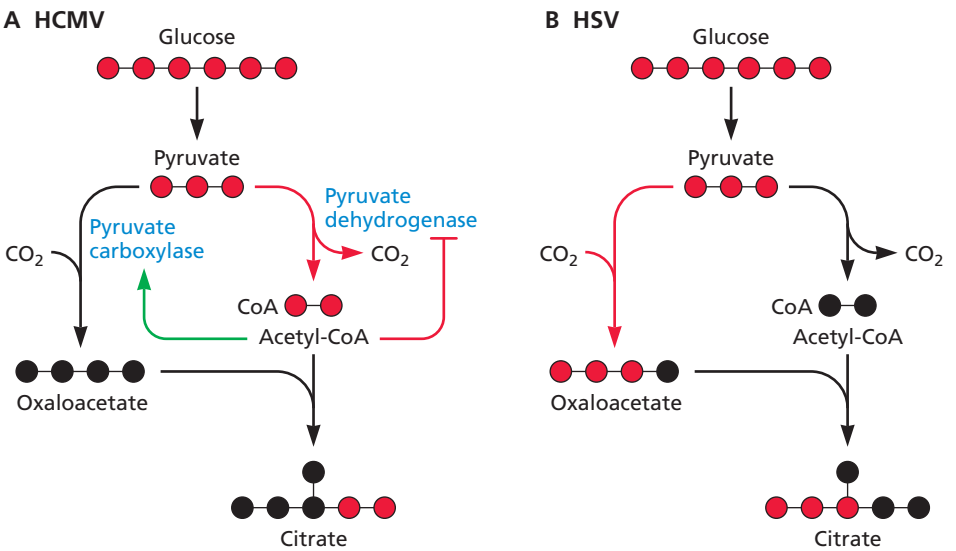


Figure 14.11 Alternative metabolism of pyruvate in alpha- and betaherpesvirus-infected cells. When all carbon atoms of glucose are labeled with ^{13}C (red), so too are all 3 carbon atoms of pyruvate. This metabolite can be converted to acetyl-CoA by pyruvate dehydrogenase or to oxaloacetate by pyruvate carboxylase to produce citrate with 2 or 3 ^{13}C-labeled carbon atoms, respectively. These compounds can be distinguished by mass spectrometry. Measurement of the pools of citrate with different numbers of ^{13}C-labeled carbon atoms indicated that citrate is produced by pyruvate dehydrogenase in cells infected by human cytomegalovirus (HCMV) **(A)**, but by pyruvate carboxylase in herpes simplex virus 1 (HSV)-infected cells **(B)**. Adapted from L. Vastag et al., *PLoS Pathog* **7:**e1002124, 2011, with permission.

Glutamine is also necessary for maximally efficient reproduction of the poxvirus vaccinia virus: its removal from the medium of infected cells reduces the yield of infectious virus particles by >3 orders of magnitude. However, the absence of glucose had no effect, indicating that vaccinia virus reproduction does not depend on catabolism of glucose. The result of experiments in which infected cells were supplied with other metabolites indicated that one fate of glutamine is replenishment of the citric acid cycle via synthesis of α-ketoglutarate. However, continual operation of the cycle to produce energy requires a source of acetyl-CoA (Fig. 14.10). The increased reduction in synthesis of acetyl-CoA from pyruvate observed in vaccinia virus-infected cells indicates that glycolysis is not the source of acetyl-CoA. Rather, it has been proposed that in cells infected by this poxvirus, this crucial compound enters the citric acid cycle by a baroque mechanism in which the fatty acid palmitate is first synthesized in the cytoplasm and then degraded to acetyl-CoA by oxidation in mitochondria (Box 14.7). Such oxidation is a process that itself generates considerable energy.

Electron Transport and Oxidative Phosphorylation

Most of the ATP consumed in a cell is produced as a result of transfer of electrons from the reduced electron carriers NADH and $FADH_2$ through a series of acceptor and donor groups to the final acceptor, molecular oxygen, which is reduced to water (Fig. 14.12). This electron transport system comprises four extremely large, multiprotein assemblies (usually named complexes I, II, III, and IV) and is located in the inner mitochondrial membrane, as is ATP synthase. Transfer of electrons through complexes I, III, and IV results in translocation of protons across the inner mitochondrial membrane, and hence generation of proton and pH gradients across this membrane. The rotary machine ATP synthase harnesses the electrochemical energy of such gradients to synthesize ATP from ADP and phosphate, as protons flow through the enzyme back into the mitochondrial matrix. Normally, there is tight coupling among the electron transfer reactions. However, when cells are hypoxic or experience some other forms of stress, such coupling is compromised, leading to increased formation of damaging reactive oxygen species, such as superoxide and hydroxyl free radical, and decreased synthesis of ATP.

Infection by several viruses has been reported to modulate one or more of these processes. For example, the rates of electron transport and ATP synthesis increase early after infection with the alphavirus Sindbis virus, but the ATP concentration then decreases as the infectious cycle progresses, and oxygen consumption, a surrogate for the rate of ATP production, is decreased following human herpesvirus 8 infection. A more common response to infection is increased production of

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BOX 14.7

EXPERIMENTS

Vaccinia virus infection stimulates both synthesis and degradation of long-chain fatty acids

Studies of fatty acid metabolism in human cells infected by the poxvirus vaccinia virus revealed an unusual, if not unique, combination of metabolic pathways to produce ATP.

Inhibition of either of two enzymes needed for synthesis of fatty acids (acetyl-CoA carboxylase or fatty acid synthase) with small-molecule inhibitors reduced substantially the yield of infectious particles from vaccinia virus-infected cells, indicating that *de novo* synthesis of long-chain fatty acids is necessary for efficient reproduction of this poxvirus. Vaccinia virus particles are enveloped, and their assembly is initiated with formation of the virus-specific crescent membrane in infected cells (Chapter 13). Consequently, the final product of fatty acid synthesis, palmitate (C_{16}), might be expected to facilitate production of membrane components such as phospholipids. However, an inhibitor of the enzyme that catalyzes the first reaction in phospholipid synthesis did not impair vaccinia virus reproduction. Rather, it was demonstrated that

- the absence of glucose from the medium had no effect on the production of infectious vaccinia virus particles, indicating that glycolysis does not provide (directly or indirectly) energy to support the infectious cycle
- inhibition of entry of palmitate into mitochondria, the site of fatty acid oxidation, **did** reduce the yield of virions, particularly when glucose was absent, indicating that fatty acid oxidation might provide acetyl-CoA for energy generation via the citric acid cycle, electron transport, and oxidative phosphorylation
- consistent with this possibility, an inhibitor of a critical enzyme in the fatty acid oxidation pathway reduced virus yield in a dose-dependent manner
- O_2 consumption, a surrogate for the rate of ATP production, increased within a short period following infection, but this increase was blocked when entry of palmitate into mitochondria was prevented

It was therefore concluded that oxidation of palmitate in mitochondria is the primary means of energy generation in vaccinia virus-infected cells, and that this palmitate is first produced in the cytoplasm (see the figure). Other experiments indicated that the citric acid cycle is maintained by uptake of glutamine and its conversion to α-ketoglutarate, a process that might provide acetyl-CoA for palmitate synthesis following shuttling to the cytoplasm in the form of citrate. Inhibition of this mechanism of energy generation specifically impaired late reactions in the infectious cycle and assembly and morphogenesis of virus particles.

The synthesis of palmitate from acetyl-CoA so that this fatty acid can then be degraded by oxidation to produce acetyl-CoA for entry into the citric acid cycle might seem to represent a nonproductive, futile process. However, complete oxidation of 1 molecule of palmitate,

$$Palmitoyl\text{-}CoA + 23\ O_2 + 108\ P_i + 108\ ADP \rightarrow CoA + 108\ ATP + 16\ CO_2 + 23\ H_2O$$

yields 108 ATP (calculated assuming 1 NADH and 1 $FADH_2$ generate 2.5 and 1.5 molecules, respectively, of ATP via the electron transport chain and oxidative phosphorylation).

Synthesis of 1 molecule of palmitate,

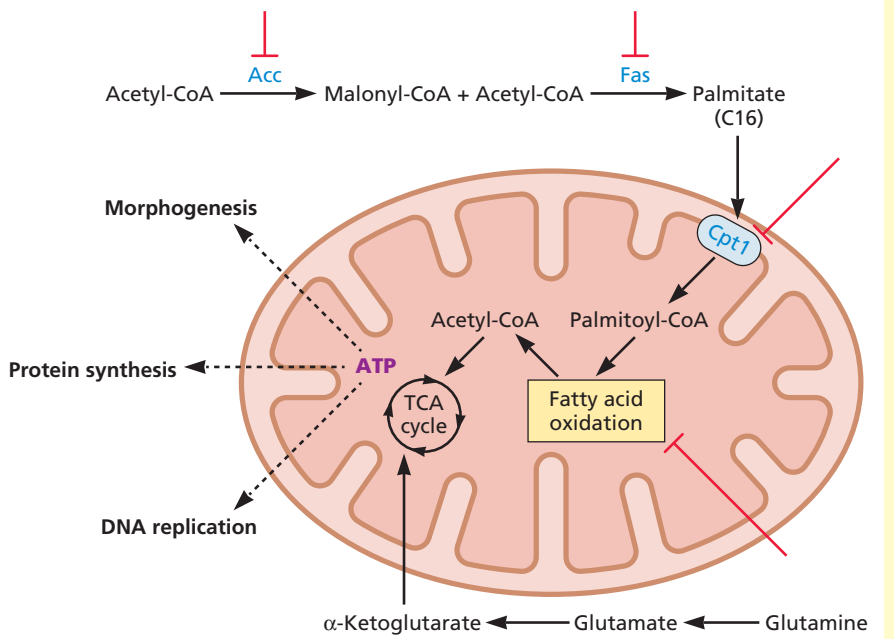
$$8\ Acetyl\text{-}CoA + 7\ ATP + 14\ NADPH + 14\ H^+ \rightarrow Palmitate + 8\ CoA + 7\ ADP + 7\ P_i + 14\ NADP^+ + 6\ H_2O$$

consumes 7 ATP directly and 49 indirectly (because 1 NADPH is equivalent to 1 NADH + 1 ATP), a total of 56 molecules of ATP.

Consequently, nearly twice as much ATP is produced as is consumed. Furthermore, the net yield from 1 molecule of palmitate, 52 molecules of ATP, is considerably greater than that generated by complete oxidation of 1 molecule of glucose, 30 to 32 molecules of ATP.

Greseth MD, Traktman P. 2014. *De novo* fatty acid biosynthesis contributes significantly to establishment of a bioenergetically favorable environment for vaccinia virus infection. *PLoS Pathog* **10:**e1004021. doi:10.1371/journal.ppat.1004021.

A model for production and utilization of palmitate in vaccinia virus-infected cells based on the observations summarized above. The red bars indicate inhibitors of reaction or pathways that reduced the yield of infectious virus particles. Acc, acetyl-CoA carboxylase; Cpt1, carnitine palmitoyltransferase 1; Fas, fatty acid synthase. Adapted from M. D. Greseth and P. Traktman, *PLoS Pathog* **10:**e1004021, 2014, with permission.



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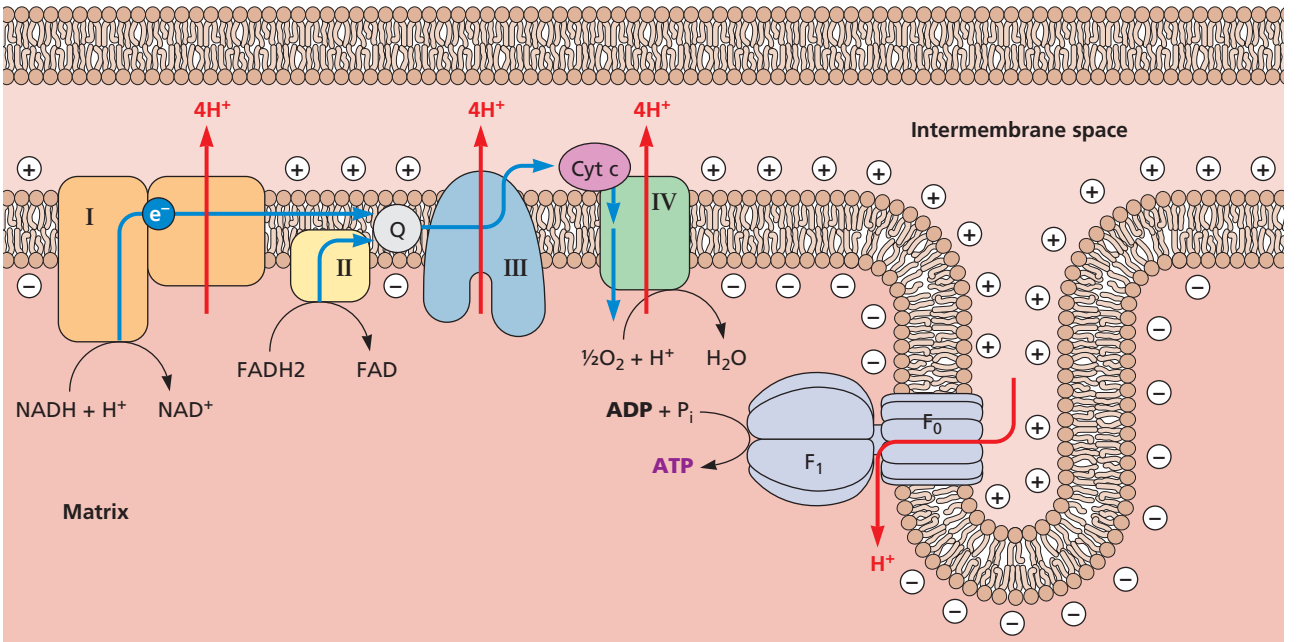


Figure 14.12 The electron transport chain and oxidative phosphorylation. The electron transport chain resides in the inner mitochondrial membrane and comprises four extremely large, multiprotein assemblies called complexes I to IV. Mammalian complex I, for example, is some 1,000 kDa. Furthermore, there is considerable evidence indicating that the complexes associate with one another to form supramolecular assemblies, an organization that would facilitate transfer of electrons among them. Electrons that enter the chain from NADH at complex I are transferred sequentially to multiple electron acceptors in each of complexes I, III, and IV to the ultimate acceptor, molecular oxygen, which is reduced to water. Such transfer of electrons to carriers of increasing reduction potential is accompanied by transfer of protons across the impermeable inner mitochondrial membrane. Electrons from reduced FAD (e.g., bound to the citric acid cycle enzyme succinate dehydrogenase) are transferred to carriers in complex II. As a result of the smaller number of electron transfer reactions between complex II and oxygen, entry of electrons at this site generates less energy for production of ATP than does entry via complex I. Shuttling of electrons from complex I or II is mediated by ubiquinone (complex Q or Q), a lipid-soluble quinone that carries an isoprenoid side chain. The accumulation of protons in the intermembrane space produces both a chemical gradient and an electrical gradient that favor flow of proteins back into the mitochondrial matrix. Because the inner mitochondrial membrane is impermeable to protons, these ions can enter only by flow through the hydrophilic, proton-specific channels present in the F_0 domain of ATP synthase. The flow of protons drives rotation of a cylinder of α-helical subunits and hence of proteins of the F_1 domain that are connected (via a shaft) to the rotary unit of F_0. The F_1 domain subunits include those with the active sites for synthesis of ATP. This reaction is driven by conformational change in the enzymatic subunits as their rotation brings them into contact with a stationary arm of the F_0 domain. ATP synthase is therefore a rotary machine that harnesses the proton motive force generated by the electron transport chain for synthesis of ATP.

reactive oxygen species. These compounds, which can oxidize and damage proteins and lipids, may promote mitochondrial dysfunction and hence contribute to virus-induced cell death. Their increased synthesis serves as a signal for oxidative stress to trigger compensating mechanisms, notably increased expression of the gene that encodes Hif-1α. This transcriptional regulator in turn activates transcription of genes that encode proteins that act either to decrease the supply of the initial electron carriers, such as an inhibitor of pyruvate dehydrogenase, or to increase synthesis of ATP by glycolysis, for example, Glut1, Glut3, and hexokinase (Fig. 14.8). As we have seen, this transcriptional regulator has been implicated in the acceleration of glycolysis observed in cells infected by a variety of viruses. Reactive oxygen species are also important in signaling pathways that activate innate immune defenses (Volume II, Chapter 3), so their increased concentrations in cells infected by these viruses may facilitate recruitment of such antiviral defenses.

Release of mitochondrial proteins, such as cytochrome *c*, into the cytoplasm initiates the protease (caspase) cascade that executes the apoptotic program. Because this process could terminate viral infectious cycles prematurely, it is targeted by proteins encoded in the genomes of many, if not all, viruses. Mechanisms by which viral proteins block apoptosis, an important antiviral defense, are described in Volume II, Chapter 3.

Lipid Metabolism

The oxidation of fatty acids, carboxylic acids with hydrocarbon chains of 4 to 35 carbon atoms, is an important source of energy, and lipids in the form of triacylglycerols are the primary energy store in most organisms. Lipids also serve as

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detergents, transporters, hormones, and intracellular signaling molecules, while phospholipids and cholesterol (a sterol) are major components of cell membranes. Consequently, most cells synthesize fatty acids and other lipids. Membranes derived from those of the host cell are the foundations of the envelopes present in many virus particles (Chapters 4, 12, and 13). Furthermore, infection by enveloped and some nonenveloped viruses leads to quite dramatic reorganization and expansion of membrane-bounded structures (see "Remodeling of Cellular Organelles" below). It is now clear that lipid metabolism is modulated following infection of mammalian cells by a number of these viruses, although to virus-specific ends.

Regulation of Fatty Acid Oxidation in Virus-Infected Cells

Lipids are stored in the form of triacylglycerols, in which 1 molecule of glycerol is esterified to 3 fatty acid chains. When an organism requires energy, these stores are mobilized with release of fatty acids for transport in the blood bound to serum albumin (Fig. 14.13). Once they enter cells (for example, of cardiac or skeletal muscle), fatty acids are linked to acetyl-CoA to form acyl-CoAs and transported into mitochondria, where they undergo repeated cycles of oxidative removal of 2 carbon units (as acetyl-CoA) and production of energy, in the form of 1 molecule each of the reduced electron carriers NADH and $FADH_2$ per cycle. Because fatty acids are highly reduced, their complete oxidation yields more than twice the energy than can be extracted from the same mass of carbohydrate.

Degradation of fatty acids is important for reproduction of vaccinia virus (Box 14.7): inhibition of the enzymes responsible for import of acyl-CoAs into mitochondria reduced the yield of virions by >10-fold. The rate of oxidation of the 16-carbon fatty acid palmitate increases during infection with human immunodeficiency virus type 1, and inhibition of this pathway impairs production of both viral genomic RNA and infectious virus particles. This process is also necessary for efficient reproduction of the flavivirus dengue virus. In this case, palmitate is obtained by an unusual mechanism of processing of intracellular triacylglycerols, which are stored in lipid droplets (Box 14.8).

Infection by Several Enveloped Viruses Stimulates Fatty Acid Synthesis

Comparison of the concentrations of enzymes of fatty acid synthesis, notably the multiple-active-site enzyme fatty acid synthase, or their mRNAs, and direct measurement of intermediates such as malonyl-CoA, have established that biogenesis of these lipids is accelerated in response to infection by several enveloped viruses, including the flaviviruses dengue and hepatitis C viruses, some herpesviruses, and human

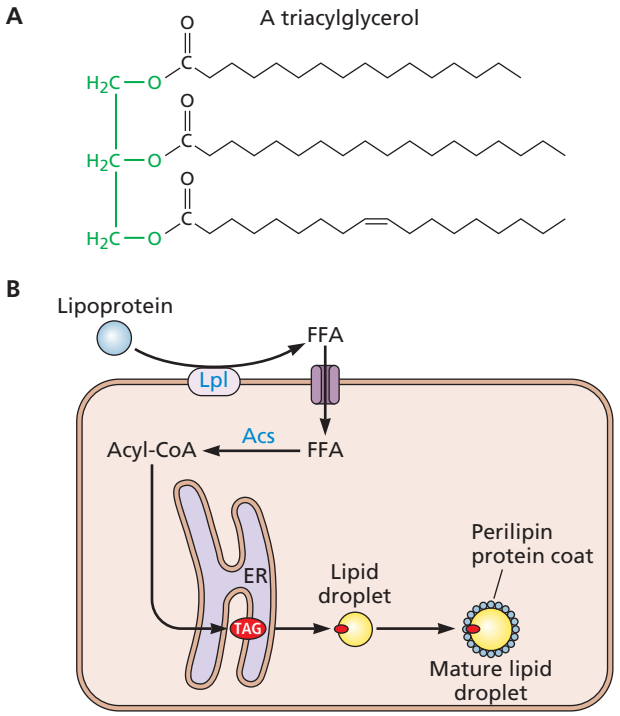


Figure 14.13 Storage and mobilization of fatty acids. (A) Fatty acids are transported and stored as triacylglycerols, in which the carbon atoms of glycerol (green) are linked to 3 fatty acid chains. As illustrated, these chains vary in length and degree of saturation. **(B)** Fatty acids in the form of triacylglycerols are transported in the blood as lipoproteins, phospholipid-bonded particles that contain lipid-binding apolipoproteins and also other lipids, notably cholesterol. At cell surfaces, plasma membrane-associated lipoprotein lipases (Lpl) hydrolyze triacylglycerols to release free fatty acids (FFA) for entry into the cell via dedicated channels. Fatty acids can also be transported bound to serum albumin. Within a cell, fatty acids are thioesterified to acetyl-CoA by acyl-CoA synthases (Acs). Acyl-CoA molecules may be transported into mitochondria for oxidation and production of energy. They can also be packaged for storage within the cell. In this process, triacylglycerols (TAG) are formed following entry of acyl-CoAs into the ER, and released from the ER associated with one of several proteins (red oval). Such immature lipid droplets coalesce and become coated by the protein perilipin to form mature lipid droplets.

immunodeficiency virus type 1. Furthermore, the major perturbations of lipid metabolism in the livers of patients infected with hepatitis B or C virus contribute to the development of such symptoms as steatosis (accumulation of fat), obesity, and hepatocellular carcinoma. We illustrate the mechanisms by which lipid synthesis is increased in virus-infected cells and the consequences, using two well-characterized examples.

Human cytomegalovirus infection induces synthesis of very-long-chain fatty acids for assembly of infectious virus particles. As discussed previously, infection of human cells with human cytomegalovirus increases the flux of carbon from glucose to acetyl-CoA, the product of the oxidation of

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BOX 14.8

DISCUSSION

Dengue virus infection induces autophagy to mobilize fatty acids for energy generation

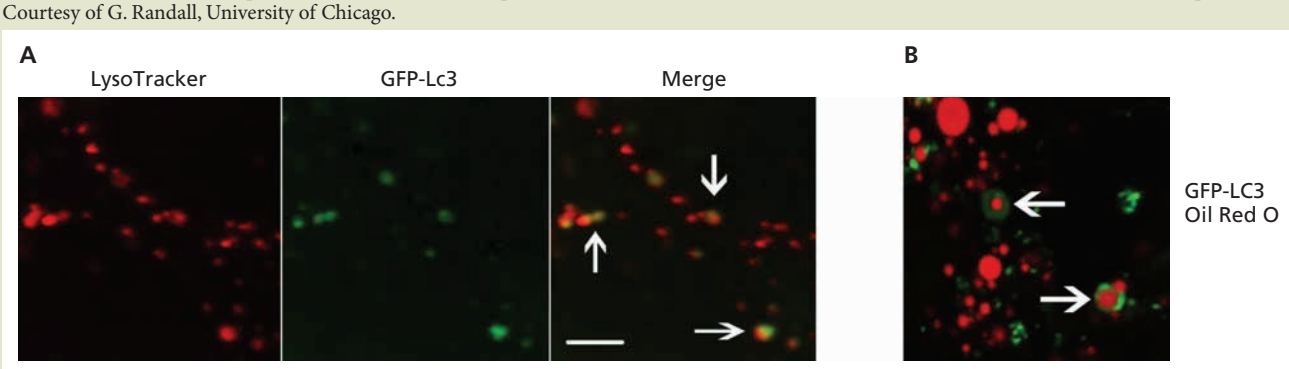
Efficient reproduction of the flaviviruses dengue virus and hepatitis C virus depends on autophagy (literally "self-eating") in infected cells. This process is normally induced in response to starvation and is characterized by the formation of autophagosomes, which are bounded by two membranes. These structures engulf cellular components and can deliver them to lysosomes for degradation and recycling of essential materials, such as amino acids. The unanticipated function of autophagy in dengue virus-infected cells became clear when the impact of infection on lipid metabolism was examined.

While autophagosomes do not appear to be co-opted to serve as viral replication centers, they are associated with lipid droplets in human hepatocytes in dengue virus-infected cells, and the area occupied by these storage depots for triacylglycerides is reduced. This decrease is the result of delivery of lipids via autophagosomes to lysosomes (see the figure), with concomitant reduction in the concentration of triglycerides (but not other lipids) in infected cells, as they are hydrolyzed by lysosomal lipases to liberate fatty acids (and glycerol). The rate of fatty acid oxidation is also increased, and inhibition of transport of fatty acids into mitochondria (the site of fatty acid oxidation) impaired replication of the viral RNA genome and production of infectious virus particles. Furthermore, addition of exogenous free fatty acids rescued the defects in virus reproduction caused by inhibition of autophagy, or of both autophagy and transport of fatty acids into mitochondria.

Infection by dengue virus therefore appears to evoke a response normally restricted to extreme conditions (e.g., starvation) to mobilize fatty acids stored as triglycerides in lipid droplets for oxidation and energy generation.

Heaton NS, Randall G. 2010. Dengue virus-induced autophagy regulates lipid metabolism. *Cell Host Microbe* **8:**422–432.

A plasmid encoding the autophagosomal protein Lc3 fused to green fluorescent protein (GFP) (green) was introduced into established human hepatocyte cells, which were then infected with dengue virus for 24 h. At that time, the cells were stained with LysoTracker (red), a dye that detects lysosomes in living cells **(A)**, or with Oil Red O, which stains neutral lipids **(B)**. White arrows indicate acidified autophagosomes (A) and localization of neutral lipids to these vesicles **(B)**. Adapted from N. S. Heaton and G. Randall, *Cell Host Microbe* **8:**422–432, 2010, with permission. Courtesy of G. Randall, University of Chicago.



pyruvate by pyruvate dehydrogenase (Fig. 14.8). However, much of this acetyl-CoA does not enter the citric acid cycle, but rather is shuttled to the cytoplasm in the form of citrate, where it is converted to malonyl-CoA, the committed precursor for synthesis of fatty acids (Fig. 14.9). Flux through this pathway is accelerated by a factor of 20. These changes are crucial for efficient virus reproduction: inhibition of either the enzymes that catalyze synthesis of malonyl-CoA (acetyl-CoA carboxylase) or fatty acid synthase reduced the yield of infectious virus particles by several orders of magnitude.

The formation of viral envelopes, and of the cytoplasmic membrane-bound compartments at which particles acquire their final envelope, imposes an increased demand for lipid synthesis. However, human cytomegalovirus infection does not simply increase production of fatty acids in infected cells, but also alters their nature: very-long-chain fatty acids (with carbon chains of $\geq$26) are increased nearly 10-fold in concentration in infected cells with no change in the abundance of the C_{14}-C_{24} fatty acids. Such a skewed distribution of fatty acids was even more pronounced in the envelope of virus particles. Inhibition of the enzymes that make long-chain fatty acids from those with shorter hydrocarbon chains (elongases) led to production of virus particles with a reduced content of long-chain fatty acids and poor infectivity. It therefore appears that the final budding of human cytomegalovirus particles is at a membrane enriched in lipids with long-chain fatty acids, but how the presence of such lipids promotes assembly and initiation of a new infectious cycle is not yet clear.

These changes in lipid metabolism can be traced to the increased availability of activators necessary for the transcription of many genes of lipid synthesis, Chrebp (described

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previously) and Srebps (sterol regulatory element-binding proteins). The latter regulators are synthesized as inactive precursors that remain associated with the endoplasmic reticulum (ER) membrane until needed, when they are transported to Golgi compartments and cleaved to release active Srebps (Fig. 14.14). Human cytomegalovirus infection leads to increased cleavage and release of Srebps, as a result of increased synthesis of the Pkr-like ER-associated kinase (Perk) described in Chapter 12. In addition, the activation of signaling via mTor inactivates a negative regulator of Srebps, lipin-1, which retains these proteins in the cytoplasm.

The net result of the many perturbations of host cell carbon metabolism characteristic of human cytomegalovirus-infected cells is to channel fuels like glucose to increased synthesis of lipids, a redirection that depends on intervention in many metabolic reactions and the coordinated modulation of multiple signal transduction pathways. Such coordination appears to be facilitated by altered properties of the antiviral protein viperin in infected cells (Box 14.9).

Hepatitis C virus infection stimulates fatty acid synthesis and increases lipid retention to induce steatosis in hepatocytes. Infection of hepatocytes with hepatitis C virus results in several perturbations in lipid metabolism. Export of fatty acids and cholesterol in the form of lipoproteins is inhibited, a response likely to be related to the reduced concentrations of serum cholesterol seen in hepatitis C virus-infected patients: normally, cholesterol and fatty acids from the diet are packaged into lipoproteins in the liver, for subsequent transport to tissues and organs where needed. Concomitantly, the synthesis of fatty acids and cholesterol is stimulated, because of increased expression of genes that encode such enzymes as fatty acid synthase and those needed for cholesterol production. The expression of the gene encoding SrebpC1, the Srebp family member required for transcription of genes for enzymes of both fatty acid and cholesterol synthesis, is also increased. These changes in lipid metabolism are important for viral reproduction. For example, inhibition of Srebp release from the ER by incubation of infected cells with a cholesterol derivative severely inhibited synthesis of viral RNA.

The viral C protein leads to elevated concentrations of active Srebp1 via the Akt signaling pathway and the transcriptional regulator forkhead box protein 1 (Foxo1) (Fig. 14.15A), as well as decreased concentration of transcriptional activators needed for transcription of genes that encode enzymes of fatty acid oxidation. The viral nonstructural proteins NS4B and NS5A have also been reported to increase the concentration of mature (active) Srebp1, but how they might do so is not known. Furthermore, the reactive oxygen species made in infected cells are thought to contribute to inhibition of lipoprotein release. *In toto*, these alterations in lipid

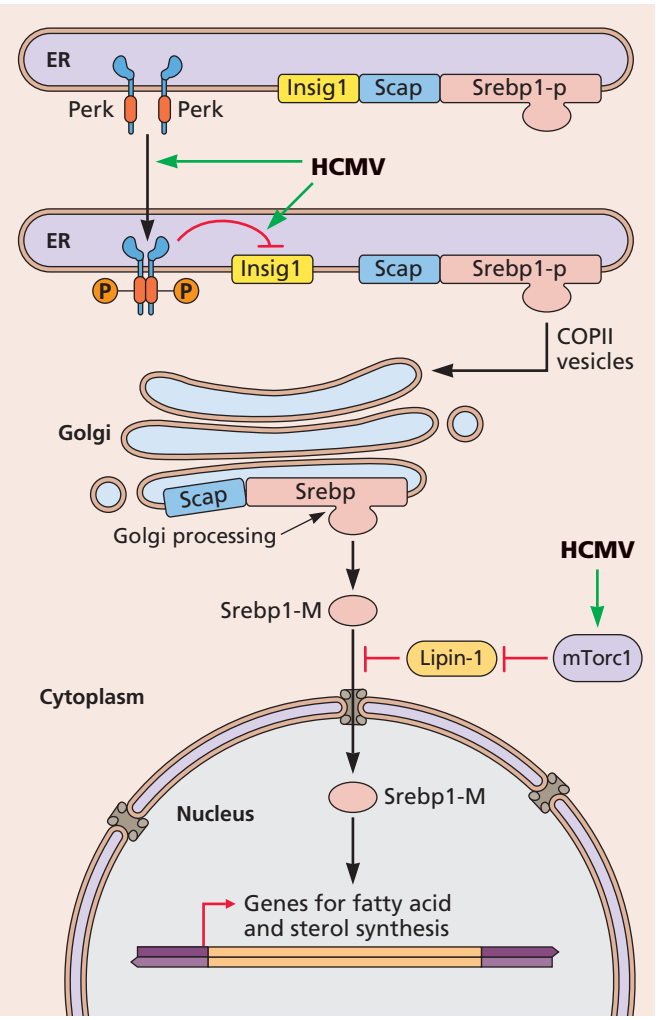


Figure 14.14 Mechanisms of stimulation of fatty acid synthesis in human cytomegalovirus (HCMV)-infected cells. Transcriptional activators of the Srebp family are required for expression of many genes that encode enzymes and other proteins required for synthesis of fatty acids. The Srebps are synthesized as inactive precursors (Srebp-ps) that are sequestered in the ER membrane by association with Srebp cleavage activation protein (Scap), which in turn binds to the protein insulin-inducible gene 1 (Insig1) when cholesterol is present. These interactions are disrupted in uninfected cells when cholesterol concentrations are low, allowing exit of Srebp-p from the ER and transport to Golgi compartments. At this location, proteolytic cleavages release active, mature Srebps (Srebp-M) into the cytoplasm for translocation into the nucleus and stimulation of transcription of Srebp-responsive genes, such as that encoding fatty acid synthase. This mechanism is overridden in human cytomegalovirus-infected cells, apparently in large part because of increased synthesis of the ER membrane enzyme Perk and its activation (see Chapter 12). The data available indicate that one consequence is reduced concentration of Insig1, and hence stimulation of production of active Srebp. Increased signaling from mTorC1 in infected cells also results in inactivation of the negative regulator of Srebps, lipin 1.

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BOX 14.9

DISCUSSION

An interferon-inducible protein initiates redirection of lipid metabolism in cells infected by human cytomegalovirus

The expression of the interferon-inducible gene viperin is increased in human cells infected by human cytomegalovirus, independently of production of interferon. Viperin includes a central region with homology to a motif present in enzymes that use *S*-adenosylmethionine as a cofactor (the radical SAM family) and binds to iron-sulfur clusters in other proteins (hence the alternative name for viperin: radical SAM domain containing 2, or Rsad2). Although viperin impairs human cytomegalovirus reproduction when its gene is artificially expressed in cells prior to infection, more-recent studies indicate a critical role in redirecting lipid metabolism.

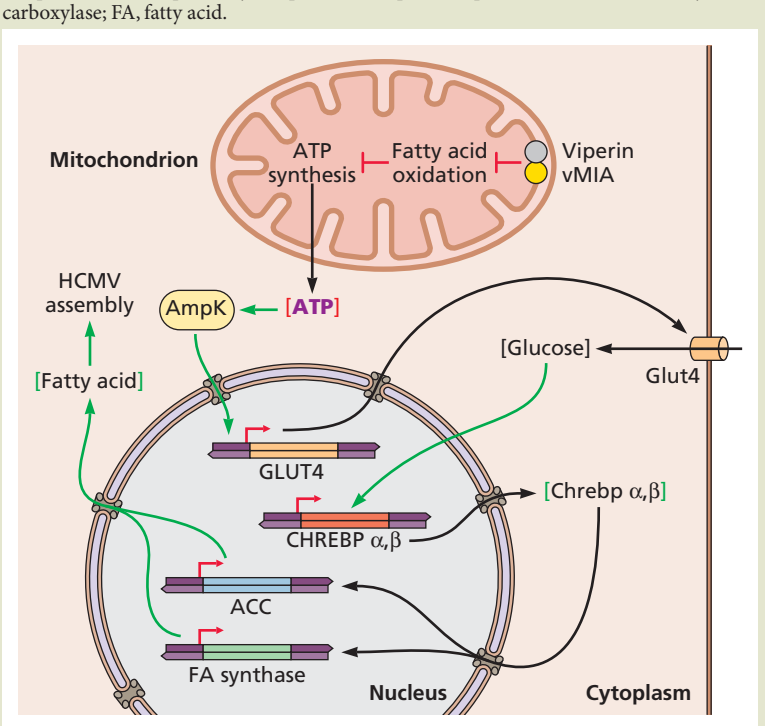
Human cytomegalovirus infection induces not only synthesis of viperin, but also its localization to mitochondria as a result of binding to the viral mitochondrial inhibitor of apoptosis (vMIA) protein. In this organelle, viperin associates with and inhibits the multienzyme assembly (the trifunctional protein) that catalyzes the last 3 reactions in the 4-reaction cycle by which 2 carbon units are removed from long-chain ($C \geq 12$) fatty acids during oxidation. Such inhibition requires the iron-sulfur cluster-binding motif of viperin. As summarized in the figure, inhibition of fatty acid oxidation and the resulting decrease in intracellular ATP concentrations activate AMP-dependent protein kinase and a subsequent regulatory cascade that leads to increased synthesis of fatty acids to facilitate assembly of infectious virus particles. The inhibitory and stimulatory responses shown are blocked when viperin production is prevented in infected cells using RNA interference, and reproduced when viperin is targeted to mitochondria in uninfected cells.

As noted in the text, formation of active Srebps, which, like Chrebp, stimulate transcription from lipogenic genes, is increased in human cytomegalovirus-infected cells. Although this response does not depend on viperin, expression of lipogenic genes is not stimulated when viperin cannot be made in infected cells. The transcriptional regulators Srebp1 and Chrebp may therefore operate synergistically to increase fatty acid synthesis.

Seo JY, Cresswell P. 2013. Viperin regulates cellular lipid metabolism during human cytomegelavirus infection. *PLoS Pathog* **9:**e1003497. doi:10.1371/journal.ppat.1003497.

Shenk T, Alwine JC. 2014. Human cytomegalovirus: coordinating cellular stress, signaling, and metabolic pathways. *Annu Rev Virol* **1:**355–374.

Regulation of lipid metabolism in human cytomegalovirus (HCMV)-infected cells, with inhibition and stimulation indicated by red bars and green arrows, respectively, and metabolites or proteins increased and decreased in concentration shown in green and red parentheses, respectively. AmpK, AMP-dependent protein kinase; ACC, acetyl-CoA carboxylase; FA, fatty acid.



metabolism greatly increase the accumulation of lipid droplets (Fig. 14.15B), which serve as platforms for assembly of virus particles (see "Remodeling of Cellular Organelles" below).

Infection by Nonenveloped Viruses Can Also Reprogram Lipid Metabolism

Even when virus particles lack an envelope, lipid metabolism can be perturbed in the infected cell, and one such nonenveloped virus has been associated with development of obesity in humans (Box 14.10). A characteristic feature of cells infected by various viruses with (+) strand RNA genomes is the formation of cytoplasmic membranous structures that are the sites of viral genome replication and/or assembly of virus particles. This process can require reshaping of the repertoire of lipids in the infected cell, a phenomenon illustrated using the picornavirus poliovirus.

Synthesis of phospholipids (major constituents of cellular membranes), particularly phosphatidylcholine, is stimulated strongly within a short period after infection with poliovirus (or other picornaviruses). This response is the result of a greatly increased rate of import of fatty acids triggered by viral protein 2A and their utilization for

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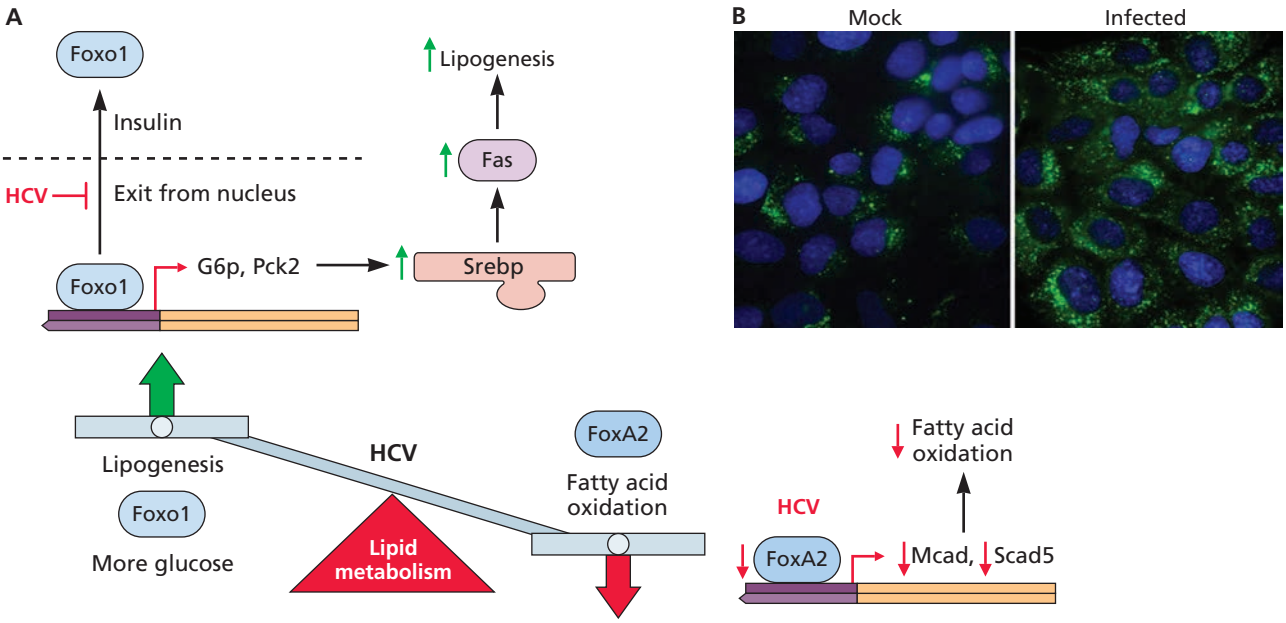


Figure 14.15 Increased synthesis and accumulation of fatty acids in hepatitis C virus-infected cells. **(A)** Model for the opposite regulation of fatty acid synthesis and breakdown in hepatitis C virus (HCV)-infected hepatocytes. Synthesis of fatty acids is stimulated, because of increased production of the enzyme fatty acid synthase (Fas). As a result of the increased availability of nuclear Srebp1 in hepatitis C virus-infected cells, translocation of the transcriptional activator Foxo1 from the nucleus to the cytoplasm (normally stimulated by insulin) is blocked. Consequently, expression of genes that encode gluconeogenic enzymes and production of glucose are increased, a response that induces increased accumulation of active Srebp1. At the same time, production of enzymes that catalyze fatty acid oxidation, such as medium- and short-chain acyl-CoA dehydrogenases (Mcad, Scad), is reduced because of decreased concentrations of FoxA2, and hence of expression of the genes that encode these enzymes. The net result is that fatty acid synthesis greatly outpaces degradation, and neutral lipids accumulate in lipid droplets. The accumulation of the protein perilipin, a component of lipid droplets that protects against removal of lipids by the action of lipases, is also increased in infected cells, while activation of lipases is prevented. As the model predicts, viral RNA genome replication is impaired by small interfering RNA-mediated knockdown of Foxo1 or overproduction of FoxA2. Adapted from S. K. Bose et al., *J Virol* **88:**4195–4203, 2014, with permission. **(B)** Human hepatocytes from a hepatocellular carcinoma were infected with hepatitis C virus or were mock-infected, and neutral lipids were examined by staining with the lipophilic fluorescent dye Bodipy 493/503 (green). Nuclei are stained blue. Courtesy of R. Ray, Washington University, St. Louis.

synthesis of phosphatidylcholine (and presumably other phospholipids). The complement of fatty acids incorporated into phospholipids is also shifted in favor of those with longer acyl chains (C_{16} or C_{18}), because the activity of a long-chain acyl-CoA synthase (Acsl3) is increased. Newly imported fatty acids are seen associated with a viral protein in structures resembling replication centers (Fig. 14.16), and inhibition of production of Ascl3 by RNA interference impairs replication of a poliovirus replicon. These observations indicate that poliovirus replication factories possess a unique lipid composition, but how this property favors their formation or function is not yet clear.

Remodeling of Cellular Organelles

Infection of cells in culture by many viruses causes changes in morphology that are obvious even when cells are observed by low-power light microscopy (see Fig. 2.8). The more dramatic changes, such as rounding up of cells and their detachment from the surfaces of tissue culture dishes, are the result of severe perturbations of cellular physiology and metabolism, and induction of cell death. They are typically seen late in infection, but even at earlier times, virus infection can induce large-scale reorganization of cellular organelles or their components. Such remodeling of host cell architecture supports fabrication of infected cell-specific structures in which replication of viral genomes and/or assembly of virus particles take place. Cytoplasmic organelles or the machinery needed for their formation may also be co-opted to facilitate release of progeny virus particles from infected cells. In this section, such alterations of cellular morphology are described in the context of individual organelles of the host cell.

The Nucleus

Altered nuclear morphology is a common feature of cells infected by viruses with DNA genomes that are replicated within nuclei. These organelles become enlarged as the infectious cycle progresses and, in many cases, filled with large arrays of mature and assembling virus particles

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BOX 14.10

DISCUSSION

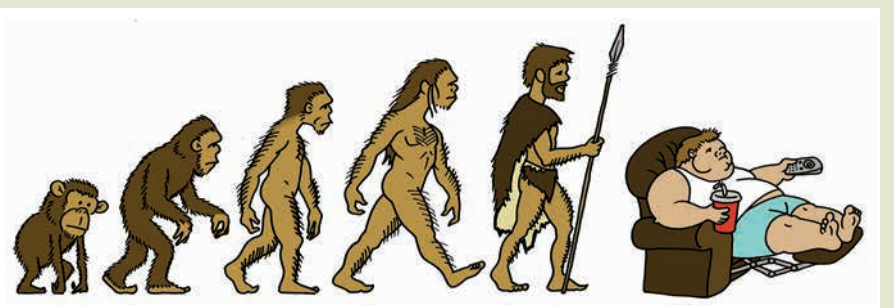
Does infection by human adenovirus type 36 contribute to obesity in humans?

Human adenovirus type 36 (Ad36), first isolated in 1979, received relatively little attention until several reports of increased body weight following infection of experimental animals. It was initially observed that Ad36-infected chickens and mice showed large increases in body weight and fat accumulation, in contrast to animals infected in parallel with an avian adenovirus. The Ad36-infected animals also exhibited decreased concentrations of serum triglycerides and improved glycemic control. Weight gain and obesity were also reported following infection of rats, hamsters, and nonhuman primates.

This response to Ad36 infections is likely to be the result of direct effects on adipocytes (fat storage cells). Infection of preadipocytes in culture induces differentiation to adipocytes that produce increased concentrations of enzymes of fatty acid synthesis, accumulate triglycerides, and exhibit increased rates of glucose uptake. Inhibition of synthesis of the viral E4 Orf1 protein in Ad36-infected cells by RNA interference blocks these changes. Furthermore, this viral protein is sufficient to stimulate glucose uptake by activation of signaling via Pi3k and Akt to increase the availability of Glut4.

Ad36 is found worldwide, with a prevalence of some 15% in the United States. Despite the consistent observations made in experimental animals, a clear connection between infection of humans with this virus and obesity has not been established. In some studies, the presence of antibodies against the virus was more common in obese than in normal individuals (e.g., 64 vs. 32% in a study of 203 adults in Italy), but no such differences were detected in several other studies (e.g., of 509 Dutch and Belgian adults). It has been suggested that these conflicting observations might reflect the multifactorial nature of obesity, and hence differences in parameters such as genetic makeup, the microbiome, diet, lifestyle, and race among the individuals participating in the various studies.

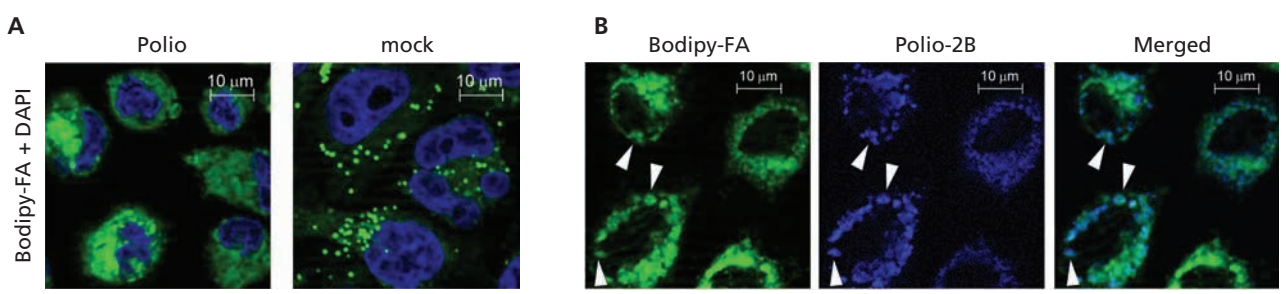
Esposito S, Preti V, Consolo S, Nazzari E, Principi N. 2012. Adenovirus 36 infection and obesity. *J Clin Virol* **55:**95–100.



(Fig. 14.17), while cellular chromatin may become condensed or dispersed to the nuclear periphery and silenced by epigenetic mechanisms. Prior to appearance of these particles, nuclear constituents are reorganized and often relocated as viral replication compartments (also called replication centers) form. These sites of viral genome replication have been observed in cells infected by all nuclear replicating DNA viruses that have been examined.

Nuclear replication centers contain incoming and replicating viral DNA genomes, proteins of both viral and cellular

Figure 14.16 Increased import of fatty acids into poliovirus-infected cells. (A) HeLa cells infected with poliovirus for 4 h or mock-infected were incubated with the fluorescent fatty acid Bodipy-FA (green), which is thought to mimic fatty acids with 18 carbon atoms, for 30 min, and nuclei were stained in blue. The greatly increased accumulation of exogenous fatty acids in infected cells is clearly evident. **(B)** Infected cells were treated as described for panel A, and the poliovirus 2B protein was then visualized by immunofluorescence (blue). This viral protein localizes to viral replication centers, the discrete domains indicated by the white arrowheads, where the fluorescent fatty acid also accumulates. Adapted from J. A. Nchoutmboube et al., *PLoS Pathog* **9:**e1003401, 2013, with permission. Courtesy of G. A. Belov, University of Maryland.



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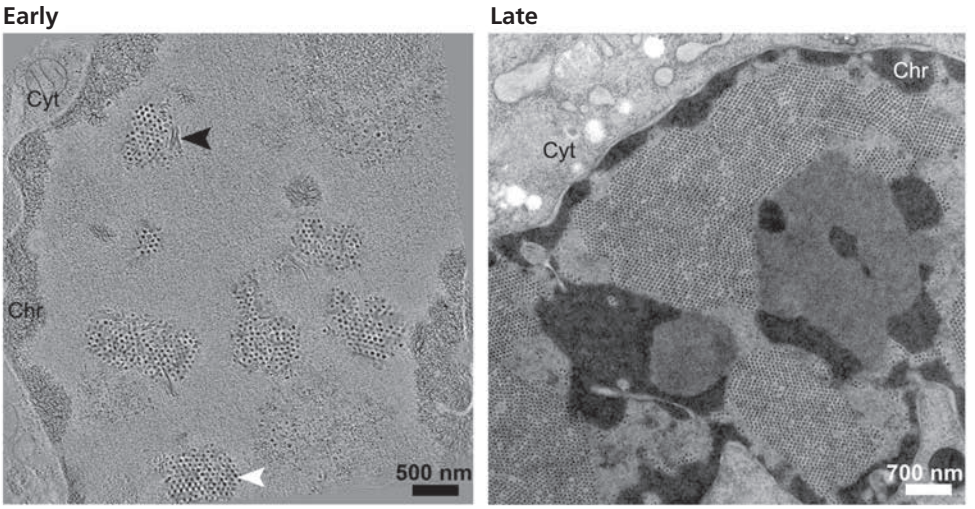
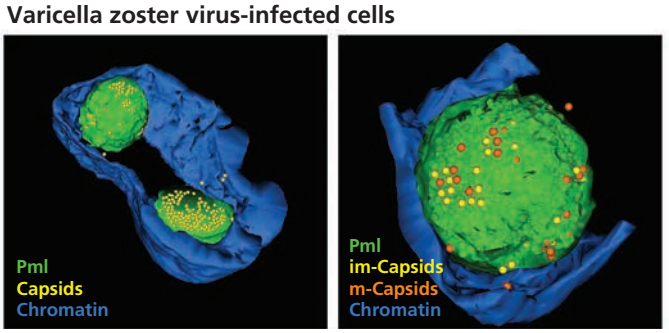


Figure 14.17 Reorganization of nuclei in polyomavirus-infected cells. Murine 3T3 cells infected with polyomavirus for 32 h were frozen under high pressure, stained at low temperature, and embedded in plastic, and sections were examined by electron tomography. Shown are 1-nm sections extracted from a 2 × 2 montage of six serial sections (1.8 μm thick) of individual infected cell nuclei. These nuclei represent earlier and later stages in the infectious cycle, as defined operationally by the sizes and numbers of clusters of virus particles present: infection of individual cells proceeds asynchronously, allowing multiple stages in the cycle to be observed in a single sample. Once the major structural protein VP1 is made (left), clusters of virus particles (white arrowhead) partially fill the interchromatin space. Each cluster is associated with tubular structures (black arrowhead) that have been shown also to be built from viral structural proteins and viral genomes. Cellular chromatin is condensed. As infection proceeds, virus particles form dense arrays that fill the interchromatin space (right). Chr, condensed chromatin, Cyt, cytoplasm. Adapted from K. D. Erickson et al., *PLoS Pathog* **8:**e1002630, 2012. Courtesy of R. L. Garcea, University of Colorado, Boulder.

origin needed for viral DNA synthesis, and a virus-specific constellation of other proteins (Chapter 9). They are also commonly associated with newly synthesized viral transcripts and the viral proteins required for efficient expression of viral genes. Although establishment of replication compartments can facilitate synthesis and transcription of viral DNA in several ways (Chapters 8 and 9), we know relatively little about how these structures are assembled in infected cells.

It has been established that one herpesviral genome is sufficient to initiate fabrication of a replication compartment. It seems likely that this is also the case for other DNA viruses with genomes replicated in the nucleus. Entering viral genomes associate with nuclear foci formed by Pml proteins (Pml bodies; Chapter 9). In many cases, viral proteins then induce degradation, dispersion, or inactivation of Pml body proteins as viral replication centers form. The latter structures enlarge as viral DNA synthesis takes place and may eventually coalesce into large reticular networks that occupy much of the nucleus. In cells infected by some herpesviruses, nuclear cages that encase assembling viral nucleocapsids are fashioned from Pml proteins late in infection (Fig. 14.18). Cellular proteins that participate in such normal processes as DNA synthesis, recombination, repair, and transcription and

Figure 14.18 Example of a Pml-containing nuclear structure in DNA virus-infected cells. Cages of Pml (green) surrounding herpesviral nucleocapsids (yellow [immature] and orange [mature]) in human melanoma cells overproducing a specific Pml isoform (Pml-IV). Cells were infected with varicella-zoster virus, the causative agent of chickenpox in humans, for 48 h, and serial sections were then examined by scanning electron microscopy. The three-dimensional reconstruction shown was produced from tracings of 18 individual sections. A larger view of the upper cage in the top panel is shown at the right, with the Pml cage in transparent green to illustrate the encasing of immature (im) and mature (m) capsids. Cellular chromatin is shown in blue. Adapted from M. Reichelt et al., *PLoS Pathog* **8:**e1002740, 2012, with permission. Courtesy of M. Reichelt, Stanford University School of Medicine.



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pre-mRNA processing are recruited to replication centers in virus-specific fashion (Chapters 8 and 9). The sequence of reactions that initiate formation of nuclear replication (or assembly) compartments and lead to recruitment of the cellular (and viral) proteins necessary for viral genome replication and expression has not been elucidated for any nuclear DNA viruses. Nevertheless, their formation can be essential for efficient viral DNA synthesis and reproduction. Interferon treatment blocks the appearance of replication centers in cells infected by certain adenovirus mutants, and the efficiency of viral genome replication is reduced considerably.

Infection can also result in the sculpting of other virus-specific nuclear domains or structures by reorganization of host cell components. For instance, nucleoli (the sites of ribosomal RNA synthesis) are disrupted in adenovirus-infected cells as several viral proteins, including the core proteins, accumulate in them. Core protein V associates with the abundant nucleolar protein nucleophosmin and induces its redistribution to the nucleoplasm. This activity of protein V is necessary for efficient assembly of virus particles in normal human cells, but how dispersal of nucleolar protein facilitates adenovirus reproduction is not clear. During the late phase of adenovirus infection, cellular small nuclear ribonucleoproteins that participate in splicing initially associate with the peripheral zones of viral replication centers, where transcription of viral DNA takes place, but then appear in distinct foci. These enlarged interchromatin granules contain spliced viral late mRNA (Fig. 14.19A), and their formation correlates with export of these viral mRNA to the cytoplasm. A very different type of infected cell-specific nuclear edifice has been observed following infection by herpes simplex virus 1, virus-induced chaperone-enriched (VICE) domains. These dynamic domains are defined by the presence of cellular chaperones, such as Hsc70, and are first seen adjacent to assembling viral replication compartments as viral early genes are expressed (Fig. 14.19B). They also contain proteasomes and ubiquitin. The viral ICP22 protein is sufficient for recruitment of Hsc70 into VICE domains and is present in these foci in virus-infected cells. These domains may serve as safe depots for storage and disposal of misfolded proteins in herpes simplex virus-infected cells, or they may be sites of storage of the cellular chaperones needed during assembly of virus particles, when large quantities of structural units must be built from individual protein subunits (Chapter 13). It seems likely that continued application of increasingly powerful methods of microscopy and proteomics will reveal new, virus-specific structures in infected cell nuclei.

The nucleus is also the site of replication of the (−) strand RNA genome of influenza A virus, as well as synthesis of viral mRNA. A characteristic feature of influenza A virus-infected cells is disruption of the architecture of the nucleolus from early in infection. The viral proteins NS1 and NP localize to nucleoli via specific targeting signals, and conversely, nucleolar proteins become associated with viral genome-containing ribonucleoproteins. Nucleolar localization of NP has been reported to be important for efficient viral genome replication and mRNA synthesis, but the molecular

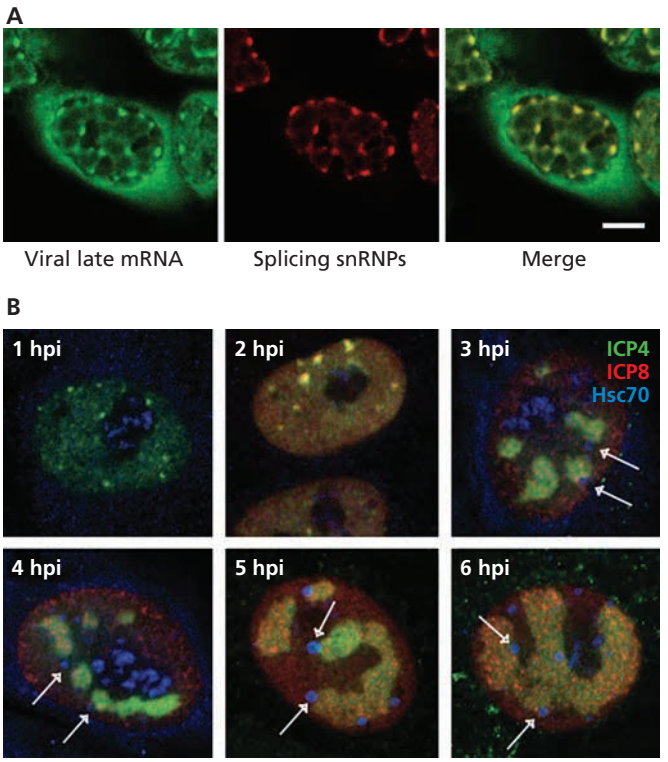


Figure 14.19 Reorganization of nuclear splicing components and chaperones in DNA virus-infected cells. (A) Formation of enlarged chromatin granules is characteristic of adenovirus-infected cell nuclei during the late phase of infection, as shown for HeLa cells infected with human adenovirus type 5 for 22 h. Infected cells were fixed and viral late mRNAs visualized by fluorescence *in situ* hybridization (green), with an oligonucleotide complementary to the sequence spanning exons 1 and 2 of the tripartite leader sequence common to all major late mRNAs (see Fig. 10.11). Small nuclear proteins (snRNPs) that participate in splicing were visualized by immunofluorescence using an antibody that recognizes a protein common to all of them (red). Bar, 10 μm. Adapted from E. Bridge et al., *Virology* **311:**40–50, 2003, with permission. Courtesy of E. Bridge, Miami University, Ohio. **(B)** Virus-induced chaperone-enriched (VICE) domains assemble as the herpes simplex virus 1 infectious cycle progresses, in this example after infection of established monkey cells with 10 PFU/cell. At the hours postinfection (hpi) indicated, cells were fixed and the viral immediate ICP4 (green) and early ICP8 (red) proteins and the cellular chaperone Hsc70 (blue) were visualized by indirect immunofluorescence. The VICE domains that form adjacent to developing replication centers, which contain the viral proteins, are indicated by white arrows. Adapted from C. M. Livingston et al., *PLoS Pathog* **5:**e1000619, 2009, with permission. Courtesy of S. Weller, University of Connecticut Health Center.

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consequences of the association of nucleolar and viral components are not known.

The Cytoplasm

Reproduction of a considerable variety of viruses is completed in the cytoplasm, and is typically accompanied by remodeling of one or more cytoplasmic components. The result may be construction of infected cell-specific platforms for replication of viral genomes, or remodeling of membrane-bound organelles for envelopment of virus particles, or their release. Such reorganization of cytoplasmic membranes also occurs in cells infected by enveloped viruses with genomes that are synthesized in the nucleus, such as herpes simplex virus 1 and human immunodeficiency virus type 1. Furthermore, cytoplasmic components can be altered even when most steps in the reproduction of nonenveloped viruses take place in the nucleus, a phenomenon illustrated by the cleavage of cytoskeletal filaments by the adenoviral protease late in infection (Chapter 13).

Cytoplasmic Viral Factories

A definitive feature of cells infected by poxvirus and other large DNA viruses that are reproduced in the host cell cytoplasm, such as mimivirus, is the establishment of sizable, viral DNA-containing foci, termed viral factories. Such viral factories contain not only the viral genome and all components of the viral DNA synthesis, transcription, and mRNA-processing machines (Chapter 8 and 9), but also cellular translation proteins, such as the initiation proteins eIF4E and eIF4G. It appears that all reactions necessary for production of progeny viral genomes and expression of viral genes take place in viral factories. A single viral genome is sufficient to seed formation of such a structure (Fig. 9.21A), but, as with nuclear viral replication compartments, it is not known how viral factories are assembled and remodeled as infection proceeds. Early in infection, vaccinia virus DNA factories are often, but not invariably, bound by rough ER. This membrane is dispersed later in infection, when assembly of virus particles is initiated by viral proteins that induce formation of crescent membranes derived from the ER membrane. This process is described in detail in Chapter 13.

Replication and Assembly Platforms

Replication of a number of viral RNA genomes (and often assembly reactions) takes place on or in infected cell-specific frameworks constructed from internal membranes or lipids of the host cell. Such structures, often called replication complexes, contain viral genomes, viral RNA polymerases, and other nonstructural proteins, and may be fashioned from the membranes of the ER, Golgi, or other cytoplasmic organelles or from lipid droplets. For instance, bunyavirus infection induces the formation of tubular-like sheets from Golgi compartment membranes, whereas cells infected by the flavivirus dengue virus are characterized by the presence of an elaborate collection of vesicles with single and double membranes and more-convoluted membranous sheets derived from the ER membrane (Fig. 14.20). These membranous elements illustrate just how great an impact virus infection can have on the morphology of the host cell cytoplasm. Their properties have been examined in some detail in mammalian cells infected by flaviviruses.

The dengue virus nonstructural proteins required for replication of the (+) strand RNA genome (the RNA polymerase and the helicase) and double-stranded RNA replication intermediates accumulate within vesicular invaginations into the ER membrane called vesicular packets (Fig. 14.20A). These properties indicate that such vesicles are sites of viral genome replication. More-convoluted membranous sheets are associated with the viral protease and have been proposed to be the sites of synthesis and processing of the viral polyprotein (Appendix, Fig. 10). The mechanisms by which membranes of the host cell ER are refashioned during dengue virus infection are not well understood. However, the important contribution of this process to viral reproduction is illustrated by the finding that replication of a viral replicon is blocked by inhibition of the lipid kinase phosphatidylinositol 1,4-phosphate, which is bound by the viral NS5A protein. The sites of budding of dengue virus particles into the ER are located close to the vesicular packets and the pores that connect them to the ER (Fig. 14.20B). This spatial arrangement may facilitate selective encapsidation of the viral RNA genome.

Although it is also a member of the *Flaviviridae*, hepatitis C virus infection leads to the appearance of a rather different membranous framework (Fig. 14.21A), larger vesicles (150 nm compared to some 90 nm in dengue virus-infected cells) with double membranes, many of which can be seen as protrusions connected to the outer membrane of the ER by a thin stalk. These vesicles contain active viral replication complexes. Their formation is thought to require the concerted action of several viral nonstructural proteins and also depends on the cellular chaperone cyclophilin A. This cellular protein interacts with the viral NS5A protein and is necessary for viral genome replication. Inhibitors of the chaperone strongly impair viral replication and are in late phases of testing as antivirals. The mechanism by which cyclophilin A promotes formation of the infected cell-specific vesicles and hence viral genome replication is not yet clear. The membranous platforms of hepatitis C virus genome replication are closely associated with lipid droplets (Fig. 4.21B) and the ER sites containing the viral C protein, at which budding of virus particles initiates.

In these examples, both viral genome replication and assembly take place in the infected cell cytoplasm. However, infected cell-specific membranous structures can also be induced when the viral genome is replicated in the nucleus, a phenomenon exemplified by formation of cytoplasmic assembly compartments in herpesvirus-infected cells. These structures are unusually large vesicles that contain cellular proteins normally

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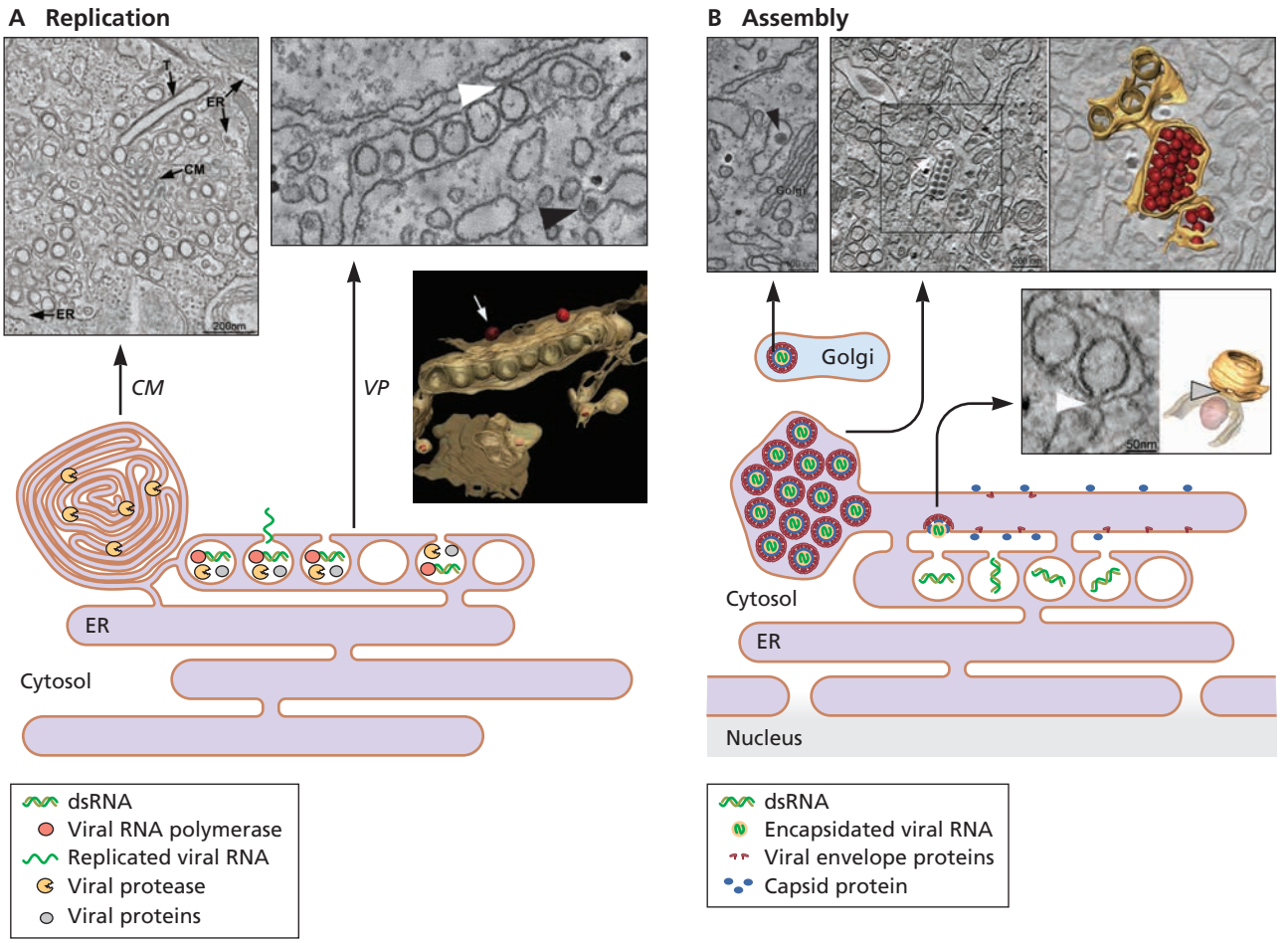


Figure 14.20 Dengue virus cytoplasmic replication and assembly compartments. Human hepatoma cells infected with dengue virus 2 for 24 h (A) or 26.5 h (B) were examined by transmission electron microscopy or by electron tomography and three-dimensional reconstruction. The latter reconstructions are shown in color. **(A)** The model for replication compartments is based on electron microscopic images like those above. The white arrowhead in the top right panel indicates a putative virus budding site shown in the tomogram in the panel below (red). CM, convoluted membranes; VP, vesicle packet; dsRNA, double-stranded RNA. **(B)** The model of assembly compartments is based on electron microscopic images like those shown above the schematic. The tomogram (far right, top) shows the reconstruction of the area boxed in the image shown to its left. The lower right panel shows a single section with a virus-induced vesicle invaginated into the ER and budding of a virus particle into the ER lumen opposite the neck of the invaginated vesicle. The three-dimensional reconstruction (right) shows the continuity between the membranes of the ER and a virus-induced vesicle (yellow) and what is probably a budding virus particle (pink). Electron micrographs and three-dimensional reconstructions reproduced from S. Welsch et al., *Cell Host Microbe* **5:**365–376, 2009, with permission. Courtesy of R. Bartenschlager, University of Heidelberg, Germany. Adapted from L. Chatel-Chaix and R. Bartenschlager, *J Virol* **88:**5907–5911, 2014, with permission.

present in Golgi compartments or endosomes, and viral late proteins. In cells infected by human cytomegalovirus, *de novo* synthesis of fatty acids is necessary for the formation of assembly compartments, and efficient envelopment of nucleocapsids to produce progeny virus particles.

The replication of the genomes of RNA viruses that do not acquire an envelope can also occur in association with host cell membranes. Infection by poliovirus (and other picornaviruses) induces inhibition of the secretory pathway (Chapter 12) and a transient increase in budding of coatomer protein II (CopII)-coated vesicles. The viral 2BC and 3A proteins co-opt membranes of the ER-Golgi intermediate compartment to establish infected cell-specific vesicles enriched in lipids that contain phosphatidylinositol 1,4-phosphate, which is bound specifically by the viral RNA polymerase. Viral genome replication and initial assembly of virus particles take place on the surfaces of these membranous replication complexes (Chapter 6). However, later in infection, double-walled autophagosomes that are associated with viral replication proteins accumulate. Inhibition of the formation of autophagosomes

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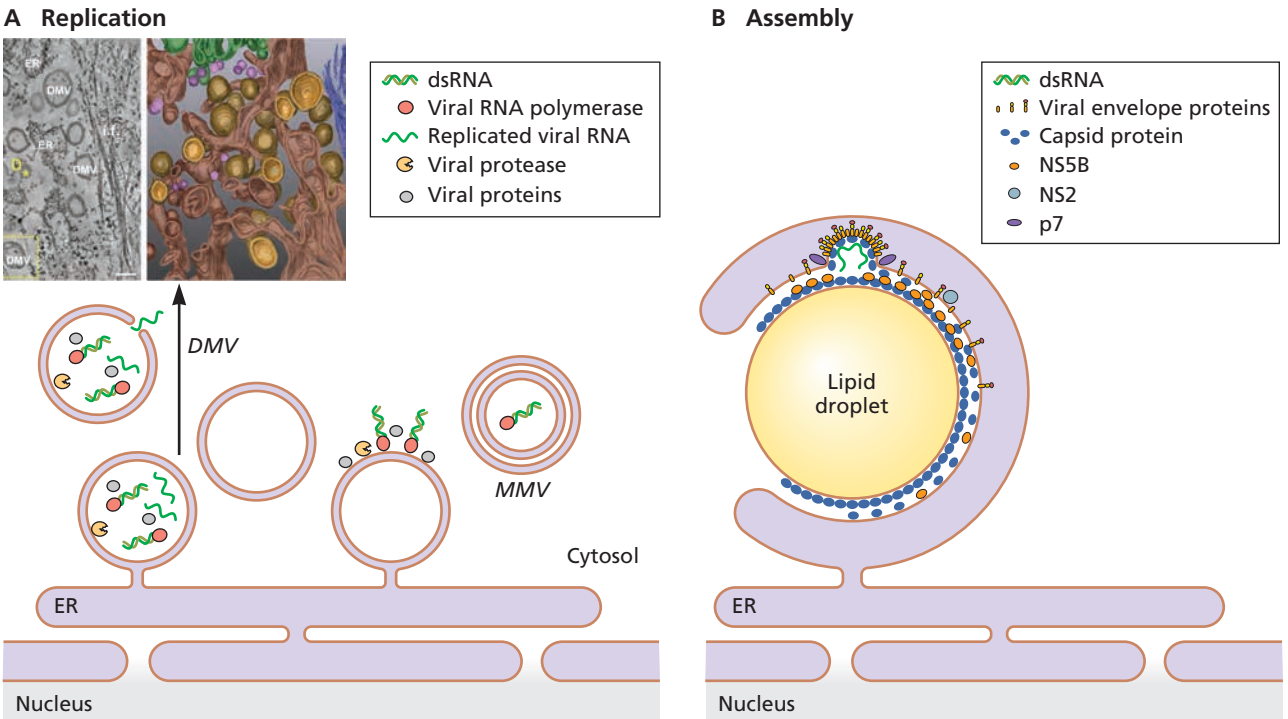


Figure 14.21 Hepatitis C virus replication and assembly compartments. (A) The model of replication compartments is based on electron tomography of human hepatoma cells infected with hepatitis C virus for 16 h. The left part of the image at the top shows one slice of a tomogram, with a three-dimensional reconstruction at the right showing a double membrane vesicle (DMV). i.f., intermediate filament. Bar, 100 nm. dsRNA, double-stranded RNA; MMV, multimembrane vesicle. Electron microscopic image reproduced from I. Romero-Brey et al., *PLoS Pathog* **8:**e1003056, 2012. Courtesy of R. Bartenschlager, University of Heidelberg, Germany. **(B)** The model of assembly compartments is based on transmission electron microscopy and indirect immunofluorescence of human hepatoma cells containing a hepatitis virus replicon that produces infectious virus particles.Adapted from L. Chatel-Chaix and R. Bartenschlager, *J Virol* **88:**5907–5911, 2014, with permission.

and their subsequent acidification reduce production of mature (infectious) virus particles, which contain VP2 and VP4 cleaved from VP0. It has therefore been proposed that the vesicles bound by a single membrane are precursors to autophagosomes, which, upon acidification, provide an environment conducive to maturation of virus particles and promote their subsequent nonlytic release (Fig. 14.22B).

Once synthesis of viral proteins begins, virus-specific inclusion bodies, termed viroplasms, are also observed in cells infected by the nonenveloped rotaviruses, which possess segmented, double-stranded RNA genomes. Viroplasms contain at least 7 (of the 12) viral proteins, and viral genomic RNA segments and mRNAs. In contrast to the virus-specific vesicular structures described above, these rotavirus-induced platforms are built of cellular lipids and proteins derived from lipid droplets. They are dynamic assemblies usually seen near the infected cell nucleus (Fig. 14.23A), and closely associated with cellular microtubules. Viroplasms first appear as small foci, but enlarge as infection progresses, because of fusion and synthesis of additional viral proteins. Their formation requires the viral NSP2 and NSP5 proteins, which are sufficient to induce assembly of viroplasm-like structures in the absence of other viral components. These proteins and VP2 recruit the other viral proteins and also cellular proteins present in lipid droplets, such as perilipin (Fig. 14.23B). Viroplasms are the sites of the initial reactions in viral genome replication and assembly of virus particles. Partially assembled, double-layered particles accumulate within these inclusions, and are then released to enter the ER for formation of the complete, three-layered particles (Chapters 4 and 6). When assembly of viroplasms is prevented, for example, by mutations in the viral genome or exposure of infected cells to inhibitors of lipid droplet formation, the yield of infectious virus particles is reduced, as is virus-induced cell death.

Cytoplasmic Vesicles and the Release of Virus Particles

In addition to viral genome replication and assembly, release of many types of virus particles depends on usurpation of cytoplasmic vesicles, or the cytoplasmic machines by which

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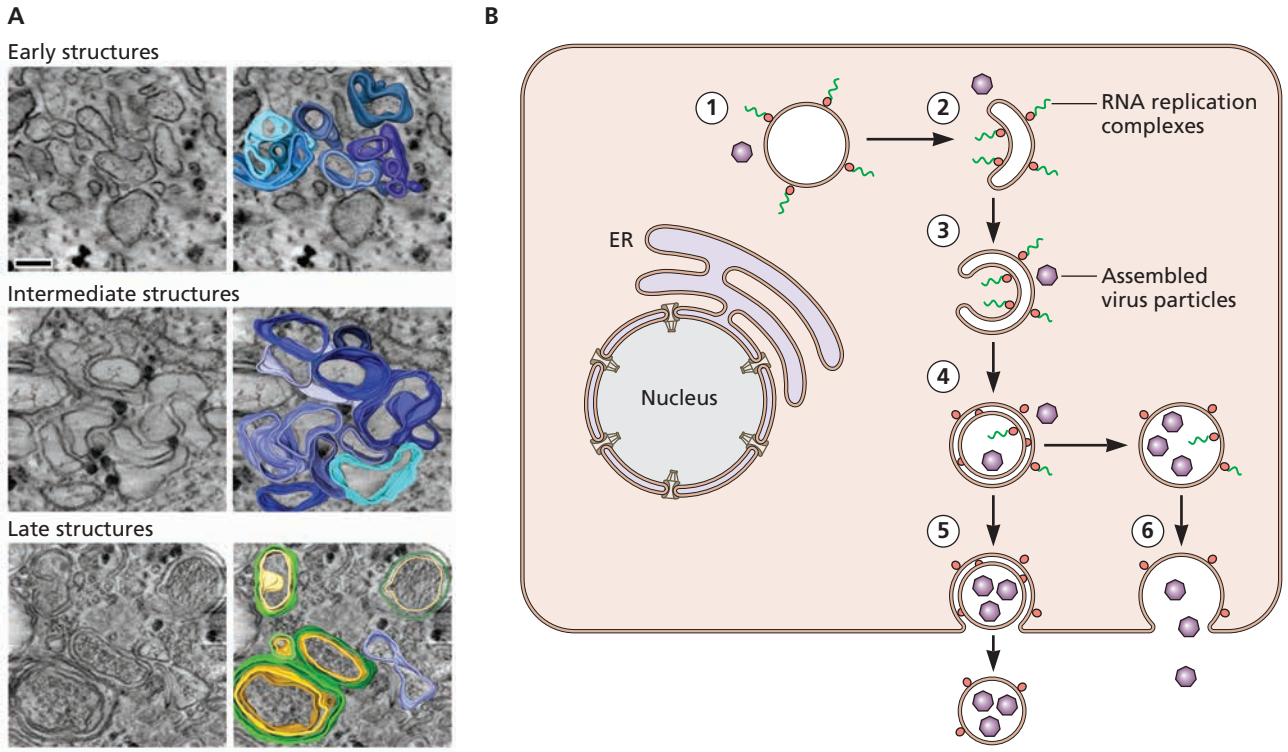


Figure 14.22 Co-option of cytoplasmic membranes in poliovirus-infected cell. (A) HeLa cells were infected with poliovirus and 260-nm-thick sections examined by electron tomography at early (3 h), intermediate (4 h), and late (7 h) times after infection. Shown are central slices in tomograms (left-hand panels) and sections with three-dimensional reconstructions overlaid (right-hand panels). In the reconstructions, single membranes are shown in blue and the inner and outer membranes of double-membrane vesicles in yellow and green. As shown, single- and double-membrane structures predominate early and late, respectively, in the infectious cycle, but occasional double-membrane vesicles can be seen from intermediate times. When sites of viral RNA synthesis were visualized using 5-bromouridine triphosphate (BrUTP) incorporation into newly synthesized viral RNA and immunoelectron microscopy with anti-BrUTP antibodies, single-membrane replication centers were seen to be most active. Adapted from G. A. Belov et al., *J Virol* **86:**302–312, 2012, with permission. Courtesy of E. Ehrenfeld, National Institutes of Health. **(B)** Model for the transition from single-membrane structures that support very active viral genome replication to double-membrane autophagic vesicles. The latter are proposed to develop upon membrane invagination into single-membrane vesicles (steps 1 to 4), so that viral genome replication and assembly can occur on and within vesicles, as has been reported. Autophagic vesicles would fuse with the plasma membrane to release vesicle-enclosed virus particles (step 5). They may also mature into autolysomes, which possess an acidic, degradative environment, with loss of one of the two autophagosomal membranes. Subsequent fusion with the plasma membrane would allow nonlytic release of mature poliovirus particles (step 6). Adapted from A. L. Richards and W. T. Jackson, *PLoS Pathog* **9:**e1003262, 2013, with permission.

they are formed. In particular, components of the multivesicular body system are co-opted to allow release of the particles of a wide variety of enveloped viruses (Chapter 13). Autophagy, a survival mechanism normally invoked under extreme conditions, may also allow nonlytic release of poliovirus and other picornaviruses.

Perspectives

Since the earliest virological experiments with host cells in culture, the considerable impact of virus infection has been documented and exploited, for example, by using cytopathic effects to search for previously unrecognized viruses. Elucidation of the molecular details of the reproduction of individual viruses established that progression through the infectious cycle is often accompanied by inhibition of fundamental cellular processes and reorganization of cellular architecture. However, a more complete appreciation of the magnitude and diversity of host cell responses has come only relatively recently, with the increasing application of the methods of systems biology.

These approaches allow the identification and quantification of very large numbers of RNAs, proteins, protein modifications, or metabolites in a single sample. Consequently, they provide extraordinarily detailed comparisons between cells infected by a particular virus and their uninfected counterparts. These methods have been applied to cells infected by a limited repertoire of viruses for only a relatively short time. Nevertheless, they have not only established the very large scale of alterations in cellular processes induced by infection, from modulation of multiple signaling pathways to stimulation or inhibition of expression of thousands of cellular genes, but also revealed unanticipated

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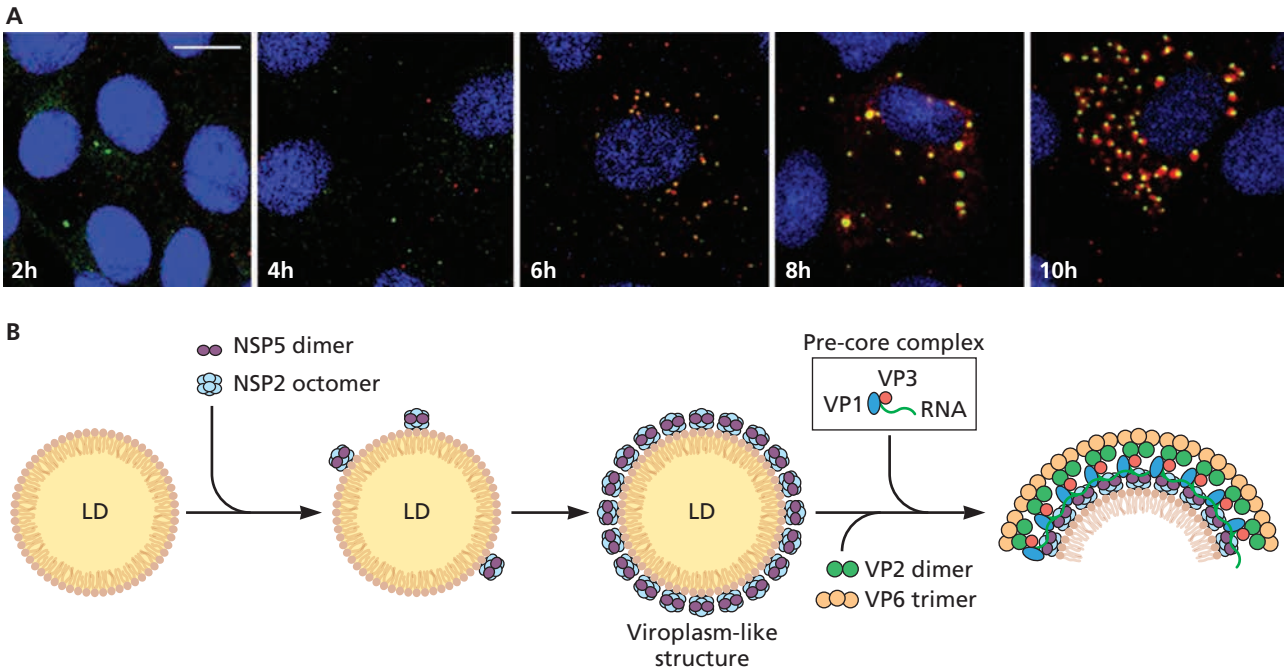


Figure 14.23 Initial rotavirus assembly on lipid droplets. (A) The kinetics of viroplasm development in established monkey cells infected with a bovine rotavirus were examined by indirect immunofluorescence using antibodies that recognize the viral protein NSP5 (green) and the cellular protein perilipin (red), a component of lipid droplets. Structures in which these proteins are both localized increase in size and number as the infectious cycle progresses. They have been termed viroplasms, and contain other viral proteins, cellular lipids, and a second cellular protein found in lipid droplets. Bar, 10 μm. Courtesy of U. Desselberger, University of Cambridge, United Kingdom. **(B)** Model for the initial assembly of rotavirus particles on lipid droplets, in which it is proposed that small, viroplasm-like structures that carry the viral NSP5 and NSP2 proteins serve as platforms for recruitment of viral structural proteins and viral single-stranded (+) RNA segments associated with VP1 and VP3 (pre-core complexes). By a poorly understood process that may include lipid degradation, such structures mature into viroplasms that contain double-layered particles. Adapted from W. Cheung et al., *J Virol* **84:**6782–6798, 2010, with permission.

redirection of cellular pathways. The latter phenomenon is epitomized by effects on cellular energy metabolism.

It might seem obvious that virus-infected cells consume large quantities of energy in the form of ATP for synthesis of viral macromolecules and intracellular transport of components of virus particles for assembly, and consequently, that infection would induce changes in cellular gene expression and metabolic pathways to promote catabolism. Cells infected by a variety of RNA and DNA viruses do indeed take up and metabolize glucose at increased rates, but this compound is not necessarily used for energy metabolism: in cells infected by human herpesviruses, it serves primarily as a source of precursors for synthesis of nucleotides or fatty acids. Furthermore, energy can also be supplied by apparently unique, virus-specific mechanisms, such as the synthesis of the fatty acid palmitate for its subsequent oxidation in poxvirus-infected cells, or the mobilization of lipid stores by induction of autophagy in cells infected by dengue virus. Additional surprises, as well as a better understanding of the mechanisms by which viral gene products directly or indirectly regulate or redirect particular cellular processes and pathways, can be anticipated.

We can now describe in considerable detail some of the striking ways in which virus reproduction and redirection is accompanied by remodeling of architectural features of the host cell. These advances are the result of improvements in the methods by which infected cells can be visualized, notably those of electron tomography and three-dimensional reconstruction. A considerable variety of infected cell-specific structures fashioned from either nuclear or cytoplasmic components have been implicated in facilitating viral genome replication and gene expression, or assembly of progeny virus particles. In some cases, viral proteins necessary for formation of such infected cell-specific platforms have been identified, but much remains to be learned about how such proteins induce reorganization of host cell components.

In this chapter, we have focused on the impact of virus infection on fundamental processes that all cells must carry out to survive and prosper, such as gene expression and generation of energy. However, the cells of multicellular organisms are specialized for particular tasks, and therefore also exhibit cell-type-specific properties and molecular functions. Virus infection can result in major perturbations, even loss,

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of such specialized functions. Such changes can result in far-reaching consequences for the host and contribute to viral pathogenesis, and are considered in Volume II.

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APPENDIX

Structure, Genome Organization, and Infectious Cycles

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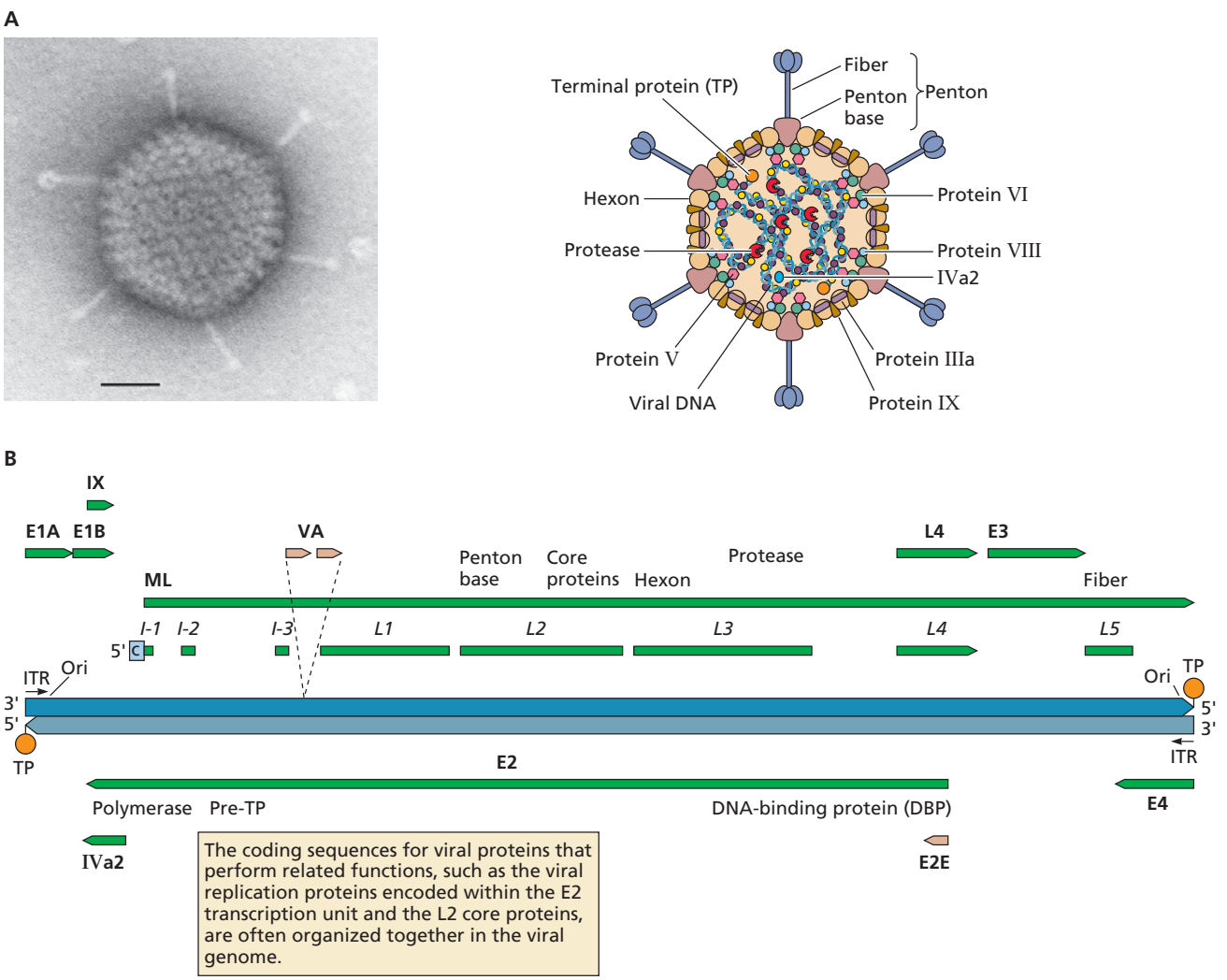
Adenoviruses

Family *Adenoviridae*

Selected Genera	Examples
Mastadenovirus	Human adenovirus type 5
Aviadenovirus	Fowl adenovirus 1

Human serotypes are very widespread in the population. Infection by these viruses is often asymptomatic but can result in respiratory disease in children (members of species B and C), conjunctivitis (members of species B and D), and gastroenteritis (species F serotypes 40 and 41). Human adenoviruses 40 and 41 are the second leading cause (after rotaviruses) of infantile viral diarrhea. Adenoviruses share capsid morphology and linear double-stranded DNA genomes, but the members of the genera differ in size, organization, and coding sequences. The *Mastadenovirinae* comprise over 65 adenoviruses of humans and other mammals, including mice, sheep, and dogs, and some are oncogenic in rodents. Study of human adenovirus transformation of cultured cells has provided fundamental information about mechanisms that control progression through the cell cycle and oncogenesis. Characteristic features of the replication of these viruses include stereotyped temporal control of viral gene expression and an unusual mechanism of initiation of viral DNA synthesis (protein priming). Mastadenoviral genomes also include genes transcribed by cellular RNA polymerase III.

Figure 1 Structure and genome organization of human adenovirus type 5. (A) Virion structure. The electron micrograph shows a negatively stained human adenovirus type 5 particle (courtesy of M. Bisher, Princeton University, Princeton, NJ). Bar = 50 nm. **(B) Genome organization.** The DNA genome length is 36 to 38 kbp. Green and tan arrows represent primary products of RNA polymerase II and III transcription, respectively, and are labeled in bold type. Coding sequences for viral proteins or families of major late mRNAs are also indicated. Hatched lines show splicing of the major late (ML) tripartite leader. ITR, inverted terminal repetition; Ori, origin of replication.



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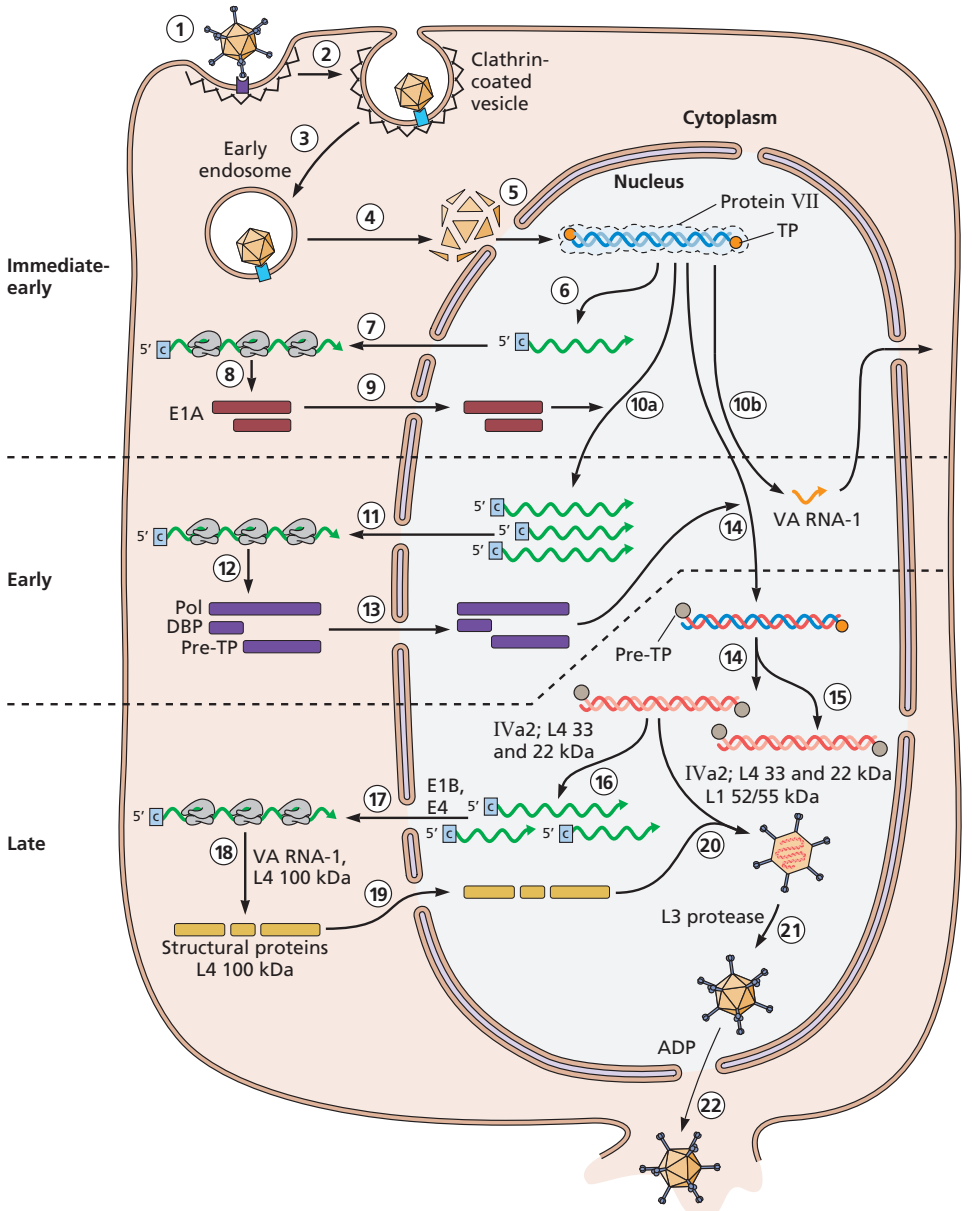


Figure 2 Single-cell reproductive cycle of human adenovirus type 5. **(1)** The virus attaches to a permissive human cell via interaction between the fiber and (with most serotypes) the coxsackie-adenovirus receptor on the cell surface (purple cylinder). **(2)** The particle then enters the cell via receptor-mediated endocytosis. **(3)** A second protein of the virus particle, penton, then interacts with a cell integrin (blue cylinder). **(4)** Partial disassembly takes place prior to entry of particles into the cytoplasm, a step that requires a membrane-lytic region of protein VI. **(5)** Following further uncoating, the viral genome associated with core protein VII is imported into the nucleus. **(6)** The host cell RNA polymerase II system transcribes the immediate early E1A gene. **(7, 8)** E1A proteins are synthesized by the cellular translation machinery, following alternative splicing and export of E1A mRNAs to the cytoplasm. **(9)** These proteins are imported into the nucleus, where they regulate transcription of both cellular and viral genes. **(10a)** The larger E1A protein stimulates transcription of the viral early genes by cellular RNA polymerase II. **(10b)** Transcription of the VA genes by host cell RNA polymerase III also begins during the early phase of infection. **(11, 12)** The early pre-mRNA species are processed, exported to the cytoplasm, and translated. **(13)** These early proteins are imported into the nucleus. **(14)** The viral replication proteins cooperate with a limited number of cellular proteins in viral DNA synthesis. **(15, 16)** Replicated viral DNA molecules can serve as templates for further rounds of replication or for transcription of late genes. Some late promoters are activated simply by viral DNA replication, but maximally efficient transcription of the major late transcription unit (Fig. 1, ML) requires the late IVa2 and L4 proteins. **(17)** Processed late mRNA species are selectively exported from the nucleus as a result of the action of the E1B 55-kDa and E4 Orf6 proteins. **(18)** Their efficient translation requires the major VA RNA, VA RNA-I, which counteracts a cellular defense mechanism, and the late L4 100-kDa protein. **(19)** The latter protein also serves as a chaperone for assembly of trimeric hexons as they and the other structural proteins are imported into the nucleus. **(20)** Within the nucleus, capsids are assembled from these proteins and the progeny viral genomes to form noninfectious immature virus particles. Assembly requires a packaging signal located near the left end of the genome, as well as the IVa2, L1 52/55-kDa, and L4 22/33-kDa proteins. Immature particles contain the precursors of the mature forms of several proteins. **(21)** Mature virions are formed when these precursor proteins are cleaved by the viral L3 protease, which is assembled into the core. **(22)** Progeny virus particles are released, usually upon destruction of the host cell via mechanisms that are not well understood, although the E3 adenovirus death protein (ADP) facilitates exit of particles from the nucleus.

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Arenaviruses

Family *Arenaviridae*

Genus	Example
Arenavirus	Lymphocytic choriomeningitis virus

The *Arenaviridae* are so called because of the sandy (*arenosus*; Latin) appearance of viral particles by electron microscopy. A prototype member of this family, lymphocytic choriomeningitis virus, has been used to elucidate essential principles of the host immune response to viral infection. Arenaviruses cause chronic, usually asymptomatic, infections in rodents, the natural host. Contact with infected mice and rats (typically by bite) can result in zoonotic transmission, with outcomes in humans ranging from asymptomatic infection to febrile illness, aseptic meningitis, and often fatal hemorrhagic fevers. Arenaviruses are categorized into Old World and New World serogroups, based on geographical and genetic parameters. Arenaviruses are enveloped and have a bisegmented RNA genome consisting of a large (L) and a small (S) segment. Only four proteins are encoded in these viral genomes, two in each segment. Two genes, one encoded in the 5′ end of each segment, are present in an ambisense orientation. Replication of arenaviruses is restricted to the cytoplasm.

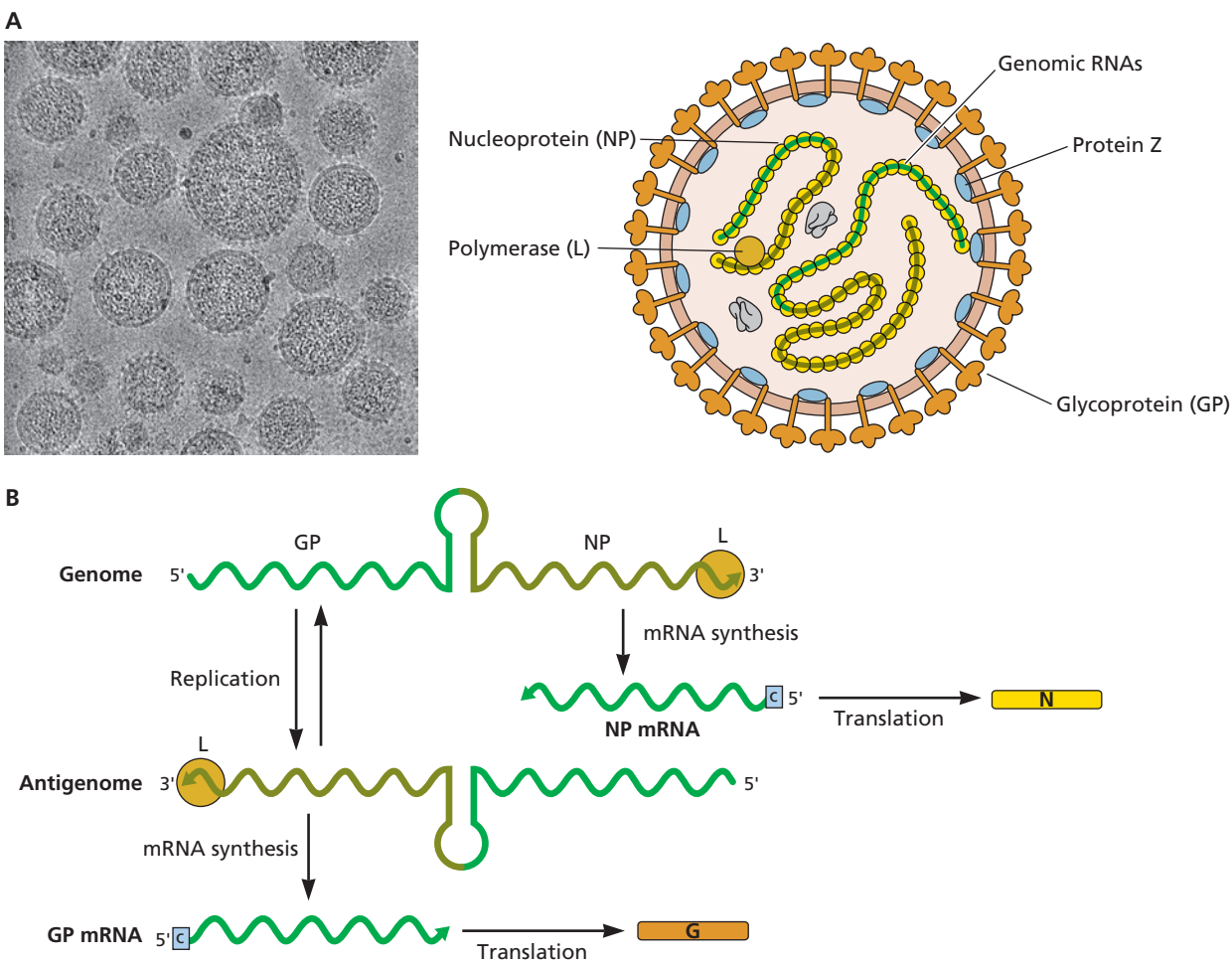


Figure 3 Structure and genome organization. (A) Virion structure. Cryo-electron micrograph of a negatively stained particle of the arenavirus lymphocytic choriomeningitis virus. These pleiomorphic particles are ~120 nm in diameter. (Image courtesy of Michael J. Buchmeier and Benjamin W. Neuman, School of Biological Sciences, University of Reading, United Kingdom.) **(B) Genome organization.** The viral genome comprises two segments: large (L; 7.2 kb) and short (S; 3.5 kb). The L segment encodes the RNA-dependent RNA polymerase (L) and an accessory protein (Z) that functions in genome packaging, particle assembly, and budding. The S segment encodes a surface glycoprotein (GP), which binds to the viral receptor and mediates target cell recognition and entry, and a histone-like nucleocapsid protein (NP) that, with the viral RNAs, forms the ribonucleocapsid. For simplicity, only expression of genes on the S segment is shown, but the same process occurs for the L segment. Upon entry of the viral RNA into the host cell cytoplasm, the viral L protein, which enters the cell with the infecting particle, binds to the 3′ end of the RNA (shown as an orange ball) and synthesizes the (+) strand NP mRNA, which is then translated. Replication of the genomic RNA into a complementary antigenome allows synthesis of GP mRNA. This mechanism of gene expression results in temporal control of viral gene expression, a common feature of the reproductive cycles of many viruses with DNA genomes. (Figure provided by Juan Carlos de la Torre, The Scripps Research Institute.)

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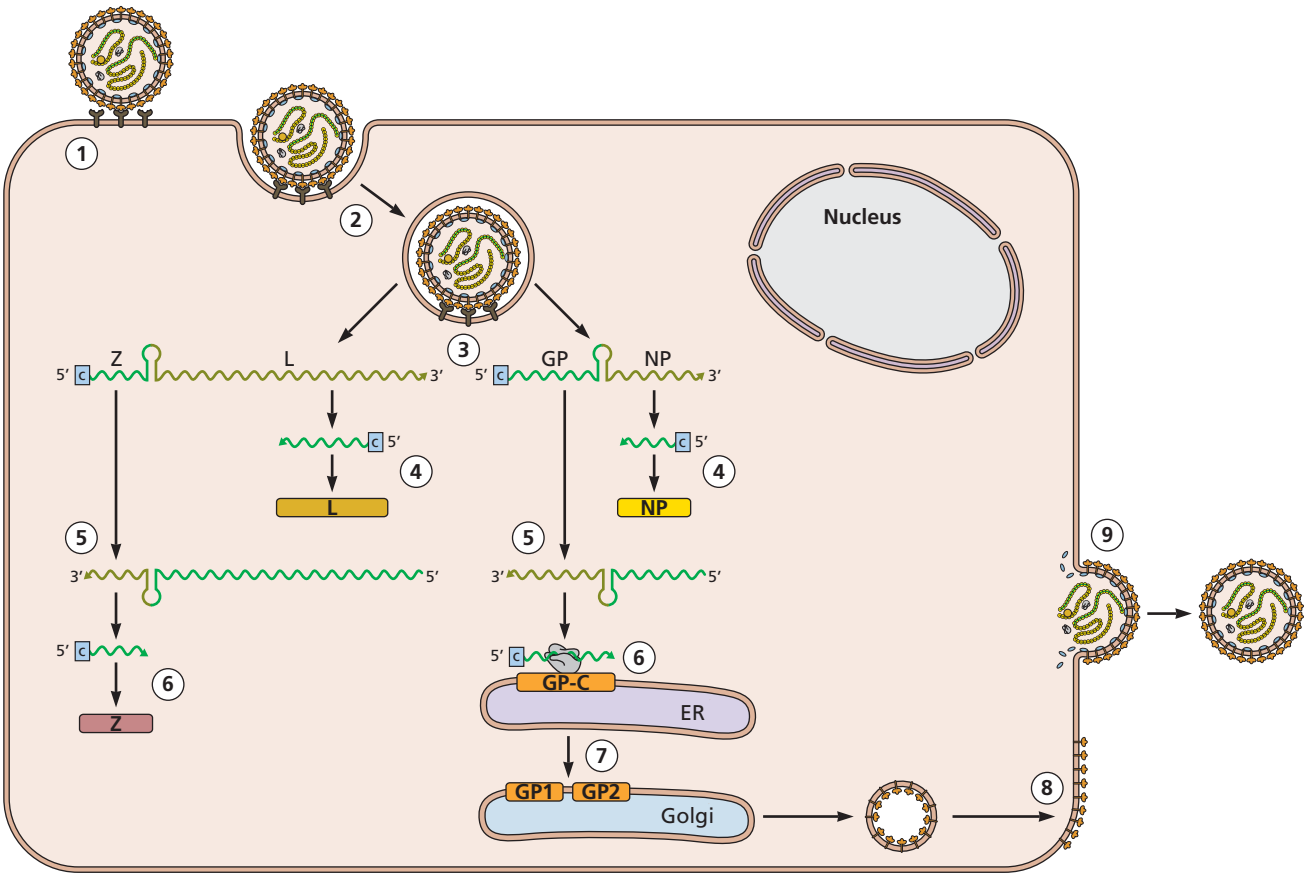


Figure 4 Single-cell reproductive cycle. **(1, 2)** The virion binds to a cellular receptor (alpha dystroglycan), which induces receptor-mediated endocytosis. **(3)** Low-pH-triggered membrane fusion between the viral and cellular membranes releases the viral genome segments into the cytoplasm. **(4)** NP and L mRNAs are synthesized using the viral genome as a template. **(5)** In addition, the ambisense viral genome (Fig. 3B) is the template for synthesis of a complementary antigenome by the viral RNA-dependent RNA polymerase (L). **(6)** The antigenome serves both as a template for production of progeny viral genomes, as well as the template for the synthesis of the other two viral mRNAs, Z and GP-C. In all cases, the intergenic region (IGR) that separates the two coding regions of each segment serves to terminate mRNA synthesis. **(7)** GP-C, which is translated by ER-bound ribosomes, is cleaved into GP-1 and GP-2 by a cellular protease (MBTPS1) as it traverses the secretory pathway. **(8)** GP-1 and GP-2 then associate to form the spikes that form the outer surface of the viral particles. **(9)** The small RING finger protein Z facilitates budding through interaction with cellular proteins, enabling release of extracellular viral particles.

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Coronaviruses

Family *Coronaviridae*

Genera	Examples
Alphacoronavirus	Human coronavirus 229E
Betacoronavirus	Murine coronavirus
	Severe acute respiratory syndrome-related coronavirus (SARS-CoV)
	Middle East respiratory syndrome coronavirus (MERS-CoV)
Gammacoronavirus	Avian coronavirus

Coronaviruses are enveloped RNA viruses that infect mammals and birds. The name derives from the fringe of club-shaped spikes observed in electron micrographs that give the virus particles the appearance of a solar corona. These viruses have the largest RNA genomes known. They cause significant respiratory and gastrointestinal disease in humans and domestic animals. They were known to cause common colds in humans, until the emergence of severe acute respiratory syndrome coronavirus in 2002, which caused a devastating human disease. Another new coronavirus, Middle East respiratory syndrome coronavirus, was first recognized in humans in April 2012.

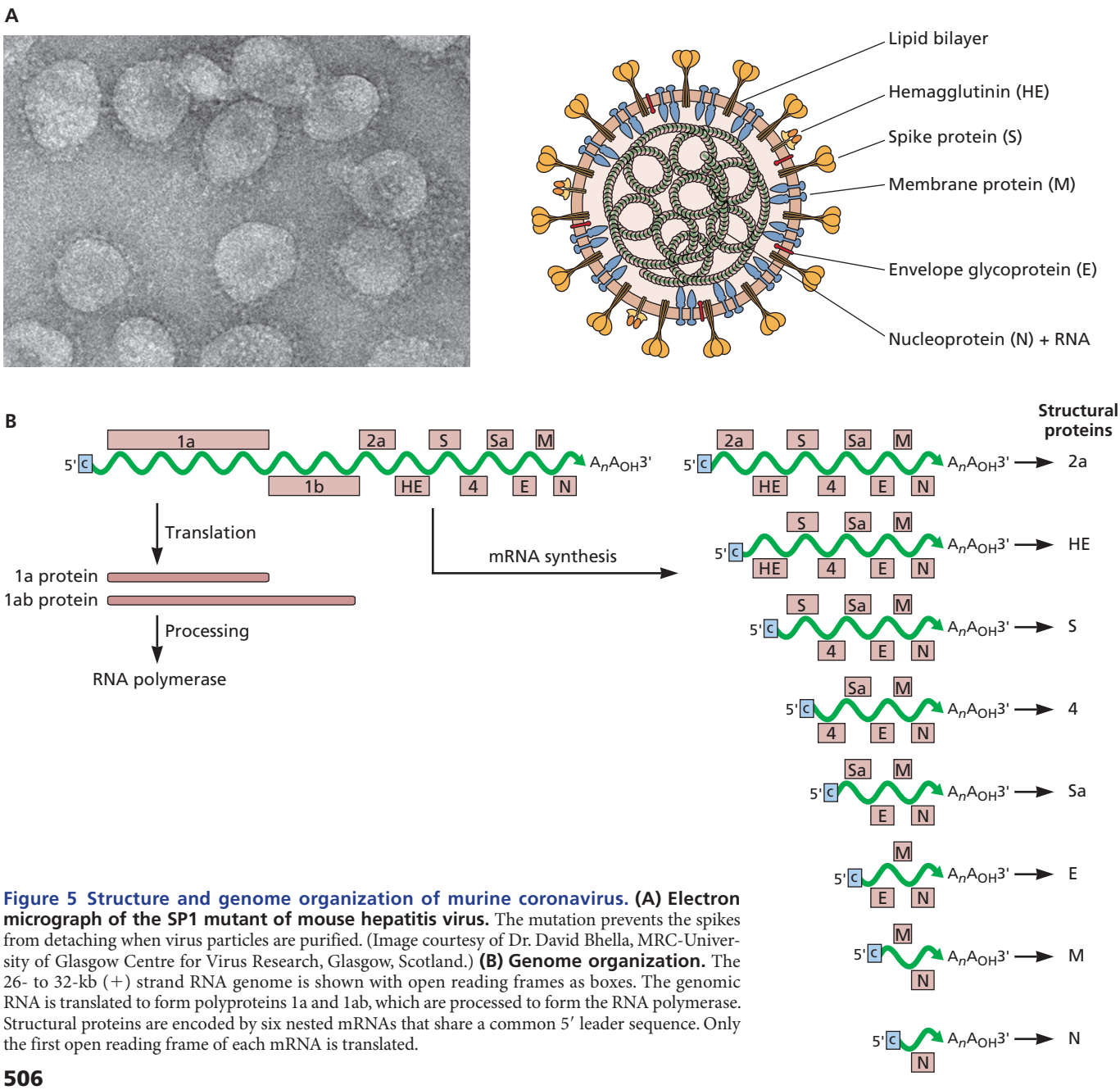


Figure 5 Structure and genome organization of murine coronavirus. (A) Electron micrograph of the SP1 mutant of mouse hepatitis virus. The mutation prevents the spikes from detaching when virus particles are purified. (Image courtesy of Dr. David Bhella, MRC-University of Glasgow Centre for Virus Research, Glasgow, Scotland.) **(B) Genome organization.** The 26- to 32-kb (+) strand RNA genome is shown with open reading frames as boxes. The genomic RNA is translated to form polyproteins 1a and 1ab, which are processed to form the RNA polymerase. Structural proteins are encoded by six nested mRNAs that share a common 5′ leader sequence. Only the first open reading frame of each mRNA is translated.

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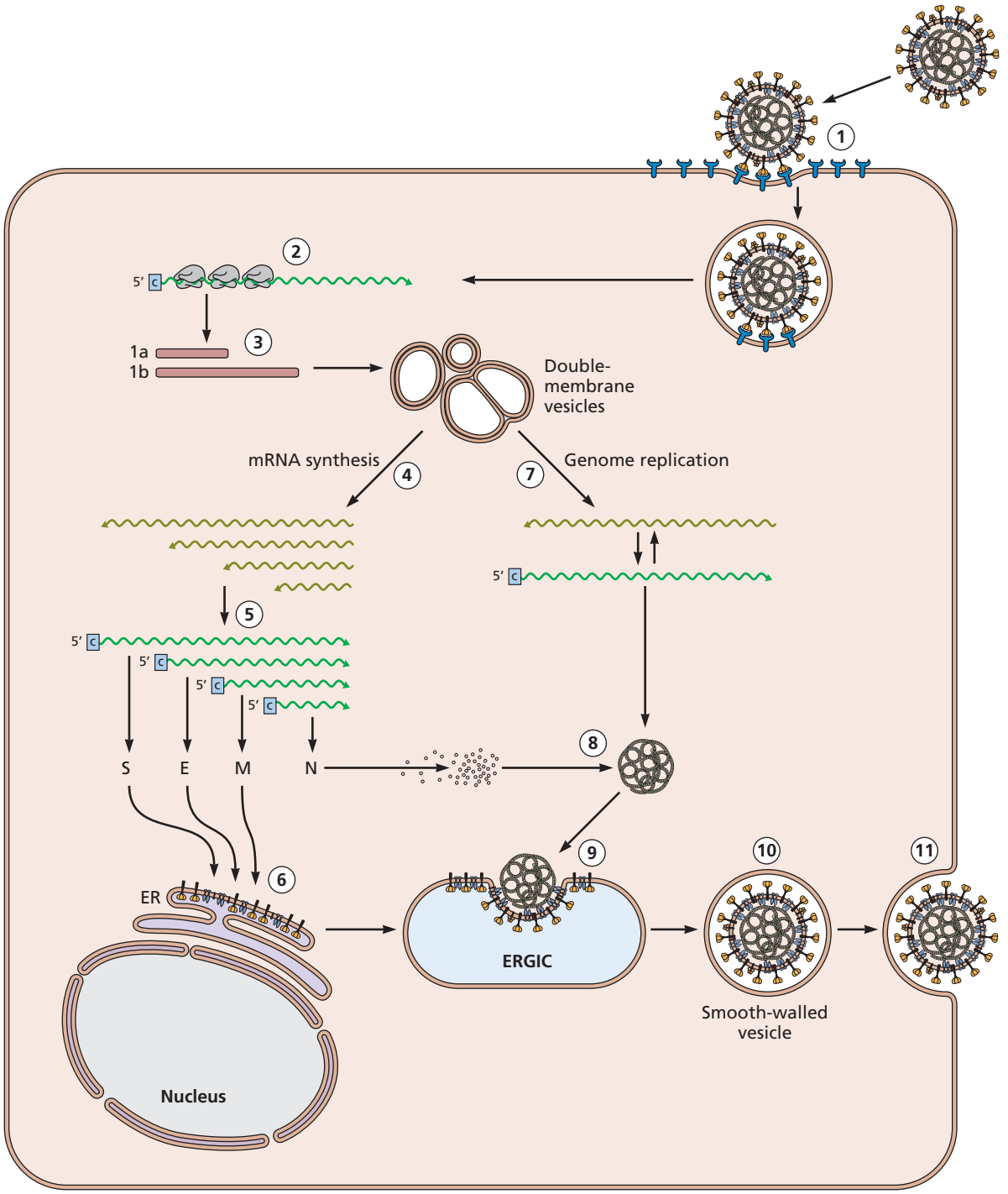


Figure 6 Single-cell reproductive cycle. **(1)** The virion binds to a cell surface receptor, and fusion of the viral and cell membrane occurs either at the cell surface or from within endosomes, depending on the virus. **(2)** Fusion is triggered by low pH, leading to delivery of the nucleocapsid into the cytoplasm. **(3)** The viral genome is translated to produce the 1a and 1ab proteins (the latter by ribosomal frameshifting). These are autoproteolytically processed by viral proteases to produce a variety of viral proteins, including the RNA-dependent RNA polymerase, proteins that remodel cellular membranes to form structures that are sites of viral RNA synthesis, enzymes that catalyze multiple steps in the synthesis of the 5′-terminal cap structure of mRNA, and an exonuclease that provides a proofreading function. **(4)** The other viral proteins are encoded by a nested set of mRNAs that share a common 5′ leader sequence. Discontinuous RNA synthesis occurs during (−) strand RNA synthesis. Most of the (+) strand template is not copied, probably because it loops out as the polymerase completes synthesis of the leader RNA. **(5)** The resulting (−) strand RNAs, with leader sequences at the 3′ ends, are then copied to form mRNAs. These mRNAs serve as templates for structural and nonstructural proteins. **(6)** The membrane-bound proteins M, S, and E are inserted into the ER, and then move to the site of viral assembly, the ER-Golgi intermediate compartment. **(7)** Full length (−) strand RNAs are produced, and these are templates for the synthesis of full length (+) strands, **(8)** which are encapsidated by N protein. **(9)** The nucleocapsid buds into the ER-Golgi intermediate compartment (ERGIC), acquiring a membrane that contains S, E, and M proteins. **(10)** Virus particles are transported to the plasma membrane in smooth-walled vesicles and **(11)** released from the cells by exocytosis as the transport vesicle fuses with the plasma membrane.

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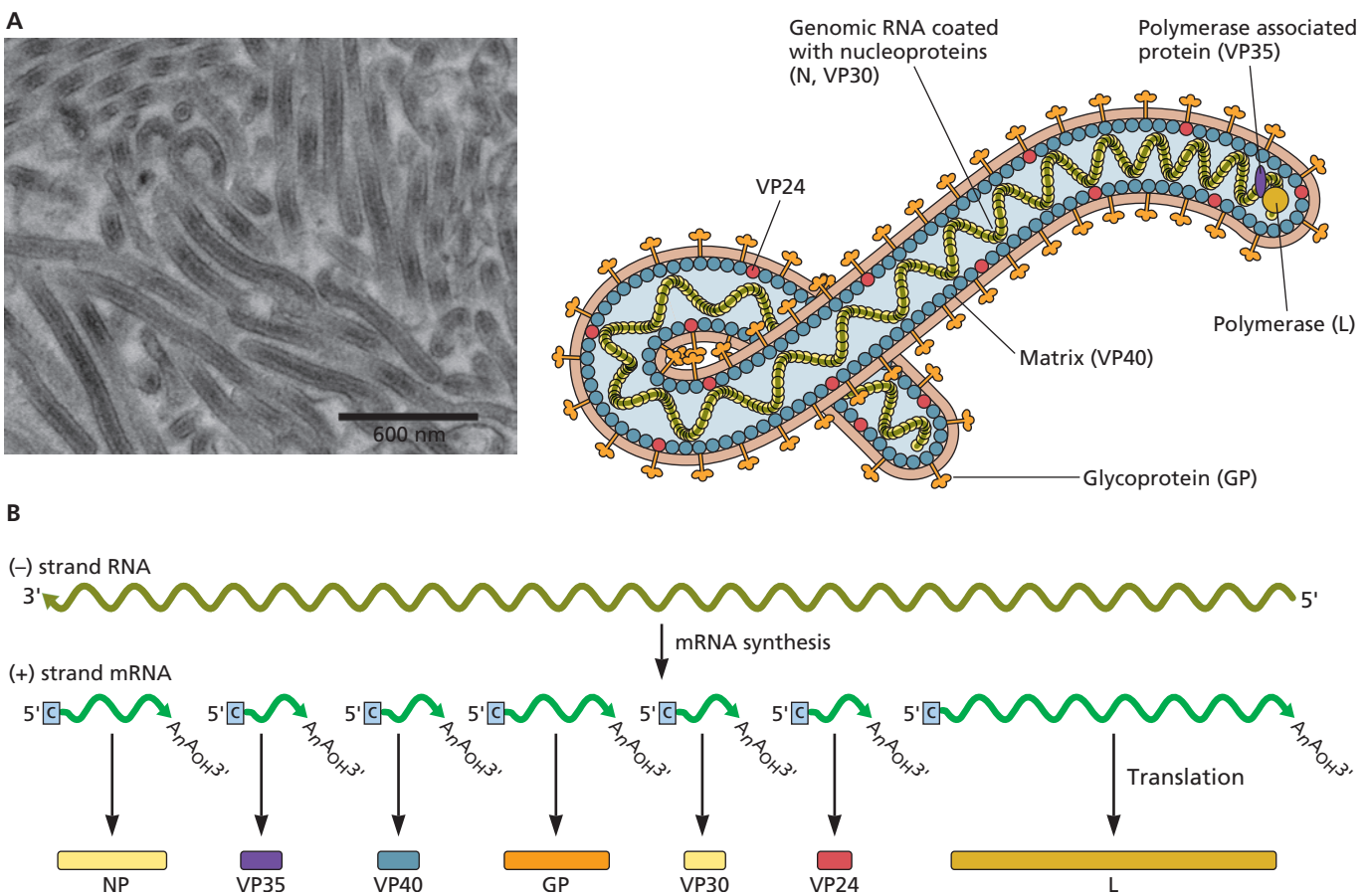
Filoviruses

Family *Filoviridae*

Genera	Examples
Marburgvirus	Lake Victoria marburgvirus
Ebolavirus	Zaire ebolavirus
	Sudan ebolavirus

Members of the *Filoviridae* are enveloped viruses with (−) strand RNA genomes. The virus particles possess an unusual filamentous morphology, which led to the name of this family (*Filum* is the Latin word for thread). They are agents of serious hemorrhagic fever in humans and primates. Because these viruses have a high case fatality ratio and can be transmitted from person to person by close contact, they have been classified as select agents by the U.S. Centers for Disease Control and Prevention. Research on these viruses must be carried out under BSL-4 containment.

Figure 7 Structure and genome organization of the filovirus Zaire ebolavirus. (A) Virion structure. The electron micrograph shows an image of ebolavirus particles inside of an infected, cultured monkey cell (courtesy of Elizabeth R. Fischer, Rocky Mountain Laboratories, NIAID, NIH). **(B) Genome organization.** The genome is ~19 kb long and contains 7 genes. Conserved sequences are present at the 3′ (leader) and 5′ (trailer) ends of the viral genome. Each gene is flanked by short, conserved sequences that specify initiation and termination of mRNA synthesis. In some cases the termination and initiation sequences of neighboring genes overlap. The (−) strand RNA is the template for synthesis of leader RNA and 7 monocistronic mRNAs (capped and polyadenylated) encoding the 7 viral proteins. The fourth gene of ebolaviruses encodes an mRNA that is translated to form a secreted, nonstructural glycoprotein (orange). Editing of the gene 4 mRNA is required to produce an mRNA encoding the membrane-associated GP. The secreted GP is not encoded by marburgvirus genomes.



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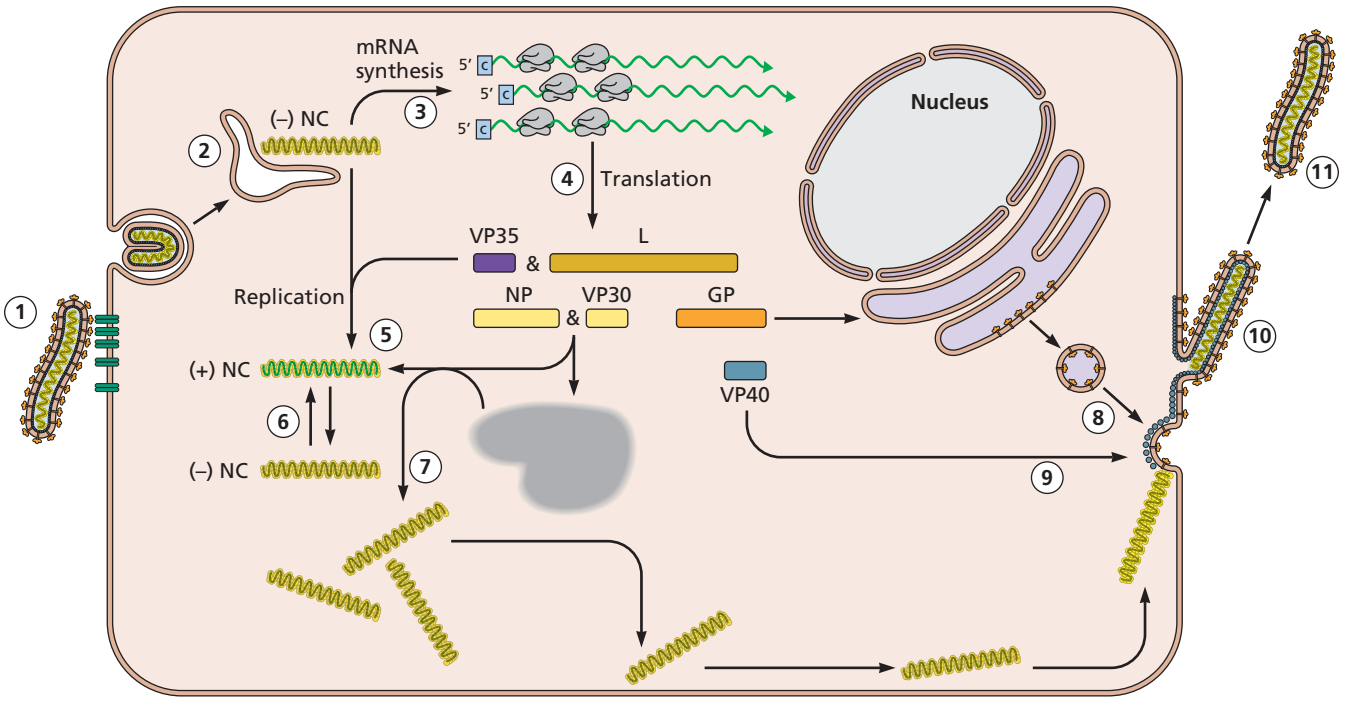


Figure 8 Single-cell reproductive cycle of ebolavirus. **(1)** Virus particles bind to a cell surface receptor, followed by uptake via macropinocytosis and trafficking to late endosomes. **(2)** Viral GP is cleaved by endosomal cysteine proteases (cathepsins) and then binds to the Niemann-Pick disease type C integral membrane protein, NPC1, which is exposed in the endosomal lumen. This interaction leads to fusion of the viral and endosomal membranes for delivery of the viral nucleocapsid into the cytoplasm. **(3)** The nucleocapsid, which is composed of the viral RNA and L (the RNA-dependent RNA polymerase), NP, and VP30 proteins, is the template for the synthesis of seven viral mRNAs in the cytoplasm. The capped and polyadenylated mRNAs are synthesized in a 3′ to 5′ direction from the (−) strand RNA template, by a process of initiation and termination as the polymerase complex recognizes conserved start and stop sequences on the template. **(4)** These mRNAs are translated. **(5)** The concentrations of viral proteins, especially NP, regulate the switch from mRNA synthesis to genome replication, which begins with synthesis of full-length (+) strand copies of the viral RNA. **(6)** These (+) strands are encapsidated by NP and, in turn, serve as templates for the synthesis of full length (−) strand RNAs. **(7)** Inclusion bodies are the sites of RNA synthesis and nucleocapsid assembly. **(8)** Assembly begins with the synthesis of GP in the ER and transport to lipid raft microdomains at the plasma membrane. **(9)** Octamers of VP40 are produced and transported to GP-containing lipid rafts by the endocytic multivesicular bodies. VP40 interacts with the C terminus of NP and serves to direct viral (−) strand nucleocapsids to sites of virus budding. **(10)** Nucleocapsids form parallel to the plasma membrane, and **(11)** virus particles are released by budding.

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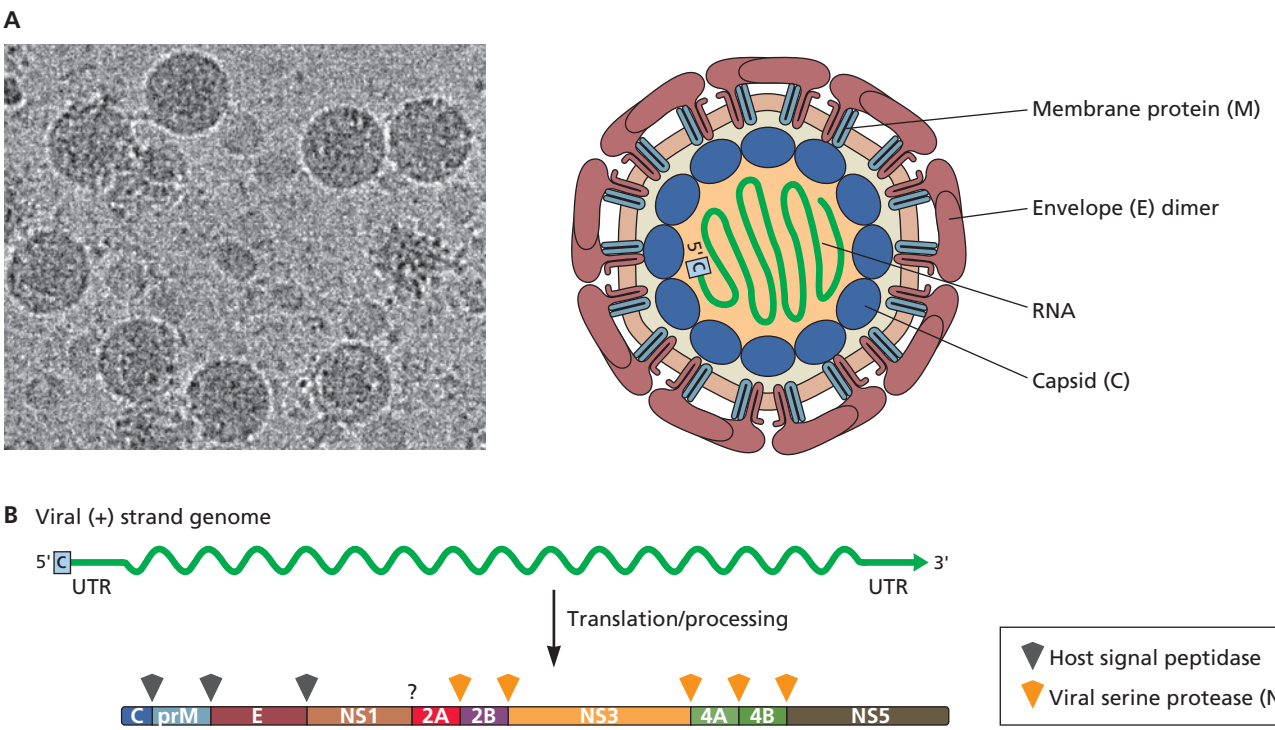
Flaviviruses

Family *Flaviviridae*

Genera	Examples
Flavivirus	Yellow fever virus
	Dengue virus
	West Nile virus
Hepacivirus	Hepatitis C virus
	GB virus B
Pestivirus	Bovine viral diarrhea virus
Pegivirus	GB virus A, C, D

The *Flaviviridae* comprises a large family of enveloped, (+) strand RNA viruses, including the first human virus discovered, yellow fever virus. There are more than 50 viral species, many of which are transmitted by arthropod vectors. Flaviviruses cause a variety of human diseases, such as encephalitis, and hemorrhagic fevers. Included in this family are major global pathogens such as dengue virus, Japanese encephalitis virus, and West Nile virus. Yellow fever virus vaccine was the first live, attenuated viral vaccine.

Figure 9 Structure and genome organization of flaviviruses. (A) Virion structure. The cryo-electron micrograph reconstruction of the flavivirus, dengue virus (50-nm) particles. (Image courtesy of Dr. Richard J. Kuhn and Valorie Bowman, Department of Biological Sciences, Purdue University.) **(B) Genome organization.** The (+) strand RNA genome is from 9.6 kb (*Hepacivirus* genus) to 12.3 kb (*Pestivirus* genus) in length. The genome RNA has a 5′ cap structure (except for hepatitis C virus) but lacks a 3′ poly(A) characteristic of cellular and viral mRNAs. The viral RNA genome has 5′ and 3′ nocoding regions and encodes a polyprotein (~3,400 amino acids) that is processed by viral and cellular proteases to produce viral structural (C, M, E) and nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). Cleavage sites for host signal peptidase and a virus-encoded serine protease, NS2B-3, are shown.



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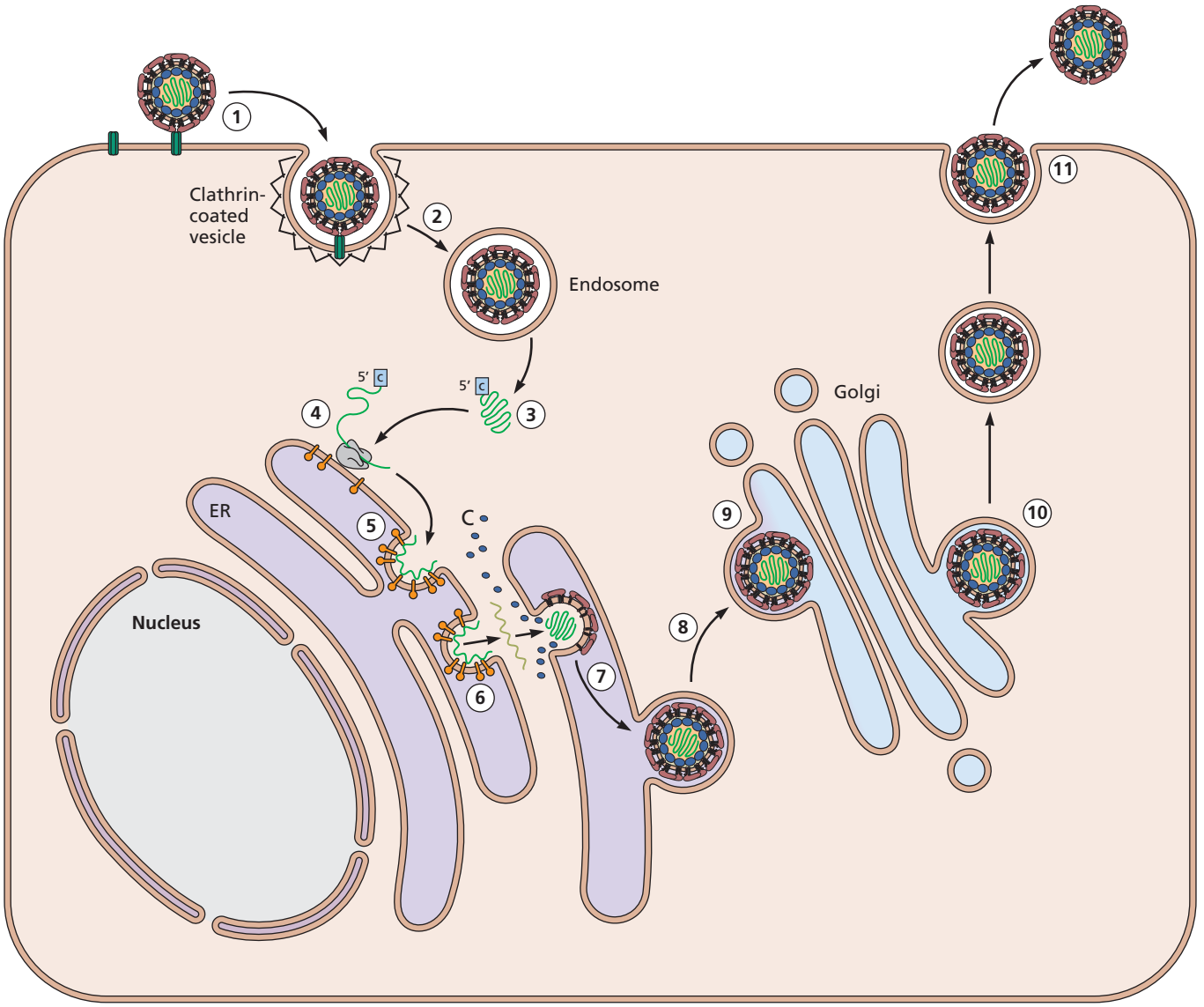


Figure 10 Single-cell reproductive cycle. **(1)** The virion binds to a cell surface receptor and **(2)** is taken into the cell by receptor-mediated endocytosis. **(3)** Fusion of the viral and cell membranes is triggered by low pH in the late endosome and the viral (+) strand RNA is released into the cytoplasm, **(4)** where it is associated with cellular membranes and translated into a polyprotein that is co- and posttranslationally cleaved into at least 10 proteins. **(5)** The viral NS proteins recruit the viral genome to a replication complex, which consists of ER-derived membrane vesicles that are invaginations of the ER that are open to the cytoplasm. **(6)** Replication begins with the synthesis of a genome-length (−) strand RNA, which is then copied to produce new (+) strand RNA genomes. These sites of RNA replication are near the sites of virus assembly; together both form a continuous network, possibly to couple replication and assembly. **(7)** The assembly process begins when C protein dimers associate with viral (+) strand RNA. This complex then buds into ER membranes containing the E-prM proteins. **(8)** The newly formed immature virus particles are transported to the cell surface by the secretory pathway. **(9)** During transport through the secretory pathway, particles undergo a series of maturation steps including glycosylation of prM and E, low-pH-induced rearrangement of E-prM, and prM cleavage. **(10)** Mature virus particles are transported to the cell surface in vesicles. **(11)** Particles are released from the cell surface by exocytosis.

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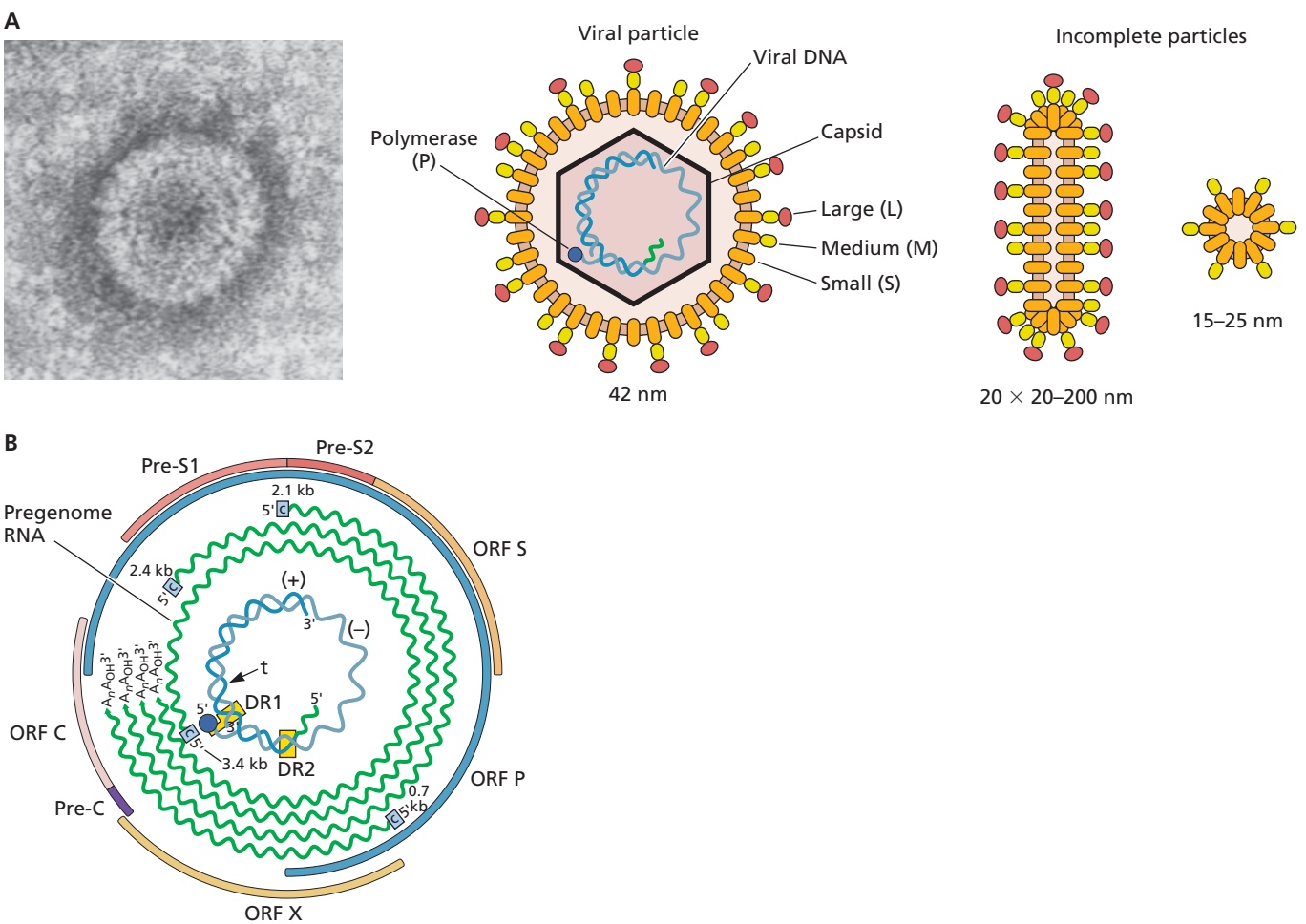
Hepadnaviruses

Family *Hepadnaviridae*

Genera	Examples
Orthohepadnavirus	Human hepatitis B virus
Avihepadnavirus	Duck hepatitis B virus

The hepadnaviruses all show very narrow host specificity and marked tropism for liver tissue. Hepadnaviruses can replicate following inoculation of primary hepatocytes with virus-containing serum, but most hepadnaviruses cannot be propagated in established cell lines. Hepadnaviruses replicate via an RNA intermediate and, like the retroviruses, encode a reverse transcriptase. Both families are included in the group called **retroid viruses**. Natural infections may be acute or persistent, depending on host age, inoculum dose, and other (undefined) parameters that influence the host immune response. Sera of infected individuals typically carry numerous small round particles and some rodlike particles, both of which include viral surface antigens but lack a capsid and genome. Relatively few mature 42-nm virions, called Dane particles, are found in these sera. Approximately 5% of the world's population has been infected with human hepatitis B virus; the World Health Organization estimates that 400 million are now chronically infected. Persistent infection with the orthohepadnaviruses but not the avihepadnaviruses confers an increased risk for hepatocellular carcinoma.

Figure 11 Structure and genome organization of orthohepadnaviruses. (A) Virion structure. The electron micrograph shows negatively stained woodchuck hepatitis virus, a mammalian hepadnavirus related to human hepatitis B virus (courtesy of W. Mason and T. Gales, Fox Chase Cancer Center, Philadelphia, PA). **(B) Genome organization.** The relaxed circular DNA genome of human hepatitis B virus is shown at the center. It comprises a complete (−) strand of 3,227 nucleotides and an incomplete (+) strand, which is only about two-thirds genome length and can have variable 3′ ends. The viral reverse transcriptase protein (indicated by a blue ball) is covalently attached to a short (8- to 9-nucleotide) single-strand terminal redundancy at the 5′ end of the (−) strand. The approximate locations of direct repeats, DR1 and DR2, in the DNA are indicted. The locations of the 5′ ends of the pregenome and mRNAs that are synthesized by host cell Pol II are shown surrounding the genome, all of which end at the same location (marked by a "t" in the genome). The outermost, colored rings show the locations of the open reading frames, which are all organized in the same direction (clockwise in the figure).



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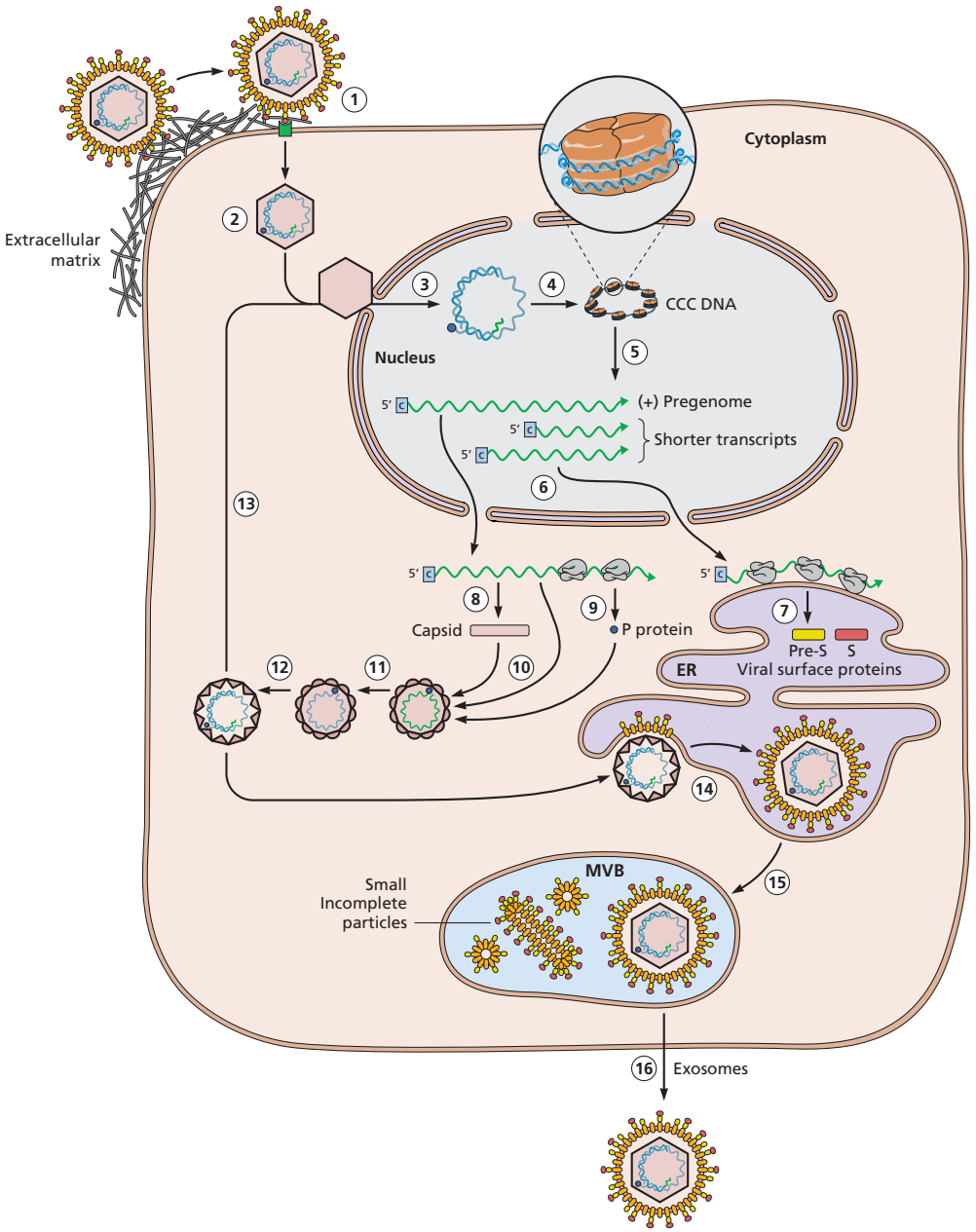


Figure 12 Single-cell reproductive cycle of hepatitis B virus. **(1)** The virion attaches to a susceptible hepatocyte, most likely via weak interaction with cell-associated heparin sulfate proteoglycans and then through recognition of a specific cell surface receptor, the sodium taurocholate-cotransporting polypeptide in human cells. **(2)** Details of entry and transport of the core (capsid encased genome) to the nucleus are unknown, although host cell calveolin-1 has been implicated in entry and the viral core is presumed to be transported along microtubules. **(3)** The core binds to proteins in the nuclear pore and the viral genome is released into the nucleus. **(4)** Repair of the gapped (+) DNA strand is likely accomplished by cellular enzymes. The product is a covalently closed circular form called CCC DNA, which associates with histones to form a minichromosome. **(5)** The (−) strand of CCC DNA is the template for transcription by cellular RNA polymerase II of a longer-than-genome-length RNA called the pregenome and shorter, subgenomic transcripts, all of which serve as mRNAs. **(6)** Viral mRNAs are transported from the nucleus. **(7)** Subgenomic viral mRNAs, which encode the viral envelope protein, are translated by ribosomes bound to the endoplasmic reticulum (ER). Proteins destined to become anchored in the viral envelope, as well as in incomplete particles, enter the secretory pathway. **(8)** The pregenome RNA is translated to produce capsid protein. **(9)** The P protein, the viral reverse transcriptase, is also produced from pregenome RNA but at low efficiency; the ratio of capsid to P protein translation is 200 to 300 to 1. Following its synthesis, P binds to the packaging signal at the 5′ end of its own transcript, where viral DNA synthesis is eventually initiated. **(10)** Concurrently with capsid formation, and aided by the host heat shock protein chaperones Hsp90/70, the RNA-P protein complex is packaged and DNA replication is primed from a tyrosine residue in the polymerase. **(11)** Reverse transcription of the pregenome occurs within the capsid. **(12)** After completion of DNA synthesis, the newly assembled "cores" acquire the ability to interact with envelope proteins. **(13)** However, at early times after infection, core particles are transported to the nucleus, where the viral genomes are deposited and give rise to additional copies of CCC DNA. Eventually, 10 to 30 molecules of CCC DNA accumulate, leading to a concomitant increase in viral mRNA concentrations. **(14)** At later times, and possibly as a consequence of the accumulation of sufficient envelope proteins, the core particles acquire envelopes as they bud into the ER, where viral surface proteins have been synthesized. **(15)** Viral assembly is believed to be completed in multivesicular bodies (MVB). **(16)** Progeny enveloped virus particles, and numerous small genome-lacking incomplete particles, are released from the cell by exocytosis.

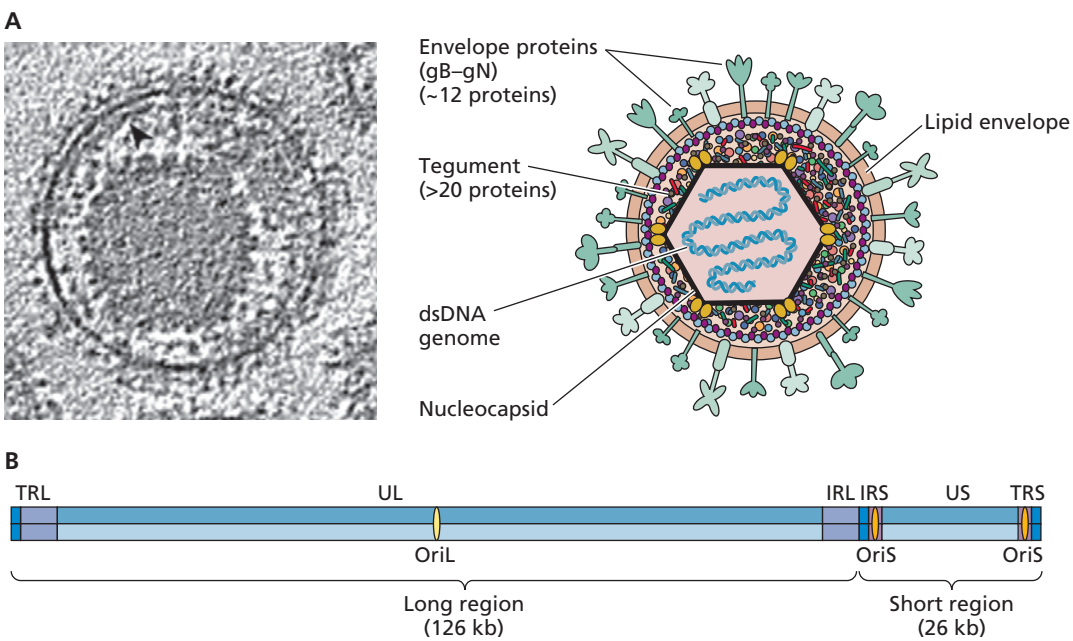
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Family *Herpesviridae*

Subfamilies and Selected Genera	Examples
Alphaherpesviruses	
Simplexvirus	Human herpes simplex virus type 1 and 2
Varicellovirus	Varicella-zoster virus
Betaherpesviruses	
Cytomegalovirus	Human cytomegalovirus
Roseolovirus	Human herpesvirus 6 and 7
Gammaherpesviruses	
Lymphocryptovirus	Epstein-Barr virus
Rhadinovirus	Human herpesvirus 8

The order *Herpesvirales* currently consists of 3 families, 3 subfamilies, 17 genera, and 90 species. The family *Alloherpesviridae* comprises fish and amphibian herpesviruses, and the family *Malacoherpesviridae* comprises viruses of oysters. The family *Herpesviridae*, listed here, includes the well-known human pathogens that belong to all three subfamilies. While some herpesviruses have broad host ranges, most are restricted to infection of a single species and spread in the population by direct contact or aerosols. The hallmark of herpesvirus infections is the establishment of a lifelong, latent or quiescent infection that can reactivate to spread to other hosts and often may cause one or more rounds of disease. Many herpesvirus infections are not apparent, but if the host's immune defenses are compromised, infections can be devastating. Some herpesviruses are pathogens of economically important animals. The study of herpesviruses has provided fundamental information about the assembly of complex virions, the regulation of gene expression and mechanisms of immune system modulation, and insight into the biology of terminally differentiated cells, such as neurons.

Figure 13 Structure and genome organization of alphaherpesviruses. (A) Virion structure. Cryo-electron tomograph of a slice through a single herpes simplex virus type 1 particle. (Adapted from E. Grunwald et al., *Science* **302:**1396–1398, with permission.) **(B) Genome organization.** The herpes simplex virus type 1 genome can "isomerize" or recombine via the large inverted repeat sequences (TRL and IRL, or IRS and TRS) such that all populations consist of four equimolar isomers in which unique long and short sequences (UL and US) are inverted with respect to each other. There are at least 84 open reading frames in this ~152-kbp genome, as well as three origins of replication (Ori).



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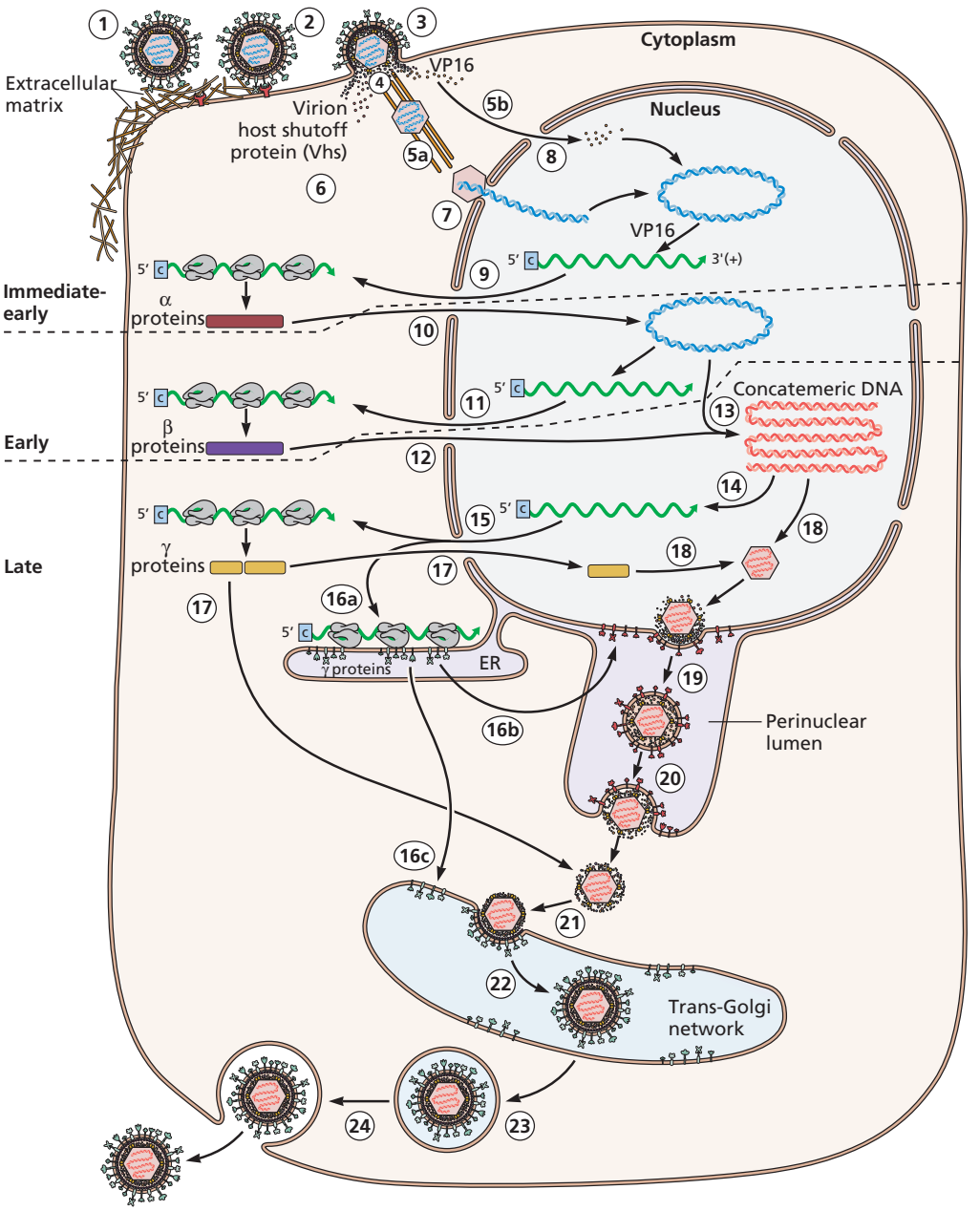


Figure 14 Single-cell reproductive cycle of herpes simplex virus type 1. **(1)** Virions bind to the extracellular matrix (heparan sulfate or chondroitin sulfate proteoglycans) via gB and gC. **(2)** Another viral membrane protein (gD) interacts with a second cellular receptor (such as nectin-1). **(3)** Particles can enter cells via a pH-independent fusion of viral envelope with the plasma membrane or alternatively (not shown) via an endocytic pathway that is similar to phagocytosis. Viral and plasma membrane fusion is mediated by viral membrane glycoproteins (gD, gB, gH, and gL). **(4)** After membrane fusion, some tegument proteins and the nucleocapsid are released into the cytoplasm. **(5a)** Viral nucleocapsids with associated inner tegument proteins attach to microtubules and are transported to the nucleus. **(5b)** Certain tegument proteins are transported to the nucleus independently of the nucleocapsids. **(6)** Other proteins, such as Vhs, remain in the cytoplasm. **(7)** Viral nucleocapsids dock at the nuclear pore, releasing DNA into the nucleus, where it is rapidly circularized. **(8)** VP16 interacts with host transcription proteins to stimulate transcription of immediate early genes by host cell RNA polymerase II. **(9)** Some immediate early mRNAs are spliced and all are transported to the cytoplasm, where they are translated. **(10)** The immediate early proteins (α proteins) are transported to the nucleus, where they activate transcription of early genes and regulate transcription of immediate early genes. **(11)** Early gene transcripts, which are rarely spliced, are transported to the cytoplasm, where they are translated. The early proteins (β proteins) function primarily in DNA replication and production of substrates for DNA synthesis. **(12)** Some early proteins function in the cytoplasm, and some are transported to the nucleus. **(13)** Viral DNA synthesis is initiated from viral origins of replication. **(14)** DNA replication and recombination produce long, concatemeric DNA, the template for late gene expression. **(15)** Most late mRNAs are not spliced but nevertheless are transported to the cytoplasm, where they are translated. Late proteins (γ proteins) are primarily structural proteins and additional proteins needed for virus assembly and particle egress. **(16a)** Some late proteins are made on, and inserted into, membranes of the rough endoplasmic reticulum. **(16b)** Many of these membrane proteins are modified by glycosylation. Some precursor viral membrane proteins are localized both to the outer and inner nuclear membranes, as well as membranes of the endoplasmic reticulum. **(16c)** The precursor glycoproteins are also transported to the Golgi apparatus for further modification and processing. **(17)** Some late proteins are transported to the nucleus for assembly of the nucleocapsid and DNA cleavage to release genomes concomitant with packaging, while some remain in the cytoplasm. **(18)** Newly replicated viral DNA is packaged into nucleocapsids. **(19)** DNA-containing nucleocapsids, together with some tegument proteins, bud from the inner nuclear membrane into the perinuclear lumen, acquiring an envelope thought to contain precursors to viral membrane proteins. **(20)** Immature enveloped particles fuse with the outer nuclear membrane from within, releasing the nucleocapsid into the cytoplasm. **(21)** This structure is transported to the *trans*-Golgi network or an endosome that contains mature viral membrane proteins. Tegument proteins added in the nucleus remain with the nucleocapsid, and others are added in the cytoplasm. **(22)** As nucleocapsids bud into the Golgi or endosome compartment, they acquire an envelope containing mature viral envelope proteins and the complete tegument layer (secondary envelopment). **(23)** The enveloped virus particle then buds into a vesicle that is transported to the plasma membrane for **(24)** release by exocytosis.

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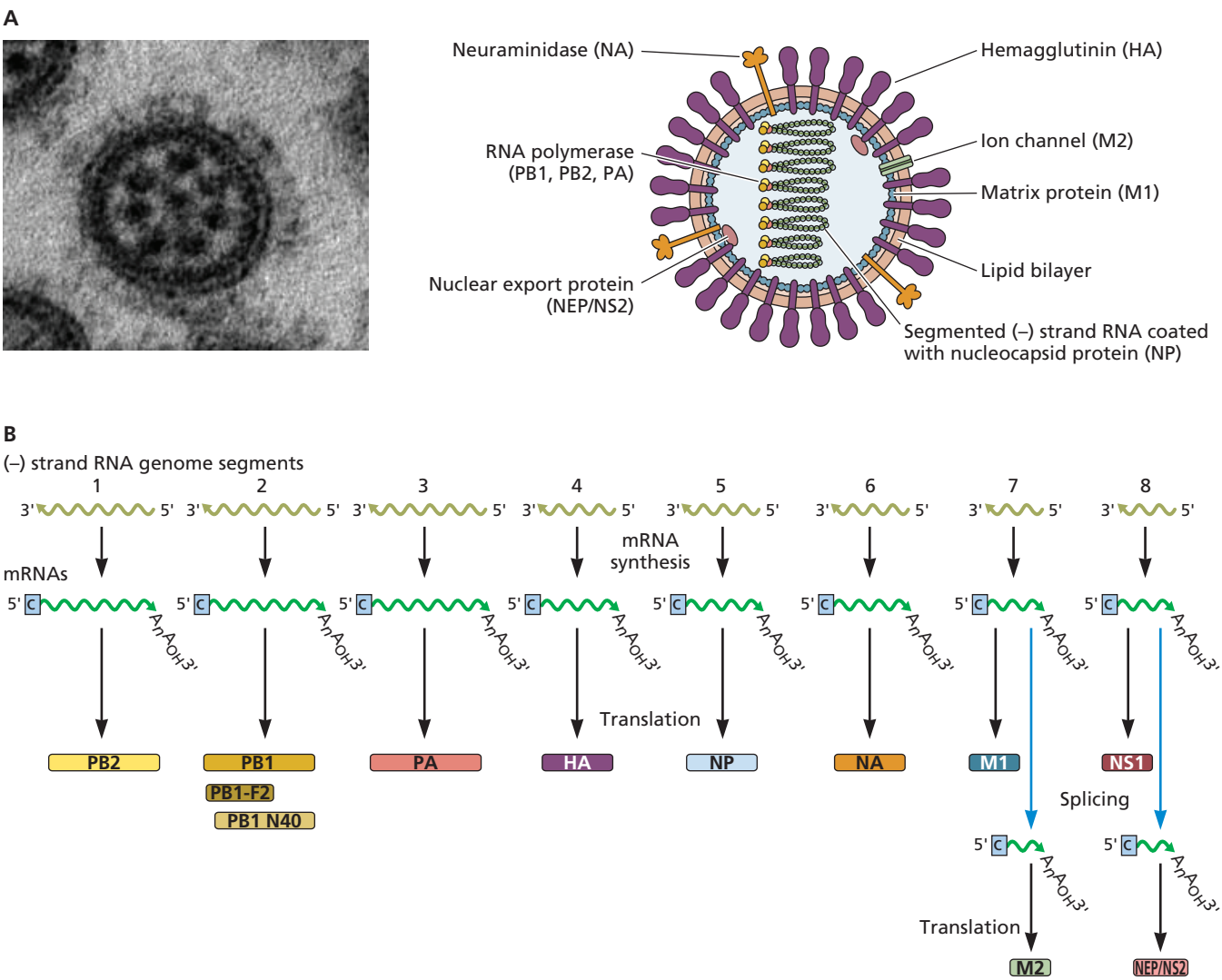
Orthomyxoviruses

Family *Orthomyxoviridae*

Selected Genera	Examples
Influenzavirus A	A/PR/8/34(H1N1)
Influenzavirus B	B/Lee/40
Influenzavirus C	C/California/78

Influenza viruses of humans are the causative agents of a highly contagious and often serious acute respiratory illness. They are unusual among RNA viruses in that all viral RNA synthesis occurs in the cell nucleus. Initiation of viral mRNA synthesis with a capped primer derived from host cell mRNA was first observed in cells infected with influenza viruses. The viral genomes undergo extensive reassortment when a host cell is infected with two distinct strains, and coding sequences are expressed via a remarkable panoply of unusual strategies, including RNA splicing, overlapping reading frames, and leaky scanning.

Figure 15 Structure and genomic organization of the orthomyxovirus influenza A virus. (A) Virion structure. Colorized negative stained transmission electron micrograph (TEM) of the influenza virus A/CA/4/09 (courtesy of CDC Public Health Image Library, ID#11214). **(B) Genome organization.** The (−) strand RNA genome comprises eight segments, each of which encodes at least one viral protein as shown. Some of the (+) strand mRNA of the smallest genomic RNA segments, 7 and 8, is spliced by host cell enzymes, allowing the production of two proteins from each. The NS (nonstructural) proteins were so named because they were thought initially not to be incorporated into virus particles. An accessory protein with proapoptotic activity, PB1-F2, is produced from the PB1 RNA by translation of an overlapping open reading frame. The PB1 N40 protein is translated from a third open reading frame in PB1 RNA.



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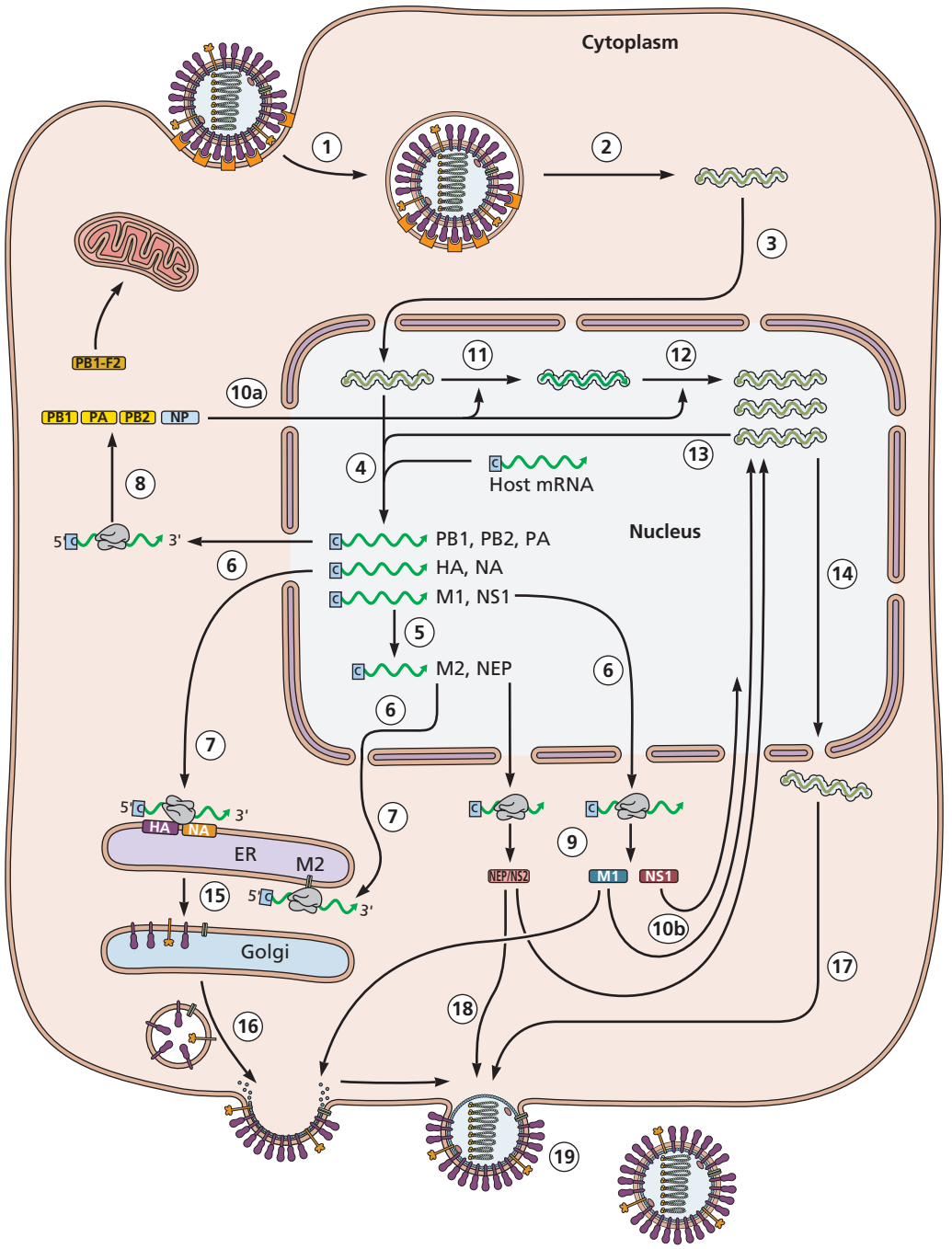


Figure 16 Single-cell reproductive cycle of influenza A virus. **(1)** The virion binds to a sialic acid-containing cellular surface protein or lipid and enters the cell via receptor-mediated endocytosis. **(2)** Upon acidification of the vesicle, the viral membrane fuses with the membrane of the vesicle, releasing the eight viral nucleocapsids into the cytoplasm (for simplicity, only one is shown). **(3)** The viral nucleocapsids containing (−) strand genomic RNA, multiple copies of the NP protein, and the P proteins are transported into the nucleus. **(4)** The (−) strand RNAs are copied by RNA polymerase entering with the virus particles into mRNAs, using the capped 5′ ends of host pre-mRNAs (or mRNAs) as primers to initiate synthesis. **(5)** Some of the mRNA encoding NS2/NEP and M2 is spliced, and **(6)** the mRNAs are transported to the cytoplasm. **(7)** The mRNAs specifying the viral membrane proteins (HA, NA, and M2) are translated by ribosomes bound to the endoplasmic reticulum (ER). These proteins enter the host cell's secretory pathway, where HA and NA are glycosylated. **(8, 9)** All other mRNAs are translated by ribosomes in the cytoplasm. **(10a)** The PA, PB1, PB2, and NP proteins are imported into the nucleus, where they participate in the synthesis of **(11)** full-length (+) strand RNAs and then of **(12)** (−) strand genomic RNAs, both of which are synthesized in the form of nucleocapsids. **(13)** Some of the newly synthesized (−) strand RNAs enter the pathway for mRNA synthesis. **(10b)** The M1 protein and the NS1 protein are transported into the nucleus. **(14)** Binding of the M1 protein to newly synthesized (−) strand RNAs shuts down viral mRNA synthesis and, in conjunction with the NS2/NEP protein, induces export of progeny nucleocapsids to the cytoplasm. **(15)** The HA, NA, and M2 proteins are transported to the cell surface and **(16)** become incorporated into the plasma membrane. **(17, 18)** The nucleocapsids associated with the M1 protein and the NS2/NEP protein are transported to the cell surface and interact with regions of the plasma membrane that contain the HA, NA, M1, and M2 proteins. **(19)** Assembly of virus particles is completed at this location by budding from the plasma membrane.

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Paramyxoviruses

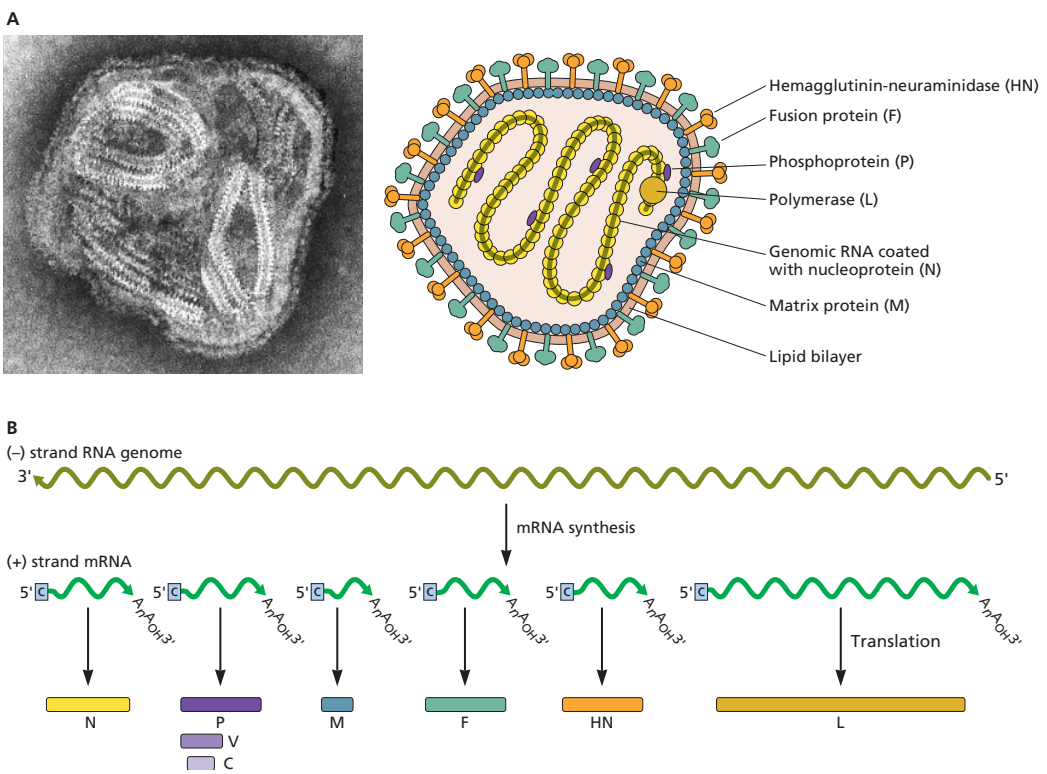
Family *Paramyxoviridae*

Selected Genera	Examples
Avulavirus	Newcastle disease virus
Henipavirus	Hendra virus, Nipah virus
Morbillivirus	Measles virus, rinderpest virus
Respirovirus	Sendai virus, human parainfluenza viruses 1 and 3
Rubulavirus	Mumps virus, human parainfluenza viruses 2 and 4

The *Paramyxoviridae* are a family within the order *Mononegavirales*. The members of this family are enveloped viruses with (−) single-stranded RNA genomes. The *Paramyxoviridae* comprise eight genera, which include human pathogens such as measles, mumps, and parainfluenza viruses.

Paramyxovirus particles have a diameter of approximately 150 nm, and are generally pleiomorphic but can be spherical or filamentous. The (−) strand RNA genome contains 6 to 10 genes arranged in the same relative order, and in the order in which the proteins are needed during the infectious cycle. A number of important human diseases are caused by paramyxoviruses, especially in infants and children. These comprise mumps and measles, as well as others that result in respiratory diseases, including pneumonia. Paramyxoviruses are also responsible for a range of diseases in other animal species, including dogs, seals, dolphins, birds, and cattle. Some, such as the henipaviruses, are zoonotic pathogens.

Figure 17 Virion structure and genome organization. (A) Virion structure. The cryo-electron micrograph shows a negatively stained paramyxovirus. The pleiomorphic particles are ~120 to 150 nm in diameter. (Courtesy of Linda Stannard/Science Photo Library, with permission.) The surface of the virus particle is studded with the attachment protein that binds to the cellular receptor. The nature of this attachment protein differs somewhat among the genera. For some, such as the morbilliviruses, this is the hemagglutinin (H). Others, such as those in the *Rubula-* and *Respirovirus* genera, possess both hemagglutination activity and the ability to cleave sialic acid (called hemagglutinin-neuraminidase [HN]). Finally, those attachment proteins that possess neither activity are simply called the glycoprotein (G), as for the henipaviruses. The RNP consists of the viral genome, which is 15 to 19 kb in length, wrapped around the virus-encoded nucleoprotein (N), the large (L) RNA-dependent RNA polymerase, and the accessory protein for RNA synthesis, the phosphoprotein (P). **(B) Genome structure.** While the number and names of the viral genes differs among the paramyxovirus genera, the order of these genes is constant. The viral RNA-dependent RNA polymerase initiates mRNA synthesis by binding to the encapsidated genome at the leader region, located at the 3′ end of the genome. RNA synthesis then proceeds as the L protein recognizes start and stop signals that flank each viral gene. After each gene is copied, the polymerase pauses to release the new mRNA and may either dissociate from the genome or go on to transcribe the next gene. If L dissociates, it must "begin again" at the 3′ leader sequence. As a result, sequentially less RNA is made for each gene as a factor of distance from the 3′ end. All viral mRNAs are capped and polyadenylated by the L protein during synthesis. Leaky scanning and mRNA editing result in the translation of two additional proteins, C and V, respectively, which are encoded in alternate reading frames within the P gene.



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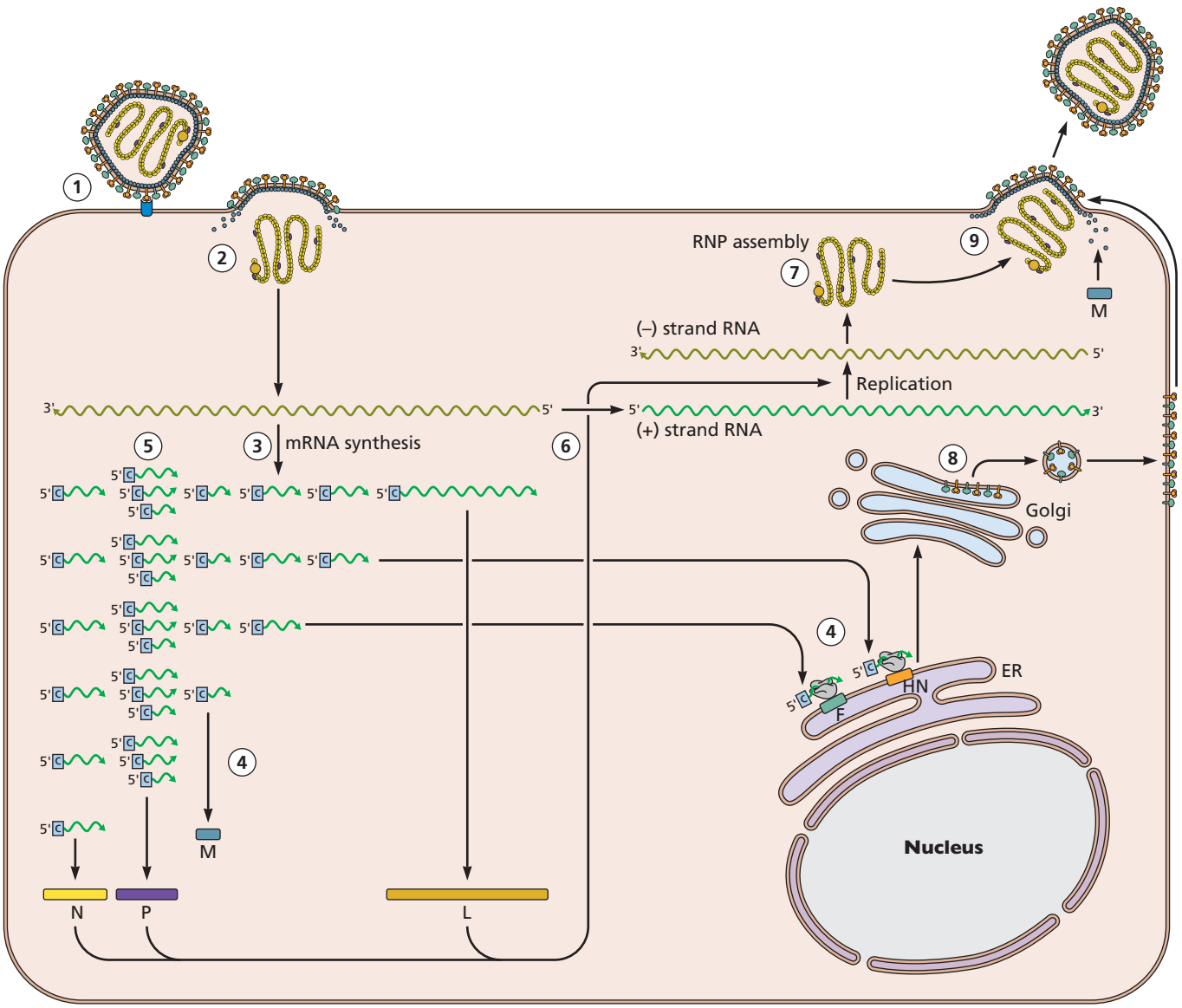


Figure 18 Single-cell reproductive cycle. **(1)** The virion attaches by binding to specific receptors on the surface of the cell. The identities of these receptors are known, and they vary among different paramyxoviruses; multiple receptors may be used by individual paramyxoviruses. **(2)** Upon binding of the virus to the receptor, the viral membrane fuses with the cellular membrane, releasing the single-stranded, negative-sense genome into the cytoplasm. **(3)** mRNAs are synthesized from this (−) strand RNA template. Separating each gene are transcription termination signals; consequently, the viral RNA-dependent RNA polymerase must re-engage with genomic RNA to continue transcription of the next downstream gene. As a result, a gradient of mRNA abundance is established, decreasing with each successive gene. **(4)** The capped mRNAs are translated. **(5)** Multiple proteins are made from the coding sequence of the P gene (Fig. 17B). **(6)** The N, P, and L proteins drive the replication of the incoming genome to produce a full-length (+) strand RNA, which then is the template for production of progeny viral genomes. **(7)** The ribonucleoprotein assembles in the cytoplasm when free N subunits associate with the genome to form a helical structure. **(8)** Viral glycoproteins are modified posttranslationally as they are transported through the endoplasmic reticulum and the Golgi network to the surface of the surface of the infected cell. **(9)** The RNP acquires its envelope at the cell surface as it buds through the plasma membrane; the viral M protein is thought to mediate association of the RNP with the viral glycoproteins.

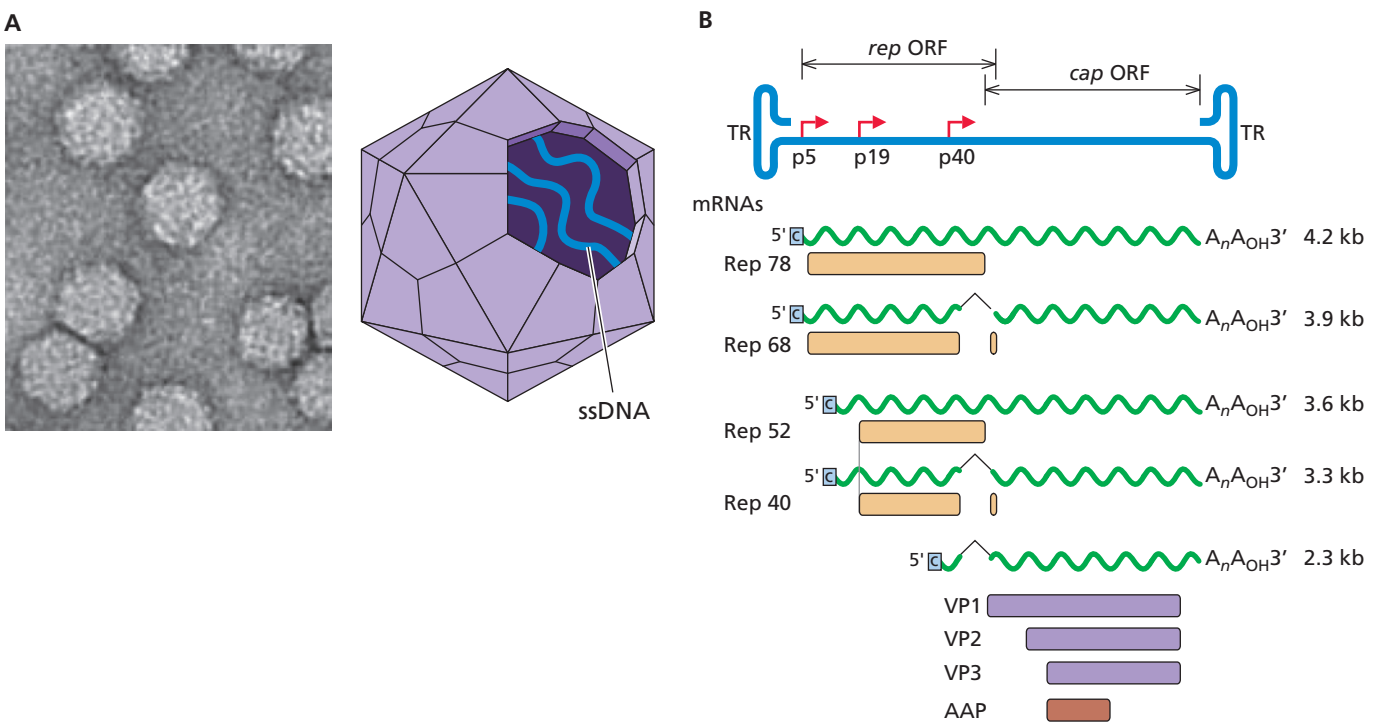
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Family *Parvoviridae*

Selected Genera	Examples
Parvovirus	Minute virus of mice
Erythrovirus	Human B19 virus
Dependovirus	Human adenovirus-associated viruses

Members of the family *Parvoviridae* are among the smallest of the animal viruses with DNA genomes. They are of particular interest because of the unique structure of their genomic DNA and its mechanism of replication. Most parvoviruses, such as the well-studied minute virus of mice, can reproduce autonomously, although they require the host cell to go through S phase in order to do so. Reproduction of dependoviruses requires a helper adenovirus or herpesvirus to induce S phase and to provide components that promote dependovirus gene expression and replication. These viruses can establish a latent infection during which their DNA is integrated into the host cell genome in an inactive state, to be activated upon subsequent infection with a helper. Because of their ability to persist and lack of pathogenicity, human adenovirus-associated viruses have been developed as vectors for gene therapy.

Figure 19 Structure and genome organization of adenovirus-associated virus (AAV). (A) Virion structure. The electron micrograph shows AAV4 (courtesy of Mavis Agbandje-McKenna, University of Florida, Gainesville, FL). Shown schematically to the right, the capsid comprises 60 protein subunits, primarily (~90%) VP3, which contains the same sequences as the C termini of VP1 and VP2. Virus particles contain either (+) or (−) single-stranded DNA. **(B) Genome organization.** The best-characterized DNA genome, that of AAV2, comprises ca. 4,600 nucleotides and includes terminal repeats (TR) of 145 nucleotides, the first 125 of which contain palindromic sequences. The TR is required in *cis* for genome replication, transcription, and encapsidation, and plays a role in integration into the host DNA during establishment of a latent infection. Use of multiple initiation codons and alternative splicing results in synthesis of multiple Rep (tan bars) and structural proteins (purple bars), respectively. ORF, open reading frame. (Adapted from R. M. Linden and K. Berns, p. 68–84, *in* S. Faisst and J. Rommelaere [ed.], *Contributions to Microbiology,* vol. 4, *Parvoviruses: from Molecular Biology to Pathology and Therapeutic Uses* [S. Karger, Basel, Switzerland, 2000] with permission.)



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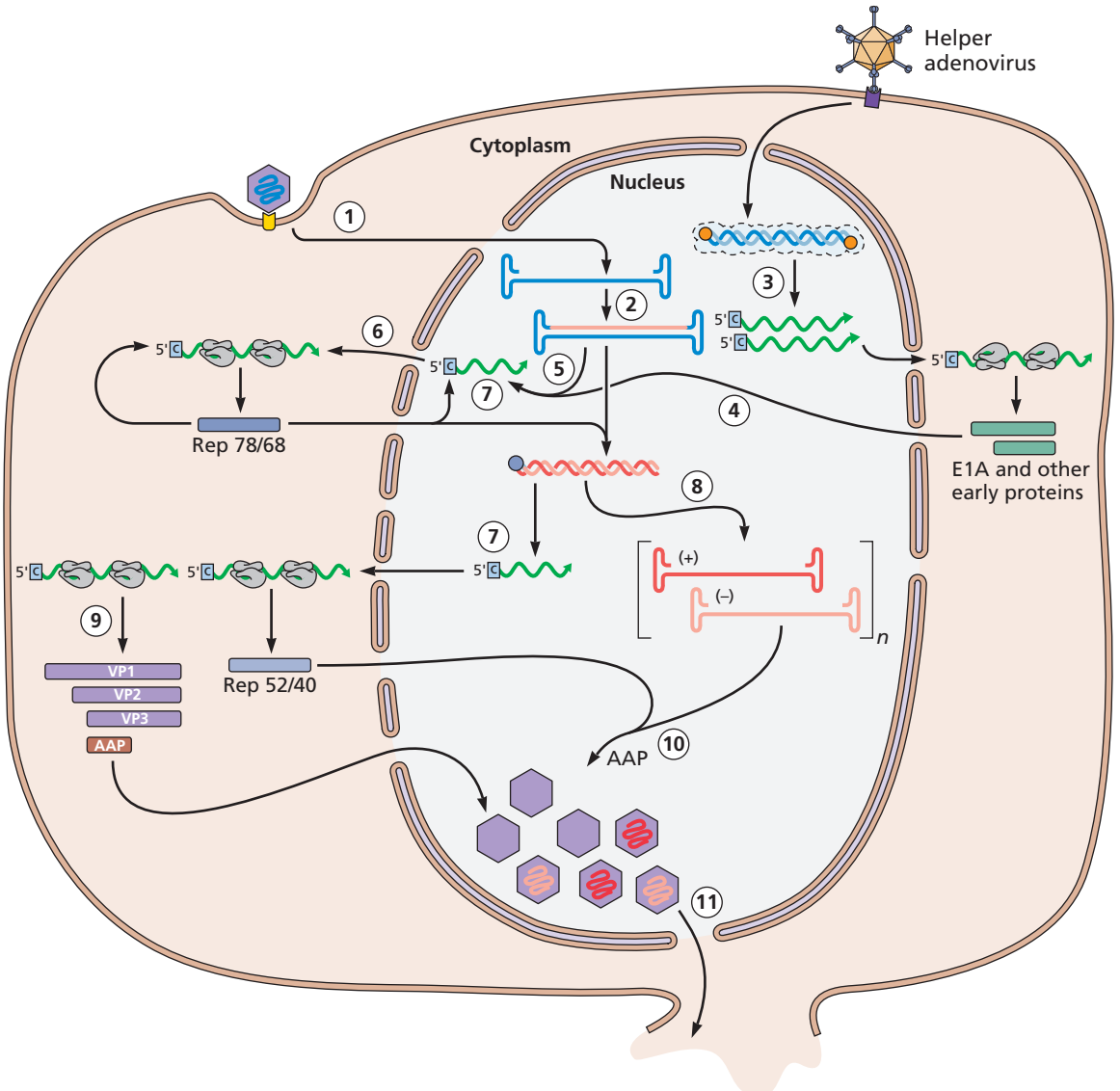


Figure 20 Single-cell reproductive cycle. Heparan sulfate proteoglycans are the primary cell surface receptors for AAV2. **(1)** However, the processes of adsorption, uncoating, and entry of the DNA into the nucleus are poorly understood for all *Parvoviridae*. **(2)** Cellular replication proteins convert the single-stranded viral DNA genome into a largely double-stranded molecule. **(3)** Upon coinfection with a helper virus, AAV undergoes a productive infection. With an adenovirus helper, this response is dependent on the expression of early genes E1A, E1B, E4, and E2A, which induce S phase and the concomitant production of cellular DNA replication proteins needed for viral DNA synthesis. **(4)** The adenovirus EIA transcriptional activator also induces transcription from the p5 promoter, **(5, 6)** leading to the production of Rep78/68 mRNA and proteins. **(7)** These proteins then function as powerful transcriptional activators (rather than repressors as in latency) and induce transcription from both the p5 and p19 promoters. **(8)** Viral DNA is replicated by a single-strand displacement mechanism that is initiated by recognition of the terminal resolution site (*trs*) by the Rep78/68 proteins, which remain linked covalently to the DNA through subsequent steps of DNA synthesis. A very large number of replicating forms (ca. 106 double-stranded genomes/cell) can be produced within a short time. **(9)** The capsid proteins produced in the cytoplasm self-associate in the nucleus during assembly of progeny particles. **(10)** Newly synthesized viral genomes are then encapsidated. The (+) or (−) strand genomes are encapsidated in equal numbers in progeny virus particles. **(11)** As with the adenovirus helper, progeny virus particles are released, usually upon destruction of the cell.

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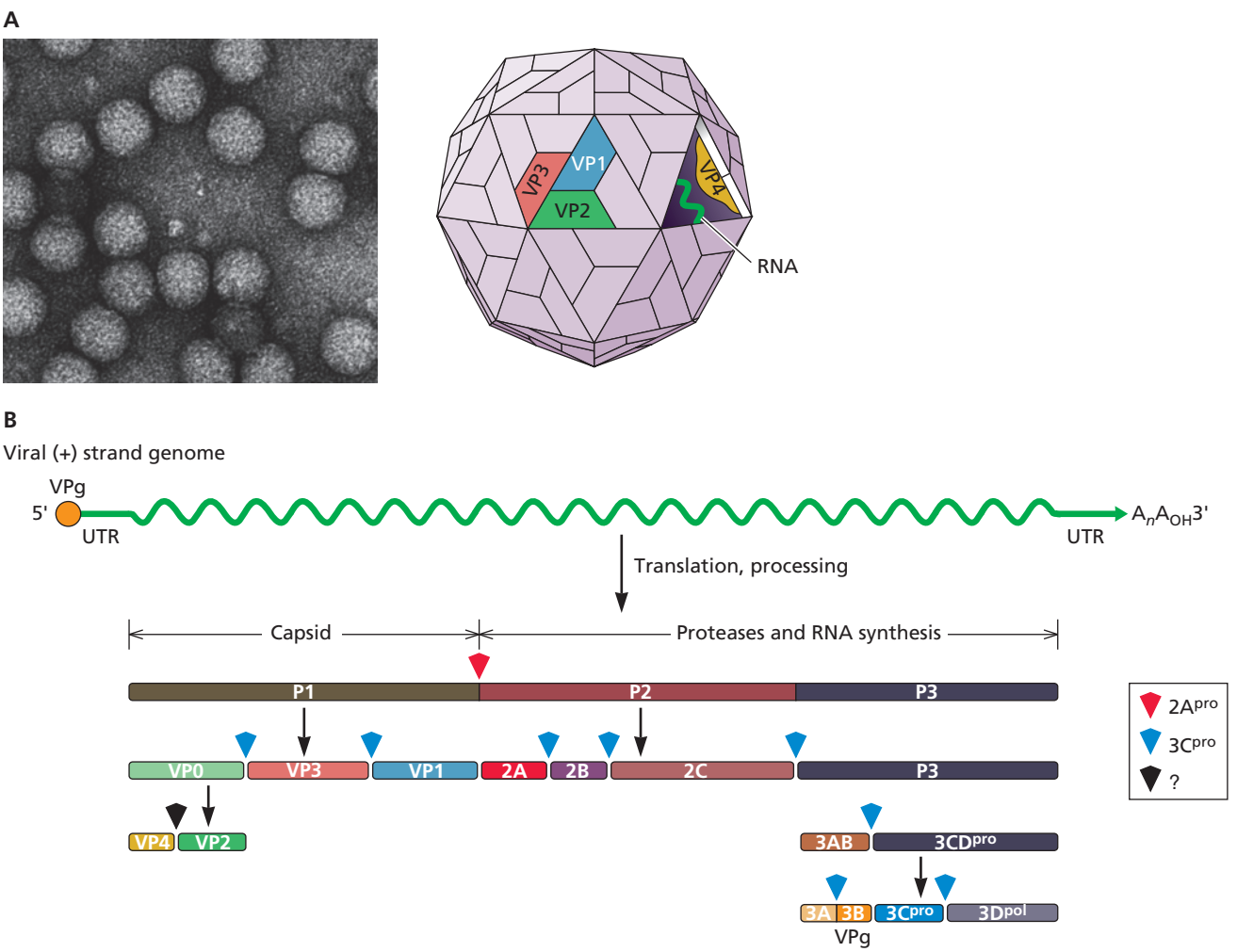
Picornaviruses

Family *Picornaviridae*

Selected Genera	Examples
Enterovirus	Poliovirus
Rhinovirus	Human rhinovirus A
Cardiovirus	Encephalomyocarditis virus
Aphthovirus	Foot-and-mouth disease virus
Hepatovirus	Hepatitis A virus

The family *Picornaviridae* includes many important human and animal pathogens. Because they cause serious disease, poliovirus and foot-and-mouth disease virus are the best-studied picornaviruses. These two viruses have had important roles in the development of virology. The first animal virus discovered, in 1898, was foot-and-mouth disease virus. The plaque assay was developed using poliovirus, and the first RNA-dependent RNA polymerase identified was poliovirus 3Dpol. Polyprotein synthesis was discovered in experiments with poliovirus-infected cells, as was translation by internal ribosome entry. The first infectious DNA clone of an animal RNA virus was that of the poliovirus genome, and the first three-dimensional structures of animal viruses determined by X-ray crystallography were those of poliovirus and rhinovirus.

Figure 21 Structure and genomic organization. (A) Virion structure. The electron micrograph shows negatively stained poliovirus. The capsid consists of 60 structural units (each made up of a single copy of VP1, VP2, VP3, and VP4, colored blue, green, red, and yellow, respectively) arranged in 12 pentamers. One of the icosahedral faces has been removed in the diagram to illustrate the locations of VP4 and the viral RNA. (Courtesy of N. Cheng and D. M. Belnap, National Institutes of Health, Bethesda, MD.) **(B) Genome organization.** Polioviral RNA is shown with the VPg protein covalently attached to the 5′ end. The genome is of (+) polarity and encodes a polyprotein precursor. The polyprotein is cleaved during translation by two virus-encoded proteases, $2A^{pro}$ and $3C^{pro}$, to produce structural and nonstructural proteins, as indicated.



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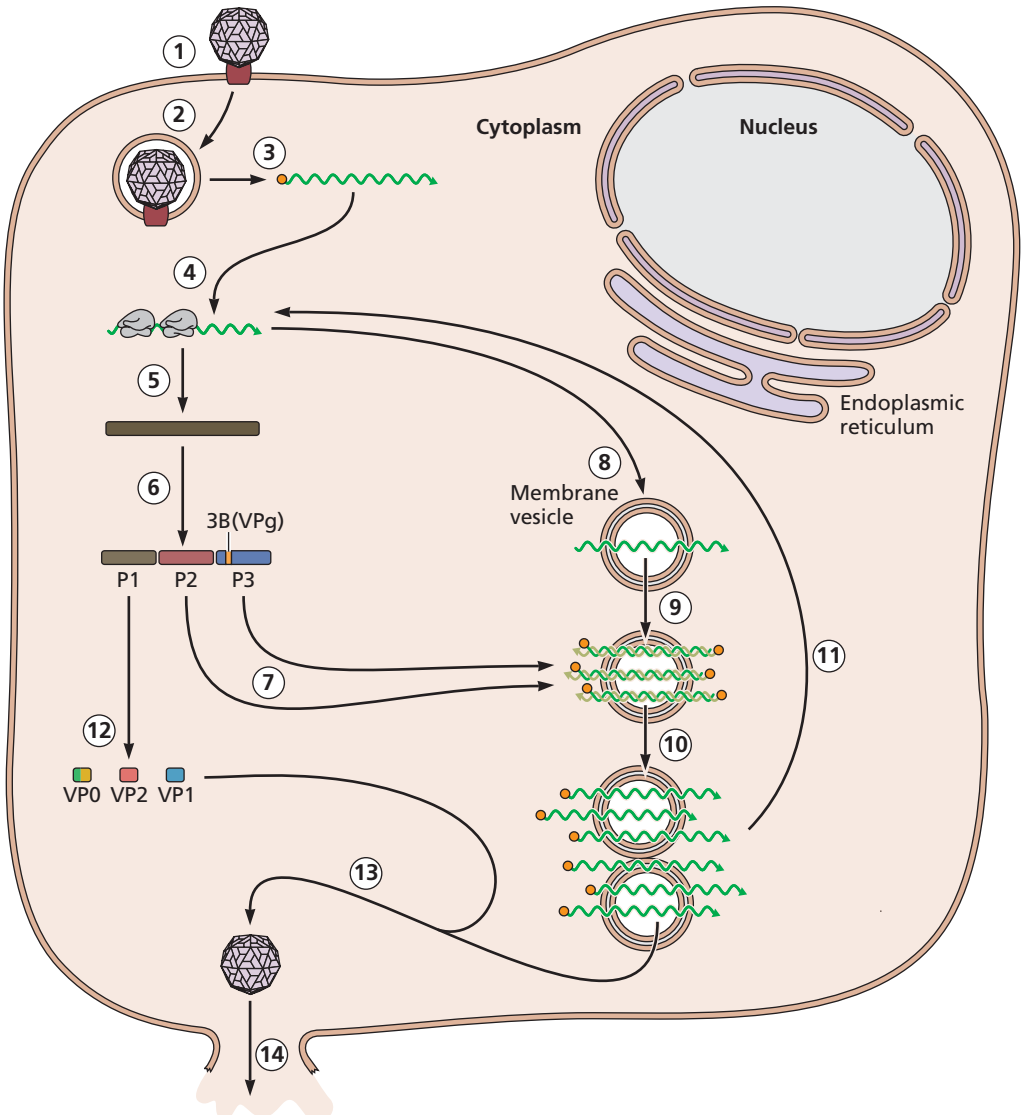


Figure 22 Single-cell reproductive cycle. **(1)** The virion binds to a cellular receptor and **(2)** enters an endosome. **(3)** Release of the poliovirus genome occurs from within early endosomes located close to the plasma membrane (within 100 to 200 nm). **(4)** The VPg protein, depicted as a small orange circle at the 5′ end of the virion RNA, is removed, and the RNA associates with ribosomes. **(5)** Translation is initiated at an internal site 741 nucleotides from the 5′ end of the viral mRNA, and a polyprotein precursor is synthesized. **(6)** The polyprotein is cleaved during and after its synthesis to yield the individual viral proteins. Only the initial cleavages are shown here. **(7)** The proteins that participate in viral RNA synthesis are transported to membrane vesicles. RNA synthesis occurs on the surfaces of these infected-cell-specific membrane vesicles. **(8)** The (+) strand RNA is transported to these membrane vesicles, **(9)** where it is copied into double-stranded RNAs. **(10)** Newly synthesized (−) strands serve as templates for the synthesis of (+) strand genomic RNAs. **(11)** Some of the newly synthesized (+) strand RNA molecules are translated after the removal of VPg. **(12)** Structural proteins are formed by partial cleavage of the P1 precursor and **(13)** associate with (+) strand RNA molecules that retain VPg to form progeny virus particles, **(14)** which are released from the cell upon lysis.

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Polyomaviruses

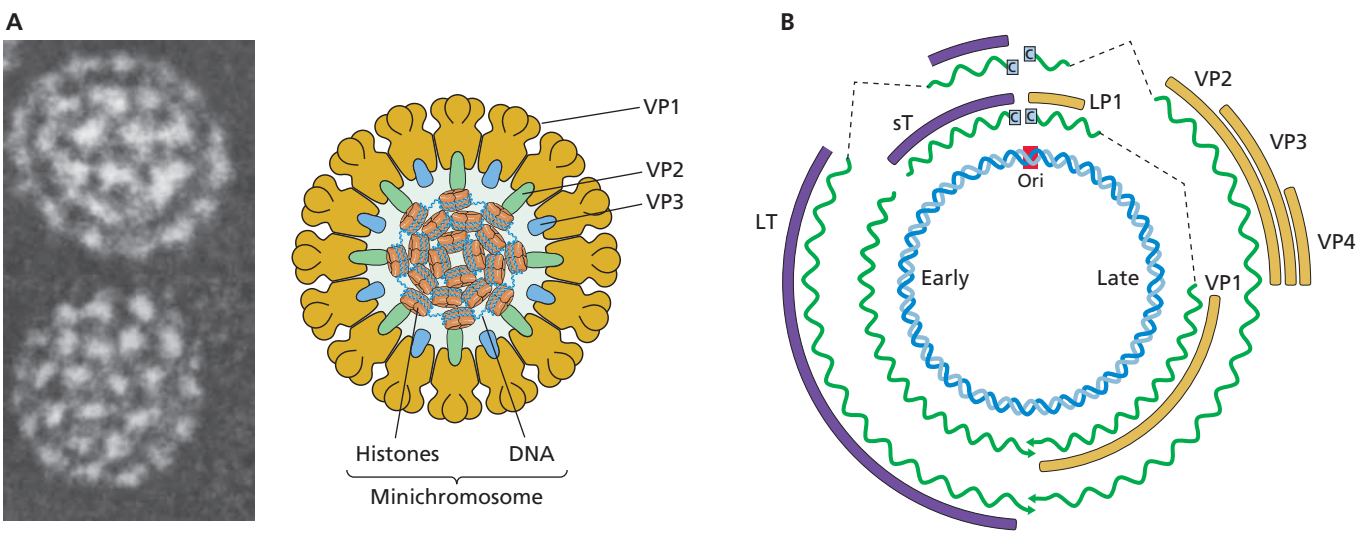
Family *Polyomaviridae*

Genera	Examples
Orthopolyomavirus	Simian virus 40
Wukipolyomavirus	KI virus

The family *Polyomaviridae* includes mouse polyomaviruses, simian virus 40, and the human JC and BK viruses, which are orthopolyomaviruses isolated from a patient with progressive multifocal leukoencephalopathy and an immunosuppressed recipient of a kidney transplant, respectively. The genus *Wukipolyomavirus* includes the human Karolinska Institute (KI) polyomavirus and human polyomaviruses 6 and 7, among others.

Under some conditions, mouse polyomavirus infection of the natural host results in formation of a wide variety of tumors (hence the name). A characteristic property of the members of this family is an ability to transform cultured cells or to induce tumors in animals. Investigation of such transforming activity has provided much information about mechanisms of oncogenesis, including the discovery of the cellular tumor suppressor protein p53. These viruses, particularly simian virus 40, have also been important in elucidation of cellular mechanisms of transcription and its regulation and characterization of the mammalian DNA synthesis machinery.

Figure 23 Structure and genome organization. (A) Virion structure. The electron micrograph shows negatively stained simian virus 40 particles (from F. A. Andered et al., *Virology* **32:**511–523, 1967, with permission). As shown on the right, the double-stranded DNA genome is organized into approximately 25 nucleosomes by the cellular core histones. One molecule of either VP2 or VP3, which possess a common C-terminal sequence, is associated with each VP1 pentamer. **(B) Genome organization.** The 5,243-bp simian virus 40 genome is shown, with locations of the origin of viral DNA synthesis (Ori) and of the early and late mRNAs indicated. The late mRNA species generally contain additional open reading frames in their 5′-terminal exons, such as that encoding the agnoprotein (LP1). The structural proteins VP2, VP3, and VP4 are encoded within the same open reading frame.



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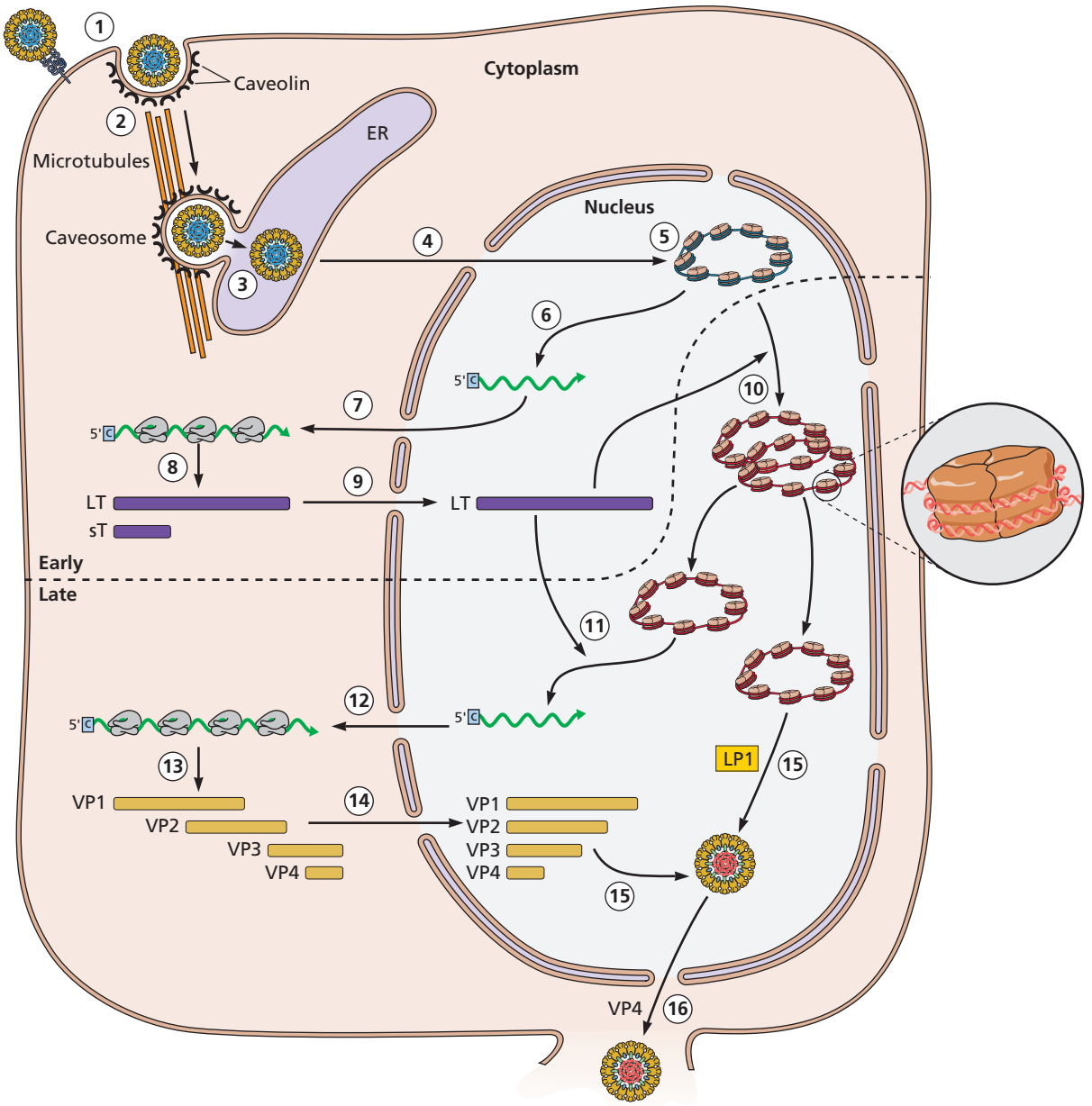


Figure 24 Single-cell reproductive cycle of simian virus 40. **(1)** The virus particle attaches to susceptible monkey cells upon binding of VP1 to the ganglioside Gm1 (a glycolipid) on the surface. **(2)** The particle is then endocytosed in caveolae, transported to the endoplasmic reticulum, and **(3)** enters that organelle. **(4)** Subsequently, it is transported to the nucleus and uncoated by unknown mechanisms. **(5)** The viral genome packaged by cellular nucleosomes is found within the nucleus. **(6)** The early transcription unit is transcribed by host cell RNA polymerase II. **(7)** After alternative splicing and export to the cytoplasm, **(8)** the early mRNAs are translated to produce the early proteins LT and sT. **(9)** The former is imported into the nucleus, **(10)** where it binds to the origin of replication to initiate DNA synthesis. Apart from LT, all components needed for viral DNA replication are provided by the host cell. As they are synthesized, daughter viral DNA molecules associate with cellular nucleosomes to form the viral nucleoproteins often called minichromosomes. **(11)** LT also stimulates transcription of the late gene from replicated viral DNA templates. **(12)** Processed late mRNAs are exported to the cytoplasm and **(13)** translated to produce the structural proteins VP1, VP2, and VP3, as well as VP4. **(14)** The structural proteins are imported into the nucleus and **(15)** assemble around viral minichromosomes to form virus particles. **(16)** Release of progeny virus particles is facilitated by VP4.

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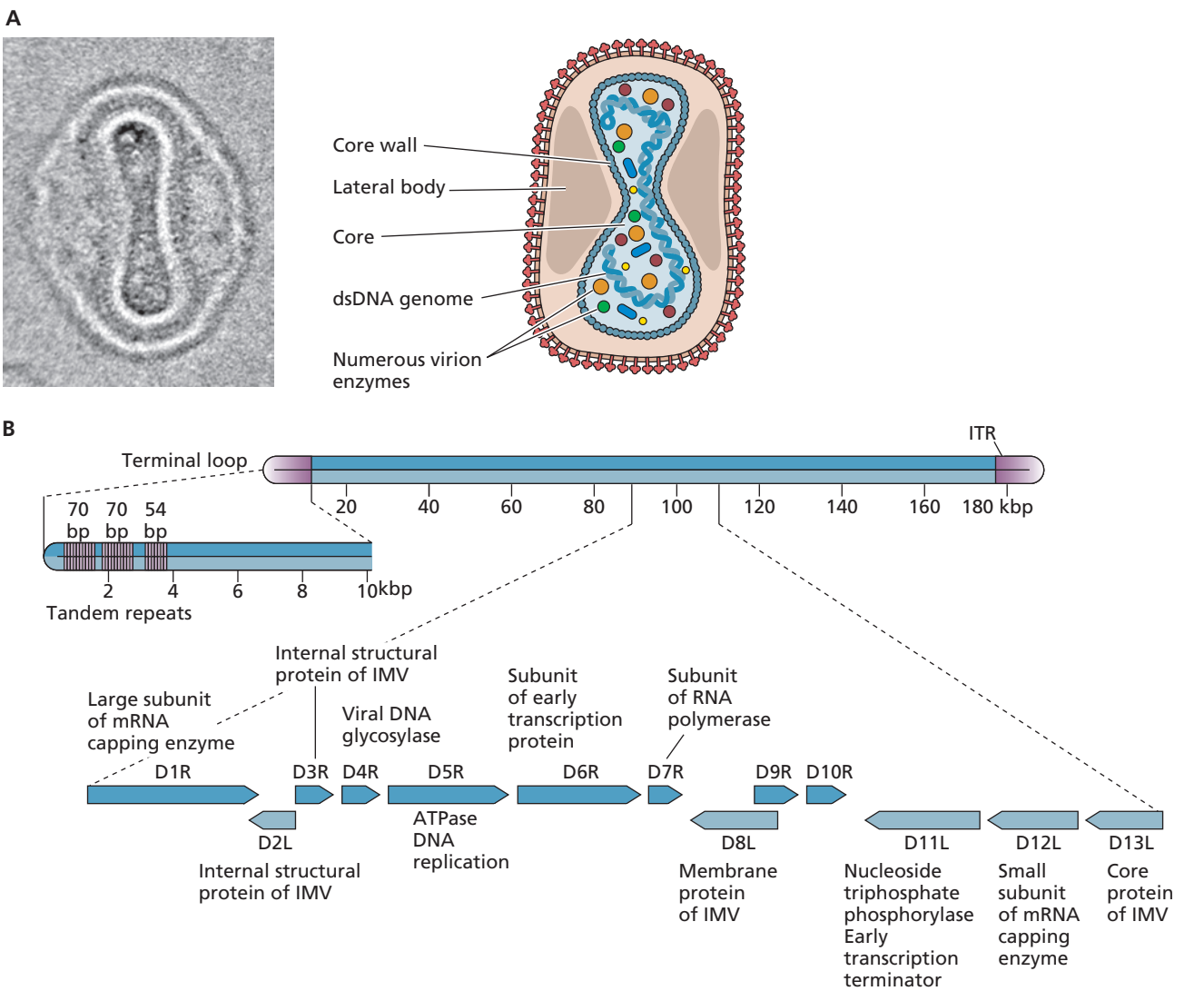
Poxviruses

Family *Poxviridae*

Selected Genera	Examples
Orthopoxvirus	Vaccinia virus
Avipoxvirus	Fowlpox virus
Leporipoxvirus	Myxoma virus
Yabapoxvirus	Yaba monkey tumor virus

Poxviruses infect most vertebrates and invertebrates, causing a variety of diseases of veterinary and medical importance. The best-known poxviral disease is smallpox, a devastating human disease that has been eradicated by vaccination. The origins of modern vaccinia virus, the virus used in smallpox virus vaccines, are obscure, but this virus is widely studied in the laboratory as a model poxvirus. Myxoma virus, which causes an important disease of domestic rabbits, was described in 1896. Rabbit fibroma virus, which was first described by Shope in 1932, was the first virus proven to cause tissue hyperplasia (warts). The genomes of poxviruses are large DNA molecules that include genes for all proteins needed for DNA synthesis and production of viral mRNAs. These viruses replicate in the cytoplasm and are minimally dependent on the host cell.

Figure 25 Structure and genome organization of the poxvirus vaccinia virus. (A) Virion structure. The electron micrograph shows the mature virion in cross section (courtesy of David J. Vaux, Sir William Dunn School of Pathology, Oxford University, Oxford, United Kingdom). **(B) Genome organization.** Shown are details for the 191-kb genome of the Copenhagen strain of vaccinia virus, with open reading frames identified in a small section of the genome. The two strands of the DNA genome are covalently connected by terminal, unpaired loops at the ends of inverted terminal repeated sequences (ITR). The genome includes ~185 unique protein-coding sequences. Those that encode structural proteins and essential enzymes are clustered in the center; those that affect virulence, host range, or immunomodulation are predominantly near the ends.



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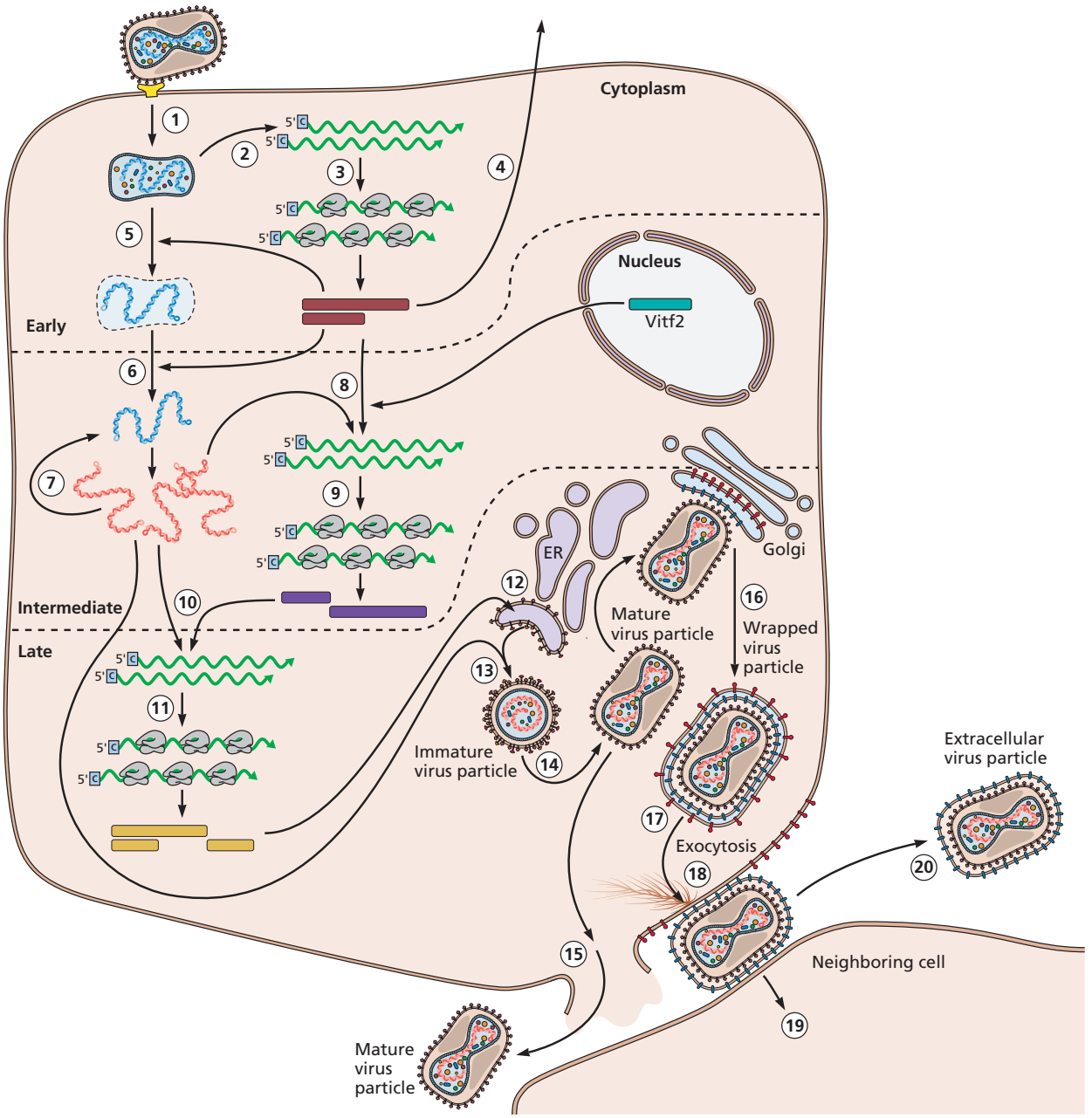


Figure 26 Single-cell reproductive cycle of vaccinia virus. **(1)** After receptor binding and fusion of viral and plasma membranes, or fusion following endocytosis, the viral core is released into the cytoplasm. **(2)** Early mRNAs are synthesized by the viral RNA polymerase aided by initiation proteins that enter the cell with virus particles. **(3)** These mRNAs are translated by the cellular protein-synthesizing machinery. **(4)** Some early proteins, which have sequence similarity to cellular growth factors and can induce proliferation of neighboring host cells, are secreted. Other early proteins counteract host immune defense mechanisms. **(5)** Some early viral proteins induce a second uncoating reaction in which the viral genome is released from the core in a nucleoprotein complex, and **(6)** others mediate replication of the genome. Newly synthesized viral DNA molecules can serve as templates **(7)** for additional cycles of genome replication and **(8)** for transcription of viral intermediate genes. Transcription of intermediate genes requires viral initiation proteins, which are products of early genes, and a cellular protein (Vitf2), which relocates from the infected cell nucleus to the cytoplasm. **(9)** Proteins made upon translation of intermediate mRNAs include those necessary for **(10)** transcription of late genes. **(11)** Late mRNAs are translated to produce viral structural proteins, enzymes, and other essential proteins that are needed early in subsequent infections and must be incorporated into virus particles during assembly. **(12)** Assembly of progeny particles begins in specialized sites, termed viral factories, that form upon viral DNA synthesis. These sites contain cellular membranes, probably derived from the endoplasmic reticulum, which are initially reorganized by specific viral protein to form crescents, **(13)** the precursor to spherical DNA-containing particles, called immature viral particles. **(14)** These particles then mature into brick-shaped intracellular mature virus particles upon proteolysis and release from viral factories. **(15)** Such particles are released only upon cell lysis. **(16)** However, they can acquire a second, double membrane from a *trans*-Golgi or early endosomal compartment to form intracellular wrapped virus particles. **(17)** The latter particles move to the cell surface on microtubules where **(18)** fusion with the plasma membrane forms cell-associated particles **(19)** that induce actin polymerization for direct transfer to surrounding cells or **(20)** that dissociate from the membrane as the extracellular virus particle. Association of the extracellular virus particle with a host cell in the next cycle of infection is thought to result in rupture of the outer membrane, giving rise to the mature virus particle.

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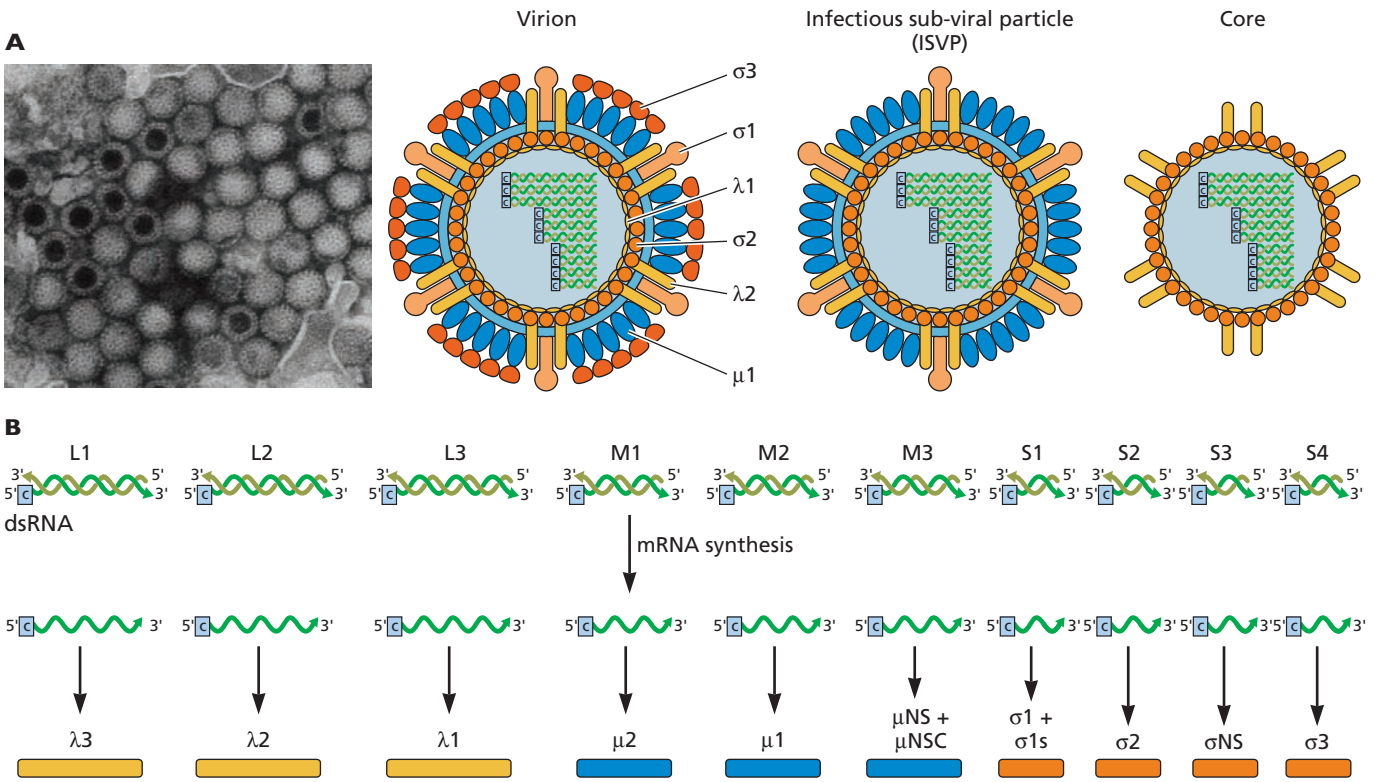
Reoviruses

Family *Reoviridae*

Selected Genera	Examples
Subfamily *Spinareovirinae*	
Orthoreovirus	Mammalian orthoreovirus
Coltivirus	Colorado tick fever virus
Subfamily *Sedoreovirinae*	
Orbivirus	Bluetongue virus
Rotavirus	Rotavirus A

Reoviridae is one of nine families of viruses with double-stranded RNA genomes. Included in this family are the human pathogens rotaviruses and Colorado tick fever virus. Reoviruses are the best studied of all the double-stranded RNA viruses. Some of the first *in vitro* research on RNA synthesis was done using reoviruses, and the 5′-terminal cap structure of mRNA was discovered in studies of reovirus mRNAs.

Figure 27 Structure and genomic organization of an orthoreovirus. (A) Virion structure. Electron micrograph of negatively stained reovirus particles (courtesy of S. McNulty, Queen's University, Belfast, United Kingdom). The locations of six virion proteins are indicated on the illustration to the right. **(B) Genome organization.** The double-stranded genome comprises 10 segments, named according to size: large (L), medium (M), and small (S). The S1 RNA encodes two proteins: σ1s protein is translated from a second initiation codon in a different reading frame from σ1. Two proteins are also produced from the M3 RNA: protein μNSC is produced by translation at a second initiation codon in the same reading frame as μNS.



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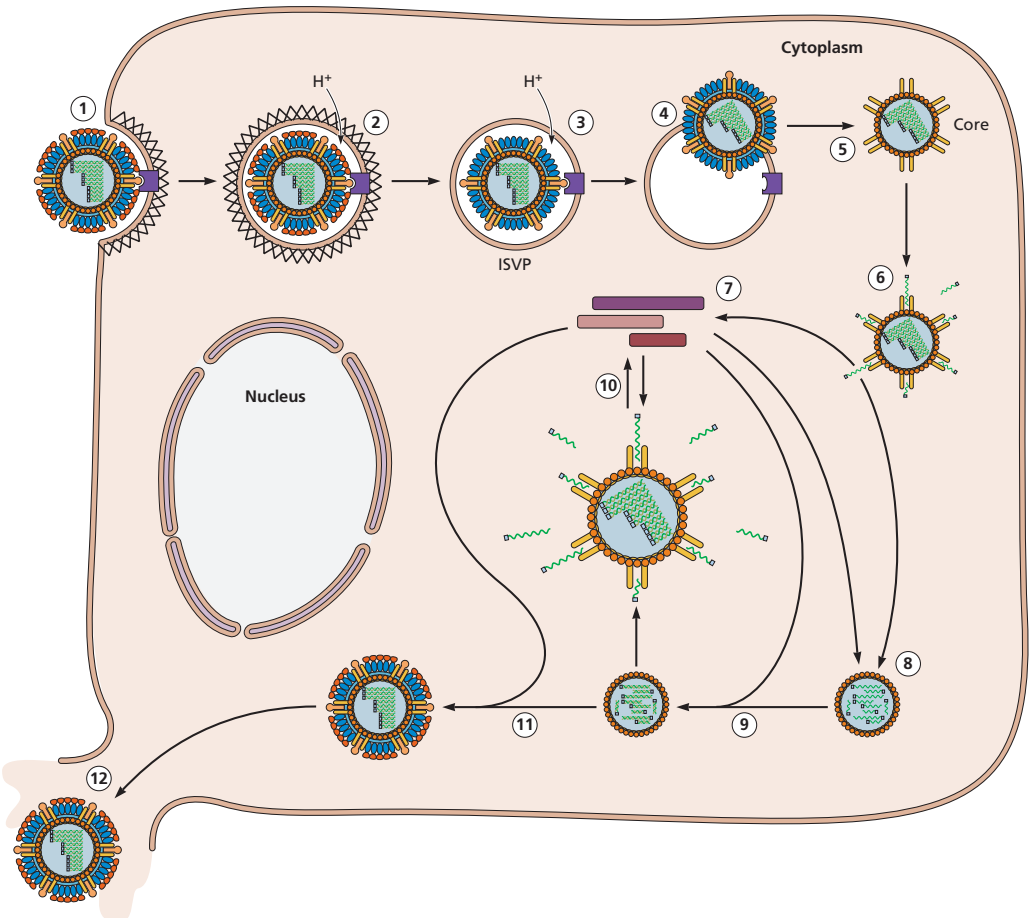


Figure 28 Single-cell reproductive cycle of orthoreovirus. **(1)** The virion binds to the cellular receptor and **(2)** enters the cell via receptor-mediated endocytosis. **(3)** In endosomes and lysosomes, the virion undergoes acid-dependent proteolytic cleavage to form the ISVP, **(4)** which penetrates the endosomal membrane, **(5)** releasing the core into the cytoplasm. **(6)** Synthesis of 10 capped viral mRNAs begins within the core particle. **(7)** These mRNAs are translated and associate with newly synthesized viral proteins **(8)** to form RNase-sensitive subviral particles in which reassortment may occur. **(9)** Each of the 10 mRNAs is a template for (−) strand RNA synthesis, leading to the production of an RNase-resistant subviral particle that contains 10 double-stranded RNAs. **(10)** Viral mRNAs produced within subviral particles are used for the synthesis of viral proteins and the assembly of additional virus particles. **(11)** In the final steps of capsid assembly, preformed complexes of outer capsid proteins are added to subviral particles. **(12)** Mature virus particles are released from the cell by lysis.

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Retroviruses

Family *Retroviridae*

Genera	Examples
Alpharetrovirus	Avian leukosis virus
Betaretrovirus	Mouse mammary tumor virus
Gammaretrovirus	Murine leukemia virus
Deltaretrovirus	Human T cell lymphotropic virus
Epsilonretrovirus	Walleye dermal sarcoma virus
Spumavirus	Chimpanzee foamy virus
Lentivirus	Human immunodeficiency virus type 1

Retrovirus particles contain the enzyme reverse transcriptase, which mediates synthesis of a double-stranded DNA copy of the viral RNA genome. Although once thought to be unique to this family, similar enzymes are now known to be encoded in other viral genomes (i.e., hepadnaviruses and caulimoviruses), and the term **retroid viruses** has been coined to include these families.

Retrovirus particles contain a second enzyme, integrase, that catalyzes the insertion of the viral DNA into many sites in host DNA. The retroviruses can be propagated as integrated elements (called proviruses) that are transmitted in the germ line or as exogenous infectious agents. Alpha- and gammaretroviruses have **simple** genomes that encode only the three genes common to all retroviruses—*gag*, *pol*, and *env*. All of the others have more **complex** genomes, which include auxiliary or accessory genes that encode nonstructural proteins that affect viral gene expression and/or pathogenesis. Five genera, *Alpha-*, *Beta-*, *Gamma-*, *Delta-*, and *Epsilonviruses*, comprising the subfamily *Orthoretroviruses*, cause cancer in their host organisms. The spumaviruses are nonpathogenic, while the lentiviruses are serious pathogens which target cells of the immune system in a number of species, including humans. The lentivirus human immunodeficiency virus type 1 is the cause of the AIDS pandemic.

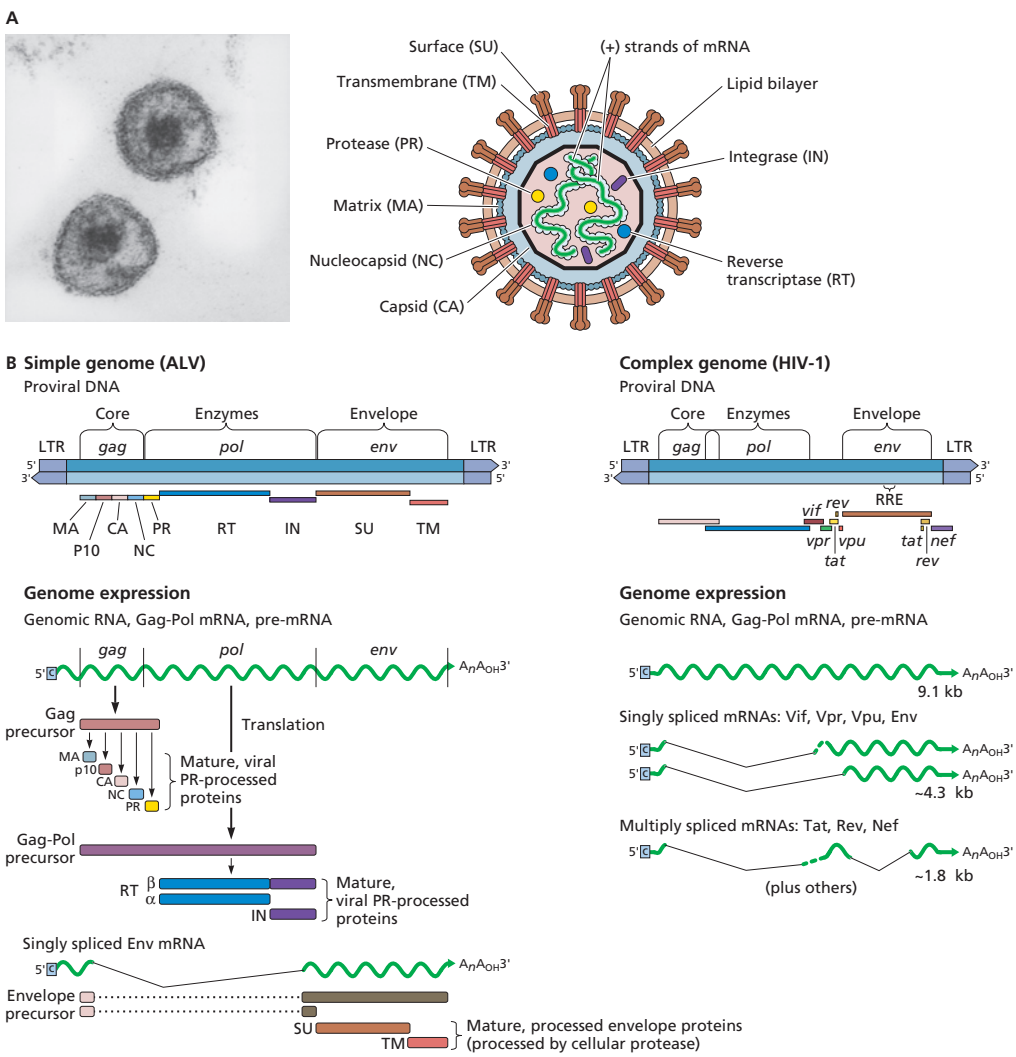


Figure 29 Structure and genomic organization. (A) Virion structure. The electron micrograph shows a negatively stained alpharetrovirus, Rous sarcoma virus (courtesy of R. Katz and T. Gales, Fox Chase Cancer Center, Philadelphia, PA). Envelope protein projections are not visible in this image. **(B) Genome organization.** (Left) A retrovirus with a simple genome (avian leukosis virus [ALV]). Proviral genes are located in different reading frames (indicated by different horizontal positions below the DNA) and are also overlapping. Colored boxes delineate open reading frames. LTR, long terminal repeats that include transcription signals. Origins of RNA and protein products are shown below. (Right) A retrovirus with a more complex genome illustrated with the lentivirus human immunodeficiency virus type 1 (HIV-1). Proviral genes are located in all three reading frames, as indicated by the overlaps. Human immunodeficiency virus type 1 mRNAs fall into one of three classes. The first type is an unspliced transcript of 9.1 kb, identical in function to that synthesized from the simple retrovirus genome shown at the left. The second type comprises singly spliced mRNAs (average length, 4.3 kb) that result from splicing from a 5′ splice site upstream of *gag* to any one of a number of 3′ splice sites near the center of the genome. One of these mRNAs specifies the Env polyprotein precursor, as illustrated for the singly spliced mRNA of the retrovirus with a simple genome. The others specify the human immunodeficiency virus type 1 accessory proteins. The third type comprises a complex class of mRNAs (average length, 1.8 kb) derived by multiple splicing from 5′ and 3′ splice sites throughout the genome. They include mRNAs that specify the regulatory proteins Tat and Rev and are the first to accumulate after infection.

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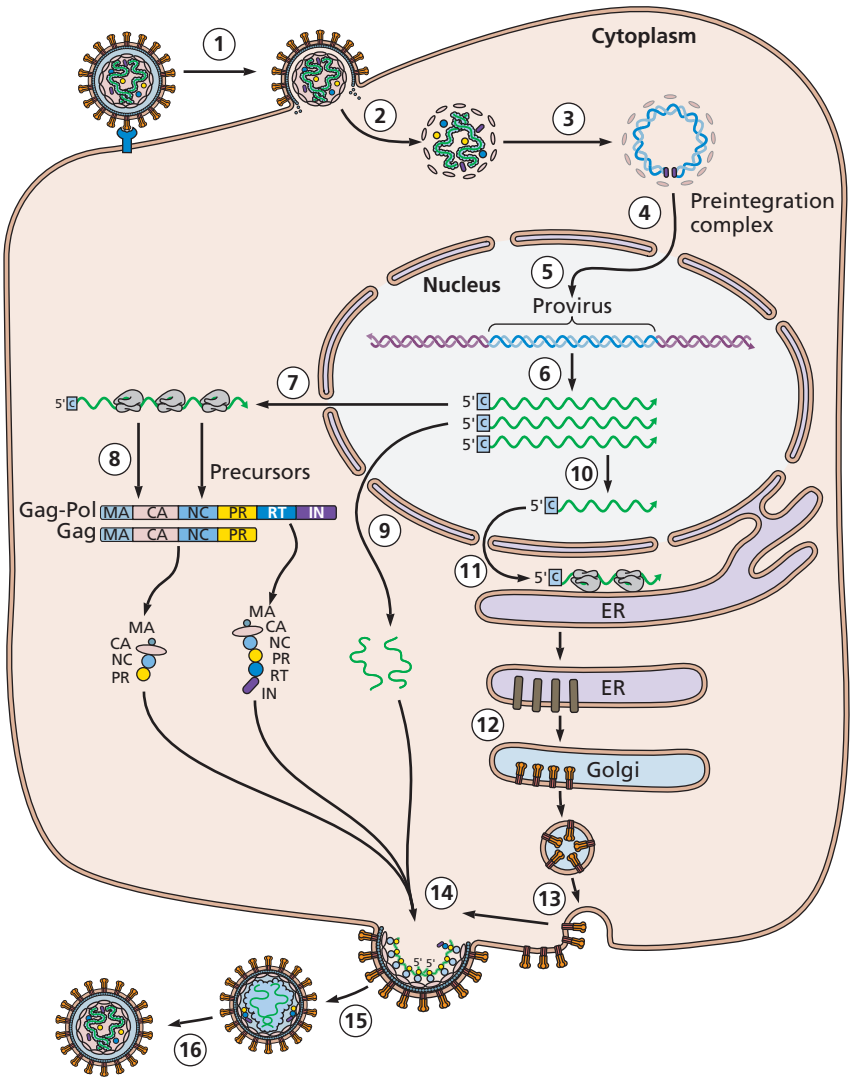


Figure 30 Single-cell reproductive cycle of a retrovirus with a simple genome. **(1)** The virus attaches by binding of the viral envelope protein to specific receptors on the surface of the cell. The identities of receptors are known for many retroviruses. **(2)** The viral core is deposited into the cytoplasm following fusion of the virion and cell membranes. Entry of some beta- and gammaretroviruses may occur via the endocytic pathways. **(3)** The viral RNA genome is reverse transcribed by the virion reverse transcriptase (RT) within a subviral particle. The product is a linear double-stranded viral DNA with ends that are shown juxtaposed in preparation for integration. **(4)** Viral DNA and integrase (IN) protein gain access to the nucleus with the help of intracellular trafficking machinery or, in some cases, by exploiting nuclear disassembly during mitosis. **(5)** Integrative recombination, catalyzed by IN, results in site-specific insertion of the viral DNA ends, which can take place at many locations in the host genome, with distinct, characteristic general preferences for different viral IN proteins. **(6)** Transcription of integrated viral DNA (the **provirus**) by the host cell RNA polymerase II system produces full-length RNA transcripts. **(7)** Some full-length RNA molecules are exported from the nucleus to the cytoplasm and serve as mRNAs. **(8)** These mRNAs are translated by cytoplasmic ribosomes to form the viral Gag and Gag-Pol polyprotein precursors at a ratio of approximately 10:1. **(9)** Some full-length RNA molecules are destined to become encapsidated as progeny viral genomes. The mechanism for sequestering RNAs for this purpose is unknown, but there is evidence that a fraction of the ASV Gag protein traffics through the nucleus, where it could perform this function. **(10)** Other full-length RNA molecules are spliced within the nucleus to form mRNA for the Env polyprotein. **(11)** Env mRNA is translated by ribosomes bound to the endoplasmic reticulum (ER). **(12)** The Env proteins are transported through the Golgi apparatus, where they are glycosylated and cleaved by cellular enzymes to form the mature SU-TM complex. **(13)** Mature envelope proteins are delivered to the surface of the infected cell. **(14)** Virion components (two copies of the viral RNA, Gag and Gag-Pol precursors, and SU-TM) assemble at budding sites with the help of *cis*-acting signals encoded in each. Type C retroviruses (e.g., alpharetroviruses and lentiviruses) assemble at the inner face of the plasma membrane, as illustrated. Other types (A, B, and D) assemble on internal cellular membranes. **(15)** The nascent particles bud from the surface of the cell. **(16)** Maturation (and infectivity) requires the action of the virus-encoded protease (PR), which is itself a component of the core precursor polyprotein. During or shortly after budding, PR cleaves at specific sites within the Gag and Gag-Pol precursors to produce the mature viral proteins. This process causes a characteristic condensation of the virus cores.

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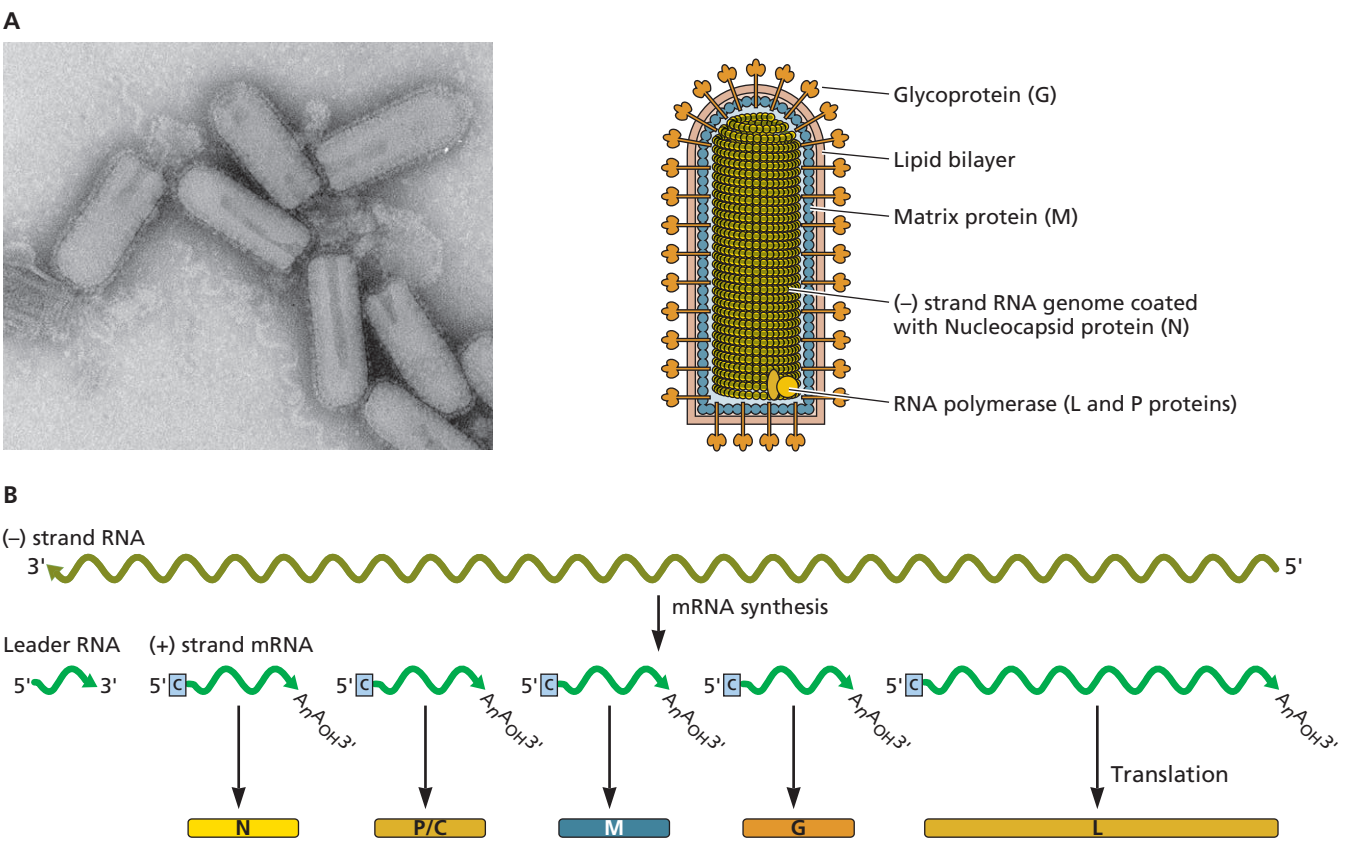
Rhabdoviruses

Family *Rhabdoviridae*

Genera	Examples
Vesiculovirus	Vesicular stomatitis virus Indiana
Lyssavirus	Rabies virus
Cytorhabdovirus	Lettuce necrotic yellows virus

Among the 175 known rhabdoviruses are the causative agents of rabies, one of the oldest recognized infectious diseases, and economically important diseases of fish. The host range of these viruses is very broad: they infect many vertebrates, invertebrates, and plants. The genome of vesicular stomatitis virus has been a model for the replication and expression of viral genomes that consist of a single molecule of (−) strand RNA. The first RNA-dependent RNA polymerase discovered in a virus particle was that of vesicular stomatitis virus.

Figure 31 Structure and genomic organization of vesicular stomatitis virus. (A) Virion structure. The electron micrograph shows negatively stained vesicular stomatitis virus (courtesy of J. Rose, Yale University School of Medicine, New Haven, CT). **(B) Genome organization.** The (−) strand RNA is the template for synthesis of leader RNA and five monocistronic mRNAs (capped and polyadenylated). Two proteins are produced from the P/C mRNA from upstream and downstream translation initiation codons.



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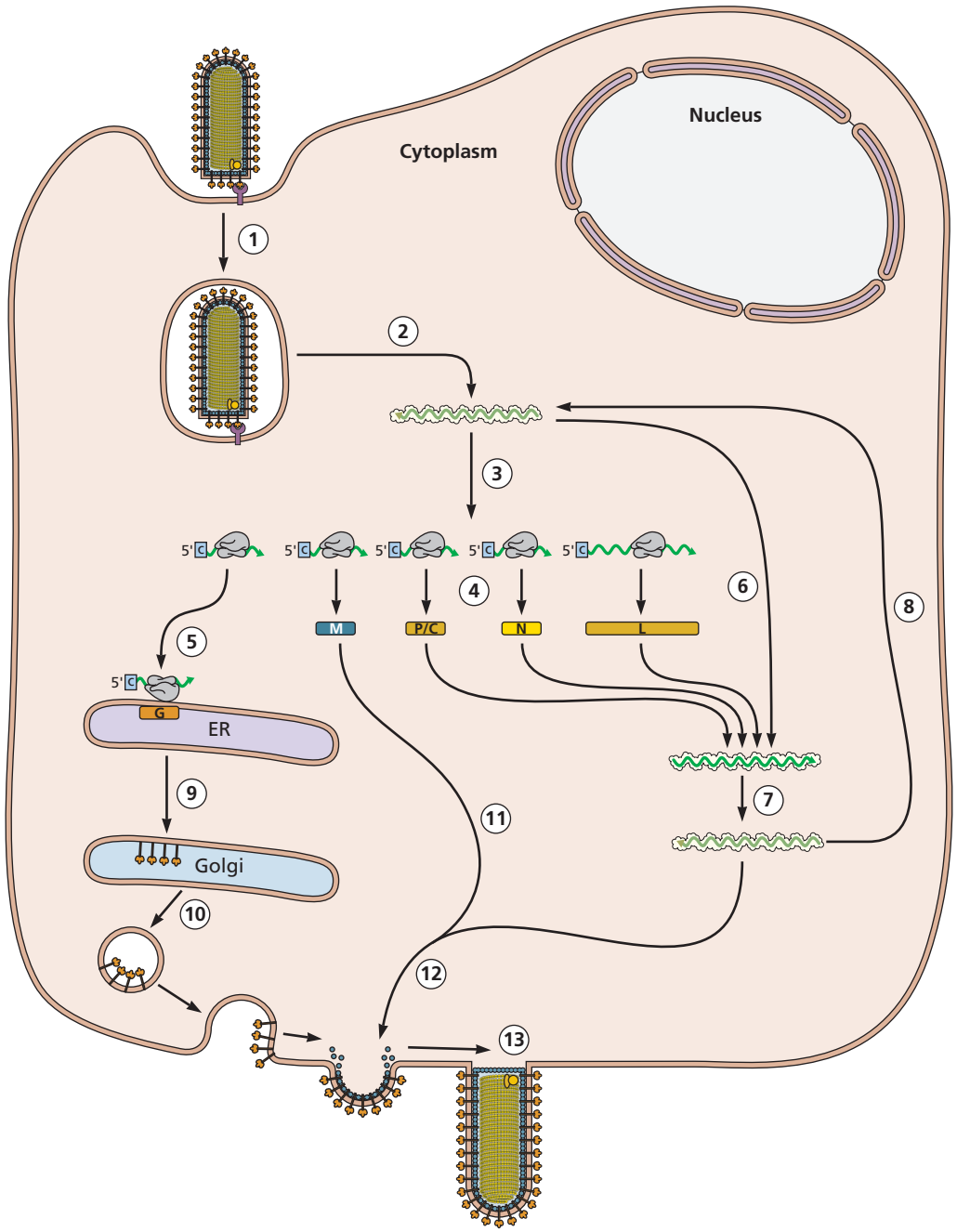


Figure 32 Single-cell reproductive cycle. (1) The virion binds to a cellular receptor and enters the cell via receptor-mediated endocytosis. **(2)** The viral membrane fuses with the membrane of the endosome, releasing the helical viral nucleocapsid. This structure comprises (−) strand RNA coated with nucleocapsid protein molecules and a small number of L and P protein molecules, which catalyze viral RNA synthesis. **(3)** The (−) strand RNA is copied into five subgenomic mRNAs by the L and P proteins. **(4)** The N, P/C, M, and L mRNAs are translated by free cytoplasmic ribosomes, **(5)** while G mRNA is translated by ribosomes bound to the endoplasmic reticulum. Newly synthesized N, P, and L proteins participate in viral RNA replication. **(6)** This process begins with synthesis of a full-length (+) strand copy of genomic RNA, which is also in the form of a ribonucleoprotein containing the N, L, and P proteins. **(7)** This RNA, in turn, serves as a template for the synthesis of progeny (−) strand RNA in the form of nucleocapsids. **(8)** Some of these newly synthesized (−) strand RNA molecules enter the pathway for viral mRNA synthesis. **(9)** Upon translation of G mRNA, the G protein enters the secretory pathway, **(10)** in which it becomes glycosylated and travels to the plasma membrane. **(11 and 12)** Progeny nucleocapsids and the M protein are transported to lipid rafts in the plasma membrane, **(13)** where association with regions containing the G protein is followed by budding to release virus particles.

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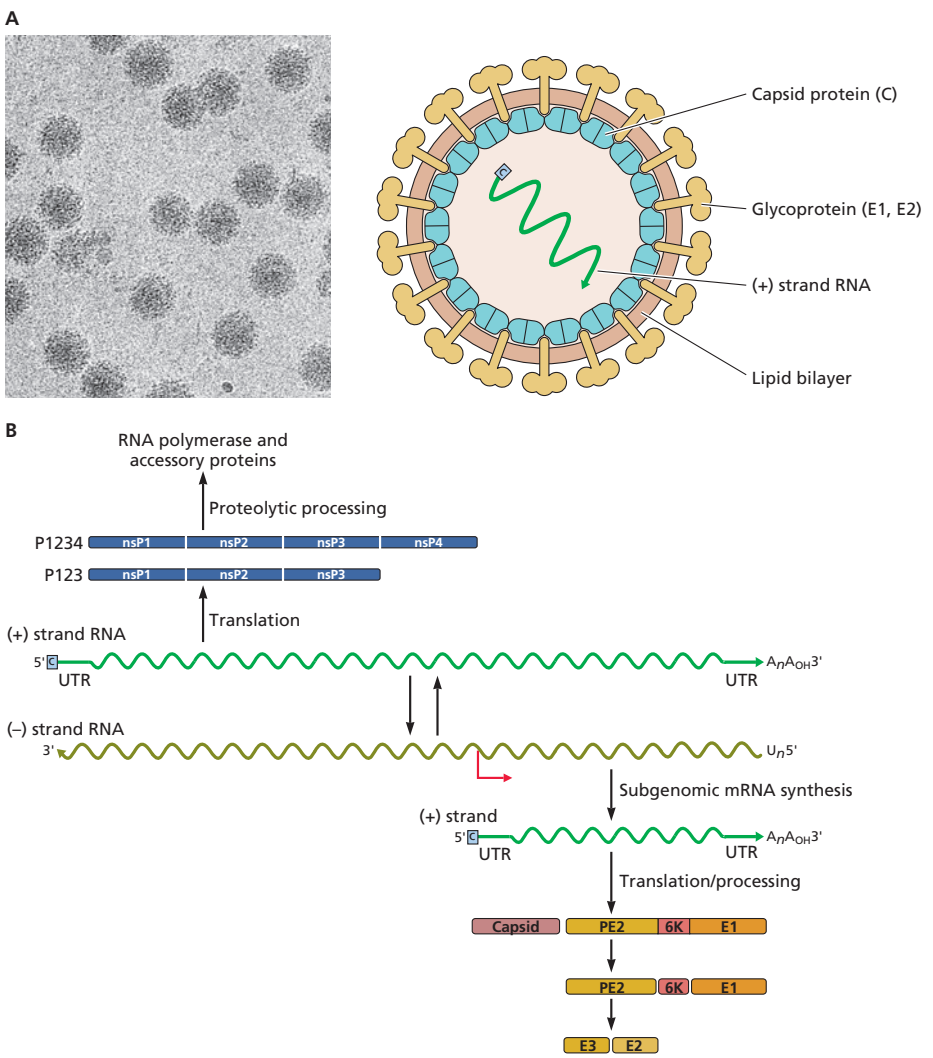
Togaviruses

Family *Togaviridae*

Genera	Examples
Alphavirus	Sindbis virus
Rubivirus	Rubella virus

Members of the *Togaviridae* are responsible for two very different kinds of human disease. All alphaviruses are transmitted by arthropods, and cause encephalitis, arthritis, and rashes. Rubella virus is the agent of a mild rash disease but can also cause congenital abnormalities in the fetus when acquired by the mother early in pregnancy. Because these virus particles have a lipid envelope, they have been important models for studying the synthesis, posttranslational modification, and localization of membrane glycoproteins.

Figure 33 Structure and genomic organization. (A) Virion structure. The cryo-electron micrograph shows the alphavirus Ross River virus (courtesy of N. Olson, Purdue University, West Lafayette, IN). **(B) Genome organization.** The (+) strand RNA genomes of alphaviruses and rubiviruses are 11.7 and 9.8 kb, respectively, in length. The first two-thirds of alphavirus genomic RNA, which is of (+) polarity and carries a 5′ cap, is translated to produce the polyproteins P123 and P1234. The latter is the precursor of the RNA polymerase. For some alphaviruses, the P1234 polyprotein is produced by translational suppression of a stop codon located at the end of the nsP3 coding region. The proteins encoded in the 3′-terminal one-third of the genome are produced from a subgenomic mRNA that is copied from a full-length (−) strand RNA intermediate. The subgenomic mRNA encodes the structural proteins.



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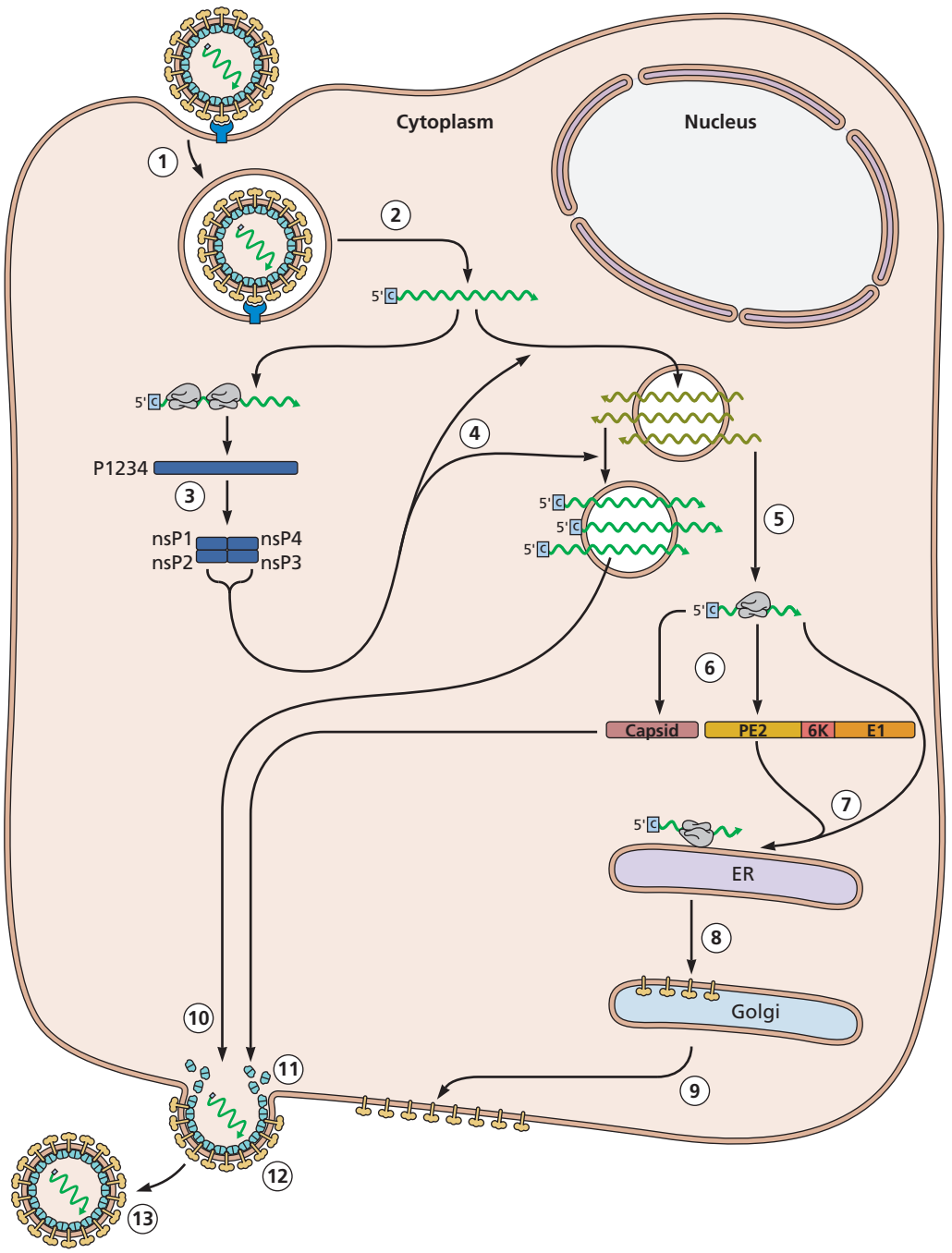


Figure 34 Single-cell reproductive cycle. **(1)** The virion binds to a cellular receptor and enters the cell via receptor-mediated endocytosis. **(2)** Upon acidification of the vesicle, the viral nucleocapsid is released into the cytoplasm and subsequently disassembled to release the (+) strand viral RNA, **(3)** which is translated to form the polyprotein P1234. **(4)** Sequential cleavage of this polyprotein at different sites by a viral proteinase produces RNA polymerases with different specificities. **(5)** These viral enzymes then copy (+) strands into full-length (−) and (+) strands and catalyze synthesis of the subgenomic mRNA. Viral RNA synthesis takes place on membranous structures that first accumulate at the plasma membrane and later move to the cell interior. **(6)** The subgenomic mRNA is translated by free cytoplasmic ribosomes to produce the capsid protein. **(7)** Proteolytic cleavage to liberate the capsid protein exposes a hydrophobic sequence of PE2 that induces the ribosomes to associate with the endoplasmic reticulum (ER). **(8)** As a result, the PE2-6K-E1 polyprotein enters the secretory pathway. **(9)** The glycoproteins are transported to the cell surface. **(10)** The capsid protein and (+) strand genomic RNA assemble to form nucleocapsids that migrate to the plasma membrane and **(11)** associate with viral glycoproteins. **(12)** The nucleocapsid acquires an envelope by budding at this site, and **(13)** virus particles are released.

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Glossary

3′ untranslated region The region of an mRNA downstream of the translation termination codon. *(Chapter 11)*

5′ untranslated region The region of an mRNA upstream of the translation initiation codon. *(Chapter 11)*

Acylation Posttranslational addition of saturated or unsaturated fatty acids to a protein. *(Chapter 12)*

Affinity The measure of the strength with which one molecule associates with another noncovalently. *(Chapter 5)*

Allele specific Complementing only a specific change; refers to suppressor mutations. *(Chapter 3)*

Alternative splicing Splicing of different combinations of exons in a pre-mRNA, generally leading to synthesis of mRNAs with different protein-coding sequences. *(Chapter 10)*

Ambisense Producing mRNAs from both (−) strand genomic RNA and the complementary (+) strand; refers to viral genomes. *(Chapter 6)*

Amphipathic Having both hydrophilic and hydrophobic portions. *(Chapter 2)*

Anabolism The metabolic reactions by which larger molecules are built from simpler ones, with the consumption of energy. *(Chapter 14)*

Aneuploid Abnormal in chromosome morphology and number. *(Chapter 2)*

Apical domain The specialized surface of an epithelial cell exposed to the environment. Also called apical surface. *(Chapters 2, 9, and 12)*

Asymmetric unit The unit from which capsids or nucleocapsids of a virus particle are built. Also called protomer or structural unit. *(Chapter 4)*

Attenuated An infection in which normally severe symptoms or pathology are mild or inconsequential; a state of reduced virulence. *(Chapter 1)*

Autophagy The controlled degradation, in response to stress, of proteins and other cellular components taken into double-membrane vesicles (autophagosomes) that fuse with lysosomes, literally self-eating. *(Chapter 14)*

Avidity The sum of the affinities (strengths) of multiple noncovalent interactions. *(Chapter 5)*

Bacteriophages Viruses that infect bacteria; derived from the Greek word *phagein,* meaning "to eat." *(Chapter 1)*

Basal lamina A thin layer of extracellular matrix bound tightly to the basolateral surface of cells; the basal lamina is linked to the basolateral membrane by integrins. *(Chapter 2)*

Basolateral domain The nonspecialized surface of an epithelial cell that contacts an internal basal lamina or adjacent or underlying cells in the tissue. Also called basolateral surface. *(Chapters 2, 9, and 12)*

Capping The addition of m^7G via a 5′–5′ phosphodiester bond to the 5′ ends of cellular and viral transcripts made in eukaryotic cells. *(Chapter 10)*

Cap structure The m^7G linked via a 5′–5′ phosphodiester bond to the 5′ ends of the majority of viral and cellular mRNAs made in eukaryotic cells. *(Chapter 11)*

Capsid The outer shell of viral proteins that surrounds the genome in a virus particle. *(Chapters 1 and 4)*

Cap snatching Cleavage of cellular RNA polymerase II transcripts by a viral endonuclease to produce capped primers for viral mRNA synthesis. *(Chapters 6 and 10)*

Catabolism The reactions that break down complex molecules into simpler ones to generate energy directly or indirectly. *(Chapter 14)*

Caveolae Flask-shaped invaginations of the plasma membrane of many types of cells that contain the protein caveolin and are rich in lipid rafts; caveolae internalize membrane components, extracellular ligands, bacterial toxins, and some animal viruses. *(Chapter 5)*

Centrosome An organelle that is the main microtubule-organizing center. *(Chapter 5)*

Chaperone A protein that facilitates the folding of other polypeptide chains, the assembly of multimeric proteins, or the formation of macromolecular assemblies (e.g., chromatin). Also called molecular chaperone. *(Chapters 4, 12, and 13)*

Chemokines Small proteins that attract and stimulate cells of the immune defense; produced by many cells in response to infection. Also called chemotactic cytokines. *(Chapter 5)*

Coactivator A protein that stimulates transcription by RNA polymerase II without binding to a specific DNA sequence; generally interacts with sequence-specific transcriptional activators. *(Chapter 8)*

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Codon Three contiguous bases in an mRNA template that specify the amino acids incorporated into protein. *(Chapter 11)*

Complementation The ability of gene products of two different, individually nonreproducing mutants to interact functionally in the same cell to permit virus reproduction. *(Chapter 3)*

Concatemer A DNA molecule comprising multiple, tandem copies of a viral genome (or other DNA sequence) joined end to end. *(Chapter 9)*

Constitutive transport elements Sequences in certain unspliced viral mRNAs that direct export from the nucleus by host cell proteins. *(Chapter 10)*

Continuous cell lines Cultures of a single cell type that can be propagated indefinitely in culture. *(Chapter 2)*

Copy choice A mechanism of recombination in which an RNA or DNA polymerase first copies the 3′ end of one parental strand and then exchanges one template for another at the corresponding position on a second parental strand. *(Chapters 6 and 7)*

Coreceptor A cell surface molecule that is required, in addition to the receptor, for entry of virus particles into cells. *(Chapter 5)*

Core promoter The minimal set of DNA sequences required for accurate initiation of transcription by RNA polymerase II. *(Chapter 8)*

Culling Removing and destroying diseased or potentially exposed animals to prevent further spread of infection. *(Chapter 1)*

Cytopathic effects The morphological changes induced in cells by viral infection. *(Chapter 2)*

Cytoskeleton The intracellular structural network composed of actin filaments, microtubules, and intermediate filaments. *(Chapter 2)*

Defective interfering RNAs Subgenomic RNAs that replicate more rapidly than full-length RNA and therefore compete for the components of the RNA synthesis machinery and interfere with the replication of full-length RNAs. *(Chapter 6)*

Deletion mutation Loss of one or more bases in a nucleic acid. *(Chapter 3)*

Diploid cell strains Cell cultures that consist of a homogeneous population of a single type and that can divide up to 100 times before dying. *(Chapter 2)*

Eclipse period The phase of viral infection during which the viral nucleic acid is uncoated from its protective shell and no infectious virus can be detected inside cells. *(Chapter 2)*

Efficiency of plating The plaque titer divided by the number of virus particles in the sample. *(Chapter 2)*

Elongation Stepwise incorporation of ribonucleoside monophosphates or deoxyribonucleoside monophosphates into the 3′-OH end of the growing RNA or DNA chain in the 5′ → 3′ direction. *(Chapter 6)*

Endemic Having a disease pattern typical of a particular geographic area; persisting in a population for a long period without reintroduction of the causative virus from outside sources. *(Chapter 1)*

Endogenous proviruses Proviruses that enter the germ line at some point in the history of an organism and are thereafter inherited in normal Mendelian fashion by every cell in that organism and by its progeny. *(Chapter 7)*

Endosome A vesicle that transports molecules from the plasma membrane to the cell interior. *(Chapter 5)*

Enhancer A DNA sequence containing multiple elements that can stimulate RNA polymerase II transcription over long distances, independently of orientation or location relative to the site of transcriptional initiation. *(Chapter 8)*

Envelope The host cell-derived lipid bilayer carrying viral glycoproteins that forms the outer layer of many virus particles. *(Chapter 4)*

Epidemic A pattern of disease characterized by rapid and sudden appearance of cases spreading over a wide area. *(Chapter 1)*

Epitope A short contiguous sequence or unique conformation of a macromolecule that can be recognized by the immune system; also called an antigenic determinant; a T cell epitope is a short peptide recognized by a particular T cell receptor, while a B cell epitope is recognized by the antigen-binding domain of antibody and is part of an intact protein. *(Chapter 2)*

Exons Blocks of noncontiguous coding sequences (generally short) present in many cellular and viral pre-mRNAs. *(Chapter 10)*

Foci (plural) Clusters of cells that are derived from a single progenitor and share properties, such as unregulated growth, that cause them to pile up on one another. One such cluster is called a **focus**. *(Chapter 2)*

Fusion peptide A short hydrophobic amino acid sequence (20 to 30 amino acids) that is thought to insert into target membranes to initiate fusion. *(Chapter 5)*

Fusion pore An opening between two lipid bilayers formed by the action of fusion proteins; it allows exchange of material across membranes. *(Chapter 5)*

Glycoforms The total set of forms of a protein that differ in the number, location, and nature of oligosaccharide chains. *(Chapter 12)*

Glycoprotein A protein carrying covalently linked sugar chains (oligosaccharides). *(Chapter 4)*

G_0 state A state in which the cell has ceased to grow and divide and has withdrawn from the cell cycle. Also called resting state. *(Chapter 9)*

Half-life The time required for decay of a molecule or macromolecule to half of the original concentration. *(Chapter 10)*

Helical symmetry The symmetry of regularly wound structures defined by the relationship $P = \mu \times \rho$, where P = pitch of the helix, μ = the number of structural units per turn, and ρ = the axial rise per unit. *(Chapter 4)*

Helper virus A virus that provides viral proteins needed for the reproduction of a coinfecting defective virus. *(Chapter 6)*

Hemagglutination The linking of multiple red blood cells by virus particles, resulting in a lattice; basis of a method to measure virus concentration. *(Chapter 2)*

Heterogeneous nuclear RNAs Nuclear precursors to mRNAs that are larger than mRNAs and heterogeneous in size. *(Chapter 10)*

Homologous recombination The exchange of genetic information between any pair of related DNAs at sites with identical sequences. *(Chapter 9)*

Host range A listing of species and cells (hosts) that are susceptible to and permissive for infection. *(Chapter 5)*

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Icosahedral symmetry The symmetry of the icosahedron, the solid with 20 faces and 12 vertices related by axes of two-, three-, and five-fold rotational symmetry. *(Chapter 4)*

Indirectly anchored proteins Proteins that are indirectly bound to the plasma membrane by interacting with either integral membrane proteins or the charged sugars of membrane glycolipids. *(Chapter 2)*

Infectious DNA clone A double-stranded DNA copy of the viral genome carried on a bacterial plasmid or other vector. *(Chapter 3)*

Initiation codon The codon at which translation of an mRNA begins, most commonly AUG. *(Chapter 11)*

Initiator A short DNA sequence that is sufficient to specify the site at which RNA polymerase II initiates transcription. *(Chapter 8)*

Insertion mutation Addition of one or more nucleotides to a nucleic acid sequence. *(Chapter 3)*

Integral membrane proteins Proteins that are embedded in a lipid bilayer, with external and internal domains connected by one or more membrane-spanning domains. *(Chapters 2 and 4)*

Internal ribosome entry site (IRES) An internal binding site for 40S ribosomal subunits and initiation of translation present in some viral and few cellular mRNAs. *(Chapter 11)*

Introns Noncoding sequences that separate coding sequences (exons) in many cellular and viral pre-mRNAs. *(Chapter 10)*

Inverted terminal repetitions Sequences that are present in the opposite orientation at the ends of certain linear viral DNA genomes. *(Chapter 9)*

Koch's postulates Criteria developed by the German physician Robert Koch in the late 1800s to determine if a given agent is the cause of a specific disease. *(Chapter 1)*

Lariat An intermediate in pre-mRNA splicing containing the intron and 3′ exon, with the branch point A residue of the intron linked via a 2′–5′ phosphodiester bond to the nucleotide at the 5′ end of the intron. *(Chapter 10)*

Latent infection Long term infection in which the viral genome is maintained with limited expression of viral genes and without loss of host cell viability. *(Chapters 8 and 9)*

Latent period The phase of viral infection during which no extracellular virus can be detected. *(Chapter 2)*

L domain sequences Short amino acid sequences required for membrane fusion during budding of enveloped viruses. *(Chapter 13)*

Lectin A protein that binds to a specific sugar. *(Chapter 5)*

Lipid raft A microdomain of the plasma membrane that is enriched in cholesterol and saturated fatty acids and is more densely packed and less fluid than other regions of the membrane. *(Chapters 2 and 12)*

Long terminal repeat A direct repeat of genetic information that is present in the proviral DNA of retroviruses; it is formed by reverse transcription of the RNA template and includes *cis*-acting elements required for viral DNA integration and its subsequent transcription. *(Chapter 7)*

Lysogenic Pertaining to a bacterium that carries the genetic information of a quiescent bacteriophage, which can be induced to reproduce, and subsequently lyse, the bacterium. *(Chapter 1)*

Lysogeny The phenomenon by which the lysogenic state is established and maintained in bacteria. *(Chapter 1)*

Lysosome A vesicle in the cell that contains enzymes that degrade sugars, proteins, nucleic acids, and lipids. *(Chapter 5)*

Marker rescue Replacement of all local nucleic acids that include a mutation with wild-type nucleic acid. *(Chapter 3)*

Marker transfer Introduction of a mutation by replacement of a segment of viral nucleic acid with one containing the mutation. *(Chapter 3)*

Membrane-spanning domain A segment of an integral membrane protein that spans the lipid bilayer; often α-helical. *(Chapters 2 and 4)*

Metagenomic analysis Sequencing of samples recovered directly from the environment, and containing many genomes. *(Chapter 1)*

Metastable structure A structure that has not attained the lowest free energy state. *(Chapter 4)*

Microdomains Regions of the plasma membrane with distinct lipid and protein composition. *(Chapter 2)*

Missense mutation A change in a single nucleotide or codon that results in the production of a protein with a single amino acid substitution. *(Chapter 3)*

Molecular chaperone *See* Chaperone.

Monocistronic Encoding one polypeptide; refers to mRNA. *(Chapter 11)*

Monoclonal antibody An antibody of a single specificity made by a clone of antibody-producing cells. *(Chapter 2)*

Monolayer A layer of cultured cells growing in a cell culture dish. *(Chapter 2)*

Multiplicity of infection The number of infectious virus particles added per cell. *(Chapter 2)*

Negative [(−)] strand The strand of DNA or RNA that is complementary in sequence to the (+) (coding) strand. *(Chapter 1)*

Neutralize To block (by antibodies) the infectivity of virus particles. *(Chapter 2)*

Nonsense mutation A substitution mutation that produces a translation termination codon. *(Chapter 3)*

Nuclear localization signal Amino acid sequence that is necessary and sufficient for import of a protein into the nucleus. *(Chapter 5)*

Nucleocapsid A nucleic acid-protein assembly packaged within the virus particle; the term is used when this complex is a discrete substructure of a complex particle. *(Chapter 4)*

Obligate parasites Organisms that are absolutely dependent on another living organism for reproduction. *(Chapter 1)*

Okazaki fragments Short (100–200 nucleotides) DNA segments elongated from RNA primers during discontinuous synthesis of the lagging strand at a replication fork. *(Chapter 9)*

Oligomerization Association of polypeptide chains, which may be the same or different, to form a protein with multiple subunits. *(Chapter 8)*

Oligosaccharide A short linear or branched chain of sugar residues (monosaccharides); also called a glycan. *(Chapter 4)*

Oncogenesis The processes leading to cancer. *(Chapter 14)*

One-hit kinetics A linear relationship between plaque count and virus concentration that indicates that one infectious particle is sufficient to initiate infection. *(Chapter 2)*

One-step growth curve A single reproduction cycle that occurs synchronously in every infected cell. *(Chapter 2)*

Origins (of replication) Specific sites at which replication of DNA begins. *(Chapter 9)*

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Packaging Incorporation of the viral genome during assembly of virus particles. *(Chapter 13)*

Packaging signal Nucleic acid sequence or structural feature directing incorporation of a viral genome into a virus particle. *(Chapter 13)*

Particle–to–plaque-forming-unit (PFU) ratio The inverse value of the absolute efficiency of plating: the ratio of the total number of particles to the number that are infectious. *(Chapter 2)*

Pathogen Disease-causing virus or microorganism. *(Chapter 1)*

Permissive Able to support virus reproduction when viral nucleic acid is introduced; refers to cells. *(Chapter 2)*

Plaque A circular zone of infected cells that can be distinguished from the surrounding monolayer. *(Chapter 2)*

Plaque-forming units per milliliter A measure of virus infectivity. *(Chapter 2)*

Plaque purified Prepared from a single plaque (refers to virus stock); when one infectious virus particle initiates a plaque, the viral progeny within the plaque are clones. *(Chapter 2)*

Polarized cells Differentiated cells with surfaces divided into functionally specialized regions. *(Chapter 12)*

Polyadenylation The addition of ~200 A residues to the 3′ ends of cellular and viral transcripts made in eukaryotic cells. *(Chapter 10)*

Poly(A) tail The segment of ~200 A residues present at the 3′ ends of most cellular and many viral mRNAs. *(Chapters 10 and 11)*

Polycistronic Encoding several polypeptides; refers to mRNA. *(Chapter 11)*

Polyclonal antibodies The antibody repertoire against the many epitopes of an antigen produced in an animal. *(Chapter 2)*

Polysome An mRNA bound to multiple ribosomes that are synthesizing proteins from the mRNA template. *(Chapter 11)*

Portal A specialized structure for entry and/or exit of a viral genome into a preassembled protein shell. *(Chapter 4)*

Positive [(+)] strand The strand of DNA or RNA that corresponds in sequence to that of the messenger RNA. Also known as the sense strand. *(Chapter 1)*

Pregenomic mRNA The hepadnaviral mRNA that is reverse transcribed to produce the DNA genome. *(Chapter 7)*

Preinitiation complex (transcription) A promoter-bound assembly of an RNA polymerase and initiator proteins competent to initiate transcription. *(Chapter 8)*

Preinitiation complex (translation) The 40S ribosomal subunit bound to translation initiation proteins and initiator tRNA. *(Chapter 11)*

Primary cell cultures Cell cultures prepared from animal tissues; these cultures include several cell types and have a limited life span, usually no more than 5 to 20 cell divisions. *(Chapter 2)*

Primary cells Cells that have been freshly derived from an organ or tissue. *(Chapter 1)*

Primase An enzyme that synthesizes RNA primers for DNA synthesis. *(Chapter 9)*

Primer A free 3′-OH group required for initiation of synthesis of DNA from DNA or RNA templates and initiation of synthesis of some viral RNA genomes. *(Chapters 6 and 9)*

Prions Infectious agents comprising an abnormal isoform of a normal cellular protein but no nucleic acid; implicated as the causative agents of transmissible spongiform encephalopathies. *(Chapter 1)*

Procapsid A closed, protein-only structure into which viral genomes are inserted; precursor to a capsid or nucleocapsid. *(Chapter 13)*

Processivity The ability of an enzyme to copy a nucleic acid template over long distances from a single site of initiation. *(Chapters 7, 8, and 9)*

Promoter A set of DNA sequences necessary for initiation of transcription by a DNA-dependent RNA polymerase. *(Chapter 8)*

Promoter occlusion The mechanism by which access to a promoter is blocked by passage of a transcribing RNA polymerase. *(Chapter 8)*

Proofreading Correction of mistakes made during chain elongation by exonuclease activities of DNA-dependent DNA polymerases. *(Chapter 6)*

Prophage The genome of the quiescent bacteriophage in a lysogenic bacterium. *(Chapter 1)*

Proteasome A complex containing multiple proteases with different specificities that is responsible for degradation of polyubiquitin-tagged proteins to amino acids and small peptides. *(Chapter 8)*

Proteoglycans Proteins linked to glycosaminoglycans, which are unbranched polysaccharides made of repeating disaccharides. *(Chapter 2)*

Proviral DNA *See* Provirus.

Provirus Retroviral DNA that is integrated into its host cell genome and is the template for formation of retroviral mRNAs and genomic RNA. Also called proviral DNA. *(Chapter 7)*

Pseudodiploid Having two RNA genomes per virus particle that give rise to only one DNA copy, as is the case for retroviruses. *(Chapter 7)*

Pseudoreversion Phenotypic reversion caused by second-site mutation; also known as suppression. *(Chapter 3)*

Quasiequivalence The arrangement of structural units in a virus particle such that similar interactions among them are allowed. *(Chapter 4)*

Quasispecies Virus populations that exist as dynamic distributions of nonidentical but related replicons. *(Chapter 6)*

Reactivation A switch from a latent to a productive infection; usually applied to herpesviruses. *(Chapter 8)*

Reassortants Viral genomes that have exchanged segments after coinfection of cells with viruses with segmented genomes. *(Chapter 3)*

Reassortment The exchange of entire RNA molecules between genetically related viruses with segmented genomes. *(Chapters 3 and 6)*

Receptor The cellular molecule to which a virus attaches to initiate infection. *(Chapter 5)*

Replication centers Specialized nuclear structures in which viral DNA genomes are replicated. Also called replication compartments. *(Chapter 9)*

Replication forks The sites of synthesis of nascent DNA chains that move away from origin as replication proceeds. *(Chapter 9)*

Replication intermediate An incompletely replicated DNA molecule containing newly synthesized DNA. *(Chapter 9)*

Replication licensing Mechanisms that ensure that replication of cellular DNA is initiated at each origin once, and only once, per cell cycle. *(Chapter 9)*

Replicon A unit of replication in large genomes, defined by discrete origin and termini. *(Chapter 9)*

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Resolution The minimal size of an object that can be distinguished by microscopy or other methods of structural analysis. *(Chapter 4)*

Resting state *See* G_0 state.

Retroelement A nucleic acid sequence that has been copied into DNA from an intermediate by reverse transcription. *(Chapter 7)*

Retroid viruses Viruses that replicate their genomes via reverse transcription. *(Chapter 7)*

Revert To change to the parental, or wild-type, genotype or phenotype. *(Chapter 3)*

Ribosome A molecular machine composed of RNA and protein that is the site of protein synthesis. *(Chapter 11)*

Ribozyme An RNA molecule with catalytic activity. *(Chapters 6 and 10)*

RNA-dependent RNA polymerase The protein assembly required to carry out RNA synthesis. *(Chapter 6)*

RNA editing The introduction into an RNA molecule of nucleotides that are not specified by a cellular or viral gene. *(Chapter 10)*

RNA interference A mechanism of posttranscriptional regulation of gene expression by small RNA molecules that induce mRNA degradation or inhibition of translation. *(Chapters 3 and 10)*

RNA processing The series of co- or posttranscriptional covalent modifications that produce mature mRNAs from primary transcripts. *(Chapter 10)*

RNA pseudoknot An RNA secondary structure formed when a single-stranded loop region base pairs with a complementary sequence outside the loop. *(Chapter 6)*

Satellites Small, single-stranded RNA molecules that lack genes required for their reproduction but do reproduce in the presence of another virus, which provides essential components (the **helper virus**). *(Chapter 1)*

Satellite virus A satellite with a genome that encodes one or two proteins. *(Chapter 1)*

Scaffolding protein A viral protein that is required for assembly of an icosahedral protein shell but is absent from mature virions. *(Chapter 13)*

Secretory pathway The series of membrane-demarcated compartments (e.g., the endoplasmic reticulum and Golgi apparatus), tubules, and vesicles through which secreted and membrane proteins travel to the cell surface. *(Chapter 12)*

Self-priming A mechanism by which some viral DNA genomes serves as primers, as well as templates, for DNA synthesis. *(Chapter 9)*

Semiconservative replication Production of two daughter DNA molecules, each containing one strand of the parental template and a newly synthesized complementary strand. *(Chapter 9)*

Serotype A virus type as defined on the basis of neutralizing antibodies. *(Chapter 2)*

Signal peptide A short sequence (generally hydrophobic) that directs nascent proteins to the endoplasmic reticulum. The signal may be removed, or retained as a transmembrane domain. *(Chapter 12)*

Signal transduction cascade or pathway A chain of sequential physical interactions among, and biochemical modification of, membrane-bound, cytoplasmic, and nuclear proteins. *(Chapter 14)*

Single-exon mRNAs mRNAs produced without splicing, because their precursors lack introns and splice sites. *(Chapter 10)*

siRNAs *See* Small interfering RNAs.

Site-specific recombination Exchange of DNA sequences at short DNA sequences that are specifically recognized by proteins that catalyze recombination. *(Chapter 9)*

Small interfering RNAs Small RNA molecules that base-pair with mRNAs to induce mRNA cleavage or inhibition of translation. Abbreviated siRNAs. *(Chapters 3 and 10)*

Small nuclear ribonucleoproteins Structures that contain small nuclear RNAs and several proteins; several participate in pre-mRNA splicing. *(Chapter 10)*

S phase The phase of the cell cycle in which the DNA genome is replicated. *(Chapter 9)*

Spliceosome The large complex that assembles on an intron-containing pre-mRNA before splicing; in mammalian cells, it comprises the small nuclear ribonucleoproteins containing U1, U2, U4, U5, and U6 small nuclear RNAs and ~150 proteins. *(Chapter 10)*

Splice sites Sites at which pre-mRNA sequences are cleaved and ligated during splicing; defined by short consensus sequences. *(Chapter 10)*

Splicing The precise ligation of blocks of noncontiguous coding sequences (exons) in cellular or viral pre-mRNAs with excision of the intervening noncoding sequences (introns). *(Chapter 10)*

Stop transfer signal A hydrophobic sequence that halts translocation of a nascent protein across the endoplasmic reticulum membrane; serves as a transmembrane domain. *(Chapter 12)*

Structural unit *See* Asymmetric unit.

Substitution mutation Replacement of one or more nucleotides in a nucleic acid. *(Chapter 3)*

Subunit A single folded protein of a multimeric protein. *(Chapter 4)*

Supercoiling The winding of one duplex DNA strand around another. *(Chapter 9)*

Suppression *See* Pseudoreversion.

Susceptible Producing the receptor(s) required for virus entry; refers to cells. *(Chapter 2)*

Tegument The layer interposed between the nucleocapsid and the envelope of herpesvirus particles. *(Chapter 4)*

Telomere A region of repeated sequences at the ends of linear, cellular chromosomes that is maintained (copied) by a specialized enzyme and that protects against loss of genetic information. *(Chapter 9)*

Termination codon Codons at which translation of an mRNA ceases, with release of both the nascent protein and ribosomes. *(Chapter 11)*

Termini Sites at which DNA replication stops. *(Chapter 9)*

Tight junctions The areas of contact between adjacent epithelial cells, circumscribing the cells at the apical edges of their lateral membranes. *(Chapter 2)*

Topology The geometric arrangement of, and connections among, secondary-structure units in a protein. *(Chapter 4)*

Transcription Copying of DNA carrying genetic information into a complementary RNA. *(Chapters 6 and 8)*

Transcriptional control region Local and distant DNA sequences necessary for initiation and regulation of transcription. *(Chapter 8)*

Transcytosis A mechanism of transport in which material in the intestinal lumen is endocytosed by M cells, transported to the basolateral surface, and released to the underlying tissues. *(Chapter 2)*

Transfection Introduction of viral nucleic acid into cells by transformation, resulting in the infection of cells. *(Chapter 3)*

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Transfer RNAs Adapter molecules that align each amino acid with its corresponding codon on the mRNA. Abbreviated tRNAs. *(Chapter 11)*

Transport vesicles Membrane-bound structures with external protein coats that bud from compartments of the secretory pathway and carry cargo in anterograde or retrograde directions. *(Chapter 12)*

tRNAs *See* Transfer RNAs.

Tropism The predilection of a virus to invade, and reproduce, in a particular cell type. *(Chapter 5)*

Tumor suppressor gene A cellular gene encoding a protein that negatively regulates cell proliferation; mutational inactivation of both copies of the genes is associated with tumor development. *(Chapter 9)*

Two-hit kinetics A parabolic relationship between plaque count and virus concentration which indicates that two different types of virus particle must infect a cell to ensure reproduction. *(Chapter 2)*

Type-specific antigens Epitopes, defined by neutralizing antibodies, that distinguish and define viral serotypes (e.g., poliovirus types 1, 2, and 3). *(Chapter 2)*

Uncoating The release of viral nucleic acid from its protective protein coat or lipid envelope; in some cases, the liberated nucleic acid is still associated with viral proteins. *(Chapter 5)*

Vaccination Inoculation of healthy individuals with attenuated or related microorganisms, or their antigenic products, to elicit an immune response that will protect against later infection by the corresponding pathogen. *(Chapter 1)*

Variolation Inoculation of healthy individuals with material from a smallpox pustule, or in modern times from a related or attenuated cowpox (vaccinia) virus preparation, through a scratch on the skin (called scarification). *(Chapter 1)*

Viral pathogenesis The processes by which viral infections cause disease. *(Chapter 2)*

Virion An infectious virus particle. *(Chapters 1 and 4)*

Viroids Unencapsidated, small, circular, single-stranded RNAs that replicate autonomously when inoculated into plant cells. *(Chapter 1)*

Viroporin Hydrophobic viral protein that forms pores in cellular membranes; many facilitate release of progeny virus particles. *(Chapter 13)*

Viruses Submicroscopic, obligate parasitic pathogens comprising genetic material (DNA or RNA) surrounded by a protective protein coat. *(Chapter 1)*

Virus reproduction The sum total of all events that occur during the infectious cycle. *(Chapter 2)*

Virus titer The concentration of a virus in a sample. *(Chapter 2)*

Wild type The original (often laboratory-adapted) virus from which mutants are selected and which is used as the basis for comparison. *(Chapter 3)*

Zoonotic Transmitted among humans and other vertebrates; refers to infections and diseases. *(Chapter 1)*

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4TH EDITION

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We dedicate this book to the students, current and future scientists, physicians, and all those with an interest in the field of virology, for whom it was written.
We kept them ever in mind.

We also dedicate it to our families:
Jonn, Gethyn, and Amy Leedham
Doris, Aidan, Devin, and Nadia
Eileen, Kelsey, and Abigail
Rudy, Jeanne, and Chris
And
Kathy and Brian

Oh, be wiser thou!
Instructed that true knowledge leads to love.

William Wordsworth
Lines left upon a Seat in a Yew-tree
1888

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Preface

The enduring goal of scientific endeavor, as of all human enterprise, I imagine, is to achieve an intelligible view of the universe. One of the great discoveries of modern science is that its goal cannot be achieved piecemeal, certainly not by the accumulation of facts. To understand a phenomenon is to understand a category of phenomena or it is nothing. Understanding is reached through creative acts.

A. D. HERSHEY
Carnegie Institution Yearbook 65

All four editions of this textbook have been written according to the authors' philosophy that the best approach to teaching introductory virology is by emphasizing shared principles. Studying the phases of the viral reproductive cycle, illustrated with a set of representative viruses, provides an overview of the steps required to maintain these infectious agents in nature. Such knowledge cannot be acquired by learning a collection of facts about individual viruses. Consequently, the major goal of this book is to define and illustrate the basic principles of animal virus biology.

In this information-rich age, the quantity of data describing any given virus can be overwhelming, if not indigestible, for student and expert alike. The urge to write more and more about less and less is the curse of reductionist science and the bane of those who write textbooks meant to be used by students. In the fourth edition, we continue to distill information with the intent of extracting essential principles, while providing descriptions of how the information was acquired. Boxes are used to emphasize major principles and to provide supplementary material of relevance, from explanations of terminology to descriptions of trail-blazing experiments. Our goal is to illuminate process and strategy as opposed to listing facts and figures. In an effort to make the book readable, rather than comprehensive, we are selective in our choice of viruses and examples. The encyclopedic *Fields Virology* (2013) is recommended as a resource for detailed reviews of specific virus families.

What's New

This edition is marked by a change in the author team. Our new member, Glenn Rall, has brought expertise in viral immunology and pathogenesis, pedagogical clarity, and down-to-earth humor to our work. Although no longer a coauthor, our colleague Lynn Enquist has continued to provide insight, advice, and comments on the chapters.

Each of the two volumes of the fourth edition has a unique appendix and a general glossary. Links to Internet resources such as websites, podcasts, blog posts, and movies are provided; the digital edition provides one-click access to these materials.

A major new feature of the fourth edition is the incorporation of in-depth video interviews with scientists who have made a major contribution to the subject of each chapter. Students will be interested in these conversations, which also explore the factors that motivated the scientists' interest in the field and the personal stories associated with their contributions.

Volume I covers the molecular biology of viral reproduction, and Volume II focuses on viral pathogenesis, control of virus infections, and virus evolution. The organization into two volumes follows a natural break in pedagogy and provides considerable flexibility and utility for students and teachers alike. The volumes can be used for two courses, or as two parts of a one-semester course. The two volumes differ in content but are integrated in style and presentation. In addition to updating the chapters and Appendices for both volumes, we have organized the material more efficiently and new chapters have been added.

As in our previous editions, we have tested ideas for inclusion in the text in our own classes. We have also received constructive comments and suggestions from other virology instructors and their students. Feedback from students was particularly useful in finding typographical errors, clarifying confusing or complicated illustrations, and pointing out inconsistencies in content.

For purposes of readability, references are generally omitted from the text, but each chapter ends with an updated list of relevant books, review articles, and selected research papers for readers who wish to pursue specific topics. In general, if an experiment is featured in a chapter, one or more references are listed to provide more detailed information.

Principles Taught in Two Distinct, but Integrated Volumes

These two volumes outline and illustrate the strategies by which all viruses reproduce, how infections spread within a host, and how they are maintained in populations. The principles of viral reproduction established in Volume I are essential for understanding the topics of viral disease, its control, and the evolution of viruses that are covered in Volume II.

Volume I The Science of Virology and the Molecular Biology of Viruses

This volume examines the molecular processes that take place in an infected host cell. It begins with a general introduction and historical perspectives, and includes descriptions of the unique properties of viruses (Chapter 1). The unifying principles that are the foundations of virology, including the concept of a common strategy for viral propagation, are then described. An introduction to cell biology, the principles of the infectious cycle, descriptions of the basic techniques for cultivating and assaying viruses, and the concept of the single-step growth cycle are presented in Chapter 2.

The fundamentals of viral genomes and genetics, and an overview of the surprisingly limited repertoire of viral strategies for genome replication and mRNA synthesis, are topics of Chapter 3. The architecture of extracellular virus particles in the context of providing both protection and delivery of the viral genome in a single vehicle are considered in Chapter 4. Chapters 5 through 13 address the broad spectrum of molecular processes that characterize the common steps of the reproductive cycle of viruses in a single cell, from decoding genetic information to genome replication and production of progeny virions. We describe how these common steps are accomplished in cells infected by diverse but representative viruses, while emphasizing common principles. Volume I concludes with a new chapter, "The Infected Cell," which presents an integrated description of cellular responses to illustrate the marked, and generally, irreversible, impact of virus infection on the host cell.

The appendix in Volume I provides concise illustrations of viral life cycles for members of the main virus families discussed in the text; five new families have been added in the fourth edition. It is intended to be a reference resource when reading individual chapters and a convenient visual means by which specific topics may be related to the overall infectious cycles of the selected viruses.

Volume II Pathogenesis, Control, and Evolution

This volume addresses the interplay between viruses and their host organisms. The first five chapters have been reorganized and rewritten to reflect our growing appreciation of the host immune response and how viruses cause disease. In Chapter 1 we introduce the discipline of epidemiology, provide historical examples of epidemics in history, and consider basic aspects that govern how the susceptibility of a population is controlled and measured. With an understanding of how viruses affect human populations, subsequent chapters focus on the impact of viral infections on hosts, tissues and individual cells. Physiological barriers to virus infections, and how viruses spread in a host, invade organs, and spread to other hosts are the topics of Chapter 2. The early host response to infection, comprising cell autonomous (intrinsic) and innate immune responses, are the topics of Chapter 3, while the next chapter considers adaptive immune defenses, that are tailored to the pathogen, and immune memory. Chapter 5 focuses on the classic patterns of virus infection within cells and hosts, the myriad ways that viruses cause illness, and the value of animal models in uncovering new principles of viral pathogenesis. In Chapter 6, we discuss virus infections that transform cells in culture and promote oncogenesis (the formation of tumors) in animals. Chapter 7 is devoted entirely to the AIDS virus, not only because it is the causative agent of the most serious current worldwide epidemic, but also because of its unique and informative interactions with the human immune defenses.

Next, we consider the principles involved in treatment and control of infection. Chapter 8 focuses on vaccines, and Chapter 9 discusses the approaches and challenges of antiviral drug discovery. The topics of viral evolution and emergence have now been divided into two chapters. The origin of viruses, the drivers of viral evolution, and host-virus conflicts are the subjects of Chapter 10. The principles of emerging virus infections, and humankind's experiences with epidemic and pandemic viral infections, are considered in Chapter 11. Volume II ends with a new chapter on unusual infectious agents, viroids, satellites, and prions.

The Appendix of Volume II provides snapshots of the pathogenesis of common human viruses. This information is presented in four illustrated panels that summarize the viruses and diseases, epidemiology, disease mechanisms, and human infections.

Reference

Knipe DM, Howley PM (ed). 2013. *Fields Virology,* 6th ed. Lippincott Williams & Wilkins, Philadelphia, PA.

For some behind-the-scenes information about how the authors created the fourth edition of *Principles of Virology*, see: http://bit.ly/Virology_MakingOf

Acknowledgments

These two volumes of *Principles* could not have been composed and revised without help and contributions from many individuals. We are most grateful for the continuing encouragement from our colleagues in virology and the students who use the text. Our sincere thanks also go to colleagues (listed in the Acknowledgments for the third edition) who have taken considerable time and effort to review the text in its evolving manifestations. Their expert knowledge and advice on issues ranging from teaching virology to organization of individual chapters and style were invaluable, and are inextricably woven into the final form of the book.

We also are grateful to those who gave so generously of their time to serve as expert reviewers of individual chapters or specific topics in these two volumes: Siddharth Balachandran (Fox Chase Cancer Center), Patrick Moore (University of Pittsburgh), Duane Grandgenett (St. Louis University), Frederick Hughson (Princeton University), Bernard Moss (Laboratory of Viral Diseases, National Institutes of Health), Christoph Seeger (Fox Chase Cancer Center), and Thomas Shenk (Princeton University). Their rapid responses to our requests for details and checks on accuracy, as well as their assistance in simplifying complex concepts, were invaluable. All remaining errors or inconsistencies are entirely ours.

Since the inception of this work, our belief has been that the illustrations must complement and enrich the text. Execution of this plan would not have been possible without the support of Christine Charlip (Director, ASM Press), and the technical expertise and craft of our illustrator. The illustrations are an integral part of the text, and credit for their execution goes to the knowledge, insight, and artistic talent of Patrick Lane of ScEYEnce Studios. We also are indebted to Jason Roberts (Victorian Infectious Diseases Reference Laboratory, Doherty Institute, Melbourne, Australia) for the computational expertise and time he devoted to producing the beautiful renditions of poliovirus particles on our new covers. As noted in the figure legends, many could not have been completed without the help and generosity of numerous colleagues who provided original images. Special thanks go to those who crafted figures or videos tailored specifically to our needs, or provided multiple pieces: Chantal Abergel (CNRS, Aix-Marseille Université, France), Mark Andrake (Fox Chase Cancer Center), Timothy Baker (University of California), Bruce Banfield (The University of Colorado), Christopher Basler and Peter Palese (Mount Sinai School of Medicine), Ralf Bartenschlager (University of Heidelberg, Germany), Eileen Bridge (Miami University, Ohio), Richard Compans (Emory University), Kartik Chandran (Albert Einstein College of Medicine), Paul Duprex (Boston University School of Medicine), Ramón González (Universidad Autónoma del Estado

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The collaborative work undertaken to prepare the fourth edition was facilitated greatly by several authors' retreats. ASM Press generously provided financial support for these retreats as well as for our many other meetings.

We thank all those who guided and assisted in the preparation and production of the book: Christine Charlip (Director, ASM Press) for steering us through the complexities inherent in a team effort, Megan Angelini and John Bell (Production Managers, ASM Press) for keeping us on track during production, and Susan Schmidler for her elegant and creative designs for the layout and cover. We are also grateful for the expert secretarial and administrative support from Ellen Brindle-Clark (Princeton University) that facilitated preparation of this text. Special thanks go to Ellen for obtaining many of the permissions required for the figures.

There is little doubt in undertaking such a massive effort that inaccuracies still remain, despite our best efforts to resolve or prevent them. We hope that the readership of this edition will draw our attention to them, so that these errors can be eliminated from future editions of this text.

This often-consuming enterprise was made possible by the emotional, intellectual, and logistical support of our families, to whom the two volumes are dedicated.

About the Authors

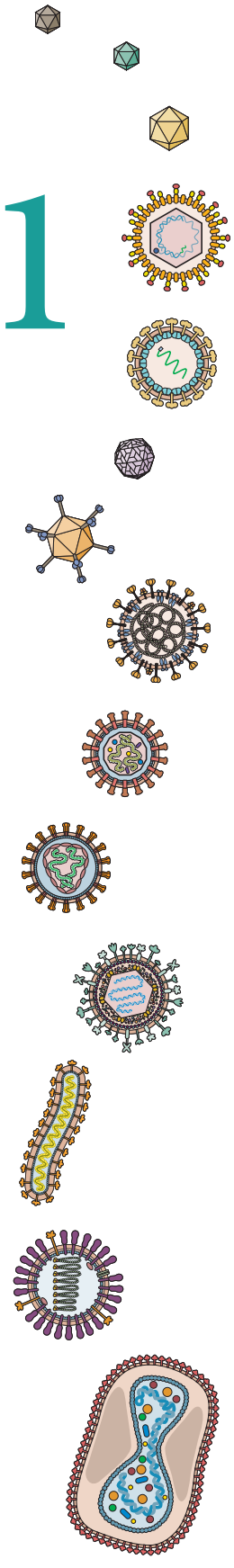


Jane Flint is a Professor of Molecular Biology at Princeton University. Dr. Flint's research focuses on investigation of the molecular mechanisms by which viral gene products modulate host cell pathways and antiviral defenses to allow efficient reproduction in normal human cells of adenoviruses, viruses that are widely used in such therapeutic applications as gene transfer and cancer treatment. Her service to the scientific community includes membership of various editorial boards and several NIH study sections and other review panels. Dr. Flint is currently a member of the Biosafety Working Group of the NIH Recombinant DNA Advisory Committee.

Vincent Racaniello is Higgins Professor of Microbiology & Immunology at Columbia University Medical Center. Dr. Racaniello has been studying viruses for over 35 years, including poliovirus, rhinovirus, enteroviruses, and hepatitis C virus. He teaches virology to graduate, medical, dental, and nursing students and uses social media to communicate the subject outside of the classroom. His Columbia University undergraduate virology lectures have been viewed by thousands at iTunes University, Coursera, and on YouTube. Vincent blogs about viruses at virology.ws and is host of the popular science program This Week in Virology.

Glenn Rall is a Professor and the Co-Program Leader of the Blood Cell Development and Function Program at the Fox Chase Cancer Center in Philadelphia. At Fox Chase, Dr. Rall is also the Associate Chief Academic Officer and Director of the Postdoctoral Program. He is an Adjunct Professor in the Microbiology and Immunology departments at the University of Pennsylvania, Thomas Jefferson, Drexel, and Temple Universities. Dr. Rall's laboratory studies viral infections of the brain and the immune responses to those infections, with the goal of defining how viruses contribute to disease in humans. His service to the scientific community includes membership on the Autism Speaks Scientific Advisory Board, Opinions Editor of *PLoS Pathogens*, chairing the Education and Career Development Committee of the American Society for Virology, and membership on multiple NIH grant review panels.

Anna Marie Skalka is a Professor and the W.W. Smith Chair in Cancer Research at Fox Chase Cancer Center in Philadelphia and an Adjunct Professor at the University of Pennsylvania. Dr. Skalka's major research interests are the molecular aspects of the replication of retroviruses. Dr. Skalka is internationally recognized for her contributions to the understanding of the biochemical mechanisms by which such viruses (including the AIDS virus) replicate and insert their genetic material into the host genome. Both an administrator and researcher, she has been deeply involved in state, national, and international advisory groups concerned with the broader, societal implications of scientific research, including the NJ Commission on Cancer Research and the U.S. Defense Science Board. Dr. Skalka has served on the editorial boards of peer-reviewed scientific journals and has been a member of scientific advisory boards including the National Cancer Institute Board of Scientific Counselors, the General Motors Cancer Research Foundation Awards Assembly, the Board of Governors of the American Academy of Microbiology, and the National Advisory Committee for the Pew Biomedical Scholars Program.



1 Infections of Populations: History and Epidemiology

Introduction to Viral Pathogenesis

A Brief History of Viral Pathogenesis

- The Relationships Between Microbes and the Diseases They Cause
- The First Human Viruses Identified and the Role of Serendipity
- New Techniques Led to the Study of Viruses as Causes of Disease

Viral Epidemics in History

- Epidemics Shaped History: the 1793 Yellow Fever Epidemic in Philadelphia
- Tracking Epidemics by Sequencing: West Nile Virus Spread to the Western Hemisphere
- The Economic Toll of Viral Epidemics in Agricultural Organisms
- Population Density and World Travel as Accelerators of Viral Transmission
- Zoonotic Infections and Viral Epidemics

Epidemiology

- Fundamental Concepts
- Tools of Epidemiology
- Surveillance

Parameters That Govern the Ability of a Virus to Infect a Population

- Environment
- Host Factors

Perspectives

References

LINKS FOR CHAPTER 1

⏭ *Video: Interview with Dr. W. Thomas London*
http://bit.ly/Virology_London

⏭ *Epidemiology causes conclusions ($p<0.05$)*
http://bit.ly/Virology_Twiv169

⏭ *Slow motion sneezing*
http://bit.ly/Virology_1-23-13

Swords, lances, arrows, machine guns, and even high explosives have had far less power over the fates of nations than the typhus louse, the plague flea, and the yellow-fever mosquito.

HANS ZINSSER
Rats, Lice and History 1934

Introduction to Viral Pathogenesis

While the title of Zinsser's classic volume *Rats, Lice and History* may trigger a wry smile, the ideas proposed in this classic volume about pathogens and the diseases they cause remain as relevant today as when they were published in 1934. As Zinsser argued, the global impact of pathogens, including viruses, has shaped human history as much as any war, natural disaster, or invention. This view may seem an exaggeration to today's student of virology, who probably perceives most viral infections as annoyances that cause unpleasant side effects and may result in a few missed classes or days of work. But in the context of history, **epidemics** of smallpox, yellow fever, human immunodeficiency virus, and influenza have resulted in an incalculable loss of life and have changed entire societies. Smallpox alone has killed over 300 million people, more than twice the number of deaths from all the wars in the 20th century. Huge empires fell to a relatively small number of invaders, in part because the conquerors inadvertently introduced viruses that crippled the empires' defense forces.

Although vaccines and antivirals have reduced, and even eliminated, some of these scourges, a recent influenza pandemic, the alarming number of human cases of Ebola virus in Africa, the lack of success in developing a human immunodeficiency virus vaccine, the resurgence of vaccine-preventable infections, and the emergence of "new" human viral pathogens, such as the coronavirus that causes Middle East Respiratory Syndrome, remind us of the challenges we still face. Of equal importance, while populations in resource-rich countries may be generally protected from some former viral foes, infections with vaccine-preventable viruses, including measles, polio, and hepatitis B virus, remain prevalent in countries that lack the money or infrastructure to ensure widespread vaccination.

The ways by which viruses cause diseases in their hosts, the tug-of-war among viruses and the host's defenses, and the impact that viral epidemics have had on human and animal populations are therefore not just interesting academic pursuits but rather life-and-death issues for all organisms. That said, it is important to bear in mind this critical fact: pathogenesis (the basis of disease) is often an unintended outcome of the parasitic lifestyle of viruses. As is true for humans, selective pressures that control viral evolution act only on the ability to survive and reproduce. From this perspective, one could argue that the most successful viruses are those that cause no apparent disease in their natural host.

In the first chapter of Volume I, we recounted an abbreviated history of virology and described milestones that established the foundation for our current understanding of viral reproduction. In this chapter, we return to history, focusing on watershed events that catalyzed the fields of viral **epidemiology** and pathogenesis. Subsequent chapters in this volume will consider the impact of viral infections on individual hosts, tissues, and cells. Our goal is to build on the principles of viral reproduction that were established in Volume I to provide a comprehensive and integrated view of how viruses cause disease in single cells, discrete hosts, and large populations.

PRINCIPLES *Introduction to viral pathogenesis*

- Koch's postulates helped to identify causal relationships between a microbe and the disease it caused in the host, though these Postulates may not always be applicable to virus infections.
- Major insights in viral pathogenesis have come from exploitation of technical advances in the fields of molecular biology and immunology.
- The increased mobility of human and animal populations on the planet has accelerated the emergence of epidemics.
- Many viruses that can infect multiple species establish a reservoir in an animal host in which the virus causes negligible disease. Spread into new human hosts, called zoonoses, are usually dead-end infections.
- Epidemiology, the study of infections in populations, is the cornerstone of public health research.
- Social interactions, individual differences among prospective hosts, group dynamics and behaviors, geography, and weather all influence how efficiently a virus can establish infection within a population.
- National and international agencies charged with monitoring outbreaks, implementing surveillance plans, disseminating vaccines and antivirals, and educating the public, must coordinate data obtained from a vast network of researchers, clinics, and physicians in the field.
- The regional occurrence of viral infections may be due to the restriction of a vector or animal reservoir to a limited geographical area.
- Seasonal differences in the appearances of some viruses may be due to variations in viral particle stability at various temperatures or humidity, changes in the integrity of host barriers (such as the skin or mucosa), or seasonal changes in the life cycles of viral vectors, such as mosquitoes.
- Susceptibility to infection and susceptibility to disease are independent.

A Brief History of Viral Pathogenesis

The Relationships between Microbes and the Diseases They Cause

Long before any disease-causing microbes were identified, poisonous air (miasma) was generally presumed to cause epidemics of contagious diseases. The association of particular microorganisms, initially bacteria, with specific diseases can be attributed to the ideas of the German physician Robert Koch. With his colleague Friedrich Loeffler, Koch developed four criteria that, if met, would prove a causal relationship between a given microbe and a particular disease. These criteria, **Koch's postulates**, were first published in 1884 and are still used today as a standard by which pathogens are identified. The postulates are as follows:

- the microorganism must be associated regularly with the disease and its characteristic lesions but should not be found in healthy individuals;
- the microorganism must be isolated from the diseased host and grown in culture;
- the disease should be reproduced when a pure preparation of the microorganism is introduced into a healthy, susceptible host; and
- the same microorganism must be reisolated from the experimentally infected host.

Guided by these postulates and the methods developed by Pasteur for the sterile culture and isolation of purified preparations of bacteria, researchers identified and classified many pathogenic bacteria (as well as yeasts and fungi) during the latter part of the 19th century. Identifying a cause-and-effect relationship between a microbe and a pathogenic outcome set the stage for transformative therapeutic advances, including the development of antibiotics.

During the last decade of the 19th century, however, it became clear that not all epidemic diseases could be attributed to bacterial or fungal agents. This breakdown of the paradigm led to the identification of a new class of infectious agents: submicroscopic particles that came to be called viruses (see Volume I, Chapter 1). Koch's postulates can often be applied to viruses, but not all virus-disease relationships meet these criteria. While compliance with Koch's principles **will** establish that a particular virus is the causative agent of a specific disease, failure to comply does not rule out a possible cause-and-effect relationship (Box 1.1).

The First Human Viruses Identified and the Role of Serendipity

The first human virus that was identified was the agent responsible for causing yellow fever. The story of its identification in 1901 is instructive, as it highlights the contributions of creative thinking, collaboration, serendipitous timing, and even heroism in identifying new pathogens.

Yellow fever, widespread in tropical countries since the 15th century, was responsible for devastating epidemics associated with extraordinary rates of mortality (for example, over a quarter of infected individuals died in the New Orleans epidemic of 1853). While the disease can be relatively mild, with transient symptoms that include fever and nausea, more-severe cases result in major organ failure. Destruction of the liver causes yellowing of the skin (jaundice), the symptom from which the disease name is derived. Despite its impact, little was known about how yellow fever was spread, although it was clear that the disease was not transferred directly from person to person. This property prompted speculation that the source of the infection was present in the atmosphere and led to desperate efforts to "purify" the air, including burning barrels of tar and firing cannons. Others believed that the pathogen was carried on **fomites**, such as bedding or clothing, although this hypothesis was disproved when volunteers remained healthy after sleeping in the nightwear of yellow fever victims.

The first real advance in establishing the origin, or **etiology**, of yellow fever came in 1880, when the Cuban physician Carlos Juan Finlay proposed that a bloodsucking insect, most likely a mosquito, played a part in the transmission of the disease. A commission to study the basis of yellow fever was

Figure 1.1 Conquerors of yellow fever. This painting by Dean Cornwell (1939) depicts the experimental exposure of James Carroll with infected mosquitoes. Walter Reed, in white, stands at the head of the table, while Jesse Lazear applies the infected mosquitoes to Carroll's arm. Also depicted in this painting is Carlos Finlay, in a dark suit. Despite the care that Cornwell took to ensure accuracy of his portrayal of the participants and their uniforms, the event documented in this painting never took place; rather, artistic license was used to place all the major players in one depiction of a watershed moment in medical history. Photo courtesy of Wyeth Pharmaceuticals.



BOX 1.1

DISCUSSION

Why viruses may not fulfill Koch's postulates

Although Koch's postulates provided a framework to identify a pathogen unambiguously as an agent of a particular disease, some infectious agents, including viruses, cause disease but do not adhere to all of the postulates. In fact, it has been argued that the rigid application of these criteria to viral agents may have impeded early progress in the field of virology. Koch himself became aware of the limitations of his postulates upon discovery that *Vibrio cholerae*, the agent of cholera, could be isolated from both sick and healthy individuals.

Application of these criteria to viruses can be particularly problematic. For example, the first postulate, which states that the microorganism must be "regularly associated" with the disease, does not hold true for many animal reservoirs, such as bats, in which the virus actively reproduces but causes no disease. Similarly, arthropod vectors, such as mosquitoes, support reproduction of a variety of hemorrhagic viruses but do not themselves die of such infections. As another example of the problem of unilaterally applying Koch's postulates to viruses, the second postulate states that the microorganism must be grown in culture. However, many viruses, including papillomaviruses that cause warts and cervical cancer and hepatitis B virus that causes liver cirrhosis and cancer, cannot be cultured, or require complex culture conditions that must mimic the tissue complexity found in the infected host. Consequently, it is generally accepted that the postulates are a guide, not an invariant set of requirements to fulfill.

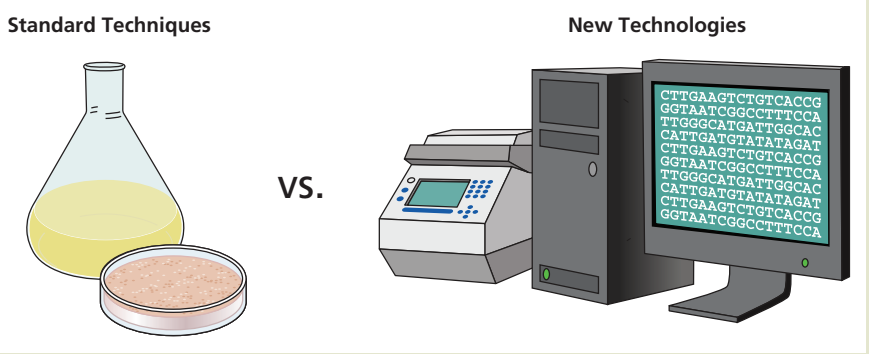
More recently, detection methods based on nucleic acid sequence have rendered Koch's original postulates even less relevant. Such approaches alleviate the requirement to culture the suspected agent and are sufficiently sensitive to detect the presence of vanishingly small quantities of viral nucleic acid in an apparently healthy individual. As such, a revised set of Koch's postulates that takes into consideration new technical capabilities has been proposed (see Volume I, Box 1.4).

Assiduously applying the postulates has been particularly problematic for identifying viruses that cause human tumors. As noted in a review by Moore and Chang, Koch's postulates "are a brilliant example of precision in scientific thinking, but they hold little practical value for 21st-century tumor virology since they cannot prove nor disprove most candidate tumor viruses to cause cancers." Whether Koch's postulates will continue to be a useful standard to identify pathogens or will become an historical footnote remains to be seen.

Fredericks, DN, Relman, DA. 1996. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clin Microbiol Rev* **9:**18–33.

Moore PS, Chang Y. 2014. The conundrum of causality in tumor virology: the cases of KSHV and MCV. *Semin Cancer Biol* **26:**4–12.

More-sensitive technologies, including DNA sequencing, have triggered a reconsideration of Koch's postulates.



established in 1899 in Cuba by the U.S. Army under Colonel Walter Reed. This commission was formed in part because of the high **incidence** of the disease among soldiers who were occupying Cuba. Jesse Lazear, a member of Reed's commission, confirmed Finlay's hypothesis when he allowed himself to be bitten by a yellow fever virus-infected mosquito. "I rather think I am on the track of the real germ," wrote Lazear to his wife, sadly just days before he died of yellow fever himself. The results of the Reed Commission's study proved conclusively that mosquitoes are the **vectors** for this disease. In retrospect, a mosquito-borne mode of transmission made sense, as the disease was predominately found in warm and humid regions of the world (e.g., Cuba, New Orleans) where mosquitoes were, and remain, abundant. The members of this courageous team, perhaps the first true epidemiologists, are depicted in a dramatic 1939 painting (Fig. 1.1).

The nature of the pathogen was established in 1901, when Reed and James Carroll injected diluted, filtered serum from the blood of a yellow fever patient into three healthy individuals. Two of the volunteers developed yellow fever, causing Reed and Carroll to conclude that a "filterable agent," which we now know as yellow fever virus, was the cause of the disease. In the same year, Juan Guiteras, a professor of pathology and tropical medicine at the University of Havana, attempted to produce immunity by exposing volunteers to mosquitoes that were allowed to take a blood meal from an individual who showed signs of yellow fever. Of 19 volunteers, 8 contracted the disease and 3 died. One of the deceased

was Clara Louise Maass, a U.S. Army nurse. Maass' story is of interest, as she had volunteered to be inoculated by infected mosquitoes some time before, developed only mild symptoms, and survived. Her agreement to be infected a second time was to test if her earlier exposure provided protection from a subsequent challenge. This was a prescient idea, because at that time, virtually nothing was known about immune memory. Maass' death prompted a public outcry and helped to end yellow fever experiments in human volunteers.

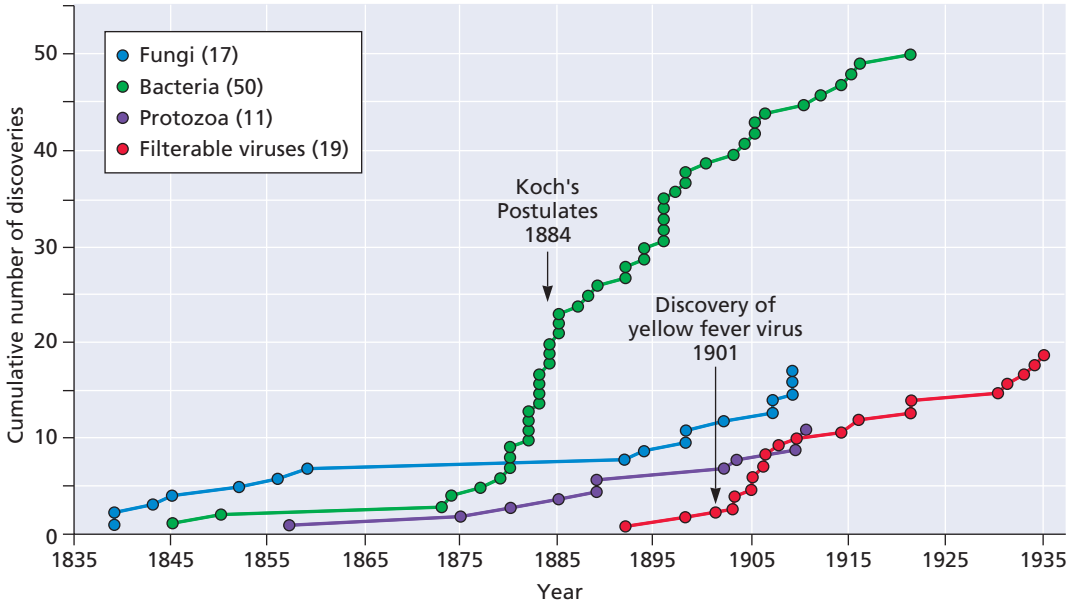
Yellow fever had been **endemic** in Havana for 150 years, but the conclusions of Reed and his colleagues about the nature of the pathogen and the vector that transmitted it led to rapid implementation of effective mosquito control measures that dramatically reduced the incidence of disease within a year. To this day, mosquito control remains an important method for preventing yellow fever, as well as other viral diseases transmitted by arthropod vectors.

Other human viruses were identified during the early decades of the 20th century (Fig. 1.2). However, the pace of discovery was slow, in great part because of the dangers and difficulties associated with experimental manipulation of human viruses so vividly illustrated by the experience with yellow fever virus. Consequently, agents of some important human diseases were not identified for many years and only then with some good luck.

A classic example is the identification of the virus responsible for influenza, a name derived in the mid-1700s from the Italian language because of the belief that the disease resulted from the "influence" of contaminated air and adverse astrological signs. Worldwide epidemics (**pandemics**) of influenza had been documented in humans for well over 100 years. Such pandemics were typically associated with mortality among the very young and the very old, but the 1918-1919 pandemic following the end of World War I was especially devastating. It is estimated that one-fifth of the world's population was infected, resulting in more than 50 million deaths, far more than were killed in the preceding war. Unlike in previous epidemics that affected the elderly and the very young, healthy young adults were often victims (Fig. 1.3).

Despite many efforts, a human influenza virus was not isolated until 1933, when Wilson Smith, Christopher Andrewes, and Patrick Laidlaw serendipitously found that the virus could be propagated in an unusual host. Laidlaw and his colleagues at Mill Hill in England were using ferrets in studies of canine distemper virus, a paramyxovirus unrelated to influenza. Despite efforts to keep these ferrets isolated from both the environment and other pathogens (for example, all ferrets were housed separately, and all laboratory personnel had to disinfect themselves before and after entering a room), it is thought that a lab worker infected with influenza transmitted the virus to a ferret. This ferret then developed a disease very similar to influenza in humans. Realizing the implications of their observation, Laidlaw and colleagues then infected naive ferrets with throat washings from sick individuals and isolated the virus now known as

Figure 1.2 Pace of discovery of new infectious agents. Koch's introduction of efficient bacteriological techniques spawned an explosion of new discoveries of bacterial agents in the early 1880s. Similarly, the discovery of filterable agents launched the field of virology in the early 1900s. Despite an early surge of virus discovery, only 19 distinct human viruses had been reported by 1935. Adapted from K. L. Burdon, *Medical Microbiology* (MacMillan Co., New York, NY, 1939, with permission.)



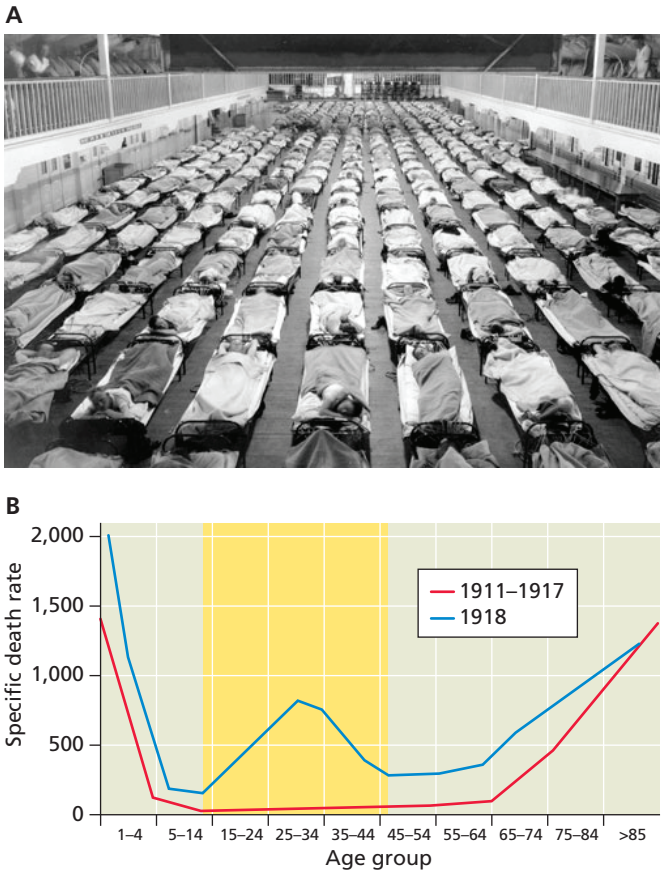


Figure 1.3 1918 flu consequences. (A) The 1918-1919 influenza pandemic infected a staggering number of people, resulting in the hasty establishment of cavernous quarantines in college gymnasia and large halls, filled with rows and rows of infected patients. Photo courtesy of the Naval History and Heritage Command. **(B)** Of particular concern, this epidemic had a high death rate among young, otherwise healthy, individuals compared to those of previous flu seasons. Adapted from R. Ahmed et al., *Nat. Immunol.* **8:**1188–1193, 2007, with permission.

influenza A virus. (Note the effective use of Koch's postulates in this study!) Subsequently, influenza A virus was shown to also infect adult mice and chicken embryos. The latter proved to be an especially valuable host system, as vast quantities of the virus are produced in the allantoic sac. Chicken eggs are still used today to produce influenza vaccines.

New Techniques Led to the Study of Viruses as Causes of Disease

Technological developments propelled advances in our understanding of how viruses are reproduced (Volume I, Chapter 1) and also paved the way for early insights into **viral pathogenesis**, the study of how viruses cause disease. The period from approximately 1950 to 1975 was marked by remarkable creativity and productivity, and many experimental procedures developed then are still in use today. With these techniques in hand, scientists performed pioneering studies that revealed how viruses, including mousepox virus, rabies virus, poliovirus, and lymphocytic choriomeningitis virus, caused illness in susceptible hosts.

Revolutionary developments in molecular biology from the mid-1970s to the end of the 20th century further accelerated the study of viral pathogenesis. Recombinant DNA technology enabled the cloning, sequencing, and manipulation of host and viral genomes. Among other benefits, these techniques allowed investigators to mutate particular viral genes and to determine how specific viral proteins influence cell pathology. The polymerase chain reaction (PCR) was first among the many new offshoots of recombinant DNA technology that transformed the field of virology. PCR is used to amplify extremely small quantities of viral nucleic acid from infected samples. Once sufficient viral DNA has been obtained and the sequence determined, the virus can be more easily identified and studied. The ability to sequence and manipulate DNA also led to major advances in the related field of immunology and, consequently, had an important impact on the investigation of viral pathogenesis. While many of the early studies in immunology focused on immune cell development, others began to address how immune cells recognized and responded to pathogens. The Nobel Prizes of the 1980s and 1990s highlight the importance of these new technologies; they include awards for the establishment of transgenic animals, gene targeting, immune cell recognition of virus-infected cells, and RNA interference. These discoveries, and the ways that they helped to shape our current view of viral disease, will be discussed in later chapters.

The emergence of molecular biology and cell biology as distinct fields marked a transition from a descriptive era to one that focused on the mechanisms by which particular viral processes were controlled, among other advances. Genomes were isolated, proteins were identified, functions were deduced by application of genetic and biochemical methods, and new animal models of disease were developed. These approaches not only defined basic steps in the viral life cycle and functions of virus-encoded proteins but also ushered in practical applications, including the development of diagnostic tests, antiviral drugs, and vaccines. As the 20th century came to a close, another paradigm shift was occurring in virology, as many scientists realized the power of a more holistic strategy to study virus-host relationships. These scientists embraced the concept of **systems biology**, the notion that all the molecules or reactions that govern a biological process could be identified and monitored during an infection, allowing discovery of new processes that were missed by the more reductionist, one-gene-at-a-time approaches. These ideas were initially developed using microarray technology, which enabled a global and unbiased snapshot of the quantities of both host and viral mRNAs under defined conditions.

New tools continue to expand our capabilities, and methods once considered cutting edge are eclipsed by more-powerful, faster, or cheaper alternatives. Parallel developments in information technology and computer analyses (often called "data mining") have been critical to infer meaningful conclusions from the massive data sets now commonly collected. Computer-aided approaches have enabled scientists to define cellular pathways that are triggered during viral infection, to identify common features among seemingly diverse viruses, and to make structural predictions about small-molecule inhibitors that could prevent infection. While these new tools are exciting and powerful, it is likely that traditional approaches will still be required to validate and advance the hypotheses that are emerging from systems biology. New technological developments should be viewed as adding to, rather than replacing, experimental strategies from the past.

While the methods that virologists employ may be ever-changing, the fundamental question asked by early pioneers is still with us: how do viruses cause disease? The remainder of this chapter focuses on the impact of viral infections in large populations and how outbreaks and epidemics begin.

Viral Epidemics in History

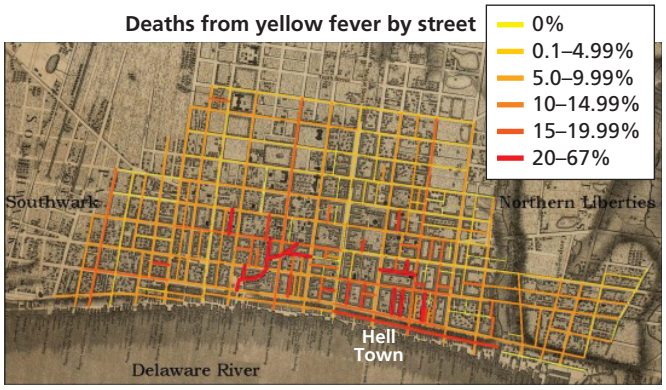
In the popular movies *Outbreak* (1995) and *Contagion* (2011), fictional epidemics were depicted following introduction of a virus into a naïve human population. Each movie included a pivotal scene in which an epidemiologist ominously described the devastating consequences of uncontrolled, exponential viral spread through a population. These movies were terrifying, exciting, and ultimately comforting, as humans, with improbable speed, developed a vaccine and gained the upper hand. But how realistic is this Hollywood vision? One could argue that proof of our triumph over viral pathogens can be found in the eradication of smallpox and the development of vaccines to prevent infection by many viruses that historically resulted in much loss of life, but there is a risk in becoming complacent. We may ignore how quickly a virus can spread in a susceptible population and forget the fear and feeling of helplessness that accompany viral epidemics. The four stories that follow highlight the financial toll, loss of life, and historical ramifications of viral epidemics and underscore a new reality: the increased mobility of human and animal populations on the planet will almost certainly accelerate the emergence of epidemics.

Epidemics Shaped History: the 1793 Yellow Fever Epidemic in Philadelphia

One powerful example of a deadly viral epidemic that influenced American history and changed how cities are managed is the yellow fever outbreak in Philadelphia, Pennsylvania. In 1793, when this epidemic occurred (and a full century before Walter Reed's commission), nothing was known about this disease or how it was spread. No one at the time knew that viruses existed, so the seemingly random way that individuals became sick compounded the confusion. Furthermore, this epidemic struck at a pivotal time for the fledgling union. At that time, Philadelphia was the new nation's temporary capital and was a city of active commerce and trade. One can easily imagine the panic in Philadelphia when scores of individuals became ill and died of this mysterious disease within a very short time frame. In the 101 days between August 1 and November 9, some 5,000 people died in a city of about 45,000, making this one of the most severe epidemics in the history of the United States (Fig. 1.4). There were few families that did not lose a relative to this disease, and many entire families were lost. Those who could flee the city did so, including the new President, George Washington, and his cabinet. Others stayed behind to aid the sick, including men of the Free African Society, who volunteered on the basis of the incorrect notion of Benjamin Rush, a prominent Philadelphia physician, that people of color were immune to infection.

Because Philadelphia was a major port city, it is likely that the agent, which we now know was the yellow fever virus, was transported by infected mosquitoes on cargo ships and that standing water in the city provided a hospitable breeding ground for the insects. Credit goes to Rush, who noticed identical symptoms in many victims and who recommended that individuals either leave the city or quarantine themselves, practices that helped to curtail the epidemic. Rush's belief that the scourge arose from a pile of rotting coffee beans left on a dock, and his treatment regimen of purging and bloodletting is less worthy of praise.

Figure 1.4 Deaths caused by the yellow fever epidemic in Philadelphia, 1793. This map records the locations of deaths due to yellow fever, with red and orange streets marking those with highest mortality. Yellow fever was most deadly near the northern wharves, where poorer people lived and where Hell Town was located. Both areas furnished breeding places for *Aedes aegypti*, the type of mosquito that transmits the disease. Adapted from Paul Sivitz and Billy G. Smith, with permission.



The city of Philadelphia was transformed after the epidemic. The outbreak, believed by many to be due to contaminated water (which was, in part, true), incentivized the local government to establish a municipal water system, the first major city in the world to do so. Infirmaries to tend to the sick (and isolate them from the healthy) were developed. Finally, the epidemic spurred a city-supported effort to keep streets free of trash, leading to the development of a sanitation program that would be a model for similar programs elsewhere.

Tracking Epidemics by Sequencing: West Nile Virus Spread to the Western Hemisphere

It took a full century to determine the cause of the Philadelphia epidemic, but technological advances have greatly accelerated our ability to understand the natural histories of some modern-day outbreaks. While the sudden appearance of West Nile virus in the Western Hemisphere in 1999 fortunately did not result in massive loss of life, this epidemic is notable for the role that viral genome sequencing played in defining its origin in the Middle East.

Prior to the summer of 1999, West Nile virus infections were restricted to Africa and the Mediterranean basin. Upon introduction to the United States, West Nile virus spread with remarkable speed; in 3 years, the incidence of infection expanded from eight cases in Queens (a borough of New York City) to virtually all of the United States and much of Canada, where it is now endemic (Fig. 1.5). The eight cases first identified in Queens held the key for major epidemiologic efforts to identify the source of this new infection. All victims had been healthy, and many had engaged in outdoor activities soon before showing signs of sickness. At about the same time, a high proportion of dead birds were found in and around New York City, prompting epidemiologists to consider that the same virus had infected both hosts. PCR and genome sequencing were used to confirm that West Nile virus was the cause of the bird deaths and the human illnesses.

How West Nile virus arrived in North America will never be known conclusively, but many think that the culprit was an infected mosquito (the natural **reservoir** for West Nile virus) that arrived as a stowaway on a flight from Israel to New York. This scenario was deduced from the remarkable similarity between genome sequences of a virus isolated in New York and an isolate obtained from an infected goose in Israel. It is sobering to contemplate that a virus that can now be found in virtually all states and provinces of North America may have begun with a single mosquito, perhaps trapped in a suitcase or purse: an invisible passenger on a trans-Atlantic flight.

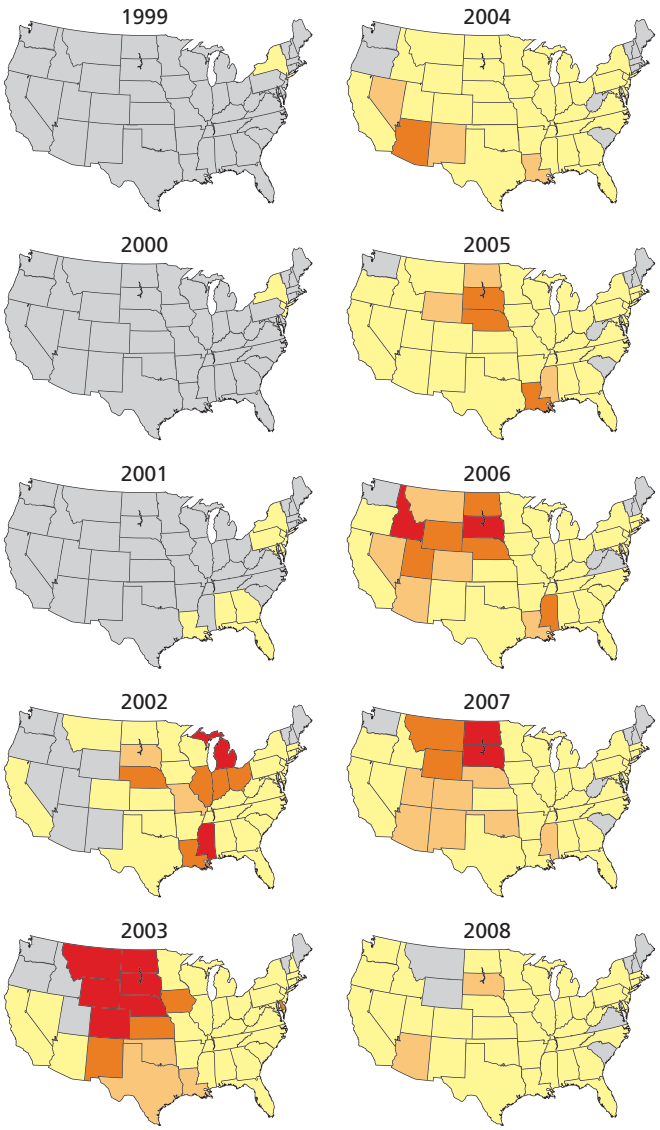


Figure 1.5 Spread of West Nile virus in the United States. The maps show West Nile virus incidence per 100,000 inhabitants in each state of the United States from 1999 to 2008. Credit: Centers for Disease Control and Prevention, with permission.

The Economic Toll of Viral Epidemics in Agricultural Organisms

Epidemics can affect animals as well, especially those in dense farming populations. The outbreak of foot-and-mouth disease in the United Kingdom in 2001 caused an agricultural crisis of historical proportions; over 10 million sheep and cattle were killed (an average of 10,000 to 13,000 per day) in an attempt to stop the infection from spreading. In this instance, the original infected animal (the "**index case**") could be traced to one pig on a specific farm in Northumberland. Unfortunately, the owner did not inform the authorities of the appearance of foot-and-mouth disease, which is relatively easy to identify, on his farm. The epidemic spread rapidly, accelerated by the use of trucks by both contaminated and noncontaminated farms to transport animals to slaughterhouses. While this outbreak

did not affect humans directly, the indirect financial impact on farming and tourism was enormous; it is estimated that this crisis cost the United Kingdom over $16 billion and almost brought down the government. A vaccine for foot-and-mouth disease virus exists and was available during the epidemic. However, at that time, vaccine use was rejected because farmers feared that they would then not be able to ship their meat to other countries, as vaccinated animals cannot be serologically distinguished from infected animals. A positive consequence of this preventable infection is that all farm animals in the United Kingdom are now vaccinated for foot-and-mouth disease virus. Other viruses of ruminants, however, including Bluetongue virus, remain threats to this day.

Population Density and World Travel as Accelerators of Viral Transmission

While the thought of an ocean cruise may evoke images of endless buffets and piña coladas by the pool, to viral epidemiologists, such pleasure ships appear as prime breeding grounds for viral epidemics. Norwalk virus, a member of the norovirus family, is most often associated with cruise ship outbreaks of gastroenteritis, although other viruses can also cause these nautical nightmares. Moreover, Norwalk virus is not restricted to ships; hot spots include any place in which many people from various locations are in close proximity for an extended period. Other high-density environments include prisons, long flights, day care facilities, dormitories, and elderly care communities. The risk of transmission is enhanced by the fact that noroviruses are quite hardy and can be transmitted either person to person or via contaminated food or surfaces, resulting in the need to decontaminate all shared surfaces with chlorine-containing solutions following an outbreak. While the gastrointestinal effects of a noroviral infection (nausea, vomiting, and diarrhea) are unpleasant, the disease is short-lived, and patients usually recover quickly. However, the frequency with which these outbreaks strike is a chilling reminder that, despite improved tools to characterize viral epidemics and reduce their spread, the ease and prevalence of world travel greatly facilitate the encounter between viruses and new hosts.

Zoonotic Infections and Viral Epidemics

Viral epidemics often appear seemingly without warning, raising questions about their origins. Some viral epidemics begin with a zoonotic infection, discussed in detail in Chapters 10 and 11. **Zoonoses** are infections transmitted between species, usually to humans from other animals. Many viruses that can infect multiple species establish a reservoir in a host in which the virus causes no disease or only nonlethal disease. When a new host is in proximity to an infected reservoir animal, a species jump may occur. While zoonotic transmission may cause disease in the new host, transspecies infection is usually a dead end for the virus. Consequently, zoonotic infections rarely spread from human to human, as is the case for rabies virus, West Nile virus, and avian influenza. Although relatively rare, zoonotic infections are a concern to epidemiologists, because the new host will not have immunity and the disease that occurs in the new host may be different (often more severe) than that in the reservoir host. The transspecies spread of a human immunodeficiency virus-like ancestor from monkeys to humans is a prime example (Box 1.2).

As increased contact between species is the predominant risk factor for zoonotic infection, one can envision how changes in the environment or ecosystems of some animals may increase the risk for contact among different species. This is of particular concern when humans invade wilderness areas. For example, it is thought that Nipah virus, a paramyxovirus for which bats are the natural reservoir, underwent species-to-species transmission in 1999 in Malaysia, when pig farming began in the habitat occupied by infected fruit bats. The virus moved from bats to pigs and ultimately to the farmers themselves. However, it is not necessary to invoke an exotic locale or complex combination of animals for zoonosis to occur; petting zoos, open markets, and state fairs provide sufficient human-animal contact to allow a virus to jump species.

Epidemiology

The study of viruses can be likened to a set of concentric circles. The most basic studies in virology comprise the detailed analyses of the genome and the structures of viral particles and proteins, which are crucial to understanding the biochemical consequences of the interaction of viral with host cell proteins. How infection of individual cells affects the tissue in which the infected cells reside and how that infected tissue disturbs the biology of the host define the landscape of the field of viral pathogenesis (discussed in the next four chapters). But if a viral population is to survive, transmission must occur from an infected host to susceptible, uninfected hosts. The study of infections of populations is the discipline of epidemiology, the cornerstone of public health research.

An epidemiologist investigates outbreaks by undertaking careful data collection in the field (that is, where the infections occur) and performing statistical analyses. Often, questions such as "how might the symptoms observed in an infected individual implicate one mode of viral transmission over another?" or "can a timeline be established to trace back the origins of an epidemic to a single event?" are asked. The answers help epidemiologists learn more about the pathogen that caused the epidemic. Social interactions, individual differences among prospective hosts, group dynamics and behaviors, geography, and weather, all influence how efficiently a virus can establish infection within a population. Epidemiologists lack the luxury of performing controlled experiments in which only one variable is manipulated. Consequently, they must consider many parameters

BOX 1.2

TRAILBLAZER

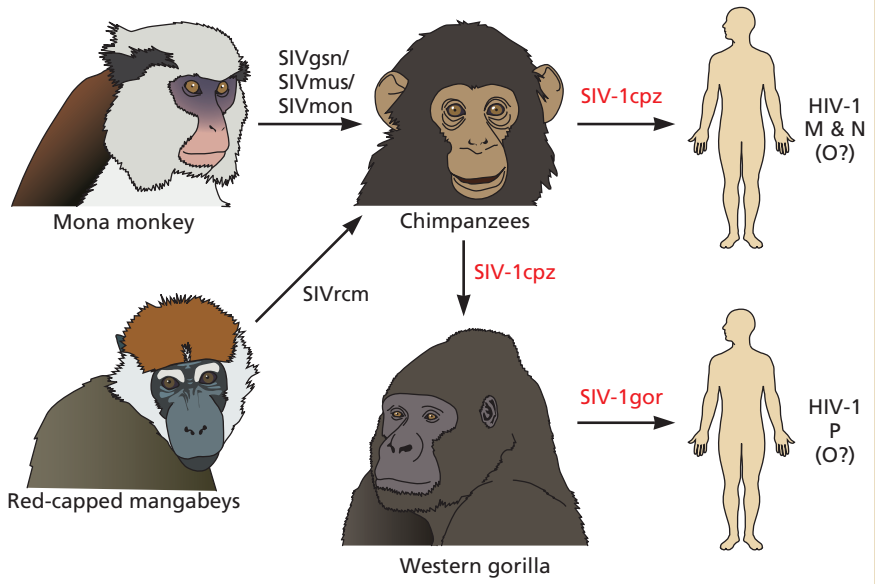
Zoonotic transmission of human immunodeficiency virus to humans

Acquired immunodeficiency syndrome (AIDS) of humans is caused by one of two lentiviruses, human immunodeficiency viruses types 1 and 2 (HIV-1 and HIV-2). Both HIV types arose as a result of multiple cross-species transmissions of simian immunodeficiency viruses (SIVs), which naturally infect African primates (see the figure). While most species-to-species transfers resulted in viruses that spread to humans to only a limited extent, a transmission of SIV from chimpanzees (SIVcpz) in southeastern Cameroon gave rise to HIV-1 group M, the principal cause of the AIDS pandemic. An AIDS-like disease likely afflicted chimpanzees for a long period before the recognition of human immunodeficiency virus as a human pathogen. Tracing the genetic changes that occurred as SIVs crossed from monkeys to apes and from apes to humans provides a new framework to examine the requirements of successful host switches and to gauge future zoonotic risk.

Sharp PM, Rayner JC, Hahn BH. 2013. Great apes and zoonoses. *Science* **340:**284–286.

Sharp PM, Hahn BH. 2011. Origins of HIV and the AIDS pandemic. *Cold Spring Harb Perspect Med* **1:**a006841.

Evolution of the human immunodeficiency virus-1 from monkey and primate hosts. Adapted from P. M. Sharp and B. H. Hahn, *Cold Spring Harb. Perspect. Med.* **1:**a006841, 2011, with permission.



simultaneously to identify the source and transmission potential of a viral pathogen within a host community. Many of these variables are captured faithfully in various video games and apps that simulate outbreaks (Box 1.3). In the next section, we identify some crucial terms and concepts used in this field (For commentary and a personal account related to the topic, see the interview with Dr. Thomas London: http://bit.ly/Virology_London).

Fundamental Concepts

Incidence versus Prevalence

Determining the number of infected individuals is a primary goal of epidemiological studies. This information is required to establish both the incidence and the **prevalence** of infection. Incidence is defined as the number of new cases within a population in a specified period. Some epidemiologists use this term to determine the number of new cases in a community during a particular period of time, while others use incidence to indicate the number of new disease cases per unit of population per period. For example, the incidence of influenza can be stated as the number of reported cases in New York City per year or the number of new cases/1,000 people/year. Disease prevalence, on the other hand, is a measure of the number of infected individuals at one moment in time divided by an appropriate measure of the population. A highly infectious and lethal disease (such as the 1793 epidemic of yellow fever in Philadelphia) may have a high incidence but a low prevalence because many of the infected individuals either died or cleared the infection. In contrast, a virus that can persist in a host for decades is likely to have high prevalence. An example of high prevalence is provided by hepatitis B virus; of the 300 to 400 million people infected globally, one-third live in China, with 130 million carriers. For this reason, incidence is an informative measure for acute infections, whereas prevalence is often used to describe persistent infections in which disease onset is not easily determined.

Prospective and Retrospective Studies

Infections of natural populations obviously differ from those under controlled conditions in the laboratory. Nevertheless, it is possible to determine if one or more variables affect disease incidence and viral transmission in nature. Two general experimental approaches are used: **prospective** (also called cohort or longitudinal) and **retrospective** (or case-controlled) studies. In prospective studies, a population is randomly divided into two groups (cohorts). One group then gets the "treatment of interest," such as a vaccine or a

BOX 1.3

DISCUSSION

Video games model infectious-disease epidemics

The hugely popular online video game *World of Warcraft* became a model for the transmission of virus infections. In this game, players interact in a fantasy world populated by humans, elves, orcs, and other exotic beasts. In late 2005, a dungeon was added in which players could confront and kill a powerful creature called Hakkar. In his death throes, Hakkar hit foes with "corrupted blood," infected with a virus that caused a fatal infection. The infection was meant to affect only those in the immediate vicinity of Hakkar's corpse, but the virus spread as players and their virtual pets traveled to other cities in the game. Within hours after the software update that installed the new dungeon, a full-blown virtual epidemic ensued as millions of characters became infected.

Although such games are meant only for entertainment, they do model disease spread in a mostly realistic manner. For example, the spread of the virus in Hakkar's blood depended on the ease of travel within the game, interspecies transmission by pets, and transmission via asymptomatic carriers. While computer models of epidemics have been developed, they lack the variability and unexpected outcomes found in real-world epidemics. Massive multiplayer online role-playing games have a large number of participants (at one point, over 10 million for *World of Warcraft*), creating an excellent community for experimental study of infectious diseases. While entertainment is the central focus of such games, the players are serious and devoted, and their responses to dangerous situations approximate real-world reactions. For example, during the "corrupted-blood" epidemic, players with healing ability were the first to rush to the aid of infected players. This action probably affected the dynamics of the epidemic because infected players survived longer and were able to travel and spread the infection. Multiplayer video games provide a nontraditional but powerful opportunity to examine the consequences of human actions within a statistically significant and danger-free computer simulation.

A more reality-based smart phone app called *Plague Inc*. asks: "Can you infect the world?" and gives players the opportunity to choose a pathogen and influence its evolution. Players compete against the clock, trying to destroy humanity before the world can develop a cure. Like *World of Warcraft*, *Plague Inc.* is immensely popular and has been downloaded over 10 million times. Despite their macabre objective, such pursuits provide an education in epidemiology. Successful players learn to integrate multiple variables simultaneously, including environment, time, and population density, and these applications demonstrate how the reproductive cycle of a virus may change over the course of an epidemic. However, the parallels to real-world epidemiology end there; a player can begin again with the click of a button or the flick of a finger. Alas, real life does not come with "do-overs."

Lofgren ET, Fefferman NH. 2007. The untapped potential of virtual game worlds to shed light on real world epidemics. *Lancet Infect Dis* **7**:625–629.



drug, and the other does not. The negative-control population often receives a placebo. Whether a person belongs to the treatment or placebo cohort is not known to either the recipient or the investigator until the data are collected and the code is broken ("**double blind**"). This strategy removes potential investigator bias and patient expectations that may otherwise skew the data. Once the data are collected, the code is broken, and the incidence of disease or side effect is determined for each cohort and compared. Prospective studies require a large number of subjects, who often are followed for months or years. The number of subjects and time required depend on the incidence of the disease or side effect under consideration and the statistical **power** required to draw conclusions.

In contrast, retrospective studies are not encumbered by the need for large numbers of subjects and long study times. Instead, some number of subjects with the disease or side effect under investigation is selected, as is an equal number of subjects who do not have the disease. The presence of the variable under study is then determined for each group. For example, in one retrospective study of measles vaccine safety and childhood autism, a cohort of vaccinated children and an equivalent cohort of age-matched unvaccinated children were chosen randomly. The proportion of children with or without autism was then calculated for each group to determine if the rate of occurrence of autism in the vaccinated group was higher, lower, or the same as in the unvaccinated group. The incidence of the side effect in each group is then calculated; the ratio of these values is the relative risk associated with vaccination. In this example, the rate of autism was not found to be different in the two groups, showing that vaccination is not a risk factor for the development of this disorder (see Chapter 8).

Mortality, Morbidity, and Case Fatality Ratios

Three other measures used in epidemiology can cause confusion because of the similarity of their definitions: **mortality**, **morbidity**, and **case fatality ratios** (Box 1.4). Mortality is expressed as a percentage of deaths in a known population

BOX 1.4

TERMINOLOGY

Morbidity, mortality, incidence, and case fatality

The terminology used to calculate the number of people who are infected and/or who become ill following a viral outbreak can be confusing. The following fictional example will be used to clarify these definitions.

Imagine that, in a city of 100,000 residents, a virus causes infection of 10,000 persons (as determined by serology). Of these 10,000, 7,000 develop signs of illness and 500 die of the infection.

- The **incidence** of this infection is the number of people infected divided by the population (10,000/100,000, or 10%).
- **Morbidity** is the number of individuals who became ill divided by the number of infected individuals (7,000/10,000, or 70%).
- **Mortality** is the number of deaths divided by the number infected (500/10,000, or 5%).
- The **case-fatality ratio** is the number of deaths divided by the number of individuals with illness (500/7,000, or 7.1%).



of infected individuals. Thus, 40 deaths in a population of 2,000 infected individuals would be expressed as 2% mortality (40/2,000). The morbidity rate is similar but refers to the number of infected individuals in a given population that show symptoms of infection per unit of time. The morbidity percentage will always be higher than the mortality percentage, of course, because not all sick individuals will die of the infection.

In contrast, a case fatality ratio is a measure of the number of deaths among clinical cases of the disease, expressed as a percentage. As an example, if 200 people are diagnosed with a respiratory tract infection and 16 of them die, the case fatality ratio would be 16/200, or 8%. In a technical sense, the use of the word "ratio" is incorrect; a case fatality ratio is actually more a measure of relative risk than a ratio between two numbers.

While statistics are crucial to all studies in virology, they are of particular value in viral epidemiology, in which outcomes and causes are rarely black or white. An understanding of terms in statistics and some essential principles concerning the use of statistics in virology are provided in Box 1.5.

Tools of Epidemiology

We have considered some of the terms that epidemiologists use, but how do these scientists monitor and develop strategies to control the spread of viruses in populations? An investigation begins at the site of an outbreak, where as much descriptive data as possible about the infected cases and the environment are gathered. In cases of viral infections in humans, information on recent travel, lifestyle, and preexisting health conditions is considered, along with the medical records of infected individuals to generate a testable hypothesis about the origin of the outbreak. The word "descriptive" can have a negative connotation in virology and is often used to mean the opposite of "mechanistic." However, in epidemiology, descriptive studies are essential to establish or exclude particular hypotheses about the origins of an outbreak. Indeed, descriptive epidemiology was the cornerstone for the discovery of human immunodeficiency virus during the AIDS epidemic in the 1980s (Box 1.6). Following the descriptive phase, analytical epidemiological methods are used to test hypotheses using control populations in either retrospectively or prospectively focused studies. Clinical epidemiology focuses on the collection of biospecimens, such as blood, sputum, urine, and feces, to search for viral agents or other pathogens and to help determine the potential route of transmission. Once specimens are collected, nucleic acid sequencing is often performed on the samples. In addition, such studies may include serological analyses, in which antibodies in the blood that implicate previous infection are identified. A timeline of the discovery of the H1N1 strain of influenza virus in 2009 illustrates the speed and coordination of epidemiological

BOX 1.5

METHODS

The use of statistics in virology

When studying viral infections in hosts, scientists do not always obtain results that are so clear and obvious that everyone agrees with the conclusions. Often the effects are subtle, or the data are highly varied from sample to sample or from study to study (sometimes referred to as "noise"). This ambiguity is particularly true in epidemiological studies, given the large number of parameters and potential outcomes. How do you know if the data that you generated (or that you are reading about in a paper) are significant?

Statistical methods, properly employed, provide the common language of critical analysis to determine whether differences observed between or among groups are significant (**Table 1.1**). Unfortunately, surveys of articles published in scientific journals indicate that statistical errors are common, making it even more difficult for the reader to interpret results. In fact, the term "significant difference" may be one of the most misused phrases in scientific papers, because the actual statistical support for the statement is often absent or incorrectly derived. While a detailed presentation of basic statistical considerations for virology experiments is beyond the scope of this text, some guiding principles are offered.

It is essential to consider experimental design carefully before going to the bench or to the field. A fundamental challenge in study design is to predict correctly the number of observations required to detect a significant difference. The significance level is defined as the probability of mistakenly saying that a difference is meaningful; typically, this probability is set at 0.05. Scientists do not usually refer to things as "true" or "false" but rather use quantitative approaches to provide a sense of the significance between two data sets (e.g., experimental versus control). An important concept is power, the probability of detecting a difference that truly is significant. In the simplest case, power can be increased by having a larger sample size (**Table 1.2**). Even when results seem black and white, having too few animals (or replicates) is insufficient for drawing a statistically meaningful conclusion.

It is critical to include a detailed description of how statistical analyses were performed in all communications linked with the data. The tests that were used to determine significance are just as important as the description of methods used to generate the data. Benjamin Disraeli, a 19th-century British Prime Minister, once said, "There are three kinds of lies: lies, damned lies, and statistics." Indeed, a gullible reader may be persuaded that a certain set of data is significant, but this conclusion depends on the stringency and appropriateness of the tests that were used, as well as the data points included in the analysis.

While this text cannot define what tests are applicable for which assays, we can make a few strong suggestions. Statistics should not be considered an afterthought or a painful process that one does in retrospect when putting data together for a publication. Reliable studies that stand the test of time have considered statistics throughout the scientific process, and good statistics are as critical as good study design. Do not be fearful of statistics. While it is true that the field can become quite complex, most of the tests used by virologists are reasonably straightforward. Computer programs such as Excel and GraphPad have made the calculations easy, but you need to know which tests to apply. Fortunately, there are excellent books available that make statistics logical and accessible (e.g., *Intuitive Biostatistics*, by Harvey Motulsky). For more-complex data, study design issues, and analyses, one may require consultation with a statistician.

Motulsky H. 2013. *Intuitive Biostatistics: a Nonmathematical Guide to Statistical Thinking*, 3rd ed. Oxford University Press, Oxford, United Kingdom.

Table 1.1 Statistical terms[a]

Term	Definition
Alternative hypothesis	Hypothesis that contradicts the null hypothesis
Binary data	Data that consist of only two values (e.g., positive and negative)
Cardinal data	Data that are on a scale in which common arithmetic is meaningful
Confidence interval	Likely range of the true value of a parameter of interest
Hypothesis testing	Use of statistical testing to objectively assess whether results seen in experiments are real or due to random chance
Nonparametric test	Statistical test that requires no assumptions regarding the underlying distribution of the data
Normally distributed data	Data which, when plotted in a histogram, look approximately like a bell-shaped curve
Null hypothesis	Hypothesis which presumes that there are no differences between treated and untreated groups; if hypothesis testing results in a statistically significant difference, the null hypothesis is rejected
P value	Probability of getting a result as extreme as or more extreme than the value obtained in one's sample, given that the null hypothesis is true
Parametric test	Statistical test that assumes that the data follow a particular distribution (e.g., normal)
Power	Probability of detecting a statistically significant difference that truly exists
Sample size	Number of experimental units in a study
Significance level	Probability of falsely finding a statistically significant difference

[a]Reprinted from B. A. Richardson and J. Overbaugh, *J. Virol.* **79:**669–676, 2005, with permission.

Table 1.2 *P* values for the differences in infection rates between experimental and control groups[a]

No. of animals per group	*P* value for indicated group[b]: All control animals infected and no experimental animals infected	All control animals and one experimental animal infected or one control animal infected and no experimental animal infected	One control animal infected and one experimental animal infected
3	0.1	0.4	1.0
4	0.03	0.1	0.5
5	0.008	0.05	0.2
6	0.002	0.02	0.08
7	<0.001	0.005	0.03
8	<0.001	0.001	0.01

[a]Reprinted from B. A. Richardson and J. Overbaugh, *J. Virol.* **79:**669–676, 2005, with permission.

[b]Determined by Fisher's exact test, using a two-sided hypothesis test with the significance level fixed at 0.05. Fisher's exact test is used because it is appropriate for experiments with small numbers of observations.

BOX 1.6

DISCUSSION

Descriptive epidemiology and the discovery of human immunodeficiency virus

Acquired immunodeficiency syndrome (AIDS) was first recognized as a new disease in the United States by physicians in New York, Los Angeles, and San Francisco, who independently noticed that some of the young homosexual male patients in their practices had developed unusual diseases, such as *Pneumocystis carinii* pneumonia (PCP) and Kaposi's sarcoma, which were typically associated with immunosuppressed patients. The first report in the medical literature that described this apparently new syndrome appeared in June 1981 and described five young homosexual men in Los Angeles with PCP. Other reports of a similar syndrome in individuals who injected drugs soon followed. While these "descriptive" observations raised many questions and incited much anxiety, they laid the foundation for the subsequent mechanistically focused work that identified the human immunodeficiency virus as a new human pathogen.

Centers for Disease Control and Prevention. 1981. Pneumocystis pneumonia—Los Angeles. *MMWR Morb Mortal Wkly Rep* **30:**250–252.

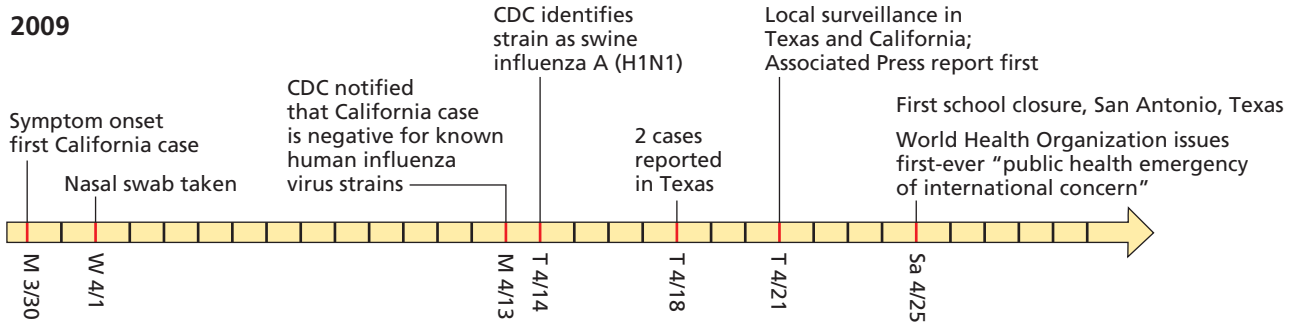
efforts to identify and thwart widespread dissemination of this virus (Fig. 1.6).

Surveillance

A final function of epidemiology is the establishment of vigilant surveillance procedures that can shorten the period between the beginning of an epidemic and its detection. One could argue that the development of worldwide surveillance programs and information sharing have had as profound an impact on limiting viral infections as antiviral medications and vaccines. The U.S. Centers for Disease Control and Prevention (CDC) was established in 1946 after World War II, with a primary mission to prevent malaria from spreading across the country. The scope of the CDC quickly expanded, and this institution is now a central repository for information and biospecimens available to epidemiologists; it also offers educational tools to foster awareness and ensure the safety of the public. The World Health Organization (WHO), founded in 1948 as an international agency of the United Nations, is charged with establishing priorities and guidelines for the worldwide eradication of viral agents. The WHO provides support to countries that may not have the resources to combat infectious diseases and coordinates results from a global network of participating laboratories. While the WHO provides coordination, the experimental work is performed in hundreds of laboratories throughout the world, often in remote locations which process samples and relay information back to the WHO. These WHO-certified laboratories adhere to stringent standards to ensure consistency of methods and interpretations. The laboratories conduct field surveillance using wild and sentinel animals and perform periodic blood screening for signs of infection or immunity. Sentinel animals ("canaries in the coal mine") allow rapid identification of new pathogens that may have entered a particular ecosystem. The chief successes of such global-surveillance efforts to date include the eradications of smallpox virus and Rinderpest virus, a relative of measles virus that causes disease in animals used in agriculture, such as cattle and sheep.

The Internet is a powerful tool for data sharing and public education. Publications and websites help to distribute consistent and timely information to health care workers across the globe. The weekly *Morbidity and Mortality Weekly Report*, published by the CDC, provides a central clearinghouse for health care providers in the United States to communicate individual cases of infectious diseases or to report unusual observations. ProMED (Program for Monitoring Emerging Diseases), sponsored by the International Society for Infectious Diseases, is a worldwide effort to promote communication among members of "the international infectious disease community, including scientists, physicians, epidemiologists, public health professionals, and others interested in infectious diseases on a global scale" (http://www.promedmail.org/aboutus/). Reporting of individual cases, when considered by epidemiologists in the aggregate, may catch an epidemic in its earliest days, when intervention is most effective. Use of

Figure 1.6 Discovery of the H1N1 strain of influenza virus (swine flu). Less than one month transpired between the first case (in San Diego County, CA) and the first press conference announcing the new strain. Adapted from ECDC Technical Emergency Team, *Eurosurveillance* **14**(18):pii=19204, 2009, with permission.



real-time data-gathering tools, such as Google Flu Trends, a Web-based application that surveys search queries from over 25 countries to predict influenza epidemics, have also emerged recently. While the predictions made from this application have been generally consistent with more-traditional surveillance data-gathering approaches, its accuracy and practical utility have not yet been proven. Nevertheless, the innovative use of keyword collection to monitor viral outbreaks underscores how collaboration between distinct fields (e.g., epidemiology and search engine design) can lead to creative ways to detect incipient outbreaks.

Parameters That Govern the Ability of a Virus To Infect a Population

One often hears that a virus is "going around," and such comments usually correlate with particular times of year (i.e., flu season). The seasonal appearance of some viruses, especially those that cause respiratory and gastrointestinal disease, raises the question of what parameters facilitate such spread in a population. This question is relevant both to viruses that cause widespread epidemics and to local, seasonal infections, such as the common cold. Identifying the variables associated with increased risk in a population has obvious value in clinical efforts to prevent outbreaks. As described below, multiple aspects of both host and environment contribute to maintaining a virus in a community.

Environment

Geography and Population Density

Some viruses are found only in specific geographical locations. The regional occurrence of viral infections may be due to the restriction of a vector or animal reservoir to a limited area. For example, most insect vectors are restricted to a specific region or ecosystem; unless this vector "escapes" its natural habitat, the viruses that it harbors will also be geographically constrained. Changes in migration routes or territory of a reservoir species may therefore influence the distribution of a virus and lead to new interactions with other species, increasing the risk of zoonotic transmission. A striking example of how a vector can change where a virus is found is provided by the global spread of the once-rare Chikungunya virus (Box 1.7).

BOX 1.7

DISCUSSION

An exotic virus on the move

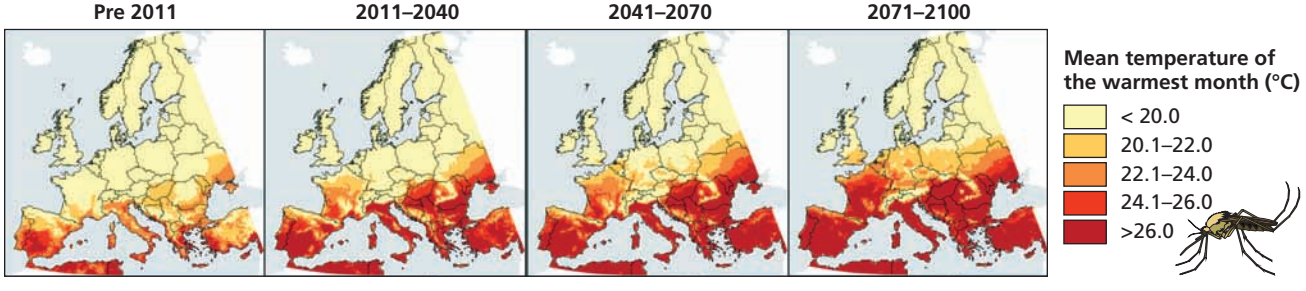
Chikungunya virus is a togavirus in the alphavirus genus. The virus is spread by mosquitoes (primarily the notorious *Aedes aegypti*). The viral disease has been known for more than 50 years in the tropics and savannahs of Asia and Africa but had never been a problem of the developed countries in Europe or the United States. The disease is uncomfortable (rashes and joint pains) but not fatal. In the last 5 years, however, something changed dramatically and brought this once exotic disease into the forefront of public concern.

In 2004, outbreaks of Chikungunya disease spread rapidly from Kenya to islands in the Indian Ocean and then to India, where it had not been reported in over 30 years. In some of the Indian Ocean islands, more than 40% of the population fell ill. In 2007, there was an outbreak in Italy, the first ever in Europe. What had happened to change the pattern of infection?

An alarming finding was that the Asian tiger mosquito (*Aedes albopictus*) became an efficient new vector for the virus. A point mutation in the viral genome appears to be the cause of the vector expansion and, perhaps, for the epidemic spread of the disease in areas where it had been unknown. *A. albopictus*, which has a greater geographical range than *A. aegypti*, is spreading across the globe from eastern Asia and is now found in mainland Europe and the United States. This mosquito is a maintenance (occasionally epidemic) vector of dengue viruses in parts of Asia and is a competent vector of several other viral diseases. Since its discovery in the United States, five arboviruses (Eastern equine encephalitis, Keystone, Tensaw, Cache Valley, and Potosi viruses) have been isolated from *A. albopictus*.

Enserink M. 2007. Chikungunya: no longer a Third World disease. *Science* **318:**1860–1861.

Projected distribution of *Aedes albopictus* in Europe, based on climate change models. Projections from two emission scenarios from the Intergovernmental Panel on Climate Change indicate that the habitat of *Aedes albopictus* will increase dramatically over the next century. From D. Fischer et al., *Int. J. Health Geogr.* **12:**51, 2013, with permission.



Host population density is a critical parameter for some virus populations to be sustained. Person-to-person transmission of some acute viral infections occurs only if the host population is large and interactive. For example, measles virus can be maintained only in human populations that exceed ~200,000, most likely because there is no animal reservoir, and infected individuals develop complete and long-lasting immunity. These infections are rarely found in isolated groups that might populate small islands or areas with extreme climates. Before global travel was possible, isolated host populations were the norm, and the distribution of viruses was far more limited. Now, however, as illustrated by the rapid colonization of the Western Hemisphere by West Nile virus, viruses are transported routinely and efficiently around the globe. In fact, epidemiologists have begun to think about the potential for epidemics in terms of the "effective distances" between airports, arguing that London is actually closer to New York than to other British towns, based upon air traffic densities; the larger the number of people that travel between airports and the cities that they serve, the smaller the effective distance.

Climate

In contrast to cultured cells that grow under conditions of invariant temperature and humidity or laboratory animals that live in strictly controlled enclosures, humans and other animals exist in ever-changing environments that directly influence viral biology. These changes include normal seasonal variations as well as progressive changes, such as global warming (Box 1.8).

Climate, including temperature and humidity, can have a profound influence on viral infections of populations. Indeed, there is a striking seasonal variation in the incidence of most acute viral diseases (Fig. 1.7). Respiratory virus infections occur more frequently in winter months, whereas infections of the gastrointestinal tract predominate in the summer. Seasonal differences in diseases caused by arthropod-borne viruses are clearly a consequence of the life cycle of the insect vector; when there are fewer mosquitos, there is a parallel reduction in the prevalence of the viruses that they harbor. However, the basis for the seasonal nature of infections by viruses that are not transmitted by arthropods is less obvious. It has been suggested that the seasonality of some infections is attributable to temperature- or humidity-based differences in the stability of virus particles. For example, poliomyelitis was known as a summertime disease in New England but not in Hawaii. The prevailing view is that poliovirus is inactivated during winter months when humidity is low, unlike other viruses, such as influenza virus, which remain infectious through the drier winter months.

A widely held belief is that large changes in temperature will increase a host's susceptibility to infection. In fact, as a parent likely warned you, transmission of "the flu" (specifically, influenza A virus particles) is more efficient at low temperature and humidity, and this property could contribute to increased rates of influenza in the winter months (Box 1.9). However, epidemiological studies with rhinoviruses that are also anecdotally associated with cold temperatures have failed to support any relationship between the cold and getting a cold; whether the "urban legends" associated with respiratory viral infections are true thus appears to depend on the virus in question (Box 1.10).

Climate-based variations in viral disease may also be caused by bodily changes in the host that influence its susceptibility. Such changes might be linked to **circadian rhythms** or be governed by alterations in the thicknesses of mucosal surfaces, production of virus receptors, or immune fitness.

BOX 1.8

DISCUSSION

How human behaviors and activities increase the risk of zoonoses

In his book *Spillover: Animal Infections and the Next Human Pandemic*, science writer David Quammen argues that the increase in zoonoses that we have seen in the past decades can be directly linked to human behavior and the ways in which we are irrevocably altering the world's ecosystems. The list of zoonotic infections that have impacted humans is impressive: Bolivian hemorrhagic fever caused by the Machupo virus (1961), Marburg hemorrhagic fever (1967), Lassa fever (1969), Ebola hemorrhagic fever (1976), HIV-1 diseases (inferred in 1981, first isolated in 1983), HIV-2 diseases (1986), hantavirus cardiopulmonary syndrome caused by Sin Nombre virus (1993), Hendra virus infection (1994), avian flu (1997), Nipah virus infection (1998), West Nile virus infection (1999), severe acute respiratory syndrome (SARS) (2003), and MERS. As Quammen notes,

> What we're doing is interacting with wild animals and disrupting the ecosystems that they inhabit—all to an unprecedented degree. Of course, humans have always killed wildlife and disrupted ecosystems, clearing and fragmenting forests, converting habitat into cropland and settlement, adding livestock to the landscape, driving native species toward extinction, introducing exotics. But now that there are seven billion of us on the planet, with greater tools, greater hungers, greater mobility, we're pressing into the wild places like never before, and one of the things that we're finding there is… new infections. And once we've acquired a new infection, the chance of spreading it globally is also greater than ever.

Quammen D. 2012. *Spillover: Animal Infections and the Next Human Pandemic*. W. W. Norton and Company, New York, NY.

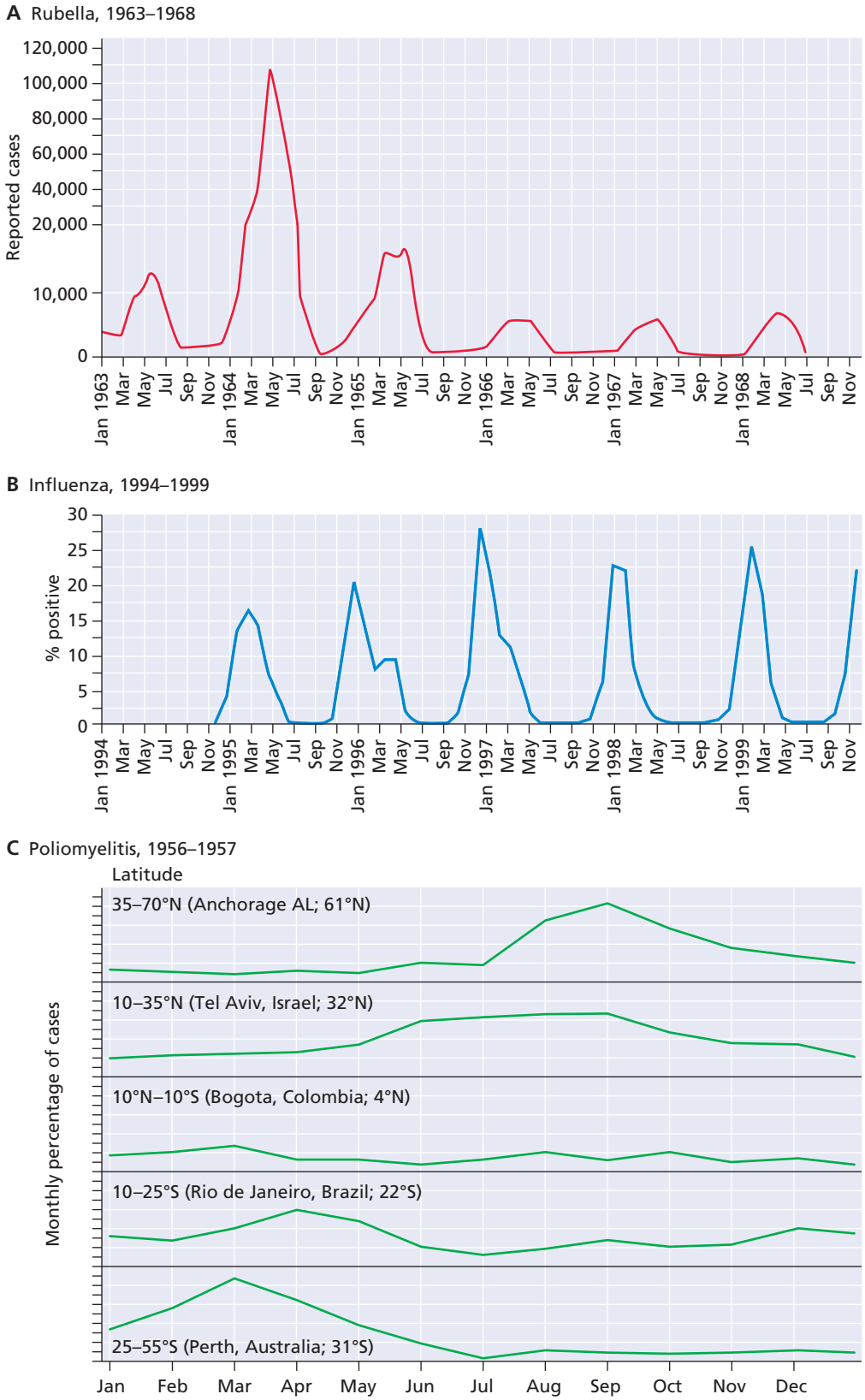


Figure 1.7 Seasonal variation in disease caused by three human pathogens in the United States. **(A)** Annual cycles of rubella between larger epidemics, which occurred every 6 to 9 years (1963 to 1968). **(B)** Annual cycles of influenza virus infection (1994 to 1999). Note the strong seasonal prevalence. **(C)** Monthly incidence of poliomyelitis at different latitudes, with representative cities denoted. Adapted from S. F. Dowell, *Emerg. Infect. Dis.* **7:**369–374, 2001, with permission.

BOX 1.9

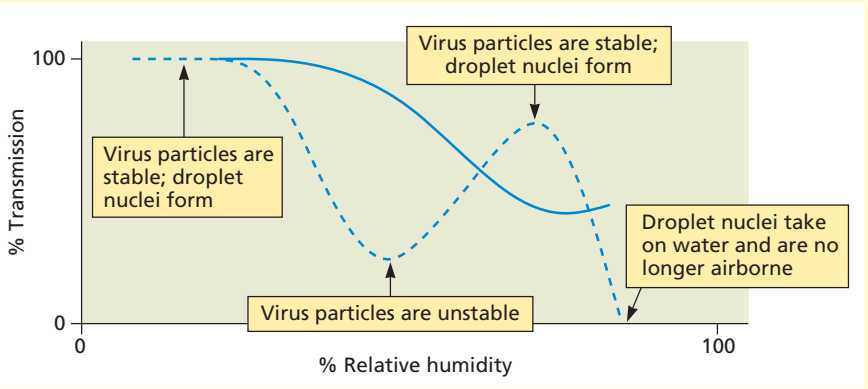
EXPERIMENTS

Seasonal factors that influence the transmission of influenza virus

Seasonality is a familiar feature of influenza: in temperate climates, the infection occurs largely from November to March in the Northern Hemisphere and from May to September in the Southern Hemisphere. There have been many hypotheses to explain this seasonality, but none had been supported by experimental data until recently. A guinea pig model was used to show that spread of the virus in aerosols is dependent upon both temperature and relative humidity.

Transmission experiments were conducted by housing infected and uninfected guinea pigs together in an environmental chamber. Transmission of infection was most effective at humidities of 20 to 35% and blocked at a humidity of 80%. In addition, transmission occurred with greater frequency when guinea pigs were housed at 5°C than at 20°C. The authors conclude that low temperature and humidity, conditions found during winter, favored influenza virus spread. The dependence of influenza virus transmission on low humidity might be related to the size of the droplets produced by coughing and sneezing (see the figure).

Model for the effect of humidity on the transmission of influenza virus. Transmission efficiency at 20°C (dashed line) or 5°C (solid line) is shown as a function of percent humidity. At 20°C, transmission is highest at low humidity, conditions which favor conversion of exhaled droplets into droplet nuclei (defined as droplets less than 5 mm in diameter and which remain airborne). Reduced particle stability at intermediate humidity is the cause of poor transmission. At high humidity, the conversion from droplets to droplet nuclei is inhibited, and the heavier droplets fall from the air, reducing transmission. At 5°C, transmission is more efficient than at 20°C, but there is a gradual loss of transmission with increasing humidity, presumably also as a consequence of the reduced formation of droplet nuclei. Adapted from A. C. Lowen et al., *PLoS Pathog.* **3**:1470–1476, 2007, with permission.



For example, if the mucosa is thinner in the winter or the skin is drier and cracked, the protective barriers that normally block viral entry into a host can become compromised.

Host Factors

The severity of disease following exposure to a viral pathogen can differ considerably from one person to another within a community in which the environmental variables discussed above are presumably the same. Some people become infected; others do not. Furthermore, among those who become infected, the severity of symptoms can vary widely, from mild to more serious, with long-lasting consequences. Susceptibility to infection and susceptibility to disease are independent; simply because someone is more susceptible to an infection does **not** mean that they will suffer more-severe illness. While we cannot yet predict who will become infected or how serious the infection will be, it is clear that both genetic and nongenetic (e.g., age) parameters determine how individuals in a population respond to viral infections (see Chapter 5).

Genetic and Immune Parameters

The basis of individual resistance or susceptibility in humans remains largely a mystery; we do not as yet understand why you always seem to catch a cold but your brother does not. However, in inbred organisms, such as laboratory mice and many plant species, proteins that make some members of a species more resistant than others have been identified. For example, the interferon-inducible myxovirus (influenza) resistance gene, *Mx1*, which is present in some strains of mice, encodes a guanosine triphosphatase (GTPase) that directly inhibits influenza virus replication. Similarly, resistance of some strains of mice to flavivirus disease has been mapped to the *Flv* locus. While virtually all mice strains lacking this locus die following infection, only ~15% of mice with this gene succumb. Flavivirus titers in resistant mice are 1,000- to 10,000-fold lower than in susceptible animals, and the infection is cleared before disease symptoms develop. The product of this gene is 2′-5′-oligo(A) synthetase, an interferon-induced enzyme that activates ribonuclease L (RNase L), leading to degradation of host and viral mRNAs (see Chapter 3). While humans possess *MX* and *FLV* genes, whether they have parallel functions has not yet been determined. These genes could have different functional roles in humans, or other host genes in outbred humans could obscure the protective effects afforded by the products of these genes.

There are few human proteins known to influence susceptibility to viral infections but not to other pathogen encounters. Those that have been identified (and that differ among susceptible and nonsusceptible humans) enable a critical step in the infectious cycle, such as viral entry. Hosts who encode

BOX 1.10

BACKGROUND

Quiz: The origins and veracity of urban legends about infections

Which of these statements about colds and the flu are true, and which are myths?

1. You can catch the flu from a flu shot.
2. Stress increases your chances of getting sick.
3. Wearing a hat will help protect you from a cold.
4. Flying on an airplane will increase your risk of getting sick.
5. Pregnant or breastfeeding women should not get vaccinated.
6. Increasing how much you sweat (e.g., using lots of blankets) will speed up how quickly you resolve an infection.
7. "Feed a cold; starve a fever."
8. Your grandmother's chicken soup can help.
9. Eating garlic can help to prevent you from getting sick.
10. Over-the-counter cold "prevention" tablets or drinks are effective.

Answers

1. ***Myth***: As discussed in Chapter 8, the injected flu vaccine is an inactivated ("dead") virus; it is therefore impossible to get the flu from the shot itself. The nasal flu mist contains a live, but drastically weakened, virus, and it is highly unlikely that someone will get influenza from the nasal vaccine.
2. Not yet proven, but probably ***fact***: Stress alters hormones, hormones affect immunity, and immunity controls your response to viral infections, so it is quite possible that stress can affect your ability to respond to an infection.
3. ***Myth***: Wearing a hat will keep your head warm, but that's it.
4. ***Fact***: Recirculated air combined with a large number of people in close quarters is a perfect recipe for transmission of respiratory infections from person to person.
5. ***Myth***: Flu symptoms are generally worse in pregnant women than in nonpregnant women, so it is of added importance that pregnant women be vaccinated. Many studies have shown that there are no adverse consequences of maternal vaccination to the fetus or the nursing neonate.
6. ***Myth***: While piling on the blankets may make you feel better, it will not make the cold go away faster; the only thing proven to alter the duration of an infection is the use of antivirals within a short (1- to 2-day) window after symptoms appear.
7. ***Myth***: How much you eat, or what you eat, will not influence how quickly you will resolve an infection. However, drinking lots of fluids **will** help, as staying hydrated, especially if you have a fever, will keep the mucus in respiratory passages loose. Moreover, colds and flu tend to cause a transient lack of appetite, so choosing food wisely (e.g., protein-rich) when recovering will hasten feeling better.
8. ***Fact!***: It is a fact that warm liquids open up nasal passages and keep the mucus moving (a good thing), and chicken soup has also been proposed to mobilize neutrophils, important virus-fighting immune cells.
9. ***Fact***: Garlic has powerful antioxidant activity, which boosts immunity. Eating **lots** of garlic will also keep away potentially infected friends and colleagues.
10. ***Myth***: The small bottles that often appear at checkout lines in supermarkets and that promise protection from catching a cold are primarily just a large dose (usually 1,000 mg) of vitamin C. While it remains controversial whether vitamin C is beneficial, daily multivitamins (or, better still, a healthy diet) can provide as much of the key ingredient and for less money.



a viral receptor are permissive; those who do not are nonpermissive. For example, most, but not all, humans synthesize the chemokine receptor CCr5, a cofactor for entry of human immunodeficiency virus type 1 into cells. Those rare few individuals with CCr5 genes that do not encode a functional protein as a result of mutation appear to be resistant to HIV-1 infection. The same cellular gene product may affect different viruses in different ways; a mutation in the gene encoding CCr5 that protects against infection with human immunodeficiency virus type 1 actually **increases** susceptibility to lethal encephalitis caused by West Nile virus.

Recently, the genes that encode Toll-like receptor 3 (Tlr3) and Unc-93B were shown to protect humans from herpes simplex virus encephalitis, as mutations in these genes were associated with elevated risk. Both gene products govern the production of **interferons**. As the importance of interferons in host defense might suggest, mutations in these genes result in broad sensitivity to many viral pathogens, as is true experimentally in mice. However, as far as can be determined, humans with Tlr3 or Unc-93B mutations showed increased susceptibility to only herpes simplex virus and not other microbial pathogens. The implication is that certain immunological defenses may be exquisitely specific for a single pathogen or reaction in a pathogen's reproductive cycle. How such precision may have evolved and is controlled is discussed in Chapters 4 and 10.

Immunity also governs the susceptibility of a host population. Viral epidemiologists divide populations into two groups, susceptible and immune (or resistant). Humans or other animals that have been infected in the past are likely to be immune. Consequently, they cannot be infected again

with the same virus and cannot transmit infection to others. Susceptible individuals are targets for infection and can develop disease and spread the virus to others. The persistence of a virus in a population depends on the presence of a sufficient number of those who are vulnerable. Immunization against viral infection by natural infection or vaccination reduces the number of potential hosts and therefore limits the foothold that a virus can establish in a community. For example, epidemics of polio were self-limiting, because the asymptomatic spread of the virus immunized the population. The competence of the immune response also determines the speed and efficiency with which an infection is spread and resolved and the severity of symptoms.

In addition to immune memory, inherent differences in an individual's ability to respond to pathogens may contribute to selective susceptibility. The class I and class II major histocompatibility complex (MHC) proteins bind to small viral peptides within an infected cell and present them on the infected cell surface to T cells (Chapter 4). Different MHC proteins bind to different peptides, and the ability of MHC proteins to interact with diverse epitopes determines, in part, the potency and quality of the antiviral response. These properties, in turn, dictate how efficiently the infection is cleared. The diversity among MHC genes increases the likelihood that there will be a suitable molecule to present peptides for any given infectious agent. An implication of this process is that some individuals may be intrinsically more or less able to mount an effective immune response to particular viral pathogens because of randomly encoded variations in the kinds of peptides that their MHC molecules can bind. This hypothesis is supported by the observations that individuals from isolated populations (e.g., dwellers of small islands) exhibit less polymorphism in these genes, a property that may account for their greater susceptibility to certain infections.

Nongenetic Risk Factors

Nongenetic risk factors include age, health, lifestyle, and occupation. In a classic story of virology history, Edward Jenner noticed that milkmaids appeared immune to smallpox, a trait that was later found to be due to "vaccination" by a relative of smallpox that infected cows. A present-day example of how one's profession influences the kinds of viruses one encounters is the careful attention paid to the health of poultry workers in China to monitor the emergence of the H5N1 or H7N9 influenza ("bird flu") strains.

The age of the host also plays an important role in determining the result of viral infections. Very young children and the elderly are generally more susceptible to disease. The increased susceptibility of these individuals can be explained by the immaturity or progressive decline, respectively, of their immune responses. However, when a virus causes **immunopathology**, infection of newborns and the elderly is **less** severe than in immunocompetent adults. For example, intracerebral inoculation of lymphocytic choriomeningitis virus in adult mice is lethal because recruitment of T cells into the brain leads to swelling and death. In contrast, infant mice survive this challenge because of their weaker response (Box 1.11). Some viral infections, including those caused by poliovirus, mumps virus, and measles virus, are less severe in children than in adults, perhaps because the robust adult response contributes to disease. The 1918-1919 influenza pandemic was particularly lethal, not only for the very young and the very old but unexpectedly also for young adults, those 18 to 30 years of age (Fig. 1.3). It has been suggested that the increased death rate in young adults was caused by an overly aggressive immune response that flooded their systems with cytokines, sometimes referred to as a "**cytokine storm**."

BOX 1.11

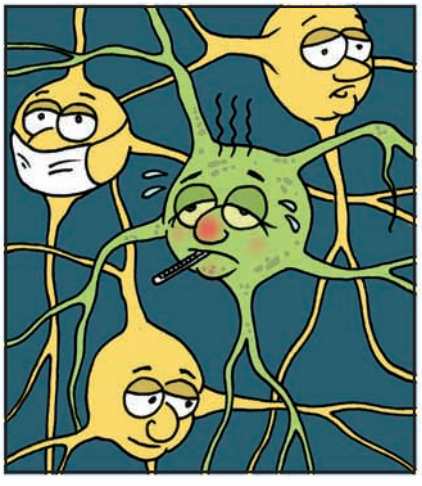
DISCUSSION

Congenital brain infection: the lymphocytic choriomeningitis virus model

During development, the fetal brain is among the most vulnerable organs; viral infections of the fetus often result in severe brain injury. Unfortunately, many animal models of congenital brain infections do not mimic human disease for a variety of poorly understood reasons.

In contrast, the neonatal rat model for congenital lymphocytic choriomeningitis virus infection reproduces virtually all the neuropathological changes observed in congenitally infected humans. Within the developing rat brain, the virus selectively infects mitotically active neuronal precursors, a fact that explains the variation in pathology with time of infection during gestation. Lymphocytic choriomeningitis virus infection results in delayed-onset neuronal loss after the virus has been cleared by the immune system. Accordingly, many researchers think that this model can be used to study neurodegenerative or psychiatric diseases associated with loss of neurons or their function.

Bonthius D, Perlman, S. 2007. Congenital viral infection of the brain: lessons learned from lymphocytic choriomeningitis virus in the neonatal rat. *PLoS Pathog* **3:**1541–1550.



Physiological differences other than immune fitness can also explain age-dependent variation in susceptibility. Infection of human infants with enteric coronaviruses is severe, because the alimentary canal is not fully active and presents a particularly hospitable niche for infection. In infants, the gastric pH tends to be less acidic, and digestive enzymes are less abundant. As animals age, the alveoli in the lungs become less elastic, the respiratory muscles weaken, and the cough reflex is diminished. These changes may explain, in part, why elderly people are at greater risk for acquiring respiratory tract infections. Other age-related variables that may be important include age-dependent changes in the tissues that a virus can infect. Respiratory syncytial virus causes severe lower respiratory tract infections in infants but only mild upper respiratory tract infections in adults. This is probably the result of both differences in the potencies of the host immune responses of children and adults and age-dependent variations in the susceptibilities of upper versus lower respiratory epithelia to viral infection.

Human males are more susceptible to viral infections than females, but the difference is slight and the reasons are not understood. Hormonal differences, which can alter the efficacy of the immune system, may be partly responsible. Pregnant women are more susceptible to infectious disease than nonpregnant women, probably for similar reasons. Moreover, the severity of disease caused by some viruses, including hepatitis A, B, and E and poliovirus, is exacerbated in pregnant women.

Malnutrition increases susceptibility to infection because the physical barriers, as well as immune fitness, are compromised. An example is the increased susceptibility to measles in children with protein deficiency. For this reason, measles is 300 times more lethal in poor countries. When children are malnourished, the small red spots inside the mouth that are hallmarks of a measles infection (called **Koplik's spots**) become massive ulcers, the skin rash is much more severe, and lethality may approach 10 to 50% (Chapter 5). Such severe measles infections are observed in children in tropical Africa and in aboriginal children in Australia.

As virologists begin to view individuals as products of their histories, genetics, environments, and life choices, rather than as masses of permissive cells and tissues, a more complete picture of susceptibility to an infection will emerge. This perspective has already yielded some insights. Corticosteroid hormones are known to affect susceptibility, because they are essential for the body's response to the stress of infection. These hormones have an anti-inflammatory effect, which is thought to limit tissue damage. Similarly, cigarette smoking increases susceptibility to respiratory infections as a result of the decreased capacity of the tar-coated lungs and airways to self-clean. Increased susceptibility can also occur in stressful life situations. We often refer to "our defenses being down," but what this probably means is that the balance of hormones that maintain homeostasis is altered, creating opportunities for a viral infection.

Perspectives

A fundamental principle of virology is that for a virus to be maintained in a host population, virus particles must be released from one infected host to infect another. This process of serial infection, while simple in principle, is difficult to study in natural systems given the mind-boggling number of host, viral, and environmental variables. Nevertheless, epidemiology, the study of this process, is evolving rapidly as new ways to track and identify infectious agents are developed. To thwart a potential epidemic, viral epidemiologists must possess the skills of a private investigator, sociologist, conductor, and chef at a popular restaurant. To track the origins of infection, epidemiologists must consider simultaneously multiple variables and clues, some of which are false leads. These investigators must understand the dynamics of the animal or human populations at risk and how aspects of behavior might increase the potential for infection. They must then integrate these diverse pieces of information, and because the investigation often begins only once an epidemic is under way and victims have been identified, epidemiologists must be able to work under great pressure, within a constrained time frame, and often under intense media scrutiny.

At the writing of this text, an Ebola outbreak in Africa has begun, killing thousands of people. Doctors cannot document all of the new cases, and many individuals who are ill are staying in their homes, fearful of the government response. Many believe that going to a clinic for care will isolate them from their families. Containing an epidemic under such pressures is a monumental challenge, especially when no certain therapy exists that can be offered to patients. The goal of epidemiology in this setting is to define the basis of the outbreak and to limit further transmission. Fortunately, Ebola virus is only transmitted via bodily fluids, such as blood and sputum; if this highly lethal virus could be acquired by means of aerosol droplets, the epidemiological challenge would obviously be far greater.

Our current understanding of the fundamental principles of viral pathogenesis comes largely from studies with animal models. For example, use of the large number of genetically identical, available mouse strains has led to the identification of many genes that confer susceptibility to particular viral infections. It is noteworthy that most of the gene products identified thus far impact a single virus or virus family, and many target a particular step in virus reproduction (Chapter 3). At one time, it seemed impossible to do similar studies with humans, because genetic techniques were not sufficiently powerful to detect rare susceptibility mutations in genetically different humans. However, several human genes that encode products that participate in intrinsic or innate defense for particular viral infections have recently been identified. Such investigations have heralded a new approach to answering fundamental questions in viral pathogenesis, the focus of the next four chapters.

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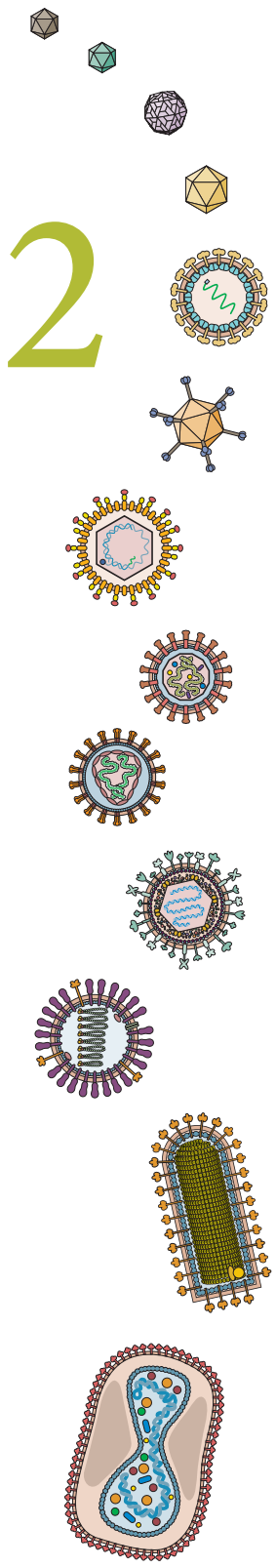
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2 Barriers to Infection

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- Initiating an Infection

Successful Infections Must Modulate or Bypass Host Defenses
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- Alimentary Tract
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Viral Tropism
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- Host Cell Proteins That Regulate the Infectious Cycle

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- Hematogenous Spread
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- Urine
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- Milk
- Skin Lesions

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LINKS FOR CHAPTER 2

⏭ *Video: Interview with Dr. Neal Nathanson*
http://bit.ly/Virology_Nathanson

⏭ *Wookie viruses*
http://bit.ly/Virology_Twiv250

This earth of majesty, this seat of Mars
This other Eden, demi-paradise
This fortress built by Nature for herself
Against infection and the hand of war.

William Shakespeare,
Richard II (Act 2, Scene 1)

Introduction

Microbes are everywhere. They are on our hands, in our food, on the lips of those we kiss, on the ground and in the oceans, filling the air we breathe. For young children who play in dirt, scrape their knees, and pick their noses, interactions with potential pathogens are even more frequent and diverse. As we begin a series of chapters dedicated to immune responses and viral diseases, perhaps the right question to ask is not "What makes us sick?" but rather, "How can we possibly manage to stay healthy?"

If students of immunology are asked to list components of the host response to infection, typical responses will include mention of professional antigen-presenting cells, antibodies, cytotoxic T lymphocytes, and interferon gamma. These answers are not incorrect *per se*, but to focus only on attributes of the immune system misses the bigger picture: by the time a virus or other microbe has been engulfed by phagocytes or stimulated a T cell response, it has already bypassed an impressive fortress of defenses. These defenses, such as skin, mucus, and stomach acid, might seem much more primitive than the elegantly coordinated innate and adaptive immune responses. Nevertheless, they block the overwhelming majority of infections.

Such sentries, while effective, are imperfect despite millions of years of evolution in the presence of microbes. When viruses breach these initial barriers, infections of host cells and attendant disease can occur. The genomes of successful viruses encode gene products that modify, redirect, or block these, as well as other, host defenses. For every host defense, there will be a viral offense. It is remarkable that the genome of every known virus on the planet today encodes countermeasures to modulate the defenses of its host, even though some viral genomes are very small. As we shall see, many of these "anti-host response" strategies (Box 2.1) are targeted at the body's physical barriers to infection. For comments and a personal account related to the chapter topic, see the interview with Dr. Neal Nathanson: http://bit.ly/Virology_Nathanson.

An Overview of Infection and Immunity

A Game of Chess Played by Age-Old Masters

Infection by viruses is often described in terms associated with warfare. There are opposing forces, each equipped with weapons to defeat the other. Once the battle ensues, each side fights with maximum force until a winner emerges. A more fitting metaphor to define the events pursuant to a viral infection would be a game of chess played by two masters. For each action, there follows a counteraction. Powerful tactics, such as induction of the adaptive immune response, may take many "moves" to be put into action. As one thinks about infection and immunity, it is imperative to bear in mind that we have coevolved with many of the viruses that infect us today. Such coevolution implies that, at a population level, **both** host and virus will survive. On an individual level, however, the consequence of infection is dictated by the host species, immune fitness, dose and strain of virus, and numerous environmental factors.

The pathogenesis of ectromelia virus, the agent of mousepox, highlights how the result of infection is affected by these variables (Fig. 2.1). Ectromelia virus is shed in the feces of its natural mouse host and gains access to naïve hosts via small abrasions in the footpad (or, in a laboratory setting, by injection into the footpad). Therefore, the first hurdle to be overcome is penetration of the dead skin of the footpad, which serves as an inhospitable barrier against infection. There is no guarantee that a mouse in a cage with infected feces will become infected. Virus particles must come in physical contact with permissive and susceptible cells for infection to occur, necessitating a break in the skin to allow access of the virus to live cells. Once the virus has gained entry, local reproduction in the epidermis and dermis of the footpad takes place. Within a day after exposure, the virus moves

PRINCIPLES ***Barriers to infection***

- Three requirements must be met to ensure successful infection in an individual host: a sufficient number of infectious virus particles, access of the virus to susceptible and permissive cells, and absent or quiescent local antiviral defenses.
- Common sites of virus entry include the respiratory, alimentary, and urogenital tracts, the outer surface of the eyes (conjunctival membranes or cornea), and the skin.
- Each of these portals is equipped with anatomical or chemical features that limit viral entry and infection.
- Spread beyond the initial site of infection depends on the initial viral dose, the presence of viral receptors on other cells, and the relative rates of immune induction and release of infectious virus particles.
- Disseminated infections typically occur by transport through the bloodstream, though some viruses can be transported by the peripheral nervous system.
- Effective transmission of virus particles from one host to another depends on the concentration of released particles and the mechanisms by which the virus particles are introduced into the next host.
- Viral transmission to a new host usually occurs through body fluids, including respiratory secretions, blood, saliva, semen, urine, and milk.

BOX 2.1

TERMINOLOGY

Is it evasion or modulation?

From the online *Merriam-Webster Dictionary*:

Evade: to elude by dexterity or stratagem

Modulate: to adjust to or keep in proper measure or proportion

The phrase "immune evasion" pervades the virology literature. It is intended to describe the viral mechanisms that thwart host immune defense systems. However, this phrase is imprecise and even misleading. The term "evasion" implies that host defenses are ineffective, similar to a bank robber evading capture by a hapless police force. In reality, a virus does not necessarily need to be invisible to the host response throughout its life cycle; it simply needs to delay or defer detection for a time sufficient to reproduce. If viruses really could evade the immune system, we might not be here discussing such semantic issues.



Perhaps a more accurate term to describe viral gene products that delay or frustrate host defenses is "immune modulators." The principle here is that, given the speed of viral reproduction, an infection can be successful even if host defenses are only suppressed transiently.

to draining lymph nodes, enters the bloodstream, and can be found in the spleen and liver by 3 days after infection. Thereafter, the virus continues to spread throughout the host, causing massive inflammation and severe skin lesions by 10 to 11 days of exposure.

From the moment of ectromelia virus entry, the host mounts a response to counteract the virus. The impact of such countermeasures is revealed by the effects of specific immune deficiencies, which lead to different kinds of disease. If the mouse lacks $CD8^+$ T lymphocytes, it will die of extensive liver destruction by 4 to 5 days after infection. If instead the host lacks the critical cytokine interferon gamma, the virus may be controlled in the liver, even though death will occur by 10 to 12 days after infection as a consequence of uncontrolled viral reproduction in the skin. Even in immunocompetent mice, viral movement from tissue to tissue means that the immune response is continually playing catch-up as infection is controlled in the liver, infection of the skin appears. Moreover, while mice of a certain strain can contain the infection, immunocompetent mice of a different strain cannot, underscoring the critical involvement of more-subtle genetic regulators of immune control.

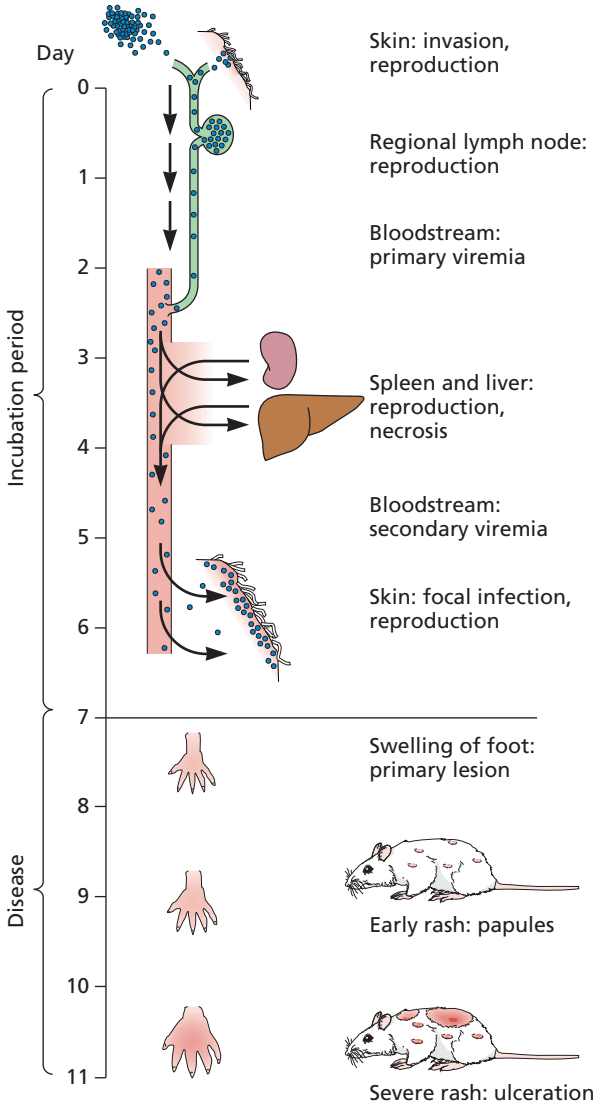


Figure 2.1 Ectromelia virus infection of mice. Infection begins through a break in the skin, allowing local viral reproduction and dissemination via the lymphatics within 1 to 2 days of exposure. Primary viremia occurs when the virus is released into the bloodstream, permitting infection of the spleen, liver, and other organs. Secondary viremia occurs due to release of virus from organs, resulting in infection of distal sites of the skin. The foot (the site of primary infection) swells due to the inflammatory response. In certain strains and wild mice, a severe rash may develop. Adapted from F. Fenner, et al., *The Biology of Animal Viruses* (Academic Press, New York, NY, 1974), with permission.

Just as ectromelia advances through various permissive tissues of the host, the host defenses are deployed in a coordinated, stepwise manner (Fig. 2.2). All surfaces of the mammalian body where pathogens may enter are protected by defensive layers provided by fur, skin, and mucus, or are protected by acidic environments. Once these barriers are

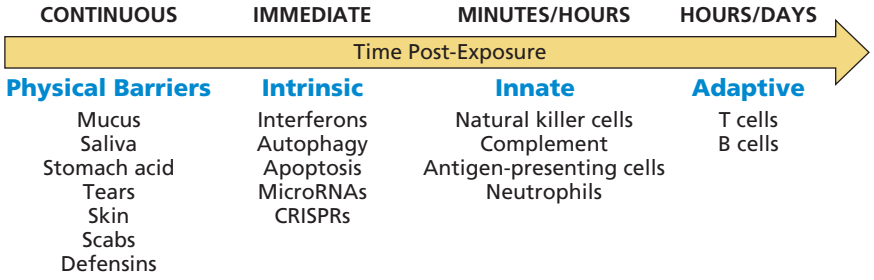


Figure 2.2 The coordinated host response to infection. In healthy individuals, anatomical and chemical barriers are in place to prevent or repel infection by microbes. When viruses successfully bypass these defenses, intrinsic responses are engaged. These responses encompass those that already exist in the infected cell or host, poised to respond without the need for new transcription or translation. Within hours following exposure, cellular components of the innate immune response migrate to the site of infection, including professional antigen-presenting cells, neutrophils, and natural killer cells. These cells elaborate chemokines and cytokines that serve as a beacon for the subsequent recruitment of the adaptive immune response and synthesis of interferon-stimulated genes that induce an antiviral state. Within days following infection, antigen-specific T and B cells will be activated and undergo massive proliferation and migration to the site of infection, where, in most cases, resolution of the infection occurs. CRISPRs, clustered regularly interspaced short palindromic repeats.

crossed and cells become infected, **instrinsic cellular defenses** including cell-autonomous responses, such as interferon production, autophagy, editing, and, as a last resort, cell suicide, or **apoptosis**, are engaged. Because the virus may reproduce faster than an infected cell can control it, the "professional" immune response is also induced, beginning with the **early innate response** (Box 2.2). Finally, virus-specific cells of the **adaptive response** arrive at the site of infection, targeting infected cells and extracellular virus particles for destruction or elimination.

While this text generally avoids imparting actions to viruses, the impression one may have from the ectromelia virus example is that viruses are on a seemingly preordained, step-by-step path to gain access to their target cells of choice (for example, hepatitis viruses in hepatocytes, measles virus in epithelia, or human immunodeficiency virus in $CD4^+$ T cells). Likewise, one might think that the immune response is deployed in a synchronized and choreographed manner, much like actors performing a play night after night. These impressions would be wrong. As every game of chess is constrained by the same rules, but each game differs in execution and outcome, so too are viral infections and host immunity influenced by random, or stochastic, events. For example, tissues and the immune system may impose bottlenecks on the dissemination of a virus population. The diversity of viral populations enables some particles to pass through the bottleneck, while others are lost as the virus spreads (Chapter 10). Such bottlenecks include not only access to tissues but also immune restriction (Fig. 2.3). The stochastic view does not reject the idea that infection is a series of defined steps but rather adds random elements to the consequence of each.

Initiating an Infection

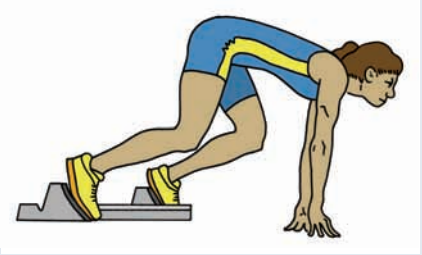
Three requirements must be met to ensure successful infection in an individual host: a sufficient number of infectious virus particles must be available to initiate infection; the cells at the site of infection must be physically accessible to the virus, **susceptible** (bear receptors for entry), and **permissive** (contain

BOX 2.2

TERMINOLOGY

Innate or intrinsic?

The line that distinguishes intrinsic immunity from innate immunity is a blurry one, and even the authors of this text disagree on some assignations. Many in the field use these terms interchangeably, adding to the confusion. For the purposes of clarity, we will define intrinsic responses to be those that preexist in the host or target cell and function without the need for new protein synthesis in response to pathogen detection.



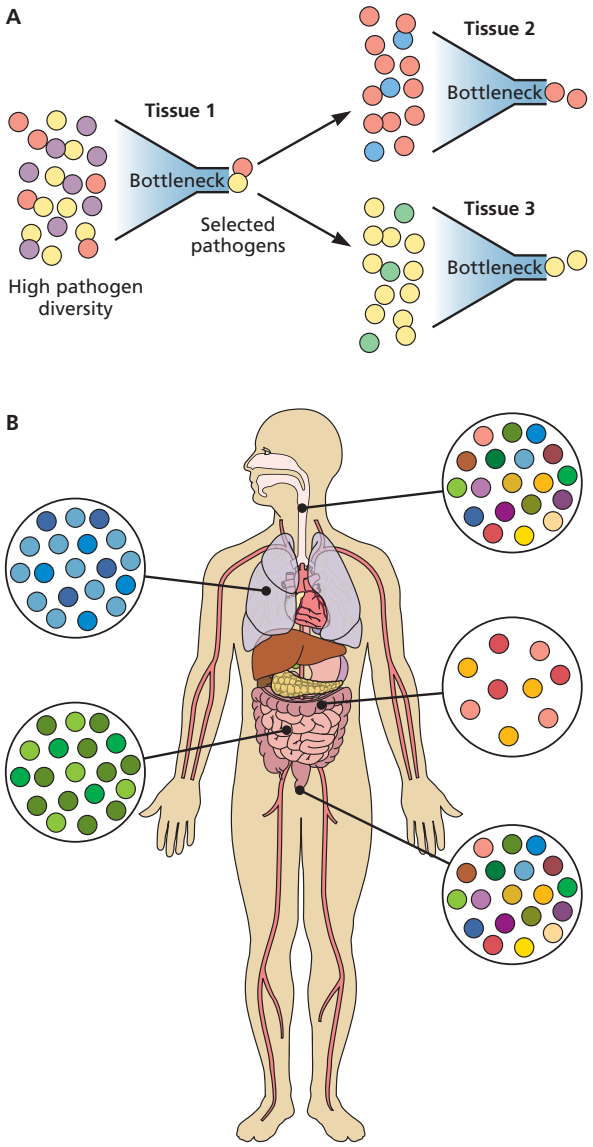


Figure 2.3 Infection seen as a series of stochastic events. **(A)** Selection of viruses that can pass through bottlenecks in cells, organs, or hosts. In this case, the viral population enters the host as a diverse quasispecies with sufficient titer to establish infection. After entry, the population may encounter a host barrier that limits diversity. Pool members with the ability to overcome this barrier may then reproduce and restore diversity. The subsequent viral population in this tissue may have high diversity but differ in overall consensus sequence from the initial infecting population. Certain tissues may be highly permissive for viral infection with limited host or viral pressures. The viral population in these tissues may become dominated by more-fit variants with enhanced replicative capacity, resulting in high titer and low diversity. **(B)** Alternatively, the viral population may enter a highly restrictive environment where host and viral factors limit productive reproduction. The resulting population may have decreased diversity and titer or be eliminated from this tissue entirely. A productive infection in a host should allow efficient reproduction in one or more tissues, facilitating spread of a viral population at high titer and high diversity to optimize spread. Adapted from H. W. Virgin, *Nat. Immunol.* **8:**1143–1147, 2007, with permission.

intracellular gene products needed for viral reproduction); and local antiviral defenses must be absent or, at least initially, quiescent.

The first requirement imposes a substantial barrier to any infection and represents a significant limitation in the transmission of virus from host to host. Free virus particles face both a harsh environment and rapid dilution that can reduce their concentration. To remain infectious, viruses that are spread in contaminated water and sewage must remain stable in the presence of osmotic shock, pH changes, proteases, and sunlight. Aerosol-dispersed virus particles must remain hydrated and highly concentrated to infect the next host. These requirements account for why respiratory viruses spread most successfully in populations in which individuals are in close contact; the time that a virus particle is outside a host is minimized. In contrast, viruses that are spread by biting insects, contact with mucosal surfaces, or other means of direct contact, including contaminated needles, have virtually no environmental exposure; the virus is transmitted directly, for example, from mosquito to human.

Even after transmission from one host to another, infection may fail simply because the concentration of infectious virus particles is too low. In principle, a single West Nile virion should be able to initiate an infection, but host physical and immune defenses, coupled with the complexity of the infection process itself, usually require the presence of many particles. Even those particles that have remained intact may not encounter a target cell following entry into a host. One can envision many paths to failure: the virus particle may adhere to a dead or dying cell, become attached to nonsusceptible cells by nonspecific protein-protein interactions, be swept away in the bloodstream, get stuck in mucus, or be delivered to a lysosome upon entry into a target cell.

In addition, populations of viruses often contain particles that are not capable of completing an infectious cycle. Defective particles can be produced by incorporation of errors during virus genome replication or by interactions with inhibitory compounds in the environment. In the laboratory, a quantitative measure of the proportion of infectious viruses is the particle-to-plaque forming unit (PFU) ratio. The number of particles in a given preparation can be counted, usually with an electron microscope, and compared with the number of infectious units per unit volume (Volume I, Chapter 2). This ratio is a useful indicator of the quality of a virus preparation, as it should be relatively constant for a given virus. Some viruses, such as Semliki Forest virus, have a very low ratio (that is, virtually all particles are infectious), while other particle-to-PFU ratios, including those for poliovirus and some papillomaviruses, exceed 1,000 or 10,000. Why these ratios differ so drastically is not known, but the main point should be clear: not every virus particle that binds to a susceptible and permissive cell can induce all the steps needed to

produce progeny virus particles, and even those that can may be thwarted at any step of the viral reproductive cycle.

Successful Infections Must Modulate or Bypass Host Defenses

In most mammals, common sites of virus entry include the mucosal linings of the respiratory, alimentary, and urogenital tracts, the outer surfaces of the eyes (conjunctival membranes or cornea), and the skin (Fig. 2.4). Each of these portals is equipped with anatomical or chemical features that limit viral entry and infection.

Skin

The skin is the largest organ of the body, weighing more than 5 kg in an average adult. It serves obvious protective functions but is also required for thermoregulation, control of hydration and evaporation, and integration of sensory information. The external surface of the skin, or epidermis, is composed of several layers, including a basal germinal layer of proliferating cells, a granular layer of dying cells, and an outer layer of dead, keratinized cells (Fig. 2.5). This outermost layer is a rather literal coat of armor against viral infection: many virus particles that land on intact skin are inactivated by desiccation, acids, or other inhibitors secreted by commensal microorganisms or are simply removed from the body when dead cells slough off. Particles may also be washed away by soap and water. However, when the integrity of the dead cell layer is compromised by cuts, abrasions, or punctures (e.g., insect bites and needle sticks), virus particles can access blood vessels, epithelial cells, endothelial cells, and neuronal processes.

Examples of viruses that can gain entry via the skin are some human papillomaviruses, certain poxviruses (e.g., myxoma virus), and all tick- or mosquito-borne viruses that are transmitted by arthropod injection below the dead cell layer (Table 2.1). Even deeper inoculation into the tissue and muscle below the dermis can occur by hypodermic needle punctures, body piercing, tattooing, or sexual contact when body fluids are mingled as a result of skin abrasions or ulcerations. Animal bites can introduce rabies virus into tissue and muscle rich with nerve endings, through which virus particles can invade motor neurons. In contrast to the strictly localized reproduction in the epidermis (e.g., papillomaviruses that cause warts), viruses that initiate infection in dermal or subdermal tissues can reach nearby blood vessels, lymphatic tissues, and cells of the nervous system. As a consequence, they may spread to other sites in the body (Box 2.3).

The body's response to a breach in the critical barrier formed by the skin is to make rapidly a hard, water-resistant

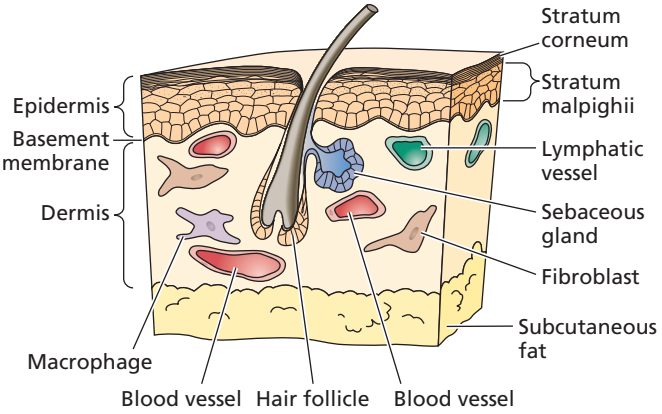


Figure 2.5 Schematic diagram of the skin. The epidermis consists of a layer of dead, keratinized cells (stratum corneum) over the stratum malpighii. The latter may have two layers of cells with increasing numbers of keratin granules and a basal layer of dividing epidermal cells. Below this is the basement membrane. The dermis contains blood vessels, lymphatic vessels, fibroblasts, nerve endings, and macrophages. A hair follicle and a sebaceous gland are shown. Adapted from F. Fenner et al., *The Biology of Animal Viruses* (Academic Press, New York, NY, 1974), with permission.

Figure 2.4 Sites of viral entry into the host. Sites of virus entry and shedding are indicated. The body is covered with skin, which has a relatively impermeable (dead) outer layer of keratinocytes covering a live layer of epithelial cells rich in capillaries. Moreover, other portals in the host, present to absorb food, exchange gases, and release urine, may allow access of viruses to host tissues.

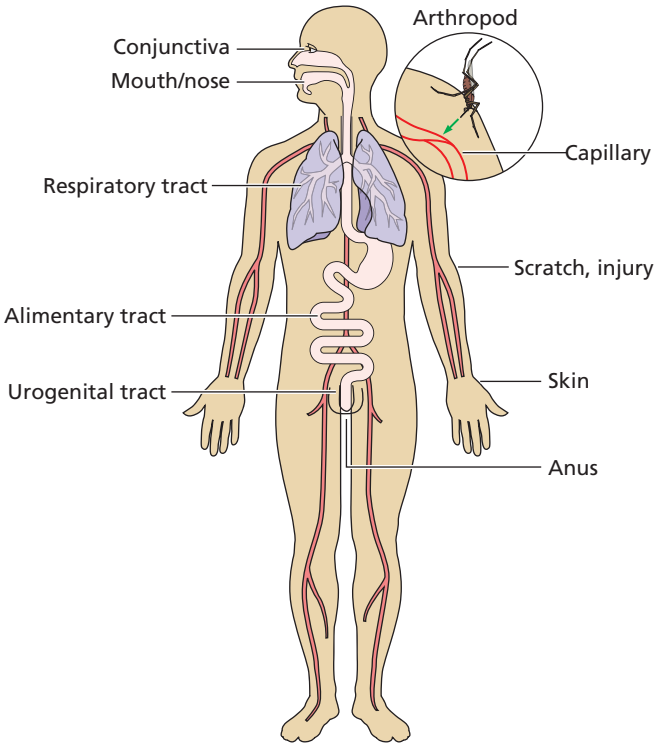


Table 2.1 Different routes of viral entry into the host

Location	Virus(es)
Skin	
Arthropod bite	Bunyavirus, flavivirus, poxvirus, reovirus, togavirus
Needle puncture, sexual contact	Hepatitis C and D viruses, cytomegalovirus, Epstein-Barr virus, hepatitis B virus, human immunodeficiency virus, papillomavirus (localized)
Animal bite	Rabies virus
Respiratory tract	
Localized upper tract	Rhinovirus; coxsackievirus; coronavirus; arenaviruses; hantavirus; parainfluenza virus types 1–4; respiratory syncytial virus; influenza A and B viruses; human adenovirus types 1–7, 14, 21
Localized lower tract	Respiratory syncytial virus; parainfluenza virus types 1–3; influenza A and B viruses; human adenovirus types 1–7, 14, 21; severe acute respiratory syndrome coronavirus
Entry via respiratory tract followed by systemic spread	Rubella virus, arenaviruses, hantavirus, mumps virus, measles virus, varicella-zoster virus, poxviruses
Alimentary tract	
Systemic	Enterovirus, reovirus, adenovirus types 40 and 41
Localized	Coronavirus, rotavirus
Urogenital tract	
Systemic	Human immunodeficiency virus type 1, hepatitis B virus, herpes simplex virus
Localized	Papillomavirus
Eyes	
Systemic	Enterovirus 70, herpes simplex virus
Localized	Adenovirus types 8, 22

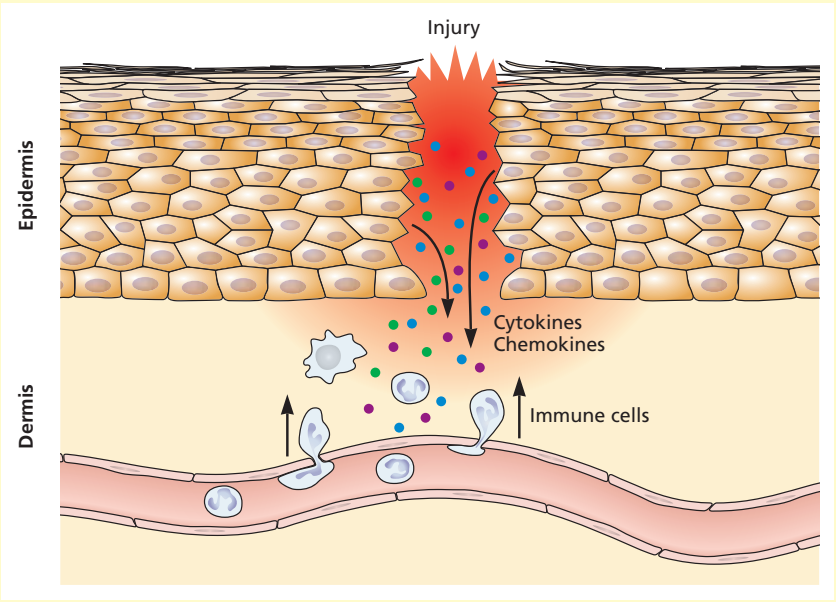
BOX 2.3

EXPERIMENTS

Dermal damage increases immunity and host survival

When it was still in use, the smallpox vaccine was delivered by a bifurcated needle (Volume I, Box 1.3), in a process referred to as scarification, that results in local damage to the skin and a subsequent and quickly resolved reaction or lesion in most individuals. Until recently, it was not appreciated that the scarification process itself was an important component of the vaccine's efficacy. Experiments using the smallpox-related virus, vaccinia virus, showed that intradermal inoculation of the virus into rabbits resulted in lethal disease by 8 days after infection, whereas delivery of the virus by scarification led to a protective host response. Scarified rabbits also responded more than a day before those inoculated by the intradermal route. Moreover, scarification in the absence of virus followed immediately by a same site intradermal challenge with virus resulted in significant protection to the infected rabbits. This dramatic difference can be attributed to the rapid induction of a non-specific host response. The act of scarification damages skin cells and the underlying epidermis, inducing the release of cytokines and chemokines that help to direct the host response to the site of infection and restrict the dissemination of the virus throughout the host.

Rice AD, Adams MM, Lindsey SF, Swetnam DM, Manning BR, Smith AJ, Burrage AM, Wallace G, MacNeill AL, Moyer RW. 2014. Protective properties of vaccinia virus-based vaccines: skin scarification promotes a nonspecific immune response that protects against orthopoxvirus disease. *J Virol* **88**: 7753–7763.



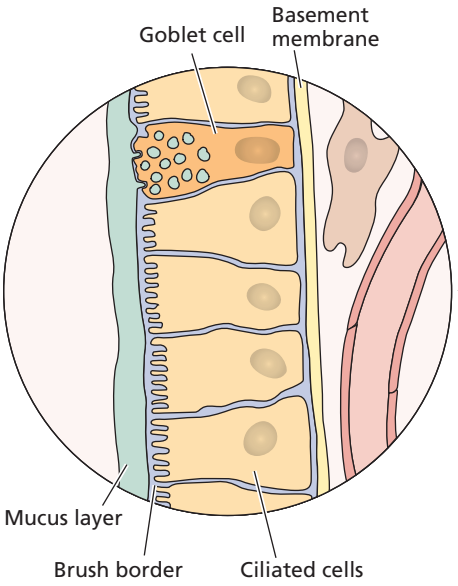
shell over the wound, called a scab. Scabs are more than just the dermis below the site of injury drying and hardening; neutrophils and macrophages are recruited in large numbers to a wound, primarily to engulf bacteria and other pathogens that may benefit from this breach in the skin to infect the host. Recruitment of immune cells before pathogen exposure is one way by which the host prepares for possible pathogen invasion. In addition to their anti-pathogen functions, macrophages recruited to the wound further aid the healing process by the production of growth factors that promote cell proliferation.

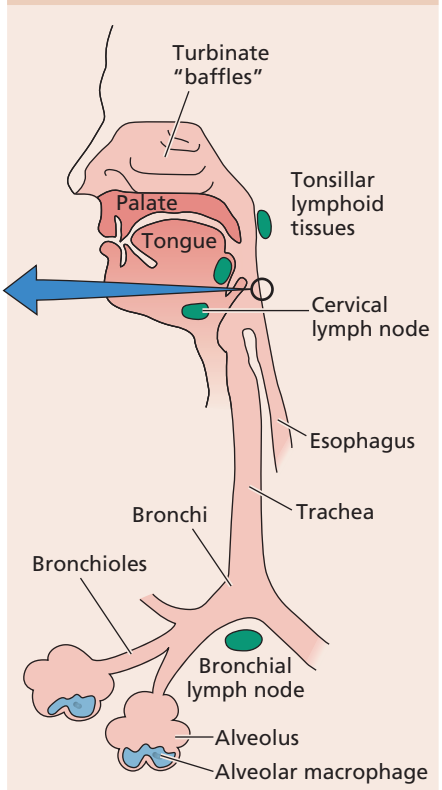
Respiratory Tract

Surfaces exposed to the environment but not covered by skin are lined by living cells and are at risk for infection despite the continuous actions of self-cleansing mechanisms. The most common route of viral entry is through the respiratory tract. In a human lung, there are about 300 million terminal sacs, called alveoli, which function in gaseous exchange between inspired air and the blood. Each sac is in close contact with capillary and lymphatic vessels. The combined surface area of the human lung is ~30 to 50 m^2, approximately the size of a studio apartment. At rest, humans inspire ~6 liters of air per minute. Together, the impressive surface area and large volumes of "miasma" that one inhales each minute imply that foreign particles, such as bacteria, allergens, and viruses, are introduced into the lungs with every breath.

Mechanical barriers play a significant role in antiviral defense in the respiratory tract. The tract is lined with a mucociliary blanket consisting of ciliated cells, mucus-secreting goblet cells, and subepithelial mucus-secreting glands (Fig. 2.6). Foreign particles

Figure 2.6 Sites of viral entry in the respiratory tract. **(Left)** A detailed view of the respiratory epithelium. A layer of mucus, produced by goblet cells, is a formidable barrier to virus particle attachment. Virus particles that traverse this layer may reproduce in ciliated cells or pass between them, reaching another physical barrier, the basement membrane. Beyond this extracellular matrix are tissue fluids, from which particles may be taken into lymphatic capillaries and reach the blood. Local macrophages patrol the tissue fluids in search of foreign particles. Adapted from C. A. Mims et al., *Mims' Pathogenesis of Infectious Disease* (Academic Press, Orlando, FL, 1995), with permission. **(Right)** Viruses that reproduce at different levels of the respiratory tract, with the associated clinical syndromes. SARS, severe acute respiratory syndrome; MERS, Middle East respiratory syndrome.





Site of reproduction	Clinical manifestation	Virus
Turbinate "baffles"	Rhinitis (common cold)	Rhinovirus Coronavirus Parainfluenza virus Respiratory syncytial virus Influenza virus Adenovirus Herpes simplex virus Epstein-Barr virus
Palate, Tongue, Tonsillar lymphoid tissues	Pharyngitis	
Cervical lymph node	Laryngitis	
Esophagus		
Trachea, Bronchi	Tracheitis	Parainfluenza virus Respiratory syncytial virus Influenza virus Adenovirus Measles SARS MERS
Bronchioles	Bronchitis	
Bronchial lymph node	Bronchiolitis	
Alveolus, Alveolar macrophage	Bronchopneumonia	

BOX 2.4

DISCUSSION

In praise of mucus

When you have a cold or sinus infection, it can be disconcerting to take a peek in your tissue after a sneeze. However, the mucus that accompanies many such infections actually serves a very important purpose. Mucus-producing cells line the mouth, nose, sinuses, throat, lungs, vagina, and entire gastrointestinal tract. In addition to its lubricant function, mucus acts as a protective blanket over these surfaces, preventing the tissue underneath from dehydrating. Mucus also acts as a sort of pathogen flypaper, trapping viruses and bacteria. More than being just a sticky goo, mucus contains antibodies, enzymes that kill the invaders it traps, and a variety of immune cells poised to respond to pathogens that attach to it.

It is a common misconception that discolored mucus is directly due to bacterial or viral presence, but the yellow or green mucus hue observed during infection is not due to bacteria or virus particles. When an individual acquires a respiratory tract infection, neutrophils, a key element of the host innate response, rush to the infected site. These cells contain an enzyme, myeloperoxidase, which is critical for the ability of neutrophils to eliminate pathogens, as individuals with a genetic loss of myeloperoxidase are immunocompromised, especially for respiratory tract infections. Myeloperoxidase is stored in azurophilic granules prior to release; these granules are naturally green or tan. Thus, when neutrophils are present in large numbers, the mucus appears green. One may indeed assume that discolored mucus is a sign of infection, as recruitment of neutrophils often accompanies infection.

One final thought that you may wish you did not know: while it is not a very socially acceptable practice, eating one's own nasal secretions (mucophagy), a habit of many young children, may have some evolutionary benefit. Some scientists argue that mucophagy provides benefits to the immune system, especially the underdeveloped host responses of children. As noted above, mucus destroys most of the pathogens that it tethers, so nasal secretions themselves are unlikely to be laden with infectious virus particles. Reintroducing these crippled microorganisms into the gut, where antigen-presenting cells are abundant, may be a form of "low-tech" vaccination or immune memory booster.

Bellows A. 2009. *Alien Hand Syndrome and Other Too-Weird-Not-To-Be-True Stories*, p 28–30. Workman Publishing, New York, NY.





deposited in the nasal cavity or upper respiratory tract are often trapped in mucus, carried to the back of the throat, swallowed, and destroyed in the low-pH environment of the gut (Box 2.4). In the lower respiratory tract, particles trapped in mucus are brought up from the lungs to the throat by ciliary action (Fig. 2.7). Cold temperatures, cigarette smoke, and very low humidity cause the cilia to stop functioning, likely accounting for the association of these environmental conditions with increased illness. When coughing occurs, both the host and the virus benefit; the host expels virus-laden mucus with each productive cough, and the virus is carried out of the host, perhaps to infect another nearby. The lowest portions of the tract, the alveoli, lack cilia or mucus, but macrophages lining the alveoli ingest and destroy virus particles.

Many viruses enter the respiratory tract in the form of aerosolized droplets expelled by an infected individual by coughing or sneezing (Table 2.1; Fig. 2.8). Infection can also spread through contact with respiratory secretions or saliva from an infected individual. Larger virus-containing droplets are deposited in the nose, while smaller droplets can penetrate deeper into the airways or the alveoli. To infect the respiratory tract successfully, virus particles must not be captured or swept away by mucus, neutralized by antibody, or destroyed by alveolar macrophages.

Figure 2.7 Cilia help to move debris trapped in the mucus of the respiratory tract out of the body. Cells in the cell membrane under the mucus have tiny hair-like projections called cilia. Usually, the mucus traps incoming particles. In coordinating waves, the cilia sweep the mucus either up to the nasal passages or back into the throat, where it is swallowed rather than inhaled into the lungs. The acid of the stomach destroys most pathogens not inactivated by the mucus.

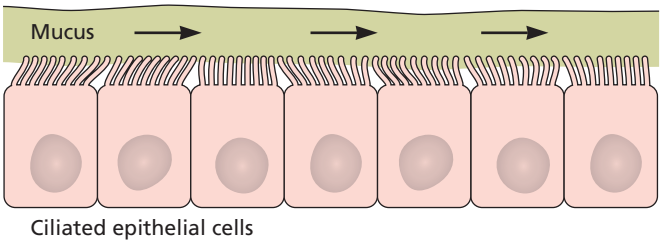




Figure 2.8 A picture is worth a thousand words. A group of applied mathematicians evaluated the distance and "hang time" of various sized droplets produced after a sneeze, using the same strategies as ballistics experts studying gunfire. As many as 40,000 droplets can be released in a single sneeze, some traveling at over 200 miles an hour. Heavier droplets (seen in the photo) succumb to gravity and fall quickly, while smaller droplets (less than 50 μm in diameter) can stay in the air until the droplet dehydrates. Courtesy of CDC/ Brian Judd, CDC-PHIL ID#11161.

Alimentary Tract

The alimentary tract is another major site of viral invasion and dissemination (Table 2.1). Eating, drinking, kissing, and sexual contact routinely place viruses in the gut. Virus particles that infect by the intestinal route must, at a minimum, be resistant to extremes of pH, proteases, and bile detergents. Many enveloped viruses do not initiate infection in the alimentary tract, because viral envelopes are susceptible to dissociation by detergents, such as bile salts.

As depicted in Fig. 2.4, the lumen of the alimentary tract, from mouth to anus, is "outside" of our bodies, and thus the anatomy of the alimentary tube possesses many of the features of the skin. Like the skin, the gut has numerous physical, chemical, and protein-based barriers that collectively limit viral survival and infection: the stomach is acidic, the intestine is alkaline, and proteases and bile detergents are present at high concentrations. In addition, mucus lines the entire tract, and the luminal surfaces of the intestines contain antibodies and phagocytic cells. Moreover, the small and large intestines are coated in a thick (50-μm) paste of symbiotic bacteria that not only aids in digestion and homeostasis but also imposes a formidable physical barrier for virus particles to access the cells beneath (Box 2.5).

BOX 2.5

EXPERIMENTS

Commensal bacteria aid enteric virus infection

On a per-cell basis, humans are more bacterial than mammalian; we are "metaorganisms." Our gastrointestinal tract teems with bacteria, most of which aid in food digestion and promote good health. Consequently, both our eukaryotic defenses and the commensal bacteria that occupy the small intestine can be barriers to viral infection.

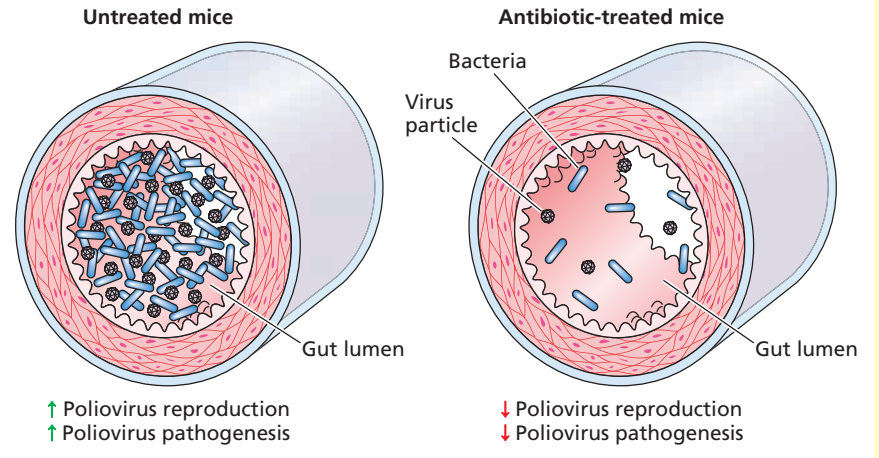
In many cases, however, commensal bacteria actually facilitate viral infection of the host. For example, when the intestinal microbiota of mice was depleted with antibiotics before inoculation with poliovirus, an enteric virus, the animals were found to be less susceptible to disease. Further investigation showed that poliovirus binds lipopolysaccharide, the major outer component of Gram-negative bacteria, and exposure of poliovirus to bacteria enhanced host cell association and infection. Furthermore, three other unrelated enteric viruses, reovirus, mouse mammary tumor virus, and murine norovirus, also have enhanced infection in the presence of intestinal bacteria. These results indicate that interactions with intestinal microbes promote enteric virus infection.

Baldridge MT, Nice TJ, McCune BT, Yokoyama CC, Kambal A, Wheadon M, Diamond MS, Ivanova Y, Artyomov M, Virgin HW. 2015. Commensal microbes and interferon-lambda determine persistence of enteric murine norovirus infection. *Science* **347:**266-269.

Jones MK, Watanabe M, Zhu S, Graves CL, Keyes LR, Grau KR, Gonzalez-Hernandez MB, Iovine NM, Wobus C, Vinje J, Tibbetts SA, Wallet SM, Karst SM. 2014. Enteric bacteria promote human and murine norovirus infection of B cells. *Science* **346:**755-759.

Kane M, Case L K, Kopaskie K, KozlovaA, MacDearmid C, Chervonsky AV, Golovkina TV. 2011. Successful transmission of a retrovirus depends on microbiota. *Science* **334:**245-249.

Kuss SK, Best GT, Etheredge CA, Pruijssers AJ, Frierson JM, Hooper LV, Dermody TS, Pfeiffer JK. 2011. Intestinal microbiota promote enteric virus replication and systemic pathogenesis. *Science* **334:**249–252.



Saliva in the mouth presents an initial obstacle to virus entry. While saliva is mostly water, it does contain lysozymes and other enzymes which aid in the breakdown of food but also can destabilize viral particles. One type of antibody found in saliva, secretory IgA (Chapter 4), may directly bind and inactivate incoming viral particles. A protein known as salivary agglutinin can directly interfere with influenza virus and human immunodeficiency virus, although ingestion is not the traditional route of infection by these viruses.

While passage from the mouth to the stomach is generally considered a quick trip following a swallow, cells in the oropharynx (for example, the tonsils and the back of the throat) appear to be permissive for human papillomaviruses, which can cause oropharyngeal squamous cell carcinoma. Papillomaviruses, traditionally thought to be restricted to the genitourinary or urogenital tract, are likely delivered to the throat during oral sex and can affect both men and women, as both semen and vaginal secretions can carry infectious papillomavirus particles.

Once in the stomach, a virus particle must endure stomach acid, which typically has a pH of 1.5 to 3.0, sufficiently low to denature most proteins of incoming food and many opportunistic viruses. Mucus is also abundant in the stomach, where it coats the lining and helps to prevent the highly corrosive gastric acid from attacking the stomach itself. Mucus also serves as a trap for virus particles, much as in the respiratory tract.

Nearly the entire small intestinal surface is covered with columnar villous epithelial cells with apical surfaces that are densely packed with microvilli (Fig. 2.9). This brush border, together with a surface coat of glycoproteins and glycolipids and the overlying mucus layer, is permeable to electrolytes and nutrients but presents a barrier to microorganisms. Once in the small intestine, pathogens can be attacked by small antimicrobial peptides called **defensins**, which are secreted by Paneth cells. These cells, which lie at the base of the microvillus crypt, secrete large granules filled with enteric alpha-defensins, also called cryptdins. These small (~30-amino-acid) peptides serve primarily to inactivate bacteria by destabilizing the bacterial cell wall or by interfering with bacterial metabolism. Recently, a role of these small peptides in antiviral defense has also been demonstrated. While a widely held view is that defensins exert their antimicrobial functions by disrupting lipid membranes, studies with viruses, including nonenveloped viruses without a lipid coat, reveal more-diverse functions of these peptides, including negative effects on viral entry and movement to the nucleus. Defensins actually **promote** the infection of some viruses, such as human immunodeficiency virus type 1 and human adenovirus, likely by increasing attachment of the virus particles to their cellular receptors.

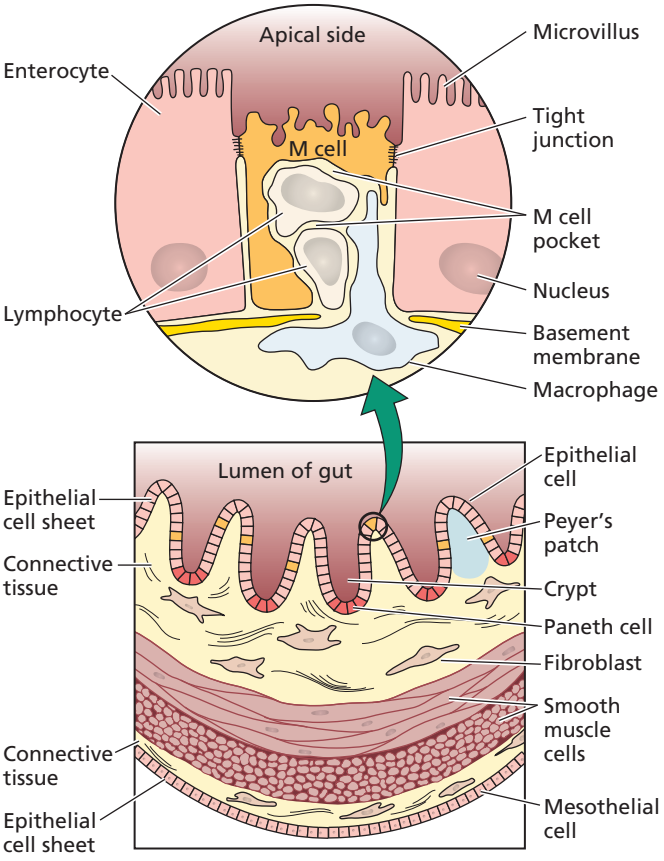


Figure 2.9 Cellular organization of the small intestine. A simplified view of the cellular composition of the small intestine is shown, with Paneth cells lining the base of the intestinal crypts and M cells providing the thin barrier between the intestinal lumen and the Peyer's patches beneath. Schematic drawing of the intestinal wall. This organ is made up of epithelial, connective, and muscle tissues. Each is formed by different cell types that are organized by cell-cell adhesion within an extracellular matrix. A section of the epithelium has been enlarged, and a typical M cell is shown surrounded by two enterocytes. Lymphocytes and macrophages move in and out of invaginations on the basolateral side of the M cell. Adapted from A. Siebers and B. B. Finlay, *Trends Microbiol.* **4:**22–28, 1996, and B. Alberts et al., *Molecular Biology of the Cell* (Garland Publishing, New York, NY, 1994), with permission.

Despite the formidable barriers, some viruses reproduce extensively in intestinal epithelial cells. Scattered throughout the intestinal mucosa are lymphoid follicles that are covered on the luminal side with a specialized follicle-associated epithelium consisting mainly of columnar absorptive cells and M (membranous epithelial) cells. The M cell cytoplasm is very thin, resulting in a membrane-like bridge that separates the intestinal lumen from the subepithelial space. M cells deliver antigens to the underlying lymphoid tissue (termed Peyer's patches) by **transcytosis**. In this process, material taken up on the luminal side of the M cell traverses the cytoplasm virtually intact and is delivered to the underlying basal membranes and

extracellular space (Fig. 2.9). It is thought that M cell transcytosis is the mechanism by which some enteric viruses gain access to deeper tissues of the host. After crossing the mucosal epithelium, a virus particle could enter lymphatic vessels and capillaries of the circulatory system, facilitating spread within the host. A particularly well-studied example is transcytosis of reovirus. After attaching to the M cell surface, reovirus subviral particles are transported to cells underlying the lymphoid follicle, where the virus is reproduced and then spreads to other tissues. Some viruses actively reproduce only within M cells and not underlying tissues. For example, infection by human rotavirus and the coronavirus, transmissible gastroenteritis virus, destroys M cells, resulting in mucosal inflammation and diarrhea, but is not spread beyond the gut.

In some cases, the hostile environment of the alimentary tract actually **facilitates** infection. For example, reovirus particles are converted by host proteases in the intestinal lumen into infectious subviral particles, the form that subsequently infects intestinal cells.

While most viruses that can infect via the alimentary tract gain access via the mouth, it is possible for virus particles to enter the body through the lower gastrointestinal tract without passing through the upper tract and its defensive barriers. Human immunodeficiency virus can be introduced efficiently as a result of anal intercourse. Anal sex can cause abrasions within the rectum, stripping away the protective mucus and damaging the epithelial lining, resulting in broken capillaries. Human immunodeficiency virus particles can pass through such damaged epithelia to gain access to the blood for efficient transport to lymph nodes, where infection and reproduction can ensue. Moreover, as M cells are abundant in the lower colon, they are likely to provide a portal of entry for this virus into susceptible lymphocytes in the underlying lymphoid follicles. Once in the follicle, the virus can infect migratory lymphoid cells and spread throughout the body.

Urogenital Tract

Some viruses enter the urogenital tract, most typically as a result of sexual practices (Table 2.1). Like the alimentary tract, the urogenital tract is well protected by mucus and low pH. The vagina maintains a pH that is typically between 3.4 and 4.5; when the pH increases toward neutrality (as a result of antibiotic use or natural changes in the menstrual cycle, for example), many pathogens, including bacteria and yeast, can flourish. Sex can result in tears or abrasions in the vaginal epithelium or the urethra, allowing virus particles to enter. Some viruses infect the epithelium and produce local lesions (for example, human papillomaviruses, which cause genital warts). Others penetrate deeper, gaining access to cells in the underlying tissues and infecting cells of the immune system (human immunodeficiency virus type 1) or the peripheral nervous system (herpes simplex virus type 2). Infection by the latter two viruses invariably spreads from the initial urogenital site to other tissues in the host, thereby establishing lifelong infections.

Viruses that gain entry by the urogenital tract are extremely common. Approximately one in six people between 15 and 50 years of age has genital herpes, and as this is a lifelong infection, the risk of transmission to future sex partners is high. Herpesvirus infection is often asymptomatic, although the virus can still be shed and infect others. Infections by these viruses pose a particular risk to the developing fetus and can result in miscarriage, early delivery, or lifelong infection that begins in the neonate. These dangers can be mitigated by Caesarian delivery. It is sobering to note that individuals may be affected by multiple sexually transmitted pathogens, and a preexisting infection with one may predispose to infection with another. For example, a genital herpes lesion provides an excellent portal for human immunodeficiency virus.

Eyes

The epithelia that cover the exposed part of the sclera (the outer fibrocollagenous coat of the eyeball) and form the inner surfaces of the eyelids (conjunctivae) provide the route of entry for several viruses. Every few seconds, the eyelid closes over the sclera, bathing it in secretions that wash away foreign particles. Like the saliva, tears that are routinely produced to keep the eye hydrated also contain small quantities of antibodies and lysozymes, which can destroy the peptidoglycan layer of some bacteria. Of interest, the chemical composition of tears differs, depending on whether they are "basal" tears produced constantly in the healthy eye, "psychic tears" produced in response to emotion or stress, or "reflex tears" produced in response to noxious irritants, such as tear gas or onion vapor. The concentration of antimicrobial molecules increases in reflex tears, but not psychic tears, underscoring the fact that host defenses are finely calibrated to respond to changes in the environment.

The primary function of tears is to wash away dust particles, viruses, and other microbes that land on the eye or under the eyelid. There is usually little opportunity for viral infection of the eye, unless it is injured by abrasion. Direct inoculation into the eye may occur during ophthalmologic procedures or from environmental contamination, such as improperly sanitized swimming pools and hot tubs. In most cases, viral reproduction is localized and results in inflammation of the conjunctiva, a condition called conjunctivitis or "pink eye." Systemic spread of the virus from the eye is rare, although it does occur; paralytic illness after enterovirus 70 conjunctivitis is one example. Herpesviruses, in particular herpes simplex virus type 1, can also infect the cornea, mainly at the site of a scratch or other injury, and immunocompromised individuals are at greater risk of retinal infection with cytomegalovirus. Such infections may lead to immune destruction of the

cornea or the retina and eventual blindness. Inevitably, herpes simplex virus infection of the cornea is followed by spread of the virus to sensory neurons and then to neuronal cell bodies in the sensory ganglia, where a latent infection is established. Injury to the eye that allows for viral entry need not be a major trauma: small dust particles or rubbing one's eyes too aggressively may be sufficient to damage the protective layer and form a doorway for virus particles to access permissive cells.

While one may not normally think of eyelashes and eyebrows as key components of host defenses, these well-placed patches of hair help to capture fomites that might invade the eye. An intriguing thought is that, as evolution progressed from apes to humans, dense hair was lost from all except a few parts of the body: on top of the head, in the pubic region, and around the eye. It is tempting to speculate that individuals who retained these patches of hair may have had an evolutionary advantage because they were more resistant to certain infections (Box 2.6).

BOX 2.6

DISCUSSION

Is intuition a host defense?

As sentient humans (and animals), we are constantly surveying our environment for dangers and opportunities. Such senses help all organisms to evade predation and to locate food sources but may also be useful in avoiding infections. For example, a rather typical human behavior upon locating some food toward the back of the refrigerator is to smell it to see if it is still "good," and people are usually quite adept at knowing when food is no longer acceptable to eat. In principle, food surveillance is a kind of quality control to ensure that the foods we eat do not carry dangerous microbes. Similarly, avoiding a murky hot tub or declining the advances of a dubious sexual partner could also be considered finely honed skills that may help to avoid contact with pathogens.



Viral Tropism

Before we describe how viruses move throughout a host, it will be useful to discuss briefly viral **tropism**: the cellular and anatomical parameters that define the cells in which a virus can reproduce *in vivo*. Most viruses do not infect all the cells of a host but are restricted to specific cell types in certain organs. For example, an enterotropic and a neurotropic virus reproduce in cells of the gut and nervous system, respectively. Some viruses are **pantropic**, infecting many cell types and tissues.

Tropism is governed by at least four parameters. It can be determined by the distribution of receptors for entry (**susceptibility**) or by a requirement for differentially produced intracellular gene products required to complete the infectious cycle (**permissivity**). However, even if the cell is susceptible and permissive, infection may not occur because virus particles are physically prevented from interacting with the tissue (**accessibility**). Finally, an infection may not occur, even when the tissue is accessible and the cells are susceptible and permissive, because of intrinsic and innate immune defenses.

Tropism influences the pattern of infection, pathogenesis, and long-term virus survival. Human herpes simplex virus is considered neurotropic because of its ability to infect, and be reactivated from, the nervous system, but in fact, this virus is pantropic and reproduces in many cells and tissues in the host. By infecting neurons, it may establish a stable latent infection, but because it is pantropic, infection may spread to other tissues. Consequently, if an infection is not contained by host defenses at the site of inoculation, the virus may cause disseminated disease, as can occur when herpes simplex virus infects infants and immunocompromised adults (Fig. 2.10). On rare occasions, this virus can enter the central nervous system and cause fatal encephalitis.

Accessibility of Viral Receptors

A cell may be susceptible to infection if the viral receptor(s) is present and functional. However, the receptor may not be accessible to the virus. If the cellular receptor is present only on the basal cell membrane of polarized epithelial cells, a virus cannot infect cells unless it first reaches that location by some means. Alternatively, if the viral receptor is located between adjacent cells (at the tight junctions), another cell surface protein may be necessary to ferry the virus particle from the apical membrane to the site of the viral receptor (Volume I, Chapter 5). Nonsusceptible (non-receptor-producing) cells can still be infected by alternative routes; for example, virus particles bound to antibodies can be taken up by Fc receptors (see "Immunopathological lesions caused by B cells" in Chapter 4).

Host Cell Proteins That Regulate the Infectious Cycle

Sequences in viral genomes that control transcription of viral genes, such as enhancers, may be determinants of viral tropism.

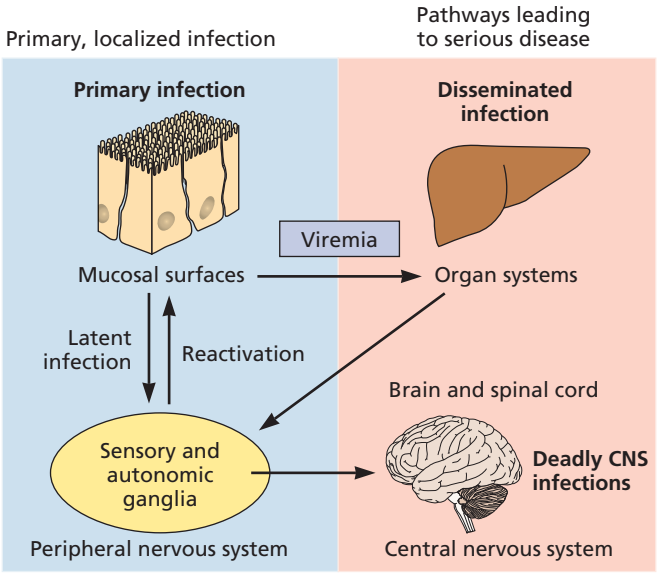


Figure 2.10 Outline of the spread of alphaherpesviruses and relationship to disease. CNS, central nervous system.

In the brain, JC polyomavirus reproduces only in oligodendrocytes (Box 2.7), because the JC virus enhancer is active only in this cell type. Other examples include the liver-specific enhancers of hepatitis B virus, the keratinocyte-specific enhancer of human papillomavirus type 11, and the enhancers in the long terminal repeat of human immunodeficiency virus type 1 that are active in cells of the immune system.

Cellular proteases are often required to cleave viral proteins to form the mature infectious virus particle (Volume I, Chapter 13). For example, a cellular protease cleaves the influenza virus HA0 precursor into two subunits so that fusion of the viral envelope and cell membrane can proceed. In mammals, the reproduction of influenza virus is restricted to epithelial cells of the upper and lower respiratory tracts. The tropism of this virus is thought to be influenced by the limited production of the protease that processes HA0. This serine protease, called tryptase, is secreted by nonciliated club cells of the bronchial and bronchiolar epithelia (Fig. 2.11). The purified enzyme can cleave and activate HA0 in virus particles *in vitro*. Alteration of the hemagglutinin (HA) cleavage site so

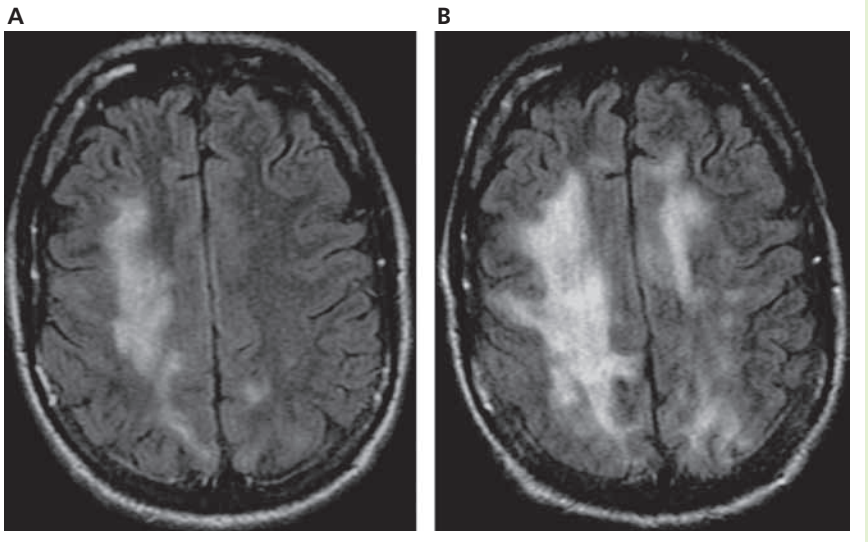
BOX 2.7

BACKGROUND

JC virus, a ubiquitous human polyomavirus

JC virus is widespread in the human population: 70 to 90% of adults are infected. Most humans experience inapparent childhood infections, but the virus then persists for life in the kidneys, brain, and gut tissue. If the immune system is compromised by pregnancy or chemotherapy, virus often reactivates from kidney tissues, and infectious virus particles can be found in the urine, approaching titers of greater than 100,000 particles/ml. On rare occasions, JC virus reactivates in the brain, causing the serious disease progressive multifocal leukoencephalopathy (PML) that affects the myelin-producing oligodendrocytes. This disease is often seen in patients with acquired immunodeficiency syndrome and after immunosuppressive therapy for organ transplants. The genome of JC virus found in the central nervous system of PML patients generally contains differences in the promoter sequence compared to those in healthy individuals. It is thought that such differences in promoter sequence contribute to the fitness of the virus in the central nervous system and thus to the development of PML. Given the rarity of the disease and the lack of suitable animal models, it has been difficult to determine how the genomes of these viruses are maintained and replication reactivated.

Progression of PML in an AIDS patient. Over the course of 4 months, the severity of white matter damage, seen in these magnetic resonance images as bright patches, increases significantly. Reprinted from A.K. Bag et al., *AJNR Am J Neuroradiol* **31:**1564-1576, 2010, with permission.



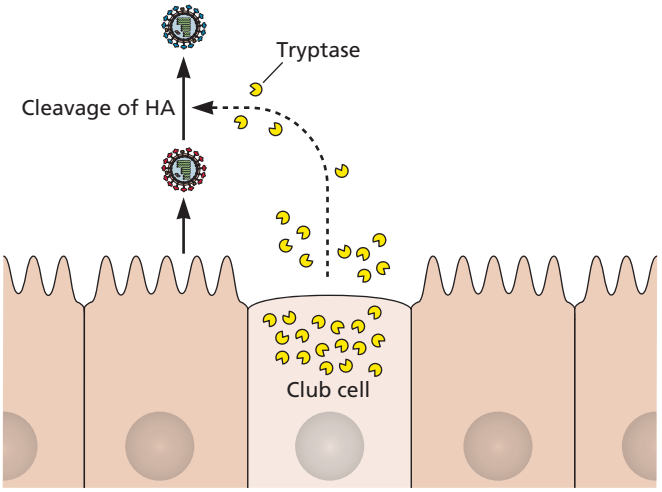


Figure 2.11 Cleavage of influenza virus HA0 by club cell tryptase. Influenza viruses reproduce in respiratory epithelial cells in humans. These virus particles contain the uncleaved form of HA (HA0) and are noninfectious. Club cells (once called Clara cells) secrete a protease, tryptase, which cleaves the HA0 of extracellular particles, thereby rendering the viral particles infectious. Adapted from M. Tashiro and R. Rott, *Semin. Virol.* **7:**237–243, 1996, with permission. Note: In previous editions of this text, club cells were referred to as "Clara cells," named after the German scientist who discovered them. Because Clara was an active member of the Nazi party, in 2013, the lung physiology community elected to change the name of these cells to "club cells." We have adopted this convention.

BOX 2.8

DISCUSSION

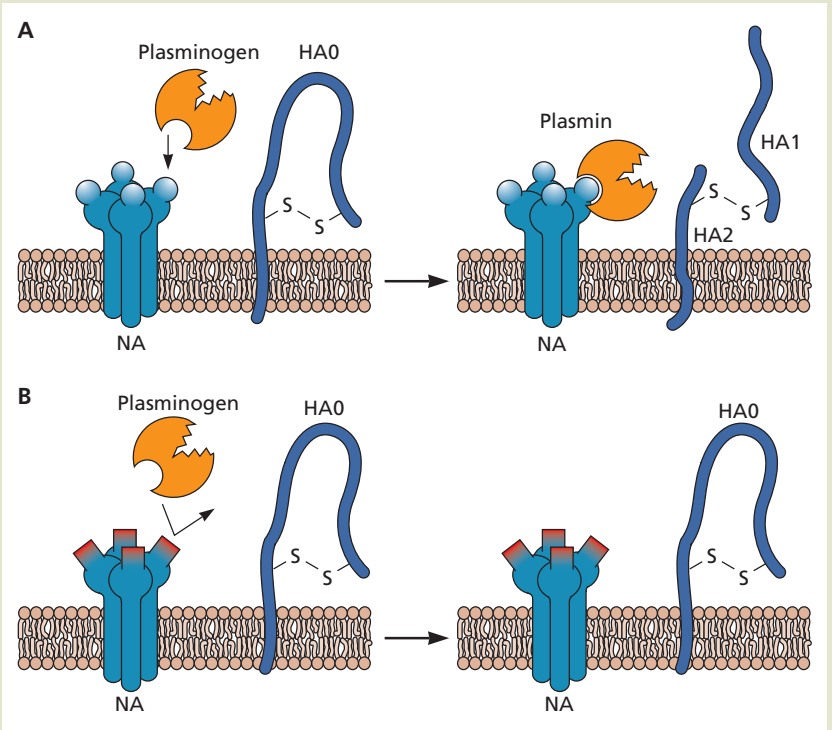
A mechanism for expanding the tropism of influenza virus is revealed by analyzing infections that occurred in 1940

Until the isolation of the H5N1 virus from 16 individuals in Hong Kong, viruses with the HA0 cleavage site mutation that permits cleavage by ubiquitous furin proteases had not been found in humans. However, the WSN/33 strain of influenza virus, produced in 1940 by passage of a human isolate in mouse brain, is pantropic in mice. Unlike most human influenza virus strains, WSN/33 can reproduce in cells in culture in the absence of added trypsin, because its HA can be cleaved by serum plasmin. Surprisingly, it was found that the NA of WSN/33 is necessary for HA0 cleavage by serum plasmin. This altered NA protein can bind plasminogen, sequestering it on the cell surface, where it is converted to the active form, plasmin (see figure, panel A). Plasmin then cleaves HA0 into HA1 and HA2. Therefore, a change in NA, not in HA, allowed cleavage of HA by a ubiquitous cellular protease. This property may, in part, explain the pantropic nature of WSN/33.

Goto H, Kawaoka Y. 1998. A novel mechanism for the acquisition of virulence by a human influenza A virus. *Proc Natl Acad Sci U S A* **95:**10224–10228.

Taubenberger JK. 1998. Influenza virus hemagglutinin cleavage into HA1 and HA2: no laughing matter. *Proc Natl Acad Sci U S A* **95:**9713–9715.

Proposed mechanism for activation of plasminogen and cleavage of HA. (A) Plasminogen binds to NA, which has a lysine at the carboxyl terminus. A cellular protein converts plasminogen to the active form, plasmin. Plasmin then cleaves HA0 into HA1 and HA2. **(B)** When NA does not contain a lysine at the carboxyl terminus, plasminogen cannot interact with NA and is not activated to plasmin. Therefore, HA is not cleaved. Adapted from H. Goto and Y. Kawaoka, *Proc. Natl. Acad. Sci. U. S. A.* **95:**10224–10228, 1998, with permission.



that it can be recognized by other cellular proteases changes the tropism of the virus and its pathogenicity dramatically; some highly virulent avian influenza virus strains contain an insertion of multiple basic amino acids at the cleavage site of HA0. This new sequence permits processing by ubiquitous intracellular proteases, such as furins. As a result, these variant viruses are released in active form and are able to infect many organs of birds, including the spleen, liver, lungs, kidneys, and brain. Naturally occurring mutants of this type cause high mortality in poultry farms. Avian influenza viruses isolated from 16 people in Hong Kong contained similar amino acid substitutions at the HA cleavage site. Indeed, many of these individuals had gastrointestinal, hepatic, and renal symptoms as well as respiratory disease. A virus with such an HA site alteration had not been previously identified in humans, and its isolation led to fears that an influenza pandemic was imminent. To prevent the virus from spreading, all chickens in Hong Kong were slaughtered. Changes in other viral proteins can influence HA cleavage indirectly (Box 2.8).

Spread throughout the Host

Following reproduction at the site of entry, virus particles can remain localized or can spread to other tissues (Table 2.1). Spread beyond the initial site of infection depends on multiple parameters, including the initial viral dose, the presence of viral receptors on other cells, and the relative rates of immune induction and release of infectious virus particles. Localized infections in the epithelium are usually limited by the physical constraints of the tissue and are brought under control by the intrinsic and innate defenses discussed in Chapter 3. An infection that spreads beyond the primary site of infection is said to be **disseminated**. If many organs are viral targets, the infection is described as **systemic.** Spread beyond the primary site requires that the host's physical barriers be breached. For example, virus particles may be able to cross the basement membrane when the integrity of that structure is compromised by inflammation and epithelial cell destruction. Below the basement membrane are subepithelial tissues, where virus particles encounter tissue fluids, the lymphatic system, and phagocytes. All three make substantial contributions in clearing foreign particles but may also allow infectious virus particles to be disseminated beyond the primary site of infection.

One important mechanism for avoiding local host defenses and facilitating spread within the body is the directional release of virus particles from polarized cells at a mucosal surface (Volume I, Chapter 12). Virus particles can be released from the apical surface, from the basolateral surface, or from both (Fig. 2.12). After reproduction, particles released from the apical surface are back where they started, that is, "outside" the host. Such directional release facilitates the dispersal of many newly synthesized enteric viruses in the feces (e.g., poliovirus)

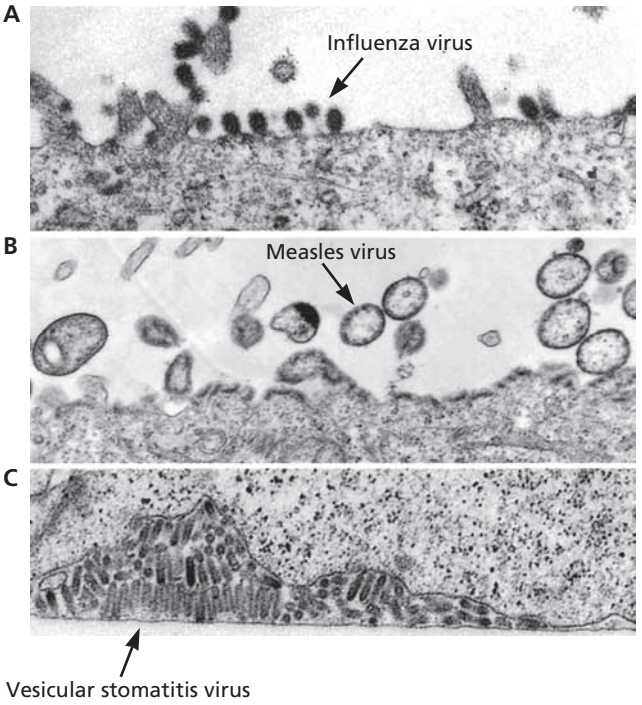


Figure 2.12 Polarized release of viruses from cultured epithelial cells visualized by electron microscopy. (A) Influenza virus released by budding from the apical surface of canine kidney cells. **(B)** Budding of measles virus on the apical surface of human colon carcinoma cells. **(C)** Release of vesicular stomatitis virus at the basal surface of canine kidney cells. Arrows indicate virus particles. Magnification, ×324,000. Reprinted from D. M. Blau and R. W. Compans, *Semin. Virol.* **7:**245–253, 1996, with permission. Courtesy of D. M. Blau and R. W. Compans, Emory University School of Medicine, Atlanta, GA.

or the respiratory tract (e.g., rhinoviruses). In general, virus particles released at apical membranes establish a localized or limited infection and do not penetrate deeply beyond the primary site of infection. In this case, local lateral spread from cell to cell may occur in the infected epithelium, but the underlying lymphatic and circulatory vessels are rarely infected. In contrast, virus particles released from the basolateral surfaces of polarized epithelial cells can access underlying tissues, facilitating systemic spread. The consequences of directional release are striking. Sendai virus, which is normally released from the apical surfaces of polarized epithelial cells, causes only a localized infection of the respiratory tract. In stark contrast, a mutant strain of this virus which is released from both apical and basal surfaces is disseminated, and the infected animals suffer higher morbidity and mortality.

When spread occurs by neural pathways, innervation at the primary site of inoculation determines which neuronal circuits will be infected. The only areas in the brain or spinal cord that are targets for herpes simplex virus infection are those that contain neurons with axon terminals or dendrites

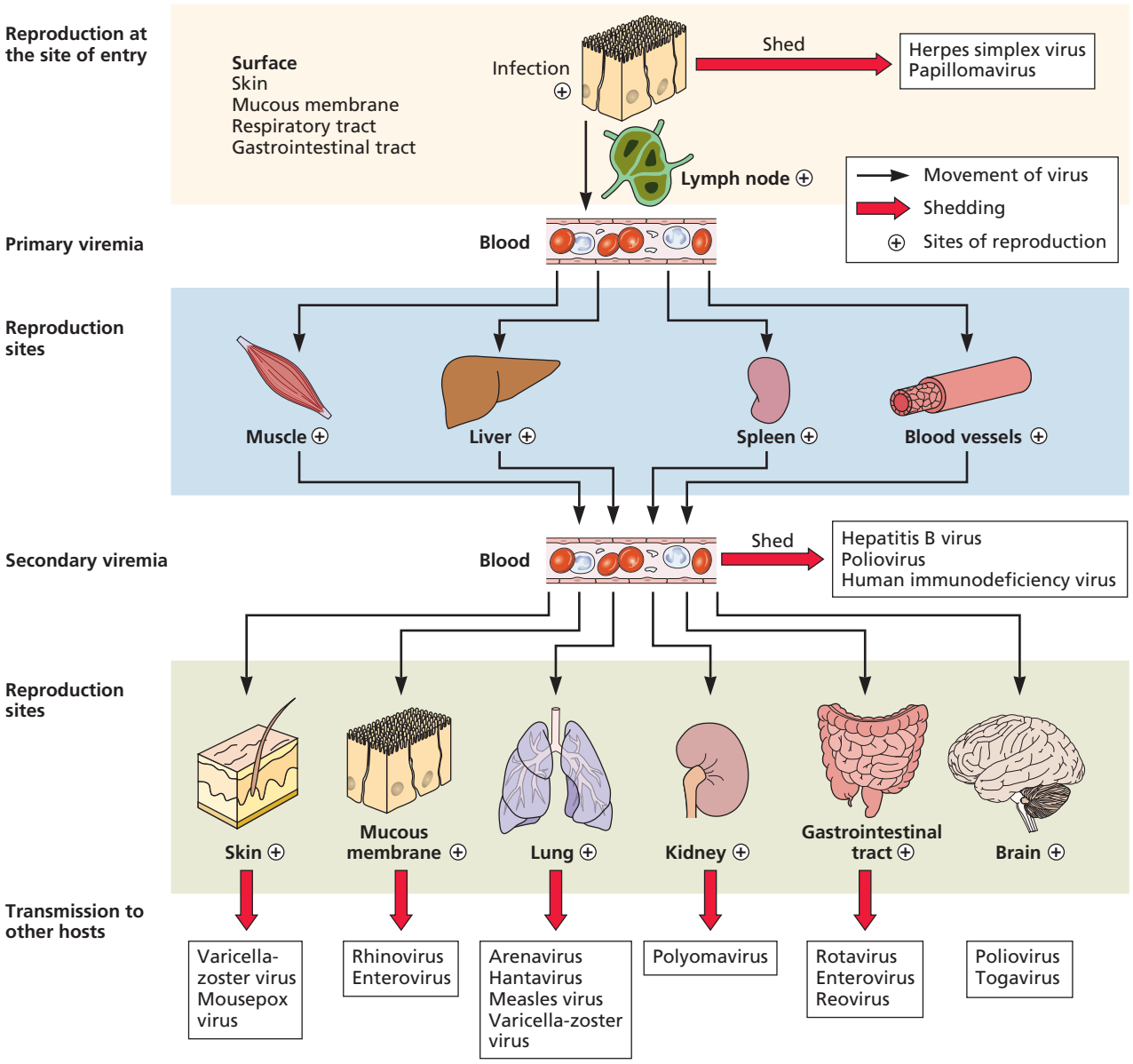
connected to common sites of inoculation in the body. Reactivated herpes simplex virus uses the same neural circuits to return to those sites, where it causes lesions (for example, cold sores in the mouth). After peripheral infection, poliovirus never reaches certain areas of the spinal cord and brain. However, reproduction occurs in these locations if the virus is inoculated directly into the brain or neural pathways.

There are two primary ways to gain access to tissues distal to the site of the inoculation: via the blood and via the nervous system.

Hematogenous Spread

Disseminated infections typically occur by transport through the bloodstream (**hematogenous spread**). Entry may occur through broken blood vessels (human immunodeficiency virus), through direct inoculation (for example, from the proboscis of an infected arthropod vector or the bite of a dog), or by basolateral release of virus particles from infected capillary endothelial cells. Because every mammalian tissue is nourished by a web of blood vessels, virus particles in the blood have access to all host organs, provided that susceptible cells exist in other tissues (Fig. 2.13).

Figure 2.13 Entry, dissemination, and shedding of blood-borne viruses. Shown are the target organs for some viruses that enter at epithelial surfaces and spread via the blood. The sites of virus shedding (red arrows), which may lead to transmission to other hosts, are shown. Adapted from N. Nathanson (ed), *Viral Pathogenesis* (Lippincott-Raven Publishers, Philadelphia, PA, 1997), with permission.



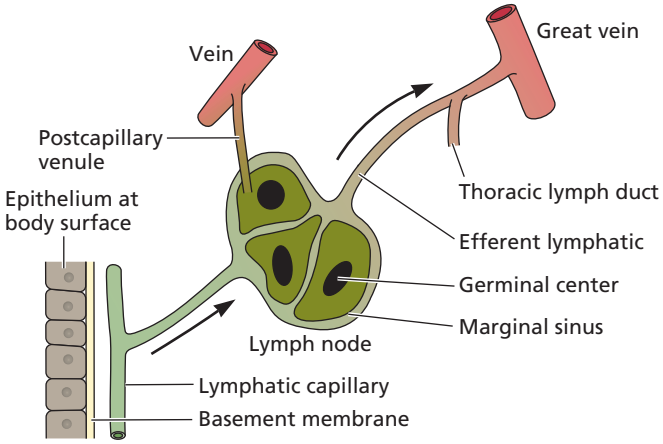


Figure 2.14 The lymphatic system. Lymphocytes flow from the blood into the lymph node through postcapillary venules. Adapted from C. A. Mims et al., *Mims' Pathogenesis of Infectious Disease* (Academic Press, Orlando, FL, 1995), with permission.

Hematogenous spread begins when newly synthesized particles produced at the entry site are released into extracellular fluids and are taken up by the local lymphatic vascular system (Fig. 2.14). Lymphatic capillaries are considerably more permeable than circulatory system capillaries, facilitating virus entry. As lymphatic vessels ultimately drain into the circulatory system, virus particles in lymph have free access to the bloodstream. In the lymphatics, virus particles pass through lymph nodes, where they encounter lymphocytes and monocytes. When viruses reproduce in cells of the immune system, such as human immunodeficiency virus and measles virus, once virus particles reach the lymph node, where susceptible cells are in abundance, they initiate a robust phase of viral reproduction.

The migratory nature of many immune cells allows some viruses to move throughout the host. Because the viral genome is inside a cell during transport, it is effectively shielded from antibody recognition. Traversing the blood-brain barrier poses a particular challenge, as the capillaries that make up this unique barrier limit the access of serum proteins to the brain. However, activated macrophages can pass through, freely delivering viruses such as human immunodeficiency virus into the brain tissue. This process is often referred to as the Trojan Horse approach, because of its similarity to the legend of how the Greeks invaded and captured the protected fortress of Troy (Box 2.9).

The term **viremia** describes the presence of infectious virus particles in the blood. Active viremia is a consequence of reproduction in the host, whereas passive viremia results when particles are introduced into the blood without viral reproduction at the site of entry (as when an infected mosquito inoculates a susceptible host with West Nile virus). Progeny virus particles released into the blood after initial reproduction at the site of entry constitute the **primary viremia** phase. The concentration of particles during this early stage of infection is usually low. However, subsequent dissemination of the virus to other sites results in the release of considerably more virus particles. The delayed appearance of a high concentration of infectious virus in the blood is termed **secondary viremia** (Fig. 2.15). The two phases of viremia were first described in classic studies of mousepox (Fig. 2.1).

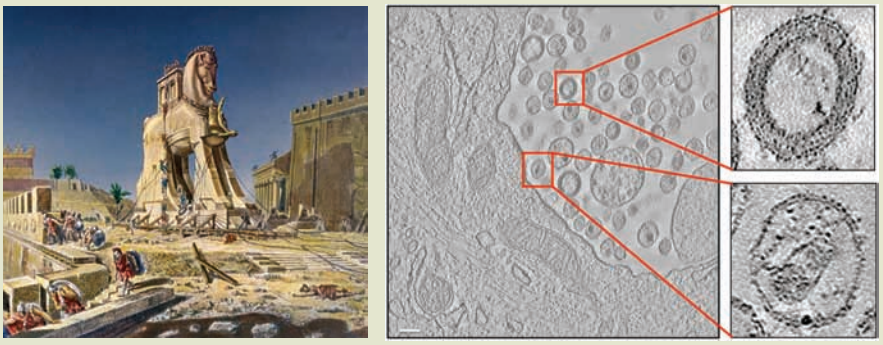
The concentration of virus particles in blood is determined by the rate of their synthesis in permissive tissues and by how

BOX 2.9

DISCUSSION

Inviting packaging may contain dangerous gifts

In the Trojan Horse myth, Greek soldiers could have easily defeated the Troy forces, were it not for the fact that Troy was a walled city, difficult for enemies to enter. The Greeks built a large wooden horse that was intended to be a victory trophy, but instead, many Greek soldiers hid within the hollow horse. Once the horse was safely inside the city walls, the soldiers emerged and quickly attained victory.



Electron tomography of a 150-nm-thick section from HIV-1 BaL-infected macrophages. A slice through a region of the cell containing a collection of viruses in an internal compartment. Scale bar, 100 nm. Reprinted from A.E. Bennett et al., *PLoS Pathog* **5:**e1000591, 2009, with permission.

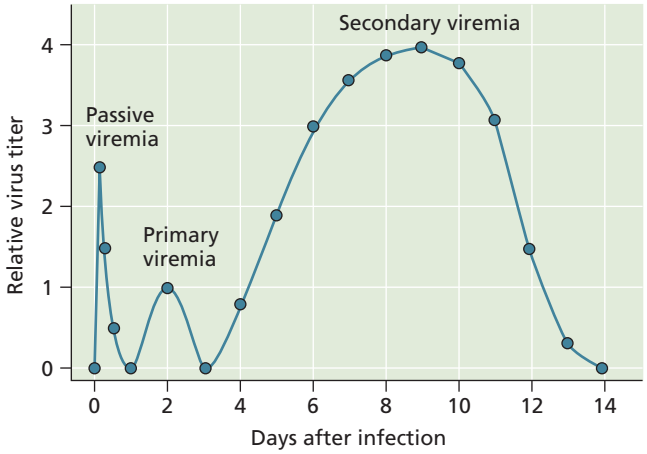


Figure 2.15 Characteristics of viremia. The graph was produced using data from different viral infections. For passive viremia, La Crosse virus, a bunyavirus, was injected into weanling mice, and virus titers in plasma, brain, and muscle were determined at different times thereafter. No virus can be detected in the blood after 1 day. For primary viremia, ectromelia virus was inoculated into the footpads of mice; after local multiplication, the virus enters the blood. For secondary viremia, viral progeny produced by reproduction of ectromelia virus in the target organs were counted. Virus reaches these organs during primary viremia. Adapted from N. Nathanson (ed.), *Viral Pathogenesis and Immunity* (Academic Press, London, United Kingdom, 2007), with permission.

quickly they are released into, and removed from, the blood. Circulating particles are engulfed and destroyed by phagocytic cells of the **reticuloendothelial system** in the liver, lungs, spleen, and lymph nodes. When serum antibodies appear, virus particles in the blood may be bound by them and be neutralized (Chapter 4). Formation of a complex of antibodies and virus particles facilitates uptake by Fc receptors carried by macrophages lining the circulatory vessels. These virus-antibody complexes can be sequestered in significant quantities in the kidneys, spleen, and liver, prior to elimination from the host via urine or feces. The average duration of an individual virus particle in the blood varies from 1 to 60 min, depending on parameters such as the physiology of the host (e.g., age and health) and the size and structural integrity of virus particles. Some viral infections are noteworthy for the long-lasting presence of infectious particles in the blood. Hosts infected with hepatitis B and C viruses or mice infected with lymphocytic choriomeningitis virus may have active viremia that persists for months to years.

Viremia is of diagnostic value to monitor the course of infection in an individual over time, and epidemiologists use the detection of viremia to identify infected individuals within a population. Frequently, it may be difficult, or technically impossible, to quantify infectious particles in the blood, as is the case for hepatitis B virus. In these situations, the presence of characteristic viral proteins, such as the reverse transcriptase for human immunodeficiency virus, provides surrogate markers for viremia.

However, the presence of infectious virus particles in the blood also presents practical problems. Infections can be spread inadvertently in the population when pooled blood from thousands of individuals is used for therapeutic purposes (transfusions) or as a source of therapeutic proteins (gamma globulin or blood-clotting factors). We have learned from unfortunate experience that blood-borne viruses, such as the hepatitis viruses and human immunodeficiency virus, can be spread by contaminated blood and blood products. The World Health Organization estimates that, as of 2000, inadequate blood screening resulted in 1 million new human immunodeficiency virus infections worldwide. Careful screening for these viruses in blood supplies before it is transfused into patients is now standard procedure. However, sensitive detection methods and stringent purification protocols are useful only when we know what we are looking for; as-yet-undiscovered viruses may still be transmitted through the blood supply.

Neural Spread

Some viruses spread from the primary site of infection by entering local nerve endings. In some cases, neuronal spread is the definitive characteristic of pathogenesis, notably by rabies virus and alphaherpesviruses, which cause infections that primarily impact neuronal function or survival. In other cases, invasion of the nervous system is a rare, typically dead-end, diversion from their normal site of reproduction (e.g., poliovirus and reovirus). Mumps virus, human immunodeficiency virus, and measles virus reproduce in the brain but access the central nervous system by the hematogenous route. The molecular mechanisms that dictate spread by neural or hematogenous pathways are not well understood. While viruses that infect the nervous system are often said to be neurotropic (Box 2.10), they are generally capable of infecting a variety of cell types. Viral reproduction usually occurs first in nonneuronal cells, with virus particles subsequently spreading into afferent (e.g., sensory) or efferent (e.g., motor) nerve fibers that innervate the infected tissue (Fig. 2.16).

Neurons are polarized cells with structurally and functionally distinct processes (axons and dendrites) that can be separated by enormous distances. For example, in adult humans, the axon terminals of motor neurons that control stomach muscles can be 50 centimeters away from the cell bodies and dendrites in the brain stem. While our understanding of how viral particles move in and among neurons of the nervous system is incomplete, what is certain is that neurotropic viruses do not traverse these great distances by Brownian (random) motion. Rather, the neuronal cytoskeleton, including microtubules and actin, provides the "train tracks" that enable movement of mitochondria, synaptic vesicles, and virus particles to and from the synapse. Molecular motor proteins, such as dynein and kinesin, are the "engines" that move along these

BOX 2.10

TERMINOLOGY

Infection of the nervous system: definitions and distinctions

A **neuroinvasive virus** can enter the central nervous system (spinal cord and brain) after infection of a peripheral site.

A **neurotropic virus** can infect neurons; infection may occur by neural or hematogenous spread from a peripheral site.

A **neurovirulent virus** can cause disease of nervous tissue, manifested by neurological symptoms and often death.

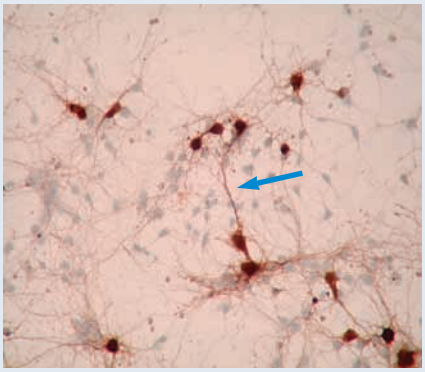
Examples:

Herpes simplex virus has low neuroinvasiveness but high neurovirulence. It always enters the peripheral nervous system but rarely gains access to the central nervous system. When it does, the consequences are severe, often fatal.

Mumps virus has high neuroinvasiveness but low neurovirulence. Most infections lead to invasion of the central nervous system, but neurological disease is mild.

Rabies virus has high neuroinvasiveness and high neurovirulence. It readily infects the peripheral nervous system and spreads to the central nervous system with 100% lethality, unless postinfection vaccination is given.

Primary mouse hippocampal neurons expressing a measles virus receptor, CD46, and infected with measles virus for 48 h. Virus-infected cells are stained brown. Original magnification = ×200.



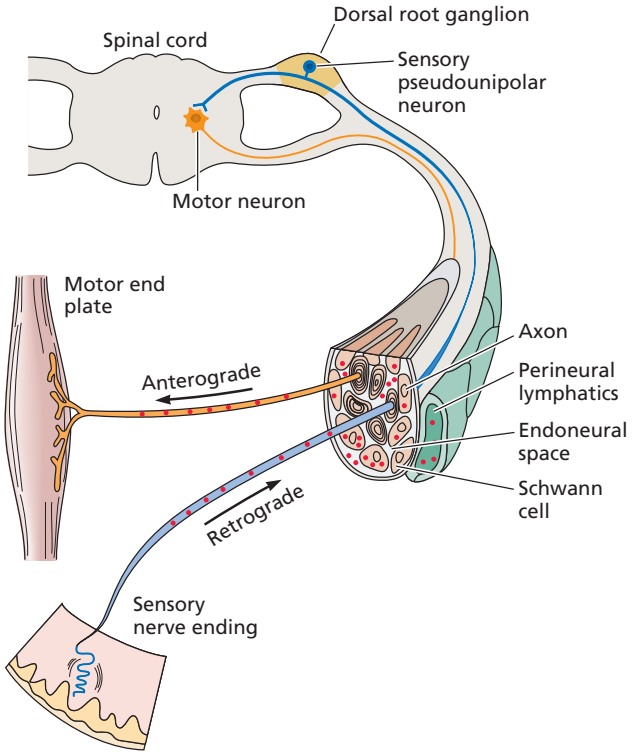


Figure 2.16 Possible pathways for the spread of infection in nerves. Virus particles may enter sensory or motor neuron endings. They may be transported within axons, in which case viruses taken up at sensory endings reach dorsal root ganglion cells. Those taken up at motor endings reach motor neurons. Viruses may also travel in the endoneural space, perineural lymphatics, or infected Schwann cells. Directional transport of virus particles inside the sensory neuron is defined as anterograde [movement from the (−) to the (+) ends of microtubules] or retrograde [movement from the (+) to the (−) ends of microtubules]. Adapted from R. T. Johnson, *Viral Infections of the Nervous System* (Raven Press, New York, NY, 1982), with permission.

thoroughfares (Box 2.11). Drugs, such as colchicine, that disrupt microtubules efficiently block the spread of many neurotropic viruses from the site of peripheral inoculation to the central nervous system (Volume I, Chapter 12).

With few exceptions (Box 2.12), cells of the peripheral nervous system are the first to be infected. These neurons represent the first cells in circuits connecting the innervated peripheral tissue with the spinal cord and brain. Once in the peripheral nervous system, alphaherpesviruses and some rhabdoviruses (e.g., rabies virus), flaviviruses (e.g., West Nile virus), and paramyxoviruses (e.g., measles and canine distemper virus) can spread among neurons connected by synapses (Box 2.13). Virus spread by this mode can continue through chains of connected neurons of the peripheral nervous system and may eventually reach the spinal cord and brain, often with devastating results (Fig. 2.10). Nonneuronal support cells and satellite cells in ganglia may also be infected.

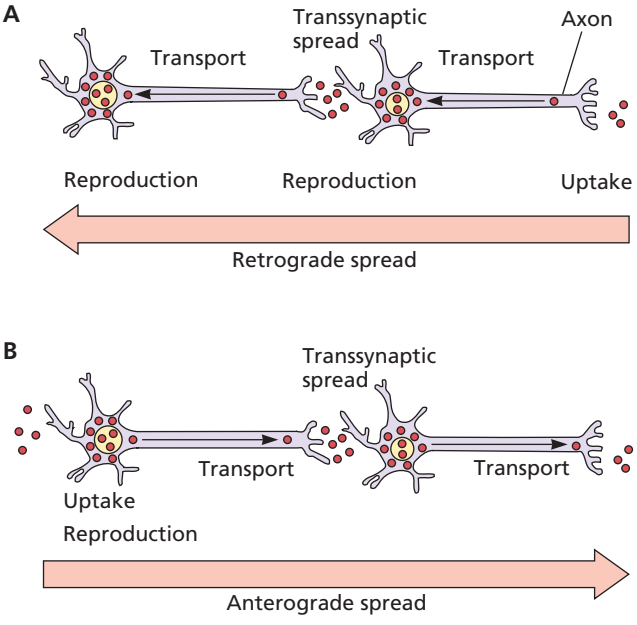
Movement of virus particles and their release from infected cells are important features of neuronal infections. As is true for polarized epithelial cells discussed earlier, directional release of virions from neurons affects the outcome of infection. Alphaherpesviruses become latent in peripheral neurons that innervate the site of infection. Reactivation from the latent state results in viral reproduction in the primary neuron and subsequent transport of progeny virus particles from the neuron cell body back to the innervated peripheral tissue where the infection originated. Alternatively, virus particles can spread from the peripheral to the central nervous system (Fig. 2.10). The direction taken is the difference between a minor local infection (a cold sore) and a life-threatening viral encephalitis. Luckily, spread back to the peripheral site is by far more common.

BOX 2.11

TERMINOLOGY

Which direction: anterograde or retrograde?

Those who study virus spread in the nervous system often use the words **retrograde** and **anterograde** to describe direction. Unfortunately, confusion arises because the terms can be used to describe directional movement of virus particles inside a cell, as well as spread between synaptically connected neurons. Spread from the primary neuron to the second-order neuron in the direction of the nerve impulse is called anterograde spread (see figure). Spread in the opposite direction is termed retrograde. Spread inside a neuron is defined by microtubule polarity. Transport on microtubules from (−) to (+) ends is anterograde, while transport on microtubules from (+) to (−) ends is retrograde.



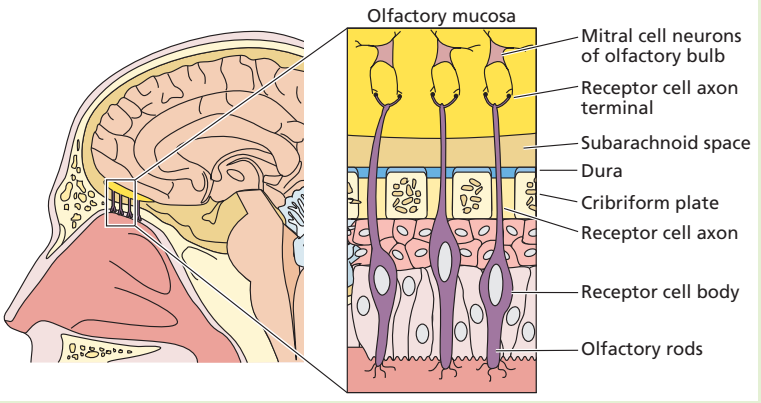
Retrograde and anterograde spread of virus in nerves. (A) Retrograde spread of infection. Virus invades at axon terminals and spreads to the cell body, where reproduction occurs. Progeny virus particles spread to a neuron at sites of synaptic contact. Particles enter the axon terminal of the second neuron to initiate a second cycle of replication and spread. **(B)** Anterograde spread of infection. Virus invades at dendrites or cell bodies and reproduces. Virus particles then spread to axon terminals, where virus particles cross synaptic contacts to invade dendrites or cell bodies of the second neuron.

BOX 2.12

BACKGROUND

The path rarely taken: direct entry into the central nervous system by olfactory routes

Olfactory neurons are unusual in that their cell bodies are present in the olfactory epithelia and their axon termini are in synaptic contact with olfactory bulb neurons. These conduits to the brain project from cells that are in direct contact with the environment. The olfactory nerve fiber passes through the skull via an opening called the arachnoid. Remarkably, few viruses enter the brain by the olfactory route, despite significant reproduction of many in the nasopharyngeal cavity. Adapted from R. T. Johnson, *Viral Infections of the Nervous System* (Raven Press, New York, NY, 1982), with permission.



BOX 2.13

DISCUSSION

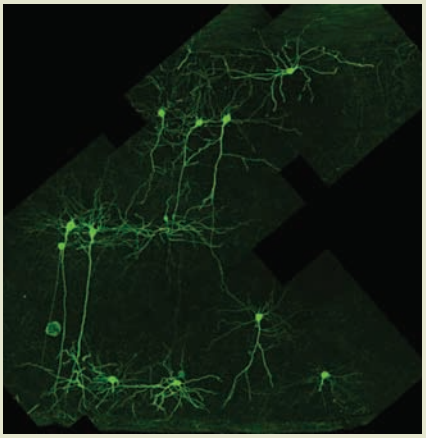
Tracing neuronal connections in the nervous system with viruses

The identification and characterization of synaptically linked multineuronal pathways in the brain are important to understanding the functional organization of neuronal circuits. Conventional tracing methodologies have relied on the use of markers, such as wheat germ agglutinin-horseradish peroxidase or fluorochrome dyes. The main limitations of these tracers are their low specificity and sensitivity. During experimental manipulation, it is difficult to restrict the diffusion of tracers to a particular cell group or nucleus, which can lead to false-positive labeling of a circuit. Furthermore, neurons located one or more synapses away from the injection site receive progressively less tracer, which is diluted at each stage of transneuronal transfer.

Some alphaherpesviruses and rhabdoviruses have considerable promise for use as self-amplifying tracers of synaptically connected neurons. Under proper conditions, second- and third-order neurons will show the same labeling intensity as those infected initially. Moreover, the specific pattern of infected neurons observed in such tracing studies is consistent with transsynaptic passage of virus rather than lytic spread through the extracellular space.

Viruses are typically detected by light microscopy using immunohistochemical staining to localize viral antigens. More recently, reporter genes, such as that encoding the green fluorescent protein (GFP) of *Aequorea victoria*, have been introduced into the genomes of neurotropic viruses for direct visualization of viral infection.

Ekstrand M, Pomeranz L, Enquist LW. 2008. The alpha-herpesviruses: molecular pathfinders in nervous system circuits. *Trends Mol Med* **14:**134–140.



Identification of a possible microcircuit in the rodent visual cortex (V2) after injection of a GFP-expressing strain of pseudorabies virus into the synaptically connected, but distant, V1 region. Infection spread via V1 axons (V1 cell bodies are located far out of the field of view) in a retrograde manner to a subset of V2 cell bodies seen here. Confocal microscopy and image reconstruction by Botond Roska, Friedrich Miescher Institute, Basel, Switzerland.

Organ Invasion

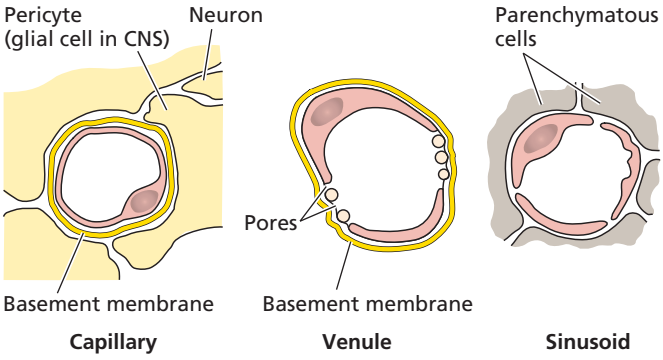
Once virus particles enter the blood or neurons and are dispersed from the primary site, any subsequent reproduction requires invasion of other cells. We have already discussed viral movement into and among neurons to access the brain and spinal cord and will return to this issue in Chapter 5 when we discuss neuropathogenesis resulting from viral infections.

There are three main types of blood vessel-tissue junctions that serve as portals for tissue invasion (Fig. 2.17). In some tissues, the endothelial cells are continuous with a dense basement membrane. At other sites, the endothelium contains gaps, and at still others, there may be **sinusoids**, in which macrophages form part of the blood-tissue junction. Viruses can traverse all three types of junctions.

Entry into Organs with Sinusoids

Organs such as the liver, spleen, bone marrow, and adrenal glands are characterized by the presence of sinusoids lined with macrophages. Such macrophages, known somewhat misleadingly as the reticuloendothelial system (these macrophages are neither endothelial nor a "system"), function to filter the blood and remove foreign particles, similar to a HEPA filter purifying incoming air. The macrophages often provide the portal

Figure 2.17 Blood-tissue junction in a capillary, venule, and sinusoid. **(Left)** Continuous endothelium and basement membrane found in the central nervous system, connective tissue, skeletal and cardiac muscle, skin, and lungs. **(Center)** fenestrated endothelium found in the choroid plexus, villi of the intestine, renal glomerulus, pancreas, and endocrine glands. **(Right)** sinusoid, lined with macrophages of the reticuloendothelial system, as found in the adrenal glands, liver, spleen, and bone marrow. Adapted from C. A. Mims et al., *Mims' Pathogenesis of Infectious Disease* (Academic Press, Orlando, FL, 1995), with permission.



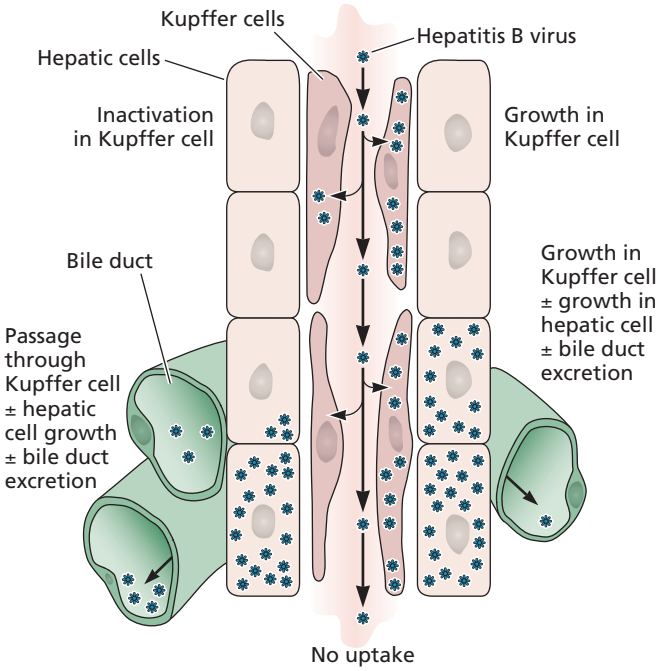


Figure 2.18 Routes of viral entry into the liver. Two layers of hepatocytes are shown, with the sinusoid at the center lined with Kupffer cells. Endothelial cells are not shown. Adapted from C. A. Mims et al., *Mims' Pathogenesis of Infectious Disease* (Academic Press, Orlando, FL, 1995), with permission.

for entry of viral particles into tissues. For example, the hepatitis viruses that infect the liver, which is the major filtering and detoxifying organ of the body, usually enter from the blood. The presence of virus particles in the blood invariably leads to the infection of **Kupffer cells**, the macrophages that line liver sinusoids (Fig. 2.18). Virus particles may be transcytosed across Kupffer and endothelial cells without reproduction to reach the underlying hepatic cells. Alternatively, viruses may multiply in these cells and then infect underlying hepatocytes. Either mechanism may induce inflammation and necrosis of liver tissue, a condition termed hepatitis.

Entry into Organs That Lack Sinusoids

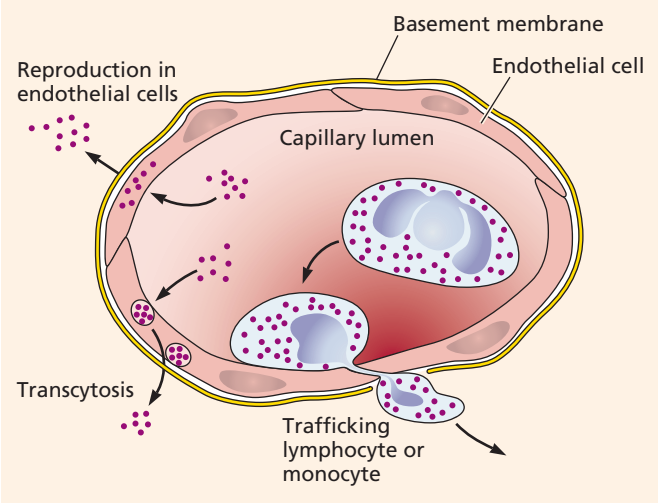
To enter tissues that lack sinusoids (Fig. 2.17), virus particles must first adhere to the endothelial cells lining capillaries or venules, where the blood flow is slowest and the walls are thinnest. To increase the chances of adhesion, virus particles must be present in a high concentration and circulate for a sufficient period. Clearly there is a "race" between adhesion and removal of virus particles by macrophages. Once blood-borne virus particles have adhered to the vessel wall, they can readily invade the renal glomerulus, pancreas, ileum, or colon, because the endothelial cells that make up the capillaries of these tissues are fenestrated, permitting virus particles or virus-infected cells to cross. Some viruses traverse the endothelium while being carried by infected monocytes or lymphocytes, a process called **diapedesis**.

Organs with Dense Basement Membranes

In the central nervous system, connective tissue, and skeletal and cardiac muscle, capillary endothelial cells are supported by a dense basement membrane, which raises an additional barrier to viral passage into the tissue (Fig. 2.17 and 2.19). In the central nervous system, the basement membrane, formed in part by astrocyte processes that align with the basolateral surface of the capillary endothelium, is the foundation of the blood-brain barrier (Fig. 2.20).

In several well-defined parts of the brain, the capillary epithelium is fenestrated (with "windows" between cells, loosely joined together), and the basement membrane is sparse, affording an easier passage for some neurotropic viruses. These highly vascularized sites include the choroid plexus, a sheet of tissue that lies within the brain ventricles and that produces more than 70% of the cerebrospinal fluid that bathes the spinal cord. Some viruses (mumps virus and certain togaviruses) pass through the capillary endothelium and enter the stroma of the choroid plexus, where they may cross the epithelium into the cerebrospinal fluid either by transcytosis or by directed release following production of progeny virus particles. Once in the cerebrospinal fluid, infection spreads to the ependymal cells lining the ventricles and the underlying brain tissue (Fig. 2.20). Other viruses (picornaviruses) may infect directly, or be transported across, the capillary endothelium.

Figure 2.19 How viruses travel from blood to tissues. Schematic of a capillary illustrating different pathways by which viruses may leave the blood and enter underlying tissues. Adapted from N. Nathanson (ed.), *Viral Pathogenesis and Immunity* (Academic Press, London, United Kingdom, 2007), with permission.



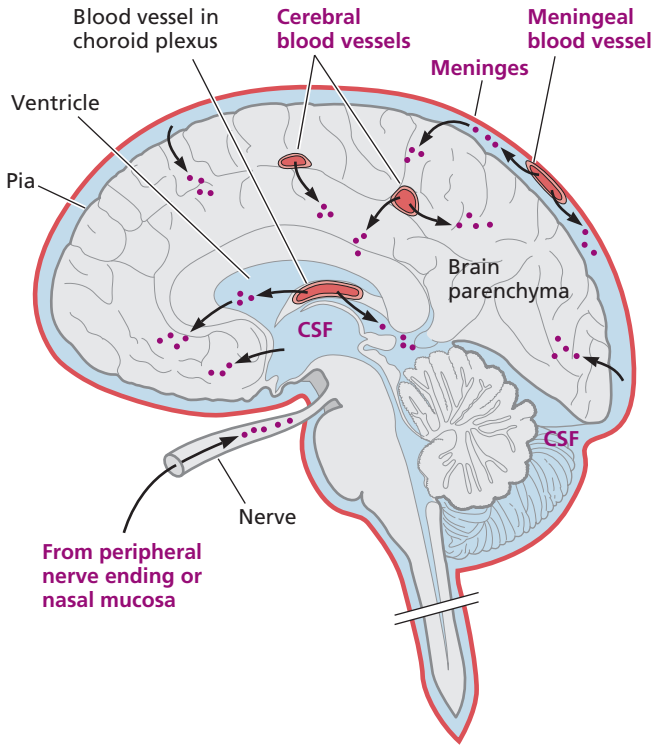


Figure 2.20 How viruses gain access to the central nervous system. A summary of the mechanisms by which viruses can enter the brain is shown. CSF, cerebrospinal fluid. Adapted from C. A. Mims et al., *Mims' Pathogenesis of Infectious Disease* (Academic Press, Orlando, FL, 1995), with permission.

Some viruses (human immunodeficiency virus and measles virus) cross the endothelium within infected monocytes or lymphocytes (the Trojan Horse approach, described earlier). Increased local permeability of the capillary endothelium, caused, for example, by certain hormones, may also facilitate virus entry into the brain and spinal cord.

Skin

In a number of systemic viral infections, rashes are produced when virus particles leave blood vessels. Viruses that cause rashes include measles virus, rubella virus (German measles), varicella-zoster virus (chicken pox and shingles), some parvoviruses (fifth disease), poxviruses (smallpox), and coxsackieviruses (hand, foot, and mouth disease). Skin lesions are distinguished by size, color, frequency, and elevation (an indication of inflammation), may appear coincident with or subsequent to an infection, and are sufficiently distinct in appearance to be easily associated with a particular virus. Destruction of cells by virus reproduction and the host immune system are the primary causes of these skin lesions.

Rashes are not restricted to the skin. Lesions may also occur in mucosal tissues, such as those in the mouth and throat. Because these surfaces are wet, vesicles break down more rapidly than on the skin. During measles infection, vesicles in the mouth become ulcers before the appearance of skin lesions. Such Koplik spots are diagnostic for measles virus infection and appear 2 to 4 days before the skin rash. After virus particles leave the subepithelial capillaries in the respiratory tract, only a single layer of cells must be traversed before particles reach the exterior. Hence, during infections with measles virus and varicella-zoster virus, particles appear in respiratory tract secretions a few days before the skin rash appears. By the time that the infection is recognized from the skin rash, viral transmission to other individuals may already have occurred.

The Fetus

Basement membranes are less well developed in the fetus, and infection can occur by invasion of the placental tissues and subsequent entry into fetal tissue. Infected circulating cells, such as monocytes, may enter the fetal bloodstream directly. In a pregnant female, viremia may result in infection of the developing fetus. Transplacental infections are distinct from perinatal infections, in which the virus is acquired via contact with maternal blood as the baby is delivered through the birth canal. Many pregnant women who are positive for human immunodeficiency virus deliver their children by C-section, but before this became a widespread practice, perinatal infection with human immunodeficiency virus was a major cause of infant morbidity, responsible for an estimated 4 million deaths since the start of the pandemic.

While perinatal infections can be avoided by Caesarian delivery, transplacental infections cannot. Historically, the primary transplacental infections of concern were rubella, cytomegalovirus, and herpes simplex. These viruses, along with the parasite *Toxoplasma*, comprise the four pathogens defined by the acronym TORCH. These pathogens pose a substantial threat to the fetus. The risk of fetal infection in infants whose mothers were infected with rubella virus during the first trimester is approximately 80%. Similarly, intrauterine transmission of human cytomegalovirus occurs in approximately 40% of pregnant women with primary infection. We now know that transplacental transfer of other viruses, including some parvoviruses, measles virus, human immunodeficiency virus, and varicella-zoster virus, can also occur.

Shedding of Virus Particles

As viruses that cannot spread from host to host face extinction, viruses must exit one host to infect another. The release of virus particles from an infected host is called **shedding**. While most transmission events are attributable to such release, there are some exceptions. These exceptions include the direct transmission to host progeny of viral genomes in the host germ line and viruses transmitted via blood transfusions or organ transplantation, such as the human immunodeficiency viruses or hepatitis viruses.

During localized infections in or near one of the body openings, shedding can occur from the primary site of virus reproduction. The papillomaviruses cause genital warts; these viruses reproduce locally in the genital epithelium and are transmitted to naïve hosts via sexual contact. In contrast, release of virus particles that cause disseminated infections can occur from many sites. Effective transmission of virus particles from one host to another depends on the concentration of released particles and the mechanisms by which the virus particles are introduced into the next host. The shedding of small quantities of virus particles may be insufficient to cause new infections, while the shedding of high concentrations may facilitate transmission via minute quantities of tissue or body fluid. For example, the concentration of hepatitis B virus particles in blood can be so high that a few microliters is sufficient to initiate an infection. The stability of virus particles in the environment also influences the efficiency of transmission.

Respiratory Secretions

Respiratory transmission depends on the incorporation of airborne particles in aerosols. Aerosols are produced during speaking and normal breathing, while coughing produces even more forceful expulsion. Transmission from the nasal cavity is facilitated by sneezing and is much more effective if infection induces the production of nasal secretions. A sneeze produces up to 20,000 droplets (in contrast to several hundred expelled by coughing), and all may contain rhinovirus if the individual has a common cold. As noted when we discussed viral entry, the size of a droplet affects its "hang time": large droplets fall to the ground, but smaller droplets (1 to 4 μm in diameter) may remain suspended in the air indefinitely. Such particles may not only come in contact with a naïve host but may be able to reach the lower respiratory tract. Nasal secretions also frequently contaminate hands or tissues. The infection may be transmitted when these objects contact another person's fingers and that person in turn touches his or her nose or conjunctiva. In today's crowded society, the physical proximity of people may select for viruses that spread efficiently by this route. Sneezing may be the body's way of trying to eliminate an irritant in the respiratory tract. Some have speculated that viruses may have been selected that induce sneezing in their hosts to ensure transmission to new hosts (Box 2.14).

Saliva

Some viruses that reproduce in the lungs, nasal mucosa, or salivary glands are shed into the oral cavity. Transmission may occur through aerosols, as discussed above, via contaminated fingers, or by kissing or spitting. Animals that lick, nibble, and groom may also transmit infections in saliva. Perhaps the best-known human virus that is transmitted via saliva is Epstein-Barr virus, which results in mononucleosis, or "kissing disease." Remarkably, the incubation time for this virus

BOX 2.14

DISCUSSION

A ferret model of influenza virus infection ignites irrational fears

Ferrets, which are carnivorous mammals, are excellent models for the study of influenza virus infection, pathogenesis, and transmission. Human and avian influenza viruses reproduce in the ferret airway, and infected animals develop many characteristic signs of the flu, including fever and sneezing. The release of infectious virus through nasal discharges allows for ferret-to-ferret transmission of influenza, an observation first reported in 1933.

In 2011, influenza virus experiments using this well-established model came under intense media scrutiny when two research groups genetically engineered an H5N1 strain of influenza virus that was suspected to be a possible origin of "pandemic" strain in humans. These investigators showed that the engineered viruses were transmissible in ferrets, raising concern that, if the viruses or an infected ferret escaped or was otherwise released, this could trigger a new influenza pandemic. The debate, which continues to impact the scientific and lay communities, centers around "dual-use" experiments: studies that have both a potential public health benefit but that could also be used for bioterrorism or could endanger humans. Some scientists have contended that work such as this should never have been done, given the risks. Others, including many virologists, counter with multiple points. First, that a virus can be transmitted in ferrets does not indicate that it will also spread in humans. However, knowing the genetic changes that affect transmission would have great benefit should an H5N1 virus infection occur in humans by enabling better monitoring of infections and more-rapid development of antivirals. Finally, high-level biocontainment facilities and procedures for such experiments have been mandated to prevent accidental release of nefarious viruses (or infected animals). While it may seem like the basis of a terrific thriller, work with these agents has been strictly controlled.

Belser JA, Katz JM, Tumpey TM. 2011. The ferret as a model organism to study influenza A virus infection. *Dis Model Mech* **4:**575–579.



(that is, the time between infection and disease) is 4 to 7 weeks. Consequently, an Epstein-Barr virus-infected Lothario has lots of time to transmit far more than a radiant smile and magnetic personality before being "found out." Human cytomegalovirus, mumps virus, and some retroviruses can also be transmitted through saliva via an oral route.

Feces

Particles of enteric and hepatic viruses that are shed into the intestine and transmitted via a fecal route are generally more resistant to inactivation by environmental conditions than those released at other sites. An important exception is hepatitis B virus, which is shed in bile that is released into the intestine but is inactivated as a consequence and therefore not transmitted in feces. Viruses transmitted by fecal spread usually survive dilution in water, as well as drying.

Inefficient sewage treatment or its absence, contaminated irrigation systems, and the use of animal manures are prime sources of fecal contamination of food, water supplies, and living areas. Any one of these conditions provides an effective mode for continual reentry of these viruses into the alimentary canals of new hosts. Two hundred years ago, such contamination was inevitable in most of the world, as disposal of human feces in the streets was a common practice. Communities downstream of sites of defecation and waste removal used contaminated water for cooking and drinking. With modern sanitation, the fecal-oral cycle has been largely interrupted in developed countries but remains a major cause of viral spread throughout the rest of the world.

Blood

Viremia is a common feature of many viral infections, and exposure to viremic blood is a primary mode of virus transmission. Arthropods acquire virus particles when they bite viremic hosts and may transmit them to subsequent hosts upon the next blood meal. Hepatitis viruses and human immunodeficiency virus can be transmitted by virus-laden blood during transfusions and injections. Virus particles may also be transmitted from blood during sex or childbirth, and consumption of raw meat may place contaminated blood in contact with the alimentary and respiratory tracts. Health care professionals, emergency rescue workers, and dentists are exposed routinely to blood from individuals who may harbor infections. Indeed, for many of the viruses that cause fatal hemorrhagic fevers (such as members of the *Bunyaviridae* and *Filoviridae*), the only mode of transmission to humans is via contaminated blood and body fluids. Consequently, health care workers often are the first people to become infected and show symptoms in an outbreak of such viral diseases.

Urine

Virus-containing urine is a common contaminant of food and water supplies. The presence of virus particles in the urine is called **viruria**. Hantaviruses and arenaviruses that infect rodents cause persistent viruria. Consequently, humans may be infected by exposure to dust that contains dried urine from infected rodents. A few human viruses, including the polyomaviruses JC and BK, reproduce in the kidneys and are shed in urine.

Semen

Some retroviruses, including human immunodeficiency virus type 1, herpesviruses, and hepatitis B virus, are shed in semen and transmitted during sex. Herpesviruses that infect the genital mucosa are shed from lesions and transmitted by genital secretions, as are papillomaviruses.

Recently, it was shown that human immunodeficiency virus in semen is different from the virus found in blood of the same patients. While sequences of genomes of viruses isolated from the blood are heterogeneous, sequences of the viruses in the semen were much more homogeneous. Two mechanisms were proposed to account for this difference: clonal amplification and compartmentalization. In the first mechanism, one to several viruses are proposed to reproduce in T cells in the seminal tract over a short period of time, such that the population detected in semen is relatively homogeneous (compared to the complex population in the blood). In the second mechanism, the virus is proposed to reproduce in these same cells but over a period of time that is long enough to allow a population genetically distinct from the virus in the blood to be selected.

Milk

Mouse mammary tumor virus is transmitted to offspring primarily via mother's milk into which the virus is shed, as are some tick-borne encephalitis viruses. Mumps virus and cytomegalovirus are shed into human milk but are probably not often transmitted by this route.

Skin Lesions

Many viruses reproduce in the skin, and the lesions that form from such infections contain virions that can be transmitted to other hosts. In these cases, the virus is usually transmitted by direct body contact. For example, herpes simplex virus causes a common rash in wrestlers, known as herpes gladiatorum. Warts caused by certain poxviruses and papillomavirus may also be transmitted by direct, skin-to-skin contact.

Varicella-zoster virus, the agent of chicken pox, is released from the skin in a particularly effective manner. The lesions that form during an acute chicken pox infection are small, lymph-filled blisters that erupt, leaving a crusty scab. Virus concentrations in this fluid are high. Despite the availability of an effective vaccine, acute infections still occur in unvaccinated individuals. Alarmingly, some parents have elected to allow their children to become infected by encouraging close exposure to acutely infected peers (Box 2.15).

BOX 2.15

DISCUSSION

Chicken pox parties

Prior to the widespread use of the varicella-zoster virus vaccine, some parents who wanted to control when their child would get chicken pox (often considered a childhood rite of passage) would host chicken pox parties, in which uninfected children would share lollipops licked by infected children. Given the presence of the virus in the oral mucosa, this ensured that the lollipop contained a high dose of the virus and virtually guaranteed infection. Moreover, because the incubation period for varicella is quite precise (about 14 days following exposure), parents could preplan days off of work to be with their sick child. Even today, there is a "black market" of virus-laced items (such as lollipops) available through the Web. Such practices are an almost inconceivably bad idea; infections by these viruses can be quite severe, and effective, safe vaccines do exist. Moreover, infected children pose risks to immunocompromised adults, such as the elderly and cancer patients receiving immunosuppressive chemotherapy.



Perspectives

Despite the complexity and diversity of viral infection cycles, at a minimum, all viruses must get in, and they must get out. This is true not only for the infected cell (a major theme of Volume I) but also for the infected host. In this chapter, we discussed the many ways by which an organism may acquire pathogens. It is not hyperbole to note that pathogens, including viruses, bacteria, parasites, and fungi, are truly everywhere, and because they have coevolved with their hosts, viruses have coopted our most intimate behaviors to ensure host-to-host transmission.

Fortunately, our counterdefenses pose formidable obstacles. Viruses are trapped in mucus, repelled by dead layers of skin, brushed away by cilia, and destroyed by stomach acid, but simply capturing a rook and a bishop does not end this age-old game of chess. Some viruses can bypass these defenses to reach target cells deep within organs. When viruses breach one of these walls, it is up to the elite forces of the host immune system, the precise strategies of the intrinsic, innate, and adaptive responses, to either end the game in checkmate or suffer the fateful capture of the King.

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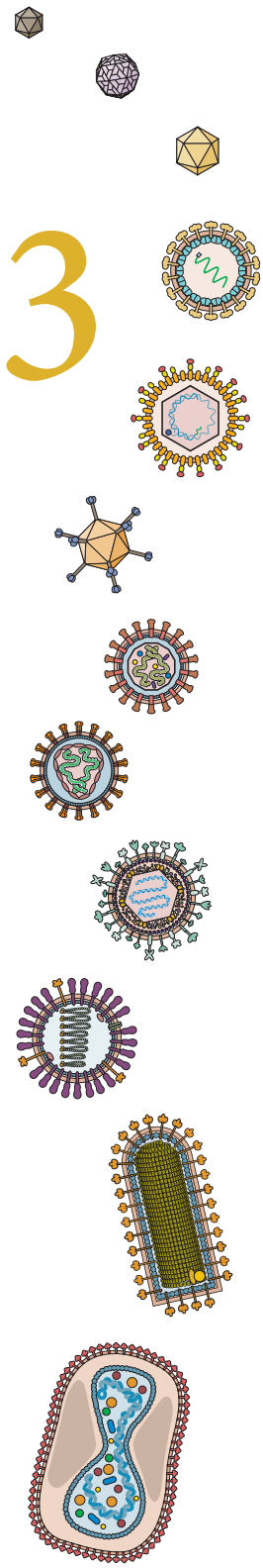
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3 The Early Host Response: Cell-Autonomous and Innate Immunity

Introduction

The First Critical Moments of Infection: How Do Individual Cells Detect a Virus Infection?
- Cell Signaling Induced by Receptor Engagement
- Receptor-Mediated Recognition of Microbe-Associated Molecular Patterns
- Cellular Changes That Occur Following Viral Infection

Intrinsic Responses to Infection
- Apoptosis (Programmed Cell Death)
- Other Intrinsic Immune Defenses
- The Continuum between Intrinsic and Innate Immunity

Soluble Immune Mediators of the Innate Immune Response
- Overview of Cytokine Functions
- Interferons, Cytokines of Early Warning and Action
- Chemokines

The Innate Immune Response
- Complement
- Natural Killer Cells
- Other Innate Immune Cells of Relevance to Viral Infections

Perspectives

References

LINKS FOR CHAPTER 3

⏭ *Video: Interview with Dr. George Stark*
http://bit.ly/Virology_Stark

⏭ *Brought to you by the letters H, P, and eye*
http://bit.ly/Virology_Twiv336

⏭ *Jumpin' Jack Flash, it's a GAS GAS GAS*
http://bit.ly/Virology_Twiv222

Organic life, we are told, has developed gradually from the protozoon to the philosopher, and this development, we are assured, is indubitably an advance. Unfortunately it is the philosopher, not the protozoan, who gives us this assurance.

BERTRAND RUSSELL

Introduction

In the previous chapter, anatomical and chemical barriers that repel the vast majority of potential pathogens were described. When viruses breach these barriers and cells become infected, a distinct and more complicated panoply of reactions is triggered. Some of these responses are deployed moments after pathogen encounter, while others are induced hours to days following infection. Collectively, this constellation of events is defined as the "immune response," but this designation is too broad to describe with any precision what actually happens in the body after viral infection. Consequently, it is helpful to consider antiviral immune responses in temporal stages. When a cell is infected, cell-intrinsic defensive actions are initiated almost immediately. These defensive actions confer antiviral protection, activating and recruiting components of the innate and adaptive immune response (Fig. 2.2). Following pathogen clearance, a memory state is established; memory T and B cells are rapidly reactivated if that same pathogen is encountered again. A corollary to immune activation and viral resolution is the importance of suppressing the host response. Moderating the swift, coordinated, and aggressive assault on a viral infection is crucial for host survival, as antiviral effector actions usually include the destruction of infected cells and production of toxic inflammatory mediators. When improperly stimulated or regulated, the immune response can lead to massive cellular damage, organ failure, chronic illness, or host death. How antiviral host defenses are induced, utilized, and integrated will be the focus of this chapter and Chapter 4.

In Chapter 5, we will turn our focus to the mechanisms by which viruses cause disease, and how improper activation of the immune cascade can lead to tissue damage. Before embarking on these thematically linked chapters, we offer four introductory points to help frame the discussion.

Conveying immunological complexity is challenging. The field of immunology is bewildering to many, even to those who work in closely related disciplines. Contemplating the sheer number and diversity of cell types, soluble proteins, signal transduction pathways, and anatomical locations can be overwhelming. Everything appears to interact with everything else, and there is no evident foundation on which subsequent layers of information can be built. In efforts to convey this complexity, textbooks and reviews often revert to comparisons either with warfare, and the notion that different aspects of the military possess different skills, or with the medical profession, in which immediate immune responders are equated with emergency medical technicians and the later adaptive response compared to specialized surgeons. While initially appealing, these metaphors fail, not simply because they anthropomorphize the immune response, but because they are inaccurate: the immune response is not merely an assortment of different cells with distinct functions, nor does it function in a prescribed sequence of events in which A leads to B and then to C, independently of the pathogen.

As metaphors can be helpful, we propose that the immune response is much like an orchestra playing a symphony: many instruments contribute at discrete times and with unique sounds to create the final piece. The bassoon may appear in both the first movement and the third, the violins may be active throughout but carrying different tunes and played at different volumes, and the cymbals may be silent until the final climactic measures. While listening to an individual part will give you an appreciation of that particular instrument's

PRINCIPLES *The early host response: cell-autonomous and innate immunity*

- The immediate response to an infection is based on two coupled processes: detection and alarm.
- Microbes contain unique components, including certain carbohydrates, nucleic acids, and proteins, that are recognized by cellular pattern recognition receptors present either on the cell surface or in the cytoplasm.
- Binding of a particular ligand to a pattern recognition receptor initiates a signal transduction cascade that results in activation of cytoplasmic transcription regulatory proteins such as Nf-κb and interferon regulatory factors.
- Apoptosis is a normal biological process that can be induced by the biochemical alterations initiated by virus infection.
- Most cells synthesize interferon when infected, and the released interferon inhibits reproduction of a wide spectrum of viruses.
- Phagocytes gather information and initiate the host immune response by taking up cellular debris and extracellular proteins released from dying or apoptosing cells.
- Mechanisms to limit viral reproduction that do not result in the death of the infected cell include autophagy, epigenetic silencing, RNA interference, cytosine deamination, and Trim protein interference.
- Infected cells, sentinel phagocytes, and cellular components of the innate and adaptive immune response secrete many different proteins that can result in activation and recruitment of immune cells, induction of signaling pathways, tissue damage, and fever.
- The innate immune response is crucial in antiviral defense because it can be activated quickly, functioning within minutes to hours of infection.

role in the symphony, only hearing them played simultaneously will allow you to appreciate what the composer hoped to achieve. Moreover, the same set of instruments can be used to play many symphonies. And so it is with immunology. As we delve into interferon γ's signaling pathways or the manner in which antibodies bind to virus particles, do not lose sight of the purpose of the host response: to eliminate a foreign invader quickly with minimal damage to host cells and tissues.

Critical elements are still unknown. The challenge of clearly explaining how the dynamic defensive response is coordinated is further compounded by the fact that we still do not know all the parameters that govern the timing of the host response. That is, if we revert to our symphony metaphor, we know of no specific conductor who leads the entire process. The field of molecular immunology is still quite new, and we have discovered critical players, including Toll-like receptors, T regulatory cells, and T_h17 cells, only within the past couple of decades. Our incomplete understanding of the cast of characters means that many important questions cannot yet be answered. Consequently, we must constantly reevaluate what we thought we understood as new principles and players are identified. Progress is rapid: since the last edition of this textbook, many critical questions have been answered or clarified.

Descriptors sometimes fail us. This text describes the immune response from a temporal viewpoint; in this chapter, we discuss the events that occur immediately after infection (the intrinsic response) through ~2 to 4 days postchallenge (the innate response). We use terms such as "intrinsic" and "innate" because they aid in telling the story, but, of course, the immune response knows no such distinctions. It will therefore be useful to focus on the larger continuum of host immunity.

Virology illuminates immunology. The coevolution of viruses with their hosts has resulted in the selection of viruses that can survive, despite host defenses. The genomes of successful pathogens, which can evolve far faster than the hosts they infect, encode proteins that modify, redirect, or block each step of host defense. Indeed, for every host defense, there will be a viral counter-offense, even for those viruses with genomes that encode a small number of proteins. Consequently, the exploration of how viruses reproduce in their hosts led to the discovery of crucial immunological principles, as we shall see throughout this chapter and the next.

The First Critical Moments of Infection: How Do Individual Cells Detect a Virus Infection?

A viral infection in a host can begin only once physical and chemical barriers are breached and virions encounter living cells that are both susceptible and permissive (Chapter 2). But once these hurdles are overcome, the host's awareness of a foreign invader must transition from a single cell sending out an "I'm infected" alarm to a full-scale, whole-body response, and these processes must be exquisitely coordinated and timed. The importance of an early and appropriate response cannot be overstated: if the response is delayed or weak, the host may die from the consequences of unrestricted infection; if it is too aggressive, the host may suffer from damage by its own immune cells and proteins.

All cells have the capacity to react defensively to various stresses, such as starvation, temperature extremes, irradiation, and infection. Some of these safeguards maintain cellular homeostasis, while others have evolved to detect cellular invaders rapidly. These cell-autonomous (that is, can be accomplished by a single cell in isolation), protective programs, which are inherent in all cells of the body, are termed **intrinsic cellular defenses** to distinguish them from the specialized defenses possessed by "professional" cells of the innate and adaptive arms of the immune system. As a quick response is key, most intrinsic defenses are "ready-to-go"; that is, they do not require transcription and translation, but rather are present in the cell, ready to act, or awaiting a signal to become activated immediately. Intrinsic defenses are among the most conserved processes in all of life, shared by humans, fruit flies, plants, and bacteria. In contrast, specialized immune cells and effector proteins appeared much later in evolution, during the emergence of multicellular organisms.

How the intrinsic defenses are induced in the first cell to be infected within a host, or in an adjacent phagocytic cell, such as a macrophage or dendritic cell, is quite similar to our own experience when something in our environment changes: we perceive a difference only in the context of what we recall as normal. As we can distinguish "familiar" and "different," the immune response distinguishes "self" from "nonself." The immediate response to an infection is based on two coupled processes: detection and alarm. First, the microbe must be recognized by the infected cell. This achievement, alone, is fascinating: in addition to the specific function(s) of any individual cell in the body, virtually **all** mammalian cells are equipped with a detection system that is triggered when a pathogen has engaged or crossed the plasma membrane. Furthermore, the cell is able to identify aspects of the intruder, including whether it is DNA based or RNA based; cytoplasmic or nuclear; an intracellular bacterium, virus, or parasite. Specific protein detectors recognize structures that are unique to microbes or their genomes.

Once a microbe has been detected, the infected cell must then sound the alarm to initiate the series of events that lead to an appropriate defense. The virus infection may be halted at any step along this continuum (Fig. 3.1). The molecular coupling between the pathogen detectors and

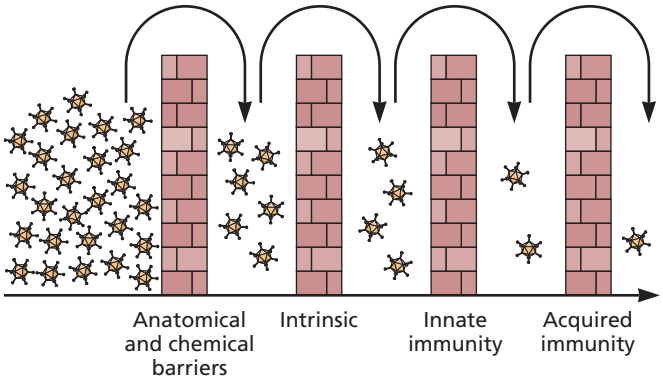


Figure 3.1 Integration of intrinsic defense with the innate and adaptive immune response. The sequential nature of host defenses is depicted as the breaching of successive barriers by viral infection. Most infections are prevented by anatomical or chemical barriers (Chapter 2). When these barriers are penetrated, additional host defenses, including intrinsic and innate defenses, come into play to contain the infection. Activation of acquired immune defenses (also called adaptive immunity) is usually sufficient to contain and clear any infections that escape intrinsic and innate defense. In rare instances, host defenses may be absent or inefficient, and severe or lethal tissue damage and host sickness or death can result. Adapted from D. T. Fearon and R. M. Locksley, *Science* **272:**50–54, 1996, with permission.

the response effectors is understood only in a broad sense, although this is an intense area of research. Tailoring the immune response to the pathogen continues as the infection proceeds, and at each critical juncture of immune defense. There are at least three ways by which a cell can become alerted to infection.

Cell Signaling Induced by Receptor Engagement

As soon as virus particles engage their receptors, cellular signal transduction pathways are activated. Remember that no cell surface protein is only a viral receptor: viruses have been selected to co-opt cellular proteins for viral entry (Volume I, Chapter 5). Many cell surface proteins are linked to intracellular molecules, such that binding of the normal ligand to the receptor triggers pathways that enable the cell to respond to changes in its environment. For example, when it is bound by a ligand, the CD46 receptor activates signaling molecules that impact cell proliferation, cellular polarity, and gene expression, including the synthesis of type I **interferons** (IFNs). Because this protein is the receptor for measles virus and some adenoviruses and herpesviruses, binding of these virus particles to CD46 can elicit the same signals, with the same consequences. Thus, even before the virus enters the cell, the dynamics of ion flow, membrane permeability, protein modification and localization, and host gene transcription may change. Note that noninfectious virus particles, which can bind to receptors but cannot reproduce, may also induce these signals.

Receptor-Mediated Recognition of Microbe-Associated Molecular Patterns

A second way that cells respond to infection is by interaction of intracellular components with microbial proteins or nucleic acids. Microbes contain unique components, including bacterial and fungal carbohydrates (e.g., lipopolysaccharide [LPS]), nucleic acids (such as single-stranded [ss] DNA or double-stranded [ds] RNA), polypeptides (such as flagellin), and lipoteichoic acids from Gram-positive bacteria. Such molecules, initially called **pathogen-associated molecular patterns (PAMPs)**, are detected by cellular **pattern recognition receptors**, which can be present either on the cell surface or in the cytoplasm. Pattern recognition receptors have been selected to be highly pathogen specific, and some also detect host components that are released upon cellular damage, including uric acid (termed **damage-associated molecular patterns [DAMPs]**). Recognition by pattern recognition receptors rapidly triggers the synthesis of antimicrobial products, including inflammatory cytokines, chemokines, and type I IFNs. While the term "PAMP" is relatively new, **all** microbes, including those that are not pathogenic, possess these molecules, prompting some to reconsider them as **microbe**-associated molecular patterns (MAMPs). This is not merely a semantic distinction, as this discovery implies that even nonthreatening entities could sound a cellular alarm.

Pattern recognition receptors were first identified in plants, which exhibit the simplest detection-to-alarm process: certain plant proteins are both the detector **and** the signal transducer that drives cell behavior. In contrast, most mammalian pattern recognition receptors transmit signals by engaging with multiple cytoplasmic adapter molecules that eventually provoke a cellular response, usually by influencing gene expression. Perhaps the use of multiple adapter proteins in animals allowed for diversification of the alarm to a common detection signal, or conversely, for the integration of diverse signals to a common node, such as nuclear factor-κb (Nf-κb). Our first insights into the immunological nature of these pathogen receptors came from *Drosophila* developmental genetics (Box 3.1). We now understand that all intrinsic and innate defense systems arose early in the evolution of multicellular organisms, and remain absolutely essential for survival of mature organisms in a microbe-filled world.

Members of different families of receptors detect specific motifs that are characteristic of invading microbes in single cells (Table 3.1 and Fig. 3.2). The Toll-like receptor (Tlr) family consists of 10 members in humans (12 in mice) that are present either on the cell surface or within lysosomes, where entering viruses first appear (Box 3.2). Tlrs 1, 2, 4, 5, and 6 are found on the extracellular membrane and recognize primarily extracellular microbes, such as bacteria, fungi,

BOX 3.1

TRAILBLAZER

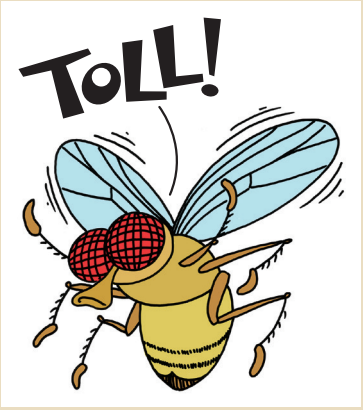
Toll receptors: the fruit fly connection

- The Toll signaling pathway was defined initially as being essential for the establishment of the dorsal-ventral axis in *Drosophila* embryos. Eric Wieschaus and Christiane Nüsslein-Volhard discovered the first Toll mutants. When Wieschaus showed the unusual mutant *Drosophila* embryos to Nüsslein-Volhard, she exclaimed, "Toll!" (a German slang term comparable to "Cool!" or "Awesome!").
- Toll signaling also initiates the response of larval and adult *Drosophila* to microbial infections.
- Toll-like receptors in both flies and mammals bind to a variety of microbe-specific components and trigger a defensive reaction via signal transduction pathways and activation of new gene expression.
- Insect Toll receptors are activated by an endogenous protein ligand produced indirectly by exposure to microbes. Vertebrate Toll receptors bind microbial ligands directly.

Anderson KV. 2000. Toll signaling pathways in the innate immune response. *Curr Opin Immunol* **12:**13–19.

Gay NJ, Gangloff M. 2007. Structure and function of Toll receptors and their ligands. *Annu Rev Biochem* **76:**141–165.

Mushegian A, Medzhitov R. 2001. Evolutionary perspective on innate immune recognition. *J Cell Biol* **155:**705–710.



and protozoa, although the coat proteins of some viruses, such as measles virus, can be recognized by these cell surface molecules. Tlrs 3, 7, 8, and 9 are present within endocytic compartments of the cell and recognize primarily nucleic acid MAMPs derived from viruses or intracellular bacteria, including unmethylated DNA and dsRNA. The specificity of these molecules can be even more precise: Tlr9 binds to DNA-binding proteins, Tlr3 binds to dsRNA, and Tlr7 binds to ssRNA.

While Tlrs are the prototype pattern recognition receptors, their synthesis is generally restricted to macrophages and dendritic cells that engulf pathogens at points of entry into the body, including the skin and mucous membranes. Other receptors are produced more ubiquitously, including RNA helicases (e.g., Rig-I and Mda5) that detect foreign RNA in the cytoplasm (Box 3.3). These RNA helicases recognize chemical features of viral RNAs that do not appear on cellular RNA, such as 5′ triphosphate groups and replication intermediates with extensive tracts of dsRNA.

Binding of a particular ligand to a pattern recognition receptor initiates a signal transduction cascade that results in activation of transcription regulatory proteins such as Nf-κb and interferon regulatory factors (Irfs). These regulatory proteins, in turn, stimulate expression of cytokine genes including those that encode IFN-α and IFN-β, as well as other proinflammatory cytokines. As we shall see later in this chapter, IFN-α and IFN-β play important roles in the recruitment of innate immune cells to the site of an infection, and the amplification of intrinsic cellular defenses by binding to IFN receptors on the infected cell surface or on the surface of adjacent, uninfected cells. For example, Rig-I and Mda5 result in the synthesis of IFNs, which in turn amplify Rig-I and Mda5 gene expression.

Table 3.1 Intracellular detectors of viral infection[a]

Receptor	Cellular compartment	Ligand(s) detected	Virus infection(s) detected
Rig-I	Cytoplasm	dsRNA; ssRNA with 5′ phosphate	Influenza virus
Mda5	Cytoplasm	dsRNA	Encephalomyocarditis virus, measles virus
Tlr2	Plasma and endosomal membranes	Measles virus HA protein	Human cytomegalovirus
Tlr4	Plasma and endosomal membranes	Mouse mammary tumor virus envelope protein	Respiratory syncytial virus
Tlr3	Plasma and endosomal membranes	dsRNA	Murine cytomegalovirus, reovirus, West Nile virus
Tlr7 and Tlr8	Plasma and endosomal membranes	ssRNA	Human immunodeficiency virus, influenza virus
Tlr9	Plasma and endosomal membranes	dsDNA; synthetic, unmethylated CpG DNA	Herpes simplex virus 1 and 2

[a]Data from T. Saito and M. Gale, Jr., *Curr Opin Immunol* **19:**17–23, 2007; G. Trinchieri and A. Sher, *Nat Rev Immunol* **7:**179–190, 2007; and N. J. Gay and M. Gangloff, *Annu Rev Biochem* **76:**141–165, 2007.

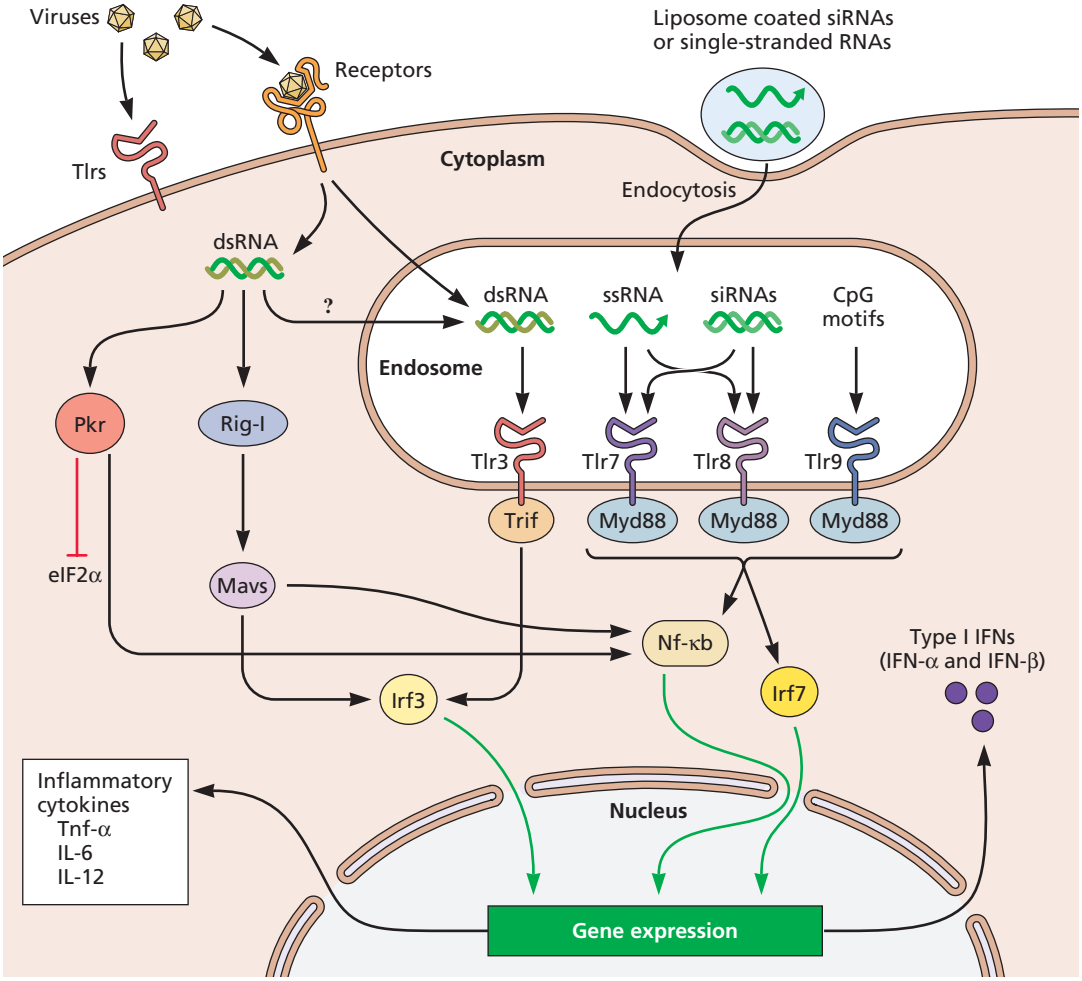


Figure 3.2 Recognition of foreign nucleic acids in mammalian cells. The Tlrs, Rig-I, and Pkr all contribute to detection of microbe-associated molecular patterns including ssRNA, dsRNA, RNA nucleotides, siRNAs, and unmethylated CpG-containing oligonucleotides. As the receptor's cognate nucleic acid is bound on the cell surface, in the cytoplasm, or in the lumen of endosomes, signal transduction leads to activation of Nf-κb, Irf3, or Irf7 to induce expression of inflammatory cytokines and IFN-α/β. Important cytoplasmic proteins in the signal transduction cascade, including Trif and Myd88, bind the cytoplasmic tails of endosomal Tlr proteins after they have engaged their cognate ligand. Viral RNA and DNA may be exposed in the lumen of endosomes after degradation or uncoating events. Pkr is autophosphorylated when dsRNA is bound, leading to phosphorylation of its substrates. One such substrate is the α subunit of eukaryotic translation initiation factor 2 (eIF2α). Phosphorylation of this protein blocks protein synthesis.

New pattern recognition receptors that recognize novel microbial products continue to be identified. For example, dsDNA is rarely found in the cytoplasm, and its presence could indicate either cellular damage or infection. Sensors of dsDNA, including cGAS, interferon-inducible protein 16 (Ifi16), and others, activate the cellular signaling molecule Sting to induce type I IFN synthesis (Fig. 3.3). The distribution and concentration of these receptors vary among different cell types and tissues.

While cytoplasmic sensors of infection are reasonably well characterized, less is known about nuclear detectors of foreign nucleic acids. It is known that a DNA damage response may ensue in the nucleus as a result of detection of single-stranded nucleic acid (e.g., from a single-stranded nuclear virus, such as influenza) or exposed double-strand ends of DNA. For example, Ifi16 binds herpesvirus DNA in the nucleus, triggering expression of antiviral cytokines.

Notwithstanding this impressive array of cell-based virus detectors, every virus that exists must reproduce to some extent in the face of these cellular responses. Clearly, viral gene products can bypass or modulate the intracellular detectors

BOX 3.2

DISCUSSION

The Toll-like receptors

Toll-like receptors (Tlrs) are the prototypical pattern recognition molecules. They are synthesized predominantly by dendritic cells and macrophages, but can be found in other cell types. Tlrs are type I transmembrane proteins that are conserved from sea urchins to humans, and they can recognize intracellular as well as extracellular microbial ligands. Endocytosed proteins and virus particles end up in lysosomal compartments of dendritic cells, where they can be digested. Some Tlrs, including Tlr3 and Tlr9, are also located in endosomes and lysosomes, perfectly placed to bind these unusual viral products. Ligands that might characterize viral infections include CpG-containing DNA, dsRNA, and unique features of ssRNA. Unmethylated CpG tracts are present in bacterial and most viral DNA genomes, while dsRNA and ssRNA are commonly found in virus-infected cells.

After ligand binding, the Tlrs, like many receptors, aggregate in the plasma or lysosomal membrane, an event that stimulates binding of adapter proteins (see the figure). Many downstream signaling steps in pattern recognition and inflammation are mediated by common components. For example, when ligands bind Tlrs, a domain on their cytoplasmic tails, called the Toll/interleukin-1 receptor (Tir) domain, binds the adapter protein myeloid differentiation primary response protein 88 (Myd88), which then in turn binds to the IL-1 receptor-associated kinase (Irak) through common death domain motifs. With the exception of Tlr3, all Tlrs engage Myd88. Irak then activates conserved downstream pathways. Nf-κb activation leads to transcription of genes encoding inflammatory cytokines and T cell costimulatory molecules, whereas extracellular signal-regulated kinase (Erk) and Jun N-terminal protein kinase (Jnk) signaling affect cytoskeletal organization, cell survival, and proliferation. The p38 pathway can lead to stabilization of short-lived mRNAs and increased production of various cytokines.

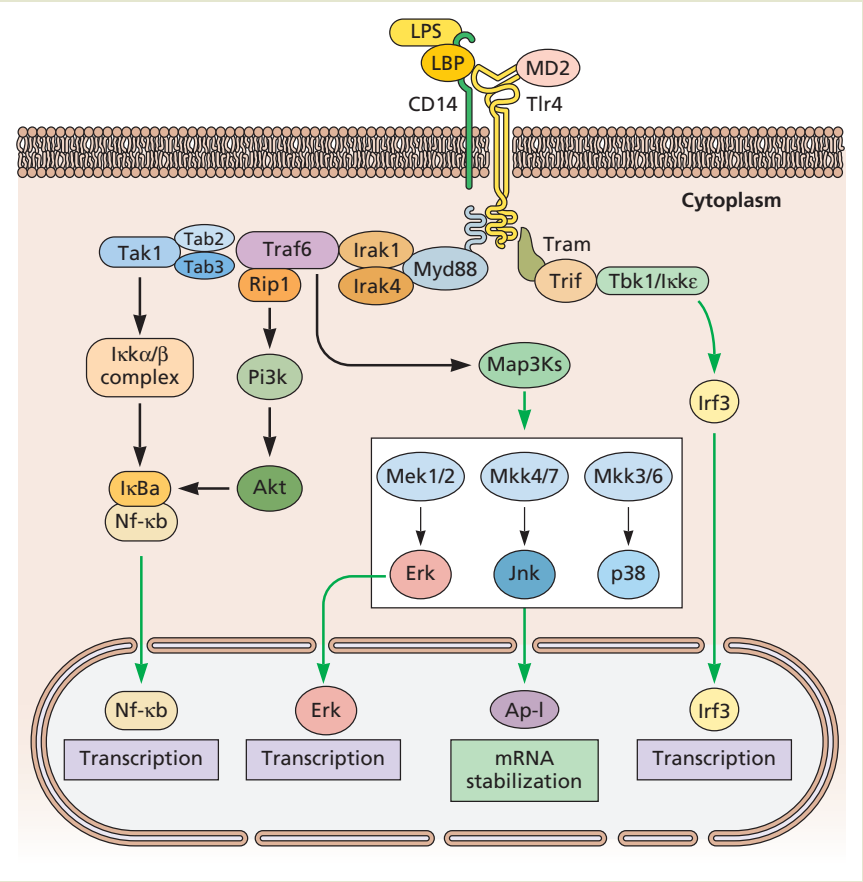
Tlrs are critical players in antiviral defense. Respiratory syncytial virus persists longer in the lungs of infected *tlr4*-null mice than in wild-type mice. Moreover, two vaccinia virus proteins, A46R and A52R, are similar in sequence to segments in the cytoplasmic domain of Tlrs and IL-1 receptors. These two viral proteins can inhibit IL-1- and Tlr4-mediated signal transduction, respectively. Vaccinia virus may modulate host immune responses by competing with this domain-dependent intracellular signaling.

Meylan E, Tschopp J, Karin M. 2006. Intracellular pattern recognition receptors in the host response. *Nature* **442:**39–44.

Quicke KM, Suthar MS. 2013. The innate immune playbook for restricting West Nile virus. *Viruses* **5:** 2643–2658.

Saito T, Gale M, Jr. 2007. Principles of intracellular viral recognition. *Curr Opin Immunol* **19:**17–23.

Signaling pathways and consequences pursuant to Tlr engagement. The cytoplasmic signaling pathways following Tlr4 binding (used as an example) are shown. These pathways lead to transcription of specific genes, including the interferons, as well as stabilization of RNA molecules.



Toll-like receptors recognize microbial macromolecular patterns[a]

Toll-like receptor	Pattern recognized
Tlr1	Bacterial lipoproteins
Tlr2	Lipoproteins, viral glycoproteins, Gram-positive peptidoglycan
Tlr3	dsRNA
Tlr4	LPS, viral glycoproteins
Tlr5	Bacterial flagellin
Tlr6	Bacterial lipoproteins
Tlr7	ssRNA
Tlr8	ssRNA
Tlr9	CpG DNA, unmethylated CpG oligonucleotides
Tlr10	Unknown
Tlr11	Profilin

[a]Data from G. Barton and R. Medzhitov, *Curr Opin Biol* **14:**380–383, 2002; and S. Uematsu and S. Akira, *Handb Exp Pharmacol* **183:**1–20, 2008.

BOX 3.3

DISCUSSION

Detecting viral invaders within the cell

A fundamental problem solved over eons of evolution is the detection by individual cells of invading viral RNA or DNA. A challenge for cells in detecting viruses is that these obligate intracellular parasites are made of the same basic building materials as the cell itself. This property imposes a significant challenge for their detection. How an invading nucleic acid is distinguished from cellular RNA and DNA remained an enigma for decades. While their names seem disconnected from their now known functions, the retinoic acid-inducible protein I (Rig-I) and melanoma differentiation-associated protein (Mda5) represent a class of virus detectors that recognize RNA in the cytoplasm. These RNA helicases of the DEXD/H box family share tandem caspase activation and recruitment domains (CARDs). After binding their ligands, Rig-I and Mda5 signal via interaction of these domains with an adapter protein. This adapter is an outer mitochondrial membrane protein (Mavs) that, when bound to the CARD domain of either Rig-I or Mda5, activates Irf3 and Nf-κb by the pathways shown in the figure. (Note that the protein we refer to as Mavs actually has three other names, all still in use: Ips1, Cardif, and Visa. Apparently, the field felt that immunology was not sufficiently complex!) Mavs also localizes to peroxisomes, and both peroxisomal and mitochondrial Mavs are required for robust antiviral responses.

Mavs binds to Traf6 and induces its polyubiquitination by E1 ligase and Ubc12/Uve1A. In turn, Tak1 kinase and Tab2 adapter protein bind and activate Jnk kinases and the Iκk complex, phosphorylating the inhibitor of Nf-κb, IκB, and causing it to be released from the complex. Free Nf-κb can enter the nucleus and induce gene expression. The common adapter Mavs integrates two different interactions with a common output. The coordination of these three signal transduction pathways leads to the assembly of a multiprotein enhancer complex in the nucleus, which drives expression of the IFN-β gene.

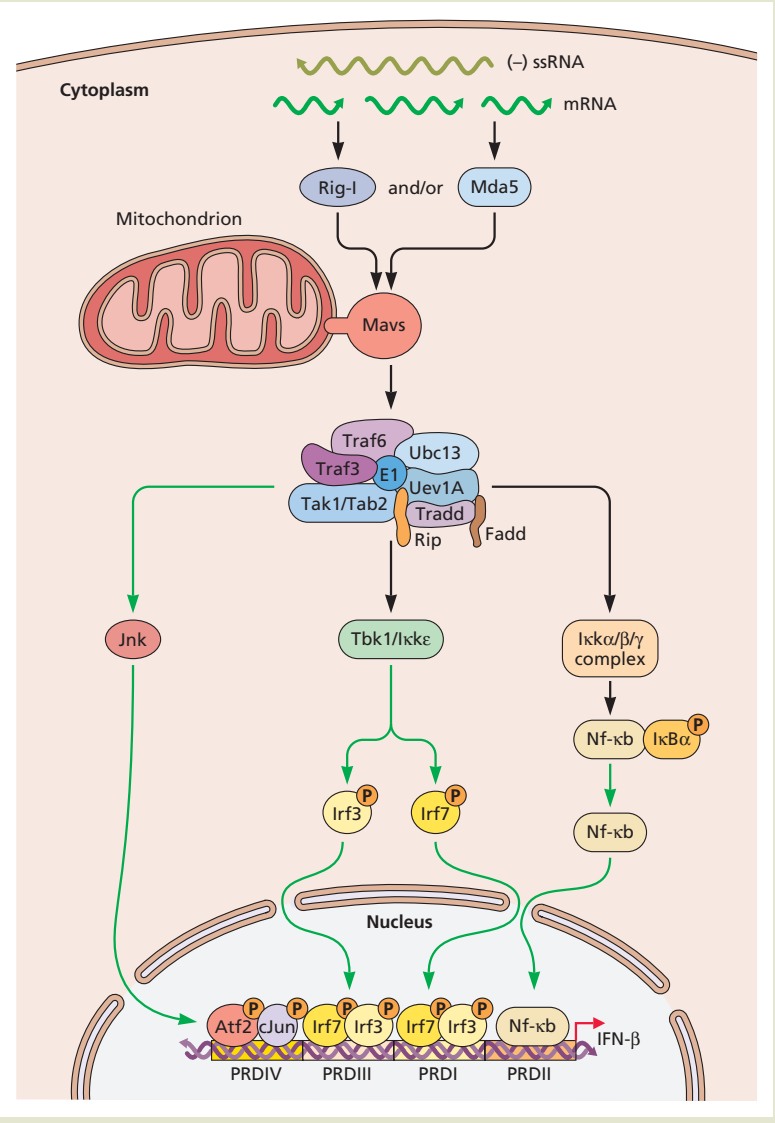
A crucial question is how Rig-I and Mda5 distinguish viral from cellular RNA. It has been clear for some time that the two receptors have different specificities and actions *in vivo*. Use of mice that lacked either of these two RNA detectors showed that Rig-I is required for the flaviviruses and influenzaviruses. The Mda5 detector seems to be essential for the antiviral response to encephalomyocarditis virus and measles virus. Rig-I binds to RNAs with a 5′ phosphate group, which is common in viral RNAs and distinguishable from the 5′ cap structures present on all cellular mRNAs. While certainly an important finding, the 5′ phosphate recognition must be just the tip of the iceberg, as the abundant human 7SL RNA has a 5′ triphosphate group yet does not activate an IFN response.

Picornaviral RNAs do not have cap structures **or** triphosphates on their 5′ ends, and cannot be detected by Rig-I, but they can be detected by Mda5. Mda5 also recognizes the synthetic dsRNA analog poly(I:C). Interestingly, short poly(I:C) is preferentially recognized by Rig-I, rather than by Mda5, suggesting that Rig-I and Mda5 recognize different lengths of dsRNA, which may be the basis of their apparently preferential recognition of viruses. Furthermore, some viruses, such as dengue virus and West Nile virus, require recognition by **both** Rig-I and Mda5 to generate a robust innate immune response.

Pichlmair A, Schulz O, Tan CP, Näslund TI, Liljeström P, Weber F, Reis e Sousa C. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. *Science* **314:**997–1001.

Saito T, Gale M, Jr. 2007. Principles of intracellular viral recognition. *Curr Opin Immunol* **19:**17–23.

Yoneyama M, Onomoto K, Jogi M, Akaboshi T, Fujita T. 2015. Viral RNA detection by RIG-I-like receptors. *Curr Opin Immunol* **32:**48–53.



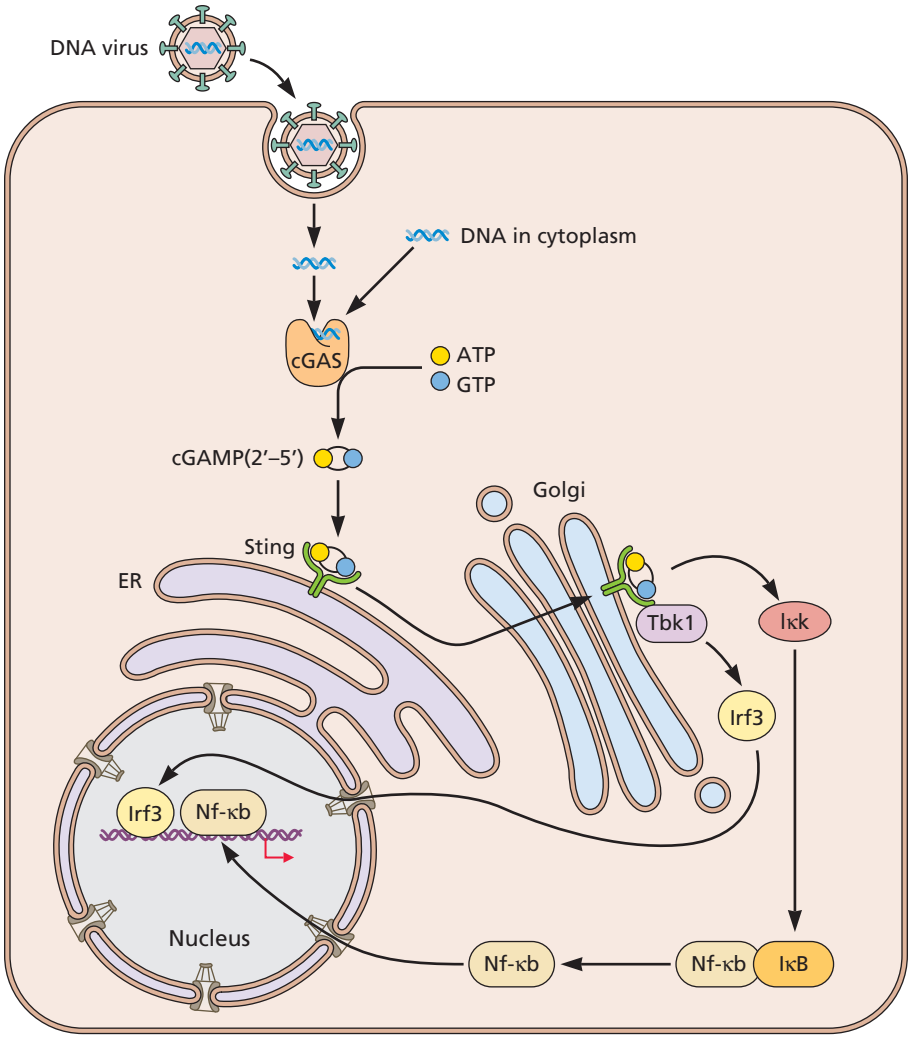


Figure 3.3 The cGAS/Sting axis in innate immunity. dsDNA in the cytoplasm (either from microbes with DNA genomes or from the cell nucleus) is detected by cyclic GMP-AMP (cGAMP) synthase (cGAS), which is activated to synthesize the cyclic dinucleotide (CDN) cGAMP(2′-5′) as its second messenger molecule (using the substrates ATP and GTP). cGAMP(2′-5′) then binds and activates the endoplasmic reticulum (ER)-resident receptor stimulator of interferon genes (Sting). Activated Sting then translocates to a perinuclear Golgi compartment, where it binds to TANK-binding kinase 1 to activate Irf3 and to induce Nf-κb activation.

of infection to enable the viral genome to be maintained in a host population. For example, herpes simplex virus 1 encodes a protein, ICP0, that suppresses Ifi16-dependent immune responses (Box 3.4).

Cellular Changes That Occur Following Viral Infection

Viral infection can also trigger the target cell to initiate a host response following viral protein production (Table 3.2). For example, viral proteins may induce alterations in cell integrity, collectively called **cytopathic effect**, causing release of intracellular components that may be engulfed by adjacent phagocytic cells (Fig. 3.4). The phagocytic cells then produce inflammatory cytokines and present the engulfed cellular and viral components as antigens to naïve T and B cells. Viral proteins may also block essential host processes such as translation, DNA and RNA synthesis, and vesicular transport. The most dramatic cellular response to infection when homeostasis is altered or when signaling cytokines bind to their receptors is that the cell undergoes suicide, a process

BOX 3.4

EXPERIMENTS

Viral proteins that block pattern receptor recognition function

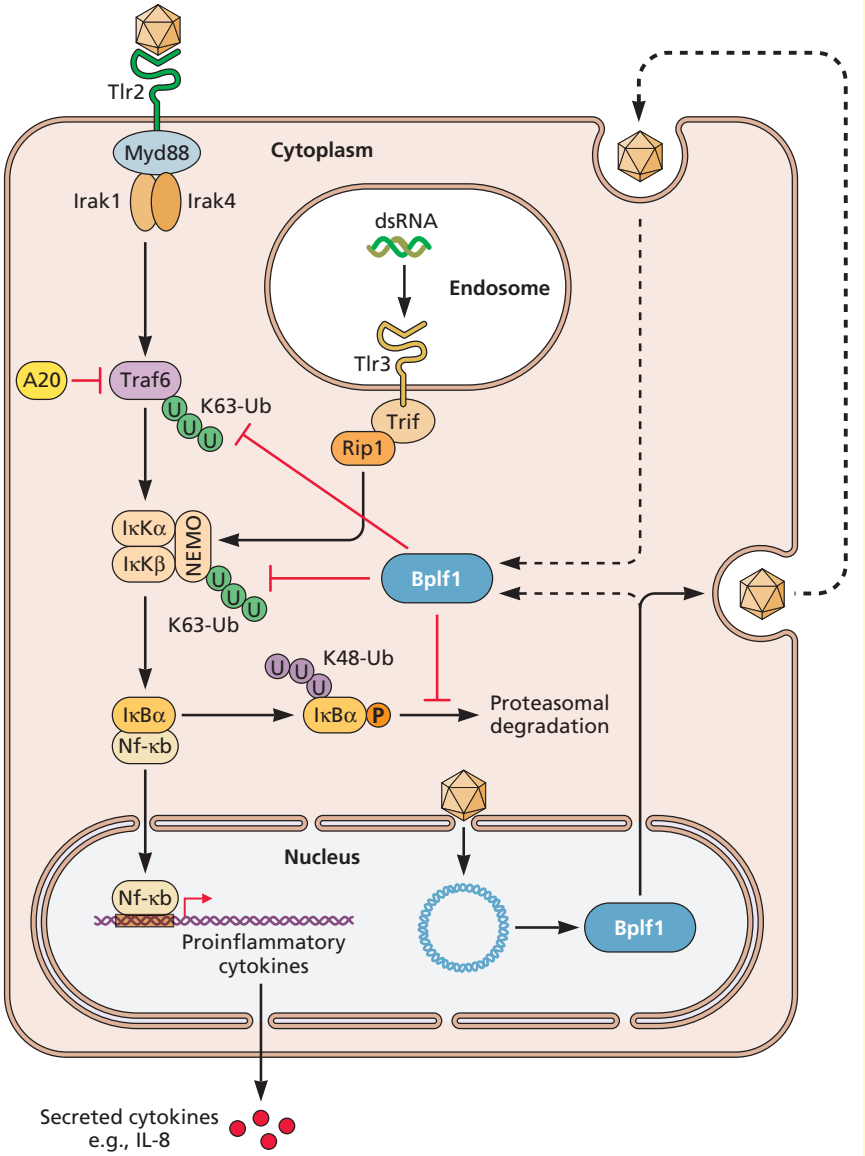
Viruses have been selected to interfere with multiple steps of the Tlr signaling cascade. For example, the V proteins of paramyxoviruses bind to Mda5, thereby inhibiting the activation of the IFN-β promoter, whereas the paramyxovirus P protein suppresses Tlr signaling through induction of the ubiquitin-modifying enzyme A20. The adenoviral E3 protein 14.7K inhibits antiviral immunity and inflammation by blocking the activity of Nf-κb. The hepatitis C virus NS3/4A protein cleaves Ips1, releasing this protein from the mitochondria and blunting subsequent pattern recognition receptor signaling.

Large DNA viruses, including vaccinia virus, encode immunomodulatory proteins that antagonize important components of the intrinsic immune response, as well. The vaccinia virus protein A52R blocks the activation of Nf-κb induced by multiple Tlrs by associating with Irak2 and Traf6 and inhibiting their activity. Likewise, the viral protein A46R targets multiple Tir adapter molecules, like Myd88 and Trif, thereby contributing to virulence. A recombinant vaccinia virus lacking A52R is attenuated in a murine intranasal model, demonstrating the importance of this protein to viral reproduction.

A recent publication underscores the effective ways by which virus-encoded proteins can blunt this critical early step in induction of immune responses. Epstein-Barr virus is a human herpesvirus that persistently infects >90% of adults worldwide. Epstein-Barr virus encodes enzymes, called deubiquitinases, that remove ubiquitin tags from substrate proteins. The activation of many proteins in the Tlr signaling cascade is regulated by the addition of ubiquitin tags. A virus-encoded deubiquitinase, Bplf1, deubiquitinylates components of the Tlr signaling pathway during Epstein-Barr virus production and is packaged into newly produced virus particles (see the figure).

van Gent M, Braem SG, de Jong A, Delagic N, Peeters JG, Boer IG, Moynagh PN, Kremmer E, Wiertz EJ, Ovaa H, Griffin BD, Ressing ME. 2014. Epstein-Barr virus large tegument protein BPLF1 contributes to innate immune evasion through interference with Toll-like receptor signaling. *PLoS Pathog* **10:**e1003960. doi:10.1371/journal.ppat.1003960.

Xagorari A, Chlichlia K. 2008. Toll-like receptors and viruses: induction of innate antiviral immune responses. *Open Microbiol J* **2:**49–59.



Schematic model of Bplf1-mediated Tlr evasion during Epstein-Barr virus infection. The Epstein Barr virus-encoded deubiquitinase Bplf1 counteracts Tlr-mediated Nf-κb activation and blocks ubiquitination of crucial signaling intermediates. Bplf1 is expressed as a full-length protein during the late phase of productive infection, is incorporated into the tegument of viral particles, and can subsequently be released into newly infected cells.

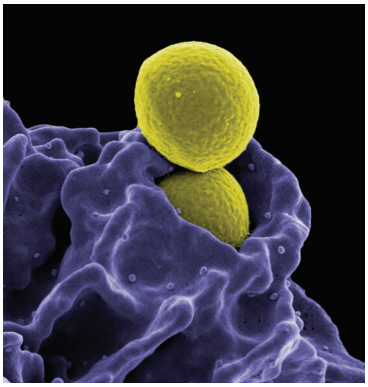


Figure 3.4 Phagocytosis. A scanning electron micrograph image of methicillin-resistant *Staphylococcus aureus* ingestion by a neutrophil. Bacteria pictured in yellow; neutrophil in purple. Courtesy of NIAID/National Institutes of Health/Science Photo Library, with permission.

called **apoptosis**. This response is remarkably effective in curtailing virus propagation, especially when it occurs early in the infection: the cellular factory required to make more infectious particles is destroyed and fragments of such dead or dying cells are taken up by tissue-resident dendritic cells, which then amplify the host response.

Even an approximation of the various ways by which the intrinsic antiviral response is induced is overwhelming (Fig. 3.5). To complicate matters further, we are beginning to appreciate that different cell populations use particular pathways selectively, and that substantial cross-talk likely exists among these various sensors and their effector responses within a responding cell. While daunting, such knowledge reminds us of the numerous mechanisms that are in place to protect the host from rampant infection. Even so, viral evolution in the face of these responses has selected for viral gene products that counter, modulate, or even bypass intrinsic cell defenses.

Intrinsic Responses to Infection

Apoptosis (Programmed Cell Death)

Apoptosis is a normal biological process used chiefly used to eliminate particular cells during development and differentiation and to maintain organ size (Box 3.5). For example, the separation of fingers and toes during fetal development is a result of apoptosis of the cells between the digits. Such programmed cell death continues throughout life: every day, in an adult human, >50 billion cells die by apoptosis.

Apoptosis can also be induced by the biochemical alterations initiated by virus infection. Cell death is the result of a cascade of reactions that ultimately leads to nuclear membrane breakdown, chromatin condensation, loss of membrane integrity (called "blebbing," in which bubbles of cytoplasm appear on the cell surface [Fig. 3.6]), and eventually DNA degradation. When a cell undergoes apoptosis, cellular debris is taken up by macrophages and dendritic cells, which then migrate to local lymph nodes and produce cytokines to amplify the host response.

Regardless of the nature of the initiation signal that triggers the apoptotic response, all converge on common effectors, the **caspases**. Caspases are members of a family of cysteine proteases that cleave after aspartate residues. These proteases are first synthesized as precursors with little or no activity, and exist in normal cells in this inactive state. A mature caspase with full activity is produced after cleavage by a protease, often another caspase. Increasing the concentration of some caspase precursors results in cleavage-independent activation. These protease cascades are not unlike the many sequential steps that result in blood clotting or the complement cascade (discussed below). This property underscores the "ready-to-go" nature of the intrinsic response: induction of apoptosis requires successive activation of proteins that are already present in the cytoplasm (a rapid event), in contrast to a more time-consuming response that includes new or enhanced gene transcription and mRNA translation.

Table 3.2 Host alterations are early signals of infection

Alteration	Virus	Viral protein	Target
Inhibition of transcription	Poliovirus	3C	Tbp-TfiIIc complex
Blocking accumulation of host mRNA in cytoplasm	Adenovirus	E1B-55K E4-34K	Cellular protein involved in mRNA transport?
Inhibition of 5′-end-dependent translation	Poliovirus	$2A^{pro}$	eIF4G
Alteration of MAP4	Poliovirus	3C	MAP4
Increased plasma membrane permeability	Sindbis virus	?	Na,K-ATPase
Fusion of cell membranes, syncytium formation	Paramyxovirus	F protein	Plasma membrane
Inhibition of transport and processing of host RNA	Herpes simplex virus	ICP27	SR splicing proteins
Depolymerization of cytoskeleton	Many viruses	?	Actin filaments, microtubules, intermediate filaments

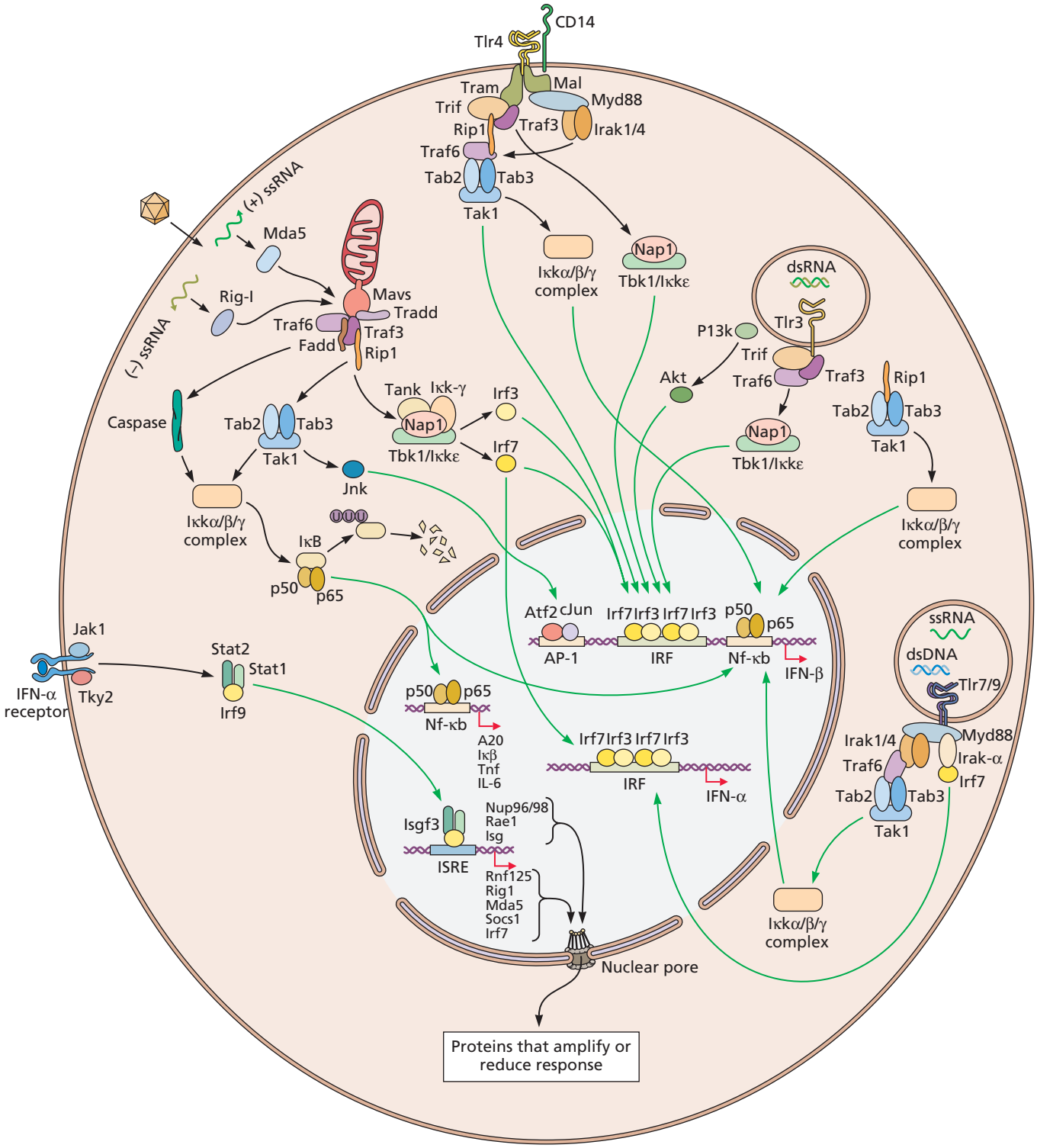


Figure 3.5 Summary of some established intrinsic defense responses. Every cell has receptors on the surface and inside that bind microbial proteins and nucleic acids. A generic cell is indicated here, but it should be clear that not all cells have the same constellation of responses. Upon binding their cognate ligand, pattern recognition receptors initiate reactions leading to production of potent cytokines. Cytokines, such as IFN, are the primary response mediators that emanate from a single infected cell.

BOX 3.5

TERMINOLOGY

What to do with the second "p"?

How does one pronounce the word "apoptosis"? The most straightforward approach, and the one that implies its actual function, is to pronounce the second "p": a-POP-tosis. But many in the field find this to be an amateur mistake, and prefer to keep the second "p" silent: a-po-tosis.

In Greek, "apoptosis" translates to the "dropping off" of petals or leaves from plants or trees. Hippocrates co-opted the term for medical use to describe "the falling off of the bones." In English, the *p* of the Greek *pt* consonant cluster is typically silent at the beginning of a word (as in "pterodactyl" or "Ptolemy"), but usually pronounced when preceded by a vowel (as in "helicopter" or "chapter"), but not always (as in "receipt"). What to do?

In the original *British Journal of Cancer* paper by Kerr, Wyllie, and Currie, there is a footnote regarding the pronunciation: "We are most grateful to Professor James Cormack of the Department of Greek, University of Aberdeen, for suggesting this term. To show the derivation clearly, we propose that the stress should be on the penultimate syllable, the second half of the word being pronounced like 'ptosis' (with the 'p' silent), which comes from the same root 'to fall' . . ."

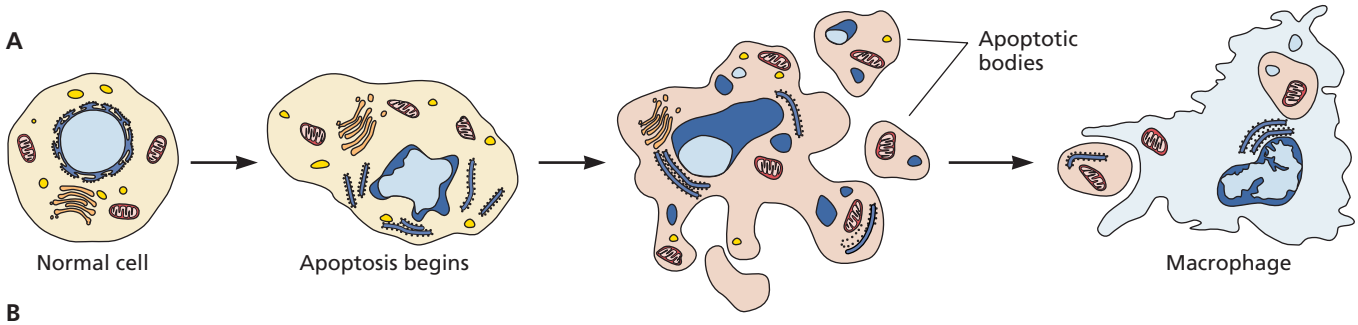
A-po-tosis, it is.

Kerr JF, Wyllie AH, Currie AR. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26:**239–257.



Two convergent caspase activation cascades are known: the extrinsic and intrinsic pathways (Fig. 3.7). The **extrinsic pathway** begins when a cell surface receptor binds a proapoptotic ligand (e.g., the cytokine tumor necrosis factor α [Tnf-α]). Ligand binding trimerizes the receptor so that death-inducing signaling proteins are recruited to the receptor's now clustered cytoplasmic domain (Fig. 3.7A). The latter proteins in turn attract procaspase-8, which becomes activated following receptor engagement. Caspase-8 cleaves and activates procaspase-3 to produce caspase-3, the final effector common for both extrinsic and intrinsic pathways.

The **intrinsic pathway**, often called the mitochondrial pathway, integrates cellular stress responses as well as internal developmental cues. Common intracellular initiators include DNA damage and ribonucleotide depletion. In these situations, the cell cycle regulatory protein p53 is activated (Chapter 6) and apoptosis typically follows in virus-infected



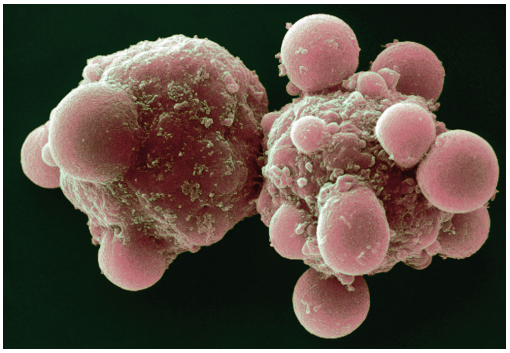


Figure 3.6 Apoptosis, the process of programmed cell death. (A) Apoptosis can be recognized by several distinct changes in cell structure. A normal cell is shown at the left. When programmed cell death is initiated, the first visible event is the compaction and segregation of chromatin into sharply delineated masses that accumulate at the nuclear envelope (dark blue shading around periphery of nucleus). The cytoplasm also condenses, resulting in shrinkage of the cell and nuclear membranes. The process can be rapid: within minutes, the nucleus fragments and the cell surface convolutes, giving rise to the characteristic "blebs" and stalked protuberances illustrated. These blebs then separate from the dying cell and are called apoptotic bodies. Macrophages (the cell at the right) engulf and destroy these apoptotic bodies. Adapted from J. A. Levy, *HIV and the Pathogenesis of AIDS*, 2nd ed. (ASM Press, Washington, DC, 1998). **(B)** A liver cell undergoing programmed cell death, with characteristic blebbing of the plasma membrane. Credit: David McCarthy/Science Photo Library, with permission.

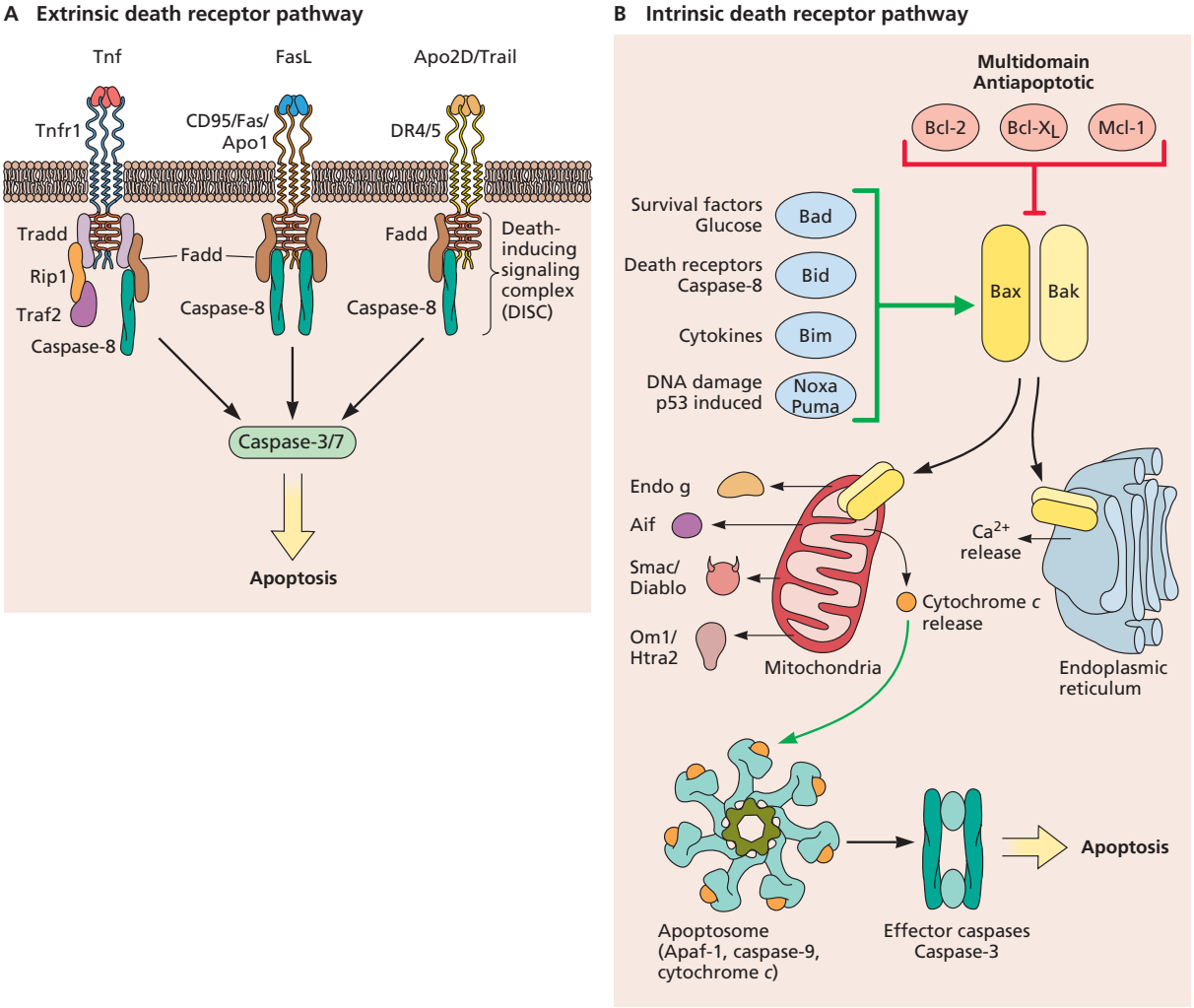


Figure 3.7 Pathways to apoptosis. (A) The extrinsic death receptors and their death-inducing signaling proteins. Three receptors found on the surfaces of cells can initiate the apoptosis pathway: the Tnf receptor, the Fas ligand (FasL) receptor (CD95), and the Apo2/Trail receptor (Dr4/5). When these receptors engage their respective ligands, the cytoplasmic domains of each protein complex form a scaffold for assembly of the death-inducing signaling complex (DISC). Important cytoplasmic proteins in this complex are shown. Caspase-8 is activated when it binds these complexes, leading to activation of caspase-3, the main effector of apoptosis. Adapted from N. N. Danial and S. J. Korsmeyer, *Cell* **116:** 205–219, 2004, with permission. **(B)** The process of intrinsic apoptosis is controlled by the Bcl-2 family of proteins. The critical antiapoptotic regulators are Bcl-2, Bcl-X_L, and Mcl-1. These proteins keep Bax and Bak (proapoptotic proteins) from assembling at the mitochondrial or endoplasmic reticulum membranes and causing release of cytochrome *c* and calcium, respectively. When not sequestered in this way, these proteins permeabilize mitochondrial membranes, and internal stores of cytochrome *c* are released. Cytochrome *c* in the cytoplasm binds to a cellular protein (Apaf-1), which oligomerizes in the presence of deoxyadenosine 5′ triphosphate (dATP) or ATP. The oligomeric assembly, called the apoptosome, then binds and cleaves procaspase-9, which in turn activates procaspase-3, the effector caspase that produces the characteristic events of controlled cell suicide. Four other classes of Bcl-2 regulatory proteins that also bind to different subsets of the Bcl-2 proteins are indicated at the top left (blue ellipses). These four classes act under conditions in which survival is threatened (Bad), when the extrinsic pathway is stimulated (Bid), when certain cytokines are produced (Bim), and when DNA damage is detected and p53 is induced (Noxa and Puma).

cells, with formation of the apoptosome and consequent activation of caspase-9. Members of a single family of proteins, the Bcl-2 family (Fig. 3.7B), are the master regulators that inhibit intrinsic apoptosis. Their chief function is to restrict **pro**apoptotic proteins, which, curiously, are also Bcl-2 family members. The differential binding of other antiapoptotic Bcl-2 proteins, including Bcl-X_L, enables tissue-specific regulation and stress-specific responses.

The extrinsic and intrinsic signaling pathways can converge in other ways. For example, if the extrinsic pathway is activated, mature caspase-8 may cleave the proapoptotic protein Bid, which then translocates to the mitochondria

to trigger the intrinsic pathway. There is ample evidence to suggest that the intrinsic pathway can amplify the extrinsic pathway. Consequently, these pathways are not discrete roads to a common effector, but rather may intersect throughout the response to cellular stress.

Apoptosis Is a Defense against Viral Infection

Because virus particles engage cell receptors, and because viral reproduction requires engagement of many or all of the host's metabolic pathways (Volume I, Chapter 14), a variety of signals can activate the extrinsic and intrinsic pathways (Box 3.6). In many infections, the target cell is quiescent and hence unable to provide the enzymes and other proteins needed by the infecting virus to reproduce. Consequently, viral proteins, including the adenoviral E1A proteins or simian virus 40 large T protein, may cause the cell to leave the resting state and enter the cell cycle. Cell cycle checkpoint proteins may then respond to this unscheduled event by inducing apoptosis, which limits the production of infectious progeny (a form of antiviral defense by suicide). Perhaps not surprisingly, to ensure that infected cells survive long enough to produce some progeny, viral genomes encode counter-response proteins that block cell-initiated apoptosis.

Viral Gene Products That Modulate Apoptosis

The discovery of viral proteins that modulate the apoptotic pathway proved exceedingly valuable in dissecting the complex interactions and regulatory circuits that control this pathway in normal cells. Apoptosis is normally held in check by viral regulatory proteins called inhibitors of apoptosis. The prototype gene in this group was described in baculovirus genomes by the late Lois Miller and colleagues in 1993. This seminal work led to the discovery of cellular orthologs in yeasts, worms, flies, and humans. Mutant viruses unable to inhibit apoptosis were detected originally because the host DNA of infected cells was unstable; the cells lysed rapidly upon infection; and, as a consequence, viral yields were reduced, resulting in small plaques. Since then, we have discovered many viral gene products that modulate apoptosis (Table 3.3).

Human cytomegalovirus encodes an abundant, 2.7-kb noncoding RNA (β2.7) that binds to and inhibits the mitochondrial protein complex that triggers apoptosis. Not only is this response blocked, but the mitochondrial membrane potential is maintained, enabling continued ATP biosynthesis, resulting in prolonged cell viability to allow for the production of infectious progeny before host cell collapse. This strategy is particularly favorable for those viruses, like human cytomegalovirus, with long infectious cycles. Alternatively, in cells infected by some viruses, including poliovirus, apoptosis provides a way for progeny virus particles to get out of the cell.

Many viral genomes that encode mimics of cellular proteins that hold apoptosis in check can contribute to transformation. For example, the human adenovirus E1B 19-kDa protein, one of the first viral homologs of cellular antiapoptotic proteins to be identified, allows survival and transformation of rodent cells that also contain the viral growth proliferation-promoting E1A protein. Similarly, human herpesvirus 8 encodes the antiapoptotic protein vFLIP, which has been associated with the development of Kaposi's sarcoma.

BOX 3.6

BACKGROUND

The many ways by which virus infections activate apoptotic pathways

At the Cell Surface

- Production of apoptosis-inducing cytokines after virus particles bind their receptors
- Alteration of membrane integrity or composition via membrane fusion or virus particle passage into the cytoplasm via receptor-mediated endocytosis.

In the Cytoplasm

- Production of inhibitors (e.g., arrest of host translation)
- Modification of cytoskeleton (e.g., disruption of actin microfilaments)
- Disruption of signal transduction pathways (e.g., death domain proteins and kinase- and phosphatase-binding proteins)

In the Nucleus

- Degradation of, and damage to, DNA
- Alteration of gene expression (e.g., increased expression of heat shock genes)
- Disruption of the cell cycle (e.g., inactivation of p53 or phosphoretinoblastoma [pRb])

Hay S, Kannourakis G. 2002. A time to kill: viral manipulation of the cell death program. *J Gen Virol* **83:**1547–1564.

Miller LK, White E. 1998. Apoptosis in viral infections. *Semin Virol* **8:**443–523.

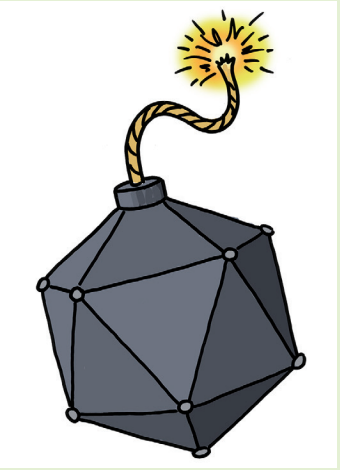


Table 3.3 Some viral regulators of apoptosis[a]

Cellular Target	Virus	Gene	Function
Bcl-2	Adenovirus	E1B 19K	Bcl-2 homolog
	Epstein-Barr virus	LMP-1	Increases synthesis of Bcl-2; mimics CD40/Tnf receptor signaling
Caspases	Adenovirus	14.7K	Inactivates caspase-8
Cell cycle	Hepatitis B virus	pX	Blocks p53-mediated apoptosis
	Human papillomavirus	E6	Targets p53 degradation
	Simian virus 40	Large T	Binds and inactivates p53
Fas/Tnf receptors	Adenovirus	E3 10.4/14.5K	Internalizes Fas
	Cowpox	CrmB	Neutralizes Tnf and LT-α
	Myxoma virus	MT-2	Secreted Tnf receptor homolog
vFLIPs; DED box-containing proteins	Human herpesvirus 8	K13	Blocks activation of caspases by death receptors
Oxidative stress	Molluscum contagiosum virus	MC066L	Inhibits UV- and peroxide-induced apoptosis; homologous to human glutathione peroxidase
Transcription	Human cytomegalovirus	IE1, IE2	Inhibits Tnf-α but not UV-induced apoptosis

[a]Data from D. Tortorella et al., *Annu Rev Immunol* **18:**861–926, 2000; S. Redpath et al., *Annu Rev Microbiol* **55:**531–560, 2001; and S. Hay and G. Kannourakis, *J Gen Virol* **83:**1547–1564, 2002.

Apoptosis Is Monitored by Sentinel Cells

Specialized phagocytes, dendritic cells and macrophages, reside in areas of the body where microbes are most likely to invade, including skin and mucous membranes. These remarkable cells, sometimes referred to as "sentinels" because they keep watch for invading microbes, are critical players in early defense as well as in activating a more global immune response. Phagocytes gather information (as packets of proteins) by taking up cellular debris and extracellular proteins released from dying or apoptosing cells. This process activates the sentinel cell to migrate to local lymph nodes, where it presents its collected peptide cargo to lymphocytes of the adaptive immune system. This cell-cell communication, the interface between the innate and adaptive arms of host immunity, informs T cells about the nature of the insult that is killing cells in peripheral tissues, and specific T cells become activated accordingly. Moreover, at the site of viral invasion, the sentinel cells, as well as the damaged and dying cells, produce cytokines, such as Tnf-α, that can induce apoptosis in nearby infected cells and contain the infection until the cells of the adaptive immune response arrive. A core principle that this process exemplifies should now be quite familiar: a modest initial signal that occurs in a single infected cell (induction of an intrinsic apoptotic pathway) leads to a cascade of subsequent events that vastly amplify the host response.

In a curious adoption of a cellular process by a virus, the vaccinia virus envelope is derived from membranes of a dying cell. These membranes have cellular markers of apoptosis, including phosphatidylserine, which normally bind to receptors that are present on the surface of phagocytic cells and that initiate endocytosis of debris. When vaccinia virus particles, with their envelopes marked by these "eat me" phospholipids, bind to the cell surface of a susceptible cell, they trigger engulfment, normally appropriate for apoptotic debris, allowing the virus to gain access to target cells.

Programmed Necrosis (Necroptosis)

As viruses can block the apoptotic cascade, alternative mechanisms are in place to ensure cell death in many cells following infection. One recently identified pathway is through programmed necrosis (necroptosis). This form of cell death is mediated by two kinases, Rip1 and Rip3. Their activation following infection leads to phosphorylation, trimerization, and activation of a pseudokinase, Mlkl, which inserts into cell membranes, including the plasma membrane, where it punches holes and leads to osmotic imbalance and necrotic death of the cell. The importance of this pathway has been shown in Rip3 knockout mice, which have a higher susceptibility to various viruses, because the host cannot clear these infections.

Other Intrinsic Immune Defenses

While killing an infected cell is a good way to contain an infection, other processes that limit infection but do not result in the death of the infected cell have also been identified. These conserved cellular processes include autophagy, epigenetic silencing, RNA interference, cytosine deamination, and Trim protein interference, described below.

Autophagy

Cells can degrade cytoplasmic contents by formation of specialized membrane compartments related to lysosomes. This process, **autophagy** (from the Greek, "to eat oneself"), allows for recycling of cellular components, but is also an effective way to target incoming viruses to the lysosomal pathway. Autophagy is evoked by stressors, such as nutrient

starvation or viral infection. In contrast to apoptosis, which results in cell death, autophagy is a cellular effort to consolidate resources and "weather the storm."

Infection by many viruses induces a state of metabolic stress that normally triggers intrinsic defenses. These include stress-induced alterations in translation that are modulated in part by eIF2α kinases. Phosphorylated eIF2α can trigger autophagy, a process that, in turn, leads to engulfment and digestion of virus particles or other viral components. Capturing virus particles and targeting them for degradation in lysosomes is called **xenophagy**. Virus reproduction can be blocked when xenophagy is induced, and some viruses encode gene products that block this process, including the herpes simplex virus 1 ICP34.5 protein and the Nef protein of human immunodeficiency virus type 1.

Autophagy can also exert antiviral functions by delivering viral genetic material to endosomes where engagement of the resident ssRNA-sensing Tlr7 leads to production of type I IFNs, or by targeting viral peptides to major histocompatibility complex (MHC) class II compartments to enhance $CD4^+$ T cell responses. These processes integrate virus-induced stress responses with the molecular detectors of viral nucleic acid (e.g., cytoplasmic Rig-I/Mda5 and endosomal Tlrs).

For some RNA viruses, including hepatitis C virus, poliovirus, and rotavirus, the autophagy machinery may be co-opted to create scaffolds that aid in viral genome replication or morphogenesis. Poliovirus may also use these structures to promote the noncytolytic release of new virus particles from cells. All of these viruses presumably have means to block the maturation of these structures into destructive autolysosomes.

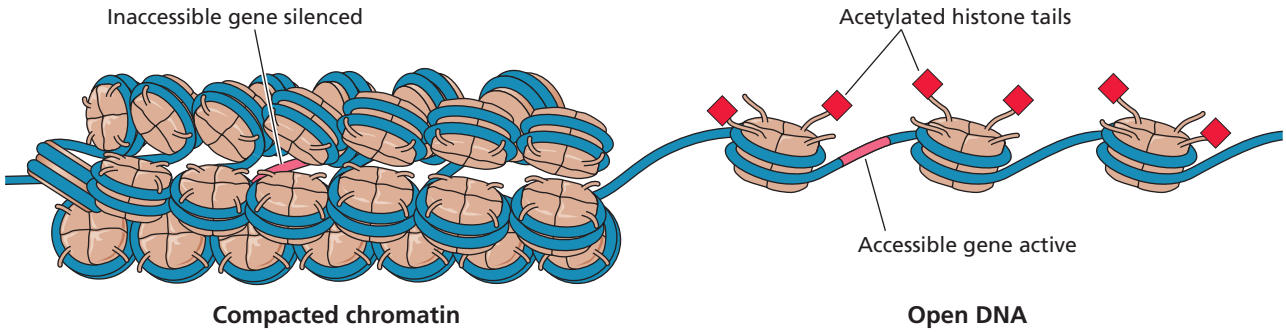
Epigenetic Silencing

Epigenetic silencing is another normal cellular process that specifies transcriptionally repressed regions of host chromatin. Epigenetic changes do not affect the nucleotide sequence, but rather add or remove modifications to the chromosomal histones that make it either more condensed (and therefore generally less accessible to the transcriptional machinery) or less compacted and more available to transcription factors. Chromatin packing is maintained, in part, by histone acetylation and deacetylation, reactions that are catalyzed by histone acetyltransferase and histone deacetylase activity, respectively. Acetylation removes the positive charge on the histones, thereby converting acetylated regions of chromatin into a more relaxed structure that is associated with easier access to the DNA and greater degrees of transcription. This relaxation can be reversed by histone deacetylase activity (as well as other histone modifications), resulting in condensed chromatin. DNA methylation at CpG sites, which is catalyzed by cellular DNA methyltransferases, is another mechanism for gene silencing (Fig. 3.8).

The genomes of viruses that reproduce in the nucleus, including those of many DNA viruses, can be susceptible to these modifications. Upon entering the nucleus, foreign DNA molecules can be quickly organized into transcriptionally silent chromatin, an intrinsic effort that limits viral replication but may also allow the virus to establish a long-term infection in the host cell. Histone deacetylation can maintain the viral genome in a quiescent state for long periods, often over many cell divisions. Organized collections of proteins in the nucleus, called **promyelocytic leukemia (Pml) bodies**, may be nuclear sites where such repression occurs. These structures are implicated in antiviral defense for many reasons, including the fact that IFN stimulates synthesis of some of the proteins that comprise them, increasing the number and size of the Pml bodies (Fig. 3.9).

As might be predicted, viral proteins that counter epigenetic silencing have been identified. The human cytomegalovirus protein pp71 binds to Daxx, a cellular protein that interacts with histone deacetylases and DNA methyltransferases to maintain transcriptional repression. By engaging Daxx, and marking it for degradation, silencing is avoided and transcription of the cytomegalovirus genome can proceed. Other DNA virus-encoded proteins can function in this manner as well: the global repression

Figure 3.8 Epigenetic silencing of DNA. Histone acetylation and deacetylation impact host chromatin condensation and the access of transcriptional regulators to cellular genes. Generally, though not always, condensed chromatin and silenced genes are associated with nonacetylated histones, whereas acetylated histones are associated with open chromatin in which DNA is transcriptionally active.



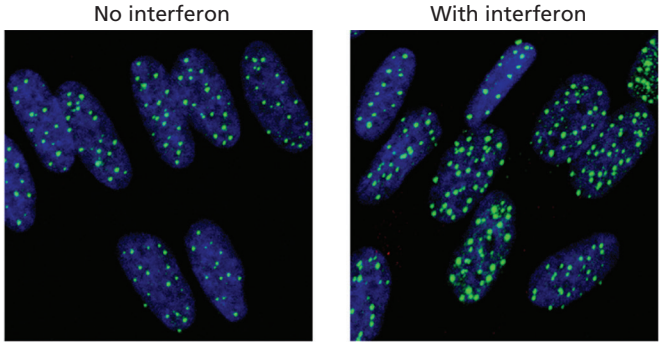


Figure 3.9 Interferon increases the number and size of Pml bodies. Human foreskin fibroblasts were treated with 500 U of type I IFN and imaged by immunofluorescence 24 h thereafter. The images are confocal z-stack projections. DAPI, 4′,6-diamidino-2-phenylindole. From J. S. Chahal et al., *PLoS Pathog* **8:**e1002853, 2012.

of Pml-bound DNA can be relieved by viral proteins such as the ICP0 protein of herpes simplex virus 1. This protein accumulates in Pml bodies and induces the proteasome-mediated degradation of several of their protein components. The human cytomegalovirus IE1 proteins, the Epstein-Barr virus nuclear antigen Ebna5 protein and the adenovirus E4 Orf3 protein all affect Pml protein localization or synthesis.

Viruses that cause long-lasting, persistent or latent infections may benefit from heritable epigenetic changes that produce an environment that is conducive to long-term infection of a host cell. For example, Epstein-Barr virus, which establishes a latent infection and is associated with Burkitt's lymphoma, nasopharyngeal carcinoma, and gastric cancers, induces hypermethylation of DNA in host cells. Methylation of distinct sets of gene promoters in Epstein-Barr viral DNA enables this virus to establish persistent infection and temporarily escape immune detection, and may also silence host tumor suppressor genes.

Epigenetic silencing manifests in many ways; those studying gene transfer with retrovirus vectors are often frustrated to find that expression of their favorite gene is low or completely turned off in the infected cell. We now understand that integrated retroviral DNA is subject to reversible epigenetic silencing, a prominent process in embryonic or adult stem cells (Volume I, Chapter 8).

RNA Interference

RNA silencing is a mechanism of sequence-specific inhibition of gene expression that operates in diverse plants and animals. It is likely to have arisen early in the evolution of eukaryotes to detect and destroy foreign nucleic acids. RNA silencing is related to a process called RNA interference (RNAi) that was identified first in petunias, and subsequently in many eukaryotes (Volume I, Chapter 10).

RNAi is mediated by microRNAs (miRNAs) or small interfering RNAs (siRNAs). While these short RNA molecules are derived from distinct RNA sources, both RNAi types can bind to mRNA molecules and decrease gene expression, for example, by blocking translation of the mRNA. Both pathways are also controlled by the enzyme Dicer, which cleaves long dsRNAs into short fragments of 20 to 25 nucleotides in length. Each siRNA is unwound into ssRNAs, and then, as part of a larger assembly of proteins called the RNA-induced silencing complex (Risc), binds to a complementary sequence on an mRNA to influence its translation or stability. RNAi is a valuable research tool because synthetic dsRNA introduced into cells can selectively suppress expression of genes of interest (Box 3.7).

When a ssRNA virus infects a cell, among the first steps is the synthesis of the viral RNA-dependent RNA polymerase, which makes a complementary strand to the incoming genome. This strand is used as a template to generate progeny genomes, but with each round of replication, the viral genome becomes transiently double stranded. This double-stranded intermediate may be targeted by the RNAi machinery, which chops dsRNA into siRNA fragments.

miRNAs are single-stranded, noncoding host RNAs of 19 to 22 nucleotides that regulate gene expression. While the contributions of cellular miRNAs to the antiviral response in plants and invertebrates have been known for some time, a role in antiviral action in mammals has been demonstrated only recently. In one study, IFN-β treatment of the human hepatoma cell line Huh7, as well as freshly isolated primary murine hepatocytes, resulted in an induction of numerous cellular miRNAs. Eight of these miRNAs targeted hepatitis C virus genomic RNA: treatment of infected cells with synthetic miRNAs of the same sequence blocked virus reproduction. IFN-β treatment also reduces expression of liver-specific miR-122, an RNA essential for hepatitis C virus reproduction (Volume I, Chapter 10). In another example, host miRNAs are required to shut down human immunodeficiency virus type 1 transcription in blood mononuclear cells from infected donors. However, in other cell types, viral infection suppresses the host miRNAs that would normally repress proviral gene expression.

We are learning more about RNAi as an antiviral defense, as many viral genomes, including those of plant, insect, fish, and human pathogens, encode suppressors of this process. Many of these suppressors are RNA-binding proteins without a preference for siRNAs. While we have known for some time that simpler organisms use RNAi to fend off invading viruses, until recently it was unclear if mammals use this strategy for fighting viruses. While RNAi may be active in embryonic stem cells and other undifferentiated cells, most evidence suggests that RNAi is not a primary antiviral response in mammalian somatic cells. Thus, whether virus-directed RNA silencing is important in human viral infections remains controversial.

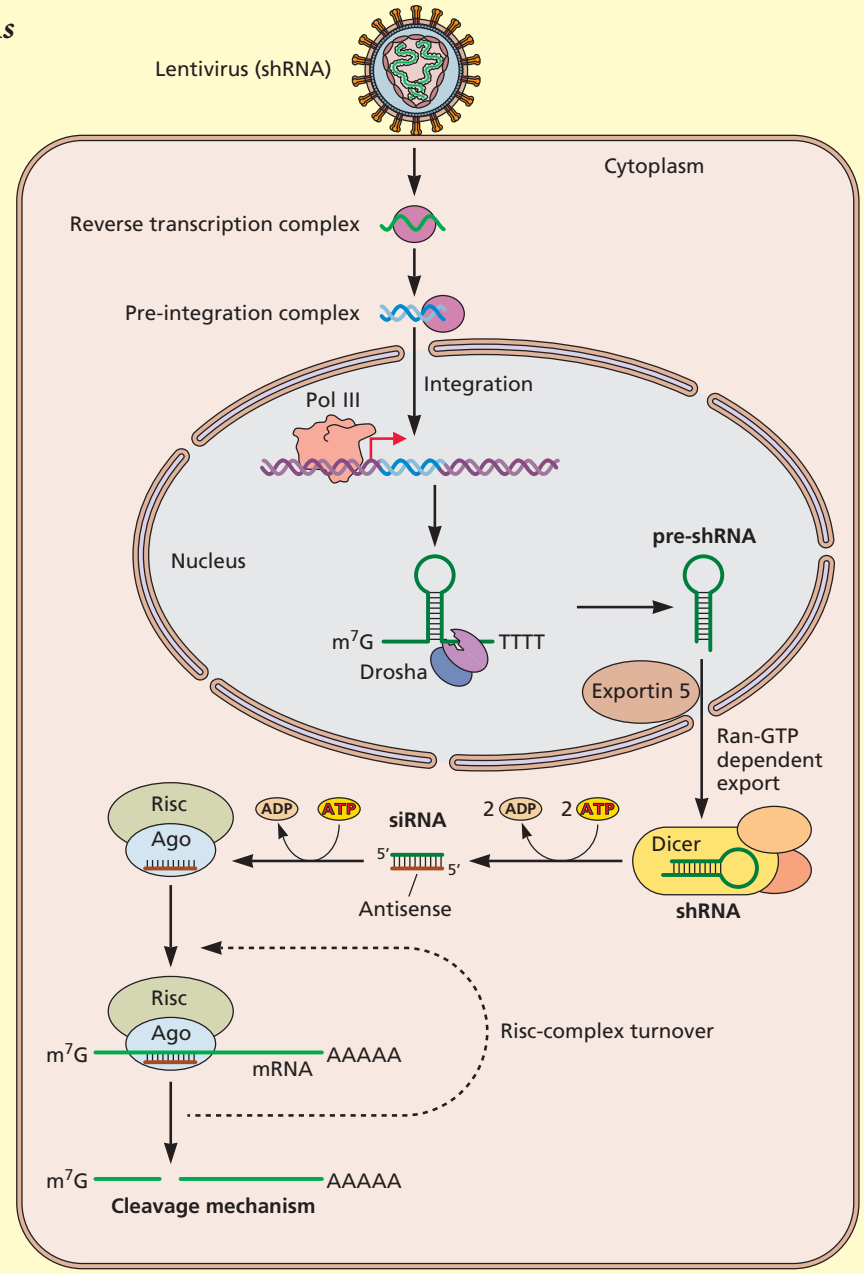
BOX 3.7

EXPERIMENTS

Use of viral vectors encoding siRNAs

Viruses can be used as delivery vehicles to shut down gene expression in cells. In 2003, a method to generate a large number of transgenic mice in which expression of specific genes could be downregulated was described. The technology built on the use of lentiviral vectors, combined with the use of RNAi. As proof of principle, a lentiviral vector capable of producing siRNA specific for green fluorescent protein (GFP) after transduction of 293T-GFP cell lines showed no GFP fluorescence. Furthermore, no GFP-specific RNA could be detected.

Tiscornia G, Singer O, Ikawa M, Verma IM. 2003. A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc Natl Acad Sci U S A* **100:**1844–1848.



Following integration of a short hairpin RNA (shRNA)-containing lentivirus, the cellular machinery that generates shRNA is activated. This results in the cleavage of the shRNA by Drosha, its nuclear export, and the formation of the cytoplasmic Risc complex that targets a designated cellular gene.

Editing and Cytosine Deamination

While genomic editing (the process of modifying specific nucleotides) was considered initially to be restricted to a small number of viruses with RNA genomes, a growing literature has shown that this is a commonly employed device in the intrinsic immunity toolkit. A number of studies have established the importance of adenosine deaminase acting on RNA (Adar) -specific enzymes that primarily lead to A-to-I editing during viral infections (Table 3.4). Somewhat surprisingly, the effect of these enzymes on the virus-host interaction can be either antiviral or proviral, dependent upon the specific virus and mammalian host cell combination and the amount of Adar protein synthesized. Our current understanding suggests that Adars may act either directly by editing a viral RNA in a manner that influences the outcome of the infection, or indirectly by editing a cellular RNA and hence a cellular protein that participates in the antiviral response. It is also possible that Adars may function in an editing-independent manner, by altering protein- or nucleic acid-binding interactions that subsequently affect the outcome of the viral infection. In the case of viruses that reproduce in the nucleus and that display less common A-to-G and U-to-C substitutions in viral sequences, either Adar1 or Adar2 could be responsible for the editing events, as these nuclear proteins are enzymatically active deaminases. Editing is not limited to viruses with a nuclear stage of their life cycle, however. Cytoplasmic viruses may be edited by the p150 isoform of Adar1, as this isoform is the only known cytoplasmic Adar in mammalian cells.

Reproduction of some viruses may be enhanced by these cellular Adars. Adar1 promotes production of human immunodeficiency virus type 1 particles by an editing-independent mechanism that does not require its deaminase activity. This effect appears to be the result of Adar1 inhibition by RNA-activated protein kinase (Pkr). The latter enzyme, which is synthesized and activated in response to IFN, phosphorylates the translation initiation protein eIF2B to inhibit translation (see Volume I, Chapter 11). It is well established that inhibition of Pkr by Adar1 increases the efficiency of reproduction of several viruses with (−) strand RNA genomes.

Table 3.4 Some viral gene products that suppress RNA interference

Virus	Gene product	Mechanism
Human adenovirus type 5	VA-RNA I and VA-RNA II[a]	Competition for binding to exportin-5 and Dicer
Ebola virus	VP35 protein	Binding to dsRNA
Influenza A virus	NS1 protein	Binding to dsRNA
Vaccinia virus	E3L protein	Binding to dsRNA
Human immunodeficiency virus type 1	Tat	Inhibition of Dicer?

[a]Both RNAs are cleaved by Dicer, and the products are incorporated into Riscs.

In contrast to the editing enzymes that can modulate infection by a broad range of viruses described above, Trim and Apobec proteins appear to function only against retroviruses. Like the cellular components already discussed, these proteins are constitutively produced, and therefore are part of the intrinsic host response.

Trim proteins. Trim (tripartite motif family) proteins prevent some cell types from being infected by certain retroviruses (see Chapter 7). The hypothesis is that *Trim* genes evolved independently in various species to protect against endemic retroviruses. We know by the presence of large numbers of retroviral proviruses in vertebrate genomes that retroviruses have existed for millions of years. We also know that many cell types are resistant to infection by some retroviruses, despite carrying functional receptors. For example, human immunodeficiency virus type 1 infections were found to be restricted in some, but not all, cell types, prompting a massive effort to identify the constitutive inhibitor, and perhaps develop it as a new antiviral. Despite its ability to enter, human immunodeficiency virus type 1 is unable to reproduce in the cells of Old World monkeys. Infection is blocked soon after entry, but before reverse transcription, resulting in an aborted infection. Introduction of a rhesus macaque cDNA library into permissive cells led to the identification of a dominant gene that blocked reproduction, tripartite interaction motif 5α (Trim5α). While humans do encode a Trim5α homolog, the human allele does not restrict human immunodeficiency virus type 1 reproduction. One could speculate that if humans had the rhesus macaque *Trim5*α gene, the AIDS pandemic might not have occurred (Chapter 10).

Rhesus macaque Trim5α targets the human immunodeficiency virus type 1 capsid protein, but not the capsid proteins of other retroviruses. When synthesis of Trim5α was reduced experimentally using siRNA molecules, the block of human immunodeficiency virus infection in monkey cells was relieved, but this treatment had no effect on a different retrovirus, murine leukemia virus. Trim5α binds to motifs within the capsid proteins and interferes with the uncoating process, preventing reverse transcription and nuclear entry of the retroviral genome. Trim5α is a ubiquitin ligase, promoting ubiquitinylation of capsids and their subsequent degradation by the proteasome. While Trim5α is made constitutively, IFN treatment increases its synthesis in both human and monkey cells.

The idea that a retroviral capsid can be a target for intrinsic defense is not new. Host restriction (or exclusion) of mouse retrovirus infection has been known for more than 30 years. The prototypical host gene blocking early retroviral replication events was identified using the Friend strain of murine leukemia virus. The locus is called Fv1 (Friend virus susceptibility). The Fv1 protein blocks reproduction of some strains of murine leukemia virus soon after reverse transcription. Surprisingly, the mouse *Fv1* gene encodes sequences related to

that of the capsid of an endogenous retrovirus resident in the mouse genome! Recent studies with wild mice have revealed that *Fv1* from various strains can recognize and restrict a wide range of retroviruses, including examples from the gammaretrovirus, lentivirus, and foamy virus genera. How Fv1 acts remains unclear, but restriction depends on a specific interaction with the capsid protein of the incoming virus. This finding is a remarkable demonstration of selection, turning endogenous retroviral gene-related expression against potential infection by other retroviruses.

Apobec proteins. Apobec (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like) proteins have various functions, including RNA editing of host genes (Fig. 7.6). Several members of the Apobec3 family are induced by IFN and are intrinsic antiretroviral proteins packaged into virus particles. After infection, some of these cellular enzymes, including Apobec3g, inhibit reverse transcription and introduce point mutations. When the viral reverse transcriptase begins to copy viral RNA into DNA, Apobec deaminates ssDNA, specifically the nascent (−) strand, which is synthesized first. The enzyme converts C's to U's with the consequence that when the deaminated (−) DNA strand is copied, the U pairs with A, producing a G-to-A transition. Consequently, the new proviral genome is mutated in a very characteristic pattern (many GC pairs become AT pairs), and some codons may be converted into stop codons. One retrovirologist called Apobec a "WMD"—a weapon of mass deamination.

The action of Apobec should be lethal for retroviruses that incorporate this enzyme into virus particles. However, human immunodeficiency virus type 1 Vif protein counters this potential lethal defense by binding to Apobecs, as well as to a host ubiquitin ligase, and promoting the ubiquitinylation and subsequent degradation of the editing enzymes by the proteasome. Interestingly, mouse Apobec3g cannot be targeted by human immunodeficiency virus type 1 Vif, a main reason why we cannot model this infection in mice.

ISGylation

Interferon-stimulated gene 15 (Isg15) is expressed primarily in monocytes and lymphocytes, and acts much like a cytokine. A 17-kDa antiviral protein encoded in the *Isg15* gene in humans shares properties with ubiquitin-like molecules that mark cellular proteins for degradation. However, in contrast to ubiquitin-like molecules, Isg15 has not been identified in yeasts, invertebrates, or plants.

The first identified viral target of Isg15 was the NS1 protein of influenza A virus (NS1A), a multifunctional protein made in influenza A virus-infected cells but not incorporated into virus particles. It was observed that multiple lysines in the Ns1A proteins of several influenza A virus strains were

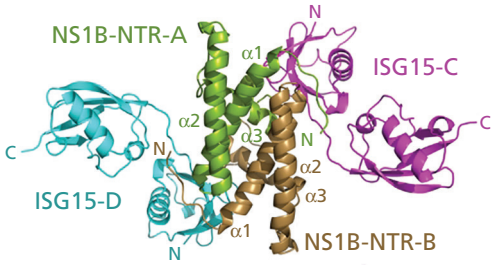


Figure 3.10 Influenza NS1B-Isg15 interactions. A heterotetramer, comprising two NS1B N-terminal region (Ntr) molecules (NS1B-Ntr-A, green; NS1B-Ntr-B, brown), which form an interwoven dimer, binds to two Isg15 molecules (Isg15-C, magenta; Isg15-D, cyan). Reprinted from R. Guan et al., *Proc Natl Acad Sci U S A* **108:**13468–13473, 2011, with permission.

modified by addition of Isg15; such changes resulted in a loss of Ns1A protein binding to critical cellular components, including importin α, and concomitant loss of infectivity. Isg15 conjugation targets newly synthesized proteins. As viral proteins constitute the majority of those newly synthesized molecules in infected cells, this property may explain the specificity with which Isg15 acts. It was subsequently shown that this protein inhibits the reproduction of a large number of other viruses, including Sindbis virus, human immunodeficiency virus type 1, and herpes simplex virus 1.

Less than 5% of the total target protein population is modified at any one time during virus infection. Some have conjectured that modification of a limited number of viral targets may confer a dominant-negative effect on the function of unconjugated potential targets, especially if the ISGylated viral protein functions as an oligomer in infected cells.

The genomes of influenza B and vaccinia viruses encode viral proteins that bind to Isg15 and prevent its conjugation with target proteins. The NS1B protein of influenza virus engages with human and nonhuman primate Isg15 (Fig. 3.10), but not with the mouse homolog, perhaps accounting for why influenza B virus does not reproduce in mice. Another countermeasure against Isg15 conjugation, encoded by both nairovirus [(−) strand RNA viruses] and arterivirus [(+) strand RNA viruses] genomes, is a viral protease that removes Isg15 from conjugated protein targets.

CRISPRs

The intrinsic antiviral defenses described above are found in mammals, and some are unique for nonhuman primates and humans. However, bacteria and archaea also mount defenses to impede or prevent invasion by foreign DNAs, including viral genomes (Box 3.8). A prokaryotic mechanism to silence exogenous DNA, also known as clustered regularly interspaced short palindromic repeats (CRISPRs), consists

BOX 3.8

BACKGROUND

Ancient mechanisms of intrinsic immunity

Antiviral defense systems in prokaryotes act at virtually all stages of the bacteriophage life cycle (see the figure). An effective and simple means of acquiring phage resistance is to block adsorption to the host cell, either by masking the receptor (for example, as a result of the expression of polysaccharides on the cell surface) or by downregulating or altering receptor molecules. Obstruction of the entry of the viral genome into the host's cytoplasm is a second line of defense. The proteins that block DNA injection are usually localized in association with or in close proximity to the membrane/cell wall and can be encoded by a plasmid or a prophage. If bacteriophage adsorption and DNA injection are not prevented, intracellular defense systems may act directly on the viral DNA. The restriction-modification (R-M) system is a broad-range prokaryotic immune system that targets DNA. A typical R-M system consists of a DNA methyltransferase, which modifies specific DNA sequences; and a restriction endonuclease, which cleaves the same sequences when unmodified. The general principle of these systems is that the host's genomic DNA is methylated and protected against cleavage, whereas exogenous DNA is unmodified and subject to degradation. Altruistic cell suicide, similar to apoptosis in eukaryotic cells, may also be triggered; the Abi system is one such pathway that can result in prokaryotic death.

An unusual structure of repetitive DNA downstream from the *Escherichia coli iap* gene consisting of invariant direct repeats (29 nucleotides) and variable spacer sequences (32 nucleotides) was discovered in 1987. This unique arrangement is now called the CRISPR system. All CRISPR/Cas systems operate in three stages: adaptation, expression, and interference. During the adaptation stage, resistance is acquired by integration of a new spacer sequence, corresponding to a section of the genome of the invading phage, into the CRISPR array. During the expression stage, *cas* genes are transcribed and translated; in addition, CRISPRs are transcribed into precursor CRISPR RNAs (pre-CrRNAs), which are subsequently cleaved by a Cas6 homolog. Mature CrRNAs contain only a single spacer sequence, and hence can recognize only a single target. During the interference stage, the CrRNA guides one or more Cas proteins to cleave the genome of an invading bacteriophage genome at the sequence that is complementary to the spacer.

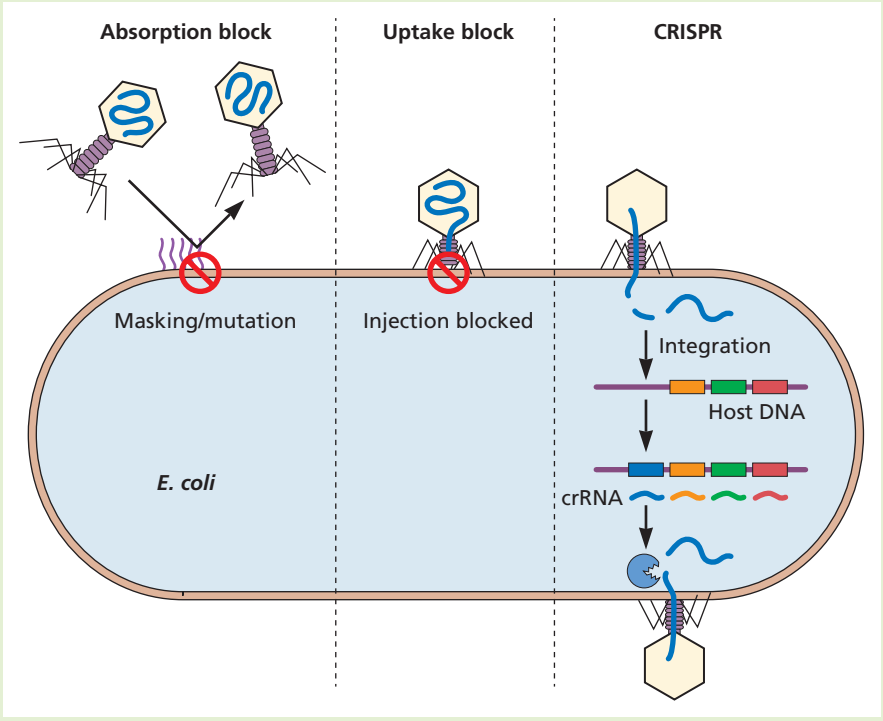
As expected, viral genomes also encode CRISPR systems of their own to antagonize those of the host. For example, it was recently shown that a bacteriophage-encoded CRISPR/Cas system can counteract a phage-inhibitory chromosomal island of the bacterial host, enabling a successful lytic infection.

Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. 1987. Nucleotide sequence of the *iap* gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. *J Bacteriol* **169:**5429–5433.

Seed KD, Lazinski DW, Calderwood SB, Camilli A. 2013. A bacteriophage encodes its own CRISPR/Cas adaptive response to evade host innate immunity. *Nature* **494:**489–491.

Westra ER, Swarts DC, Staals RH, Jore MM, Brouns SJ, van der Oost J. 2012. The CRISPRs, they are a-changin': how prokaryotes generate adaptive immunity. *Annu Rev Genet* **46:**311–339.

Overview of bacterial defense systems. Bacterial cells possess several mechanisms to defend against bacteriophage infection. Such strategies include blocking of phage adsorption or DNA injection or those that act directly on the phage DNA, such as R-M and CRISPR/Cas.



of short repetitions of nucleotide sequences in about half of eubacterial genomes and virtually all archaeal genomes. CRISPRs are associated with **cas genes**, which code for proteins related to CRISPRs. The CRISPR/Cas system confers prokaryotic resistance to foreign genetic elements such as bacteriophages. In a manner analogous to RNAi, CRISPR/Cas systems include an RNA-guided DNA endonuclease, Cas9, to generate double-strand breaks in invasive DNA during the bacterial immune response. The ability to program Cas9 for DNA cleavage at specific sites defined by guide RNAs has led

to its adoption as a versatile platform for genome engineering and gene regulation. By delivering the Cas9 protein and appropriate guide RNAs into a cell, the organism's genome can be cut at any desired location.

The Continuum between Intrinsic and Innate Immunity

At the beginning of this section, we noted that intrinsic immune responses are "ready-to-go" when a virus infection occurs and do not require new protein synthesis. The attentive reader may have noted that some of the systems we described above depend on IFN stimulation, and do require transcription and translation to produce the antiviral molecules.

As is discussed in the following section, IFNs released from infected cells can bind to receptors on adjacent, uninfected cells as a warning of a pending possible infection. Binding of IFN to uninfected cells stimulates the production of antiviral defenses in advance of viral infection, such that if the virus **does** infect the cell, these defenses are ready to deploy. One could argue that IFN-stimulated genes, such as *Isg15*, encode molecules that are "innate" in already-infected cells, but "intrinsic" in those that have yet to be infected.

A warning at the beginning of this chapter noted that distinctions such as these are often imprecise, and that, despite our efforts to impose order on the host immune response to make it easier to explain, the reality is far more complex. This is especially true when considering the soluble proteins of the host immune response.

Soluble Immune Mediators of the Innate Immune Response

Infected cells, sentinel phagocytes, and cellular components of the innate and adaptive immune response secrete many different proteins that can result in activation and recruitment of immune cells, induction of signaling pathways, tissue damage, and fever. The presence of cytokines in the blood is one of the first indications that a host has been infected and that immune defenses have been called into play. Traditionally, soluble mediators that chiefly influence the migration of immune cells to sites of infection are called **chemokines**, those that activate antiviral programs are called **interferons** (for their ability to interfere with viral infection), and the diverse collection of other soluble molecules are traditionally referred to as **cytokines**, although members of this latter category share few properties, either in sequence, structure, or function.

For purposes of clarity, we will adhere to these traditions, though a few introductory comments are warranted. With the potential exception of type I IFNs, these soluble products rarely, if ever, operate alone: the inflammatory response at the site of a viral infection comprises a heterogeneous mix of many different cell types and cytokines, and it is possible that many cytokines can bind to their receptors on a single cell, transducing distinct (perhaps conflicting) signals. Scientists often refer to the cytokine response as a "storm," though most laboratory-based experiments assess the function of only one cytokine at a time for simplicity. Whether cytokine A behaves the same when it is used alone in a controlled laboratory experiment as when it is part of a storm of cytokines A to Z in an infected host is not yet known.

In addition, while immunologists refer to soluble effector proteins as "cytokines," neurobiologists call them "neurotransmitters" and endocrinologists refer to them as "hormones." We are beginning to appreciate that these distinctions are artificial, made in an effort by humans to comprehend a complex topic. But neurotransmitters have been shown to possess immune cell-activating properties; hormones can alter neuronal behavior; and cytokines, such as interleukin-1β (IL-1β), can act on the central nervous system. An interested student would find a fascinating literature developing in the area of "cross-disciplinary" soluble molecules.

Overview of Cytokine Functions

IFN production is the initial response when a single cell detects a foreign nucleic acid or when a Tlr is engaged (Fig. 3.2). In turn, locally released IFN activates a more global innate immune response, should viral reproduction continue unabated. Most of these pattern recognition receptor-stimulated pathways converge on two critical cellular transcriptional activators, Irfs and Nf-κb (Fig. 3.11). Secreted IFNs engage receptors on sentinel dendritic cells, macrophages, and adjacent uninfected cells, which then synthesize a new burst of cytokines, amplifying the initial response. The first cytokines to appear in high concentrations are IFN-α and IFN-β, followed by Tnf-α, IL-6, IL-12, and IFN-γ. While their individual functions vary, all are potent molecules, capable of inducing a response at nanograms-per-milliliter concentrations. More than 100 cytokines are known, and the list continues to grow.

Consequences of massive cytokine induction include many of the clinical symptoms we typically associate with viral infection (Fig. 3.12). Some cytokines act directly on cells of the nervous system to produce fever, sleepiness, lethargy, muscle pain (myalgia), appetite suppression, and nausea. Proinflammatory cytokines stimulate the liver to synthesize acute-phase proteins, many of which are required to repair tissue damage and to clear the infection. Members of the colony-stimulating factor class of cytokines, which are made in the bone marrow after an inflammatory response,

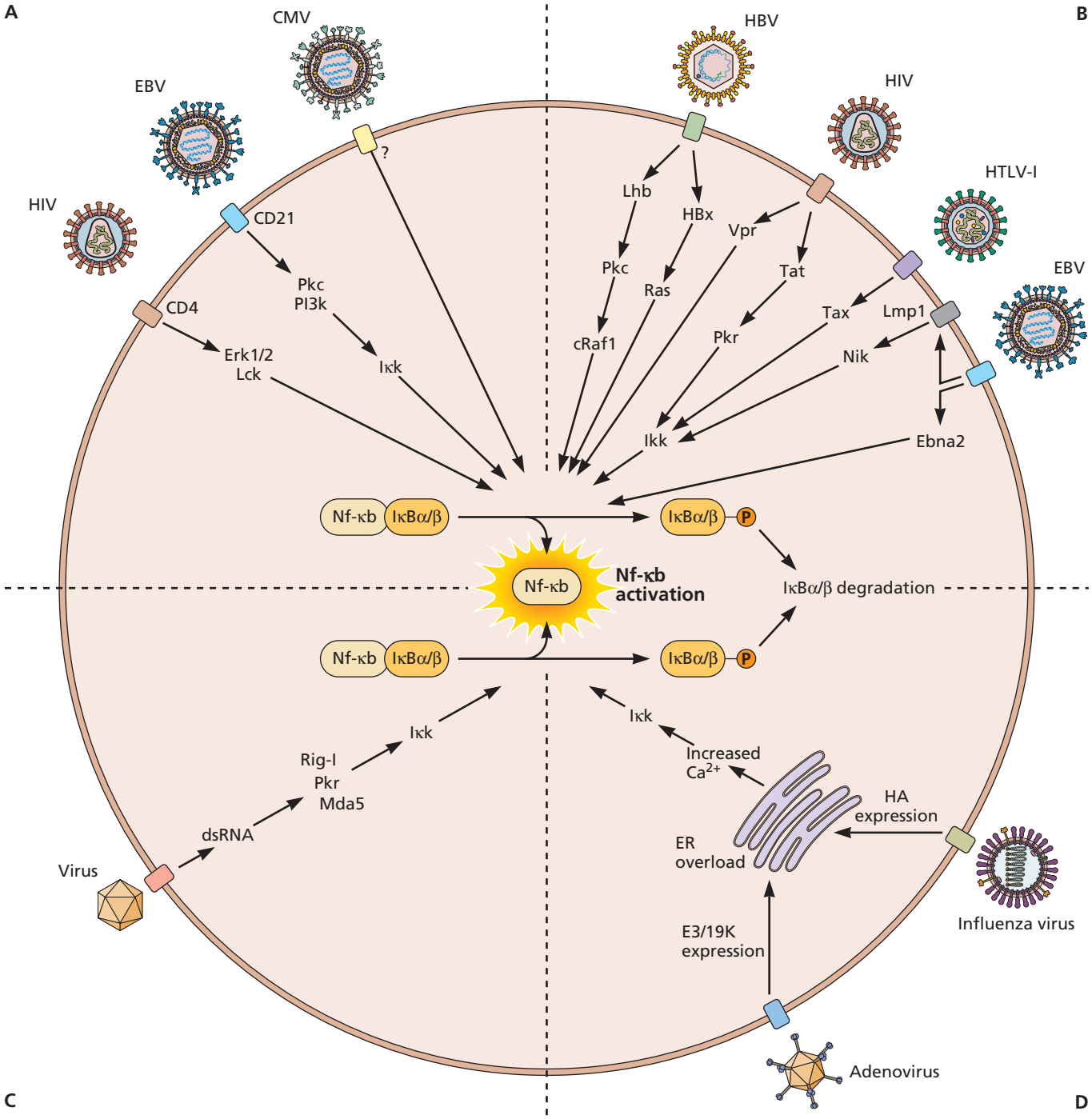


Figure 3.11 Activation of the transcription regulator Nf-kB by viral infection. Nf-κB is a transcription control protein important in the initial response to viral infection. Four diverse mechanisms that result in activation of Nf-κB are illustrated. In all cases, activation of these pathways leads to destruction of the inhibitor of Nf-κB, IκB, freeing Nf-κB to transit to the nucleus and initiate transcription of IFN genes. **(A)** Signal transduction pathways are activated upon binding of a virus particle to its receptor; **(B)** viral proteins synthesized in the infected cell directly engage signal transduction pathways that culminate in Nf-κB activation; **(C)** Pkr binds double-stranded viral RNA, or Rig-I/Mda5 bind single-stranded viral RNA, leading to activation of Nf-κB; and **(D)** overproduction of viral proteins in the endoplasmic reticulum (ER) leads to calcium release, which, in turn, activates Nf-κB. HIV, human immunodeficiency virus; EBV, Epstein-Barr virus; CMV, cytomegalovirus; HBV, hepatitis B virus; HTLV, human T cell lymphotropic virus.

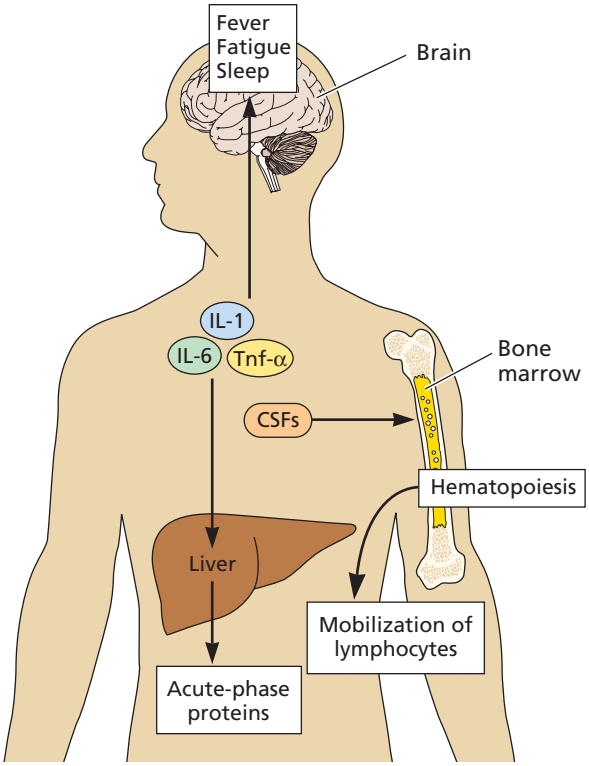


Figure 3.12 Systemic effects of cytokines in inflammation. A localized viral infection often produces global effects, including fever and lethargy, lymphocyte mobilization (swollen glands), and appearance of new proteins in the blood. The proinflammatory cytokines IL-1, IL-6, and Tnf all act on the brain (particularly the hypothalamus) to produce a variety of effects, including fever and fatigue. These cytokines also act in the liver to cause the release of iron, zinc, and acute-phase proteins, including mannose-binding protein, fibrinogen, C-reactive protein, and serum amyloid protein. These acute-phase proteins have innate immune defense capabilities: e.g., C-reactive protein binds phosphorylcholine on microbial surfaces and activates complement. The colony-stimulating factors (CSFs) activated by an inflammatory response have long-range effects in the bone marrow on hematopoiesis and lymphocyte mobilization. Adapted from A. S. Hamblin, *Cytokines and Cytokine Receptors* (IRL Press, Oxford, United Kingdom, 1993), with permission.

control the proliferation and maturation of lymphocytes and other cells essential in antiviral defense (Box 3.9).

Predictably, many viral gene products can mimic or modulate cytokine responses. The former proteins have been called **virokines** if they mimic host cytokines, or **viroceptors** if they mimic host cytokine receptors. The arsenal includes remarkable proteins such as soluble IL-1 receptors, a variety of chemokine antagonists, and functional homologs of IL-10 and IL-17. Viral DNA genomes encode most of the well-known virokines and viroceptors, but smaller viral RNA genomes contain some surprises. For example, the envelope protein of respiratory syncytial virus is a mimic of fractalkine, the only known chemokine that is a non-secreted membrane protein. The viral envelope protein competes with the binding of cellular fractalkine to its receptor, which also functions as a receptor for the virus.

In this section, we focus on two cytokine groups, type I IFNs and chemokines. Other cytokines, including the interleukins and the type II IFN, IFN-γ, are primarily produced by cells of the adaptive immune response, and will be discussed in Chapter 4.

BOX 3.9

TERMINOLOGY

Infiltration and inflammation

While these words sound somewhat similar, and perhaps imply similar processes, the distinction between them is important. **Infiltration** is defined as the accumulation of substances in a tissue that are not normally present, such as the infiltration of T cells to the site of a viral infection. Infiltration of cells into a target tissue may or may not result in symptoms. **Inflammation** refers to the response of a tissue to damage or infection, usually accompanied by swelling, heat, and redness. Certainly infiltration of immune mediators may accompany an inflammatory response, but cells within the affected tissue (for example, tissue-resident dendritic cells) and the cytokines they produce are sufficient to generate an inflammatory response.

Interferons, Cytokines of Early Warning and Action

IFNs are synthesized by mammals, birds, reptiles, amphibians, and fish, and are critical signaling proteins of the host frontline defense (Box 3.10). The discovery of IFN was first reported almost simultaneously in the 1950s by two groups of investigators. One group observed that chicken cells exposed to inactivated influenza virus contained a substance that interfered with the infection of other chicken cells by infectious influenza virus. The second group made their discovery using vaccinia virus. We now know that most cells synthesize IFN when infected, and the released IFN inhibits reproduction of a wide spectrum of viruses.

There are three types of IFN: types I, II, and III. All type I IFNs bind to a specific cell surface receptor known as the IFN-α receptor (IFNar) that consists of IFNar1 and IFNar2 chains (Fig. 3.13). The type I IFNs present in humans are IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω, though this text will primarily focus on IFN-α and IFN-β. The sole type II IFN, IFN-γ, binds to the heterodimeric IFN-γ receptor. Less is known about the third type of IFN, which includes IFN-λ1, -2, and -3. (Rather unhelpfully, these also have interleukin designations: IL-29, IL-28A, and IL-28B, respectively.)

BOX 3.10

EXPERIMENTS

The interferon system is crucial for antiviral defense

Many steps in a viral infectious cycle can be inhibited by IFN, depending on the virus family and cell type. Binding of type I IFN to its receptor leads to increased transcription of nearly 2,000 genes. A website, called interferome (http://interferome.its.monash.edu.au/interferome/home.jspx), maintains a searchable index of the known IFN gene targets. The proteins produced inhibit viral penetration and uncoating, synthesis of viral mRNAs or viral proteins, replication of the viral genome, and assembly and release of progeny virus particles. Multiple steps in virus reproduction can be inhibited, providing a strong cumulative effect.

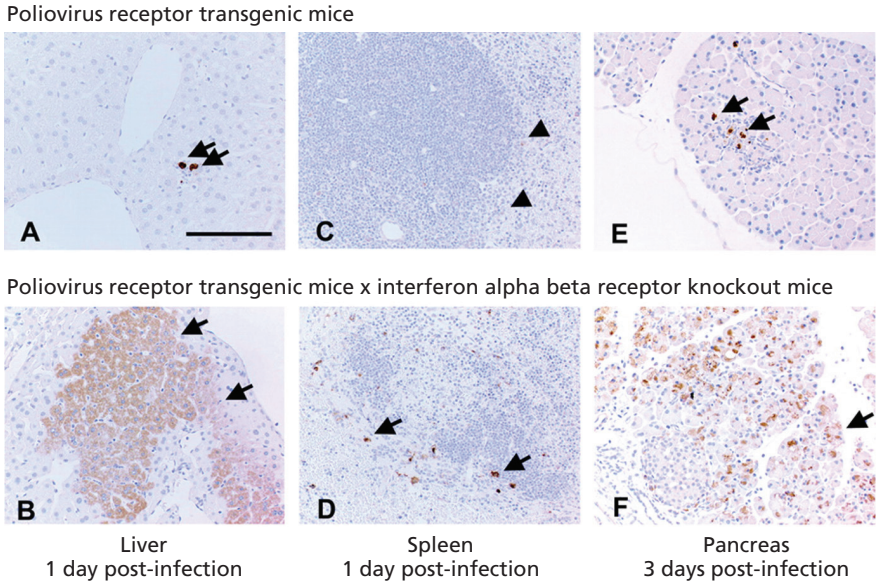
The contribution of IFN can be demonstrated in animal models in which the antiviral response is reduced by treatment with anti-IFN antibodies, or in mice harboring mutations that delete or inactivate IFN genes, IFN receptor genes, genes that regulate the IFN response, or genes that are induced by IFNs. Animals with a defective IFN response typically exhibit a reduced ability to contain viral infections, and often show an increased incidence of illness or death. When the IFN-α/β response is impaired, there is a global increase in susceptibility to most viruses (see the figure). These observations indicate that IFN-α/β is crucial as a general antiviral defense.

Huang S, Hendriks W, Althage A, Hemmi S, Bluethmann H, Kamijo R, Vilcek J, Zinkernagel RM, Aguet M. 1993. Immune response in mice that lack the interferon-gamma receptor. *Science* **259:**1742–1745.

Ida-Hosonuma M, Iwasaki T, Yoshikawa T, Nagata N, Sato Y, Sata T, Yoneyama M, Fujita T, Taya C, Yonekawa H, Koike S. 2005. The alpha/beta interferon response controls tissue tropism and pathogenicity of poliovirus. *J Virol* **79:**4460–4469.

Stojdl DF, Abraham N, Knowles S, Marius R, Brasey A, Lichty BD, Brown EG, Sonenberg N, Bell JC. 2000. The murine double-stranded RNA-dependent protein kinase PKR is required for resistance to vesicular stomatitis virus. *J Virol* **74:**9580–9585.

Zhou A, Paranjape JM, Der SD, Williams BR, Silverman RH. 1999. Interferon action in triply deficient mice reveals the existence of alternative antiviral pathways. *Virology* **258:**435–440.



Immunohistochemical detection of poliovirus proteins in infected poliovirus receptor (Pvr) transgenic mice (top panels) and Pvr mice crossed to IFN-α/β receptor knockout mice (bottom panels). (A/B) Representative liver sections, day 1 postinfection. **(C/D)** Representative spleen sections, day 1 postinfection. **(E/F)** Representative pancreas sections, day 3 postinfection. Bar, 125 μm. From M. Ida-Hosonuma et al. *J Virol* **79:**4460–4469, with permission.

Type I IFN Synthesis

While type I IFNs are induced principally following detection of infection and signaling by pattern recognition receptors and their downstream partners, other signals can also lead to IFN production. Structural proteins of some viruses stimulate IFN synthesis upon binding of virus particles to cells. For example, engagement of CD46, which is an entry receptor for vaccine strains of measles virus, induces potent IFN responses. In other cases, virus-induced degradation of the inhibitor of Nf-κb (Iκbα) leads to transcription of the genes encoding IFNs (Fig. 3.14).

Virus-infected cells usually produce IFN, but uninfected macrophages and dendritic cells that patrol tissues also make this cytokine when their Tlrs bind products released from infected cells (by apoptosis, for example). Such products include viral proteins, viral nucleic acids, and cellular stress proteins (e.g., heat shock proteins). If the infection is not contained at this stage and spreads to more cells, large quantities of IFN may be synthesized by specialized dendritic cell precursors in the blood called plasmacytoid dendritic cells. Systemic circulation of IFN leads to many of the general symptoms we associate with feeling sick following viral infection.

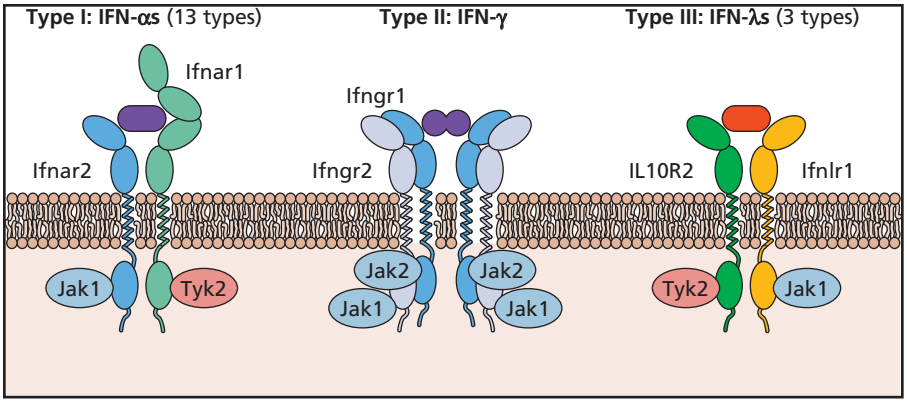
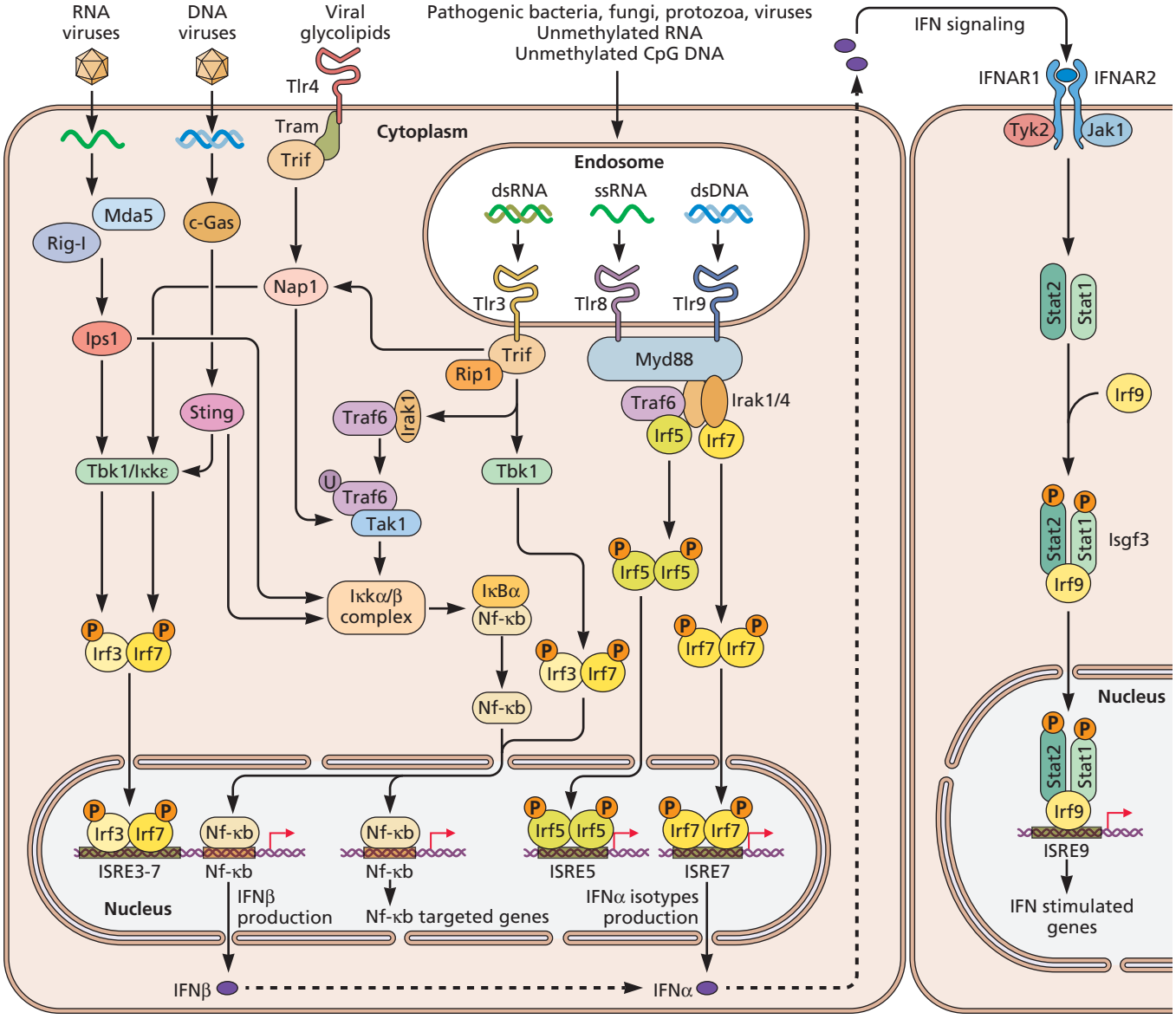


Figure 3.13 Interferon receptors. Type I IFNs interact with IFN-α receptors 1 and 2 (Ifnar1 and Ifnar2); type II IFN with IFN-γ receptors 1 and 2 (Ifngr1 and Ifngr2); and type III IFNs with IFN-λ receptor 1 (Ifnlr1) and IL-10 receptor 2 (IL10R2). Important cytoplasmic proteins that bind to the intracellular domains of each of these receptors are indicated.

Figure 3.14 Type I interferon synthesis, secretion, receptor binding, and signal transduction. Viruses or viral components are bound by Tlrs that trigger downstream signaling cascades leading to the production of type I IFNs (α and β) and Nf-κB-regulated genes. The type I IFNs are released from the cell, and can then bind to IFN receptors on the surfaces of adjacent cells to stimulate synthesis of IFN-responsive genes.



We tend to describe the two main type I IFNs, α and β, in similar terms, but they are quite different. There is only ~50% homology between α and β proteins, and while there is only one type I IFN-β gene, there are at least 20 IFN-α genes in the mouse and 13 in humans. Although these different type I IFN-α genes appear to be redundant (they are >80% identical to each other), they can be expressed differentially upon infection. The selective pressures that led to the diversity of type I IFNs remain to be discovered. In addition, transcription of the human *IFN-β* gene precedes expression of the IFN-α genes. The *IFN-β* enhancer possesses several remarkable properties that allow precise temporal control of transcription (Box 3.11).

The production of IFN by infected cells and uninfected, immature dendritic cells at the site of infection is rapid and robust, but transient; it occurs within hours of infection and generally declines by 10 h postexposure. Furthermore, the quantity of IFN released from cells infected by different isolates of a particular virus is astonishingly variable. In the case of vesicular stomatitis virus infection, the released IFN concentration can vary over a 10,000-fold range, depending on the serotype of the virus. As discussed later, many viral proteins affect the quantity of IFN made, as well as its action.

IFN Affects Only Cells with IFN Receptors

IFN functions only when it occupies its receptor on the surfaces of cells. Cells that produce IFN may also respond to IFN, but it must first be secreted, and then bind to cellular receptors (Fig. 3.14). A cell without IFN receptors may synthesize IFN, but cannot be affected by this cytokine. Binding of IFN to its receptor initiates a signal transduction cascade that culminates in increased transcription of many genes. A simplified outline of this signaling pathway is shown in Fig. 3.15.

IFNs affect gene expression by signaling via the Jak/Stat pathway. Members of this signal transduction family can also respond to IL-6 and other cytokines. There are four known Jak kinases and seven structurally and functionally related Stat proteins. Their targeted disruption of the respective genes in mice has revealed much about their functions. For example, a mouse in which the *stat1* gene has been deleted has no innate response to viral or bacterial infection, whereas deletion of *stat4* and *stat6* leads to inhibition of specific functions of the adaptive response. *Stat* gene homologs are encoded in the genomes of *Drosophila melanogaster* and *Dictyostelium discoideum*, underscoring the ancient evolutionary origin of this pathway. Signaling via Jak/Stat activates transcription dependent on specific promoter sequences. These sequences are found in the promoters of ~300 well-characterized IFN-activated genes, though more sensitive methods to detect gene expression changes have shown that 1,000 to 2,000 genes may be affected by this critical early cytokine.

IFN Action Produces an Antiviral State

As the name aptly indicates, IFN interferes with the reproduction of a wide variety of viruses in cells in culture and animals. Shortly after infection of the host, newly made IFN released from infected cells and local immature dendritic cells can be found circulating in the body, but its concentration is highest at the site of infection, where it is bound by any cell with the appropriate receptor. Cells that bind and respond to IFN do not support propagation of many different viruses; they are said to be in an **antiviral state**.

Many genes are induced by IFN signaling, but their mix and concentrations vary according to cell type and specific IFN. Which subset of the hundreds of IFN-inducible proteins establishes the antiviral state in any given cell remains unknown. Many of the products of IFN-inducible genes possess potent, broad-spectrum antiviral activities, but the relevant molecular mechanisms of only a few are understood. IFN not only induces death of the infected cells, but also ensures that uninfected cells in the vicinity are prepared to kill themselves should they become infected. Such a local cauterizing response has led some to characterize IFN action as a molecular firebreak to infection: IFNs define the boundary of infection by inducing either the death of infected cells or the antiviral state to prevent the virus from spreading beyond the local region of infection (Fig. 3.16).

IFN-induced proteins are functionally diverse and participate in signal transduction, chemokine action, antigen presentation, regulation of transcription, the stress response, and control of apoptosis. Some of these proteins are induced by other stimuli, including dsRNA, bacterial LPSs, Tnf-α, or IL-1. Because IFN induces the synthesis of many cytotoxic gene products, a common outcome of IFN signaling is cell death. We next describe some of the better-characterized IFN-stimulated gene products and their specific contributions to the antiviral state.

Some IFN-Induced Gene Products and Their Antiviral Actions

dsRNA-activated protein kinase. Viral and cellular protein synthesis in infected cells is often stopped abruptly. In many cases, this phenomenon, mediated by a cellular dsRNA-activated protein kinase (Pkr), is lethal to both the virus and the infected cell (also described in Volume I, Chapter 11). Establishment of the Pkr-mediated antiviral state is a two-step process, in which IFN promotes the increased production and accumulation of an inactive protein that can become activated only when it encounters double-stranded viral RNA.

All mammalian cells contain low concentrations of inactive Pkr, a serine/threonine kinase with antiviral, antiproliferative, and antitumor activities. The signal transduction cascade initiated by IFN binding to its receptor leads to a dramatic increase in the concentration of inactive Pkr. Metaphorically, this

BOX 3.11

DISCUSSION

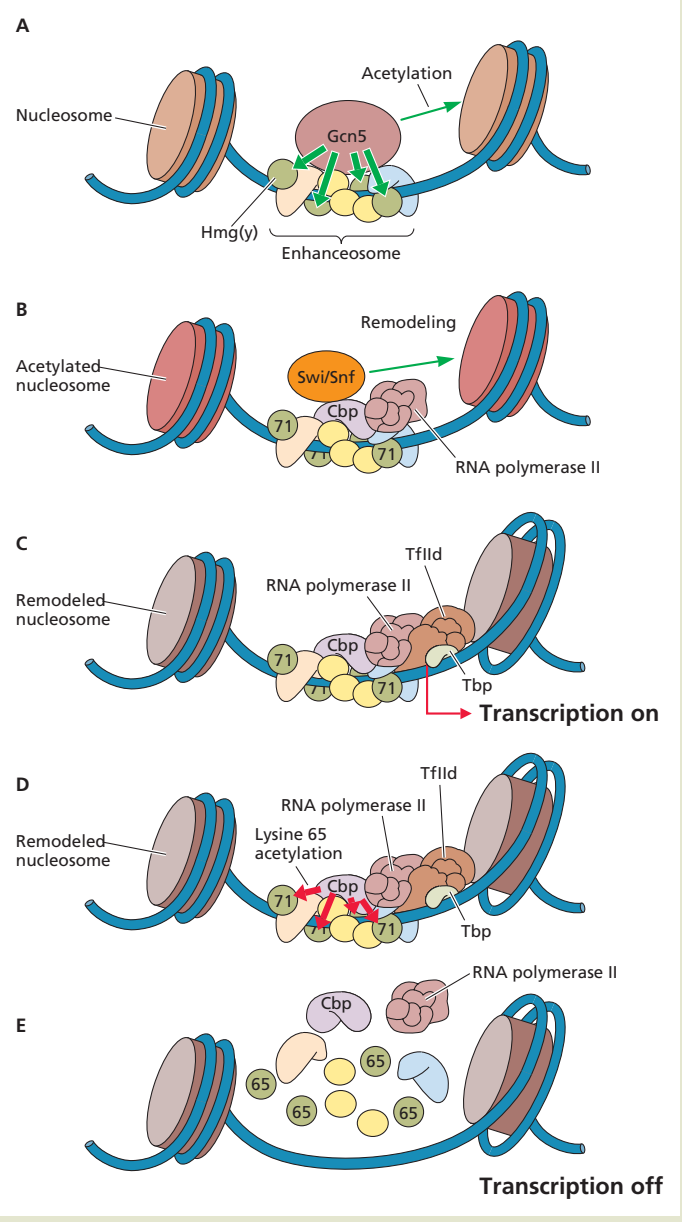
Switching IFN-β transcription on and off

The regulation of the process that controls transcriptional activation and cessation of the *IFN-β* gene is the result of the coordinated action of many cellular proteins. It is therefore a significant scientific achievement that we have been able to understand this process.

Viral infection activates transcription of the human *IFN-β* gene, but only for a short period. This on-off response is controlled by an enhancer located immediately upstream of the core promoter. Like other enhancers, this regulatory sequence contains binding sites for multiple transcriptional activators, including Nf-κb and members of the Ap-1 and Atf families. However, the *IFN-β* enhancer possesses several remarkable properties that allow precise temporal control of transcription.

- The enhancer contains four binding sites for the architectural protein Hmg1(Y), which alters DNA conformation to direct the assembly of a precisely organized nucleoprotein complex on the enhancer.
- In contrast to typical modular enhancers, all binding sites **and** their natural arrangement are essential for activation of *IFN-β* transcription.
- Formation of the complex takes place in stages, and is not complete until several hours after infection.
- Activation of transcription requires sequential recruitment of the histone acetylase general control nonderepressible 5 (Gcn5), the coactivator cellular cAMP response element binding protein (Creb)-binding protein (Cbp), RNA polymerase II, and the chromatin-remodeling complex Swi/Snf.
- In addition to modifying nucleosomes, Gcn5 acetylates one of the Hmg proteins (A1) at Lys71. This modification stabilizes the complex.
- The Hmg(A1) protein is also acetylated by Cbp at another residue, Lys65. However, **this** modification impairs DNA-binding activity and results in disruption of the complex and cessation of *IFN-β* transcription.
- Remarkably, this inhibitory modification by Cbp is blocked for several hours by prior Gcn5 acetylation of Hmg(A1). As a result, the "off" switch is delayed for a sufficient period to allow a burst of *IFN-β* transcription.

Munshi N, Agalioti T, Lomvardas S, Merika M, Chen G, Thanos D. 2001. Coordination of a transcriptional switch by HMG1(Y) acetylation. *Science* **293:** 1133–1136.



(A) Viral infection of human cells leads to assembly of multiple proteins on the IFN-β enhancer, which lies in a nucleosome-free region of the gene. The signals that direct binding of transcriptional activators (blue, yellow, and tan, collectively called the enhanceosome in the figure) and Hmg(A1) (green) are not fully understood. The precisely organized surface of the complex allows binding of Gcn5, which acetylates both histones in nearby nucleosomes and Lys71 of bound Hmg(A1) molecules (green arrows). The latter modification stabilizes the enhanceosome. **(B)** A complex of Cbp, RNA polymerase II, and the chromatin-remodeling protein Swi/Snf binds sequentially to the stabilized complex. The Swi/Snf complex alters the adjacent nucleosome that contains the core promoter DNA (green arrow). **(C)** Such alteration allows binding of TfIId and activation of transcription. Because Lys71 of Hmg(A1) is acetylated, Cbp cannot acetylate Lys65. **(D)** Eventually, Cbp does acetylate Lys65 of Hmg(A1) (red arrows), but how the inhibition induced by Lys71 acetylation is overcome is not yet clear. **(E)** Hmg(A1) modification by Cbp disrupts the complex and switches off transcription. Adapted from K. Struhl, *Science* **293:** 1054–1055, 2001, with permission.

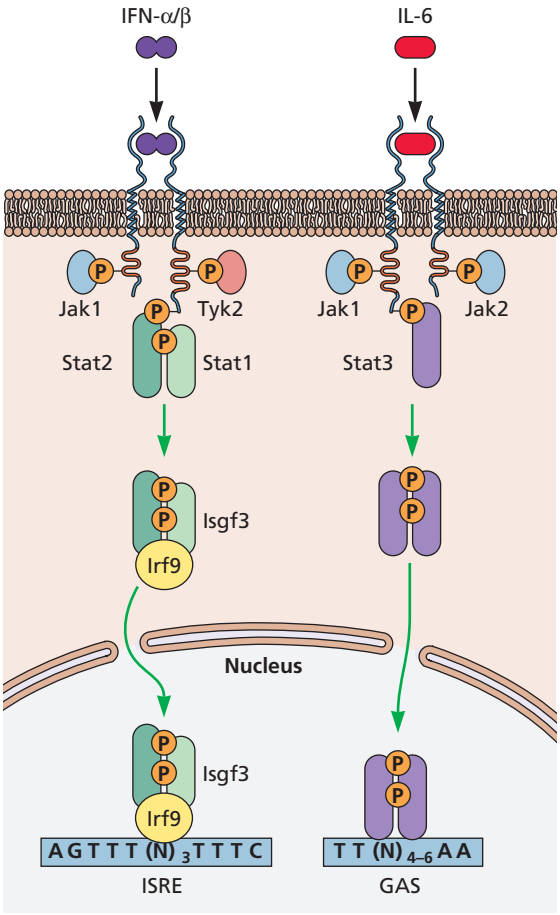


Figure 3.15 Common signal transduction pathways for IFN-α/β and IL-6. IFN signals via the Jak/Stat pathway, characterized by a family of tyrosine kinases given the acronym Jak (Janus kinases; Janus, a Roman god, guardian of gates and doorways, is represented with two faces and therefore faces in two directions at once) and a set of transcription proteins named Stat (signal transduction and activators of transcription). The receptors for IFN-α/β and IL-6 are different, but all affect components of the Jak/Stat signal transduction pathway. Type I IFNs and IL-6 bind to their receptors with high affinity (equilibrium dissociation constant [K_d] of $\sim 10^{-10}$ M). Binding of IFN or IL-6 to the appropriate receptor leads to the phosphorylation of tyrosine in tyrosine kinases as well as in the receptor itself. These modifications are followed by phosphorylation of tyrosine in the Stat proteins. In mammals there are seven *Stat* genes. The phosphorylated Stat proteins then form a variety of dimers that enter the nucleus. Within that organelle, Stat dimers bind, in some cases in conjunction with other proteins (e.g., Irf9), to specific transcriptional control sequences of IFN-α/β- and IL-6-inducible genes called interferon-stimulated response elements (ISREs) and IFN-gamma activated sequence (GAS) elements, respectively. Later in the transcriptional response to IFN, a second transcriptional activator called Irf1 replaces Isgf3.

means that the cell may go from 5 hand grenades to 50, but the pin is still in place, and the hand grenades are therefore not dangerous. If the cell is infected, this enzyme becomes activated by binding viral dsRNA; the pin is pulled. Active Pkr phosphorylates the α subunit of the eIF2 translation initiation



Figure 3.16 The interferon-induced firebreak that restricts viral spread beyond the site of infection.

protein (eIF2α), rendering it incapable of supporting protein synthesis in the cell (see Volume I, Chapter 11). Phosphorylated eIF2α does not invariably lead to cell death, as this modified protein can also trigger autophagy.

Many viral genomes encode proteins that can block the lethal actions of Pkr (Table 3.5). For example, a herpes simplex virus 1 protein (ICP34.5) redirects the cellular protein phosphatase 1 to dephosphorylate eIF2α after it has been phosphorylated and inactivated by Pkr. While wild-type virus is fully virulent in mice, ICP34.5-null mutants are markedly attenuated, particularly in brain infections. Significantly, this mutant regains wild-type virulence in mice lacking the *pkr* gene. This observation provides convincing evidence that Pkr mediates defense against herpes simplex virus infection in mice.

RNase L and 2′-5′-oligo(A) synthetase. Another well-studied antiviral response induced by IFN is mediated by two enzymes and dsRNA. RNase L can degrade most cellular and viral RNA species. Its concentration increases 10- to 1,000-fold after IFN treatment, but the protein remains inactive unless a second enzyme is synthesized. This enzyme, 2′-5′-oligo(A) synthetase, makes oligomers of adenylic acid, but only when triggered by dsRNA. These unusual nucleotide oligomers then activate RNase L, which in turn begins to degrade all host and viral mRNA. We now know from studies of mouse mutants defective in RNase L that this enzyme is important for the IFN-β response to viral infection. RNA fragments produced by RNAse L have double-stranded regions that allow them to be identified by RNAse L and Mda5, enhancing the production of IFN-β.

Mx proteins. Unlike the broad-spectrum antiviral effects of Pkr and RNase L, at least one IFN-induced mouse protein and two related human proteins appear to be directed against specific viruses. Mouse strains that have an IFN-inducible

Table 3.5 Some viral modulators of the interferon response[a]

Type of modulation	Representative viruses	Viral protein, if known	Mechanism of action
Inhibition of IFN synthesis	Epstein-Barr virus	Bcrf1	IL-10 homolog, inhibits production of IFN-γ
	Vaccinia virus	A18R	Regulates dsRNA production
	Foot-and-mouth disease virus	L	Host protein synthesis block
IFN receptor decoys	Vaccinia virus	B18R	Soluble IFN-α/β decoy receptor
Inhibition of IFN signaling	Adenovirus	E1A	Decreases quantity of Stat1 and P48, blocks Isgf3 formation, interferes with Stat1 and Cbp/p300 interactions
	Vaccinia virus	VH1	Viral phosphatase reverses Stat1 activation
	Human papillomavirus 16	E7	Binds p48
	Hepatitis C virus	NS5a	Blocks formation of Isgf3 and Stat dimers
	Nipah virus	V protein	Prevents Stat1 and Stat2 activation and nuclear accumulation
Block function of IFN-induced proteins	Adenovirus	VA-RNA I	Binds dsRNA, blocks Pkr
	Herpes simplex virus 1	US11	Blocks Pkr activation
		ICP34.5	Redirects protein phosphatase 1α to dephosphorylate eIF2α, reverses Pkr action
	Vaccinia virus	E3L	Binds dsRNA and blocks Pkr
		K3L	Pkr pseudosubstrate, decoy
	Human immunodeficiency virus type 1	TAR RNA	Blocks activation of Pkr
		Tat	Pkr decoy
	Hepatitis B virus	Capsid protein	Inhibits MxA
	Influenza virus	NS1	Binds dsRNA and Pkr, blocks action of Isg15
	Reovirus	σ3	Binds dsRNA, inhibits Pkr and 2′-5′-oligo(A) synthase

[a]For further examples and details, see B. B. Finlay and G. McFadden, *Cell* **124:**767–782, 2006.

gene called *mx1* are completely resistant to influenza virus infection. (The name, Mx1, is from the former name for influenza, myxovirus.) The Mx1 protein is part of a small family of IFN-inducible guanosine triphosphatases (GTPases) with potent activities against various (−) strand RNA viruses. After IFN induction, this protein accumulates in the nucleus and inhibits the unusual influenza virus "cap-snatching" mechanism (Volume I, Chapter 6). It is likely that the Mx1 protein interferes with the function of the viral polymerase subunit PB2, as overproduction of this viral protein overcomes the antiviral effect of Mx1. The significance of the *mx1* gene in the biology of influenza virus or of mice is not clear, as influenza virus does not circulate among wild mice. Moreover, about one-quarter of the mouse population (including many inbred strains used in laboratory research) lacks a functional *mx1* gene, with no obvious deleterious consequences.

The two human genes related to the murine *mx1* gene are termed *mxA* and *mxB*. Expression of these genes is also induced by IFN, but unlike the murine protein, the human proteins reside in the cytoplasm. MxA, but not MxB, blocks reproduction of influenza virus. In contrast to murine Mx1, which inhibits only influenza virus, the human MxA protein also prevents reproduction of vesicular stomatitis virus, measles virus, and other (−) strand RNA viruses. These human Mx proteins are related to members of the dynamin superfamily of GTPases, which regulate endocytosis and vesicle transport, but how this property relates to their antiviral activities is unknown.

Promyelocytic leukemia proteins. The promyelocytic leukemia (Pml) proteins are present in both the nucleoplasm and discrete multiprotein complexes known as nuclear bodies or Pml bodies (discussed earlier, and in Volume I, Chapter 9). These structures are important in the intrinsic cellular response to infection because their components bind foreign DNA that enters the nucleus. Pml and other proteins present in the complexes are thought to exert their antiviral effects by transcriptional repression and nucleosome remodeling. Many viral infections promote the dismantling of Pml bodies, in part as a measure to override global repression.

Ubiquitin-proteasome pathway components. The proteasome is a large, multisubunit protease that degrades cytoplasmic and nuclear proteins targeted for proteolysis by

polyubiquitylation. Such degradation is important for the removal of abnormal or damaged proteins, the turnover of short-lived regulatory proteins, and the production of peptides for assembly of MHC class I proteins that are critical for induction of adaptive immunity. All IFNs induce transcription of a number of genes that encode proteins of the ubiquitin-proteasome pathway. In fact, many IFN-stimulated genes encode ubiquitin ligases. Increased protein degradation may contribute to the antiviral response to some viruses. For example, proteasome inhibitors block the anti-hepatitis B virus action of type I IFN. In this case, activation of the proteasome may be **the** major antiviral effect, because the results of other experiments demonstrate that the Pkr and RNase L systems are completely ineffective.

Tetherin/Bst2. Tetherin, or bone marrow stromal antigen 2 (Bst2), is a lipid raft-associated protein encoded by the *Bst2* gene in humans. This protein is constitutively made in some cells of the immune system, but can be induced by IFN in many others. Most of what is known about this protein relates to its antiviral properties, though recently its role has been identified in uninfected cells (Chapter 7). Tetherin blocks many enveloped viruses from budding from the infected cell surface by tethering the budding viral membranes to each other and to the plasma membrane. Tetherin protein spans the plasma membrane at one end and is attached to membranes by a glycosylphosphatidylinositol anchor at the other end. This unique topology and the tendency of tetherin to form dimers aid in retention of enveloped viral particles at the plasma membrane and prevent their separation (Fig. 3.17).

The human immunodeficiency virus type 1 protein Vpu can overcome this restriction by ubiquitinylating tetherin, leading to its degradation. Consistent with its importance in preventing human immunodeficiency virus type 1 transmission, tetherin gene variants are associated with disease progression.

IFN regulatory proteins. Members of the Irf protein family are required for sustained transcription of the IFN genes after induction. Mice lacking the *irf1* gene are incapable of mounting an effective IFN response to viral infection. Other members of this gene family (*irf2* to *irf9*) were discovered because their protein products bound to the interferon-stimulated response element (ISRE) in promoters of IFN-regulated genes. Irf4 is synthesized only in T and B cells, and Irf8 is made only in cells of the macrophage lineage. Mice defective for *irf8* gene expression are markedly more susceptible to infection and cannot synthesize proinflammatory cytokines. The protein Irf9 is the DNA-binding component of the transcriptional regulator IFN-stimulated gene factor 3 (Isgf3) (Fig. 3.15). Several viral Irf-like proteins that block IFN action have been identified (Table 3.5).

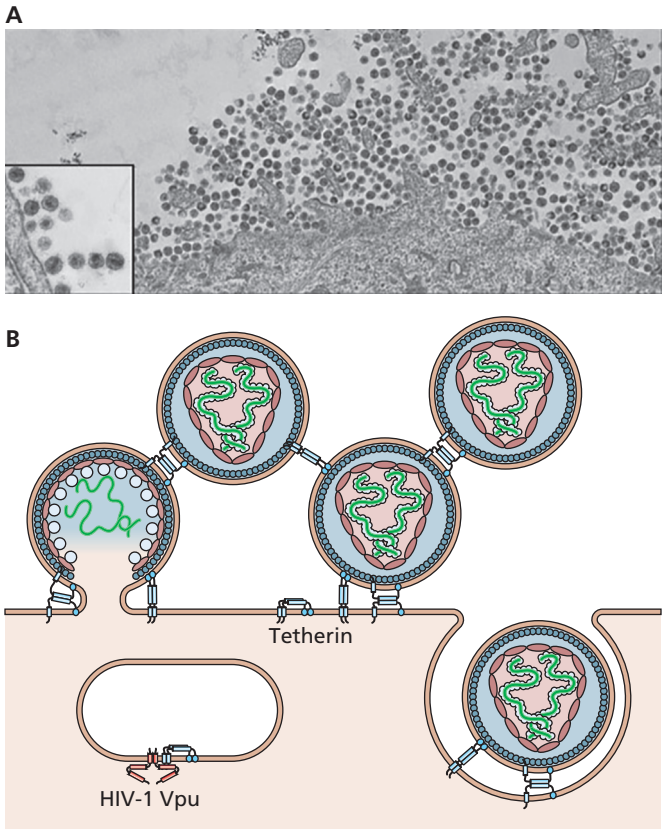


Figure 3.17 Tetherin prevents budding of enveloped viruses. Human immunodeficiency virus type 1 (HIV-1) virus particles lacking a functional Vpu protein are trapped at the surface of a tetherin-expressing cell by apparent particle-to-particle, as well as particle-to-cell, tethering. Panel A: from S. J. Neil et al., *Nature* **451:**425–430, 2008. **(B)** Tetherin anchors enveloped virus particles to the cell membrane, and to each other, preventing release. Human immunodeficiency virus type 1 protein Vpu antagonizes this intrinsic restriction mechanism.

Other IFN-induced proteins. Other proteins with antiviral effects surely remain to be discovered among the many IFN-induced genes that have been identified. For example, the IFN response is required to clear human cytomegalovirus infections, but Pkr, Mx, and RNase L proteins are not. Similarly, uncharacterized IFN-induced proteins block penetration and uncoating of simian virus 40 and some retroviruses. Others impair the maturation, assembly, and release of vesicular stomatitis virus, herpes simplex virus, and some retroviruses by unknown mechanisms. Specific combinations of the products of Isgs are probably needed to control viral reproduction, and this likely depends on the nature of the infecting virus.

Regulators of the IFN Response

As many of the gene products characteristic of the antiviral state are highly cytotoxic, it is imperative to suppress the

response once viral reproduction has been controlled. Such containment is accomplished by the action of members of the suppressor of cytokine signaling (Socs) protein family, which act in a classical negative-feedback loop to attenuate cytokine signal transduction (Fig. 3.18). The SH2 domains of Socs proteins interact with activated cytokine receptors, including the Jaks, blocking their ability to activate Stat molecules. The Socs proteins combine specific inhibitory interactions with a general mechanism of targeting associated signaling molecules for degradation.

Gene-knockout studies have shown that Socs proteins are indispensable regulators of important physiological systems: Socs1 is an essential homeostatic regulator of IFN signaling that is crucial to allow the beneficial immunological effects of IFN without the damaging pathological responses. Mice that lack Socs1 die early in life, even in the absence of viral infections; they have liver disease, inflammatory lesions, lymphopenia, apoptosis of lymphoid organs, and anomalous T cell activation, all probably the result of unrestricted IFN signaling.

Viral Gene Products That Counter the IFN Response

The term "antiviral state" implies that the IFN response confers complete resistance to virus infection. However, viruses vary considerably in their sensitivity to the effects of this cytokine. The reproduction of some viruses, such as vesicular stomatitis virus, is so sensitive to IFN that this property is used to titrate the cytokine. Other viruses can be more resistant to IFN. We now know that numerous viral mechanisms confound IFN production or action.

Many viral genomes encode dsRNA-binding proteins that interfere with detection by pattern recognition receptors and IFN induction. The reovirus σ3 protein, the multifunctional influenza virus NS1 protein, and the hepatitis B virus core antigen are all well-characterized dsRNA-binding proteins with anti-IFN effects. The vaccinia virus E3L protein and the herpes simplex virus 1 US11 protein also have binding properties that correlate with inhibition of IFN induction. Adenovirus VA-RNA I acts as a dsRNA decoy and blocks the activation of Pkr by binding to this enzyme directly.

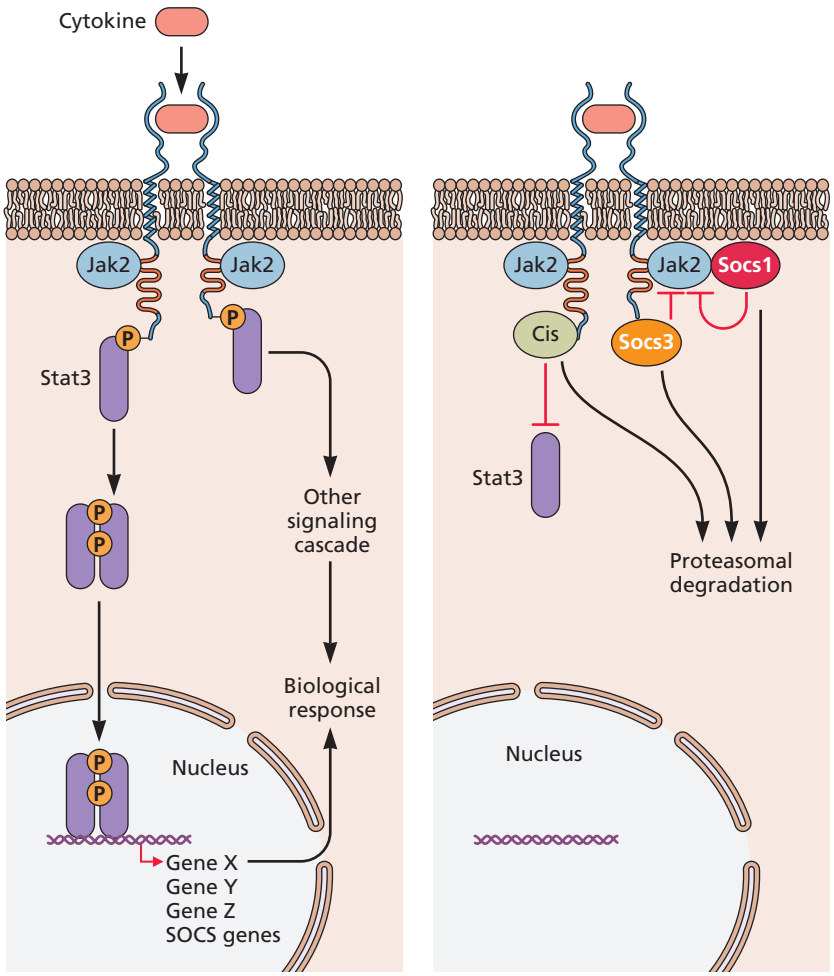
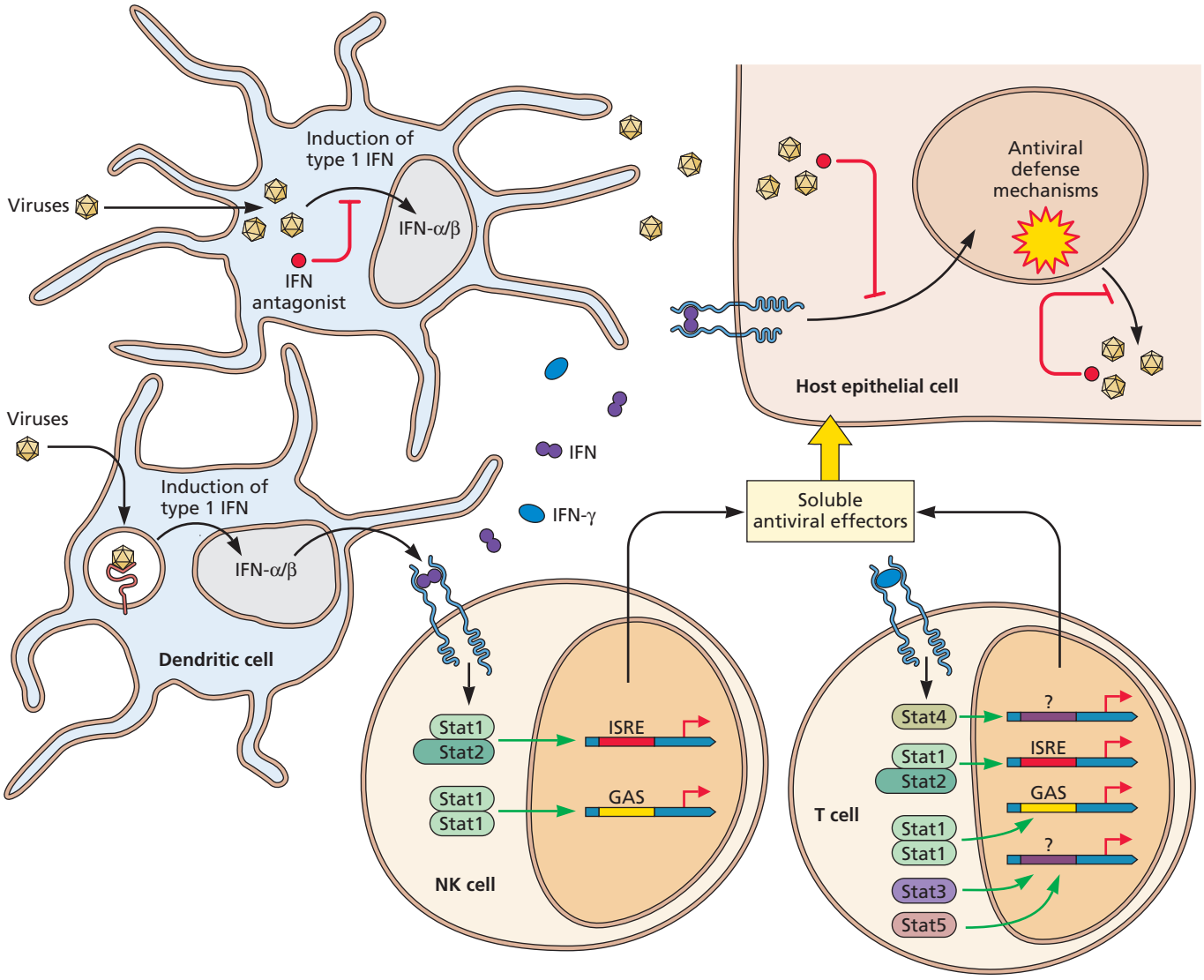


Figure 3.18 Suppressors of cytokine signaling. In unstimulated cells, Socs genes are not expressed. However, when IFN is present, Socs proteins are among the genes induced, which then act in a negative-feedback loop to block signal transduction. Socs1 interacts directly with Jaks and Socs3 inhibits Jaks after gaining access by receptor binding. In addition, Socs proteins interact with the cellular ubiquitination machinery through the Socs box and might direct associated proteins, such as Jaks or receptors, for ubiquitin-mediated proteasomal degradation.

An inescapable inference from the various countermeasures encoded by the genomes of diverse viruses is that IFN is an essential host defense component (Fig. 3.19). But numerous questions remain. For example, infections by some viruses (e.g., Newcastle disease virus) are inhibited only by IFN-α, while others (e.g., herpes simplex virus 1) are inhibited primarily by IFN-β. IFN synthesis is induced after infection by vaccine strains of measles virus, while little IFN is made after wild-type virus infection. Perhaps most fascinating, the IFN response varies depending on the route of infection when animals are inoculated experimentally (Box 2.3). Despite much progress, and elucidation of many details of

Figure 3.19 Virus-mediated modulation of interferon production and action. Viral gene products modulate most steps in the IFN response from the infected cell to the responding cell. Such modulation affects the dynamics of cytokine production and action in ways that are not fully understood. For example, dendritic cells detect viral infection or products of viral infection and produce type I IFN and IFN-γ. However, viral infection may lead to reduction of IFN production in these primary defense cells (red line, IFN antagonist). The IFN produced by dendritic cells can bind to receptors on innate immune cells (e.g., NK cells) or T cells, leading to production of IFNs and other IFN-inducible gene products (indicated by the question mark). The combination of NK cell and T cell action should produce soluble antiviral effectors, leading to destruction of other infected host cells (e.g., epithelial cells). However, viral gene products produced in these infected target cells can impair IFN signaling or block recognition of the infected cell by NK cells or T cells. As a result, virus-infected cells are exposed to a rapidly changing cytokine array, not only by the infected cell, but also by innate and adaptive immune cells reacting to the infection. Adapted from A. García-Sastre and C. A. Biron, *Science* **312:**879–882, 2006, with permission.



IFN biology (for example, how the *IFN-β* gene is turned on and off), it is likely that major principles of IFN synthesis, activity, and regulation remain to be discovered.

Chemokines

Imagine that, somewhere in a large U.S. city, a person has just started a fire while cooking dinner. His efforts to put out the fire, perhaps dousing it with water, may limit the blaze, but these localized efforts on the part of the chef may not be completely successful; the professionals, who offer tools, expertise, and experience, must be called in. But how, in this vast city, are the firefighters alerted? In this example, one would call 911. For the host response, chemokines are the emergency alert, attracting circulating immune cells to the specific site of damage.

The ability of the immune system to respond to the presence of foreign antigens, tissue damage, and other physiological insults depends on chemokine gradients to recruit lymphocytes to the right place and to activate these cells at the right time. Chemokines also coordinate cellular movement in normal processes, including lymphocyte and neural development and new blood cell formation. Chemokines, secreted by local macrophages and some infected cells, bind to G protein-coupled receptors on circulating lymphocytes, inducing signaling pathways involved in cell movement and activation.

A representation of the process by which chemokines aid the migration of a white blood cell from the blood, across the endothelium, and into an affected tissue during an inflammatory response is shown in Fig. 3.20. First, selectins on the endothelium interact with mucin receptors on the leukocyte, causing it to roll along the cell surface, slowing its transit, and enabling it to migrate through the blood vessel. Chemokines bind glucoasminoglycans on the endothelial cell surface to induce production of additional adhesion molecules, including integrins, which further retain lymphocytes near the site of viral infection. The cells then pass from the blood into tissue, squeezing between endothelial cells that comprise blood vessels, following chemokine gradients.

Approximately 50 human chemokines and 20 receptors have been discovered (Table 3.6). Early in the chemokine literature, these molecules and their receptors were given names based on their presumed functions. This quickly became confusing, because the names reflected only some of the actual functions of these molecules. Since 2000, chemokines are named based on the number and location of conserved cysteine residues. There are four families: CXC, CC, C, and CX3C (in which "X/X3" represents one or three noncysteine amino acids). Chemokine ligands are denoted with an "l" (as in CCl2), and their receptors are designated with an "r" (as in CCr2).

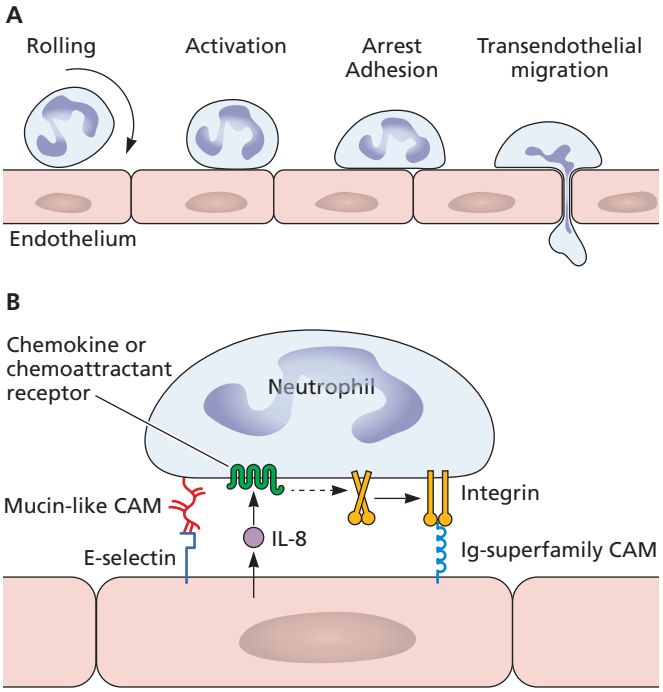


Figure 3.20 Steps in immune cell extravasation into tissues, and the role of chemokines. (A) The sequential steps of lymphocyte migration from the blood into a tissue parenchyma. **(B)** The critical cell adhesion molecules that result in anchoring of a blood cell (here, a neutrophil) to the endothelium. CAM: cell adhesion molecule.

Although chemokines were selected to benefit the host, inappropriate regulation or utilization of these proteins can contribute to or cause many diseases, including autoimmune disorders (e.g., psoriasis, rheumatoid arthritis, and multiple sclerosis), pulmonary diseases (asthma and chronic obstructive pulmonary disease), cancer, and vascular disease, presumably by disrupting cell mobility within the host. In addition, the CXCr4 and CCr5 receptors serve as coreceptors by human immunodeficiency virus type 1 for entry into cells.

Table 3.6 Some chemokine receptors and their ligands[a]

Receptor[b]	Old chemokine ligand name	New chemokine ligand name
CCr1	Mip1α, Rantes	CCl3
CCr2	Mcp1	CCl2
CCr5	Rantes	CCl5
CXCr2	IL-8	CXCl8
CXCr3	Ip-10	CXCl10

[a]Data from C. R. Mackay, *Curr Biol* **7**:R384–R386, 1997.

[b]The four families of chemokine receptors are distinguished by the pattern of cysteine residues near the amino terminus and are abbreviated CXC, CC, C, and CX3C. Only two types are listed in this table. The CXC family has an amino acid between two cysteines; the CC family has none; the C family has only one cysteine; and the CX3C family has three amino acids between two cysteines. Subfamilies of these major four groups also exist.

The Innate Immune Response

The staggering number and diversity of local mechanisms to contain or eliminate viruses underscores the importance of a powerful frontline defense. While this text focuses on viruses, almost all of these mechanisms also operate against other types of microbial challenges.

When intrinsic cell defenses are unable to stop the spread of infection, the combination of cell death, local increasing concentrations of cytokines, and release of other stress-related molecules around the area of infection leads to activation of the next phase of host defense, the innate immune response. We have already introduced some of the critical players in this response: the local **sentinel cells** (dendritic cells and macrophages), which bring peptides derived from viral proteins to the lymph node to induce the adaptive response, and which synthesize soluble, antiviral mediators. These mediators, chemokines and type I IFNs, dampen viral spread, forewarn uninfected cells that are adjacent to infected areas, and are a beacon for the subsequent recruitment of components of the innate and adaptive responses. In addition to these effectors, the innate response also incorporates a large collection of serum proteins termed **complement**, and cytolytic lymphocytes called **natural killer cells (NK cells)**. Neutrophils and other granulocytic white blood cells are also important in innate defense in response to the initial burst of cytokines from dendritic cells, macrophages, and infected cells.

The innate immune response is crucial in antiviral defense because it can be activated quickly, functioning within minutes to hours of infection. Such rapid action contrasts with the activation of the adaptive response, which is far slower than the infectious cycles of some viruses. It takes days to weeks to orchestrate the effective response of antibodies and activated lymphocytes specifically tailored to the infecting virus. While the speed and potency of innate immunity is important, this response must also be transient, because its continued activity is damaging to the host.

Complement

The **complement system** was identified in 1890 as a heat-labile serum component that lysed bacteria in the presence of antibody. The name "complement" derived from the ability of this blood component to cooperate with antibodies and phagocytic cells to clear an infection (Box 3.12). We now know that the complement system comprises many proteins that function in a complex cascade, in which inactive precursors are sequentially triggered, leading to the massive amplification of the response and the activation of the membrane attack complex. More than 30 proteins and protein fragments make up the three distinct pathways in the complement system: the **classical pathway**, **alternative pathway**, and **lectin pathway**. Unfortunately, the nomenclature of the complement proteins can be confusing, as they were named in order of their discovery.

BOX 3.12

DISCUSSION

The complement cascade has four major biological functions

Lysis

Membrane disruption and lysis occur when specific activated complement components (C6, C7, C8, and C9) polymerize on a foreign cell or enveloped virus, forming pores or holes that disrupt the lipid bilayer and compromise its function. The cell or virus is disrupted by osmotic effects.

Activation of Inflammation

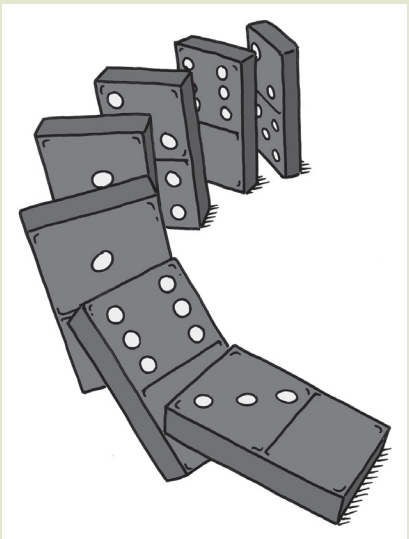
Inflammation is stimulated by several peptide products of complement proteins produced during the complement cascade. These peptides (C3a, C4a, and C5a) bind to vascular endothelial cells and various classes of lymphocytes to stimulate the inflammatory response and to enhance responses to foreign antigens.

Opsonization

Complement proteins (typically C3b and C1q) can bind to virus particles so that phagocytic cells carrying appropriate receptors can then engulf the coated viruses and destroy them; this process is called opsonization. Complement receptors such as Cr1 present on phagocyte surfaces bind C3b-coated particles and initiate their endocytosis.

Solubilization of Immune Complexes

Noncytopathic viral infections commonly result in pathological accumulations of antigen-antibody complexes in lymphoid organs and kidneys. Complement proteins can disrupt these complexes, by binding to both antigen and antibody, and facilitate their clearance from the circulatory system.



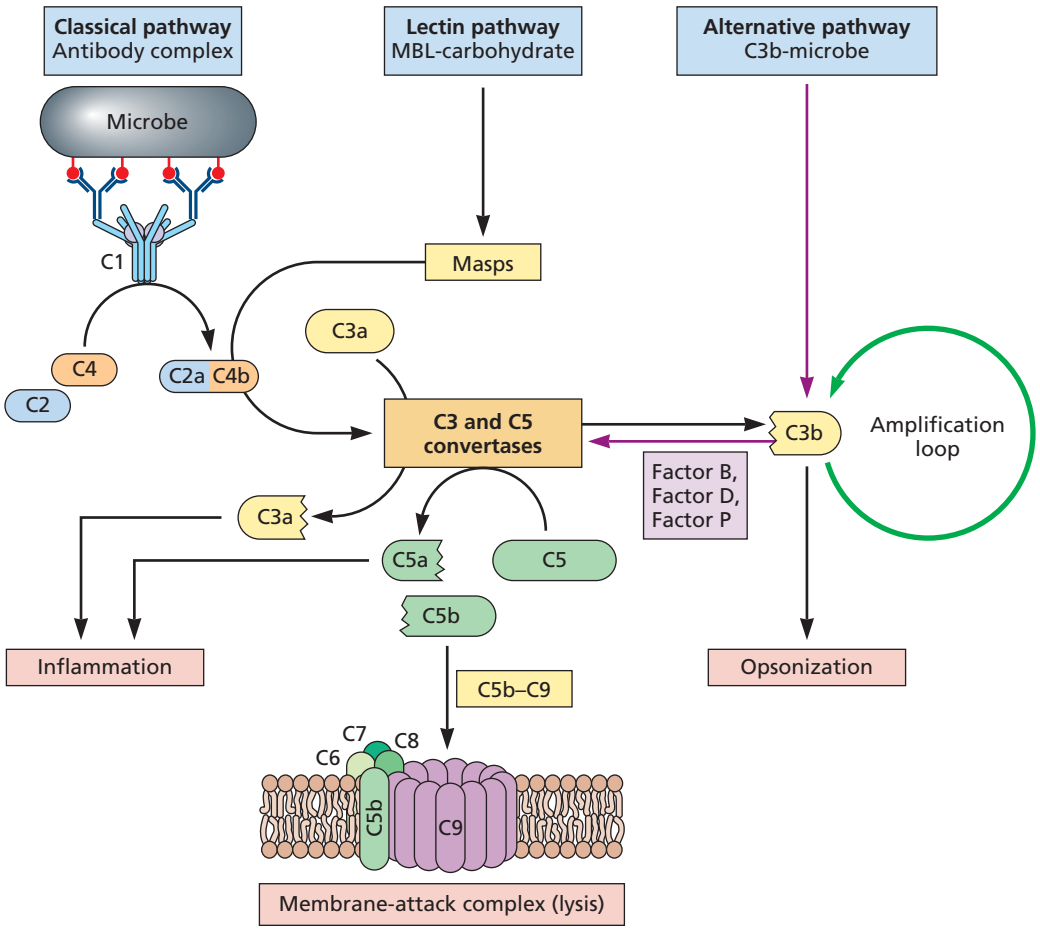
One push triggers an inevitable cascade.

Complement proteins are present in the blood and in various tissues in uncleaved, inactivated forms. Complement action can be initiated by direct recognition of a microbial invader by C1q (a component of C1) in the classical pathway, or by recognition of cleaved C3b proteins in the alternative pathway (Fig. 3.21). The mannan-binding lectin pathway triggers complement action upon binding of a lectin similar to C1q to mannose-containing carbohydrates on bacteria or viruses. Importantly, complement can also function as an effector of the adaptive defense system by the binding of C1q to antigen-antibody complexes on the surface of a microbe or infected cell (the classical pathway).

The Complement Cascade

In all three pathways, a protease cascade leads to the activation of two critical proteases called **C3 convertase** and

Figure 3.21 Activation and regulation of the complement system. The complement system can be activated through three pathways: classical, lectin, and alternative. Complement component 1 (C1) comprises C1q (a pattern recognition protein), C1r, and C1s. The complement cascade is activated when C1 binds an antigen-antibody complex on the surface of an infected cell or a virus particle; C1 also links the classical and lectin activation pathways by interacting with the mannose-binding lectin (MBL)-associated serine protease (Masp). These complexes contain proteases that cleave complement proteins C2 and C4, which then form the C3 and C5 convertases for the classical and lectin pathways. The alternative pathway activates complement without going through the C1-C2-C4 complex. For the alternative pathway, factor B is the C2 equivalent. Factor B is cleaved by factor D. Factor P (properdin) stabilizes the alternative pathway convertases. All three pathways culminate in the formation of the C3 and C5 convertases (orange box), which produce the three primary actions of activated complement: inflammation, cell lysis, and coating of foreign antigens so that they can be taken up by phagocytes (opsonization). The C3a and C5a proteins are potent stimulators of the inflammatory response (also called anaphylatoxins). The membrane attack complex is formed by the complement proteins C5b, C6, C7, C8 and C9 and forms a hole in membranes, leading to lysis of cells. The C3b (opsonin) coats bacteria and virions and also amplifies the alternative pathway. See C. Kemper and J. P. Atkinson, *Nat Rev Immunol* **7**:9–18, 2007.



C5 convertase (note that the three pathways yield the same enzyme activity, but the proteins comprising each convertase are different). A crucial property of C3 and C5 convertase enzymes is that they are bound covalently to the surface of the pathogen or the infected cell. The action of surface-bound C3 convertase on its substrate yields C3b, the primary effector of all three complement pathways, and C3a, a potent soluble mediator of inflammation. C3b remains on the pathogen surface, where it binds more complement components to stimulate a protease cascade that produces other bioactive proteins. The protease cleavage products stimulate inflammation, attract lymphocytes, potentiate the adaptive response, and kill infected cells. C3b also stimulates phagocytic cells to take up the C3b-coated complex.

More than 90% of plasma complement components are made in the liver. Other sites of synthesis include the major portals of pathogen entry. For example, the initiator complex C1 is synthesized mainly in the gut epithelium, and mannan-binding lectin is found in the respiratory tract. In addition, monocytes, macrophages, lymphocytes, fibroblasts, endothelial cells, and cells lining kidney glomeruli or synovial cavities all make most proteins of the complement system.

One important consequence of complement cascade activation is the initiation of a local, broad-spectrum defense. Complement components released locally aid in recruitment of monocytes and neutrophils to the site of infection, stimulate their activities, and increase vascular permeability (Table 3.7). The antiviral effects of complement are both direct and indirect. The membrane attack complex lyses infected cells and inactivates enveloped viruses, while phagocytes engulf and destroy virus particles coated with C3b protein. Complement components stimulate a local inflammatory response that can limit infection, and aid in presenting signals of the invader to the adaptive immune system. The activated complement system "instructs" the humoral and T cell responses much as activated dendritic cells communicate with T cells, and is one of the bridges between frontline early defenses and adaptive immunity.

Table 3.7 Biological activities of proteins and peptides released during the complement cascade

Substance	Biological activity
C5b, C6, C7, C8, and C9	Lytic membrane attack complex
C3a	Peptide mediator of inflammation, smooth-muscle contraction; vascular permeability increase; degranulation of mast cells, eosinophils, and basophils; histamine release; platelet aggregation
C3b	Opsonization of particles and solubilization of immune complexes; facilitation of phagocytosis
C3c	Neutrophil release from bone marrow; leukocyte lysis
C3dg	Molecular adjuvant; profound influence on adaptive response
C4a	Smooth-muscle contraction; vascular permeability increase
C4b	Opsonin for phagocytosis, processing, and clearance of antibody-antigen immune complexes
C5a	Peptide mediator of inflammation, smooth-muscle contraction; vascular permeability increase; degranulation of mast cells, basophils, and eosinophils; histamine release; platelet aggregation; chemotaxis of basophils, eosinophils, neutrophils, and monocytes; hydrolytic enzyme release from neutrophils
Bb	Inhibition of migration and induction of monocyte and macrophage spreading
C1q	Opsonin for phagocytosis, clearance of apoptotic cells, and processing and clearance of antibody-antigen immune complexes

"Natural Antibody" Protects against Infection

The classical complement pathway of humans and higher primates can be activated by a particular collection of antibodies present in serum prior to viral infection (historically called "natural antibody"). Synthesis of some of these antibodies is triggered by the antigen galactose α(1,3)-galactose (α-Gal) found as a terminal sugar on glycosylated cell surface proteins. Lower primates, most other animals, and bacteria synthesize the enzyme galactosyltransferase, which attaches α-Gal to membrane proteins, but humans and higher primates lack the enzyme and do not make this antigen. Because of constant exposure to bacteria producing α-Gal in the gut, human serum contains high levels of antibodies specific for this antigen. Indeed, >2% of the IgM and IgG populations is directed against this sugar. It is this antibody that triggers the complement cascade and subsequent lysis of foreign cells and enveloped viruses bearing α-Gal antigens. The anti-α-Gal antibody-complement reaction is probably the primary reason why humans and higher primates are resistant to infection by enveloped viruses of other animals, despite the ability of many of these viruses to infect human cells efficiently in culture. Consistent with this view, when such viruses are grown in nonhuman cells, they are sensitive to inactivation by human serum. Anti-α-Gal antibodies provide a mechanism for cooperation of the adaptive immune system and the innate complement cascade to provide immediate, "uninstructed" action.

Regulation of the Complement Cascade

Any amplified antiviral defense system as lethal as the complement cascade must be regulated with precision.

Spontaneous activation of any one of the three pathways must be blocked, and triggering by minor infections, nonpathogenic microbes, or noninfectious proteins avoided. Some regulation is intrinsic to the complement proteins themselves. For example, many are large and cannot leave blood vessels to attack infected tissues unless there is localized tissue damage and capillary wall breakdown that exposes cells directly to blood. Consequently, minor infections do not activate a substantial complement response. Moreover, many cascade intermediates do not exist long enough to diffuse far from the site of infection: they are short-lived, with millisecond half-lives. Further control is maintained by complement-inhibitory proteins present in the serum and on the surface of many cells (e.g., the complement receptor type 1 protein [Cr1], decay-accelerating protein [Daf, or CD55], protectin [CD59], and membrane cofactor protein [CD46]). These proteins are regulators that can limit the alternative-pathway cascade by binding to complement components such as C3b and C4b and preventing the accidental deposition of these cascade triggers on host cells. Some viruses have co-opted these molecules to protect themselves from complement-mediated lysis. Human immunodeficiency virus type 1 and the extracellular form of vaccinia virus incorporate CD46, CD55, and CD59 in their envelopes, providing protection from complement-mediated lysis.

Many viral genomes encode proteins that interfere with the complement cascade. For example, alphaherpesvirus glycoprotein C binds the C3b component, and several poxvirus proteins bind C3b and C4. The variola Spice protein (smallpox inhibitor of complement enzymes) inactivates human C3b and C4b and is a major contributor to the high mortality caused by this virus.

Several viral receptors, including those for measles virus and certain picornaviruses, are complement control proteins. Epstein-Barr virus particles bind to CD21 (the Cr2 complement receptor), with profound consequences for the host and virus: this interaction activates the Nf-κb pathway, which then allows transcription from an important viral promoter. Epstein-Barr virus binding to the complement receptor enables viral reproduction in resting B cells that would otherwise be incapable of supporting viral transcription. Moreover, binding of measles virus to the complement regulatory protein CD46 induces an IFN response. As only vaccine strains bind to this receptor, this interaction may be the reason why these measles strains induce a protective response, rather than initiate a pathogenic infection.

Pattern Recognition by C1q, the Collectins, and the Defensins

The action of the complement initiator protein C1q exemplifies a definitive property of intrinsic and innate defense: C1q can recognize molecular patterns characteristic of pathogens, much like the Tlrs. C1q is a calcium-dependent, sugar-binding protein (a **lectin**) in the collectin family of proteins. These proteins bind to polysaccharides on a wide variety of microbes and act as opsonins or activators of the complement cascade. Defensins represent another class of antimicrobial lectins. They are small (29- to 51-residue), cysteine-rich, cationic proteins produced by lymphocytes and epithelial cells that are active against bacteria, fungi, and enveloped viruses. Collectins and defensins bind the glycoproteins of a number of enveloped viruses, including human immunodeficiency virus type 1, herpes simplex viruses, Sindbis virus, and influenza virus. The antiviral activity of some collectins and defensins can be observed with cells in culture. The basis for such activity appears to be inhibition of membrane fusion. An attractive hypothesis is that they function by cross-linking viral glycoproteins and blocking displacement of other proteins from the fusion site. While these lectins display antiviral activity in the laboratory, their physiological contributions have not been well studied. Some have been modified for testing as antiviral compounds to be delivered systemically or topically.

It is useful to reiterate the multiple parts that certain immune proteins play in host defense. Complement can be considered a component of the intrinsic, innate, or adaptive response. Some complement proteins are pattern recognition receptors, others need to be activated by upstream signals, and still others can distinguish self from nonself.

Natural Killer Cells

Natural killer cells are at the front line of innate defense: they are ready to recognize and kill some virus-infected cells, and do not need selection or stimulation to do so. Like T cells, NK cells can distinguish infected cells amidst vast numbers of uninfected cells. However, the mechanism of recognition is completely different: NK cells recognize "missing self." We previously introduced the concept that humans recognize that something is different in their environment based on their recollection of what was there before. For NK cells, "different" means an **absence** of something familiar.

NK cells are abundant, representing ~2% of circulating lymphocytes. They are large, granular cells, distinguished by the absence of the antigen receptors found on B and T cells (Chapter 4). When an NK cell binds an infected target cell, it releases a mix of cytokines (notably IFN-γ and Tnf-α) that contribute to a local inflammatory response and alert cells of the adaptive immune system. They also can produce prodigious quantities of IL-4 and IL-13, the major cytokines that stimulate antibody production. In addition, NK cells

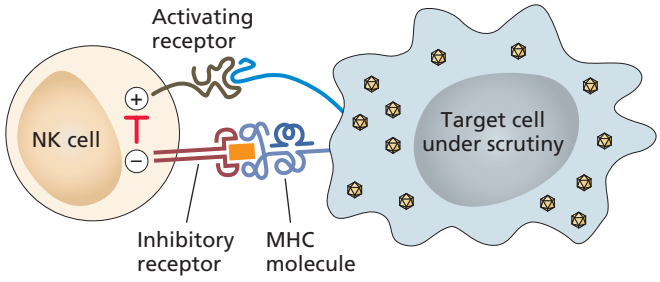
participate later in adaptive defense by binding to infected cells coated with IgG antibody and inducing antibody-dependent cell-mediated cytotoxicity.

The number of NK cells increases quickly after viral infection and then declines as the adaptive immune response is educated and amplified. NK cells are stimulated to divide whenever infected cells and sentinel dendritic cells make IFN. The NK cells kill after contact with the target by releasing perforins and granzymes that perforate membranes and trigger caspase-mediated cell death, respectively, in a process identical to how cytotoxic T lymphocytes kill their targets. In humans, NK cells are particularly important in controlling primary infection by many herpesviruses, as patients with NK cell deficiencies suffer from severe infections with varicella-zoster virus, human cytomegalovirus, and herpes simplex viruses. While a role for direct NK cell-mediated killing in antiviral defense is difficult to establish experimentally, NK cell production of IFN-γ clearly provides significant antiviral action.

NK Cell Recognition of Infected Cells: Detection of "Missing Self" or "Altered Self" Signals

A collection of cell surface proteins called the MHC proteins are important receptors in the adaptive immune response (Chapter 4). MHC class I proteins are found on the surfaces of most cells of the body and "present" microbial peptides to T cells. The MHC class I molecules are the self antigens that, when missing, cause the NK cell to kill the target cell. A mechanism for detection of missing self is illustrated in Fig. 3.22. At least two receptor-binding interactions that cooperate to send either a "go" or "stop" signal to the prearmed NK cell are required for such discrimination.

Figure 3.22 NK cells distinguish normal, healthy target cells by a two-receptor mechanism. Both positive (stimulating) and negative (inhibiting) signals may be received when an NK cell contacts a target cell. The converging signal transduction cascades from the two classes of receptor regulate NK cell cytotoxicity and release of cytokines. The inhibitory receptors dominate all interactions with normal, healthy cells. Their ligands are the MHC class I proteins. When NK cells contact MHC class I molecules on the surface of the target cell, signal transduction blocks the response of activating receptors.



The "go" signal is delivered when an NK activating receptor binds a pathogen-specific ligand (e.g., virus-infected cells may present new glycoproteins on their surface). As a consequence, a signal transduction cascade is initiated and the NK cell is stimulated to secrete cytokines and kill the cell. However, a dominant-negative regulatory signal is produced when an inhibitory receptor on the NK cell engages MHC class I molecules on the surface of the same target cell. Because many infected cells carry fewer MHC class I molecules on their surfaces (Chapter 5), they are prime NK cell targets. In essence, NK cells serve a counter-counter-response to those viruses that downregulate MHC molecules on the infected cell surface to escape T cell detection. The two-receptor recognition system employed by NK cells ensures that normal cells that synthesize MHC class I proteins, even those that may be virus infected, are not killed by NK cells.

MHC Class I Receptors on NK Cells Produce Inhibitory Signals

Human NK cells synthesize two inhibitory MHC class I receptors of either the C-type lectin family or the immunoglobulin family (called killer cell immunoglobulin-like inhibitory receptors, or Kirs). NK cells also can recognize and spare target cells carrying HLA-E, an unusual MHC class I protein that binds peptides derived from the signal sequences of other MHC class I molecules. The presence of HLA-E protein bound to signal peptide informs the NK cell that MHC class I synthesis is normal. An intriguing finding is that infection by human cytomegalovirus induces synthesis of HLA-E protein, thereby escaping potential NK cell recognition and lysis.

Viral Proteins Modulate NK Cell Actions

Many viral genomes encode proteins that block or confound NK cell recognition and killing (Fig. 3.23). At least five distinct categories of modulation can be described. NK modulators have been identified in the genomes of several virus families including *Flaviviridae*, *Papillomaviridae*, *Herpesviridae*, *Retroviridae*, and *Poxviridae*. Some viral genomes encode more than one distinct NK modulator. For example, human cytomegalovirus encodes at least seven such gene products that modulate the NK cell response. One striking example of viral interference with NK cell activity is provided by the hepatitis C virus E2 envelope protein, which binds to CD81, a protein on the surface of NK cells, and blocks activation signals. As a result, the NK cell no longer recognizes infected cells.

NK Cell Memory

NK cells may have a "memory" state, a property normally thought to be unique to cells of the adaptive immune

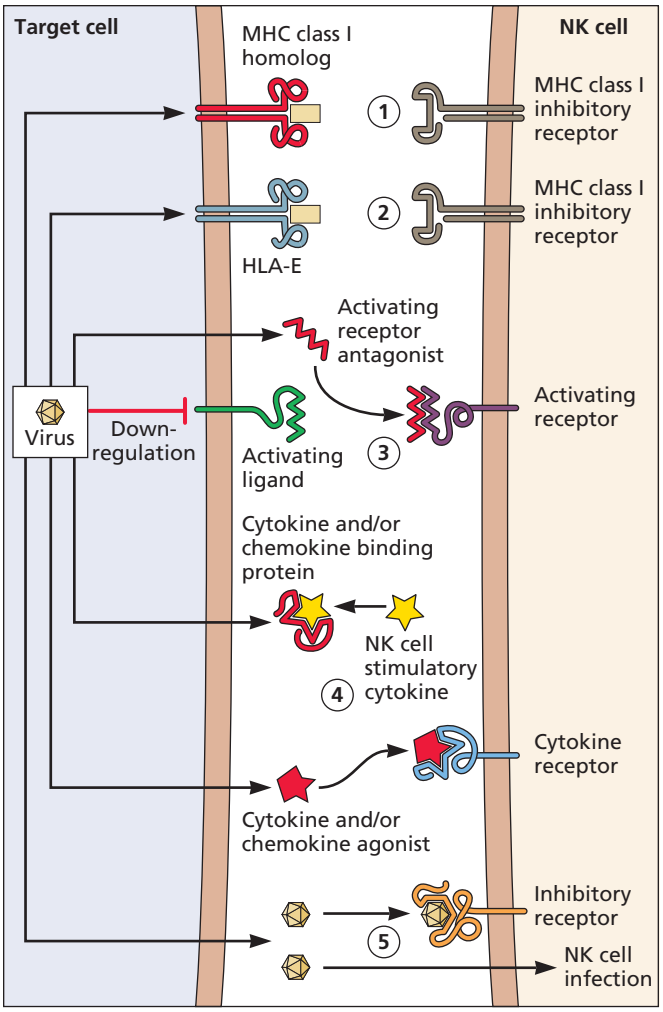


Figure 3.23 Virus-encoded mechanisms for modulation of NK cell activity. (Left) An infected target cell. (Right) An NK cell. The infected target cell should be lysed by an activated NK cell. However, five categories of NK cell-modulating strategies are illustrated (circled numbers). Viral proteins produced in the infected cell are labeled in red. (1) Inhibition by a viral protein with homology to cellular MHC class I proteins. (2) Inhibition of expression or cell surface localization of host HLA-A or HLA-B (human MHC class I homologs) resulting in an increase in the amount of host HLA-E (or HLA-C) on the target cell surface. (3) Release of virus-encoded cytokine-binding proteins that block the action of NK cell-activating cytokines (also, viral proteins can reduce the amount of the activating ligand on the surface of the infected cell so that the NK cell is not activated). (4) Inhibition of action of NK cell-stimulating cytokines by binding these cytokines or by producing a chemokine antagonist. (5) Effect of newly produced virus particles, which can engage the NK cell, block an inhibitory NK cell receptor, or infect the NK cell itself to disrupt various effector functions or even kill the cell.

response. Exposure to activating cytokines, such as IL-12 and IL-18, elicits a form of memory in splenic NK cells whereby the primed cells mediate enhanced IFN-γ responses after restimulation by cytokines or by antibody-mediated ligation of activating receptors. Memory NK cells are found in the liver, where this memory pool can access the circulation and maintain immune surveillance at a low but constant level. When the same antigen is encountered in the periphery, antigen-specific NK cells then accumulate at the site of challenge, where they orchestrate local effector responses. Moreover, most NK cells can acquire certain memory-like properties even without exposure to a specific antigen, similar to the cytokine-driven, antigen-independent "bystander" response of $CD8^+$ T cells (Chapter 4).

Other Innate Immune Cells of Relevance to Viral Infections

While the main cellular actors that govern the outcome of a viral infection are T cells, B cells, and NK cells, the heterogeneity of blood cells and the presence of multiple "minor players" at sites of infection indicate that other blood-borne cells likely also contribute to antiviral immunity.

Neutrophils

Neutrophils are, by far, the most abundant type of cell in the blood, comprising >50% of the circulating white blood cells. These cells also produce soluble mediators, such as cytokines, reactive oxygen species, and perforating granules. Neutrophils participate primarily in the resolution of bacterial infections, in part as a result of the release of nets that capture extracellular bacteria, much like a spiderweb. These neutrophil extracellular networks (NETs), comprising DNA decorated with cellular histones, are highly charged, making them "sticky" (Fig. 3.24). But such innovative strategies

Figure 3.24 Neutrophils produce a "net" to capture extracellular pathogens. In this image, a *Klebsiella pneumoniae* bacterium is captured in the extracellular chromatin net produced by neutrophils within the lung. Credit: Science Photo Library.



would presumably not work for viruses, which spend much of their life cycle within cells, or would likely be too small to be ensnared by these mesh-like DNA structures derived from decondensed chromatin DNA within the cell. It had therefore been a puzzle why neutrophils are found at sites of viral infections.

A study using the poxvirus vaccinia virus showed that these same structures may, in fact, have antiviral properties. Following infection, NETs within the liver microvasculature were found to significantly reduce the number of infected host cells: a direct role for the NETs was shown using DNase treatment (destroying the NETs, but not the neutrophils), which abrogated their protective effect. Other studies indicate that such sticky solutions may be operative for other viruses as well, including human immunodeficiency virus type 1 and influenza virus. At present, we understand little about this remarkable defense mechanism.

NKT Cells

NKT cells are so named because they share features of both NK and T cells. They make an αβ T cell receptor, but also possess cell surface markers that are found primarily on NK cells. These cells are rare, constituting only 0.1% of peripheral blood T cells. This T cell receptor recognizes the nonpolymorphic molecule CD1d, an antigen-presenting molecule that binds to lipids and glycolipids. This interaction may be important for recognizing infections by bacteria that have glycolipids on their surfaces, but a contribution of these cells in viral resolution has yet to be defined.

γδ Cells

As we will discuss in Chapter 4, conventional T cells are characterized by the dimeric T cell receptor, which comprises α and β chains. γδ cells possess many of the same T cell markers, but have a distinct receptor, called γδ to distinguish them from their more abundant and well-characterized cousins. These T cells are highly prevalent in the gut mucosa: a clue that they may be critical for early recognition of invading microbes. Conventional T cells recognize peptides in the context of class I MHC determinants, but γδ T cells do not, although some recognize class Ib MHC molecules. It is thought that these cells are particularly suited to bind to lipid (as opposed to protein-based) antigens.

These unconventional cells lie at the intersection of the innate and adaptive response. They can be considered adaptive, in that they rearrange their T cell receptors and establish memory, but much like NK cells, they do not recognize processed antigen nor need extensive education and amplification to be functionally active. Their specific contributions to antiviral immunity have not yet been explored.

Perspectives

This chapter began with some warnings: the immune response is elaborate, and defies our efforts to order neatly where each effector process "belongs" in the overall response. From an evolutionary perspective, one must marvel at the number and diversity of ways in which our body's defenses continually try to keep us safe from pathogens. Intrinsic and innate defenses are always on high alert: unlike the cells of the adaptive response, which sit patiently in the spleen or lymph node waiting for their cognate antigen to appear, these defenses are constitutively surveying all possible portals of microbial invasion.

To illuminate important principles in this chapter, it is useful to consider a hypothetical acute viral infection that is cleared by the host response (Fig. 3.25). To initiate the primary infection, physical barriers are breached and virus particles enter permissive cells. Almost immediately, viral proteins and viral nucleic acids are bound by pattern recognition receptors. Signal transduction cascades then result in the activation of transcriptional regulators that drive the production of cytokines, such as IFN. As new viral proteins are produced, the cell initiates other intrinsic defenses, such as apoptosis or autophagy. Local sentinel cells (the immature dendritic cells and macrophages) respond to the locally released cytokines and internalize viral proteins produced by infected cells. The first response of the immature dendritic cell is to produce massive quantities of IFN and other cytokines. If viral anti-IFN or antiapoptotic gene products are made, progeny virus particles are released. If the newly infected cells have already bound IFN, protein synthesis is inhibited when viral nucleic acid is produced. Soon thereafter, NK cells recognize the infected cells because of new surface antigens and a low or aberrant display of MHC class I proteins. The IFN produced by infected cells stimulates the NK cells to intensify their activities, which include target cell destruction and synthesis of IFN-γ. In some cases, serum complement can be activated to destroy enveloped viruses and infected cells. In general, the intrinsic and innate defenses bring most viral infections to an uneventful close before the adaptive response is required. Even if all these responses prove insufficient, the immune response still has one powerful trick up its sleeve, as we shall see in the next chapter.

One cannot help but be impressed by such a swift, diverse, coordinated, integrated response. But bear in mind this central fact: no matter how adept our host defense may be at detecting and neutralizing viruses, all successful viruses encode gene products that frustrate their host's defenses, and many cause disease (Chapter 5). In these instances, the struggle has barely begun.

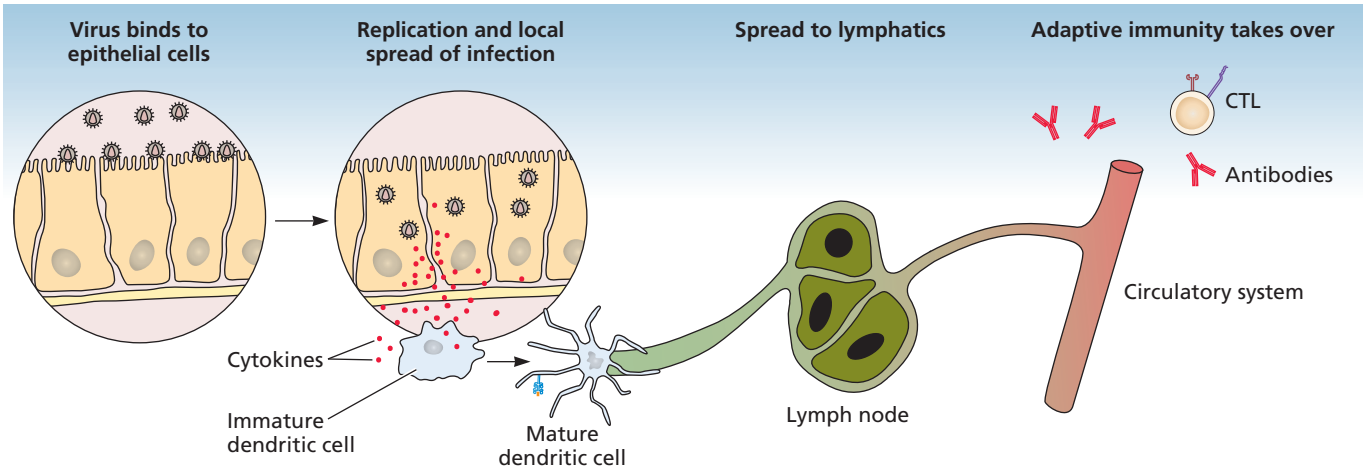


Figure 3.25 Critical events in a hypothetical acute virus infection. CTL, cytotoxic T lymphocyte.

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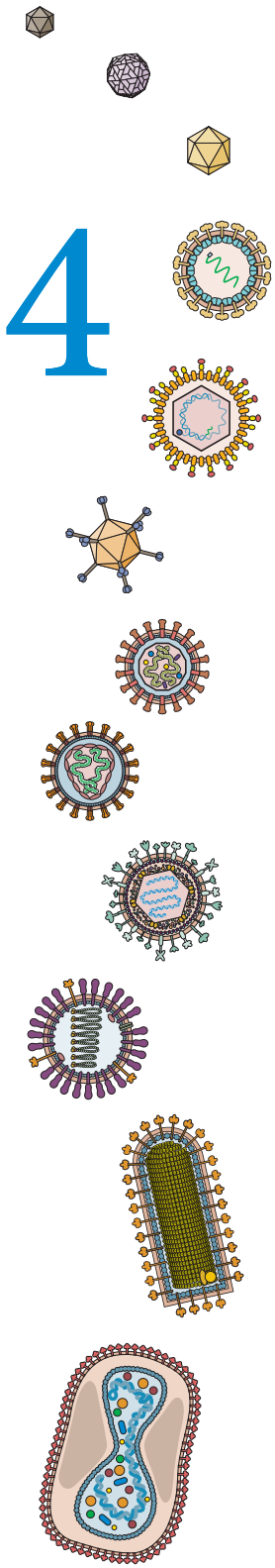
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4 Adaptive Immunity and the Establishment of Memory

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- Speed
- Diversity and Specificity
- Memory
- Self-Control

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- The Two Arms of Adaptive Immunity
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Perspectives

References

LINKS FOR CHAPTER 4

- ⏭ *Video: Interview with Dr. Peter Doherty*
  http://bit.ly/Virology_Doherty
- ⏭ *More than one way to skin a virus*
  http://bit.ly/Virology_Twiv175
- ⏭ *Concerto in B*
  http://bit.ly/Virology_Twiv161
- ⏭ *How ZMapp antibodies bind to Ebola virus*
  http://bit.ly/Virology_11-25-14
- ⏭ *Viruses might provide mucosal immunity*
  http://bit.ly/Virology_7-2-13

Life is simple, but we insist on making it complicated.
CONFUCIUS

Introduction

There are some who find the study of immunology confounding. It can be dizzying to try to comprehend the many types of T cells, markers, cytokines, and signaling pathways, especially for a student new to the field. For example, immunologists often identify particular immune cell subtypes on the basis of the presence or absence of a panel of proteins. A regulatory T cell may therefore be referred to as "$CD4^+/CD8^-/CD25^+/Foxp3^+$." Such lists of protein markers can be perplexing, but they are simply a means to distinguish one cell population from another, much as humans are distinguished by traits such as hair color, height, and voice. Compounding the challenge of mastering the jargon of immunology are these additional attributes: the immune response is dynamic and dependent on diverse cell-cell interactions, the tissues and cells that produce the host response are scattered throughout the body, and many lymphocytes can morph from one functional state to another during their lifetimes. But this complexity is also one of the most fascinating aspects of immunology: that so many diverse, potent, interacting, and overlapping strategies are in place to thwart pathogenic encounters underscores the importance of a formidable host defense. As many students of immunology appreciate, the more one knows, the more amazing the immune system seems.

In this text, we have divided our discussion of the antiviral response into distinct chapters: the physical barriers to infection (Chapter 2), the cell-intrinsic and innate immunity (Chapter 3), and, in this chapter, adaptive immunity. These distinctions, however, are not meant to imply that the immune response happens in discrete temporal phases. Indeed, interferons are present and functional throughout the host response, memory T and B cells may be called into play soon after exposure to a pathogen, and neutrophils are prevalent and active at the sites of infection during the peak of the adaptive response. Nevertheless, the ways by which the innate and adaptive arms of host defense recognize and control a virus infection are quite distinct. The innate immune system, which recognizes pathogen-specific properties, provides critical frontline control to limit viral spread, but it is the adaptive response that executes the highly specific assault on virus particles and infected cells. This system is called "adaptive" because it not only differentiates between infected and uninfected cells, but also is tailored to the particular microbe or antigen. Understanding how such precision is achieved was among the most important advances in immunology, and defining which viral proteins are important for eliciting this response remains an essential step in modern vaccine design.

Attributes of the Host Response

Speed

The interval between viral infection and immune-mediated resolution defines the window during which disease may occur. (Exceptions are those virus infections that result in immunopathology, in which case the host response itself is the cause of tissue damage; see Chapter 5). The consequences of infection and the development of immunity are often described as a race against the clock: the virus replication rate, yield, and distribution in the host are pitted against the efficiency of detection and clearance by host defenses. While elements of

PRINCIPLES ***Adaptive immunity and the establishment of memory***

- The adaptive response is characterized by speed, antigen specificity, memory, and self control.
- The degradation of "foreign" proteins (e.g., viral proteins) by professional antigen presenting cells such as dendritic cells, and their presentation to naive lymphocytes, are the critical steps that bridge the innate and the adaptive responses.
- Activation of tissue-resident dendritic cells causes them to leave the site of infection and migrate to lymphoid tissues, where naïve T and B cells are found.
- The cell-mediated response (chiefly, T cells) facilitates recovery from a viral infection primarily because it eliminates virus-infected cells without damaging uninfected cells.
- The humoral immune response (chiefly, antibodies produced by B cells) contributes to antiviral defense by binding to, and causing the elimination of, free virus particles.
- Viral peptides can be presented on the cell surface in the groove of either a class I or a class II major histocompatibility complex protein. Class I proteins present internally synthesized antigens; class II MHC proteins present antigens that were phagocytosed.
- Two primary types of T cells exist: $CD4^+$ T cells and $CD8^+$ T cells.
- $CD4^+$ T cells interact with MHC class II-expressing cells (including professional antigen-presenting cells and B cells), and synthesize cytokines and growth factors that stimulate ("help") the specific classes of lymphocytes with which they interact.
- $CD8^+$ T cells, also called cytotoxic T lymphocytes (CTLs), interact with antigen presented in the context of MHC class I proteins; when productively engaged, CTLs can destroy the cell presenting the peptides.
- Once a specific adaptive response has been established and the viral infection is resolved, the individual is immune to subsequent infection by the same pathogen; this is the core principle of vaccination.

intrinsic and innate immunity usually keep the virus in check during the critical early days following infection, the subsequent massive clonal expansion of antigen-specific T and B cells is often the fatal blow to a virus infection: individuals with mutations that affect T or B cell function fare poorly following most infections. The conversion of a small number of quiescent, naive lymphocytes into a mob of activated, cytokine-producing effector cells over such a short period is a marvel of cell biology. For example, $CD8^+$ T cells specific for the lymphocytic choriomeningitis virus divide as many as 20 times following infection of the host, resulting in up to a 50,000-fold increase in total number in just a few days.

Rapid induction of the adaptive response is also facilitated by the presence of lymph nodes throughout the body. These "immunological chat rooms" are the sites at which antigen-presenting cells, transported in the lymph, encounter naive lymphocytes that circulate in the blood. Lymph nodes are located strategically near areas of the body that are sites of virus entry, including the respiratory and gastrointestinal tracts, thereby minimizing the distance an antigen-presenting cell must travel from the tissue. The intersection between lymphatic circulation and the blood that occurs in the lymph node increases the probability that a dendritic cell that presents viral antigens will find the appropriate naive T lymphocytes, and, as a consequence, accelerates the activation and amplification of antigen-specific T and B cells.

Diversity and Specificity

Naive T cells specific for every possible pathogen circulate in all humans: while your chances of contracting Ebola virus are vanishingly small, rest assured that there are naive T cells capable of recognizing Ebola virus antigens circulating in you now, at the ready. Most of these naive cells will never encounter their cognate antigen, and consequently their numbers will never be increased. But the process of generating this astounding diversity is one of the more interesting properties of immune cell development. T and B cells possess receptors on their surface that can recognize small portions of a viral protein (or three-dimensional facets of a protein) termed **epitopes**. Receptor diversity accounts for the capacity of these cells to recognize and respond to virtually any pathogen: for example, it has been estimated that there are >20 million distinct T cell specificities. As T and B cell receptors are encoded by host genes (of which humans have only ~30,000), it is not possible that each of the millions of T and B cell receptors are encoded by a discrete gene. Rather, random, somatic rearrangements of a limited number of segments of lymphocyte receptor genes create many putative receptors that then pass through a process of quality control before release into the circulation. An interesting consequence of this stochastic process is that everyone's T and B cell repertoire is distinct, even among closely related individuals. The abundance and diversity of the immune repertoire may explain why otherwise healthy individuals may respond differently to an encounter with the same pathogen.

Memory

Once T and B cells have become activated by interaction with a cognate antigen, a small number are retained as memory cells, equivalent to an idling car stopped at a red light. While these cells do not produce the effector functions of the majority of the activated cells, they are poised to reenter the cell cycle rapidly and to expand clonally immediately upon reexposure to their cognate antigen (the "green light"). At the next encounter with the virus, preservation of memory cells dramatically skews the immune response race in favor of the host: while the initial conversion of naive to activated cells requires multiple steps and a few days, the amplification of memory cells begins almost immediately. Consequently, subsequent infections with the same agent are met with a robust and highly specific defense that usually stops the infection as soon as it starts, with minimal reliance on the innate response. As discussed later in this chapter, and in Chapter 8, this property is the basis of vaccination.

Self-Control

Mounting an immune defense results in the production of large quantities of cytokines and expansion of immune cells that can make the host quite ill: most of the unpleasant symptoms of infections (fever, muscle aches) result from the host response (e.g., fever-inducing cytokines) rather than from the virus itself. Therefore, once the pathogen has been vanquished, this response must be blunted quickly to avoid further risk to the host. Processes that are intrinsic to activated lymphocytes ensure their demise within a short period after activation: the life of an activated immune cell is exciting, but brief. The need to dampen an activated immune response has been explored in detail only recently, but it is clear from these studies that turning off the antiviral cascade is as important as turning it on.

Lymphocyte Development, Diversity, and Activation

Some knowledge of developmental immunology will be useful as we explore how lymphocytes recognize antigens presented by infected cells or by professional antigen-presenting cells (Box 4.1). In this section, we introduce the developmental origins of the cells in the blood, describe how lymphocyte receptor diversity is generated, and introduce the two major players in the adaptive response: T cells and B cells.

All Blood Cells Derive from a Common Hematopoietic Stem Cell

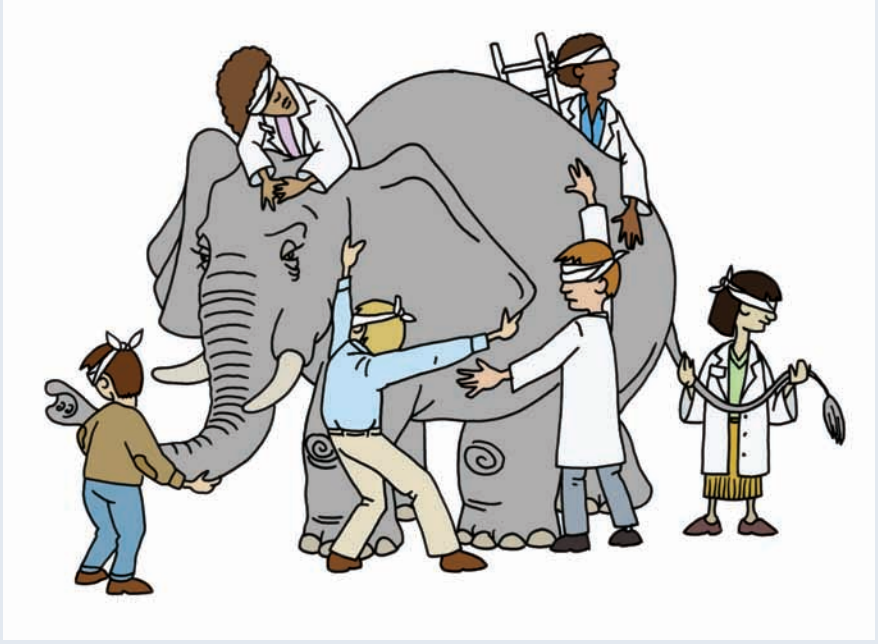
All cells in the blood are derived from a common lineage, originating with a multipotential hematopoietic stem cell

BOX 4.1

TERMINOLOGY

Pathogens, antigens, and epitopes

T and B lymphocytes do not recognize complete virus particles, but rather small, linear pieces of a viral protein (generally true for T cells) or three-dimensional facets of folded viral proteins (generally true for B cells and antibodies). However, the terminology to distinguish what these lymphocytes recognize can be confusing. A **pathogen** is a microbe, such as a virus particle, that can cause disease (hence, "pathology"). Not all microbes are pathogens: the normal gut flora includes many types of bacteria, but in most cases, these microbes do not make the host sick or they contribute positively to the host's welfare. Proteins made by pathogens that are capable of inducing a host immune response are called **antigens**. The term was originally derived from "antibody-generating" proteins, although antigens can also be bound by T cells. Moreover, antigens can be DNA, polysaccharides, or lipids. An **epitope** is the portion of the antigen that is bound by an antibody or that is recognized by a T cell receptor. Consequently, a protein antigen (for example, the measles virus hemagglutinin protein) may have multiple epitopes to which different T cells and antibodies can bind.



that differentiates into two discrete progenitor populations, the common myeloid stem cell and the common lymphoid stem cell. These two precursors subsequently give rise to all blood cell types (Fig. 4.1). The parental hematopoietic stem cells reside in the bone marrow and are self-renewing. Lymphocyte differentiation is marked by an orchestrated loss of stem cell-specific proteins and a concomitant acquisition of those that are characteristic of fully differentiated leukocytes (Box 4.2). Although new immune cells are generated throughout life, the rate of production declines with age, a property that is generally considered a major contributor to the greater vulnerability of the elderly to infection. Given the common ancestry of all blood cells, the process of distinguishing among them was a notable challenge for early immunologists. The advent of flow cytometry, combined with the development of fluorescent antibody reagents specific for leukocyte signature proteins, were crucial technical accomplishments that enabled immunologists to define the functional contributions of the diverse family of blood cells.

The Two Arms of Adaptive Immunity

The adaptive response comprises two complementary actions, the **humoral response** (B cells and the antibodies they produce) and the **cell-mediated response** (helper and effector T cells) (Fig. 4.2). As we discuss the features of these lymphocytes and the processes that characterize each component, it is important to understand that **both** are essential in antiviral defense, and they function in concert. In general, antibodies bind and inactivate virus particles in the bloodstream and at mucosal surfaces, whereas T cells recognize and kill infected cells, the "factories" that generate new virus particles. The relative contribution of each in any given infection varies with the nature of the virus, as well as with host parameters including age, organs infected, and previous immunological exposures.

The Major Effectors of the Adaptive Response: B Cells and T Cells

B Cells

B cells develop in the bone marrow. As they mature, each synthesizes an antigen receptor, which is a membrane-bound antibody. Antigen binding in a specific manner to a membrane-bound antibody on an immature B cell initiates a signal transduction cascade. As a consequence, new gene products are made and the cell begins to divide rapidly. The daughter cells produced by each division differentiate into effector **plasma cells** and a small number of memory B cells.

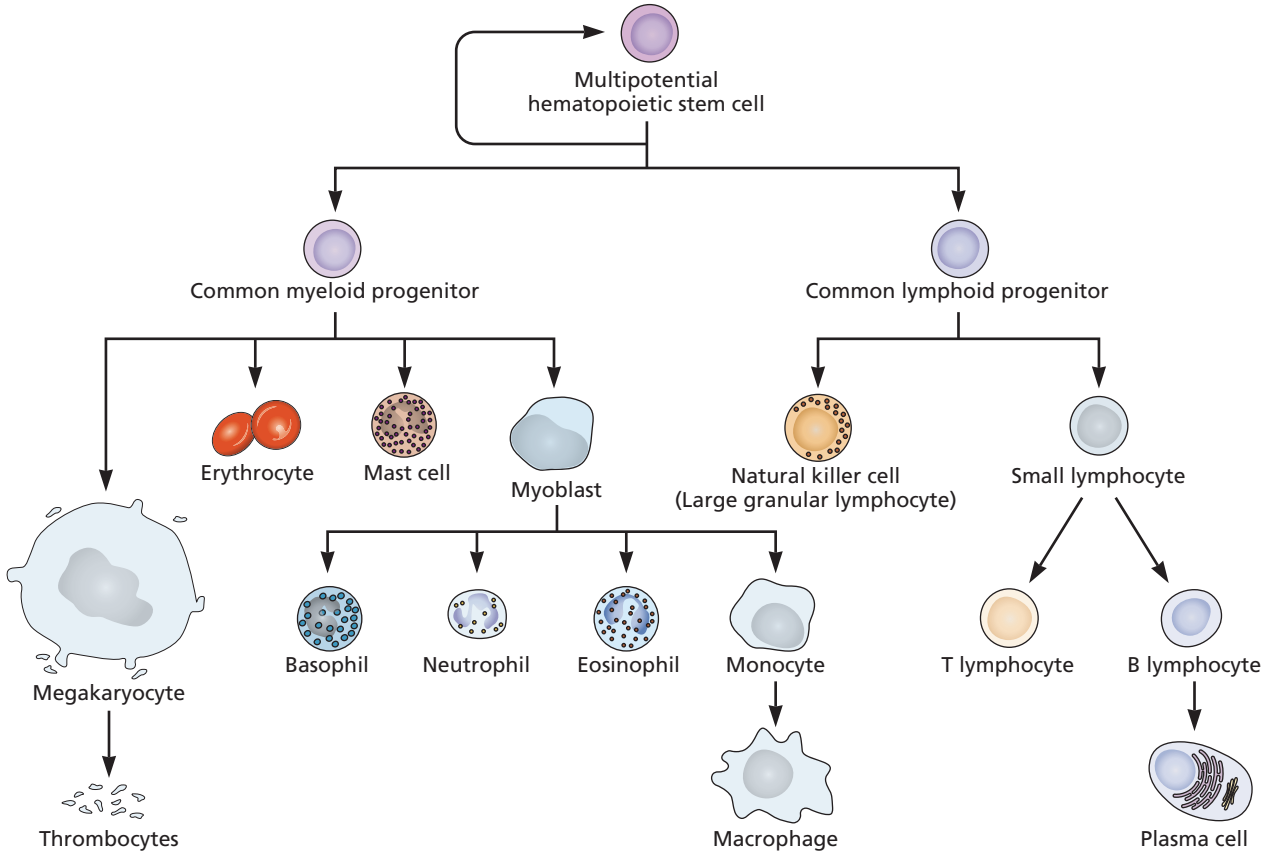


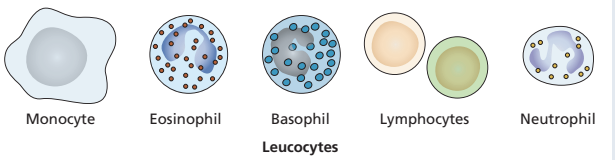
Figure 4.1 Development of leukocytes from a common stem cell precursor. All cells within the blood are derived from a common stem cell precursor, termed the multipotential hematopoietic stem cell. This self-renewing cell population, which exists in the bone marrow, generates two additional precursors. The common myeloid progenitor can differentiate further into red blood cells (erythrocytes), mast cells, and myeloblasts, which give rise to basophils, neutrophils, eosinophils, and monocytes. The common lymphoid progenitor differentiates into natural killer cells, T lymphocytes, and B lymphocytes.

BOX 4.2

TERMINOLOGY

Leukocytes and lymphocytes

While these names sound similar, lymphocytes and leukocytes are not synonymous. "Leukocyte" is a general term for a white blood cell, and includes lymphocytes, neutrophils, eosinophils, and macrophages. Lymphocytes are a subset of leukocytes, specifically T and B cells and NK cells, that possess variable antigen-detecting cell surface receptors (the T cell receptor and the B cell receptor).



As their name implies, memory B cells, and their clonal progeny, are long-lived and continue to produce the parental, membrane-bound antibody receptor. In contrast, plasma cells live for only a few days and no longer make membrane-bound antibody, but instead synthesize the same antibody in secreted form. These antibodies can bind and inactivate extracellular pathogens, including virus particles. A single plasma cell can secrete >2,000 antibody molecules per second. Like dendritic cells, the B cell is an antigen-presenting cell that uses the major histocompatibility complex (MHC) class II system and exogenous antigen processing.

T Cells

T cell precursors are also produced in the bone marrow, but in contrast to those of B cells, a T cell precursor must migrate to the thymus gland to mature; hence the "T" in "T cell." The thymus gland is located in the thoracic cavity, above the heart. Subsets of T cells have distinct functions.

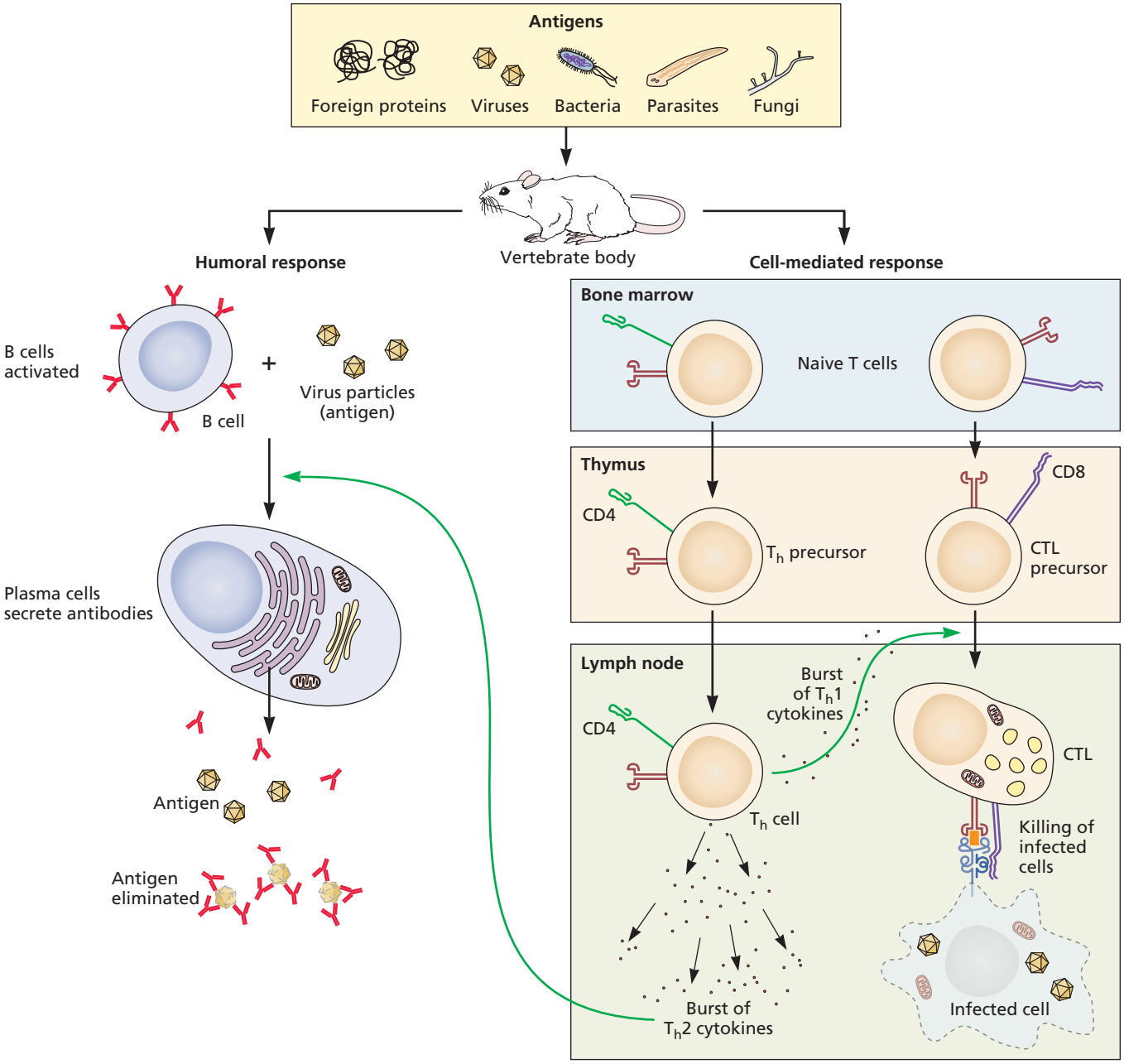


Figure 4.2 The humoral and cell-mediated branches of the adaptive immune system. A variety of foreign proteins and particles (antigens) may stimulate adaptive immune responses after recognition by intrinsic and innate defense systems. (Left) The humoral branch comprises lymphocytes of the B cell lineage, which produce antibodies, the important effector molecules of this response. The process begins with the interaction of a specific receptor on precursor B lymphocytes with an antigen. Binding of antigen promotes differentiation into antibody-secreting cells (plasma cells). (Right) The cell-mediated branch comprises lymphocytes of the T cell lineage that arise in the bone marrow and are differentiated further in the thymus. The activation process is initiated in lymph nodes when the T cell receptor on the surface of naive T lymphocytes binds viral peptides that are in a complex with MHC class II protein on the surface of dendritic cells or B cells. Two subpopulations of naive T cells are illustrated: the T helper (T_h)-cell precursor and the cytotoxic T lymphocyte (CTL) precursor. The T_h cell recognizes antigens bound to MHC class II molecules and produces cytokines that "help" activated B cells to differentiate into antibody-producing plasma cells (T_h2 cytokines), or CTL precursors (T_h1 cytokines) to differentiate into CTLs capable of recognizing and killing virus-infected cells. The T_h1 or T_h2 cytokines are produced by different subsets of T_h cells and promote or inhibit cell division and gene activity of B cell or CTL precursors.

The T cell receptor is a disulfide-linked heterodimer composed of either α and β or γ and δ protein chains. The peptide-binding site of the T cell receptor and the epitope-binding site of the B cell receptor are very similar structures, formed by the folding of three regions in the amino-terminal domains of the proteins that participate in epitope recognition (the so-called hypervariable regions). However, unlike the B cell receptor, which can recognize the epitope as part of an intact folded protein, the T cell receptor can recognize **only** a peptide fragment produced by proteolysis. Furthermore, the peptide must be bound to MHC cell surface proteins (see below). When the T cell receptor engages an MHC molecule carrying the appropriate antigenic peptide, a signal transduction cascade that leads to gene expression is initiated. As a result, the stimulated T cell is capable of differentiating to form various effector T cells, as well as long-lived memory cells.

T helper cells and cytotoxic T lymphocytes are distinguished by unique cell surface proteins. In general, lymphocytes can be distinguished by the presence of specific cell surface proteins called **cluster-of-differentiation (CD) markers** (e.g., CD3, CD4, and CD8). The presence of these proteins can be detected with antibodies raised against them in heterologous organisms. The >350 individual CD markers known (to date!) are invaluable in identifying lymphocytes of a particular lineage or differentiation stage. Two well-known subpopulations of T cells are defined by the presence of either the CD4 or the CD8 surface proteins (Fig. 4.3), which are coreceptors for MHC class II and MHC class I, respectively. When immature T cells leave the bone marrow, they do not synthesize either CD4 or CD8 proteins (they are said to be "double-negative"). They differentiate sequentially in the thymus, initially producing both CD4 and CD8 proteins ("double-positive") and then either CD8 or CD4 ("single-positive"). These single-positive cells are the naive T cells that migrate to peripheral sites.

CD4⁺ T cells, or T helper (T_h) cells, are generally capable of interacting with B cells and antigen-presenting cells that have MHC class II proteins on their surfaces. After such interactions, $CD4^+$ T_h cells mature into T_h1 or T_h2 cells (see below). T_h cells synthesize cytokines and growth factors that stimulate ("help") the specific classes of lymphocytes with which they interact. **$CD8^+$ T cells** differentiate into cytotoxic T lymphocytes (CTLs) that can interact with MHC class I proteins, found on almost all cells of the body. CTLs recognize foreign peptides bound to MHC class I proteins and, when productively engaged, destroy the cell presenting the peptides. Mature cytotoxic T cells play important roles in eliminating virus-infected cells from the body by cell lysis and by production of interferon γ (IFN-γ) and tumor necrosis factor α (Tnf-α).

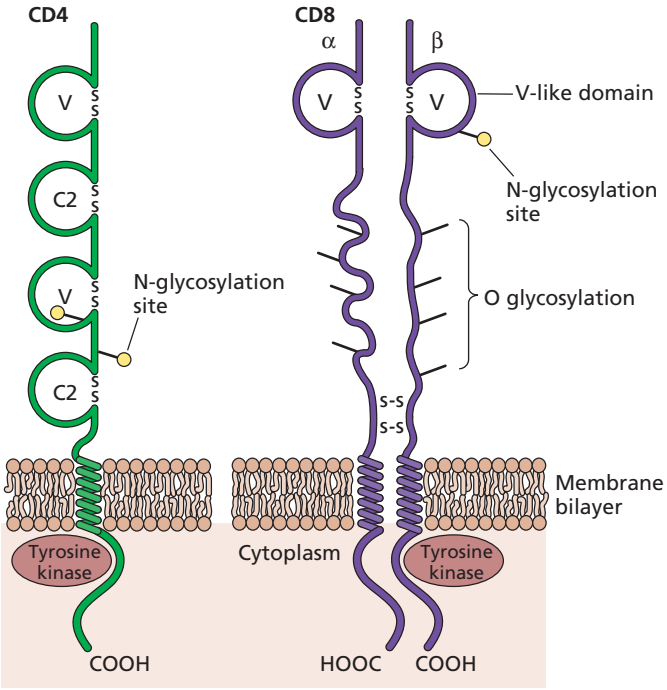
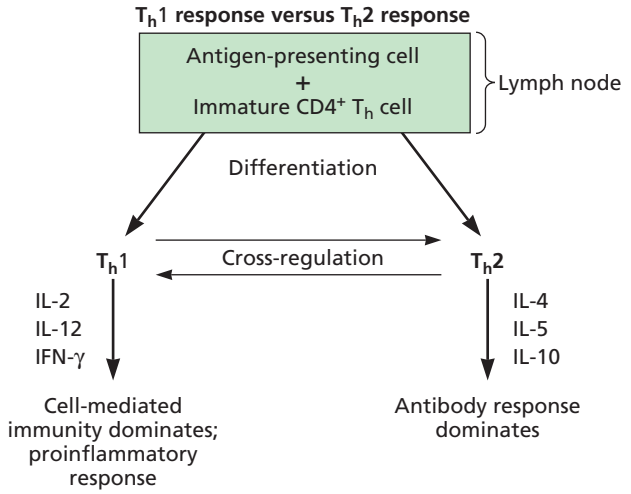


Figure 4.3 Simplified representations of CD4 and CD8 coreceptor molecules. These two molecules associate with the T cell receptor on the surface of T cells. The CD4 molecule is a glycosylated type 1 membrane protein and exists as a monomer in membranes of T cells. It has four characteristic immunoglobulin-like domains labeled V and C2. The V domains in the tertiary structure are similar to the variable domain of immunoglobulin. The first two domains form a binding site for a region on MHC class II proteins. The cytoplasmic domain interacts with specific tyrosine kinases, endowing CD4 with signal transduction properties. The CD8 molecule is a type 1 membrane protein with both N- and O-glycosylation sites. It is a heterodimer of an α chain and a β chain covalently linked by disulfide bonds, and interacts with a region on MHC class I proteins. The two polypeptides are quite similar in sequence, each having an immunoglobulin-like V domain thought to exist in an extended conformation. Tyrosine kinases also associate with the CD8 cytoplasmic domain and participate in signal transduction reactions.

T_h1 and T_h2 cells. Recall that tissue-resident antigen-presenting cells, usually dendritic cells, engulf antigens at the site of pathogen entry and then move to local lymph nodes to present these antigens to naive T cells. When naive T_h cells engage mature dendritic cells in lymphoid tissue, cytokines and receptor-ligand interactions stimulate the T cell to differentiate into one of two T_h cell types, called T_h1 and T_h2 (Fig. 4.4). These two cell types can be distinguished by the cytokines they produce and the processes they invoke. T_h1 cells are important for controlling most, but not all, viral infections; such cells promote the cell-mediated response by stimulating the maturation of cytotoxic-T cell precursors. T_h1 cells accomplish this, in part, by producing interleukin-2 (IL-2) and IFN-γ, cytokines that stimulate inflammation (the



Immune cross-regulation by cytokines

T_h1 response		T_h2 response	
Enhance	Suppress	Enhance	Suppress
IL-2 IL-12 IFN-γ	IL-4 IL-10	IL-4 IL-5 IL-6 IL-10	IFN-γ IL-12

Figure 4.4 The T_h1 versus the T_h2 response. Immature CD4+ T_h cells differentiate into two general subtypes called T_h1 and T_h2, defined functionally according to the cytokines they secrete. T_h1 cells produce cytokines that promote the inflammatory response and activity of cytotoxic T cells, and T_h2 cells synthesize cytokines that stimulate the antibody response. The cytokines made by one class of T_h cell tend to suppress production of those of the other class.

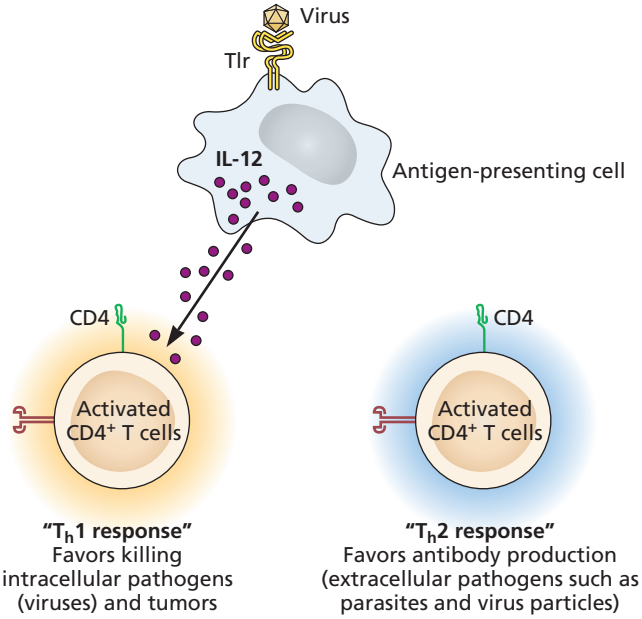


Figure 4.5 Interleukin-12 skews the T cell response toward a T_h1 profile. Engagement of Toll-like receptors (Tlrs) on the antigen-presenting cell surface drive the expression of IL-12, which promotes a T_h1 T cell response.

proinflammatory response). In addition, T_h1 cells provide stimulating cytokines to the antigen-presenting dendritic cell so that it can communicate with naive CD8+ T cells. If IL-12 is secreted by antigen-presenting cells at the time of antigen recognition, immature T_h cells differentiate into T_h1 cells. IL-12 also stimulates natural killer (NK) and T_h1 cells to secrete IFN-γ, thereby increasing the activity of macrophages at sites of inflammation (Fig. 4.5).

In the presence of IL-4, perhaps secreted by innate immune cells such as NKT cells (Chapter 3), immature T_h cells differentiate into T_h2 cells, which stimulate the antibody response rather than the cell-mediated, proinflammatory response. T_h2 cells promote the antibody response by inducing maturation of immature B cells and resting macrophages. They also reduce the inflammatory response by producing IL-4, IL-6, and IL-10, but not IL-2 or IFN-γ. T_h2 cells are more active after invasion by extracellular bacteria or multicellular parasites. Nevertheless, the T_h2 response is critical for controlling infections that result in accumulation of large quantities of virus particles in the blood.

In general, T_h1 and T_h2 responses coexist in a carefully orchestrated balance: as one increases, the other decreases (Fig. 4.4). While IFN-γ turns up the T_h1 response, it also inhibits the synthesis of IL-4 and IL-5 by T_h2 cells. On the other hand, production of T_h2 cytokines is an important mechanism to shut off the proinflammatory and potentially dangerous T_h1 response. An added complexity is that these two cell populations are distinguished only by the types of cytokines they secrete, not by particular cell surface receptors. Many immunologists believe that an individual T cell's subtype may vary based on the tissue and cytokine environment in which it exists.

How a particular type of pathogen triggers synthesis of interleukins that skew the T helper response toward either a T_h1 or T_h2 profile remains unknown, but one idea is that mature dendritic cells produce proinflammatory cytokines (e.g., IFN-γ) as their default pathway, poised to activate a T_h1 response unless appropriate T_h2 signals are provided. An alternative view is that when dendritic cells detect CpG sequences, single-stranded RNA, or double-stranded RNA via their Toll-like receptors, nuclear factor-κb (Nf-κb) is activated and T_h1 cytokine genes are transcribed.

We know that many viral proteins modulate the T_h1-T_h2 balance in interesting ways. For example, infection of B cells by Epstein-Barr virus and equine herpesvirus 2 should stimulate an active T_h1 response. However, both viral genomes encode proteins homologous to IL-10,

a regulatory cytokine that represses the T_h1 response. Viral IL-10 foils the T_h1 antiviral defense that would kill infected B cells, while promoting their differentiation into memory B cells that are important for long-term survival of the viral genome. Measles virus, which can infect antigen-presenting cells such as macrophages, may blunt production of IL-12, a crucial driver of a T_h1 response (Chapter 5). These properties identified with cells in culture are borne out in human studies: measles virus-infected patients have large quantities of IL-10 in their serum, indicative of a skewed T_h1-T_h2 balance. This shift from a (protective) T_h1 response to a less appropriate T_h2 response may, in part, account for the transient immunosuppression associated with measles virus infection and mortality.

For most viral infections, a given T_h response represents a spectrum of some T_h1 and some T_h2 cells, and consequently a mixture of cytokines. Establishment of the proper repertoire of T_h cells is therefore an important early event in host defense; an inappropriate response has far-reaching consequences. For example, synthesis of the T_h2 cytokine IL-4 by an attenuated strain of mousepox virus resulted in lethal, uncontained spread of this virus in vaccinated animals. As the design of potent and effective vaccines depends on stimulating the appropriate spectrum of response, understanding how this balance of cytokines is achieved has direct therapeutic implications.

T_h17 cells. In the past decade, a new class of $CD4^+$ helper cells that plays a central role in control of the inflammatory response was identified (Fig. 4.6). These cells are found in the skin and in the lining of the gastrointestinal tract and at other interfaces between the external and internal environments. When dendritic cells present antigens to them in the presence of transforming growth factor β (Tgf-β) and IL-6, these T cells secrete IL-17 and IL-21. In addition, the stimulated T_h17 cells now produce the receptor for IL-23, which leads to their massive proliferation. Such activated cells stimulate a strong inflammatory response, secrete defensins, and recruit neutrophils to the site of activation. T_h17 cells are probably important in the control of bacterial infections, as hosts that lack these cells are susceptible to opportunistic infections. However, because these cells are potent inducers of inflammation, they can exacerbate autoimmune diseases that lead to chronic inflammation, including psoriasis, Crohn's disease, multiple sclerosis, and rheumatoid arthritis. Their importance in controlling viral infections is only now being understood. For example, individuals with large numbers of T_h17 cells in their gut mucosa appear to be able to control human immunodeficiency virus type 1 infections much better than individuals with reduced numbers of these lymphocytes. T_h17 cells are also important in the fatal central nervous system infection caused by arboviruses.

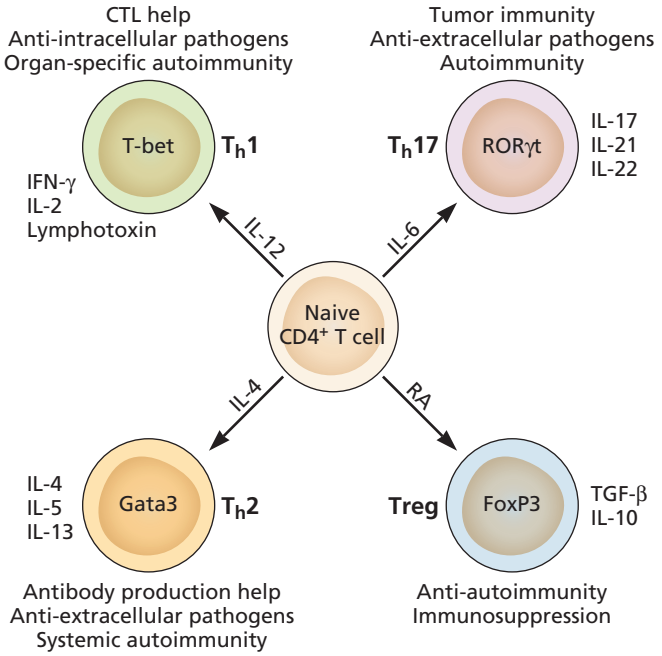


Figure 4.6 Differentiation of T helper subsets. T cell subset differentiation is modulated by cytokines released from dendritic cells and other immune cells. T_h1 cells are induced in the presence of IL-12 via induction of the transcriptional activator T-bet, and aid the resolution of intracellular pathogens. T_h1 cells are also the primary effectors of autoimmunity. T_h2 cells promote production of antibodies, contributing to the clearance of extracellular pathogens and systemic autoimmunity, and are induced by IL-4 and the transcription factor Gata3. T_h1 and T_h2 differentiation is inhibited by Tgf-β. Both Treg and T_h17 cells are induced by Tgf-β. In addition, Tregs require RA, and T_h17 cells require IL-6. Treg cells make the transcription activator forkhead box protein 3 (Foxp3) and secrete the anti-inflammatory cytokines Tgf-β and IL-10, which can suppress autoimmunity and immune responses to pathogens. T_h17 cells synthesize the transcriptional regulator Rorc2/Rorγt (humans/mice) and contribute to defense against extracellular pathogens, tumor immunity, and autoimmunity. RA, retinoic acid.

Regulatory T cells. The regulatory T cell (Treg) subset of T cells (once called suppressor T cells) has been recognized for some time, but their importance in controlling antiviral immunity has become a subject of intense study only recently. Tregs are pivotal players in the end-stage immune response to most, if not all, infectious agents. Their primary function is to terminate the response and return the immune system to a quiescent state (Fig. 4.7). As noted in the discussion of critical attributes of the adaptive response, curtailing an aggressive antiviral response is needed to minimize immunopathology.

Treg cells are important for immune suppression, self-tolerance, and control of the inflammatory response. These cells serve to maintain a balance between protection and immune pathology, but in some cases, Treg action also may limit the effectiveness of vaccines because they shut down the immune response.

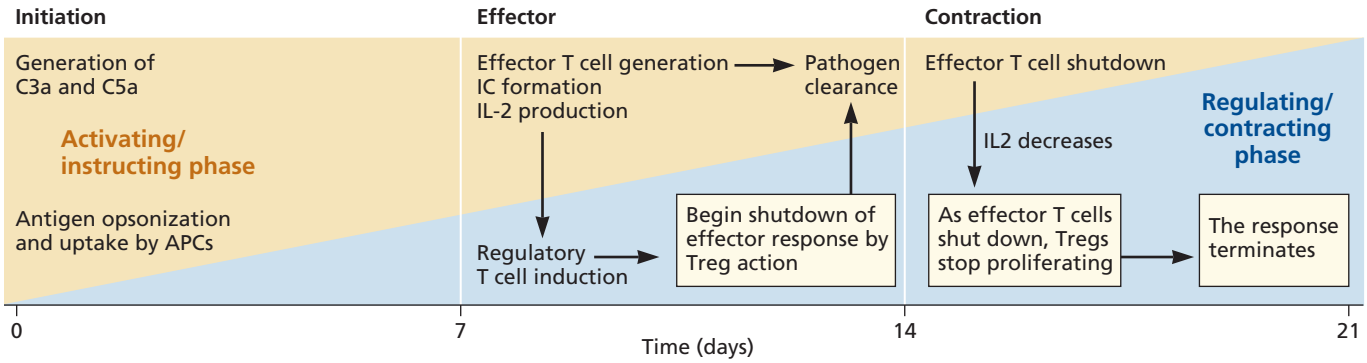


Figure 4.7 Regulation of the T cell response by complement and regulatory T cells. The magnitude and duration of the adaptive immune response is controlled in part by regulatory T cells (Treg cells). This model of an acute viral infection provides a view of how complement (part of the innate immune response; Chapter 3) may regulate the three phases of a T cell-mediated response. (Initiation) Soon after infection, antigen-presenting cells (APCs) take up viral proteins and make their way to local lymph nodes, where the T cell response is initiated. The complement cascade stimulated at the site of infection produces a variety of effector proteins, including C3a, C5a, and C3b. The C3b opsonin facilitates the uptake of C3b-coated antigens by APCs, while C3a and C5a stimulate their maturation. (Effector) Mature APCs then engage potential effector T cells in lymph nodes, resulting in production of IL-2 and activation of Treg cells. Ligands for CD46 include C3b-opsonized immune complexes (IC formation). Effector CTLs and T_h1 cells are stimulated by the APCs and leave the lymph node to resolve the infection at sites of virus reproduction. The balance between activated CTLs and Treg cells determines the extent of CTL action as well as the degree of immunopathology. Too many CTL cells can cause damage, but too few cannot clear the viral infection; conversely, too many Treg cells shut down the effector response prematurely, while too few Treg cells promote continued CTL action and potential immunopathology. (Contraction) The relative dynamics of CTL and Treg cell proliferation promote the controlled contraction of the CTL response. Both CTLs and Treg cells rapidly decline in numbers at this stage. The contraction occurs in part because CD46-stimulated Treg cells divide more quickly than CTLs, and through the action of Treg cytokines, the CTL response shuts down. Because the activated CTLs and T_h cells produce IL-2 necessary for Treg cell replication, the pool of Treg cells then diminishes as the system returns to its unstimulated state. During this phase, memory CTL and memory Treg cells are also produced. Adapted from C. Kemper and J. P. Atkinson, *Nat Rev Immunol* 7:9–18, 2007, with permission.

Diverse Receptors Impart Antigen Specificity to B and T Cells

Like the innate response, the adaptive response must distinguish infected from uninfected cells. However, this feat is accomplished in a markedly different fashion than in the innate immune system. Highly specific molecular recognition is mediated by two antigen receptors. Membrane-bound antibody on B cells and the T cell receptor on T lymphocytes both bind foreign antigens, but they do so in different ways. The B cell receptor engages discrete epitopes in intact proteins. In contrast, the T cell receptor binds short, linear peptides derived from proteolytically processed proteins, presented in the context of a class I MHC protein. The binding to an epitope has profound effects on the lymphocyte bearing that receptor: the T or B lymphocyte may respond by producing cytokines, entering a period of rapid cell division, killing the cell that bears the foreign protein or peptide, or synthesizing antibodies. The events catalyzed by epitope binding comprise the adaptive immune response.

The diversity of the B and T cell receptors is generated during the process of differentiation into mature naive cells in the bone marrow (for B cells) or the thymus (for T cells). Much is known about how such receptor diversity is generated. In brief, each transmembrane cell surface receptor possesses a constant (C) region that transduces critical signals following antigen engagement and a variable (V) region that engages the epitope for which it is specific. The diversity of these receptors enables the lymphocyte to distinguish among an extraordinary number of potential epitopes.

During the development of the individual T and B cell, DNA rearrangements generate this diversity. The genetic locus of the variable domain of T and B cell receptors comprises three main protein-coding regions, variable (V), diversity (D), and joining (J), each of which comprises many small modules. As T and B cells develop, DNA rearrangements occur in this region of the genome, in which a given allele from each of the V, D, and J regions is randomly selected, and the selected alleles for that particular lymphocyte are then spliced together during DNA recombination (Fig. 4.8). Because the DNA splicing event is inherently imprecise, additional nucleotides may be added or removed from each splice junction. From a limited number of modules, it is possible to generate extraordinary diversity: for example, the estimated total number of antibody specificities in a human is 10^{11}.

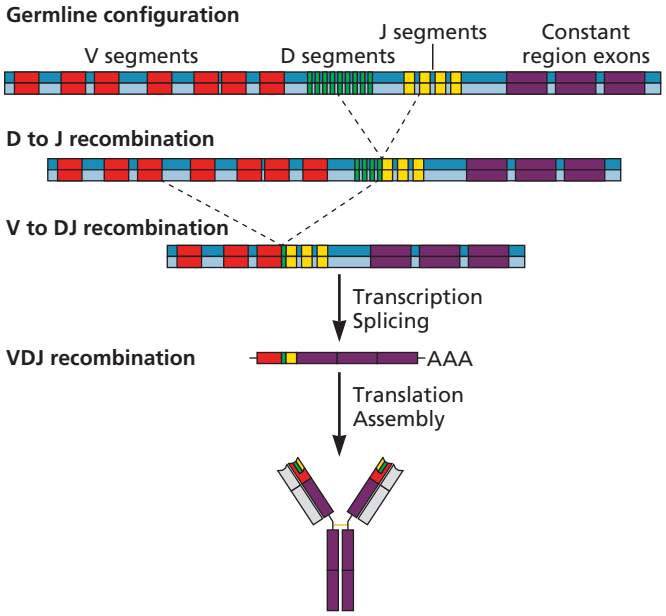


Figure 4.8 Generation of receptor diversity. The T and B cell receptor alleles in developing lymphocytes comprise small modules, called segments, that are clustered into three regions: variable (V), diversity (D), and joining (J). During development, the DNA of each lymphocyte undergoes recombination in which a module from each region is selected and spliced with a selected module from another region. D-to-J splicing occurs first, followed by V-to-DJ splicing. Incorporation of random nucleotides at these splice junctions further contributes to receptor diversity.

A large number of enzymes that mediate DNA cleavage, nucleotide additions, and ligation participate in this process, including the recombinase-activating genes, or RAGs. Many laboratories that study viral pathogenesis use RAG knockout mice to determine the contributions of the adaptive immune response, because mice lacking RAG genes cannot recombine their T and B cell receptors. As a result, lymphocyte development is blocked, and the mice are unable to mount defenses to many viral challenges, despite possessing intact intrinsic and innate immune responses.

Because the process of shuffling and recombining these sequence modules is random (in terms of which alleles are chosen, and the extent of the interjunctional nucleotide additions or deletions), many of the pre-T cells and B cells that are generated are flawed because they encode proteins that comprise rearrangements that do not form functional receptors or, worse, that form receptors that can bind to **host**-encoded epitopes. Such host-specific lymphocytes could lead to autoimmune diseases. Consequently, two concurrent quality control processes, **positive selection** and **negative selection**, weed out T cells and B cells with dysfunctional receptors. Positive selection allows the survival of those T cells that can bind appropriate surface molecules via the rearranged T cell receptor to survive, whereas negative selection efficiently kills those T cells that recognize target cells displaying host (or "self") peptides on their surfaces. As a result of these strict quality control processes that occur in the thymus, only 1 to 2% of all immature T cells that enter the thymus from the bone marrow are released into the circulation as mature T cells. Similar mechanisms exist for generation of B cells from the bone marrow. Of note, while a dual selection mechanism eliminates most autoreactive lymphocytes, many still escape into the peripheral circulation. As we will see, a second quality control checkpoint, costimulation, reduces the risk that such circulating, naive lymphocytes will become activated against host tissues.

Events at the Site of Infection Set the Stage for the Adaptive Response

At the conclusion of Chapter 3, we summarized the preexisting intrinsic processes that are deployed following infection, the critical contributions of interferons in restricting viral reproduction, and the influx of innate immune cells to the site of viral reproduction that limit viral spread. But at this stage, all the action is occurring at the site of the infection; rather literally, "naive" T cells wait in lymph nodes or circulate in the blood, unaware that a virus has entered the host. How, then, are T cells in the blood and lymph tissues alerted to an infection so that adaptive immunity can be initiated? Bridging this divide is one of the critical jobs of the antigen-presenting cell.

Acquisition of Viral Proteins by Professional Antigen-Presenting Cells Enables Production of Proinflammatory Cytokines and Establishment of Inflammation

A consequence of local innate defense is that tissue-resident myeloid cells, which include dendritic cells, engulf remnants of dying cells and virus particles, a process called **phagocytosis** ("phago-": "to devour"). Phagocytosis is a specific form of endocytosis that leads to the vesicular internalization of cellular debris, bacteria, and nutrients. This process is initiated when dendritic cells are activated by Toll-like receptor attachment to microbe-associated molecular patterns on infected cells (Chapter 3), although nonspecific activation may occur following tissue damage or local inflammation. Dendritic cell maturation triggers Nf-κb activation (Fig. 3.9), which, among other functions, results in the induction of the actin-myosin contractile system that is required for phagocytosis (Fig. 4.9). When a dendritic cell ingests a virus particle or a portion of a dying cell that contains the virus, the pathogen becomes trapped in an intracellular phagosome, which then fuses with a lysosome. Enzymes within these specialized vesicles digest viral proteins. Immature B cells and cells of the monocyte lineage (e.g., macrophages) are also considered to be professional antigen-presenting cells. Importantly, phagocytosis by these cells is about more than cleaning up debris: the degradation

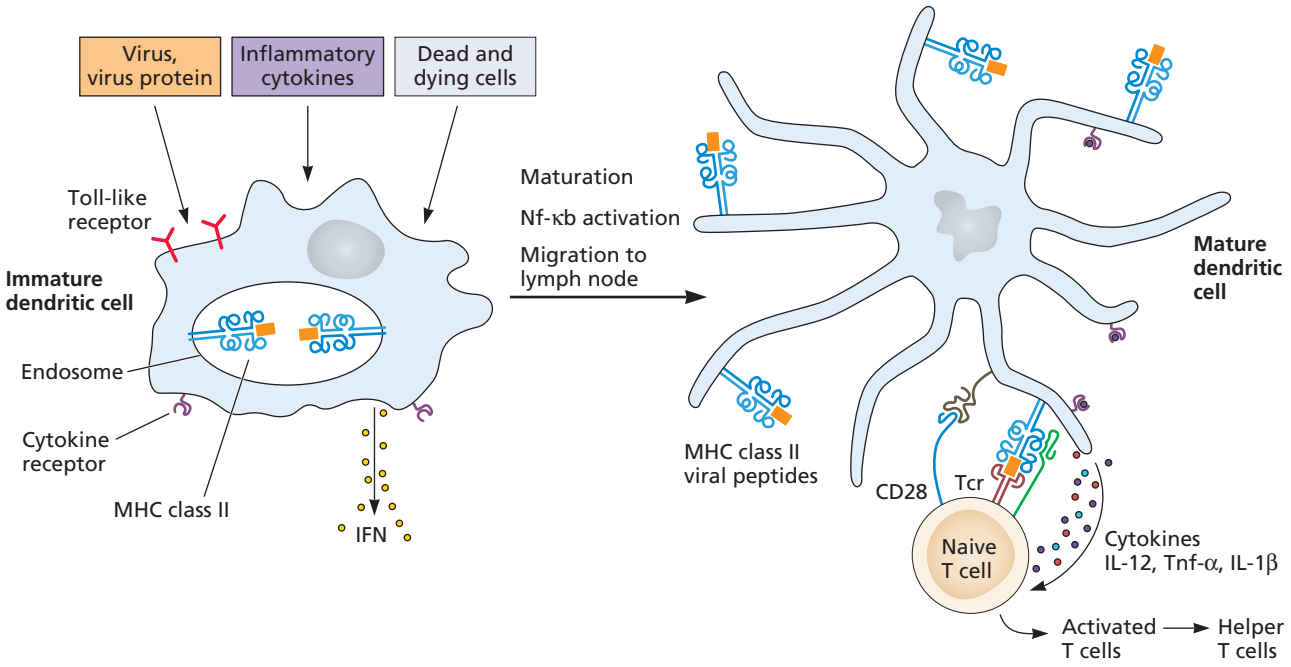


Figure 4.9 Dendritic cells provide cytokine signals and packets of protein information to naive T cells. Immature dendritic cells actively take up extracellular proteins by endocytosis and store the proteins internally. They do not present MHC class II proteins on their surfaces. Binding of ligands to the Toll-like receptors or cytokine receptors induces differentiation into mature dendritic cells. These cells no longer have the capacity for endocytosis of proteins and display a new repertoire of cell surface receptors. Some of these are chemokine receptors that enable the dendritic cell to migrate to local lymph nodes. The proteins ingested by the immature cell are now processed into peptides and loaded onto MHC class II proteins for subsequent transport to the cell surface. Mature cells extend long dendritic processes to increase the surface area for binding of naive T cells in the lymph node. Mature dendritic cells release proinflammatory cytokines to stimulate T cell differentiation. Naive but antigen-specific T cells bind to the MHC class II-peptide assemblies via recognition of specific peptides by their T cell receptors. The interaction is strengthened by the presence of increased concentrations of costimulatory ligands (e.g., CD28) on the mature dendritic cell. The T cell is activated, begins the maturation process into its final effector state, and moves out of the lymph node into the circulation.

of viral proteins and their presentation to naive lymphocytes is the critical bridge between the innate and the adaptive responses.

The Inflammasome and Cytokine Release

In addition to phagocytosis, dendritic cell activation triggers two additional reactions: cytokine release and migration to a local lymph node. Initiation of the inflammatory response is held under strict control by many regulatory proteins, as an overexuberant or inappropriately triggered proinflammatory response may set in motion a cascade of cytopathic events. Important checkpoints that require multiple, independent switches to be engaged to result in a "go" signal help to guard against faulty or premature induction of these powerful pathways.

One such checkpoint is a cytoplasmic protein complex in antigen-presenting cells called the "inflammasome." This multiprotein assembly of >700 kDa links the sensing of microbial products with the activation of proinflammatory cytokines (Fig. 4.10). When a microbe-associated molecular pattern is engaged by a Toll-like receptor, a signal transduction cascade results in the activation of Nf-κb, and the synthesis of two precursor cytokine molecules, pro-IL-1β and pro-IL-18, is triggered. These precursor proteins must be cleaved for release from the dendritic cell as functional cytokines. Cleavage is achieved via a second signal, the inflammasome, which results from pathogen-nonspecific stimuli such as potassium release or elevated intracellular reactive oxygen species. The induction of the inflammasome leads to the synthesis of caspases that cleave the pro- forms of IL-1β and IL-18 to create secreted and functional cytokines. Secretion of these potent **interleukins** (so named because they enable communication among leukocytes) catalyzes production of a number of proinflammatory cytokines and chemokines, resulting in the further recruitment of immune cells to the site of infection.

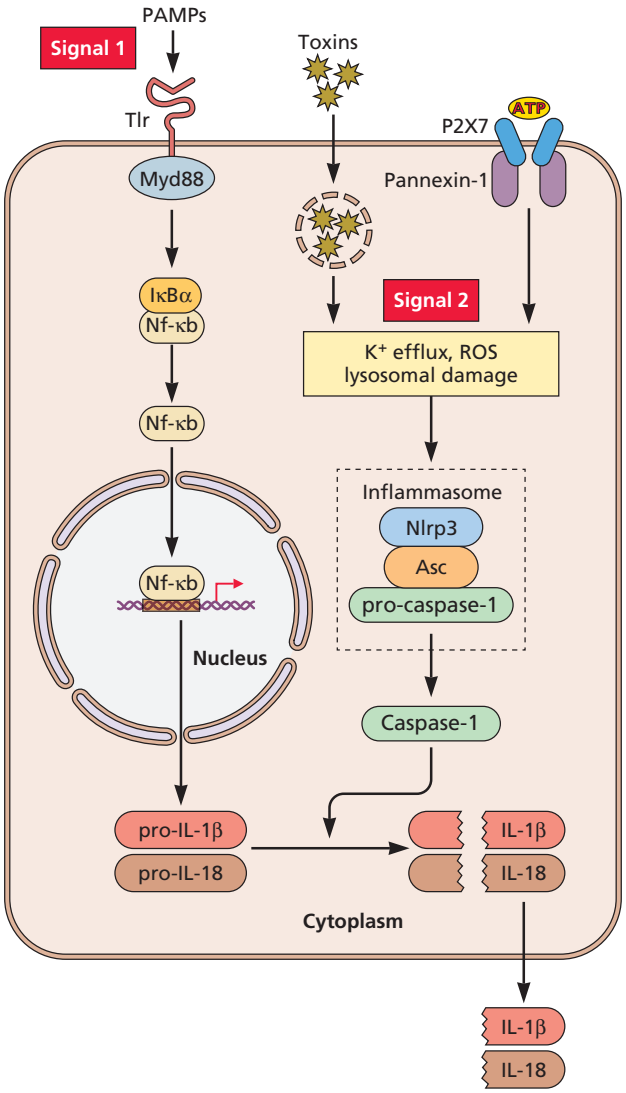


Figure 4.10 The inflammasome. The best characterized inflammasome is the Nlrp3 inflammasome which comprises the Nlrp3 protein (Nacht, Lrr, and PYD domain-containing protein 3), the adapter Asc (apoptosis-associated speck like protein containing a CARD domain), and procaspase-1. Maturation and release of IL-1β requires two distinct signals: the first signal leads to synthesis of pro-IL-1β and other components of the inflammasome, such as Nlrp3 itself; and the second signal results in the assembly of the Nlrp3 inflammasome, caspase-1 activation, and IL-1β secretion.

Inflammation

The rapid release of cytokines and the appearance of soluble mediators of the complement cascade at the site of infection have far-reaching consequences. Multifunctional Tnf-α, one of the signals of early warning, is produced by activated antigen-presenting cells. Tnf-α changes the local permeability of capillaries that attract, and facilitate entry of, circulating leukocytes to the site of infection. Tnf-α also can induce an antiviral response directly when it binds to receptors on infected cells. Within seconds, the combination of infection and binding of Tnf-α to its receptor initiates a signal transduction cascade that activates caspases, resulting in apoptotic cell death. Viral proteins that modulate the function of Tnf-α have been identified, including the core (capsid) protein of hepatitis C virus. Many DNA viruses that encode homologs of a cellular protein, cellular FLICE-like inhibitory protein (cFLIP), inhibit Tnf-α-mediated apoptosis. These viral counterparts, called vFLIPs, inhibit caspase activation in infected cells (Chapter 3). However, even when the caspase-dependent cell death pathway is blocked by viral proteins, infected cells can still induce their own demise via caspase-independent, programmed necrosis. This thrust-and-parry relationship between the altruistic suicide of the host cell and the virus-encoded proteins that keep the cell alive to prolong virus reproduction underscores the "chess match between masters" tension between host and virus.

One visible response to Tnf-α is **inflammation**. The four classical signs of inflammation are redness, heat, swelling, and pain. Such symptoms result from increased blood flow and capillary permeability, influx of phagocytic cells, and tissue damage. While unpleasant, these signs of infection are important to localize the host response to the site of damage. Local inflammation causes blood vessels to constrict, resulting in swelling of the capillary network in the area of infection. This response produces redness (erythema) and an increase in local tissue temperature. Capillary permeability increases, further facilitating an efflux of fluid and cells from the engorged capillaries into the surrounding tissue. The cells that migrate into the damaged area are largely mononuclear phagocytes. These circulating antigen-presenting cells are attracted by chemokines synthesized by virus-infected cells; cytokines elaborated by local defensive systems; and secondary reactions that facilitate adherence of phagocytic cells and other cells of the innate response, including neutrophils, to capillary walls near sites of damage (Fig. 4.11). Infiltrating monocytes are also important in the healing reactions that take place after the infection is resolved.

In the past, investigators experimentally induced inflammation by the use of adjuvants, such as Freund's adjuvant (killed mycobacterial cells in oil emulsion) or aluminum hydroxide gels, at sites of antigen administration. The adjuvant-induced inflammation mimics an infection to cultivate an environment conducive for the induction of a strong immune response to injected viral proteins in vaccines. Indeed, most vaccines would not work without adjuvants to stimulate the adaptive immune response (Chapter 8).

The nature and extent of the inflammatory response to viral infection depend on the tissue that is infected, as well as whether the virus is cytopathic; in general, noncytopathic viruses do not induce a strong inflammatory response. The early reactions at the site of infection dictate the type

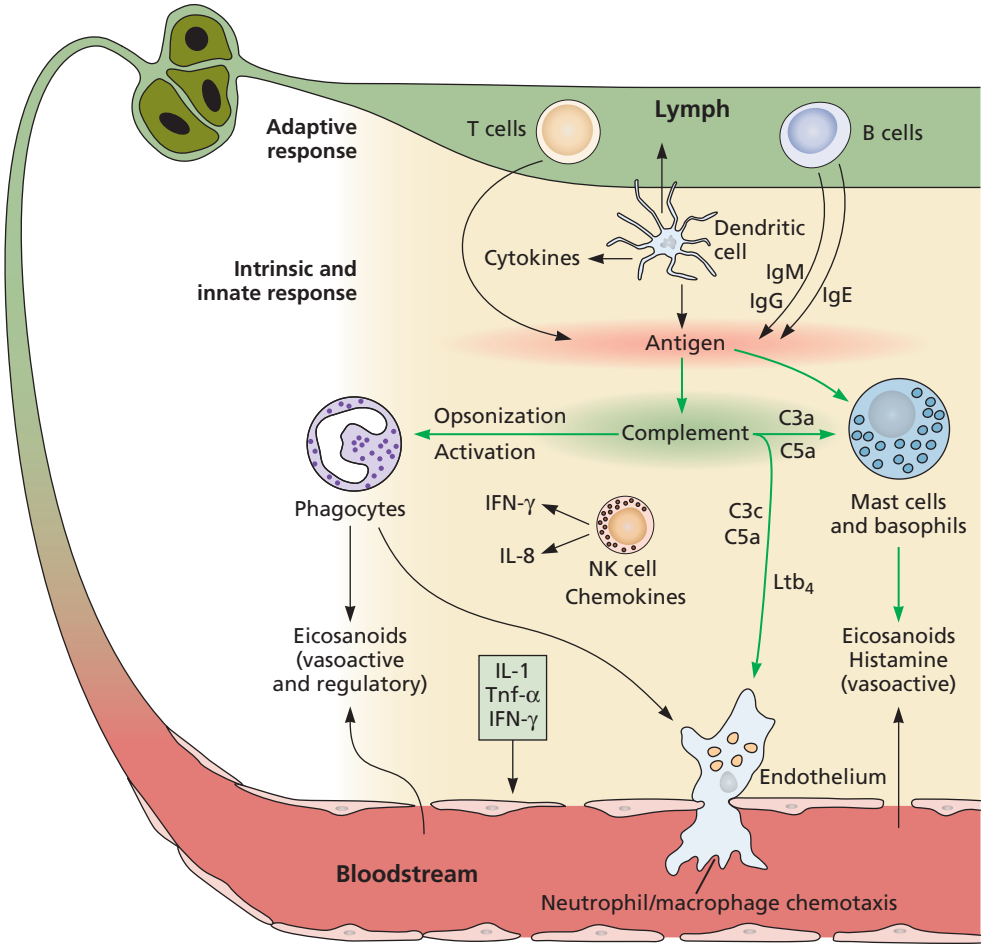


Figure 4.11 Inflammation provides integration and synergy with the main components of the immune system. Viral infection at entry sites in the body often triggers an inflammatory response. A stylized section of infected tissue served by the lymphoid system (top, green) and the circulatory system (bottom, red) is shown. Inflammation reactions can be initiated in several ways, for example, by cytokines such as IFN that are released by immature dendritic cells as they detect infection; by the classical or alternative pathway of complement activation; or by mast cells that migrate to sites of cell damage in response to cytokine release, where they can be activated by IgE antibody and antigen. C3a, C3c, and C5a are protease digestion products of the complement cascade that stimulate the inflammatory response. C3a increases vascular permeability and activates mast cells and basophils; C3c stimulates neutrophil release; and C5a increases vascular permeability and chemotaxis of basophils, eosinophils, neutrophils, and monocytes and stimulates neutrophils. The cytokines IL-1, IFN-γ, and Tnf-α act on the local capillary endothelium to enhance leukocyte adhesion and migration. IL-8 and other chemokines promote lymphocyte and monocyte chemotaxis. IL-1 and Tnf-α bind to receptors on epithelial and mesenchymal cells to induce division and collagen synthesis and to stimulate prostaglandin and leukotriene synthesis. Ltb_4 is a particularly active leukotriene that is vasoactive and chemotactic. The activities of cells that enter an infected site where inflammation reactions are occurring are controlled by locally produced cytokines, particularly Tnf-α, IL-1, and IFN-γ. Adapted from D. Male et al., *Advanced Immunology*, 3rd ed. (Mosby, St. Louis, MO, 1996), with permission.

of adaptive response that will predominate, and hence can influence the outcome of a viral infection. Tissues that have reduced access to the circulatory system (e.g., the brain and the interior of the eye) are less accessible to mediators of inflammation. As a result, the kinetics, extent, and final outcome of viral infections of these tissues are often markedly different from those of more-vascularized tissues.

Antigen-Presenting Cells Leave the Site of Infection and Migrate to Lymph Nodes

Activation of tissue-resident dendritic cells as a result of cytokine production and local inflammation causes them to leave the site of infection and migrate to lymphoid tissues, which include the extensive network of lymph nodes strategically located throughout the body (Fig. 4.12).

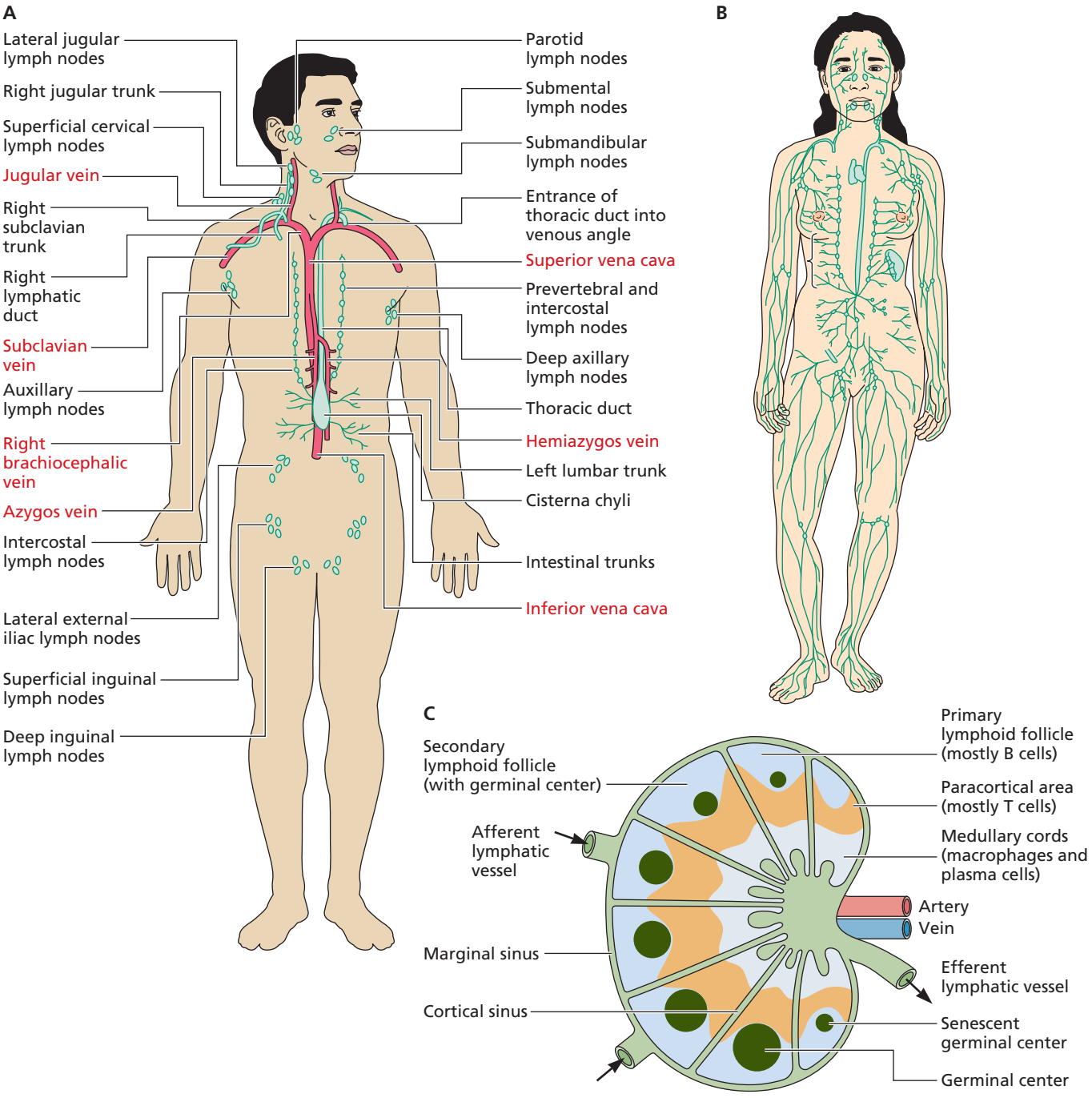
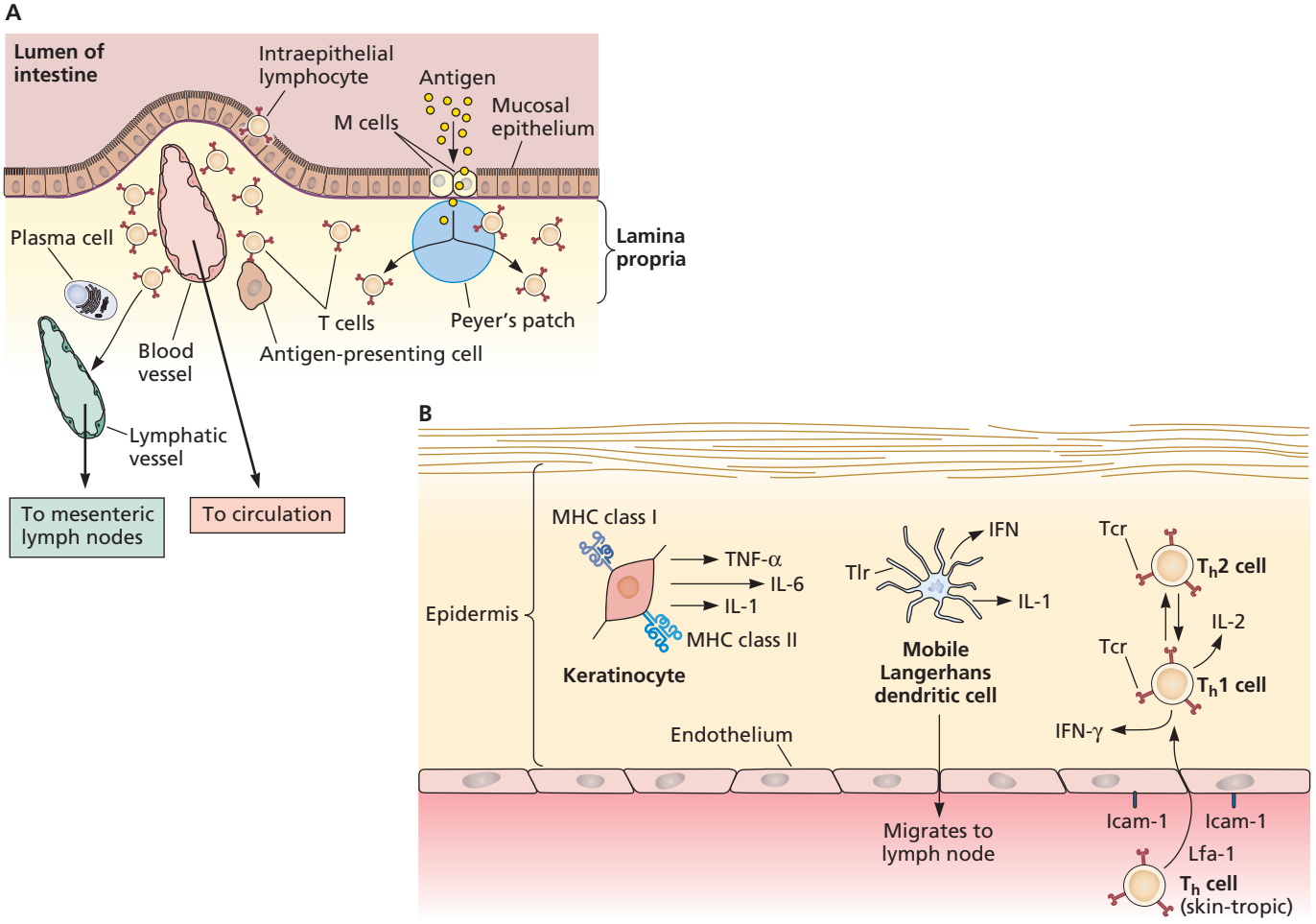


Figure 4.12 Lymph node anatomy. Lymph from the extracellular space carries antigens and antigen-presenting cells such as dendritic cells and macrophages from the tissues to the lymph nodes. The lymph enters the node at several points along the lymphatic system through afferent lymphatic vessels. The blood supply enters and leaves the lymph node at the hilum via small arteries that create a capillary network within the node. Lymphocytes in the blood can then enter the lymph node across the walls of postcapillary venules, which are also known as high endothelial venules (HEVs). These HEVs merge into small veins, which then carry blood away from the node. **(A)** Major lymph nodes in humans and their relation to major blood vessels; **(B)** the lymphatic system; **(C)** the anatomy of a lymph node.

Mucosal immunity is usually the first adaptive defense to be engaged. The lymphoid tissues below the mucosa of the respiratory and gastrointestinal tracts (mucosa-associated lymphoid tissue [MALT] and gut-associated lymphoid tissue [GALT]) (Fig. 4.13A) are vital in antiviral defense. These clusters of lymphoid cells include the tonsils in the pharynx, the submucosal follicles of the upper airways, Peyer's patches in the lamina propria of the small intestine, and the appendix. A specialized epithelial cell of mucosal surfaces is the **M cell** (microfold or membranous epithelial cell), which samples and delivers antigens to the underlying lymphoid tissue by transcytosis (Chapter 2). M cells have invaginations (pockets) of their

Figure 4.13 Components of the human lymphatic and mucosal immune systems. (A) Cellular components of the mucosal immune system in the gut (gut-associated lymphoid tissue). The lumen of the small intestine is depicted at the top of the figure. The mucosal epithelial cells are shown with their basal surface oriented toward the lamina propria. Cross sections of a lymphatic vessel and a capillary are shown, illustrating their juxtaposition to cells of the mucosal immune system. M cells have large intraepithelial pockets filled with B and $CD4^+$ T lymphocytes, macrophages, and dendritic cells. M cells and intraepithelial lymphocytes are important in the transfer of antigen from the intestinal lumen to the lymphoid tissue in Peyer's patches, where an immune response can be initiated. **(B)** The cutaneous immune system (skin-associated lymphoid tissue) comprises three cell types: keratinocytes, Langerhans cells, and T cells. Keratinocytes secrete various cytokines, including Tnf-α, IL-1, and IL-6, and have phagocytic activity. They also synthesize both MHC class I and MHC class II proteins and can present antigens to T and B cells if stimulated by IFN-γ. Langerhans cells are migratory dendritic cells and are the major antigen-presenting cells in the epidermis. When products of viral infections in the skin are detected, Langerhans cells secrete IFN and undergo maturation. Mature dendritic cells migrate to the local draining lymph node, where they present viral peptides on both MHC class I and MHC class II proteins to antigen-specific T cells. Dedicated skin-tropic T cells can cross the endothelium to enter the epidermis, where they can mature into T_h1 or T_h2 cells depending on the antigen and cytokine milieu. Activated T cells synthesize cytokines, including IFN-γ, that increase antigen presentation via MHC expression from keratinocytes and Langerhans cells. Tcr, T cell receptor. Adapted from A. K. Abbas et al., *Cellular and Molecular Immunology* (The W. B. Saunders Co., Philadelphia, PA, 1994), with permission.



membranes that harbor immature dendritic cells, B and $CD4^+$ T lymphocytes, and macrophages. The secreted antibody IgA (important in antiviral defense at mucosal surfaces) is made by B cells that accumulate at adhesion sites in these M-cell membrane pockets. After viruses or viral components transit through M cells, they emerge to come into intimate contact with all the appropriate immune cells. This process represents an essential step for the development of mucosal immune responses.

The skin, the largest organ of the body, possesses its own diverse community of organized immune cells. Lymphocytes and Langerhans cells comprise the **cutaneous immune system** (also called skin-associated lymphoid tissue) (Fig. 4.13B). These cells are important in the initial response and resolution of viral infections of the skin. In particular, Langerhans cells, the predominant scavenger antigen-presenting cells of the epidermis, function as the sentinels or outposts of early warning and reaction. These abundant, mobile cells sample antigens and migrate to regional lymph nodes to transfer information to T cells, and to activate B lymphocytes directly. Certain T cells in the circulation have tropism for the skin and, after binding to the vascular endothelium, enter the epidermis to interact with Langerhans cells and keratinocytes. These skin-tropic T cells are important for the production of the virus-specific skin rashes and poxes characteristic of measles virus and varicella-zoster virus infections.

Virus particles at the primary site of infection can interact with lymphoid cells that are associated with the mucosal and cutaneous immune systems to suppress their responses by inducing lysis or misregulation of such cells. These interactions can govern the outcome of the primary infection and often establish the pattern of infection that is characteristic of a given virus. The M cells in the mucosal epithelium have been implicated in the spread from the pharynx and the gut to the lymphoid system of a variety of viruses, including poliovirus, enteric adenoviruses, human immunodeficiency virus type 1, and reovirus. M cells have also been suggested to be sites of the persistent or latent infection of a number of other viruses, including herpes simplex virus.

In some cases, such as infections with herpesviruses or influenza virus, the dendritic cells that migrate from the periphery to the lymph node are not those that eventually present antigen to naive lymphocytes. In herpes simplex virus infection, for example, Langerhans cells in the skin phagocytose debris from dying infected cells and migrate to local nodes. Thereafter, some transfer of antigen occurs between the Langerhans cell and a dendritic cell that is resident in the lymph node, a process called cross-presentation. Such complexity may be important following infection by those viruses that can enter and kill dendritic cells: antigen presentation by uninfected dendritic cells, and the eventual induction of adaptive immunity, can still occur, despite the loss of the original antigen-presenting cell (Box 4.3).

BOX 4.3

BACKGROUND

Infection of the sentinels: dysfunctional immune modulation

When viruses infect immature or mature dendritic cells, the immune system's first command-and-control link is compromised. Some of the many possible consequences of such infection, any one of which could lead to suppression of the immune response locally or systemically, include

- destruction of immature dendritic cells
- interference with their maturation
- impairment of antigen uptake or processing
- inhibition of migration of dendritic cells to lymphoid tissue
- prevention of their activation of T cells

López CB, Yount JS, Moran TM. 2006. Toll-like receptor-independent triggering of dendritic cell maturation by viruses. *J Virol* **80:**3128–3134.

Mellman I, Steinman RM. 2001. Dendritic cells: specialized and regulated antigen processing machines. *Cell* **106:**255–258.

Finally, some viruses take advantage of the migration of dendritic cells to lymph nodes. Human immunodeficiency virus type 1 binds to a protein on the antigen-presenting cell surface, called Dc-Sign, to be ferried to the lymph node, where millions of naive T cells, the target cell for this virus, await.

Antigen Processing and Presentation

Professional Antigen-Presenting Cells Induce Activation via Costimulation

Dendritic cells and macrophages are generally referred to as "professional" antigen-presenting cells, implying that they have enhanced capabilities as compared to other cell types. Like virtually all cells in the body, dendritic cells present antigens in the groove of an MHC molecule, but they alone are able to activate naive lymphocytes, a process called **priming**. The ability to activate naive cells is mediated by cell surface proteins that enable costimulation: two protein-protein interactions between an antigen-presenting cell and a quiescent, naive lymphocyte must happen simultaneously to induce activation. The first of these signals is antigen specific (the MHC class II-epitope complex interacting with the T cell receptor), while the second signal is mediated via interactions of molecules such as CD80 and CD86 on the dendritic cell with CD28 on the naive T cell (Fig. 4.14). This two-trigger process is called costimulation. Other interactions include those that facilitate adhesion of T cell and target cell to one another, such as binding of CD2 or leukocyte function antigen 1 (Lfa1) on the CTL with Lfa-3 or intercellular adhesion molecule 1 (Icam1), respectively, on the infected cell. This fail-safe ensures that naive T cells are not activated in error: engagement of naive T cells in the absence of costimulation leads to T cell anergy or tolerance, a response in which these cells do not proliferate further. B cells, which can also present

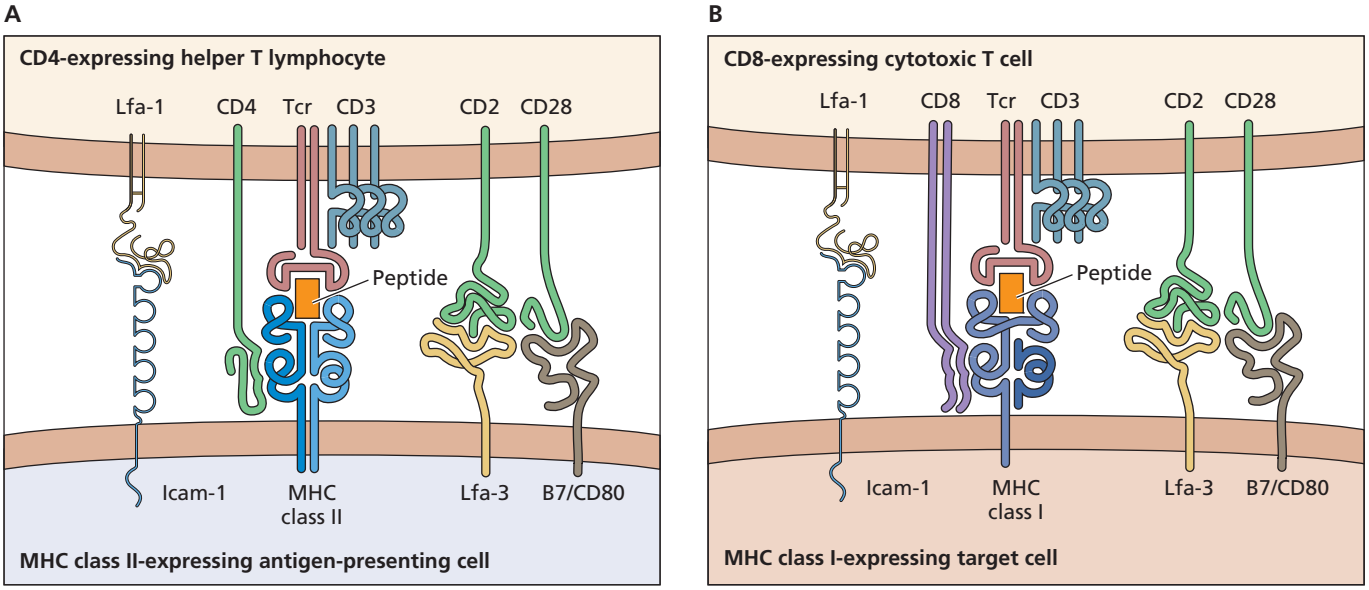


Figure 4.14 T cell surface molecules and ligands. The interactions of these receptors and ligands are important for antigen recognition and initiation of signal transduction and other T cell responses. **(A)** Interaction of a CD4 coreceptor-expressing T_h cell with an antigen-presenting cell. The antigen-presenting cell exhibits an MHC class II-peptide complex in addition to Icam-1, Lfa-3, and CD80 (B7) membrane proteins. These cell surface proteins all are capable of binding cognate receptors on the T_h cell as illustrated. **(B)** Interaction of a CTL bearing the CD8 coreceptor with its target cell. The target cell exhibits an MHC class I-peptide complex in addition to Icam-1, Lfa-3, and CD80 (B7) membrane proteins. Tcr, T cell receptor.

antigen, are costimulated in a similar way during engagement with T cells via a CD40-CD40L interaction.

Presentation of Antigens by Class I and Class II MHC Proteins

Earlier in this chapter, the generation of T and B cell receptor diversity was introduced, a process that enables receptor-bearing lymphocytes to "recognize" their cognate antigen. Antigens on infected cells or professional antigen-presenting cells can be displayed by one of two related but distinct protein assemblies: class I and class II MHC proteins. MHC class I proteins display protein fragments on the surface of almost all cells, whereas MHC class II proteins are generally found only on the surfaces of mature dendritic cells, macrophages, and B lymphocytes (the professional antigen-presenting cells). How antigens are presented by these proteins differs, but they have one major feature in common: fragments of proteins are presented in an outward-facing groove of the MHC molecule, similar to a hot dog in a hot dog bun. In this way, the T or B cell receptor detects the presence of "altered self": a familiar "self" protein (the MHC molecule) that is modified by the virus-specific epitope.

MHC class I proteins present antigens from intracellular pathogens, whereas MHC II molecules display antigens from extracellular pathogens or those that have been engulfed by phagocytosis. Corresponding to the two classes of MHC molecules, there are two types of effector T lymphocytes. $CD8^+$ CTLs defend against intracellular infections (e.g., viruses) and chiefly recognize class I-presented epitopes. $CD4^+$ T_h lymphocytes recognize class II-presented peptides and afford protection against extracellular pathogens (e.g., many bacteria and parasites) as a result of the activation of B cells and the production of antibodies. Elucidation of the basis of MHC recognition systems, defined by immunologists as MHC restriction, was a major step forward, not only explaining how T cells recognize their targets, but also providing broader insights into how cells communicate with one other (Box 4.4).

Cytotoxic T Cells Recognize Infected Cells by Engaging MHC Class I Receptors

An imperative of the host response is to destroy virus-infected while ignoring uninfected cells. The former are identified, in part, because they display small viral peptides in association with MHC class I proteins on their surfaces. These viral (and cellular) peptides are produced and displayed via a pathway called **endogenous antigen presentation** (Fig. 4.15).

In all uninfected and infected cells, a fraction of most newly synthesized proteins is degraded by the proteasome. The targeted protein is marked for destruction by the covalent attachment of multiple copies of the small protein

BOX 4.4

TRAILBLAZER

Virology provides Nobel Prize-winning insight: MHC restriction

In 1974, Rolf Zinkernagel and Peter Doherty performed a classic experiment that provided insight into how CTLs recognize virus-infected cells. Initially, they teamed up to determine the mechanism of the lethal brain destruction observed when mice are infected with lymphocytic choriomeningitis virus, a noncytolytic arenavirus. They anticipated that the brain damage was due to CTLs responding to replication of the noncytopathic virus in the brain.

When they infected mice of a particular MHC type with the virus and then isolated T cells, these cells lysed virus-infected target cells *in vitro* **only** when the target cells and the T cells were of identical MHC type. Uninfected target cells were not lysed, even when they shared identical MHC alleles. This requirement for MHC matching was called **MHC restriction**.

The Nobel Prize-winning insight was that a CTL must recognize two determinants present on a virus-infected cell: one specific for the virus and one specific for the MHC of the host. We now know that CTLs recognize a short peptide derived from viral proteins and only engage the peptide when it is bound to MHC class I proteins present on the surface of target cells. (For an interview with Dr. Peter Doherty, see: http://bit.ly/Virology_Doherty)

Zinkernagel RM, Doherty PC. 1974. Restriction of *in vitro* T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* **248:**701–702.

ubiquitin, and following ATP-dependent unfolding, the protein is broken down in the inner chamber of the proteasome. The peptide products are released and transported into the endoplasmic reticulum (ER) by proteins that span the ER membrane, called transport-associated proteins (Taps). Within the ER, binding of peptides to newly synthesized MHC class I proteins allows these MHC molecules to adopt their native conformation and to be transported to the cell surface via the secretory pathway. In this manner, MHC class I proteins constitutively "present," or display, a representative sampling of intracellular epitopes on their surface; when a cell is infected, the sampling includes epitopes from the virus. Binding of a viral peptide-MHC class I complex on the surface of an infected cell by the T cell receptor triggers a series of reactions that activate the CTL for killing (see below). Presentation of cellular proteins is usually ignored, because such autoreactive T cells were either deleted during development or were never appropriately costimulated.

MHC class I proteins are found on the surfaces of nearly all nucleated cells. These proteins comprise two subunits called the α chain (often called the heavy chain) and β_2-microglobulin (the light chain). Lymphocytes possess the highest concentration, with about 5×10^5 molecules per cell. In contrast, fibroblasts, muscle cells, hepatocytes, and neurons carry much smaller quantities, sometimes 100 or fewer molecules per cell. There are three MHC class I loci in humans (*A*, *B*, and *C*) and two in mice (*K* and *D*). Because there are many allelic forms of these genes in outbred populations, they are said to be **polymorphic genes**. For example, at the human MHC class I locus *hla-B*, >149 alleles with pairwise differences ranging from 1 to 49 amino acids have been identified. When cells bind cytokines such as type 1 IFN and IFN-γ, transcription of the MHC class I α chains, β_2-microglobulin, and the linked proteasome and peptide transporter genes is markedly increased.

T_h Cells Recognize Professional Antigen-Presenting Cells by Engaging MHC Class II Receptors

Both antibody and CTL responses are controlled by cytokines produced by T_h cells, which are activated upon antigen recognition via MHC class II proteins presented on the surfaces of professional antigen-presenting cells. As dendritic cells mature, MHC class II glycoproteins loaded with peptides produced from their stores of endocytosed antigens appear on their surfaces. Mature antigen-presenting cells also carry high surface concentrations of costimulatory T cell adhesion molecules that bind receptors on naive T_h cells in the lymphoid tissue.

The process by which viral proteins are taken up from the outside of the cell and digested, and by which resulting peptides are loaded onto MHC class II molecules, is called **exogenous antigen presentation** (Fig. 4.16). Unlike proteins processed by the endogenous pathway, viral proteins presented by MHC class II are not produced inside the cell, as the cell is not infected. Rather, phagocytosed viral particles are broken down and their proteins digested in endosomes rather than in the proteasome. Viral peptides and MHC class II molecules are then brought together by vesicular fusion. Similar to peptide-bound MHC class I presentation, the assembly is then transported to the surface of the antigen-presenting cell, where it is available to interact with appropriate T cells in the lymph node. Interaction of T cell receptors on the naive $CD4^+$ T_h cell with the MHC class II-peptide induces concerted changes in the T_h cell, leading to its activation and differentiation.

T_h cells activated in this fashion produce IL-2, as well as a high-affinity receptor for this cytokine. The secreted IL-2 binds to the newly synthesized receptors to induce autostimulation and proliferation of T_h cells. Such clonal expansion of specific T_h1 or T_h2 cells then promotes the activation of CTLs and B lymphocytes (Fig. 4.2).

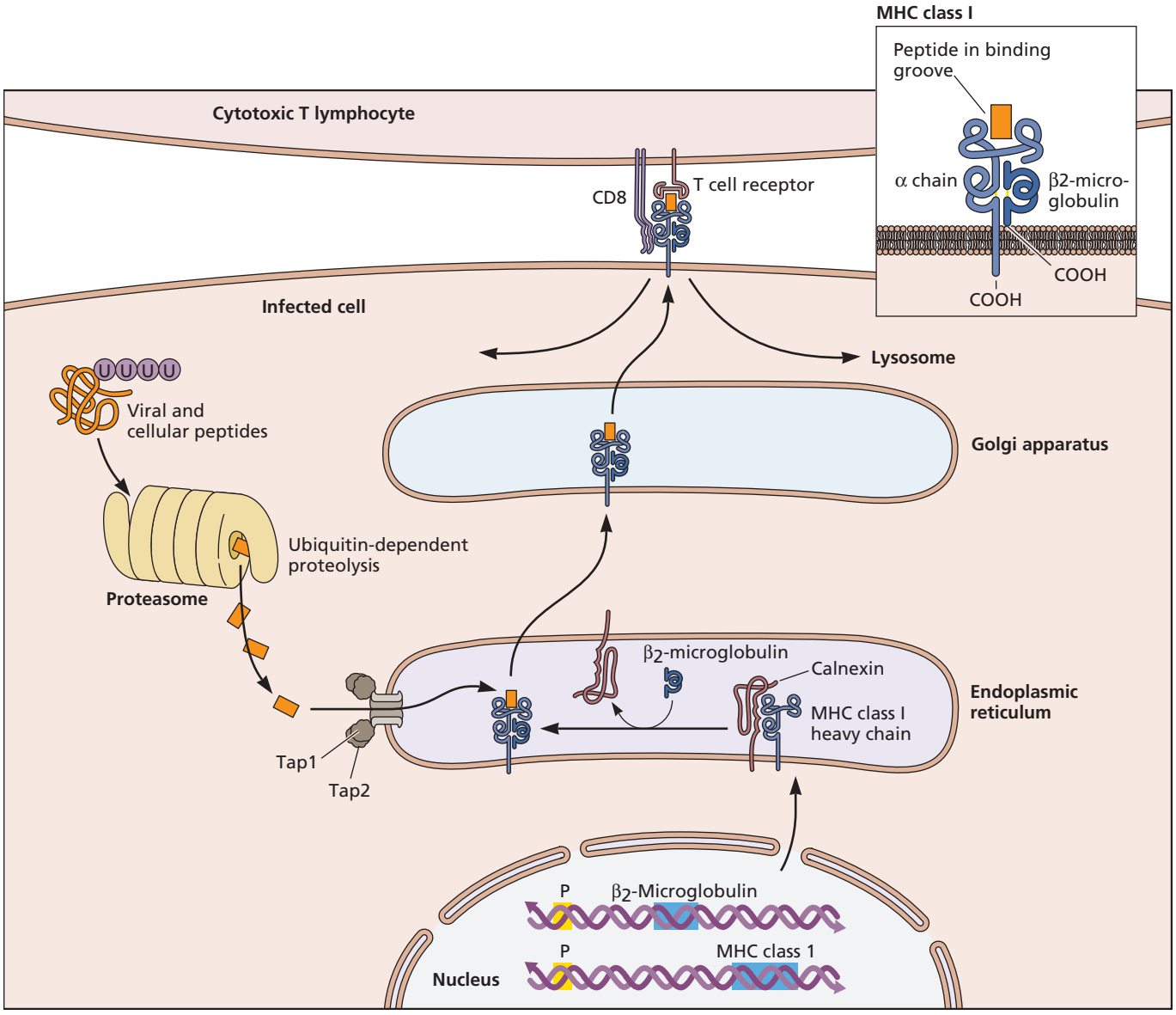


Figure 4.15 Endogenous antigen processing: the pathway for MHC class I peptide presentation. Intracellular proteins derived from both the host cell and the virus are degraded in the cytoplasm. Proteins are marked for destruction by polyubiquitinylation and are then taken up and degraded by the proteasome. The resulting short peptides are transported into the lumen of the ER by the Tap1-Tap2 heterodimeric transporter in a reaction requiring ATP. Once in the ER lumen, the peptides associate with newly synthesized MHC class I molecules that bind weakly to the Tap transporter. Assembly of the α chain and β_2-microglobulin (β_2m) of the MHC class I molecule is facilitated by the ER chaperone calnexin, but formation of the final native structure requires peptide loading. The MHC class I complex loaded with peptide is released from the ER to be transported via the Golgi compartments to the cell surface, where it is available for interaction with the T cell receptor of a cytotoxic T cell carrying the CD8 coreceptor. (Inset) The MHC class I molecule is a heterodimer of the membrane-spanning type I glycoprotein α chain (43 kDa) and β_2-microglobulin (12 kDa) that does not span the membrane. The α chain folds into three domains, 1, 2, and 3. Domains 2 and 3 fold together to form the groove where peptide binds, and domain 1 folds into an immunoglobulin-like structure. Adapted from D. Male et al., *Advanced Immunology*, 3rd ed. (Mosby, St. Louis, MO, 1996), with permission.

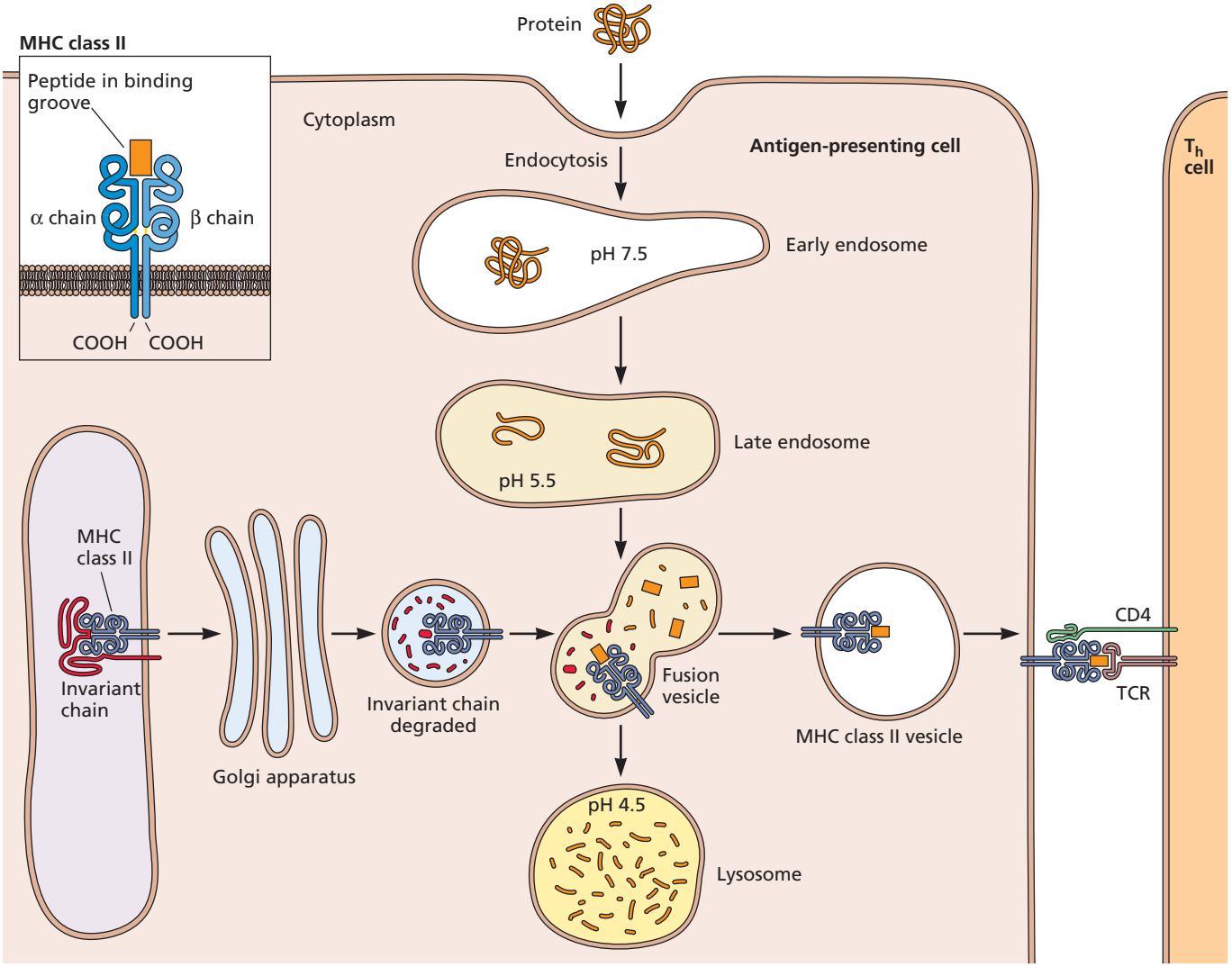


Figure 4.16 Exogenous antigen processing in the antigen-presenting cell: the pathway for MHC class II peptide presentation. Peptides in the ER lumen of the antigen-presenting cell are prevented from binding to the MHC class II peptide groove by association of MHC class II molecules with a protein called the invariant chain. The complex is transported through the Golgi compartments to a post-Golgi vesicle, where the invariant chain is removed by proteolysis. This reaction frees MHC class II molecules to accept peptides. The peptides are derived not from endogenous proteins but from extracellular proteins that enter the antigen-presenting cell. In some cells, the proteins enter by endocytosis (top) and are internalized to early endosomes with neutral luminal pH. Endocytotic vesicles traveling to the lysosome via this pathway are characterized by a decrease in pH as they "mature" into late endosomes. The lower pH activates vesicle proteases that degrade the exogenous protein into peptides. Internalized endosomes with their peptides fuse at some point with the vesicles containing activated MHC class II. The newly formed peptide-MHC class II assembly then becomes competent for transport to the cell surface, where it is available for interaction with the T cell receptor (TCR) of a T_h cell carrying the CD4 coreceptor. (Inset) The MHC class II molecule is a heterodimer of the membrane-spanning type I α-chain (34-kDa) and β-chain (29-kDa) glycoproteins. Each chain folds into two domains, 1 and 2, and together the α and β chains fold into a structure similar to that of MHC class I. The two amino-terminal domains from α and β chains form the groove in which peptide binds. Unlike the closed MHC class I peptide groove, the MHC class II peptide-binding groove is open at both ends. The second domain of each chain folds into an immunoglobulin-like structure. Human genomes contain three MHC class II loci (*DR*, *DP*, and *DQ*), and mouse genomes have two (*IA* and *IE*). The three sets of human genes give rise to four types of MHC class II molecules. Adapted from D. Male et al., *Advanced Immunology*, 3rd ed. (Mosby, St. Louis, MO, 1996), with permission.

Although synthesis of MHC class II proteins occurs primarily in professional antigen-presenting cells, other cell types, including fibroblasts, pancreatic β cells, endothelial cells, and astrocytes, can synthesize MHC class II molecules, but only after exposure to IFN-γ. As with MHC class I, there are many alleles of MHC class II genes.

Both classes of MHC protein have a peptide-binding cleft that is sufficiently flexible to accommodate many epitopes. Even so, not all possible peptides are bound. The ability of MHC molecules to bind and display particular epitopes on the cell surface varies from individual to individual as a result of the many MHC alleles that exist within the human population. Such allelic diversity plays an important role in an individual's capacity to respond to various infections: the greater the diversity, the wider the capacity to respond. This fact has dramatic consequences for the spread of viral diseases. For example, individuals in inbred populations lose MHC diversity over time and have a concomitantly limited capacity to respond to infections. Protective immunity is difficult to establish in such populations, increasing the risk of epidemics.

Lymphocyte Activation Triggers Massive Cell Proliferation

Following a productive interaction between an antigen-presenting cell and a naive T cell, a massive expansion of the naive population ensues. Only a few cells in this population, whether in lymphoid tissues or elsewhere in the body, participate in the initial encounter with any foreign epitope. For example, the frequency of B or T lymphocytes that recognize infected cells on first exposure is as few as 1 in 10,000 to 1 in 100,000. Following activation, this precursor population is amplified substantially during the next 1 or 2 weeks: the number of virus-specific lymphocytes increases >1,000-fold, in some cases by as much as 50,000-fold. Because much of this expansion occurs in lymph nodes, individuals suffering from virus infection often note swelling in the neck or the groin, areas of high lymph node density. Activation is accompanied by differentiation such that each daughter cell has the same specific immune reactivity as the original parent (often called a clonal response).

Before discussing how activated lymphocytes resolve a virus infection, we pause here to note that many of the fundamental discoveries described above were accelerated by the development of powerful methodologies. As examples, restriction enzymes and DNA sequencing enabled precise determination of the genetic origins of the T and B cell receptors, flow cytometry allowed isolation of lymphocytes at distinct stages of maturation, and recombinant DNA approaches made possible the generation of transgenic and knockout mice with genetically altered immune systems. T_h17 and Treg cells are fairly new discoveries in immunology, but their identification prompted a reevaluation of what we thought we understood when $CD4^+$ T cells were "simply" either T_h1 or T_h2. As new technologies come along, it is exciting, and a bit intimidating, to consider how our current understanding of the host response will change based on the discoveries that lie ahead.

The Cell-Mediated Response

The cell-mediated response facilitates recovery from a viral infection primarily because it eliminates virus-infected cells without damaging uninfected cells. While the T_h2-promoted antibody response is important for some infections in which virus particles spread in the blood, antibody alone is often unable to contain and clear an infection. Indeed, antibodies have little or no effect in many natural infections that spread by cell-to-cell contact, including those caused by many neurotropic viruses that spread transsynaptically, or by viruses that establish long-term or noncytolytic infections, such as the hepatitis viruses. These infections can be stopped only by CTL-produced antiviral cytokines and direct killing of infected cells.

CTLs Lyse Virus-Infected Cells

CTLs are superbly equipped to kill virus-infected cells, and following lysis of an infected cell, they can detach and kill again. Signaling from the T cell receptor pursuant to engagement of the peptide antigen-MHC complex requires clustering (aggregation) of a number of T cell receptors and reorganization of the T cell cytoskeleton to form a specialized structure called the **immunological synapse** (Fig. 4.17; see also Fig. 7.14). Only after these reactions have taken place can the CTL lyse an infected cell.

The term "immunological synapse" was coined because the proteins that mediate target and T cell recognition show an unexpected degree of spatial organization at the site of T cell–target cell contact, not unlike a neuronal synapse. The synapse structure contributes to stabilizing signal transduction by the T cell receptor for the prolonged periods required for activation of gene expression. In addition, membrane proteins in the synapse engage the underlying cytoskeleton and polarize the secretion apparatus so that a high local concentration of effector molecules is attained. Small numbers of peptide ligands bound to MHC class I molecules apparently can stimulate a T cell because they serially engage many T cell receptors on the opposing cell surface within the synapse. Unengaged T cell receptors subsequently entering this zone have an increased likelihood of binding a specific ligand and amplifying a signal.

Given the central roles of the T cell receptor and formation of the immunological synapse in adaptive immune defense, it should come as no surprise that viral gene products can alter the structure, function, and localization of the T cell receptor and the various coreceptors. Indeed, infection by

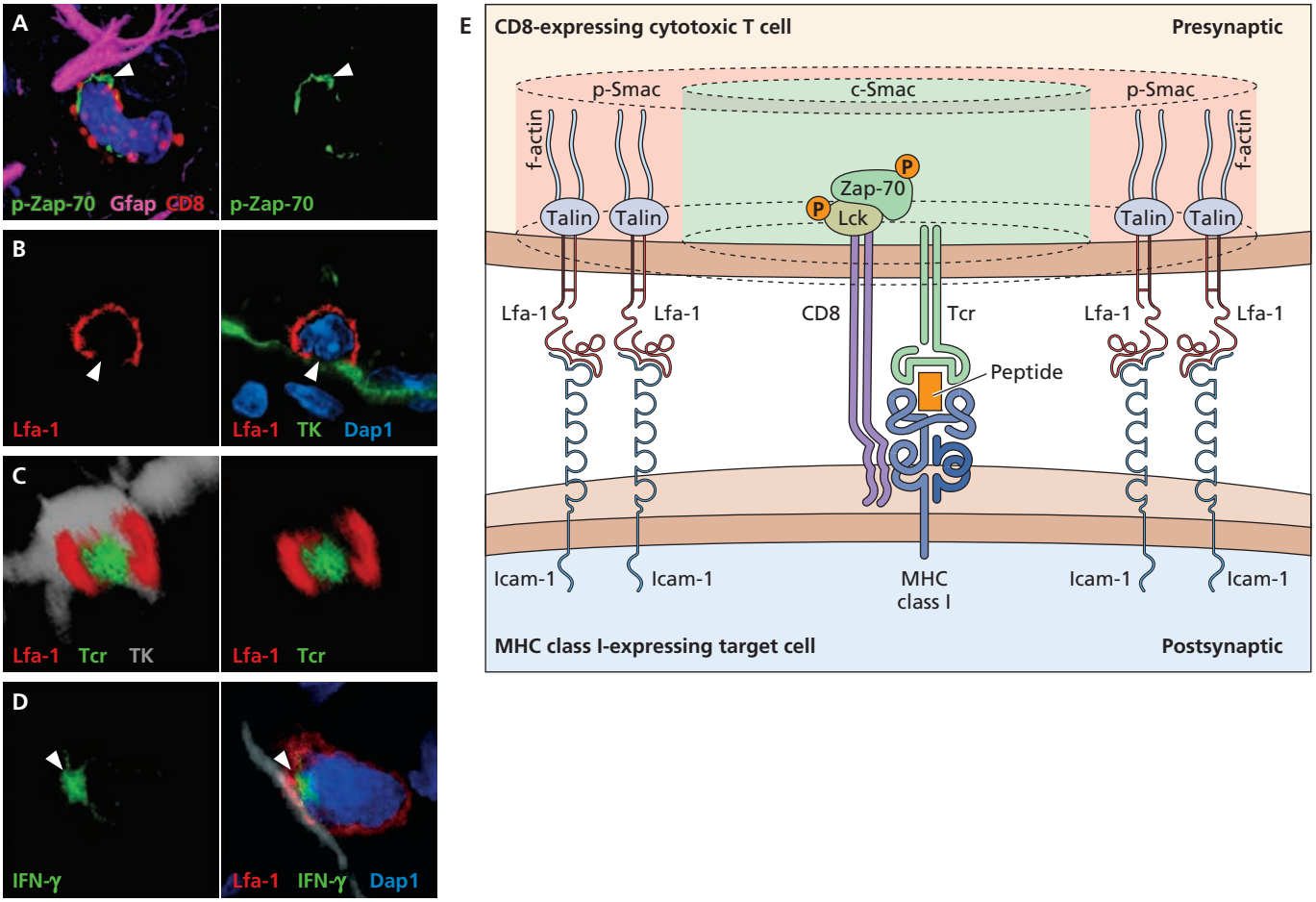


Figure 4.17 The immunological synapse. (A–D) The morphological characteristics of an *in vivo* immunological synapse between $CD8^+$ CTLs and adenovirus-infected astrocytes is illustrated. The striatum of rats was injected with a recombinant adenovirus vector carrying an expression cassette for the herpes simplex virus thymidine kinase (TK) gene. **(A)** Interaction between a $CD8^+$ CTL (red) and an infected astrocyte (Gfap, magenta [which marks astrocytes]) stimulates T cell receptor (Tcr) signaling, resulting in phosphorylation and polarization of tyrosine kinases such as Zap70 (green) toward the interface with the infected cell. The white arrow indicates polarized pZap70; blue stain (Dap1) indicates nuclei. **(B)** Adhesion molecules such as Lfa-1 (red) aggregate to form a peripheral ring (p-SMAC [peripheral supramolecular activation cluster]) at the junction formed by the immunological synapse. The postsynaptic astrocyte process can be identified by staining with antibody to TK, a marker of adenovirus infection (green). Note the characteristic absence of Lfa-1 at the central portion of the immunological synapse between the T cell and the infected astrocyte (white arrow). **(C)** A rotated image from a three-dimensional reconstruction demonstrates the typical central polarization (c-SMAC) of Tcr molecules (green) toward the infected astrocyte (TK, white) and the peripheral distribution of Lfa-1 in the p-SMAC (red). **(D)** The effector molecule IFN-γ (green) within a Tcr^+ (red) CTL is directed toward the site of close contact with an infected target cell (TK, white); the white arrow indicates the T-target cell contact zone. The diameter of a CTL is ~10 μm. **(E)** Schematic cross section of an immunological synapse showing the characteristic polarized arrangement of the cytoskeleton (actin and talin proteins indicated) and organization of the adhesion molecule Lfa-1 toward the p-SMAC. The Tcr molecules are directed toward the c-SMAC. The phosphorylated TKs (Zap70 and Lck) and effector IFN-γ molecules (not shown) are in the center of the immunological synapse. See C. Barcia et al., *J Exp Med* **203:**2095–2107, 2006. Figure coutesy of Pedro Lowenstein, Kurt Kroeger, and Maria Castro.

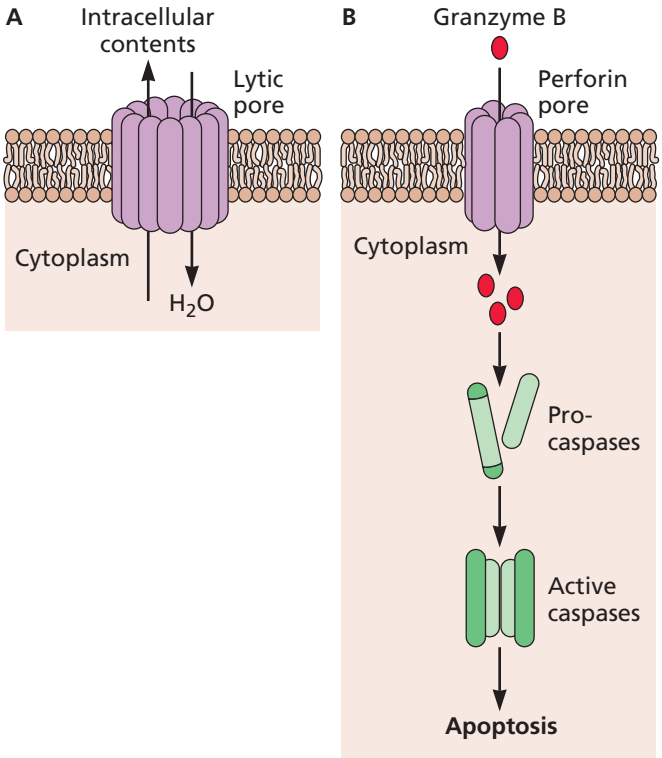
several members of the *Retroviridae* and *Herpesviridae* leads to reduction of T cell receptor function by inhibiting the synthesis of one or more of the T cell receptor protein subunits. Viral infection can also modulate the abundance of various accessory molecules on cell surfaces and therefore alter CTL recognition and subsequent effector function.

CTLs kill by two primary mechanisms: transfer of cytoplasmic granules from the CTL to the target cell, and induction of apoptosis. These killing systems develop during cellular differentiation. The maturing CTL fills with cytoplasmic granules that contain macromolecules required for lysis of target cells, such as perforin, a membrane pore-forming

protein; and granzymes, members of a family of serine proteases. Granules are released by CTLs in a directed fashion when in membrane contact with the target cell, and are taken up by that cell via receptor-mediated endocytosis. Perforin, as its name implies, punctures holes in the plasma membrane, allowing access of granzymes that induce apoptosis in the infected cell (Fig. 4.18). CTL killing by the perforin pathway is rapid, occurring within minutes after contact and recognition. Activated CTLs can also induce apoptotic cell death via binding of the Fas ligand on their surfaces to the Fas receptor on target cells, although this pathway is much slower than perforin-mediated killing. Many activated CTLs also secrete IFN-γ, which, as we have seen, is a potent inducer of both the antiviral state in neighboring cells and synthesis of MHC class I and II proteins (Box 4.5). Activated CTLs also secrete powerful cytokines, including IL-16, and chemokines such as CCl5. Their release by virus-specific CTLs following recognition of an infected target cell may assist in coordination of the antiviral response.

Typically, following infection by a cytopathic virus, CTL activity appears within 3 to 5 days, peaks in about a week,

Figure 4.18 CTL lysis. Granzymes participate in target cell apoptosis in association with perforin. Granzymes diffuse into the target cell cytoplasm upon perforin "puncturing," where they activate caspases, leading to apoptotic death. Pores can either allow release of cytoplasmic contents **(A)** or injection of granzymes that result in cleavage of procaspases, leading to cell death **(B)**.



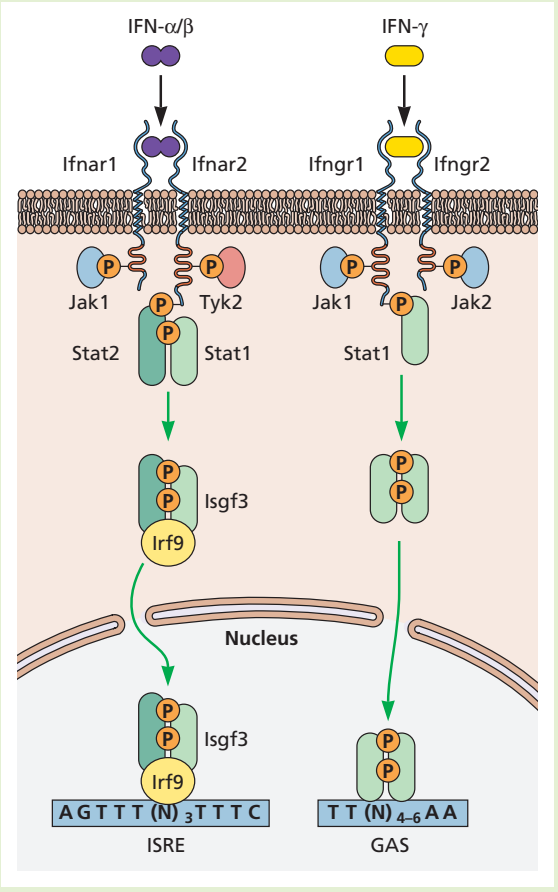
BOX 4.5

BACKGROUND

Interferon γ signaling

IFN-γ is the only type II member of the interferon family, which also comprises type I (IFN-α/β) and type III IFNs. Unlike type I IFNs, which are expressed by most cells soon after infection, IFN-γ is produced chiefly by activated cells of the immune system such as NK cells and T cells. IFN-γ initiates a cellular response by binding to the IFN-γ receptor, Ifngr (a heterotetramer of the Ifngr1 and Ifngr2 subunits). Binding triggers activation of receptor-associated Janus kinases (Jaks) 1/2 and subsequent phosphorylation of tyrosine in the cytoplasmic tail of the Ifngr1 subunits. Signal transducer and activator of transcription (Stat) 1 is recruited to the phosphorylated Ifngr1 subunit, where Stat1 becomes phosphorylated, homodimerizes, and translocates to the nucleus. The phosphorylated Stat1 homodimer binds to IFN-γ-activated site (GAS) elements within IFN-γ-responsive genes (Isgs) to initiate transcription. More than 250 genes are induced in this manner to inhibit viral spread. While Stat1 is required for a classical IFN-γ response, a substantial number of studies have also demonstrated the presence of IFN-γ-dependent, Stat1-independent pathways.

The classical pathways of type I and type II IFN signaling are shown for comparison. Irf9, IFN regulatory factor 9; Isgf3, IFN-stimulated gene factor 3; ISRE, IFN-stimulated response element; P, phosphate.



and declines thereafter. The magnitude of the CTL response depends on such variables as viral titer, route of infection, and age of the host. The critical contribution of CTLs to antiviral defense is demonstrated by **adoptive transfer** experiments in which virus-specific CTLs from an infected animal can confer protection to nonimmunized recipients following virus inoculation. However, CTLs can also cause direct harm by large-scale cell killing. Such immunopathology often follows infection by noncytopathic viruses, when cells can be infected yet still function. For example, the liver damage, cirrhosis, and hepatocellular cancer associated with infection by hepatitis viruses is actually caused by CTL killing of persistently infected liver cells and the consequent necessity for their constant regeneration (Chapter 5).

Control of CTL Proliferation

Massive CTL precursor expansions after acute primary infections by viruses such as lymphocytic choriomeningitis virus and Epstein-Barr virus have been identified (Box 4.6). For example, >50% of CTLs from the spleen of a lymphocytic choriomeningitis virus-infected mouse were specific for a **single** viral peptide. The response reached a maximum 8 days after infection, but up to 10% of virus-specific T cells were still detectable after a year postchallenge. Such results are in contrast to those for hepatitis B virus or human immunodeficiency virus infection, in which <1% of the CTLs from spleens of infected patients are specific for a single viral peptide. The basis for this large range in CTL epitope recognition is not understood.

Viral proteins can blunt the CTL response, with far-reaching effects, ranging from rapid onset of severe symptoms and death of the host to long-lived, persistent infections (Chapter 5). Many of these proteins confound CTL recognition by disguising or reducing antigen presentation by MHC class I molecules. In the case of human immunodeficiency virus type 1, the viral genome encodes three proteins that interfere with CTL action: Nef and Tat induce Fas ligand production and subsequent Fas-mediated apoptosis of CTLs, whereas Env engages the CXCr4 chemokine receptor, triggering the death of the CTL. Nef also reduces MHC class I presentation by cell surface depletion and lysosomal degradation of the protein (Fig. 7.10). Human cytomegalovirus-infected cells contain at least six viral proteins that interfere with the MHC class I pathway and also evoke apoptosis of virus-specific CTLs by increasing synthesis of Fas ligand (Chapter 5).

Noncytolytic Control of Infection by T Cells

Complete clearance of intracellular viruses by the adaptive immune system does not depend solely on the destruction of infected cells by CTLs. The production of cytokines, such as IFN-γ and Tnf-α, by these cells can lead to purging of viruses from infected cells without cell lysis. This mechanism requires that the infected cell retain the ability to activate antiviral pathways induced by binding of these cytokines to their receptors, and that viral reproduction is sensitive to the resulting antiviral response.

In certain circumstances, such as infection of the liver by hepatitis B and C viruses, there are orders of magnitude more infected cells than there are virus-specific CTLs. Furthermore, when nonrenewable cell populations, such as neurons, are infected, CTL killing would do more harm to the host than good (Box 4.7). In such circumstances, cytokine-mediated viral clearance represents an optimal strategy, and the results can be highly effective: when hepatitis B virus-specific CTLs are experimentally transferred to another animal (via adoptive transfer), the IFN-γ and Tnf-α produced appear to clear the infection from thousands of cells without their destruction.

The resolution of infections by noncytolytic viruses via CTL-produced cytokines such as IFN-γ and Tnf-α has been documented for many DNA and RNA viruses. Indeed, CTL production of powerful, secreted, antiviral cytokines provides a simple explanation for how CTLs are able to control massive numbers of infected cells. Additional cytokines produced by a variety of immune system cells are likely to participate in such viral clearance. $CD4^+$ T cells can also clear some infections with noncytolytic viruses with little involvement of CTLs. Such cases include infections by vaccinia virus, vesicular stomatitis virus, and Semliki Forest virus.

Rashes and Poxes

Many infections, including those caused by measles virus, smallpox virus, and varicella-zoster virus, produce a characteristic rash or lesion over extensive areas of the body (Fig. 4.19), even though the primary infection began at a distant mucosal surface. This phenomenon results when the primary infection escapes the local defenses and virus particles or infected cells spread in the circulatory system to initiate foci of infected cells in the skin. T_h1 cells and macrophages activated by the initial infection home to these secondary sites and respond by aggressive synthesis of cytokines, including IL-2 and IFN-γ. Such cytokines then act locally to increase capillary permeability, which is partially responsible for a characteristic local immune response referred to as **delayed-type hypersensitivity**. This response, which usually requires 2 to 3 days to develop, is the basis of many virus-promoted rashes and lesions with fluid-filled vesicles.

The Humoral (Antibody) Response

Antibodies Are Made by Plasma Cells

After a B cell emerges from the bone marrow into the circulation, it travels to lymph and lymphoid organs and differentiates and synthesizes antibody only if its surface antibody receptor is bound to the cognate antigen. Antigen binding causes clustering of receptors on the B cell surface, which then

BOX 4.6

METHODS

Measuring the antiviral cellular immune response

The Classic Assays: Limiting Dilution and Chromium Release

For more than 50 years, virologists have assessed the presence of CTLs in blood, spleen, or lymphoid tissues of immune animals by using the limiting-dilution assay and radioactive chromium release from lysed target cells. This assay measures $CD8^+$ CTL precursors, or memory CTLs, based on two attributes: (i) the ability of foreign proteins and peptides to stimulate CTL proliferation and (ii) the capacity of activated CTLs to lyse target cells.

Lymphocytes are obtained from an animal that has survived virus infection and are cultured for 1 to 2 weeks in the presence of whole inactivated virus, its proteins, or synthetic viral peptides. Under these conditions, virus-specific CTL precursors are induced to divide (clonal expansion). The expanded CTL population is then tested for its ability to destroy target cells that display viral peptides on MHC class I molecules. The target cells loaded with the viral antigen in question are then exposed to chromium-51, a radioactive isotope that binds to most intracellular proteins (Panel A). After being washed to remove external isotope, cultured CTLs are incubated with the target cells, and lysis is measured by the release of chromium-51 into the supernatant. Within the last decade, nonradioactive substrates have become commercially available; these alternatives function in the same manner and offer many of the same attributes, but are much safer than ^{51}Cr.

Serial dilution before assay provides an estimate of the number of $CD8^+$ CTL precursors in the original cell suspension (providing the name of the assay: "limiting dilution"). This assay provides a quantitative measure of cellular immunity, but it is time-consuming, technically demanding, and expensive.

Identifying and Counting Virus-Specific T Cells

The limiting-dilution assay defines T cells by function, but not by their peptide specificity. Until recently, investigators have tried with little success to identify individual T cells based on their peptide recognition properties. This failure has been attributed to the low affinity and high "off" rates of the MHC–peptide–T cell receptor interaction. Without this information, it was difficult, if not impossible, to measure antigen-specific T cell responses.

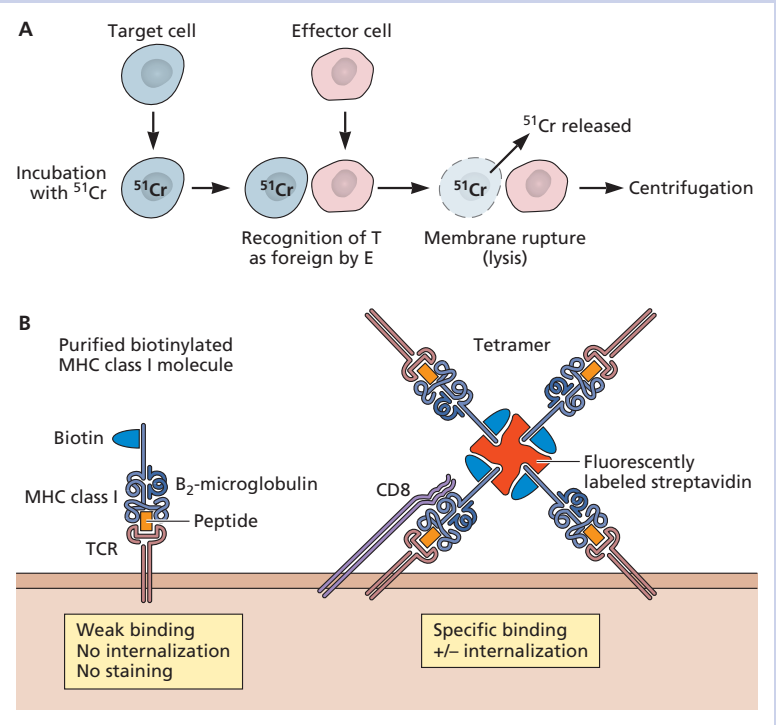
An important advance is the use of an **artificial MHC tetramer** as an epitope-specific, T cell-staining reagent (Panel B). The extracellular domains of MHC class I proteins are produced in *Escherichia coli*. These engineered MHC class I molecules have an artificial C-terminal 13-amino-acid sequence that enables them to be biotinylated. The truncated MHC class I proteins are folded *in vitro* with a synthetic peptide that will be recognized by a specific T cell. Biotinylated tetrameric complexes are purified and mixed with isolated T cell pools from virus-infected animals. Individual T cells that bind the biotinylated MHC class I-peptide complex are detected by a variety of immunohistochemical techniques. Cell-sorting techniques can be used, and stained cells are viable.

The **intracellular cytokine assay** is a relatively rapid method to count specific CTLs. Fresh lymphoid cells are treated with brefeldin A. This fungal metabolite blocks the secretory pathway and prevents the secretion of cytokines. The cells are then fixed with a mild cross-linking chemical that preserves protein antigenicity, such as glutaraldehyde. Treated cells are permeabilized so that a specific antibody for a given cytokine can react with the intracellular cytokines. Cells that react with the antibody can be quantified by fluorescence-activated cell sorting. With appropriate software and calibration, the staining intensity corresponds to cytokine concentration, and the number of cells responding to a particular epitope and MHC class I molecule can be determined.

Measuring the Antiviral Antibody Response

Antibodies are the primary effector molecules of the humoral response. There are many methods to detect antibodies. However, a standard method in virology is the **neutralization assay**: viral infectivity is determined in the presence and absence of antibody. Two variations on this general theme include the **plaque reduction assay** and the **neutralization index**. In the former, a known number of PFU are exposed to serial dilutions of the antibody or serum in question. The highest dilution that will reduce the plaque count by 50% is taken as the plaque reduction titer of the serum or antibody. The index is calculated as the difference in viral titers in the presence and absence of test antibody or serum. Obvious requirements for these assays are that the virus in question can be propagated in cultured cells and that a measure of virus infectivity such as plaque formation is available.

Klenerman P, Cerundolo V, Dunbar PR. 2002. Tracking T cells with tetramers: new tales from new tools. *Nat Rev Immunol* 2:263–272.



BOX 4.7

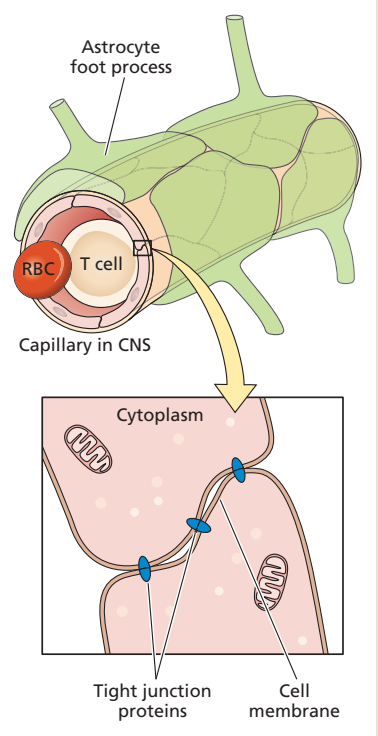
DISCUSSION

The immune system and the brain

Cells of the central nervous system (CNS; brain and spinal cord) can initiate a robust and transient intrinsic defense (for example, by the production of type I IFNs), but, surprisingly, cannot mount an adaptive response. A primary reason for this deficit is that the CNS lacks lymphoid tissue and dendritic cells. In addition, the CNS of vertebrates is separated from many cells and proteins of the bloodstream by tight endothelial cell junctions that comprise the so-called blood-brain barrier (see the figure). As a consequence of these features, viral infections of the CNS can have unexpected outcomes. For example, if virus particles are injected directly into the ventricles or membranes covering the brain, which are in contact with the bloodstream, the innate immune system is activated and a strong inflammatory and an adaptive response occur. In contrast, if virus particles are injected directly into brain tissue, avoiding the blood vessels and ventricles, only a transient inflammatory response is produced. The adaptive response is not activated; antibodies and antigen-activated T cells are not produced.

Although the CNS is unable to initiate an adaptive immune response, it is not isolated from the immune system, and hence an earlier concept of the brain being "immune privileged" has fallen out of favor. Indeed, the blood-brain barrier is open to entry of activated immune cells circulating in the periphery. Antigen-specific T cells regularly enter and travel through the brain, performing immune surveillance. Moreover, at least two glial cell types, astrocytes and microglia, have a variety of cell surface receptors that can engage the T cells. Astrocytes, the most numerous cell type in the central nervous system, respond to a variety of cytokines made by immune system cells. All natural brain infections begin in peripheral tissue, and any infection that begins outside the CNS activates the adaptive immune response. However, if the infection spreads to the brain, the resulting immune attack on this organ can be devastating.

Virus particles can be injected experimentally into the brain directly without infecting peripheral tissues; in such cases, an adaptive response is completely avoided. On the other hand, if the animal is first immunized by injecting virus particles into a peripheral tissue, such that the adaptive response is activated, subsequent injection of the same virus into the brain of the immunized animal elicits massive immune attack on that organ. Any virus-infected target in the brain is recognized and destroyed by the peripherally activated T cells. In both natural and experimental infections, the inflammatory response is not transient but sustained, resulting in capillary leakage, swelling, and cell death. Uncontrolled inflammation in the closed confinement of the skull has many deleterious consequences, and when it is coupled with bleeding and cell death, the results are disastrous. In such extreme cases, the swollen brain has nowhere to go but to be extruded out the foramen at the base of the skull.



Cross section of a brain capillary. Capillaries within the central nervous system are comprised of tightly packed endothelial cells and astrocyte processes on the "brain" side of the capillary. This barrier prevents free access of blood-borne proteins and cells from the blood to the brain (termed the "blood-brain barrier"), though this barrier is permeable to activated lymphocytes.

Figure 4.19 Virus-induced rashes and poxes.



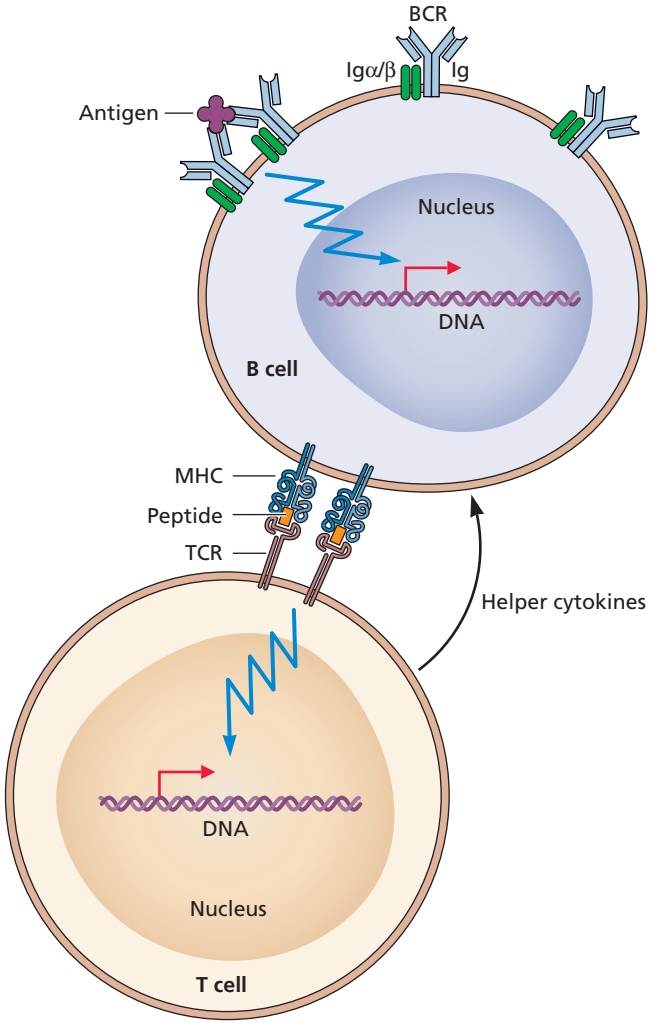


Figure 4.20 Activation of B cells to produce antibodies. When antigen binds and causes clustering of B cell receptors (BCRs), a signal transduction cascade is triggered that leads to the activation of the B cell and production of soluble antibodies. Peptides derived from the antigen bound to MHC class II are transported to the cell surface, where they are bound by T_h-cell receptors, leading to T cell activation. In turn, the activated T cell provides cytokine "help."

activates signaling via Src family tyrosine kinases that associate with the cytoplasmic domains of the closely opposed receptors. B cell coreceptors, such as CD19, CD21, and CD8, enhance signaling by recruiting tyrosine kinases to clustered antigen receptors and coreceptors (Fig. 4.20).

Binding of antigen to the B cell receptor is only part of the activation process: cytokines from T_h cells are also required. When the T cell receptor of T_h2 cells recognizes MHC class II-peptide complexes present on the B cell surface, the T_h2 cells produce a locally high concentration of stimulatory cytokines, as well as CD40 ligand (a protein homologous to Tnf). The engagement of CD40 ligand with its B cell receptor facilitates a local exchange of cytokines that further stimulates proliferation of the activated B cell and promotes its differentiation. Fully differentiated, antibody-producing plasma cells make huge quantities of specific antibodies: the rate of synthesis of IgG can be as high as 30 mg/kg of body weight/day. For a human of ~50 kg, this value equates to >1 g of antibody made every 24 h.

Types and Functions of Antibodies

All antibodies (immunoglobulins) have common structural features, illustrated in Fig. 4.21. Five classes of immunoglobulin (IgA, IgD, IgE, IgG, and IgM) are defined by their distinctive heavy chains (α, δ, ϵ, γ, and μ, respectively). Their properties are summarized in Table 4.1. IgG, IgA, and IgM are commonly produced after viral infection. During B cell differentiation, "switching" of the constant region of heavy-chain genes occurs by somatic recombination and is regulated in part by specific cytokines, including IL-4 and IL-5, which bind to their receptors on the target B cell. Importantly, while the Fc region of these antibodies changes, the antigen-binding region (the Fab portion of the antibody) does not, and hence the specificity of the antigen remains the same. Class switching allows for changes in the effector functions of the antibodies and increases their functional diversity.

During the **primary antibody response**, which follows initial contact with antigen, the production of antibodies follows a characteristic sequence. The IgM antibody appears first, followed by IgA on mucosal surfaces or IgG in the serum. The IgG antibody is the major antibody of the response and is remarkably stable, with a half-life of 7 to 21 days. Specific IgG molecules remain detectable for years, because of the presence (and occasional reactivation) of memory B cells. A subsequent challenge with the same antigen or viral infection promotes a rapid secondary antibody response (Fig. 4.22).

Virus Neutralization by Antibodies

Antibodies contribute to antiviral defense chiefly by binding to, and causing the elimination of, free virus particles. Viruses that infect mucosal surfaces or that circulate in the blood will be exposed to IgA antibodies and IgG and IgM antibody molecules, respectively. For example, immunodeficient animals can be protected from some lethal viral infections by injection with virus-specific antiserum or purified monoclonal antibodies (also called passive immunization). A therapy based on this principle was used in the recent Ebola outbreak in 2014. The experimental therapeutic, ZMapp, comprises a cocktail of three Ebola virus-specific monoclonal antibodies that was shown to have efficacy in preventing Ebola virus disease in macaques experimentally infected with Ebola virus. At the time of the outbreak, it had not been tested in humans. Because no other therapeutic options were available, ZMapp was used to treat

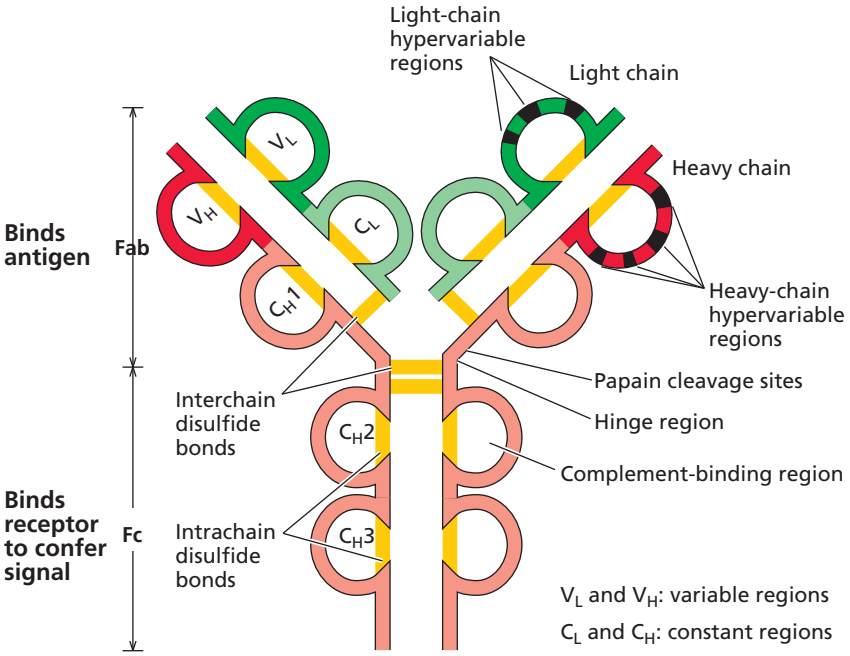


Figure 4.21 The structure of an antibody molecule. This is a schematic representation of an IgG molecule delineating the subunit and domain structures. The light and heavy chains are held together by disulfide bonds (yellow bars). The variable regions of the heavy (V_H) and light (V_L) chains, as well as the constant regions of the heavy (C_H) and light (C_L) chains, are indicated at the left part of the molecule. The hypervariable regions and invariable regions of the antigen-binding domain (Fab) are emphasized. The constant region (Fc) performs many important functions, including complement binding (activation of the classical pathway) and binding to Fc receptors found on macrophages and other cells. Clusters of papain protease cleavage sites are indicated, as this enzyme is used to separate the Fab and Fc domains.

seven Ebola virus-infected Americans; of these, five individuals survived. While these data are not sufficiently robust to show efficacy, many believe that postexposure treatment with such preparations or derivatives will be an effective method to prevent disease in Ebola virus-exposed individuals.

Perhaps the best example of the importance of antibodies in antiviral defense is the success of the poliovirus vaccine in preventing poliomyelitis, as the type of antibody produced can significantly influence the outcome of a poliovirus infection. Poliovirus infection stimulates strong IgM and IgG responses in the blood, but it is mucosal IgA that is vital in defense. This antibody isotype can neutralize poliovirus directly in the gut, the site of primary infection. The live attenuated Sabin poliovirus vaccine is effective because it elicits a strong mucosal IgA response. This antibody type is synthesized by plasma cells that underlie the mucosal epithelium. This antibody is secreted as dimers of two conventional immunoglobulin subunits. The dimers then bind the polymeric immunoglobulin receptor on the basolateral surface of epithelial cells (Fig. 4.23). This complex is then internalized by endocytosis and moved across the cell (**transcytosis**) to the apical surface. Protease cleavage of the receptor releases dimeric IgA into mucosal secretions, where it can interact with incoming virus particles.

IgA may also block viral reproduction inside infected mucosal epithelial cells (Fig. 4.23). Because IgA molecules

Table 4.1 The five classes of immunoglobulins

Property	IgA	IgD	IgE	IgG	IgM
Function	Mucosal; secretory	Surface of B cell	Allergy; anaphylaxis; epithelial surfaces	Major systemic immunity; memory responses	Major systemic immunity; primary response; agglutination
Subclasses	2	1	1	4	1
Light chain	κ, λ	κ, λ	κ, λ	κ, λ	κ, λ
Heavy chain	α	δ	ϵ	γ	μ
Concn in serum (mg/ml)	3.5	0.03	0.00005	13	1.5
Half-life (days)	6	2.8	2	25	5
Complement activation					
Classical	−	−	−	+	++
Alternative	−/+	−	+	−	−

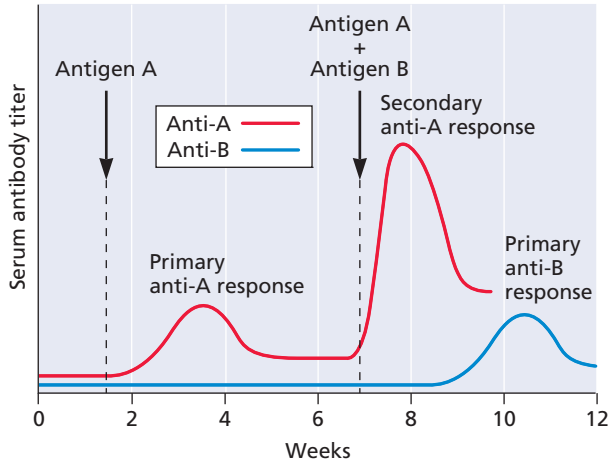


Figure 4.22 The specificity, self-limitation, and memory of the adaptive immune response. This general profile of a typical adaptive antibody response demonstrates the relative concentration of serum antibodies after time (weeks) of exposure to antigen A or a mixture of antigens A and B. The antibodies that recognize antigens A and B are indicated by the red and blue lines, respectively. The primary response to antigen A takes about 3 to 4 weeks to reach a maximum. When the animal is injected with a mixture of both antigens A and B at 7 weeks, the secondary response to antigen A is more rapid and more robust than the primary response. However, the primary response to antigen B again takes about 3 to 4 weeks. These properties demonstrate immunological memory. Antibody levels (also termed titers) decline with time after each immunization. This property is called self-limitation or resolution. Reprinted from A. K. Abbas et al., *Cellular and Molecular Immunology* (The W. B. Saunders Co., Philadelphia, PA, 1994), with permission.

must pass through such a cell en route to secretion from the apical surface, they are available during transit for interaction with viral proteins produced within the cell. The antigen-binding domain of intracellular IgA lies in the lumen of the ER, the Golgi compartment, and any transport vesicles of the secretory pathway. It can therefore bind to the external domain of any type I viral membrane protein that has the cognate epitope of that IgA molecule. Such interactions have been demonstrated with Sendai virus and influenza virus proteins during infection of cells in culture. In these experiments, antibodies colocalized with viral antigen only when the IgA could bind to the particular viral envelope protein. These studies suggest that clearing viral infection from mucosal surfaces need not be limited to the lymphoid cells of the adaptive immune system.

It is widely assumed that the primary mechanism of antibody-mediated neutralization of viruses is via steric blocking of virus particle-receptor interaction (Fig. 4.24). While some antibodies do prevent virus particles from attaching to cell receptors, the vast majority of virus-specific antibodies are likely to interfere with the concerted structural changes that are required for entry. Antibodies can also promote aggregation of virus particles, thereby reducing the effective concentration of viruses that can initiate infection. Many enveloped viruses can be destroyed *in vitro* when antiviral antibodies and serum complement disrupt membranes (the classical complement activation pathway).

Much of what we know about antibody neutralization comes from the isolation and characterization of "antibody escape" mutants or **monoclonal antibody-resistant mutants**. These mutants are selected by propagating virus in the presence of neutralizing antibody. The analysis of the mutant viruses allows a precise molecular definition, not only of antibody-binding sites but also of parts of viral proteins important for entry. Antigenic drift (see Chapters 5 and 10) is a consequence of selection and establishment of antibody escape mutants in viral populations.

Antibodies can provoke other remarkable responses in virus-infected cells. For example, in a process analogous to CTL lysis, antibodies that bind to the surface proteins of many enveloped viruses can clear the particles from persistently infected cells. This process is noncytolytic and complement independent. In this case, antibodies act synergistically with IFN and other cytokines. Virus-specific antibodies bound to surfaces of infected cells can inhibit virus budding at the plasma membrane and also reduce surface expression of viral membrane proteins by inducing endocytosis.

Nonneutralizing antibodies are also prevalent after infection: they bind specifically to virus particles, but do not interfere with infectivity. In some cases, such antibodies can even enhance infectivity: antibody bound to virus particles is recognized by Fc receptors on macrophages, and the entire complex is brought into the cell by endocytosis. This process, antibody-dependent enhancement, is the basis of disease following a secondary exposure to dengue virus (Chapter 5).

Antibody-Dependent Cell-Mediated Cytotoxicity: Specific Killing by Nonspecific Cells

T_h1 stimulation results in production of a particular isotype of IgG in B cells that can bind to antibody receptors on macrophages and some NK cells. These receptors are specific for the more conserved Fc region of an antibody molecule (Fig. 4.21). If an antiviral antibody is bound in this manner, the amino-terminal antigen-binding site is still free to bind viral antigen on the surface of the infected cell. In this way, the antiviral antibody targets the infected cell for elimination by macrophages or NK cells. This process is called **antibody-dependent cell-mediated cytotoxicity** (often referred to as ADCC). The antibody provides the specificity for killing by a less discriminating NK cell. Efforts are under way to harness the power of this process in the development of a universal influenza virus vaccine.

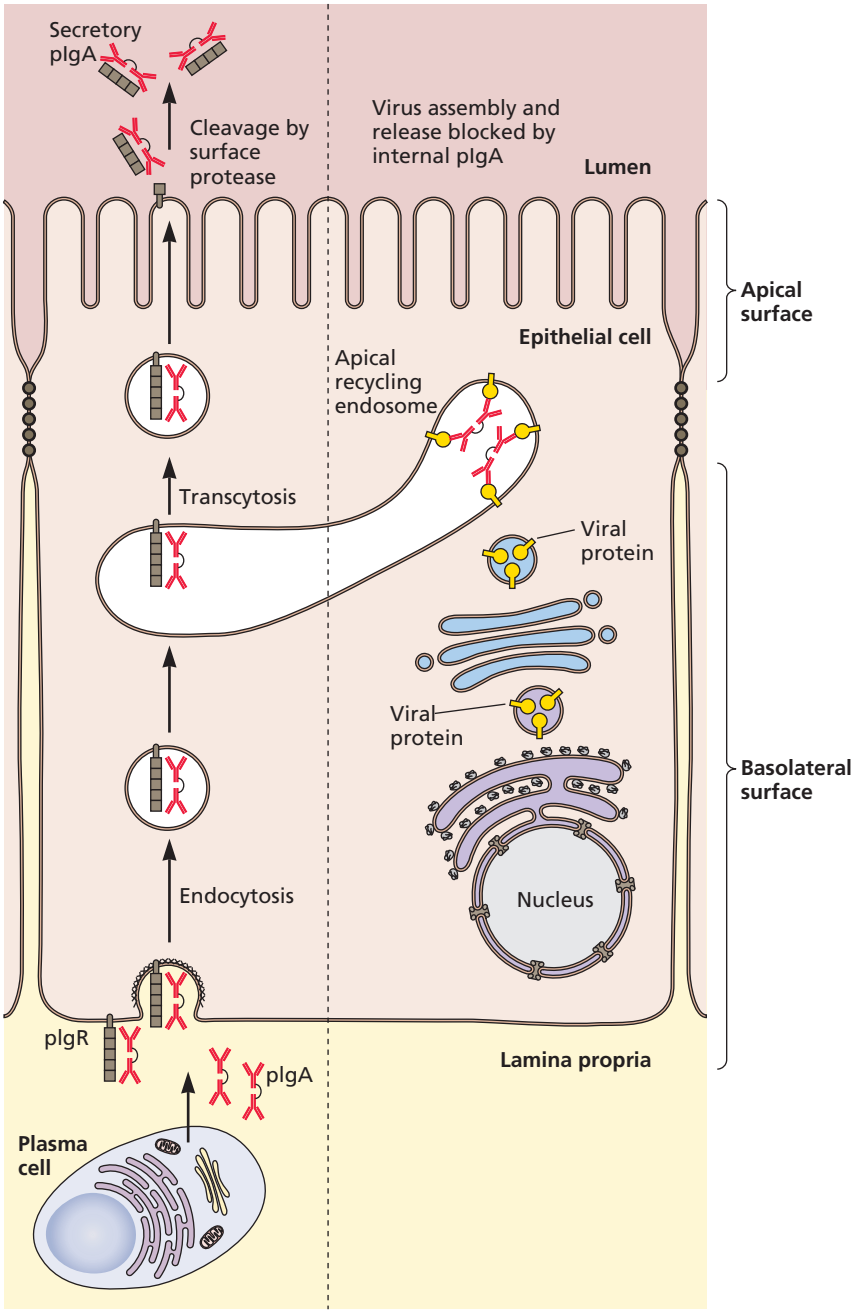


Figure 4.23 Secretory antibody, IgA, is critical for antiviral defense at mucosal surfaces. A single polarized epithelial cell is illustrated. The apical surface is shown at the top, and the basal surface is shown at the bottom. (Left) Antibody-producing B cells (plasma cells) in the lamina propria of a mucous membrane secrete the IgA antibody (also called polymeric IgA [pIgA]). pIgA is a dimer, joined at its Fc ends. For IgA to be effective in defense, it must be moved to the surface of the epithelial cells that line body cavities. This process is called transcytosis. (Right) A virus particle infecting an epithelial cell potentially can be bound by internal IgA if virus components intersect with the IgA in the lumen of vesicles during transcytosis. This process is likely to occur for enveloped viruses, as their membrane proteins are processed in many of the same compartments as those mediating transcytosis. Adapted from M. E. Lamm, *Annu Rev Microbiol* 51:311–340, 1997, with permission.

Immunological Memory

Once a specific response has been established and the viral infection is subdued, the individual is immune to subsequent infection by the same pathogen. Immunological memory of previous infections is one of the most powerful features of the adaptive immune system, and makes vaccines possible. While the primary adaptive response takes days to reach its antiviral potential, a subsequent encounter with the same pathogen awakens a massive reaction that occurs within hours of pathogen entry. This process occurs because a subset of B and T lymphocytes, called **memory cells**, is maintained after each encounter with a foreign antigen. These cells survive for years and are ready to respond immediately to any subsequent encounter by rapid proliferation and synthesis of their antiviral effector functions. Because such a secondary response is usually stronger than the primary one, childhood infection protects adults, and immunity conferred by vaccination can last for years, sometimes for a lifetime. An important

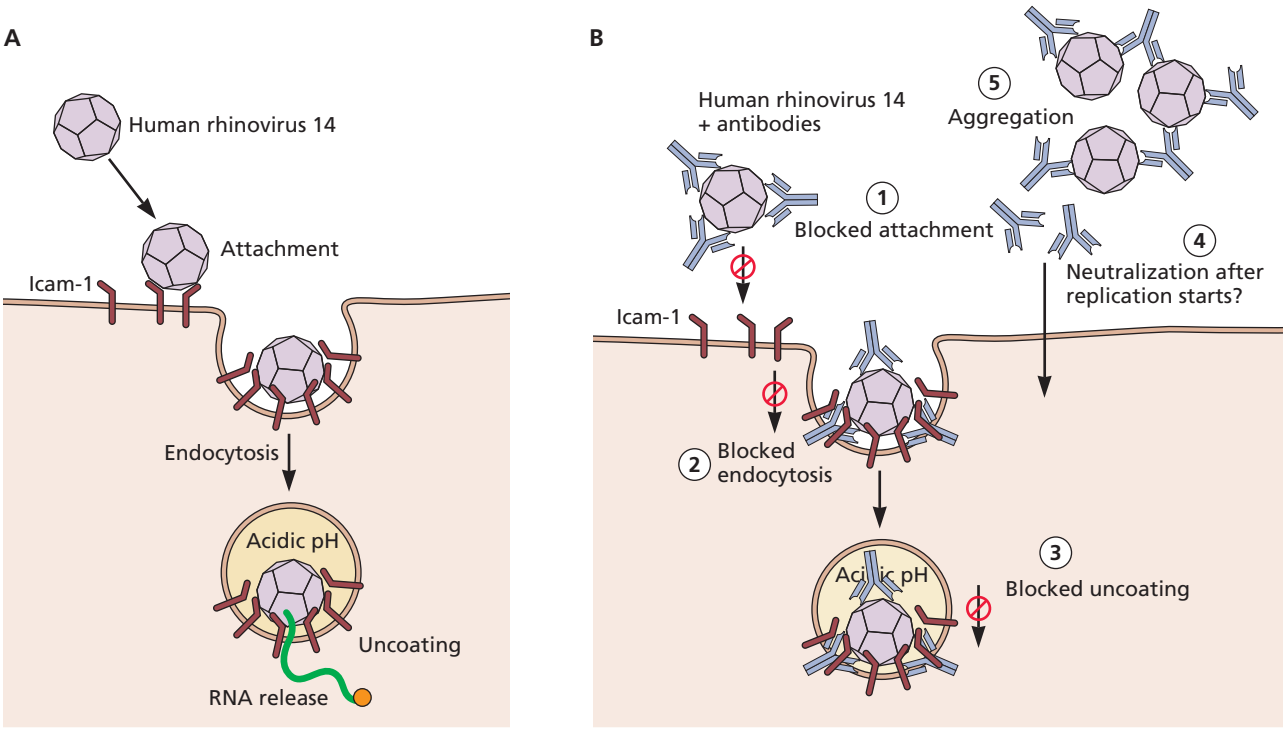


Figure 4.24 Interactions of neutralizing antibodies with human rhinovirus 14. (A) The normal route of infection. The virus attaches to the Icam-1 receptor and enters by endocytosis. As the internal pH of the endosome decreases, the particle uncoats and releases its RNA genome into the cytoplasm. **(B)** Possible mechanisms of neutralization of human rhinovirus 14 by antibodies. With well-characterized monoclonal antibodies, at least five modes of neutralization have been proposed and are illustrated: (1) blocked attachment—binding of antibody molecules to virus results in steric interference with virus-receptor binding; (2) blocked endocytosis—antibody molecules binding to the capsid can alter the capsid structure, affecting the process of endocytosis; (3) blocked uncoating—antibodies bound to the particle fix the capsid in a stable conformation so that pH-dependent uncoating is not possible; (4) blocked uncoating, inside cell—antibodies themselves may be taken up by endocytosis and interact with virions inside the cell after infection starts; (5) aggregation—because all antibodies are divalent, they can aggregate virus particles, facilitating their destruction by phagocytes. (A) Adapted from T. J. Smith et al., *Semin Virol* 6:233–242, 1995, with permission.

concept is that a memory response does not protect against reinfection, but rather against the symptoms that arise following unrestricted infection. Consequently, an individual may be exposed repeatedly to particular pathogens and never be aware of it, because the memory response eliminates the virus before illness appears.

The events that give rise to a memory response are quite similar to those that induce a primary response. The primary differences are that memory T and B cells are more abundant and more easily activated. Moreover, memory B cells produce more-effective antibodies (e.g., IgG) than the low-affinity IgM made at the beginning of a primary infection. This property, which is unique to the humoral arm of the adaptive response, is the result of somatic hypermutation of the genes encoding the virus-specific antibodies that refine and focus the antibody response after each successive exposure to the pathogen. As a result, repeated exposures strengthen B cell memory and antibody affinity.

Unlike B cells, which undergo isotype switching that distinguishes them from the primary response, the expression of the T cell receptor on memory T cells does not change. Although a comparison of the proteins that are differentially synthesized by effector and memory T cells does reveal some subtle differences in their gene expression profiles, the differences between these populations was poorly understood until recently. A major current focus in immunology is to define different types of memory T cells and to ascertain how each contributes to long-term protection of the host. Two sets of memory T cells, with distinct activation requirements, have been identified: **effector memory T cells** and **central memory T cells**. On encountering a specific viral antigen, effector memory cells quickly produce cytokines of either a T_h1 or T_h2 response. These cells are generally found in the circulatory system, and have higher concentrations of particular adhesion molecules that equip them to readily enter peripheral tissues. Central memory T cells, by contrast, are more abundant in

lymph nodes and other lymphoid organs, and have the capacity for self-renewal. Their restricted localization may ensure that a depot of memory cells is preserved in the lymph node "library" for future exposures.

Both T and B cell memory are maintained without the need for persisting antigen. As discussed in Chapter 8, it was once thought that the host may preserve a small "reminder" of previous virus encounters to restimulate memory, but this has since been shown not to be the case. While most memory cells are found in a quiescent state in an uninfected host, a small proportion are dividing. Some immunologists have hypothesized that cytokines, produced constitutively or during infections with other pathogens, may help to maintain these cells by causing some to enter the cell cycle.

Perspectives

We began this chapter with a comment on the complexity of the adaptive immune response. But as Confucius noted, "Life is simple, but we insist on making it complicated"—although the details of how virus particles are recognized and eliminated, how T cells "see" their cognate antigen, and how memory is retained for many decades are surely complicated concepts, the host defense serves to do one thing only: protect the host from pathogens. One of the features that makes the study of viruses so fascinating is how well these microbes subvert host immunity: for almost every host defense, there is a virus counter-response. In fact, much of what we know about host immunity was gleaned from the careful observation of viruses: if a viral protein that thwarts host immunity is made, it must point to an essential element of the host response.

As this third chapter focused on the host response ends, we return to the hypothetical virus infection introduced at the end of Chapter 3 (Fig. 3.25). In general, the intrinsic and innate defenses bring most viral infections to an uneventful close before the adaptive response is required. However, if viral reproduction outpaces the innate defense, a critical threshold is reached: increased IFN production by circulating immature dendritic cells elicits a more global host response, and flu-like symptoms are experienced by the infected host. As viral reproduction continues, viral antigens are delivered by mature dendritic cells to the local lymph nodes or spleen to establish sites of information exchange with T cells. T cell recirculation is shut down because of the massive recruitment of lymphocytes into lymphoid tissue. The swelling of lymph nodes that is so often characteristic of infection is a sign of this stage of immune action.

Within days, T_h cells and CTLs appear; these cells are the first signs of activation of the adaptive immune response. T_h cells produce cytokines that begin to direct the amplification of this response. The synthesis of antibodies, first of IgM and then of other isotypes, follows quickly. The relative concentrations of antibody isotypes are governed by the route of infection and the pattern of cytokines produced by the T_h cells. As the immune response is amplified, CTLs kill infected cells or purge virus from them, and antibodies bind to, and inactivate, virus particles. Specific antibody-virus complexes can be recognized by macrophages and NK cells, which induce antibody-dependent cell-mediated cytotoxicity and can also activate the classical complement pathway. Both of these processes lead to the directed killing of infected cells and virus particles by macrophages and NK cells.

An inflammatory response often occurs as infected cells die and innate and adaptive responses develop. Cytokines, chemotactic proteins, and vasodilators are released at the site of infection. These proteins, invading white blood cells, and various complement components all contribute to the swelling, redness, heat, and pain characteristic of the inflammatory response. Many viral proteins modulate this response and the subsequent activity of immune cells.

If infection spreads from the primary site, second and third rounds of virus reproduction can occur in other organs. T cells that were activated at the initial site of infection can cause delayed-type hypersensitivity (usually evident as a characteristic rash or lesion) at these later sites of infection. Immunopathology, particularly after infection by noncytopathic viruses, can result from an overly exuberant host response. Most infections are resolved: the combination of innate and adaptive responses clears the infection, and the host becomes immune because of the presence of memory T and B cells and antibodies. The high concentrations of lymphocytes drop dramatically as these cells die by apoptosis, and the system eventually returns to its preinfection state. The adaptive response can be avoided completely, or in part, when organs or tissues that have poor or nonexistent immune responses are infected, when new viral variants are produced rapidly because of high mutation rates, or when progeny virus particles spread directly from cell to cell.

Despite these formidable defenses, viruses can make us sick, and, in infections caused by human immunodeficiency virus, Ebola virus, influenza virus, and others, can result in substantial loss of life. The various ways by which viruses contribute to human disease and, in some cases, fatality are considered in Chapter 5.

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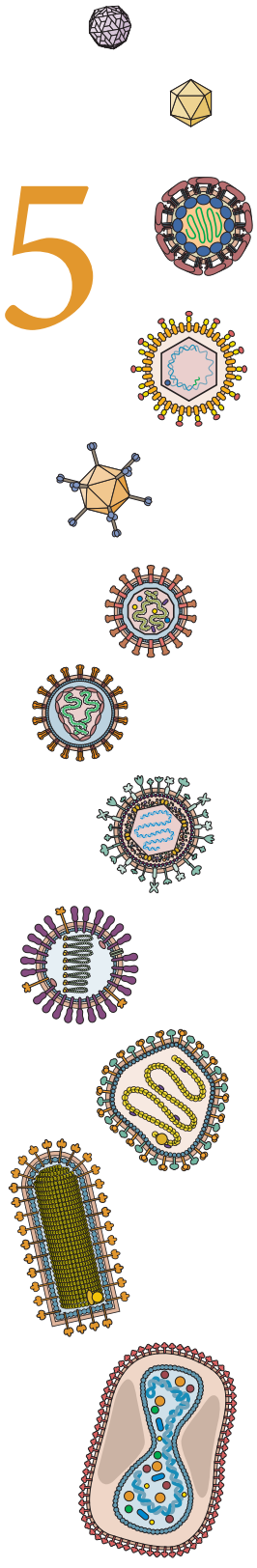
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5 Mechanisms of Pathogenesis

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LINKS FOR CHAPTER 5

- ⏭ *Video: Interview with Dr. Rafi Ahmed*
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Man is an intelligence in servitude to his organs.
ALDOUS HUXLEY

Introduction

The study of viruses has been instrumental in a multitude of fundamental discoveries in science, including revelations in basic cell biology, structural biology, the origins of cancer, and the function of immune responses. We have described the amazing diversity of viruses in terms of their structures, reproduction strategies, and methods of counteracting host defenses. Furthermore, we have emphasized that not all viruses are "bad": indeed, viruses can be used in gene therapy, or for the precise delivery of toxic payloads to cancer cells.

But for most people, the value of learning more about viruses is based on a somewhat less academic viewpoint: viruses scare us. Smallpox has killed 1 in every 20 people that have ever lived, scientists warn of the global impact of the next influenza pandemic, human immunodeficiency virus continues to be a modern plague, and Ebola virus epidemics result in high mortality and global anxiety. Virus infections of animals and crops have led to billions of dollars in lost products, and the vaccine industry has invested equally impressive resources in the development of vaccines and antivirals. Some of our fears are justified, of course, as viruses **can** kill their hosts. But it is worth reiterating that causing disease is not the purpose of any viral reproduction strategy. For example, in animal infections, cell lysis is a common mechanism for exit of virus particles from infected cells; that the loss of this particular cell may have deleterious consequences for the host is generally irrelevant for viral propagation. In some cases, virus-triggered disease is more a result of changes in the host immune response rather than the virus infection itself. Immunosuppressive viruses prolong the period in which they can reproduce unchecked: that the host may now be more vulnerable to other infections is collateral damage. Similarly, an overly aggressive antiviral response can result in immunopathology or autoimmunity. Still other viruses cause disease as a consequence of interference with normal cellular processes. For example, some nonlytic viruses inhibit specific functions of differentiated cells, such as the ability of a neuron to synthesize a particular neurotransmitter. While this effect would have little bearing on the infected cell, the consequences for the host could be considerable.

Despite the distinct ways by which viruses cause disease, infection must be viewed from a holistic perspective to understand viral pathogenesis: cell culture systems are valuable for understanding basic aspects of viral reproduction, but cannot model the complexities of infections in the host that will include numerous cells and tissues that participate in the response to the infection. In this chapter, we focus on the classic patterns of virus infection within cells and hosts, the myriad ways that viruses cause illness, and the value of animal models in uncovering new principles of viral pathogenesis.

Animal Models of Human Diseases

Viral pathogenesis refers to the adverse physiological consequences that occur as a result of viral infection of a host organism: in essence, the study of the origins of viral disease. Pathogenesis following infection is determined by many parameters in addition to the impact on the infected cells themselves. The tissues in which those cells reside, the fitness of the host response, the age, gender, health, and immunological history of the host, the size of the host population, and the environment in which it resides all are contributing components. Conclusions about the nature of pathogenesis that are derived from reductionist approaches, such as focusing on the function of a viral receptor protein in cultured cells, are frequently called into question when tested in animals (Box 5.1).

PRINCIPLES *Mechanisms of pathogenesis*

- Cell culture systems cannot replicate the complexities of infections in the host that will include numerous cells and tissues that participate in the response to the infection.
- Viral pathogenesis refers to the adverse physiological consequences that occur as a result of viral infection of a host organism.
- The laboratory mouse has been particularly useful in viral pathogenesis studies, owing to its similar physiology to humans, and our ability to manipulate the mouse genome.
- Some virus infections kill the cell rapidly (cytopathic viruses), others result in the release of virus particles without causing immediate host cell death (noncytopathic viruses), and still others remain dormant in the host cell, neither killing it nor producing any progeny.
- Antigenic variation refers to changes in virus proteins in response to antibody selection, and can arise by two distinct processes: antigenic drift that results from selection of virus particles with slightly altered surface proteins, and antigenic shift, in which particles have a major change in the surface protein(s).
- Viruses have multiple strategies to establish persistent infections, including modulation of the host response and selective reproduction in tissues with limited immune surveillance.
- Latent infections are characterized by an intact, but transcriptionally quiescent, viral genome that results in poor recognition by the host immune response.
- Viruses can cause disease by direct cell death, immunopathology, immunosuppression, oncogenesis, or more recently recognized mechanisms including molecular mimicry.
- For many noncytolytic viruses, including the hepatitis viruses and some herpesviruses, immunopathology is the primary basis of disease.

BOX 5.1

EXPERIMENTS

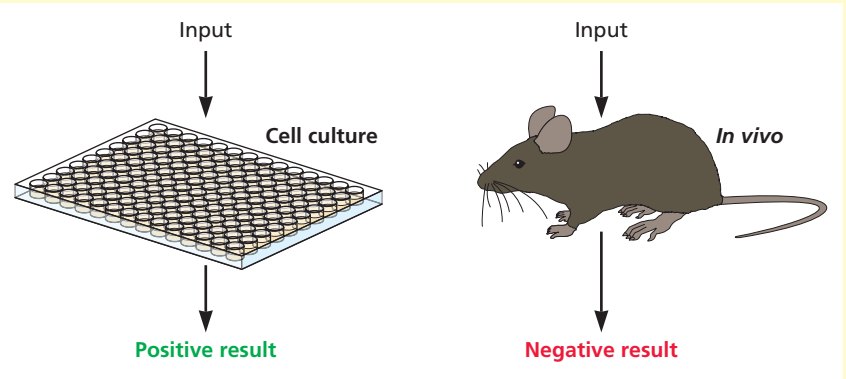
Of mice and humans

The conclusion that human influenza virus strains are preferentially bound by sialic acids attached to galactose via an α(2,6) linkage was derived by studying the binding of virus particles to cells in culture and to purified sugars. As this sugar is the major sialic acid present on the surface of cells of the human respiratory epithelium, it was thought that it was the receptor bound by virus during infection of most animals. This hypothesis was tested using mice that lack the gene encoding the sialyltransferase, ST6Gal I, the enzyme used for linking α(2,6) sialic acid to glycoproteins. Such mice have no detectable α(2,6) sialic acid in the respiratory tract. Nevertheless, human influenza viruses replicated efficiently in the lung and trachea of these mice, indicating that α(2,6) sialic acid is **not** essential for influenza virus infection, at least in mice.

The lesson to be learned is clear: even when the results of experiments performed in tissue culture seem to have obvious relevance to infection in the host, such notions must always be validated *in vivo*.

Glaser L, Conenello G, Paulson J, Palese P. 2007. Effective replication of human influenza viruses in mice lacking a major α(2,6) sialyltransferase. *Virus Res* **126:**9–18.

Experiments that provide one answer in cell culture may result in quite different outcomes when done *in vivo*.



Studying pathogenesis in animals can be challenging, because so many variables come into play that it is often impossible to define precise mechanisms of disease. To quote a famous U.S. politician, there are probably many "unknown unknowns" when studying complex organisms such as humans, or even mice. Infection of inbred littermates in the same cage can lead to different pathogenic outcomes, despite identical histories, environment, and genetics: if there is such discrepancy in response even within inbred mouse populations, imagine how difficult it is to dissect the variables that result in different outcomes in humans. Consequently, viral pathogenesis has often been called a phenomenological discipline, in which observations are many, but mechanistic insights are few. In the past two decades, however, this view has been changing, thanks to the development of new experimental tools and more precise animal models, coupled with improved studies in humans and with human tissues.

Some viruses that infect humans have a broad host range and can infect other animals such as monkeys, ferrets, and guinea pigs. As we shall see later in this chapter, these various animal models have been invaluable for understanding viral pathogenesis. The laboratory mouse has been particularly useful: because the mouse genome can be manipulated readily, and the physiology of the mouse and the human are similar, it is possible to engineer mice to allow susceptibility to some human viruses (Box 5.2). Likewise, the ability to add, modify, or delete specific genes in mice enables the assessment of the function of individual proteins in pathogenesis. For example, we have learned a lot about immunity from the use of mice with targeted deletions of specific immune cell populations or effector proteins. In some cases, insights into human disease are gleaned by studying relatives of the human viruses. An example is simian immunodeficiency virus infection of monkeys, a useful surrogate to study the pathogenesis of human immunodeficiency virus infections. Although the knowledge obtained from animal models is essential for understanding how viruses cause disease in humans, the results of such studies must be interpreted with caution. No human disease is completely reproduced in an animal model: what is true for a mouse is not always (perhaps even rarely) true for a human. Differences in size, metabolism, organ systems, aging, immune histories, and developmental program bear substantially on pathogenesis. Furthermore, as most mice used in viral pathogenesis studies are heavily inbred, they cannot provide much insight into the subtle effects of human genetic diversity. Nevertheless, the study of animal models of virus infections has yielded many principles and mechanistic insights that were corroborated in follow-up studies with human tissues.

Patterns of Infection

Studying the biology of an infected cell is a useful first step in understanding what kind of pathology the virus will cause in the host. Some virus infections kill the cell rapidly, producing a burst of new particles (**cytopathic viruses**), while other infections result in the release of virus particles without

BOX 5.2

METHODS

Transgenic and knockout mice for studying viral pathogenesis

Mice have always played an important role in the study of viral pathogenesis (see figure). Because it is possible to manipulate this animal genetically, much new information about how viruses cause disease has been collected. Introducing a gene into the mouse germ line to produce a transgenic mouse and ablating specific genes (gene knockouts) both have wide use in virology. The application of Cre-lox technology allows selective removal of a host gene within a particular cell type or at a chosen time in development.

Mice are not susceptible to infection by all viruses, however. In those cases in which mice lack virus receptors, it has been possible to engineer transgenic mice that produce the human receptor, with the goal of enabling a human virus to infect mouse cells. A model of disease can be established, assuming all the other necessary cellular proteins are present to allow reproduction of the human virus. In cases where viral receptors have not been identified, or are not sufficient for infection, an alternative approach is to express either the entire viral genome or a selected viral gene in mice. For example, transgenic mice that express the hepatitis B virus genome have been used to study interactions between the virus and the host immune response. Transgenic mice that express T cell receptor transgenes or genes encoding soluble immune mediators have also been produced. Such mice have been used to study the effect of immune cells on virus clearance, as well as the protective and deleterious effects of cytokines.

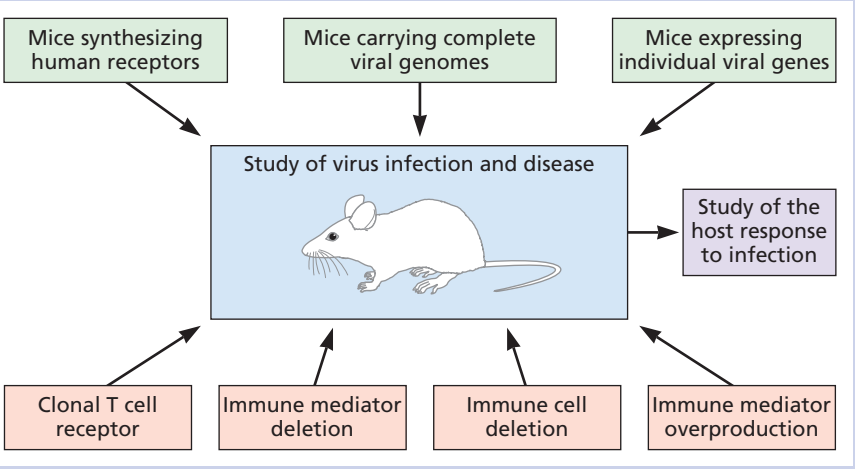
Mice lacking specific components of the immune response have proven invaluable for studying immunity and immunopathogenesis. For example, mice lacking the gene encoding perforin, a molecule essential for the ability of cytotoxic T lymphocytes to lyse target cells (Chapter 4), cannot clear infection by lymphocytic choriomeningitis virus, despite the presence of an otherwise intact immune response.

More recently, "humanized" mice have been developed to study viruses that are specific to humans. These mice carry human genes, cells, or tissues; when human cells or tissues are transplanted, the recipient mice are usually immunodeficient to prevent them from mounting an antigraft immune response. Such "Frankenstein" mice have been used for the study of human immunodeficiency virus and Epstein-Barr virus infections, among others.

Denton PW, Garcia JV. 2011. Humanized mouse models of HIV infection. *AIDS Rev* **13:**135–148.

Rall GF, Lawrence DMP, Patterson CE. 2000. The application of transgenic and knockout mouse technology for the study of viral pathogenesis. *Virology* **271:**220–226.

Various approaches to the use of transgenic and knockout mice in the study of viral reproduction and pathogenesis.



causing immediate host cell death (**noncytopathic viruses**). Alternatively, some infections neither kill the cell, nor produce any progeny, but rather remain dormant or become **abortive infections**.

Infections in host organisms can be categorized based on their duration: rapid and self-limiting (**acute infections**) or long-term (**persistent infections**). Variations and combinations of these two modes are common (Fig. 5.1). It can be argued that all virus-host encounters begin with an acute infection, and that differences in the subsequent management of that infection diversify the ultimate outcome. For example, most **latent infections**, in which no infectious particles are produced, begin as an acute infection. Conversion to a latent infection enables the viral genome to persist undetected, perhaps to be reactivated in the future. Intermediate patterns that lie between rapid viral growth and latent infection can be thought of as "smoldering infections" in which low-level viral reproduction occurs in the face of a strong immune response.

Incubation Periods

Once anatomical and chemical barriers to infection have been breached and an infection is established, a cascade of new defensive reactions occurs in the host (see Chapters 3 and 4). Symptoms and pathologies may or may not be obvious, depending upon the virus, the infected tissue and host, and the antiviral immune response. The period before the symptoms of a disease are obvious is called the **incubation period.** During this window, viral genomes are replicating and the local induction of the innate immune response produces cytokines such as interferon (IFN). Often, the initial symptoms (fever, malaise, aches, pains, and nausea) detected pursuant to infection are

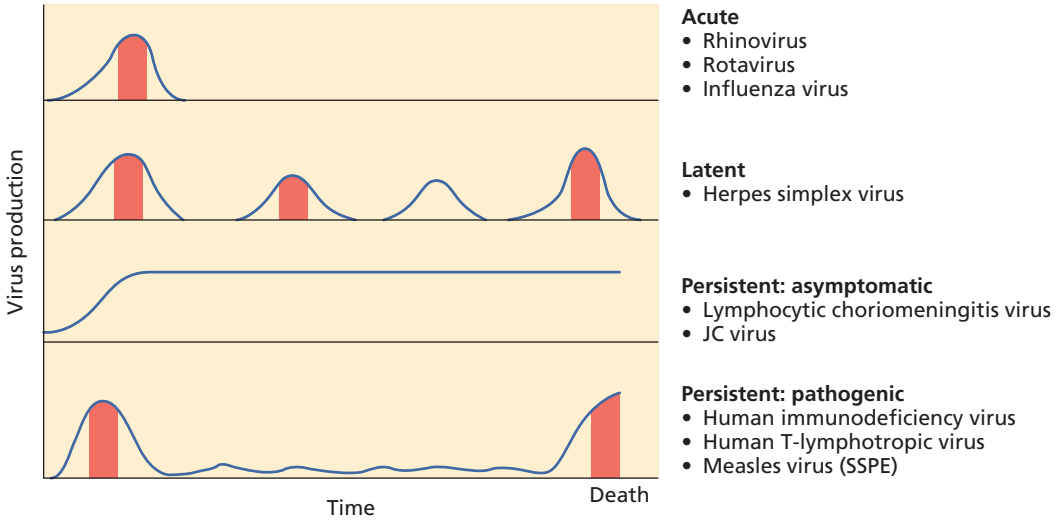


Figure 5.1 General patterns of infection. Relative virus particle production is plotted as a function of time after infection (blue line). The time during which symptoms are present is indicated by the orange shaded area. The top panel is the typical profile of an acute infection, in which virus particles are produced, symptoms appear, and the infection is cleared, often within 7 to 10 days. The second panel depicts a latent infection in which an initial acute infection is followed by a quiescent phase and repeated rounds of reactivation. Reactivation may or may not be accompanied by symptoms, but generally results in the production of infectious virus particles. The bottom two panels are variations of the profile of a persistent infection. The third panel is the typical profile of a persistent infection, in which virus production continues for the life of the host, often at low levels, or in tissues that immune cells do not routinely patrol. Symptoms may or may not be apparent, depending upon the virus. The fourth panel depicts a persistent infection, in which a period of years intervenes between a typical primary acute infection and the usually fatal appearance of symptoms. The production of infectious particles during the long period between primary infection and fatal outcome may be continuous (e.g., human immunodeficiency virus) or not detectable (e.g., measles virus, in the case of subacute sclerosing panencephalitis). Adapted from F. J. Fenner et al., *Veterinary Virology* (Academic Press, Inc., Orlando, FL, 1993), with permission.

consequences of type I IFN production. Remarkably, incubation periods can vary greatly (Table 5.1).

The intrinsic and innate responses limit and contain many acute infections. When these defenses are lacking or compromised, acute infections can be become disastrous, primarily because the infection becomes systemic and multiple organs can be damaged (provided that receptor-positive, susceptible cells are present in other tissues). If the infection spreads to multiple organs quickly, the host adaptive response may not be able to contain the infection in these various sites.

Mathematics of Growth Correlate with Patterns of Infection

Before discussing the various patterns of viral infection, it will be informative to consider the constraints of viral reproduction based on the populations that are infected. In ecology, this concept is often referred to as the r/K selection theory, in which the principle is quite simple: an organism can focus upon either increased number of offspring (with minimal attention to offspring quality), or reduced number of offspring with a corresponding increased parental investment. ***r*-selection** favors large numbers of offspring with low cost per individual, while ***K*-selected species** devote high cost in reproduction to produce

Table 5.1 Incubation periods of some common viral infections

Disease	Incubation period (days)[a]
Influenza virus	1–2
Rhinovirus	1–3
Ebola virus	2–21
Acute respiratory disease (adenoviruses)	5–7
Dengue	5–8
Herpes simplex	5–8
Coxsackievirus	6–12
Poliovirus	5–20
Human immunodeficiency virus	8–21
Measles	9–12
Smallpox	12–14
Varicella-zoster virus	13–17
Mumps	16–20
Rubella	17–20
Epstein-Barr virus	30–50
Hepatitis A	15–40
Hepatitis B and C	50–150
Rabies	30–100
Papilloma (warts)	50–150

[a]Until first appearance of prodromal symptoms.

BOX 5.3

METHODS

Mathematical approaches to understanding viral population dynamics

The changes in the size of a viral population can be described by a single, simple concept: the rate of increase in the size is the difference between the rate of reproduction and the rate of elimination. We can write this statement as

$$dN/dt = (b - d)N$$

where dN/dt is the rate of change of the population (N) with respect to time (t). The terms b and d are the average rates of birth and death, respectively (although, of course, virus particles are neither born nor die). The term $(b - d)$ is usually written as a constant r, the intrinsic rate of increase. Therefore, we obtain equation 5.1:

$$dN/dt = rN \qquad \textbf{(5.1)}$$

$$\textit{and } \ln N = rt$$

This is the equation for exponential population growth. Plotting $\ln N$ versus t yields a straight line with slope r (Fig. 5.2A).

If b far exceeds d (as is the case for infections in cultured cells), progeny accumulate. When b equals d, the population maintains a stable size. If we assume a linear relationship for increase and decrease of the population, then the slope of the increase of reproduction rate is equal to k_b and the slope of death or removal rate is equal to k_d. The stability of the population N then can be written as follows:

$$b_0 - k_b N = d_0 + k_d N$$

or

$$N = (b_0 - d_0)/(k_d + k_b)$$

This description of N, the viral population, is called the **carrying capacity (*K*)** of the environment. The term "environment" can define a single cell, an individual, or the entire host population. For any value of N greater than K, the viral population will decrease, and for any value of N less than K, the viral population will increase. The carrying capacity K is of particular interest in virology, as it defines the upper boundary of the growing population and influences patterns of infection.

Therefore, by knowing that $r = (b_0 - d_0)$ and $K = (b_0 - d_0)/(k_b + k_d)$, we can substitute these values in equation 5.1 to obtain the basic equation for growth and regulation of a population, sometimes called the logistic growth equation (equation 5.2).

$$dN/dt = rN(K - N/K) \qquad \textbf{(5.2)}$$

Plotting $\ln N$ versus t yields the curve illustrated in Fig 5.2B. Here, K is easily seen to be the upper limit to growth, and the rate of increase is r.

a low number of offspring. One strategy is not necessarily better than the other: the environment determines which of them will predominate. For example, the average gestation period for a mouse is 21 days, an average litter is 7 to 12 pups, and the female can become impregnated again on the same day she delivers her litter. This example is an r-selection strategy, in which volume (and not quality) is evolutionarily favored, likely because mice have many natural predators. Compare this to humans, with a gestation period of 9 months, and an average litter size of one. Such a strategy may be favored in humans because of the relative paucity of predators.

How can this ecological principle be applied to viruses? Production of large numbers of viral progeny maintained by a steady, unbroken lineage of serial infections is consistent with the *r*-replication strategy. Such viral reproduction will never reach a limit as long as susceptible hosts are available (Box 5.3, equation 5.1; Fig. 5.2A). The alternative is the ***K*-replication strategy**, in which the host population is at or close to its saturation density (e.g., new susceptible hosts are rare or nonexistent, or for which rates of viral propagation may be slow or very low [Box 5.3, equation 5.2; Fig. 5.2B]).

r-replication strategies often manifest as acute infections characterized by short reproductive cycles with production of many progeny, and extensive viral spread. Acute infections following an *r*-replication strategy will "burn out" if the number of susceptible hosts becomes limiting. One can mimic an *r*-selection environment in cell culture by low multiplicity-of-infection (MOI) infections: in this case, permissive cells sustain multiple rounds of replication, but transmission stops when all the cells become infected. *K*-replication strategies often appear as persistent or latent infections. In this case, infected hosts survive for extended times and faster viral reproduction confers no selective advantage. Viruses and their hosts exist along a continuum of values for *r* and *K*.

The growth equations, as written in their simplest form, can be used to model replication in identical cells in culture. However, to describe accurately how a viral infection is propagated and maintained in a large population of host organisms, more variables must be considered. These additional parameters include the rate of shedding from infected individuals, the rate of transmission to other hosts, the probability that one infected individual will infect more than one other, and the number and density of susceptible individuals. Some of these parameters are discussed in greater detail in Chapter 10.

Acute Infections

The term "acute" refers to rapid onset of viral reproduction that may be accompanied by disease with a short, but occasionally severe, course. Hallmarks of an acute viral infection include the rapid production of large numbers of virus particles (hence: an *r*-replication strategy), followed by immune-mediated destruction of the particles and virus-infected cells. Acute infections are the typical, expected course for agents such as influenza virus, norovirus, and rhinovirus (Fig. 5.3). The disease symptoms tend to resolve over a period of days. Nevertheless, during the rapid reproduction

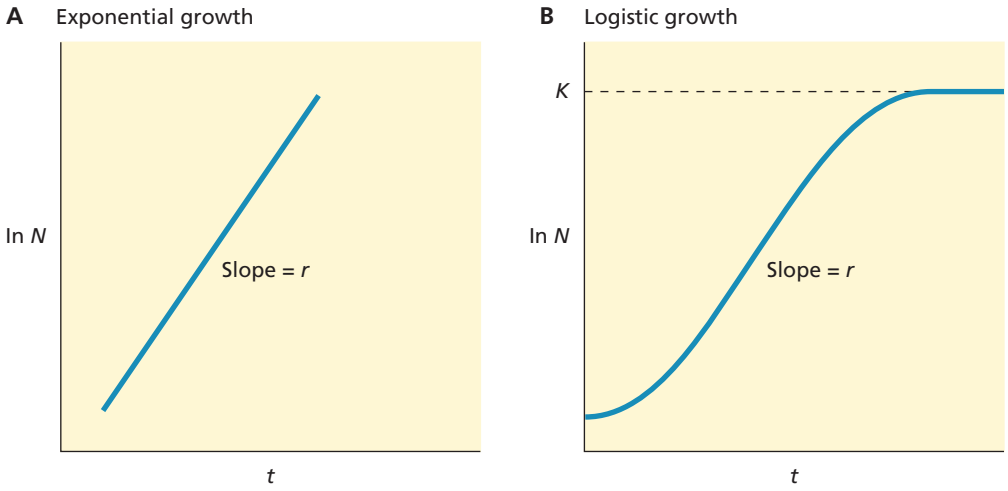


Figure 5.2 Two plots of standard growth equations. (A) A graph of simple exponential reproduction. **(B)** A graph of the pattern termed logistic growth illustrating *K*, the limit to reproduction. *r* is the slope in both plot types.

phase, some progeny are invariably shed and spread to other hosts before the infection is contained. If the initial infection modulates local immune defenses and virus spreads via hematogenous or neural routes to other parts of the body, several rounds of reproduction may occur in different tissues within the same animal, with new and distinctive symptoms. A classic example is varicella-zoster virus, an alphaherpesvirus that causes the childhood disease chickenpox, but that can recur later in life to cause a different (and far more painful) skin rash, shingles (Fig. 5.4).

An acute infection may result in limited or no obvious symptoms. Indeed, inapparent (or asymptomatic) acute infections are quite common, and can be major sources of transmission within populations. They are recognized by the presence of virus-specific antibodies with no reported history of disease. For example, over 95% of the unvaccinated population of the United States has antibody to varicella-zoster virus, but fewer than half of these individuals report that they have had chickenpox. In such infections, sufficient virus particles are made to maintain the virus in the host population, but the quantity is below the threshold required to induce symptoms. The usual way an inapparent infection is detected is by elevated antiviral antibody concentrations in an otherwise healthy individual. Well-adapted pathogens often cause asymptomatic infections, as demonstrated by poliovirus, in which more than 90% of infections are inapparent.

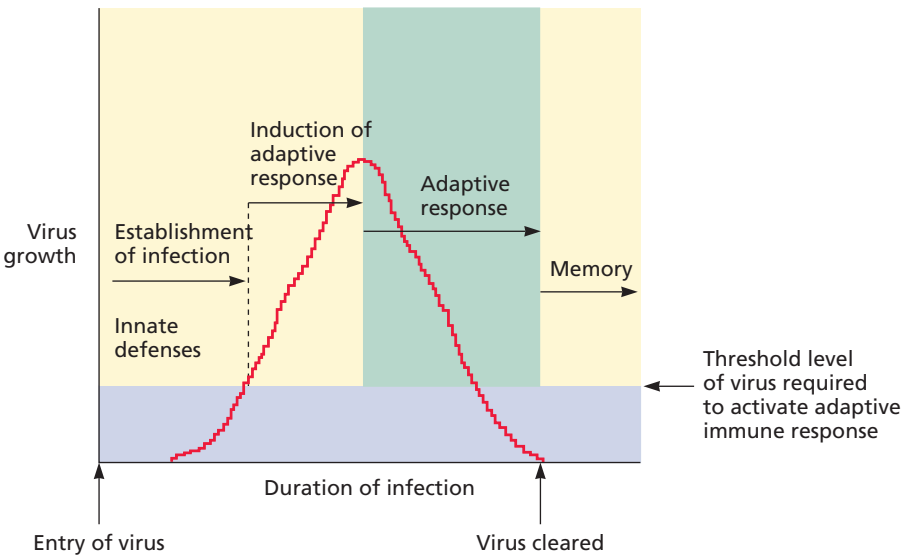


Figure 5.3 The course of a typical acute infection. Relative virus reproduction plotted as a function of time after infection. The concentration of virus particles increases with time, as indicated by the red line. During the establishment of infection, only intrinsic and innate defenses are at work. If the infection reaches a certain threshold (which is specific to the virus and host [purple]), adaptive immunity is initiated. After 4 to 5 days, effector cells and molecules of the adaptive response begin to clear infected tissues and virus particles (green shaded area). After this action, memory cells are produced, and the adaptive response is suppressed. Antibodies and memory cells provide lasting protection should the host be reinfected at a later date. Redrawn from C. A. Janeway, Jr., and P. Travers, *Immunobiology: the Immune System in Health and Disease* (Current Biology Ltd. and Garland Publishing, New York, NY, 1996), with permission.

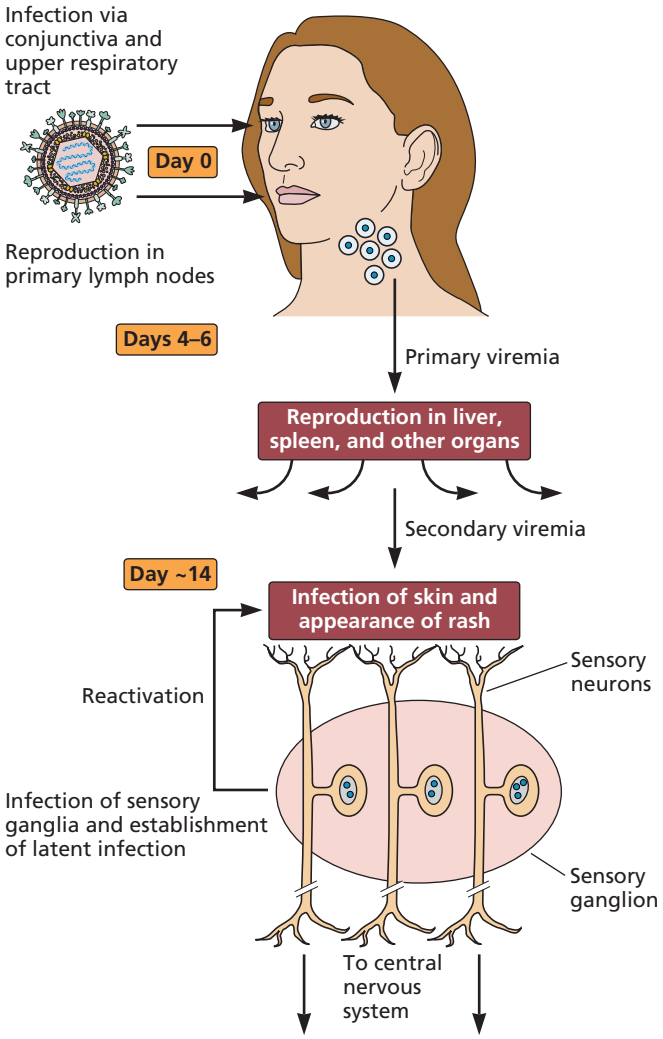


Figure 5.4 Model of varicella-zoster virus infection and spread. Infection initiated on the conjunctiva or mucosa of the upper respiratory tract spreads to regional lymph nodes. After 4 to 6 days, infected T cells enter the bloodstream, causing a primary viremia. These infected cells subsequently invade the liver, spleen, and other organs, initiating a second round of infection. Virus particles and infected cells are then released into the bloodstream in a secondary viremia. Infected skin-homing T cells efficiently invade the skin and initiate a third round of infection about 2 weeks after the initial infection. The characteristic vesicular rash of chicken pox appears as a result of immune defensive action. Next, virus particles produced in the skin infect sensory nerve terminals and spread to dorsal root ganglia of the peripheral nervous system, where a latent infection is established. The latent infection is maintained by active immune surveillance. Later in life, perhaps as the robustness of the immune system wanes, viral reactivation can occur, in which another infectious cycle is initiated. In this case, virus particles leave the peripheral neurons to infect the skin. The characteristic recurrent disease, called shingles, is often accompanied by a long-lasting painful condition called postherpetic neuralgia. Normally, an infected individual experiences only one visible reactivation event, probably because reactivation stimulates the immune system. Such restimulation of the immune system is the rationale for administering the varicella-zoster live vaccine to adults to prevent reactivation and shingles.

Antigenic Variation Facilitates Repeated Acute Infections

If an individual survives a typical acute infection, he or she is often immune to rechallenge by the same virus. Nevertheless, some acute infections occur repeatedly, despite the host mounting a robust immune response to them. These recurring infections are possible because selection pressures during the initial acute infections lead to release of virus particles that are resistant to immune clearance. Mutations in the genome may affect the structural properties of the virus and the capacity of neutralizing antibodies to block infectivity, or of T cells to recognize particular viral epitopes (Chapter 4).

Viral particles that can tolerate many amino acid substitutions in their structural proteins and remain infectious are said to have **structural plasticity** (e.g., influenza virus and human immunodeficiency virus). Populations of virus particles can include antibody-resistant mutants that are selected in the presence of neutralizing antibody. Such altered particles can reinfect individuals, even when there is preexisting immunity to the original virus.

Other viruses cannot tolerate many amino acid changes in their structural proteins (e.g., those of poliovirus, measles virus, and yellow fever virus). In these cases, even if the mutation rate is high, antibody-resistant infectious particles have a low probability of being generated, but have been observed (Box 5.4). This property ensured that vaccines effective in the 1950s are just as potent in the 21st century.

In contrast, the structural plasticity of rhinoviruses is a manifestation of the circulation of over 100 serotypes in the human population at any one time. This property accounts for the fact that individuals may contract more than one common cold each year, and also explains why it is difficult to produce a vaccine to prevent this disease.

Similarly, enveloped influenza virus particles that are resistant to antibodies are readily selected, whereas the enveloped particles of measles virus and yellow fever virus exhibit little variation in membrane protein amino acid sequence, and antibody-resistant variants are rarely observed. Consequently, an influenza vaccine is required every year, while a single measles virus vaccination typically lasts a lifetime.

Antigenic variation refers to changes in virus proteins in response to antibody selection. In an immunocompetent host, antigenic variation arises by two distinct processes. **Antigenic drift** is the appearance of virus particles with slightly altered surface proteins (antigen) following passage in the natural host (Fig. 5.5). In contrast, **antigenic shift** denotes a major change in the surface protein(s) of a virus particle, as genes encoding novel surface proteins (or substantial variants of known proteins) are acquired (Chapter 10). Fortunately, most year-to-year changes in the circulating influenza strains are due to antigenic drift. Consequently, last year's vaccine generally confers some protection against this year's virus. More

BOX 5.4

DISCUSSION

Poliovirus escapes antibodies

(This Box is adapted from a blog post by Dr. Vincent Racaniello (www.virology.ws) on August 29, 2014.)

Antigenic variation is a hallmark of influenza virus that allows host defenses to be circumvented. Consequently, influenza vaccines need to be reformulated frequently to keep up with changing viruses. In contrast, antigenic variation is not a hallmark of poliovirus, and the same poliovirus vaccines have been used for nearly 60 years to control infections by this virus. An exception is poliovirus type I that caused a 2010 outbreak in the Republic of Congo.

The 2010 outbreak (445 paralytic cases) was unusual because the case fatality ratio of 47% was higher than typically observed (usually less than 10% of patients with confirmed disease die). The first clue that something was different in this outbreak was the finding that sera from some of the fatal cases failed to neutralize effectively infection of cells by the strain of poliovirus isolated during this outbreak (the strain is called PV-RC2010). The same sera effectively neutralized the three Sabin vaccine viruses as well as wild type 1 polioviruses isolated from previous outbreaks. Therefore, gaps in vaccination coverage were not solely responsible for this outbreak.

Examination of the nucleotide sequence of the genome of type I polioviruses isolated from 12 fatal cases identified two amino acid changes within a site on surface of the viral capsid that is bound by neutralizing antibodies (illustration). The sequence of this site, called 2a, was changed from **Ser**-Ala-**Ala**-Leu to **Pro**-Ala-**Asp**-Leu. This particular combination of amino acid substitutions has never been seen before in poliovirus. Virus PV-RC2010, which also contains these two amino acid mutations, is completely resistant to neutralization with monoclonal antibodies that recognize antigenic site 2.

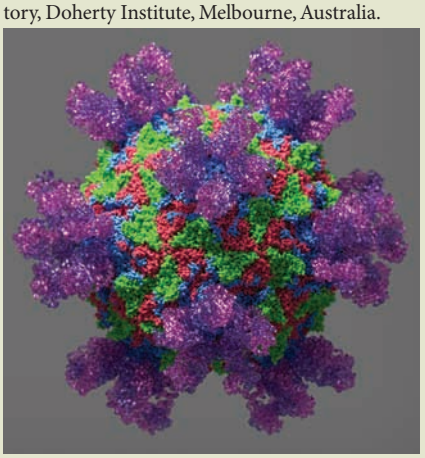
Poliovirus neutralization titers were determined using sera from Gabonese and German individuals who had been immunized with Sabin vaccine. These sera effectively neutralized the type I strain of Sabin poliovirus, as well as type 1 polioviruses isolated from recent outbreaks. However, the sera had substantially lower neutralization activity against PV-RC2010. Nucleotide sequence analysis of PV-RC2010 showed that it is related to a poliovirus strain isolated in Angola in 2009, the year before the Republic of Congo outbreak. The Angolan virus had just one of the two amino acid changes in antigenic site 2a found in PV-RC2010.

It is possible that the relative resistance of the polioviruses to antibody neutralization might have been an important contributor to the high virulence observed during the Republic of Congo outbreak. The reduced ability of serum antibodies to neutralize virus would have led to higher concentrations of virus particles in the blood and a greater chance of entering the central nervous system. Another factor could also be that many of the cases of poliomyelitis were in adults, in which the disease is known to be more severe.

An important question is whether poliovirus strains such as PV-RC2010 pose a global threat. Typically, the fitness of antigenically variant viruses is not the same as wild type, and therefore such viruses are not likely to spread in well immunized populations. The incomplete poliovirus immunization coverage in some parts of the world, together with the reduced circulation of wild-type polioviruses leads to reduced population immunity. Such a situation could lead to the evolution of antigenic variants. This situation occurred in Finland in 1984, when an outbreak caused by type 3 poliovirus took place. The responsible strains were antigenic variants that evolved as a result of use of a suboptimal poliovirus vaccine in that country.

The poliovirus outbreaks in the Republic of Congo and Finland were stopped by immunization with poliovirus vaccines, which boosted the population immunity. These experiences show that poliovirus antigenic variants such as PV-RC2010 will not cause outbreaks as long as we continue extensive immunization with poliovirus vaccines, coupled with environmental and clinical testing for the presence of such viruses.

Reconstruction of a poliovirus particle bound by antibodies. Figure courtesy of Jason Roberts, Victorian Infectious Diseases Reference Laboratory, Doherty Institute, Melbourne, Australia.



rarely, antigenic shift occurs, but when it does, it is accompanied by a huge increase in the number of cases, as few individuals have existing immunity to the reassortant virus.

Acute Infections Pose Common Public Health Problems

An acute infection is most frequently associated with serious outbreaks or epidemics, affecting millions of individuals every year (e.g., influenza virus, norovirus). The nature of an acute infection presents serious obstacles for physicians, epidemiologists, drug companies, and public health officials: by the time people report symptoms, they will probably have transmitted the virus to a naïve host. Such infections can be difficult to diagnose retrospectively, or to control in large populations or crowded environments (such as day care centers, military camps, college dormitories, and nursing homes). When distrust in the government and public health care are also present, as in the 2014 Ebola virus outbreak in Liberia, Sierra Leone, and Guinea, limiting the exposure of naïve individuals to those who are infected becomes a Herculean task. Effective antiviral drug therapy requires treatment early in the infection, often before symptoms are apparent. Antiviral drugs can be given in anticipation of an infection, but this strategy demands that the drugs be affordable, safe, and free of side effects. Moreover, our arsenal of antiviral drugs

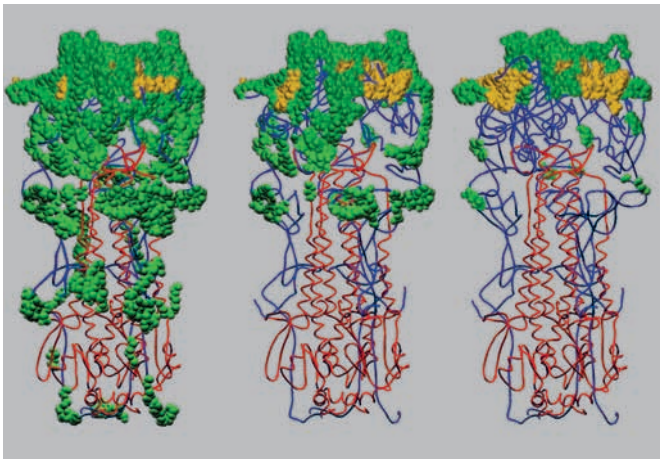


Figure 5.5 Antigenic drift: distribution of amino acid residue changes in hemagglutinins (HA) of influenza viruses isolated during the Hong Kong pandemic era (1968 to 1995). The space-filling models represent the virus-receptor binding site (yellow) and the substituted amino acids (green). **(Left)** All substitutions in HAs of virus particles isolated between 1968 and 1995; **(middle)** amino acid substitutions that were retained in subsequent years; **(right)** amino acid substitutions detected in monoclonal antibody-selected variants of A/Hong Kong/68 HA. The α-carbon tracings of the HA1 and HA2 chains are shown in blue and red, respectively. Adapted from T. Bizebard et al., *Curr. Top. Microbiol. Immunol.* **260:**55–64, 2001, with permission.

is modest (Chapter 9), and drugs effective for most common acute viral diseases do not exist.

Persistent Infections

Persistent infections occur when the primary infection is not cleared by the host immune response. Instead, virus particles, proteins, and genomes continue to be produced or persist for long periods, often for the life of the host. Virus particles may be produced continuously or intermittently for months or years, even in the presence of an ongoing immune response. In some instances, viral genomes remain after viral proteins can no longer be detected.

The persistent pattern is particularly common for noncytopathic viruses (Table 5.2). Some viruses, including the arenavirus, lymphocytic choriomeningitis virus, are inherently noncytopathic in their natural hosts and maintain a persistent infection if the host cannot mount a sufficient immune response. Other viruses toggle between a cytolytic phase and a noncytopathic phase. Epstein-Barr virus infections are typified by alternative transcription and replication programs that maintain the viral genome in some cell types with no production of viral particles. In other infections, such as those of some adenoviruses, circoviruses, polyomaviruses, and human herpesvirus 7, viral reproduction and shedding take place, but are uneventful in most individuals. What is clear from these examples is that no single mechanism is responsible for establishing a persistent viral infection. However, when viral cytopathic effects are minimized, and host defenses are suppressed, a persistent infection is likely.

Multiple Cellular Mechanisms Promote Viral Persistence

Whether viral infection leads to multiple rounds of reproduction or persistence may be based on the behavior of the infected cell. The alphavirus, Sindbis virus, provides a good example. Apoptosis is a common intrinsic cellular defense that can limit or expand viral reproduction and spread (Chapter 3). In some vertebrate cell lines, Sindbis virus infection is acute and cytopathic because apoptosis is induced.

Table 5.2 Some persistent viral infections of humans

Virus	Site(s) of persistence	Consequence(s)
Adenovirus	Adenoids, tonsils, lymphocytes	None known
Epstein-Barr virus	B cells, nasopharyngeal epithelia	Burkitt's lymphoma, Hodgkin's disease
Human cytomegalovirus	Kidneys, salivary gland, lymphocytes,[a] macrophages,[a] stem cells,[a] stromal cells[a]	Pneumonia, retinitis
Hepatitis B virus	Liver, lymphocytes	Cirrhosis, hepatocellular carcinoma
Hepatitis C virus	Liver	Cirrhosis, hepatocellular carcinoma
Human immunodeficiency virus	$CD4^+$ T cells, macrophages, microglia	AIDS
Herpes simplex virus types 1 and 2	Sensory and autonomic ganglia	Cold sore, genital herpes
Human T lymphotropic virus types 1 and 2	T cells	Leukemia, brain infections
Papillomavirus	Skin, epithelial cells	Papillomas, carcinomas
Polyomavirus BK	Kidneys	Hemorrhagic cystitis
Polyomavirus JC	Kidneys, central nervous system	Progressive multifocal leukoencephalopathy
Measles virus	Central nervous system	Subacute sclerosing panencephalitis, measles inclusion body encephalitis
Rubella virus	Central nervous system	Progressive rubella panencephalitis
Varicella-zoster virus	Sensory ganglia	Zoster (shingles), postherpetic neuralgia

[a]Proposed but not certain.

However, Sindbis virus causes a persistent infection of cultured postmitotic neurons because these cells synthesize Bcl2, a cellular protein that blocks apoptosis, and are therefore intrinsically resistant to virus-induced apoptosis. The *in vitro* studies are recapitulated in host animals. When Sindbis virus is injected into an adult mouse brain, a persistent noncytopathic infection is established. In contrast, when the same inoculum is injected into neonatal mouse brains, the infection is cytopathic and lethal, because neonatal neurons do not synthesize the gene products that block virus-induced apoptosis.

The intrinsic IFN response can also be important in determining patterns of infection. For example, bovine viral diarrhea virus, a pestivirus in the family *Flaviviridae*, establishes a lifelong persistent infection in a large proportion of cattle around the world. Persistently infected animals have no detectable antibody or T cell responses to viral antigens. Cytopathic and noncytopathic strains have been useful in understanding how persistence is established in the apparent absence of a host response. Infection of pregnant cattle by a cytopathic strain is contained quickly and eliminated. This phenotype depends on a rapid fetal IFN response that clears the infection. In contrast, infection of pregnant cattle with the noncytopathic virus during the first half of gestation results in birth of sickly, but viable, persistently infected calves. Noncytopathic infection of fetal tissue does not stimulate production of IFN, presumably because the virus is perceived as "self" during development, and does not invoke immunity. Consequently, the adaptive immune system is not activated, and because the virus does not kill cells, a persistent infection is established.

Modulation of the Adaptive Immune Response Can Perpetuate a Persistent Infection

Interference with Toll-like receptor detection and signaling. Viral infection triggers an early host response through activation of pattern recognition receptors, including Toll-like receptors (Tlr). Given the central role of Tlrs in the early immune response, it should not be surprising that these pathways are modulated following viral infection. Epstein-Barr virus activates Tlrs, including Tlr2, Tlr3, and Tlr9, but the expression of, and signaling by, Tlrs is attenuated during productive infection, probably as a result of the action of at least three viral deubiquitinases, including the tegument protein Bplf1, which suppresses Tlr-mediated activation of Nf-κB.

While DNA viruses that encode many "nonessential" genes have the greatest number and diversity of these immune-interfering proteins, small RNA viruses with more limited coding capacity can also block the host response. For example, hepatitis C virus, notorious for its ability to establish persistent liver infection, encodes the NS3/4A serine protease which degrades Trif, an adapter protein that is essential for signaling from Tlr3 to induce a multitude of antiviral defenses. Consequently, hepatitis C virus establishes a persistent infection in hepatocytes by interfering with a critical early step in the antiviral immune response, and by affecting the synthesis of numerous antiviral proteins.

Interference with production and function of MHC proteins. Cell lysis and production of inflammatory cytokines by cytotoxic T lymphocytes (CTL) are among the most powerful weapons in the antiviral arsenal (Chapter 4). CTLs make cytokines and cause cytolysis following engagement of the T cell receptor with viral peptides presented by major histocompatibility complex (MHC) class I proteins on the surface of the infected cell. Consequently, any mechanism that prevents viral peptides from binding to MHC class I molecules, even transiently, provides a potential selective advantage for the virus. Not surprisingly, then, the production of MHC class I proteins is modulated after many acute infections. Presumably such modulation prevents or delays elimination by CTLs so that sufficient progeny can be disseminated.

Many of the MHC-processing or regulatory steps were not known until the viral proteins that interfere with them were elucidated: several viral proteins block presentation of MHC class I molecules at the cell surface by interfering with various steps in the pathway (Fig. 5.6). Peptide presentation by MHC class I proteins can be reduced by lowering the expression of the MHC genes, blocking the production of peptides by the proteasome, or interfering with subsequent assembly and transport of the MHC-peptide complex to the cell surface.

Human cytomegalovirus deserves special mention, because MHC class I presentation of viral antigens is inhibited by multiple mechanisms during an acute infection. This betaherpesvirus causes a common childhood infection with inapparent to mild effects in healthy individuals. These infections are not cleared, and a persistent infection is established in salivary and mammary glands as well as the kidneys. Virus particles are secreted in saliva, milk, and urine. In addition, a latent infection is established in early precursor cells of the monocyte/macrophage lineage; no virus particles are produced from these cells. When latently infected individuals become immunosuppressed, cytomegalovirus reproduction resumes, often causing a life-threatening disease. The cytomegalovirus US6 protein inhibits translocation of viral peptides into the endoplasmic reticulum lumen, while the viral US3 protein binds to, and detains, MHC class I proteins in the endoplasmic reticulum, preventing their transport to the cell surface. Simultaneously, the US11 and US2 proteins eject MHC class I molecules from the endoplasmic reticulum lumen into the cytoplasm, where they are degraded by the proteasome. One possible reason that the genome of this virus encodes so many proteins to block antigen presentation is that multiple gene products act additively or synergistically to delay immune clearance until macrophage/monocyte precursors are infected, and a latent infection established.

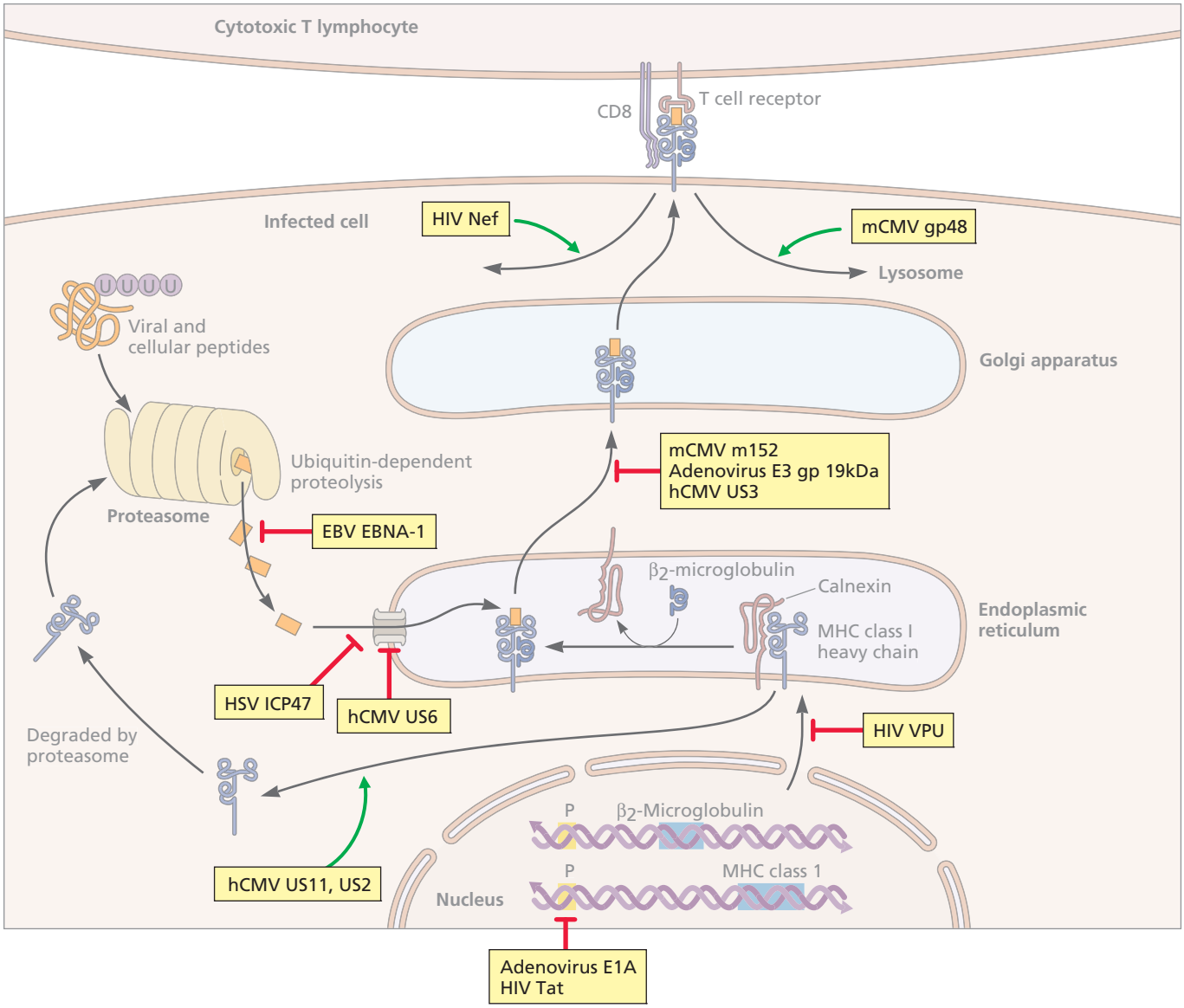


Figure 5.6 Viral proteins block cell surface antigen presentation by the MHC class I system. Specific gene products from diverse virus families block presentation of viral peptides with class I major histocompatibility complex molecules at almost every step of antigen processing and MHC assembly. Green arrows indicate stimulation; red bars indicate inhibition. HSV, herpes simplex virus; hCMV, human cytomegalovirus; mCMV, mouse cytomegalovirus; HIV, human immunodeficiency virus; EBV, Epstein-Barr virus.

Ubiquitinylation of proteins is an important regulatory mechanism that governs endocytosis, sorting, and degradation (Volume 1, Box 9.10). The genomes of many gammaherpesviruses and poxviruses encode a class of zinc-binding RING finger proteins with E3 ubiquitin ligase activity, which can interfere with class I MHC antigen presentation, stimulate viral replication, and inhibit apoptosis. The K3 and K5 genes of human herpesvirus 8 and the MK3 gene of murine gammaherpesvirus 68 encode such proteins. The K5 and MK3 proteins are related type III transmembrane proteins but, unexpectedly, act at different steps in the MHC class I antigen presentation pathway: the K5 protein targets MHC class I proteins and costimulatory molecules for degradation by adding ubiquitin to the cytoplasmic domains of these integral membrane proteins and stimulating their endocytosis and destruction. In contrast, ubiquitinylation by the related MK3 protein promotes proteasomal destruction of MHC class I proteins soon after they appear in the endoplasmic reticulum. The genome of myxoma virus (a poxvirus) encodes a similar RING finger E3 ligase called MV-LAP that also directs prote-

asomal destruction by a mechanism analogous to that used by the K5 protein. Importantly, while the effects of K5 protein on human infections cannot be assessed, myxoma virus mutants that lack the MV-LAP gene are markedly attenuated in rabbits, the natural host.

Epstein-Barr virus, a member of the herpesvirus family, is among the most common human infections, responsible for infectious mononucleosis as well as certain cancers, including Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's lymphoma. Early observations indicated that Epstein-Barr virus-infected individuals do not produce CTLs capable of recognizing the viral protein EBNA-1. This phosphoprotein is found in nuclei of latently infected cells, and is regularly detected in malignancies associated with the virus. T cells specific for other Epstein-Barr virus proteins are amplified, indicating that EBNA-1 must possess some intrinsic feature that allows escape from T cell detection. In fact, this protein contains an amino acid sequence that renders it invisible to the host proteasome. As a result, no EBNA-1 epitopes are produced. Remarkably, this inhibitory sequence can be fused to other proteins to inhibit their processing and the subsequent presentation of peptide antigens normally produced from them. The biological relevance of this mechanism is evident after acute infection of B cells: T cells kill all productively infected cells, sparing only those rare cells that produce EBNA-1, which harbor a latent viral genome.

MHC class II modulation after infection. In the exogenous pathway of antigen presentation, proteins are internalized and degraded, producing peptides that can bind to MHC class II molecules (Chapter 4). These complexes are transported to the cell surface, where they can be recognized by the $CD4^+$ T cell receptor. Activated $CD4^+$ T helper (T_h) cells stimulate the development of CTLs, and help coordinate an antiviral response to the pathogen. In many respects, they are the master regulators of the adaptive response. Consequently, any viral protein that modulates the MHC class II antigen presentation pathway would interfere with T_h cell activation, and subsequent coordination of the adaptive immune response. Numerous mechanisms of viral interference in MHC class II presentation have been identified. The human cytomegalovirus US2 protein promotes proteasomal destruction of class II molecules. A protein encoded by a less pathogenic herpes simplex virus removes the MHC class II complex from the endocytic compartment.

Bypassing CTL lysis by mutation of immunodominant epitopes. Although many peptides are generated following proteolysis, T cells respond to very few viral peptides. These peptides are said to be **immunodominant**. An extreme example of a limited CTL response is observed after infection of C57BL/6 mice with herpes simplex virus type 1. The virus-specific CTLs respond almost exclusively to a **single** peptide in the viral envelope protein gB.

A narrow repertoire of viral peptides to which immune cells can respond provides a ready opportunity for avoidance of T cell recognition, as a limited number of mutations in the coding sequence for the immunodominant peptides will render the infected cell virtually invisible to the T cell response. Viruses with these mutations, called CTL escape mutants, are thought to contribute to the accumulation of virus particles as a result of decreased immune efficacy. For example, CTL escape mutants, which are of central importance in the pathogenesis of human immunodeficiency virus type 1, arise as a consequence of error-prone genome replication and the selective pressure from constant exposure of the virus population to an activated immune response. In some cases, the sequence encoding the T cell epitope is completely deleted from the viral gene. Understanding how immunodominant peptides are selected, maintained, and bypassed is essential if effective vaccines against human immunodeficiency virus are to be developed. For example, a vaccine designed to target a dominant T cell peptide that is part of a critical structural motif in a viral protein may be useful, because CTL escape mutants (in which the critical motif is altered) will be less likely to be maintained and participate in subsequent spread of the virus.

Immunodominant epitopes and CTL escape mutants are crucial players in the common and dangerous infection caused by hepatitis C virus. The CTL response stimulated by acute infection is effective in fewer than 20 to 30% of individuals, and the majority of patients become persistently infected. After several years, this persistent infection can lead to serious liver damage and fatal hepatocellular carcinoma. Persistently infected chimpanzees harbor hepatitis C viruses with CTL escape mutations in their genomes. In contrast, the viral population isolated from animals that resolved the infection during the acute phase includes no such mutants. The principle derived from these observations is clear: if CTL escape mutations are present or arise early in the infection, a persistent infection is likely, but if CTLs clear the infection before escape mutants arise, persistent infection cannot occur.

The CTL epitope need not be deleted or radically altered to escape CTL recognition. Single nucleotide changes in the gene, which alter the protein coding sequence by only one amino acid, can be sufficient to evade detection by an activated T cell. This inherent vulnerability in the host response is particularly important for immune modulation by RNA viruses. Given their reduced coding capacity relative to the larger DNA viruses, RNA virus genomes rarely encode immune modifying proteins. However, as their RNA-dependent RNA polymerases lack the error correction mechanisms found in DNA-dependent RNA polymerases, they can survive by producing large numbers of viral mutants, some of which may be shielded from T cell recognition: in essence, a virological invisibility cloak.

Destruction of activated T cells. In some instances, when a CTL engages with an infected cell, the CTL dies instead of the infected target. This unexpected turn of events is another remarkable example of a viral counteroffense to the host defense. Activated T cells carry a membrane receptor called Fas on their surfaces, which is related to the Tnf family of membrane-associated cytokine receptors, and binds a membrane protein called Fas ligand (FasL). When Fas on activated T cells binds FasL on target cells, the receptor trimerizes, triggering a signal transduction cascade that results in apoptosis of the T cell. Consequently, if viral proteins increase the concentration of FasL on the cell surface, any T cell ($CD4^+$ or $CD8^+$) that engages it will undergo cell suicide. This mechanism has been proposed to explain the relatively high frequency of "spontaneous" T cell apoptosis that occurs in human immunodeficiency virus-infected patients. The viral Nef, Tat, and SU proteins, human T lymphotropic virus Tax protein, and the human cytomegalovirus IE2 protein have all been implicated in promoting increased synthesis of FasL within infected cells. Of note, this insidious mechanism for killing T cells is co-opted from an important host process to limit immunopathology: normal Fas-mediated CTL killing removes activated T cells when they are no longer needed after infection, or when their presence in a tissue may be detrimental. For example, certain irreplaceable organs or tissues, such as those in the eye, remain free of potentially destructive T cells by maintaining a high concentration of FasL on cell surfaces.

Persistent Infections May Be Established in Tissues with Reduced Immune Surveillance

Cells and organs of the body differ in how extensively they are patrolled by circulating immune cells. Those with less surveillance may be a fortuitous site for establishment of a persistent infection. Possibly the most extreme example of a virus family that escapes immune detection are the papillomaviruses that cause skin warts. Production of infectious particles occurs only in the outer, terminally differentiated skin layer, where an immune response is impossible because of the absence of capillaries at the skin surface. Moreover, dry skin continually flakes off, ensuring efficient spread of infection. The dust on your desk or the particles that catch the sunlight beaming through a window are most likely keratin from human skin.

Certain compartments of the body, such as the central nervous system and vitreous humor of the eye, lack initiators and effectors of the inflammatory response, because these tissues can be damaged by the fluid accumulation, swelling, and ionic imbalances that accompany inflammation. The brain, for example, is shielded by the skull that provides protection against blunt injury, but also constrains the tissue it surrounds. Consequently, even modest inflammation in the brain would be dangerous, as the brain has no "room" in which to expand. In addition, because most neurons do not regenerate, cytolytic immune defenses could be catastrophic. Because of these unique aspects of the central nervous system, the antiviral response to viral infections is notably distinct in the brain, favoring noncytolytic clearance via cytokine release. Persistent infections occur in such tissues more frequently than in those such as the lung and gastrointestinal tract with extensive immune surveillance (Table 5.2).

Persistent Infections May be Established in Cells of the Immune System

Some viruses such as measles virus, Epstein-Barr virus and human immunodeficiency virus, can infect cells of the immune system. Infected lymphocytes and monocytes rapidly disseminate throughout the host, providing efficient delivery of virus particles to new tissues. If infected immune cells die or become impaired during an acute infection, the host response could be rendered ineffective, and a persistent infection may be established. Systemic immunosuppression as a result of viral infection is discussed later in this chapter.

Human immunodeficiency virus type 1 infects not only $CD4^+$ Th cells, but also enters monocytes, dendritic cells, and macrophages, all of which can transport the virus to lymph nodes, the brain, and other organs. One might expect that the immune system would crash within a few days of the initial infection, but this does not happen, primarily because immune cells are continuously replenished. The new cells can be infected subsequently and die, but on average, the immune system remains functional for years following seroconversion: virus-triggered death is balanced by immune cell replacement. As a result, an untreated individual with human immunodeficiency virus continues to produce very large quantities of virus particles in the face of a highly activated immune system. It is only at the end stage of disease, when viral reproduction finally outpaces replenishment, that massive and fatal immune collapse occurs.

Two Viruses That Cause Persistent Infections

Measles virus infection in humans and lymphocytic choriomeningitis virus infection in mice are two well-studied examples that illustrate the establishment of persistence and the diseases associated with chronic virus reproduction.

Measles virus. This member of the family *Paramyxoviridae* is a common human pathogen with no known animal reservoir. Measles is one of the most contagious human viruses, and each year ~20 to 30 million infections occur worldwide, resulting in more than 100,000 deaths (predominantly of children). The incidence of measles varies widely in the world: most cases and fatalities occur in Southeast Asia and Africa, in contrast to Europe and the United States, where measles virus is generally well-controlled (Fig. 5.7A). Fortunately, aggressive vaccination campaigns over the past decade have reduced the global incidence of measles virus infection (Fig. 5.7B).

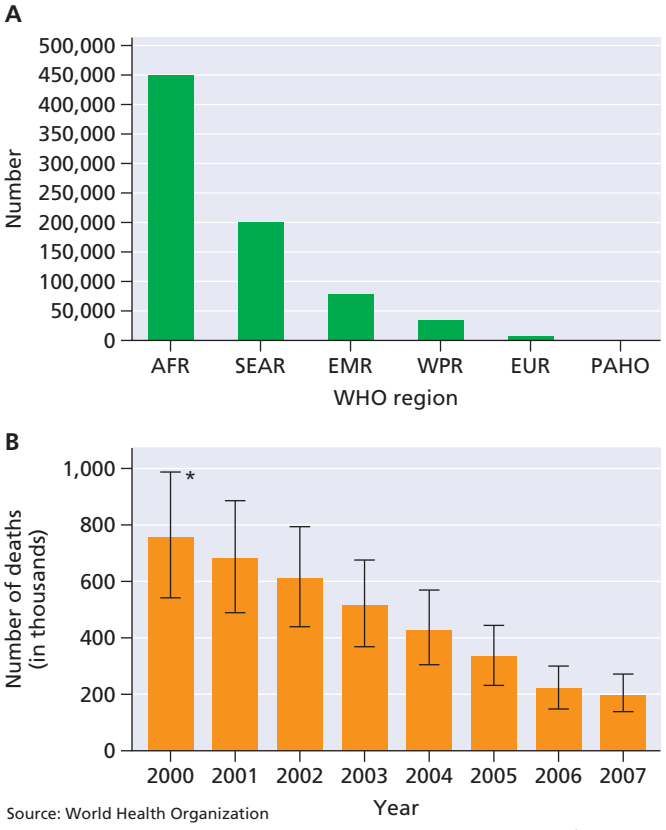


Figure 5.7 Worldwide burden of measles virus. (A) Deaths due to measles virus based on geographical region. **(B)** Reduced mortality due to measles virus as a consequence of an effective worldwide vaccination campaign. AFR, African Region; SEAR, South Eastern Asian Region; EMR, Eastern Mediterranean Region; WPR, Western Pacific Region; EUR, European Region; PAHO, Pan American Health Organization. Data from CDC, *Morb Mortal Wkly Rep* **52:**471-475 and **57:**1303-1306.

After primary reproduction in the respiratory tract, measles virus infects resident monocytes and lymphoid cells that migrate to draining lymph nodes, where a small proportion enter the circulation. Infection of lymph tissues results in a secondary viremia that leads to epithelial cell infection in the lungs and skin. The course of an uncomplicated acute infection runs about two weeks, and is associated with cough, fever, the characteristic rash, and conjunctivitis (Fig. 5.8).

The vast majority of measles-infected individuals have an uneventful recovery, and lifelong immunity is established. However, during the course of acute infection, and for about two weeks after the infection is resolved, the host is transiently immunosuppressed. Consequently, secondary infections by other pathogens during this period may be uncontested by host defenses, and the results can be serious or fatal, if immediate intervention and care are not provided (see "Immunosuppression induced by viral infection" below).

In rare cases, severe, life-threatening diseases can occur when measles virus enters the brain, carried by infected lymphocytes that traverse the blood-brain barrier. The most common central nervous system complication is acute postinfectious encephalitis, which occurs in about 1 in 3,000 infections. The other is a very rare, but often lethal, brain infection called subacute sclerosing panencephalitis (SSPE). About one in 10 to 100,000 individuals with acute measles infection eventually develop SSPE, within a 6- to 8-year incubation period (Box 5.5). SSPE is most prevalent in children, especially those infected in their first year or two of life. Although the brains of SSPE victims are described histologically as "decorticated" because of massive cell loss, fully assembled particles cannot be detected in brains from autopsy specimens, perhaps because alterations in envelope proteins lead to ineffective particle assembly. Nevertheless, viral nucleoprotein complexes are produced, and infectious genomes probably spread between synaptically connected neurons. It is thought that the viral fusion protein, but not any of the known viral receptors, is necessary for spread of such complexes in the absence of assembled virus particles.

A long-standing mystery is the state of measles virus in the brain during the multiyear period between acute infection and the clinical appearance of SSPE. One possibility is that a true latency is established, in which no viral genomes are made. Alternatively, there may be a slow accumulation of progeny, with disease apparent only after a sufficient number of neurons are infected or a particular brain substructure is reached. In support of this latter hypothesis, a large number of brain samples taken from elderly individuals who had died of non-viral- and non-brain-related causes (for example, heart attacks), were positive for measles virus RNA, indicating that this virus may be able to establish life-long, central nervous system infections. It could therefore be surmised that some viral genome replication had to occur in order to sustain viral RNA for decades following acute challenge. If this were the case, the implication would be that not all viruses that enter the brain are necessarily pathogenic. However, more subtle long-term consequences of central nervous system infection by viruses has not been explored in any detail. Why, in some cases, measles infection of the brain leads to devastating diseases such as SSPE remains unknown, in part, because these diseases are so rare that they are difficult to study.

Lymphocytic choriomeningitis virus. This member of the family *Arenaviridae* was the first virus associated with aseptic meningitis in humans, although it has been most valuable as a model infection in mice. Use of this animal model has illuminated fundamental principles of immunology and viral pathogenesis, including insights into persistent infection, CTL recognition and MHC production, and immunopathogenesis. Early in the study of this virus, it was found

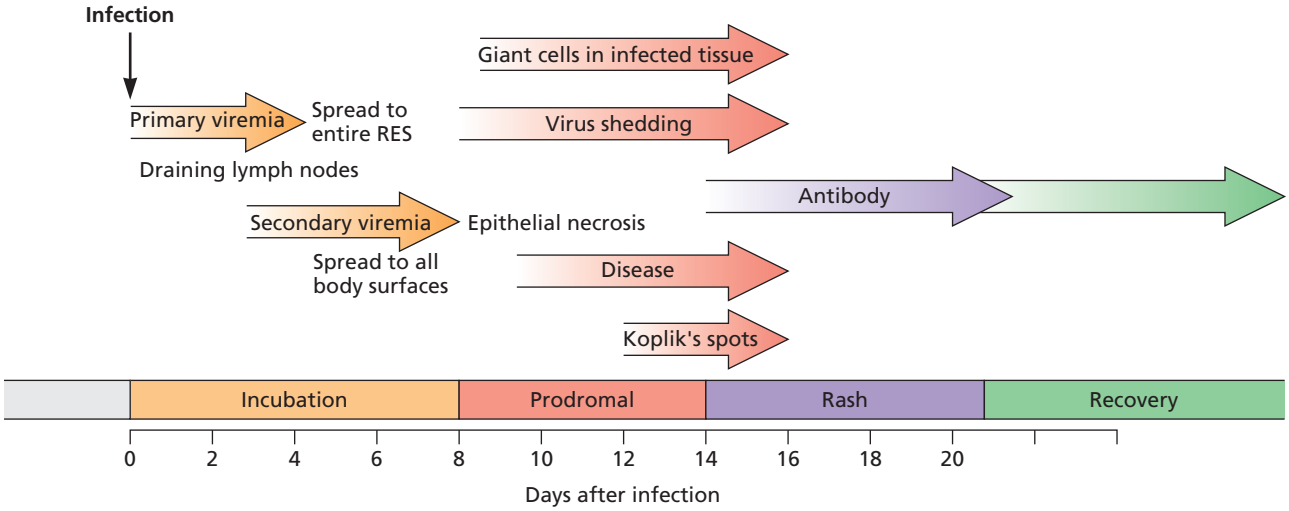


Figure 5.8 Infection by measles virus. Course of clinical measles infection and spread within the body. Four clinically defined temporal stages occur as infection proceeds (illustrated at the bottom). Characteristic symptoms appear as infection spreads by primary and secondary viremia from the lymph node to phagocytic cells, and finally to all body surfaces. The timing of typical reactions that correspond to the clinical stages is shown by the colored arrows. The telltale spots on the inside of the mouth (Koplik's spots) and the skin lesions of measles consist of pinhead-sized papules on a reddened, raised area. RES, reticuloendothelial system. Adapted from A. J. Zuckerman et al., *Principles and Practice of Clinical Virology*, 3rd ed. (John Wiley & Sons, Inc., New York, NY, 1994), with permission.

that the infection can spread zoonotically from rodents (the natural host) to humans, resulting in severe neurological and developmental damage. Infected rodent carriers excrete large quantities of virus particles in feces and urine throughout their lives without any apparent pathogenic consequence. The carrier state is established because the virus is not cytopathic and, if introduced to mice congenitally or immediately after birth (the main route of infection in the wild), viral peptides cannot be recognized as foreign ("non-self") by the developing immune response. In sharp contrast, if as few as 1 to 2 plaque-forming units are introduced intracerebrally into adult mice, the animals die of massive edema

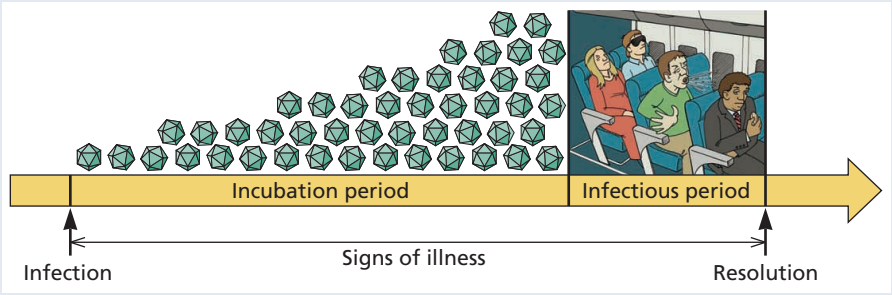
BOX 5.5

TERMINOLOGY

Incubation period and infectious period

These concepts are often used interchangeably, but they represent distinct processes that may, or may not, overlap temporally. In this text, we use "incubation period" to refer to the time interval between when a host becomes infected, and the appearance of symptoms of infection. This is distinct from the "infectious period," which defines the period during which an individual is shedding virus that can be transmitted to others.

In some cases, such as Ebola, virus is not shed until symptoms appear. In others, such as varicella-zoster virus and measles virus, the host may be infectious for some period (days) before symptoms are evident.



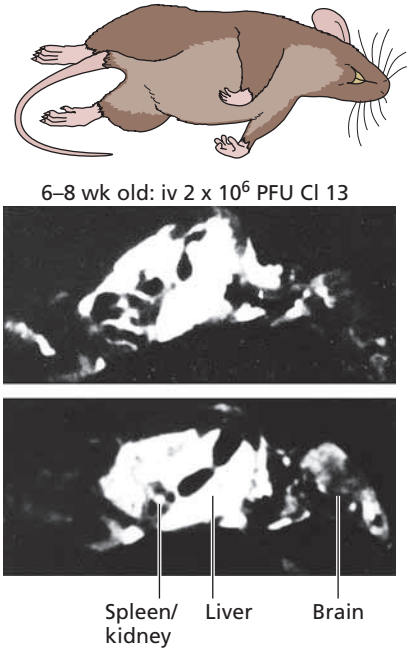


Figure 5.9 Persistent infection with lymphocytic choriomeningitis virus. Mice challenged with lymphocytic choriomeningitis virus as neonates were collected as adults, and whole body sections were made and subjected to RNA hybridization using radiolabeled probes. All "white" areas indicate active presence of viral nucleic acid. Adapted from M.B.A. Oldstone, *PLoS Pathog* **5(7):**e1000523 doi:10.1371/journal.ppat.1000523, 2009, with permission.

and encephalitis. The cerebral disease is immunopathological, as infection of adult mice lacking a functional immune response leads to persistence. In such persistently infected mice, virtually all tissues contain infectious virus, although most animals show no outward signs of illness (Fig. 5.9). More careful analyses have revealed that persistently infected mice are less "smart" than their uninfected peers, leading to the idea that persistent infections may cause nonlethal (that is, more subtle) forms of disease. The flexibility of this model system has paved the way for substantial insights into the long-term consequences of persistent infection within a host, and immune exhaustion resulting from chronic immune activation (Chapter 4).

Latent Infections

Latent infections are characterized by three general properties: viral gene products that promote virus reproduction are not made, or are synthesized in only small quantities; cells harboring the latent genome are poorly recognized by the immune system; and the viral genome persists intact so that a productive infection can be initiated at some later time to ensure the spread of viral progeny to new hosts (Fig. 5.1). The latent genome can be maintained as a nonreplicating chromosome in a nondividing cell (neuronal infection with herpes simplex virus or varicella-zoster virus), as an autonomous, self-replicating chromosome in a dividing cell (Epstein-Barr virus infection in B cells or cytomegalovirus infection in salivary and mammary glands), or be integrated into a host chromosome, where it is replicated in concert with the host genome (adenovirus-associated virus).

There is no single mechanism to account for how all viruses can establish and maintain a latent infection. An emerging principle is that epigenetic alterations of viral genomes may facilitate the switch from productive reproduction to a latent state. Reactivation may be spontaneous (stochastic) or may follow trauma, stress, or other insults. While members of other virus families can establish latency, this property is a cardinal feature of the herpesviruses, and much is known about the establishment, maintenance, and reactivation of latency in this group of human pathogens. We therefore discuss the biology of herpes simplex type 1 and Epstein-Barr virus in some detail. How latency is established and reactivated following infection with these two herpesviruses, and the diseases associated with them, are remarkably distinct. These examples illustrate that, even within the same virus family, a common outcome can result from very different strategies.

Herpes Simplex Virus

The vast majority of adults in the United States have antibodies to herpes simplex virus type 1 or 2 and harbor latent viral genomes in their peripheral nervous systems. Approximately 40 million infected individuals will experience recurrent herpes disease as a result of virus reactivation at some point in their lifetimes. Many millions more carry latent viral genomes in their nervous systems, but never report reactivated infections. Why some people are more likely to suffer from the consequences of reactivation is poorly understood (Box 5.6). Although no animal reservoirs are known, several animals, including rats, mice, guinea pigs, and rabbits, can be infected experimentally. The alphaherpesviruses, of which herpes simplex virus type 1 is the type species, are unique in establishing latent infections predominantly in terminally differentiated, nondividing neurons of the peripheral nervous system.

The primary infection. Herpes simplex virus infections usually begin in epithelial cells at mucosal surfaces (Fig. 5.10). Virus particles are released from the basal surface in close proximity to sensory nerve endings. Because sensory terminals are abundant, they are easily infected, but autonomic nerve terminals may also be infected if deeper layers of the skin, including those containing endothelial cells of capillaries, are exposed to viral particles. If infection occurs in the eye, parasympathetic and cranial nerve endings may be invaded. Fusion of the viral envelope with any of these nerve endings releases the nucleocapsid with inner tegument proteins into

BOX 5.6

DISCUSSION

The hygiene hypothesis: why people vary in their response to herpes simplex virus infection

More than 80% of the adult population in the developed world harbor latent herpesviral genomes in their peripheral nervous system. Some individuals suffer from lesions after reactivation while others do not, although what accounts for the high infectivity yet marked diversity in host response to infection remains obscure.

It has been hypothesized that the capacity of the intrinsic and innate immune responses to stimulate appropriate adaptive immunity (T_h1 versus T_h2) is shaped by the individual's exposure to microbes early in life. A highly sanitized environment may lead to reduced stimulation of innate immunity during this critical period when the immune response learns to differentiate harmless substances (e.g., allergens) from those that can cause illness. Lack of immune education early in life may result in the reduced capacity to control infections later.

According to this hypothesis, the rising incidence of allergy and asthma, as well as of herpes simplex virus infections, in Western societies results from "hypersanitized" living conditions. Such conditions arise from use of sterilized baby food, excessive application of germicidal soaps, antibiotics, and cleaners, and limited exposure of newborns to other individuals. Individuals who had limited exposure to microbes in early life will experience more reactivations of latent herpesvirus with severe symptoms because of their inability to mount an effective T_h1-dominated response. Instead, with inadequate early stimulation of innate immunity by microbial infections, subsequent exposure to foreign antigens may stimulate an inappropriate T_h2 response. Testing the hygiene hypothesis is not an easy matter; many observations that apparently support or refute the hypothesis are anecdotal or poorly controlled. Nevertheless, the idea has stimulated considerable research and debate. Get out there and make some mud pies!

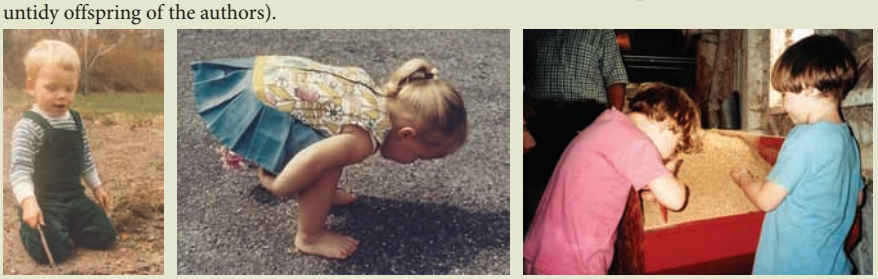
Camateros P, Moisan J, Henault J, De S, Skamene E, Radzioch D. 2006. Toll-like receptors, cytokines and the immunotherapeutics of asthma. *Curr Pharm Des* **12:**2365–2374.

Rouse BT, Gierynska M. 2001. Immunity to herpes simplex virus: a hypothesis. *Herpes* **8**(Suppl. 1)**:**2A–5A.

Strachan D. 1989. Hay fever, hygiene, and household size. *Br Med J* **299:**1259–1260.

Zock JB, Plana E, Jarvis D, Anto JM, Kromhout H, Kennedy SM, Kunzli N, Villani S, Olivieri M, Toren K, Radon K, Sunyer J, Dahlman-Hoglund A, Norback D, Kogevinas M. 2007. The use of household cleaning sprays and adult asthma: an international longitudinal study. *Am J Respir Crit Care Med* **176:**735–741.

A few not-so-representative examples of messy kids. (These examples are some of the rather untidy offspring of the authors).



the axoplasm. Dynein motors then move the internalized nucleocapsid on microtubules over long distances to the cell bodies of the neurons that innervate the infected peripheral tissue. A productive infection may be initiated in these neurons when the viral DNA enters the nucleus.

While it is commonplace to focus on neurons in this pattern of infection by herpes simplex virus, only 10% of the cells in a typical sensory ganglion are neurons; the remaining 90% are nonneuronal satellite cells and Schwann cells associated with a fibrocollagenous matrix. These nonneuronal cells are in intimate contact with neurons within ganglia. Some of the nonneuronal cells are infected during initial invasion of the ganglion, and may be the major source of infectious particles isolated from infected ganglia.

Establishment and maintenance of the latent infection. Soon after infection in neurons, the viral genome is coated with nucleosomes and may be silenced. In this case, transcription of viral genetic information is limited, and a quiescent, latent infection is established (Volume I, Chapter 8). As we will see, the establishment of this latent state is likely to depend both on viral regulatory proteins and RNA, and the intrinsic and innate immune defenses that protect these tissues.

In general, most neurons neither replicate their genomes nor divide, and so once a silenced viral genome is established in the nucleus, no further viral reproduction is required for it to persist. Standard antiviral drugs and vaccines cannot cure a latent infection. Consequently, latency is sustained for the life of the host, or, as one herpesvirologist put it, "herpes is forever."

In several animal models and presumably in infected humans, peripheral ganglia support a robust acute infection with production of appreciable numbers of virus particles followed by a strong inflammatory response. Nevertheless, after 1 or 2 weeks, infectious particles can no longer be isolated from the ganglia, and establishment of the latent infection is inevitable. Inflammatory cells may persist in the latently infected ganglia for months or years, perhaps as a result of continuous or frequent low-level reactivation and production of viral proteins in latently infected tissue. Reactivated virus could come from either infected satellite cells or neurons.

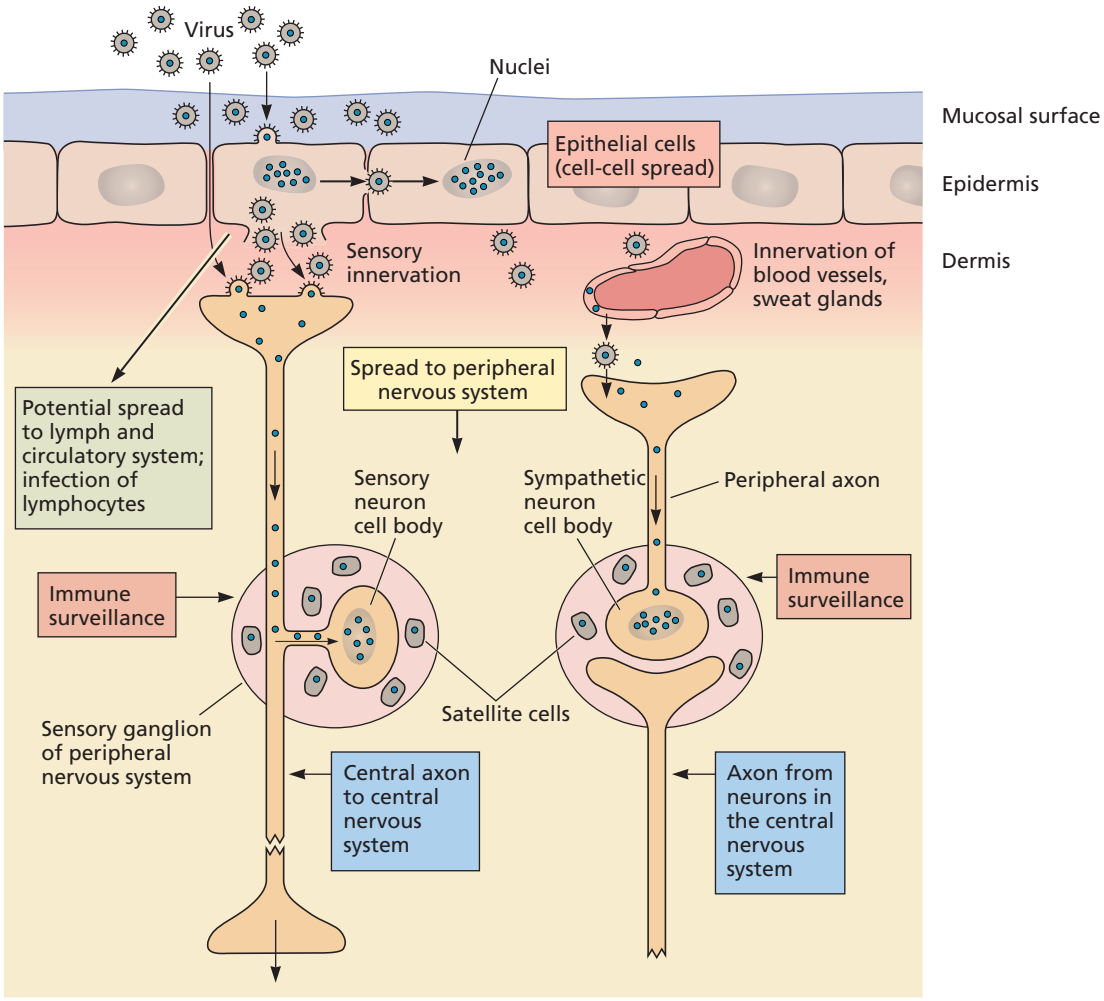


Figure 5.10 Herpes simplex virus primary infection of sensory and sympathetic ganglia. Viral reproduction occurs at the site of infection, usually in the mucosal epithelium. The infection may or may not manifest as a lesion. Host intrinsic and innate defenses, including type I IFN and other cytokines, normally limit the spread of infection at this stage. Virions may infect local immune effector cells, including dendritic cells and infiltrating natural killer cells. The infection also spreads locally between epithelial cells and may spread to deeper layers to engage fibroblasts, capillary endothelial cells, sweat glands, and other dermal cells such as those present in piloerector muscles around hair follicles. Particles that are released from basal surfaces infect nerve terminals in close contact. These axon terminals can derive from sensory neurons in dorsal root ganglia **(Left)** or from autonomic neurons in sympathetic ganglia **(Right)**. Viral envelopes fuse with neuron axonal membranes, and the nucleocapsid with outer tegument proteins is transported within the axon to the neuronal cell body by microtubule-based systems (dynein motors), where viral DNA is delivered to the neuronal nucleus. Spread of productive infection to the central nervous system from these peripheral nervous system ganglia is rare. Unlike the brain and spinal cord, peripheral nervous system ganglia are in close contact with the bloodstream and are exposed to lymphocytes and humoral effectors of the immune system (immune surveillance). Consequently, infected ganglia become inflamed and populated with lymphocytes and macrophages. Infection of the ganglion is usually resolved within 7 to 14 days after primary infection, virus particles are cleared, and a latent infection of some neurons in the ganglion is established.

We do not understand why neurons are the favored site for a latent infection. Furthermore, it is difficult to explain how neurons in ganglia survive the primary infection by this markedly cytolytic virus. Most relevant to human disease, we do not understand why the infection stops in the first-order neurons of the peripheral nervous system and rarely spreads to the central nervous system, which is in direct synaptic contact with peripheral neurons. Were this not to be the case, devastating encephalitis would presumably be much more commonplace.

The latency-associated transcripts. Many latently infected neurons synthesize RNA molecules termed **latency-associated**

transcripts (LATs) (discussed in Volume I, Chapter 8). Some researchers argue that all latently infected neurons synthesize LATs, while others report that only 5 to 30% do so. As in many studies of the latent state, the results depend on the animal model.

After infection of rabbits, viral mutants that do not synthesize LATs establish a latent infection, but spontaneous reactivation is markedly reduced. Despite this observation, which suggested that LATs contribute to reactivation, identifying molecular functions for the LATs continues to be a challenge. The major LAT contains two prominent open reading frames with potential to encode two proteins, but there is little evidence that these proteins are produced. Furthermore, disruption of these open reading frames has no effect on latency establishment or reactivation, and the sequences are not conserved in the closely related herpes simplex type 2 genome.

If the herpes simplex virus LATs are not translated, then the RNA molecules themselves may have biological activity. One hypothesis is that they are microRNA precursors that lead to degradation or reduced translation of host mRNAs. MicroRNAs may be a common feature of herpesvirus latency systems, as they now are suspected to be important for latent infections caused by the betaherpesviruses and gammaherpesviruses as well. Another proposal is that the herpes simplex virus type 1 LATs block apoptosis upon primary infection of neurons (or following reactivation). Some have contended that LATs maintain the latent state through antisense inhibition of the translation of ICP0 (a crucial viral transcription activator). Finally, it has been shown that herpes simplex virus type 1 LATs mediate the transition to latency by altering chromatin structure, perhaps by a process similar to mammalian X chromosome inactivation by the Xist RNA.

Reactivation. After reactivation of a latent infection in sensory ganglia, virus particles appear in the mucosal tissues innervated by that particular ganglion, an effective means of ensuring transmission of virus particles after reactivation, because mucosal contact is common among affectionate humans. However, in order to infect another person, sufficient virus particles must be produced in an individual who has already generated an antiviral response. Virus progeny can be produced in the face of existing immunity, in part, because the viral protein ICP47 blocks MHC class I presentation of viral antigens to T cells and facilitates spread of infection within epithelia. Such activity may provide sufficient time for virus reproduction to occur before the infected cell is eliminated by activated CTLs.

Murine models have been used to show efficient establishment of latency in neurons even in the presence of an antibody response in vaccinated animals, or in animals that receive passive immunization with virus-specific antibodies prior to infection. The immune response after reactivation is usually robust and clears the infected epithelial cells in a few days, but not before virus particles are shed. The typical "cold sore" lesion of herpes labialis is the result of the inflammatory immune response attacking the infected epithelial cells that were in contact with axon terminals of reactivating neurons. Some individuals with latent herpes simplex virus experience reactivation every 2 to 3 weeks, while others report rare (or no) episodes of reactivation. Indeed, reactivation may result in the shedding of infectious particles in the absence of obvious lesions or symptoms (Fig. 5.1). A final aspect of this reactivation phenomenon is that the virus can move directly from latently infected neurons to epithelial cells without the release of infectious progeny. Consequently, the host response would not be alerted until productive infection of epithelial cells occurs. This feature of herpesvirus reactivation presents extreme difficulties to those who strive to produce efficacious vaccines.

Reactivation from ganglia: not "all or none." The triggers that reactivate a latent infection include sunburn, stress, nerve damage, steroid use, heavy metals (the chemicals, not the music), and trauma, including dental surgery. Despite the apparent systemic nature of most reactivation stimuli, when reactivation does occur in animal models, only about 0.1% of neurons in a ganglion that contain the viral genome synthesize viral proteins and produce virus particles. The regulatory network in operation does not include an "on or off" circuit that affects all latently infected neurons, but rather may be sensitive to some nonuniformity within the latent population. Not only are different types of neurons infected in peripheral ganglia, but also the number of viral genomes in a given neuron varies dramatically (Box 5.7). Indeed, it is likely that one facet of competency for reactivation is the number of viral genomes within a given neuron: the more genomes, the more likely to reactivate.

Signaling pathways in reactivation. The diversity of potential reactivation signals may be surprising. However, it is likely that they all converge to stimulate production or action of specific cellular proteins needed for transcription of the herpes simplex virus immediate-early genes, and consequently activate the productive transcriptional program. Indeed, all of these exogenous signals have the capacity to induce the synthesis of cell cycle and transcriptional regulatory proteins that may render neurons permissive for viral reproduction. The synthesis of the viral immediate-early protein ICP0 is sufficient to reactivate a latent infection in model systems, and this viral protein has opposing functions to LATs in modulation of chromatin structure. In a single latently infected neuron, reactivation may be an all-or-none process requiring but a single reaction such as chromatin structural changes to "flip the switch" that triggers the cascade of gene expression of the

BOX 5.7

DISCUSSION

Neurons harboring latent herpes simplex virus often contain hundreds of viral genomes

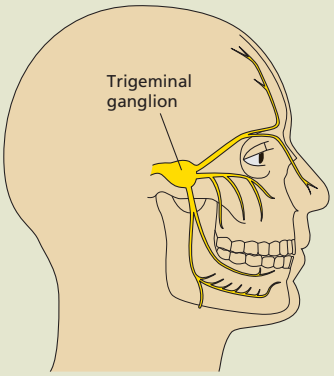
The number of neurons in a ganglion that will ultimately harbor latent genomes following primary infection depends upon the host, the strain of virus, the concentration of infecting virus particles, and the conditions at the time of infection. A mouse trigeminal ganglion contains about 20,000 neurons. It is possible to infect as few as 1% to as many as 50% of the neurons in a ganglion. In controlled experiments with mice, the number of latently infected neurons increases with the dose.

Many infected neurons contain multiple copies of the latent viral genome, from fewer than 10 to more than 1,000; a small number have more than 10,000 copies. The significance of such variation in copy number has been enigmatic. Does it reflect multiple infections of a single neuron, or is it the result of replication in a stimulated permissive neuron after infection by one particle? If it is the latter, how does the neuron recover from what should be an irreversible commitment to the productive cycle?

When virus reproduction is blocked by mutation or antiviral drugs, the number of latently infected neurons with multiple genomes is reduced significantly. Therefore, a single neuron may be infected by multiple virus particles, each of which participates in the latent infection.

Sawtell NM. 1997. Comprehensive quantification of herpes simplex virus latency at the single-cell level. *J Virol* **71:**5423–5431.

Localization of the trigeminal ganglia, also called the semilunar ganglia.



productive pathway. Glucocorticoids are excellent examples of such activators, as they stimulate transcription rapidly and efficiently while inducing an immunosuppressive response. These properties explain the observation that clinical administration of glucocorticoids frequently results in reactivation of latent herpesviruses. While reactivation from a single neuron is likely all or none, the virus is reactivated in only a small number of neurons within an infected ganglia. Why so few of such neurons are affected, especially as glucocorticoids or trauma must impact all neurons within the ganglia, remains an important question in this field.

Epstein-Barr Virus

Epstein-Barr virus, also called human herpesvirus 4, is the type species of the gamma subfamily of herpesviruses, and is one of the most common viruses to infect humans (its only host). Indeed, in the United States, up to 95% of adults are seropositive and carry the viral genome in latently infected B cells. Two strains of Epstein-Barr virus that differ in their terminal repeats, as well as in production of nuclear antigens and small RNAs during latent infection, are recognized. Epstein-Barr virus 1 is about 10 times more prevalent in the United States and Europe than is Epstein-Barr virus 2, while both strains are equally represented in Africa. Most people are infected with the virus at an early age and have no symptoms, but some develop **infectious mononucleosis** ("mono") (Box 5.8).

Epstein-Barr virus establishes latent infections in B lymphocytes, and is one of the herpesviruses consistently associated with human cancers (Table 5.2; Appendix). As we will learn in Chapter 6, B cell immortalization is a consequence of the mechanisms by which a latent infection is established, but the fact that the patient develops B cell lymphoma provides no selective advantage to the virus. In contrast to the nonpathogenic latent state of herpes simplex virus, the latent state of Epstein-Barr virus has been implicated in several serious diseases.

The primary infection. Epstein-Barr virus particles infect both epithelial cells and B cells. Some have proposed that distinct viral ligands engage different entry receptors on the surfaces of these two cell types, but this issue remains to be demonstrated. In any case, infection initiates in epithelial cells, usually those of the mucosal epithelia in the oropharyngeal cavity, which are sites for shedding of virus particles. Oral epithelium and tonsil tissue are rich in lymphoid cells and provide the perfect environment for the next stage of infection. After productive infection of epithelial cells, released particles can infect B lymphocytes, in which a modified transcriptional program can lead to establishment of a latent infection. The viral DNA genome exists as a circular, self-replicating episome in the B cell nucleus (Volume I, Chapter 9). This episome becomes associated with nucleosomes and undergoes progressive methylation at CpG residues. When latently infected B cells are in contact with epithelial cells, the virus may be reactivated, resulting in production of progeny particles that can infect epithelial cells. Infectious particles are shed predominantly in the saliva, but shedding from lung and cervical epithelia has also been reported.

BOX 5.8

DISCUSSION

Epstein-Barr virus, depression, and pregnancy

Reactivation of Epstein-Barr virus has been linked with depression both in late-term pregnant women, and in mothers soon after delivery. Up to 25% of women will experience depression either before or after delivery, and many of these individuals have a higher prevalence of Epstein-Barr virus reactivation. In addition to the challenges of the depression itself, the consequences for the developing fetus could be substantial: short-term depression in the pregnant female could irrevocably alter critical glucocorticoid signaling pathways.

Studies such as these, although provocative, can be interpreted in a number of ways. First, as Epstein-Barr virus is abundant within the human population, ascribing a direct cause-and-effect relationship is difficult; the correlation could be purely circumstantial. Alternatively, a common trigger (such as stress) may cause both depression and virus reactivation, as opposed to virus reactivation being the cause of the depression. Regardless of the nature of the link, studies such as these serve as interesting reminders that viruses may be associated with symptoms or outcomes that we typically do not attribute to them.

Haeri S, Johnson N, Baker AM, Stuebe AM, Raines C, Barrow DA, Boggess KA. 2011. Maternal depression and Epstein-Barr virus reactivation in early pregnancy. *Obstet Gynecol* **117:**862–866.

Zhu P, Chen Y-J, Hao J-H, Ge J-F, Huang K, Tao R-X, Jiang X-M, Tao F-B. 2013. Maternal depressive symptoms related to Epstein-Barr virus reactivation in late pregnancy. *Sci Rep* **3:**3096.



Persistent infection. Both latently infected and productively infected B cells circulate among activated, virus-specific CTLs in the blood of infected individuals, and antibodies specific for viral proteins are abundant. How latency is maintained in B cells in the face of an active immune response is consequently a critical issue.

Children and teenagers are commonly infected, usually after oral contact (hence the name "kissing disease"). The acute infection requires expression of most viral genes and rapidly stimulates a potent immune response. Spread of infection to B cells in an individual with a competent immune system induces the infected cells to divide, augmenting immune and cytokine responses. The resulting disease is called infectious mononucleosis. The ensuing immune response destroys most infected cells, but approximately 1 in 100,000 survive. They persist as small, nonproliferating memory B cells that make **only** latent membrane protein 2A (LMP-2A) mRNA. They home to lymphoid organs and bone marrow, where they are maintained. These cells do not produce the B7 coactivator receptor, and therefore are not recognized or killed by CTLs (see Chapter 4).

When peripheral blood cells of an infected individual are cultured, growth factors in the medium stimulate proliferation of the rare, latently infected B cells, while uninfected B cells die. It is important to understand that these cultured immortal B cells (lymphoblasts) are not the same as latently infected cells that circulate *in vivo*. Nevertheless, because they can be propagated indefinitely, these cells comprise the best-understood model of Epstein-Barr virus latent infection. These immortal lymphoblasts synthesize a set of at least 10 viral proteins, including six nuclear proteins (termed EBNAs), three viral membrane proteins (LMPs), small RNA molecules called EBER-1 and EBER-2, and at least 20 microRNAs. With the exception of LMP-1, the contributions of these viral products to transformation are unknown, as many are not synthesized in human cancers associated with Epstein-Barr virus infection. At least three distinct phenotypes or programs can be distinguished according to the viral gene products made in an infected B cell (called latency I to III). Synthesis of distinct sets of viral proteins and RNA in these latency types are also linked to particular Epstein-Barr virus associated diseases (Table 5.3).

The complicated collection of different B cell phenotypes is best understood in the context of normal B cell biology. To enter the resting state and become a memory cell, an uninfected B cell must have bound its cognate antigen and received appropriate signals from helper T cells in germinal centers of lymphoid tissue. During latent infection, the viral LMP-1 and LMP-2a proteins mimic all of these steps, such that the infected B cell can differentiate into a memory cell in the absence of external cues.

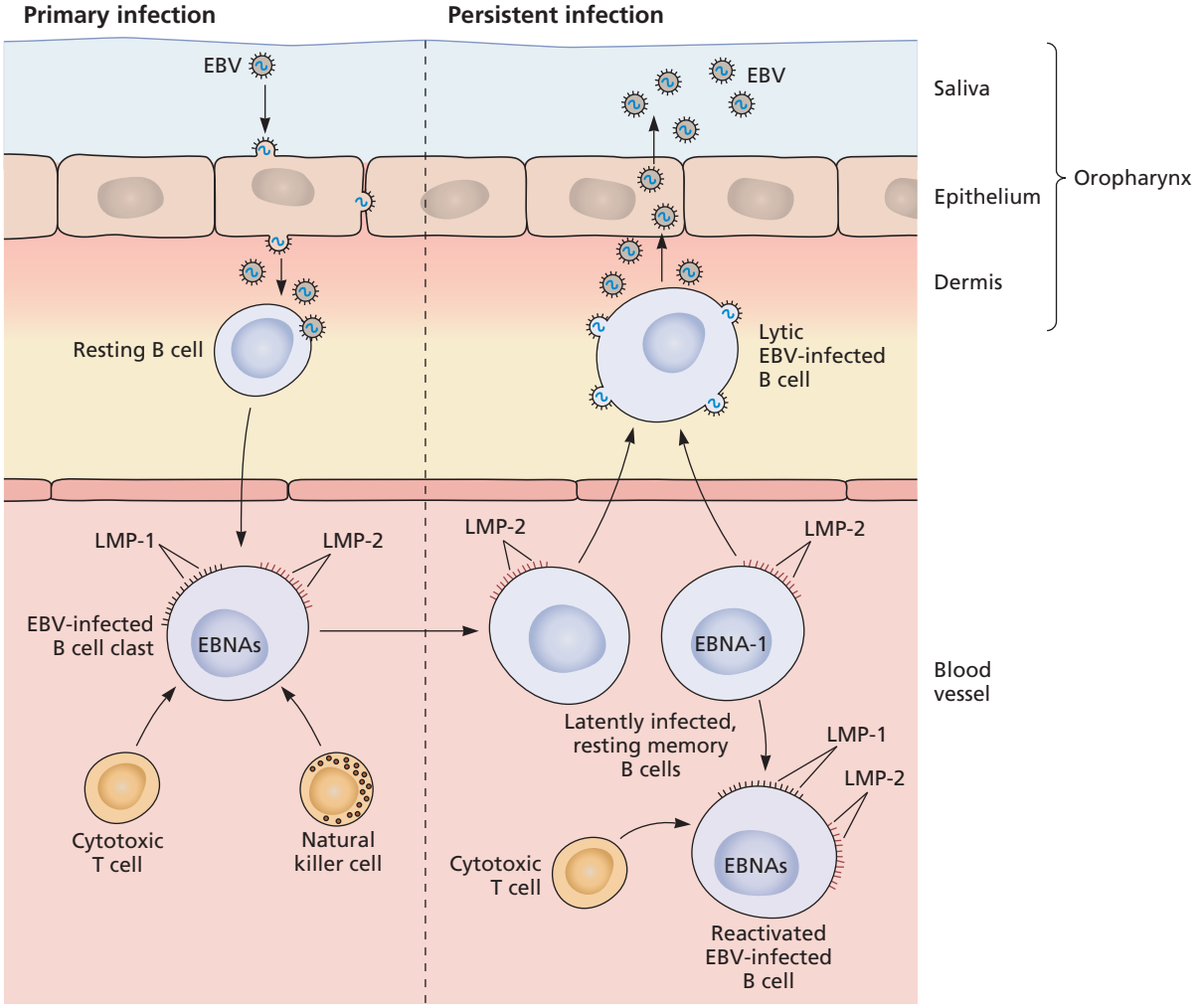
Table 5.3 Epstein-Barr latency programs

Latency program	Expressed viral genes	Disease(s)
0	None	None
I	LMP-2A/EBNA-1	Burkitt's Lymphoma
II	EBNA-1, LMP-1, LMP-2A, -2B	Hodgkin's disease, nasopharyngeal carcinoma
III	EBNA-1, -2, -3, -4, -5, -6, LMP-1, -2A, 2B	Infectious mononucleosis, AIDS-related immunoblastic B cell lymphoma

Although immunocompetent individuals maintain CTLs directed against many of the viral proteins synthesized in latently infected B cells, these cells are not eliminated. Some viral proteins, such as LMP-1, inhibit apoptosis or immune recognition of the latently infected cells. Moreover, EBNA-1 peptides are not presented to T cells, as discussed previously in this chapter. When the equilibrium between proliferation of latently infected B cells and the immune response that kills them is altered (e.g., upon immunosuppression), the immortalized B cells can form lymphomas (Fig. 5.11; see also Chapter 6).

Reactivation. The signals that reactivate latent Epstein-Barr virus reproduction in humans are not well understood,

Figure 5.11 Epstein-Barr virus primary and persistent infection. **(Left)** Primary infection. Epstein-Barr virus infects epithelial cells in the oropharynx (e.g., the tonsils). Virus particles can then infect resting B cells in the lymphoid tissue. Virus-infected B cells produce the full complement of latent viral proteins and RNAs (e.g., LMP-1 and LMP-2), and are stimulated to enter mitosis and proliferate. They produce antibody and function as B cell blasts. The latently infected B cells are attacked by natural killer cells and CTLs. **(Right)** Persistent infection. Most infected B cells are killed as a result of innate and immune defenses, but a few (approximately 1 in 100,000) persist in the blood as small, nonproliferating memory B cells that synthesize only LMP-2A mRNA. These memory B cells are presumably the long-term reservoir of Epstein-Barr virus *in vivo* and the source of infectious virus when peripheral blood cells are removed and cultured. A limited immune response to these infected B cells leads to self-limiting proliferation, infectious mononucleosis, or unlimited proliferation (polyclonal B cell lymphoma). When stimulated or propagated in culture, viral proteins needed to replicate and maintain the viral genome are again produced. Some latently infected B cells traffic to lymphoid tissues in close proximity to epithelial cells in the oropharynx. Here, the B cells are stimulated to produce particles capable of infecting and replicating in epithelial cells. Virus particles are produced and shed into the saliva for transmission to another host.



but considerable information has been obtained from studies of cultured cells. Certain signal transduction cascades that result in the production of the essential viral transcriptional activator, Zta (also called Z or zebra protein), reinitiates productive infection (Volume I, Chapter 8). However, Zta induces the full productive program only when specific promoters are methylated at CpG residues. Recall that in latently infected cells, the viral genome slowly acquires methylated cytosine residues, thereby facilitating reactivation when Zta is made. In essence, the very modifications that enable the transition to latency are also those that are critical for viral reactivation. Zta also represses the latency-associated promoters, and is responsible for recognition of the lytic origin of replication.

Many signal transduction pathways cooperate to reactivate Epstein-Barr virus from the latent state. Given this fact, it is surprising that latent infection is so stable. We now know that virus-encoded LMP-2A makes an important contribution to maintaining the latent infection by inhibiting tyrosine kinase signal transduction pathways. It is the first example of a viral protein that blocks reactivation of a latent infection. While the parameters that cause Epstein-Barr virus reactivation are less well defined than those that cause herpes simplex virus reactivation, many of the same conditions, including stress, have been implicated.

"Slow" Infections

Some fatal brain diseases of mammals are characterized by ataxia (movement disorders) or dementia (severe cognitive impairment), and likely to be a result of a variation of persistent infection, called **slow infection** (Fig. 5.1). In these instances, it may be years from the time of initial contact of the infectious agent with the host until the appearance of recognizable symptoms, but death then usually follows quickly.

Elucidating the molecular mechanisms responsible for an infectious-disease process of such long duration is a formidable challenge. Experimental analysis of these unusual diseases began in the 1930s, when a flock of Karakul sheep was imported from Germany to Iceland, where they infected the native sheep, causing a disease called maedi/visna. Thanks to careful work of Bjorn Sigurdsson, we now know that the maedi/visna syndrome is caused by a lentivirus. The striking feature that Sigurdsson discovered is the slow progression to disease after primary infection, often taking more than 10 years. He developed a framework of experimentation for studying these slow, progressive, and fatal brain infections that laid the foundation for later studies on human immunodeficiency virus, as well as prions and other "infectious" diseases of protein misfolding (Chapter 14).

Viruses such as measles virus, the polyomavirus JC virus, and retroviruses such as human immunodeficiency virus and human T lymphotropic virus can establish slow infections with severe nervous system pathogenesis at the end stage of disease. In many cases, the persistent infection is maintained in peripheral compartments with no apparent effect, and only enters the brain after many years. Consequently, whether "slow infection" is an accurate descriptor is of some debate: it may be that the virus is sequestered in a peripheral tissue in a latent state, reactivates, and transits to the brain where a more "typical" acute infection occurs. As mentioned earlier in this chapter, it is hard to imagine that the virus reproduces at such a slow pace that years are required for symptoms to be manifest. It is perhaps more meaningful to think of "slow" in relation to the incubation period rather than the actual rate of viral reproduction.

Abortive Infections

In an abortive infection, virus particles infect susceptible cells or hosts, but reproduction is not completed, usually because an essential viral or cellular gene is not expressed. Clearly, an abortive infection is nonproductive. Even so, it is not necessarily uneventful or benign for the infected host. Viral interactions at the cell surface and subsequent uncoating can initiate membrane damage, disrupt endosomes, or activate signaling pathways that cause apoptosis or cytokine production. In some instances, abortively infected cells may not be recognized by the immune system, and if they do not divide, the viral genome may persist as long as the cell survives. In some cases, an infection may proceed far enough that the infected cell is recognized by CTLs. Even though this scenario would not result in infectious progeny, an inflammatory response may nevertheless damage the host if sufficient cells participate.

With the advent of modern viral genetics, virologists can construct intentionally defective viral genomes, which initiate an abortive infection in the absence of a complementing gene product. One approach has been to use such defective genomes as vectors for gene therapy or as vaccines. For this idea to be effective, cytopathic genes of a prospective viral vector certainly must be eliminated. Many of the well-known, defective viral vectors lack essential genes and are designed to express only the therapeutic cloned gene. Given that intrinsic and innate defenses can be activated in response to replication-defective particles, prudence in assuming the safety of viral vectors is essential (Box 5.9). Cytotoxicity and inflammatory host responses are of particular concern if the therapeutic gene is to be delivered to a substantial number of cells, a process that requires administration of many virus particles.

Transforming Infections

A **transforming infection** is a special type of persistent infection. A cell infected by certain DNA viruses or retroviruses may exhibit altered growth properties and begin to proliferate faster than uninfected cells. In some cases, this change is

BOX 5.9

DISCUSSION

A viral vector leads to lethal immunopathology

In September 1999, an 18-year-old man, Jesse Gelsinger, participated in a clinical trial at the University of Pennsylvania to test the safety of a defective adenovirus designed as a gene delivery vector. It seemed like a routine procedure: normally, even replication-competent adenoviruses cause only mild respiratory disease. Most humans harbor adenoviruses as persistent colonizers of the respiratory tract and produce antibodies against the virus. The young man was injected with a very large dose of the viral vector. Four days after the injection, he died of multiple-organ failure. What caused this devastating response to such an apparently benign virus?

Some relevant facts:

- A very large dose of virus was injected directly into his bloodstream.
- Natural adenovirus infection never occurs by direct introduction of virus particles into the circulation. Most infections occur at mucosal surfaces with rather small numbers of infecting virus particles.
- Most humans have antibodies to the adenoviral vectors used for gene therapy.

One compelling idea is that most of the infecting defective virus particles were bound by antibody present in the young man's blood. As a consequence, the innate immune system, primarily complement proteins, responded to the antibody-virus complex, resulting in massive activation of the complement cascade, and hence widespread inflammation in the vessel walls of the liver, lungs, and kidneys, and resulting in multiple-organ failure. This tragic end to a young man's life led to a large-scale reassessment of gene delivery methods and clinical protocols.

accompanied by integration of viral genetic information into the host genome. In others, viral genome replication occurs in concert with that of the cell. Virus particles may no longer be produced, but some or all of their genetic material generally persists. We characterize this pattern of persistent infection as transforming because of the change in cell behavior. Some transformed cells cause cancer in animals. This important infection pattern is discussed in detail in Chapter 6.

Viral Virulence

In the previous section, we discussed patterns of viral infection within individual cells and host organisms, and considered some of the diseases that may result from such infections. The manifestation of disease is an expression of viral **virulence**: a virulent virus causes disease, whereas an avirulent virus does not.

From the earliest days of experimental virology, it was recognized that viral strains often differ in virulence despite having similar reproduction rates. Virologists correctly hypothesized that the study of viruses with reduced virulence (**attenuated**), especially when compared with more virulent relatives, would provide insights into how viruses cause disease. This approach is still widely used in viral pathogenesis studies. We can experimentally alter viral genomes, and produce viruses of such limited virulence that they can be used as replication-competent vaccines (Chapter 8). Today, the methods of recombinant DNA technology allow us to mutate all genes in an unbiased way to accelerate the discovery of virulence genes.

Measuring Viral Virulence

Virulence can be quantified in a number of ways. One approach is to determine the quantity of virus that causes death or disease in 50% of the infected animals. This parameter is called the 50% lethal dose (LD_{50}), the 50% paralytic dose (PD_{50}), or the 50% infectious dose (ID_{50}), depending on the outcome that is measured (Box 5.10). Other measurements of virulence include time to death (Fig. 5.12A), the appearance of symptoms (such as a rash), the degree of fever, and weight loss. Virus-induced tissue damage can be measured directly by examining histological sections or blood (Fig. 5.12B). For example, the safety of replication-competent, attenuated poliovirus vaccine strains is determined by assessing the extent of pathological lesions in the central nervous system of experimentally inoculated animals. The reduction in the concentration of $CD4^+$ lymphocytes in blood as a result of human immunodeficiency virus type 1 infection is another example. Indirect measures of virulence include assays for concentrations of liver enzymes (alanine or aspartate aminotransferases) that are released into the blood following infection with liver-tropic viruses such as the hepatitis viruses.

It is important to recognize that virulence is relative, and that the pathogenesis resulting from infection with a single virus strain may vary dramatically depending on the route of infection, as well as on the species, age, gender, and susceptibility of the host (Box 5.11). Consequently, the assays must be identical when comparing the virulence of two similar viruses. Furthermore, quantitative terms such as LD_{50} cannot be used to compare virulence among different viruses.

BOX 5.10

TERMINOLOGY

Measures of viral virulence

LD_{50}: Median Lethal Dose: the number of infectious particles that will kill 50% of the infected recipients.

ID_{50}: Median Infectious Dose: the number of infectious particles that will establish an infection in 50% of the challenged recipients.

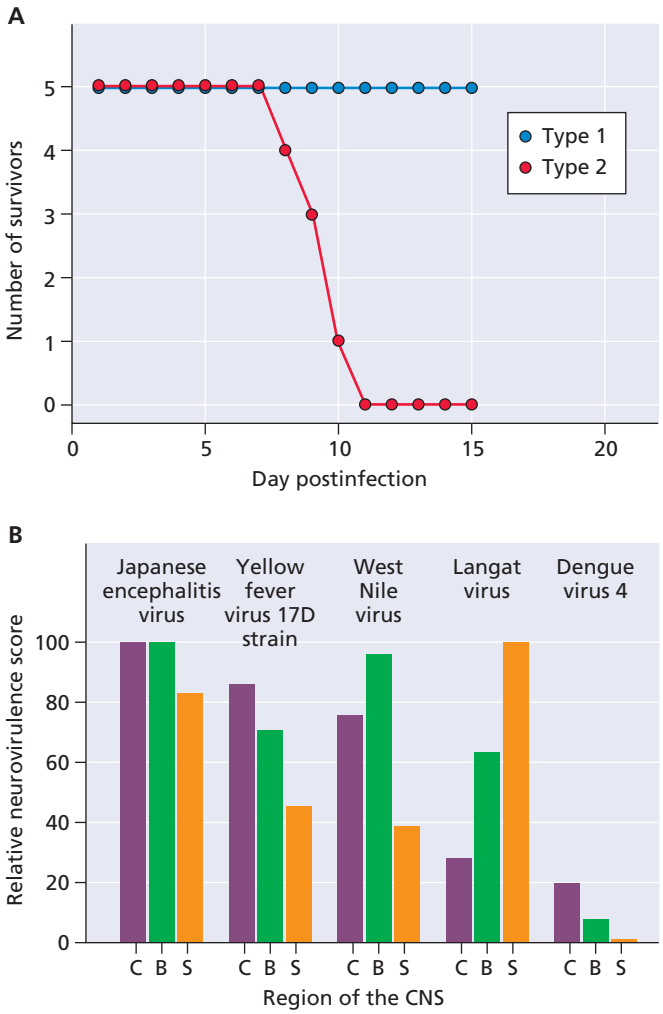


Figure 5.12 Two methods for measuring viral virulence. **(A)** Measurement of survival. Mice (5 per virus) were inoculated intracerebrally with either type 1 or type 2 poliovirus, and observed daily for survival. **(B)** Measurement of pathological lesions. Monkeys were inoculated intracerebrally with different viruses, and lesions in different areas of the central nervous system were assigned numerical values. C, cerebrum; B, brain stem; S, spinal cord. **(A)** Adapted from V. Racaniello, *Virus Res.* **1**:669–675, 1984, with permission. **(B)** Adapted from N. Nathanson (ed.), *Viral Pathogenesis and Immunity* (Academic Press, London, United Kingdom, 2007) with permission.

Alteration of Viral Virulence

Before the era of modern virology, several approaches were used to identify viral virulence genes. Occasionally, avirulent viruses were isolated from clinical specimens. For example, although wild-type strains of poliovirus type 2 readily cause paralysis after intracerebral inoculation into monkeys, an isolate from the feces of healthy children was shown to be completely avirulent after inoculation by the same route. A second approach to isolate viruses with reduced virulence was to serially passage viruses either in animal hosts or in cell culture (Chapter 8).

Although these approaches were useful, their success was unpredictable. To overcome this limitation, viral genomes were often altered experimentally by exposing the viruses to mutagens (as described in Volume I, Chapter 2), and the mutant viruses were then assayed for virulence in animals. However, controlling the degree of mutagenesis was difficult, and multiple mutations were often introduced. Until the

BOX 5.11

EXPERIMENTS

Viral virulence is dependent on multiple parameters

Lymphocytic choriomeningitis virus, a member of the arenavirus family, has been used extensively in studies of viral pathogenesis, in part owing to the distinct outcomes that occur following infection of mice via different routes. When adult immunocompetent mice are infected by a peripheral route (e.g., subcutaneously or intraperitoneally), virus reproduction is restricted to peripheral organs, the mouse mounts a robust immune response, and the virus is cleared with all mice surviving. Impressively, this outcome is independent of the original viral dose: mice can survive delivery of as many as 100,000 plaque-forming units. In sharp contrast, delivery of even 1 plaque-forming unit by an intracranial route kills all challenged mice. In these mice, the same robust immune response is made, but localized to infected cells within the brain, including the meninges, where it causes massive destruction and edema, leading to seizures that precede death (see Figure). Because disease is due solely to immunopathology, challenge of immunodeficient mice (such as recombinase-activating gene knockout mice) results in a third outcome: lifelong viral persistence throughout the mouse with no overt signs of illness.

Oldstone MBA. 2007. A suspenseful game of "hide and seek" between virus and host. *Nat Immunol* **8**:325–327.

Inoculation of mice by an intraperitoneal route with as many as 100,000 infectious units results in immunity and survival in most mice, whereas inoculation of as few as 1 infectious unit by an intracerebral route results in mortality in all challenged mice. LCMV, lymphocytic choriomeningitis virus.



Virus	LCMV-Armstrong	LCMV-Armstrong
Dose	100,000 PFU	1 PFU
Route	Intraperitoneal	Intracranial

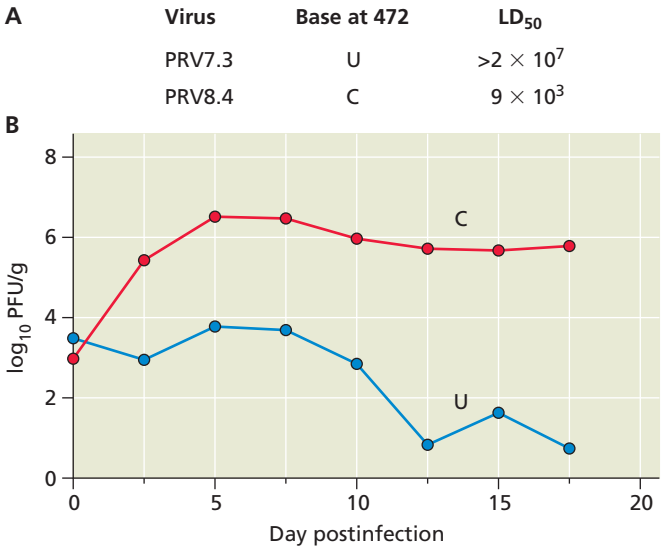


Figure 5.13 Attenuation of viral virulence by a point mutation. Mice were inoculated intracerebrally with two strains of poliovirus that differ by a single base change at nucleotide 472. **(A)** The dose of virus causing death in 50% of the animals (LD_{50}) was determined. The change from C to U is accompanied by a large increase in LD_{50}. **(B)** Viral reproduction in mice was determined by plaque assay of spinal cord homogenates. The change from C to U decreases viral replication in the spinal cord. Adapted from N. La Monica, J. W. Almond, and V. R. Racaniello, *J. Virol.* **61:**2917–2920, 1987, with permission.

advent of recombinant DNA technology, the ability to identify precisely (or create) mutations in a candidate virulence gene was limited. With the ability to sequence entire viral genomes, amplify specific genomic segments by polymerase chain reaction, and perform site-directed mutagenesis, the progress in identifying candidate viral virulence genes and their products has been greatly accelerated (Fig. 5.13).

Viral Virulence Genes

Despite these powerful technological advances, the identification and analysis of virulence genes in a systematic way has not been straightforward. Part of the problem is that no tissue culture assays that recapitulate the virulence observed in infected hosts exist. For example, many of the pathogenic effects caused by viral infections are a result of the host inflammatory response, and it is not possible to reproduce their complicated actions in a tissue culture dish. Additionally, it is not obvious which viral genes contribute to disease. There are no common "signatures" or motifs, and many so-called virulence genes encode proteins with multiple functions. A final challenge is that our expectations for viral virulence include major cytopathic effect and overt signs of cellular damage, but virulence can be subtle, affecting expression of host genes that would be difficult to appreciate in a standard cell culture-based analysis. Relevant animal models of disease are preferred for studying virulence and pathogenesis, but as noted earlier, such models are not always readily available. Nevertheless, considerable progress has been made in recent years. In the following sections, we discuss examples of viral virulence genes that can be placed in one of four general classes (Box 5.12).

Although this discussion focuses on producing viruses that are less virulent, the opposite approach, producing viruses that are **more** virulent than the wild type, is possible. The approach is rarely used, simply because unknown risks are involved (Box 11.5). An example of the inadvertent production of a more virulent pathogen is the isolation of a recombinant ectromelia virus containing the gene encoding interleukin-4 (IL-4) (Box 5.13).

Gene Products That Alter Virus Replication

Mutations in putative viral virulence genes can have one of two effects: some lead to poor reproduction of the virus, while others allow efficient reproduction, but reduced virulence (Fig. 5.14). Viral mutants that exhibit reduced or no reproduction in the animal host (or in culture) rarely cause disease, simply because they fail to produce sufficient viral progeny: this phenotype may be caused by mutations in virtually any viral gene. Some investigators mistake reduced reproduction for reduced virulence. Alternatively, some viruses exhibit

BOX 5.12

TERMINOLOGY

Four classes of viral virulence genes

The viral genes affecting virulence can be sorted into four general classes (and some may be included in more than one). The genes in these classes specify proteins that

- affect the ability of the virus to reproduce,
- modify the host's defense mechanisms,
- facilitate virus spread in and among hosts,
- are directly toxic.

As might be expected, mutations in these genes often have little or no effect on virus reproduction in cell culture and, as a consequence, are often called "nonessential genes," an exceedingly misleading appellation.

Virulence genes require careful definition, as exemplified by the first general class listed above (ability of the virus to reproduce). **Any** defect that impairs virus reproduction or propagation often results in reduced virulence. In many cases, this observation is not particularly insightful or useful. The difficulty in distinguishing an indirect effect caused by inefficient reproduction from an effect directly relevant to disease has plagued the study of viral pathogenesis for years.

BOX 5.13

EXPERIMENTS

Inadvertent creation of a more virulent poxvirus

Australia had a wild mouse infestation, and scientists were attempting to attack this problem with a genetically engineered ectromelia virus, a member of the family *Poxviridae*. The idea was to introduce the gene for the mouse egg shell protein zona pellucida 3 into a recombinant ectromelia virus: when the virus infected mice, the animals would mount an antibody response that would destroy eggs in female mice. Unfortunately, the strategy did not work in all the mouse strains that were tested, and it was decided to incorporate the gene for IL-4 into the recombinant virus. This strategy was based on the previous observation that incorporation of this gene into vaccinia virus boosts antibody production in mice. The presence of IL-4 was therefore expected to increase the immune response against zona pellucida.

To the researchers' great surprise, rampant reproduction of the recombinant virus in inoculated mice destroyed their livers and killed them. Moreover, even mice that were vaccinated against ectromelia could not survive infection with the recombinant virus; half of them died. Essentially, they had shown that the common laboratory technique of recombinant DNA technology could be used to overcome the host immune response and create a more virulent poxvirus. Those who conducted this work debated whether to publish their findings, but eventually did so. Their results raised alarms about whether such technology could be used to produce biological weapons, and the incident was widely reported in the press.

Jackson RJ, Ramsay AJ, Christensen CD, Beaton S, Hall DF, Ramshaw IA. 2001. Expression of mouse interleukin-4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox. *J Virol* **75:**1205–1210.

Müllbacher A, Lobigs M. 2001. Creation of killer poxvirus could have been predicted. *J Virol* **75:**8353–8355.



impaired virulence in animals, but show no defects in cells in culture (except perhaps in cells of the tissue in which disease develops). Such mutants should provide valuable insight into the basis of viral pathogenesis, because they allow identification of genes specifically required for disease.

A primary requirement for genome replication of DNA viruses is access to large pools of deoxyribonucleoside triphosphates. This need poses a significant obstacle for viruses that infect terminally differentiated, nondividing cells such as neurons or epithelial cells. The genomes of many small

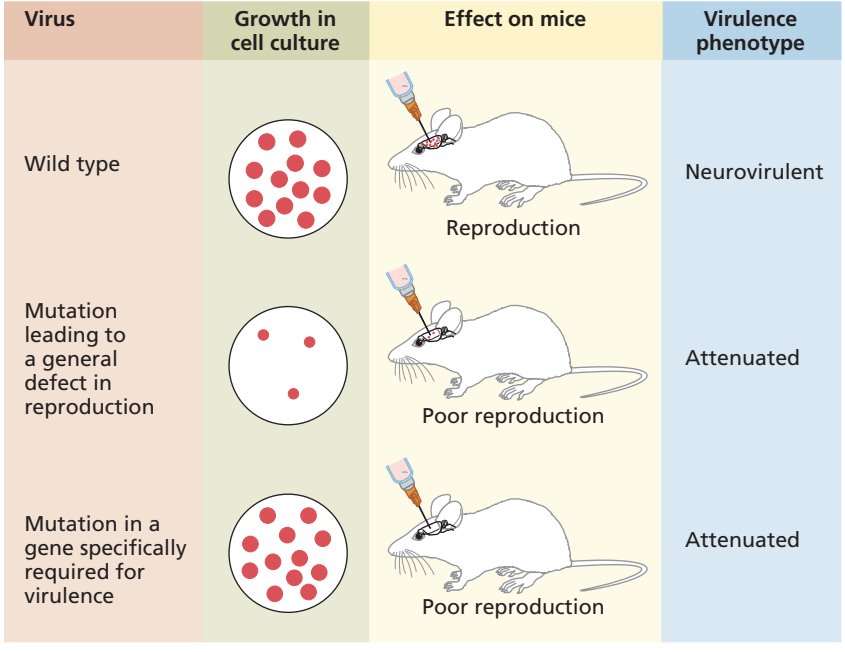


Figure 5.14 Different types of virulence genes. Examples of virulence genes that affect viral reproduction, using intracerebral neurovirulence in adult mice as an example. Wild-type viruses reproduce well in cell culture, and after inoculation into the mouse brain, they reproduce and are virulent. Mutants with replication defects do not grow well in cultured cells, or in mouse brain, and are attenuated. Mutants with a defect in a gene specifically required for virulence reproduce well in certain cultured cells, but not in the mouse brain, and are attenuated. Adapted from N. Nathanson (ed.), *Viral Pathogenesis* (Lippincott-Raven Publishers, Philadelphia, PA, 1997), with permission.

DNA viruses encode proteins that alter the cell cycle; by forcing the cell to enter the cell cycle, substrates for DNA synthesis are produced. Another solution, exemplified by alphaherpesviruses, is to encode enzymes that function in nucleotide metabolism, such as thymidine kinase and ribonucleotide reductase. These virally encoded proteins help to increase the availability of nucleotides within infected cells. Mutations in these genes often reduce the neurovirulence of herpes simplex virus because the mutants cannot reproduce in neurons or in any other cell unable to complement the deficiency. However, because reproduction of these viruses is not affected in all cells, such genes can be classified as virulence genes.

Noncoding Sequences That Affect Virus Replication

The attenuated strains that comprise the live Sabin poliovirus vaccine are examples of viruses with mutations that are not in protein-coding sequences (Chapter 8). Each of the three serotypes in the vaccine contains a mutation in the 5′ noncoding region of the viral RNA that impairs virus reproduction in the brain (Fig. 5.15). These mutations also reduce translation of viral messenger RNA in cultured cells of neuronal origin, but not in other cell types. An interesting finding is that attenuated viruses bearing these mutations apparently do not reproduce efficiently at the primary site of infection in the gut. Consequently, many fewer virus particles are available for hematogenous or neural spread to the brain. Mutations in the 5′ noncoding regions of other picornaviruses also affect virulence in animal models. For example, deletions in the long poly(C) tract within the 5′ noncoding region of mengovirus reduce disease in mice without affecting viral reproduction in cell culture.

Gene Products That Modify Host Defense Mechanisms

The study of viral virulence genes has identified a diverse array of viral proteins that sabotage the host's intrinsic, innate, and adaptive defenses. Some of these viral proteins are called **virokines** (secreted viral proteins that mimic cytokines and growth factors, but that do not transduce the same signals) or **viroceptors** (homologs of host receptors for these proteins). In most cases, these proteins are decoys that bind to cellular receptors, or that engage soluble immune mediators, and prevent them from performing their specific function, acting as a "sink" to delay the host immune response. Mutations in genes encoding either class of protein affect virulence, but these genes are **not** required for virus reproduction in cell culture.

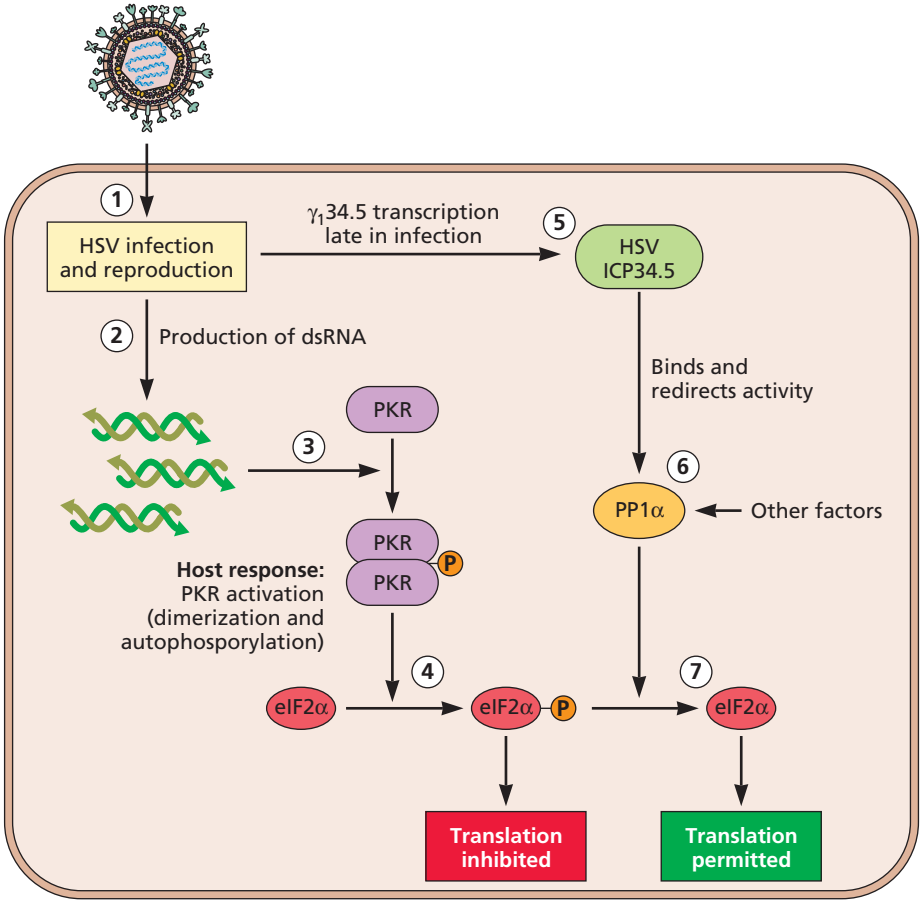


Figure 5.15 Summary of protein kinase R (PKR)-mediated protein shut-off and HSV ICP34.5 defense. Upon entry, herpes simplex virus type 1 produces double-stranded RNA molecules that are detected by the cellular PKR response. Activated PKR then phosphorylates eIF-2α, inhibiting protein translation. However, herpes simplex virus produces ICP34.5 which associates with cellular phosphatase PP1α, leading to the dephosphorylation of eIF2α and an induction of translation.

BOX 5.14

DISCUSSION

Poxviruses encode very efficient immune-modulating proteins that affect viral virulence

Variola virus, which causes the human disease smallpox, is the most virulent member of the *Orthopoxvirus* genus. The prototype poxvirus, vaccinia virus, does not cause disease in immunocompetent humans, and is used to vaccinate against smallpox. Both viral genomes encode inhibitors of the complement pathway. The vaccinia virus complement control protein is secreted from infected cells and functions as a cofactor for the serine protease factor I. The variola virus homolog, called smallpox inhibitor of complement, differs from the vaccinia virus protein by 11 amino acids. Because the variola virus protein had not been studied, it was produced by changing the 11 codons in DNA encoding the vaccinia virus homolog. The variola virus protein produced in this way was found to be 100 times more potent than the vaccinia virus protein at inactivating human complement. These findings suggest that the virulence of variola virus, and the avirulence of vaccinia virus, might be controlled in part by complement inhibitors encoded in the viral genome. Furthermore, if smallpox should reemerge, the smallpox inhibitor of complement might be a useful target for intervention.

Poxviruses encode other immune-modifying proteins, including a decoy receptor for IFN type 1, the T1-IFN binding protein, which is essential for virulence. This protein attaches to uninfected cells surrounding infected foci in the liver and the spleen, thereby impairing their ability to receive T1-IFN signaling, and facilitating virus spread. Remarkably, this process can be reversed; mousepox infection can be cured late in infection by treating with antibodies that block the biological function of the T1-IFNbp.

Rosengard AM, Liu Y, Zhiping N, Jimenez R. 2002. Variola virus immune evasion design: expression of a highly efficient inhibitor of human complement. *Proc Natl Acad Sci USA* **99:**8808–8813.

Xu RH, Rubio D, Roscoe F, Krouse TE, Truckenmiller ME, Norbury CC, Hudson PN, Damon IK, Alcami A, Sigal LJ. 2012. Antibody inhibition of a viral type 1 interferon decoy receptor cures a viral disease by restoring interferon signaling in the liver. *PLoS Pathog* **8:**e1002475.

Most virokines and viroceptors are encoded in the genomes of large DNA viruses (Box 5.14).

Other viral proteins interfere with the cellular intrinsic host response. Deletion of the herpes simplex virus gene encoding the ICP34.5 protein produces a mutant virus so dramatically attenuated that it is difficult to determine an LD_{50}, even when injected directly into the brain. Such mutants can reproduce in some cell types within the brain, but are unable to grow in postmitotic neurons. ICP34.5 has multiple functions, including counteracting the activation of the IFNβ gene and opposing the innate antiviral activity of Pkr (Chapter 3). Viral ICP34.5 mutants are attenuated because they cannot prevent the cell from inducing the intrinsic responses of Pkr-dependent inhibition of translation (Fig. 5.15). Such cell type-selective attenuated viruses are under consideration as agents to selectively kill brain tumor cells (Box 5.15).

BOX 5.15

DISCUSSION

The use of attenuated herpes simplex viruses to clear human brain tumors

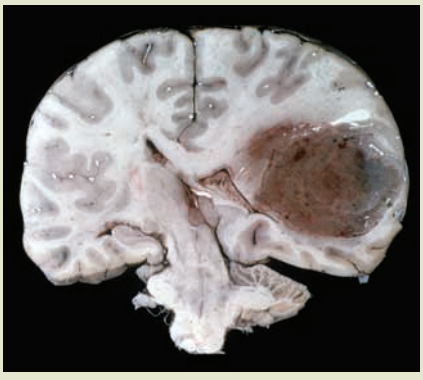
Malignant glioma, a prevalent type of brain tumor, is almost universally fatal within a year after diagnosis, despite clinical advances. Several groups have proposed the use of cell-specific replication mutants of herpes simplex virus to kill glioma cells *in situ*. One such virus under study carries a deletion of the ICP34.5 gene and the gene encoding the large subunit of ribonucleotide reductase. These mutant viruses reproduce efficiently in dividing cells, such as glioma cells, but not in nondividing cells, such as neurons. The hypothesis is that attenuated virus injected into the glioma will replicate and kill the dividing tumor cells, but will not impair nondividing neurons.

This idea works in principle: studies of mice have indicated that direct injection of this mutant virus into human gliomas transplanted into mice clears the tumor. The virus is attenuated and safe: injection of 1 billion virus particles into the brain of a monkey that is highly sensitive to herpes simplex virus had no pathogenic effect. This degree of attenuation is remarkable.

Several human trials are in progress to test safety and dosage. In one study, up to 10^5 PFU was inoculated directly into the brain tumors of nine patients. No encephalitis, adverse clinical symptoms, or reactivation of latent herpes simplex virus were observed. Higher concentrations will be used until a therapeutic effect is attained.

Ning J, Wakimoto H. 2014. Oncolytic herpes simplex virus based strategies: toward a breakthrough in glioblastoma therapy. *Front Microbiol* **5:**303.

Rampling R, Cruickshank G, Papanastassiou V, Nicoll J, Hadley D, Brennan D, Petty R, MacLean A, Harland J, McKie E, Mabbs R, Brown M. 2000. Toxicity evaluation of replication-competent herpes simplex virus (ICP 34.5 null mutant 1716) in patients with recurrent malignant glioma. *Gene Ther* **7:**859–866.



Gross anatomy of a glioblastoma. Credit: CNRI/Science Photo Library.

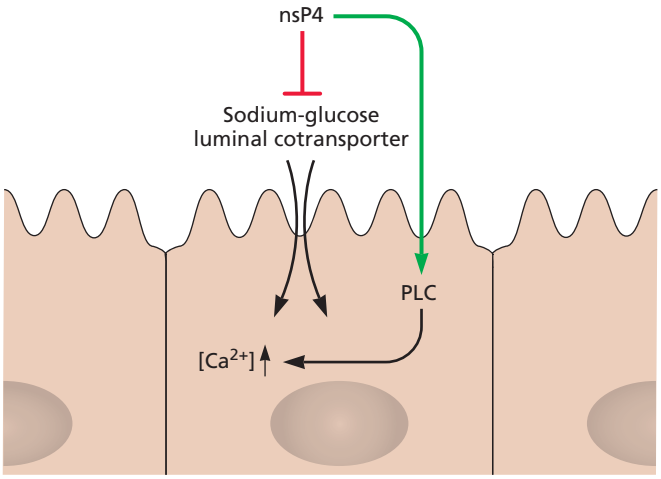


Figure 5.16 Model for rotavirus-induced diarrhea. nsP4, produced during rotavirus reproduction in intestinal epithelial cells, inhibits the sodium-glucose luminal cotransporter. Because this transporter is required for water reabsorption in the intestine, its inhibition by nsP4 could be one mechanism of diarrhea induction. nsP4 also induces a phospholipase C (PLC)-dependent calcium signaling pathway. The increase in the concentration of intracellular calcium could induce calcium-dependent chloride secretion. Adapted from M. Lorrot and M. Vasseur, *Virol. J.* **4**:31–36, 2007, with permission.

Gene Products That Enable the Virus to Spread in the Host

The mutation of some viral genes disrupts the spread from peripheral sites of inoculation to the organ in which disease is manifested (Fig. 5.16). For example, after intramuscular inoculation in mice, reovirus type 1 spreads to the central nervous system through the blood, while type 3 spreads by neural routes. Studies of recombinants between types 1 and 3 indicate that the gene encoding the viral outer capsid protein s1, which recognizes the cell receptor, determines the route of spread, and viruses with alterations in this protein are attenuated for neuroinvasion and neurovirulence.

Other viral membrane proteins have also been implicated in neuroinvasiveness. For instance, the change of a single amino acid in the gD glycoprotein of herpes simplex virus type 1 blocks neuron-to-neuron spread to the central nervous system following footpad inoculation. Similarly, studies of neuroinvasive and nonneuroinvasive strains of bunyaviruses have shown that the G1 glycoprotein is an important determinant of entry into the central nervous system from the periphery. Although it is tempting to speculate that these viral glycoproteins, which participate in entry into other cells, facilitate direct access of the virus to the nerve termini, the mechanisms by which they govern neuroinvasiveness are unknown.

Pathogenesis

We have discussed different patterns of viral infection, distinct ways by which viral virulence occurs, and the diversity of viral proteins associated with virulence. But how, ultimately, do viruses make us ill? The rapidly expanding field of viral pathogenesis attempts to integrate viral biology, cell biology, and host physiology (such as immunocompetence, age, and previous exposures) to elucidate the origins of viral disease. Some signs of virus-induced disease have been known for many centuries (as in the "dropped foot" consequence of poliovirus infection [Volume I, Chapter 1]). Other, more recently identified manifestations interfere with the health of the host in subtle ways, perhaps affecting synthesis or function of a small number of cellular proteins without overt cell destruction. We will first discuss the overt signs or symptoms of virus infection, and conclude this section with more recently identified—and more covert—ways by which viruses can cause illness in human hosts.

Infected Cell Lysis

Cell lysis is a common outcome of viral infection by most nonenveloped viruses, and some enveloped viruses. The destruction of the host cell membrane permits the release of viral progeny, and causes the death of the infected cell. There are multiple mechanisms whereby loss of membrane integrity can occur, including the production of **viroporins**.

Viroporins are hydrophobic, virus-encoded proteins that promote the release of virus particles from infected cells by associating with, and disrupting, the cellular plasma membrane. As their name implies, these proteins form pores in the membrane. Examples of viroporins include the influenza virus M2 protein, the picornavirus 2B protein, and the hepatitis C virus p7 protein. This growing family of virus proteins can accelerate the production of viral progeny either directly (by facilitating viral release) or indirectly, by destabilizing membrane polarity and integrity, allowing viruses to escape from the dying host cell.

Immunopathology

The clinical signs and symptoms of viral disease (e.g., fever, tissue damage, aches, pains, and nausea) result primarily from the host's immune response to infection (Table 5.4). This damage is called **immunopathology**, and it may be the price paid by the host to eliminate a viral infection. In fact, for noncytolytic viruses, including the hepatitis viruses and some herpesviruses, it is likely that immunopathology is the primary basis of disease. Most virus-triggered immunopathology is caused by activated T cells, but there are examples in which B cells, antibodies, or an excessive innate response are the source of disease. Because immunopathology is the result of an uncontrolled host reaction, the consequences can be severe, even life threatening (Box 5.9).

Immunopathological Lesions

Lesions caused by cytotoxic T lymphocytes. Infection of mice with lymphocytic choriomeningitis virus provides one of the most extensively characterized experimental examples

Table 5.4 Cells and mechanisms associated with immunopathology

Proposed mechanism	Virus
$CD8^+$ T cell mediated	Coxsackievirus B
	Lymphocytic choriomeningitis virus
	Sin Nombre virus
	Human immunodeficiency virus type 1
	Hepatitis B virus
$CD4^+$ T cell mediated	Theiler's virus
T_h1	Mouse coronavirus
	Semliki Forest virus
	Measles virus
	Visna virus
	Herpes simplex virus
T_h2	Respiratory syncytial virus
B cell mediated (antibody)	Dengue virus
	Feline infectious peritonitis virus

of cytotoxic T lymphocyte (CTL)-mediated immunopathology. The virus itself is noncytopathic and induces tissue damage only in immunocompetent animals. Experiments using adoptive transfer of T cell subtypes, depletion of cells, and gene knockout and transgenic mice, showed that the tissue damage following infection requires CTLs. Mice lacking CTLs, as well as perforin, the major cytolytic protein of CTLs, develop negligible disease after infection, whereas wild-type animals inoculated intracerebrally develop rapid rupturing of the cells that line the ventricles, resulting in massive edema, seizures, and death (choriomeningitis). The CTLs may also contribute to immunopathology indirectly, by releasing proteins that recruit inflammatory cells to the site of infection, which in turn elaborate proinflammatory cytokines.

Liver damage caused by hepatitis B virus also appears to depend on the action of CTLs. Production of the viral envelope proteins in transgenic mice has no effect. When the mice are injected with hepatitis B virus-specific CTLs, liver lesions that resemble those observed in acute human viral hepatitis develop. In this model, CTLs attach to the viral envelope protein-expressing hepatocytes and induce apoptosis. Cytokines released by these lymphocytes recruit neutrophils and monocytes, which exacerbate cell damage.

Lesions caused by $CD4^+$ T cells. $CD4^+$ T lymphocytes secrete larger quantities and a greater diversity of cytokines than do CTLs, resulting in the recruitment and activation of nonspecific effector cells. Such inflammatory reactions are usually called "delayed-type" hypersensitivity reactions, because of the longer period of time that must elapse for the reaction to occur, as compared to other, more immediate, hypersensitivity reactions. Most of the recruited cells are neutrophils and mononuclear cells, which can cause tissue damage as a result of release of proteolytic enzymes, reactive free radicals such as peroxide and nitric oxide (see below), and cytokines such as Tnf-α. For noncytopathic persisting viruses, the $CD4^+$-mediated inflammatory reaction is largely immunopathological.

$CD4^+$ T_h1 cells. The cytokines produced by $CD4^+$ T_h1 cells facilitate the cell-mediated response, but not the antibody response. These cytokines include IL-2, IFN-β and tumor necrosis factor-alpha. $CD4^+$ T_h1 cells are necessary for the demyelination seen in several rodent models of virally induced multiple sclerosis. When mice are inoculated with Theiler's murine encephalomyelitis virus (a picornavirus), proinflammatory cytokines produced by infected cells activate macrophages and microglial cells that mediate neuronal demyelination. It has been proposed that the activated phagocytic cells release superoxide and nitric oxide free radicals that, in combination with the T_h1 cell proinflammatory cytokines, destroys oligodendrocytes, which are the source of myelin. Similar observations have been made following infection with mouse hepatitis virus, a coronavirus. The similar demyelinating pathology caused by very different viruses suggests that these viruses may trigger a common immunopathological host reaction.

Herpes stromal keratitis is one of the most common causes of vision impairment in developed countries. The eye damage results almost entirely from immunopathology. In humans, herpes simplex virus infection of the eye induces lesions on the corneal epithelium, and repeated infections result in opacity and reduced vision. Studies of a mouse model for this disease demonstrated that $CD4^+$ T_h1 cells contribute to immunopathology, but in an unusual manner. The surprise was that while viral reproduction occurs in the corneal epithelium, $CD4^+$ T cell-mediated inflammation was restricted to the underlying, and uninfected, stromal cells. In fact, viral reproduction in the cornea had ceased by the time that $CD4^+$ T cells attacked the stromal cells. It is thought that the damage to uninfected cells in the stroma is stimulated by secreted cytokines produced by infected cells in the corneal epithelium. Binding of the $CD4^+$ T cells may be due to errant recognition of host proteins on the stromal cells by virus-specific T cells, a process called **molecular mimicry**, discussed in more detail later in this chapter.

$CD4^+$ T_h2 cells. The cytokines produced by $CD4^+$ T_h2 cells, including IL-4, IL-5, and IL-10, evoke strong antibody responses and eosinophil accumulation, typical responses to extracellular pathogens such as parasites and some bacteria. However, such cytokines have been implicated in some viral respiratory diseases. Respiratory syncytial virus is an important cause of lower respiratory tract disease in infants and the elderly. Models for this particular disease have been difficult to establish, but there has been some success using immunosuppressed mice. When these animals are infected, lesions of the respiratory tract are minor, but they become severe after

adoptive transfer of viral antigen-specific, $CD4^+$ T_h2 cells. The respiratory tract lesions contain many eosinophils, which may be responsible for pathology.

The balance of T_h1 and T_h2 cells. T_h1 and T_h2 cytokine responses are not all or none, and both can be made following viral challenge. As a result, changes in the optimal balance of these powerful immune inducers can also result in immunopathology. For example, infection with respiratory syncytial virus induces a predominately T_h1 response in young children. However, when children were vaccinated with a formalin-inactivated whole-virus vaccine that elicited a primarily T_h2 response, they not only remained susceptible to infection but also developed an atypically severe disease, characterized by increased eosinophil infiltration into the lungs. This particular pathology had been predicted by adoptive transfer of $CD4^+$ T_h2 cells in mice.

Immunopathological lesions caused by B cells. Antibodies neutralize virus particles by binding and targeting them for elimination. Virus-antibody complexes accumulate to high concentrations when extensive viral reproduction occurs at sites that are inaccessible to the cellular immune system or continues in the presence of an inadequate immune response. Such complexes are not cleared efficiently by the reticuloendothelial system and continue to circulate in the blood. These large complexes can become deposited in small capillaries and cause lesions that are exacerbated when the complement system is activated (Fig. 5.17). Deposition of such immune complexes in blood vessels, kidneys, and brain may result in vasculitis, glomerulonephritis, and neuroinflammation, respectively. This type of immunopathology was first described in mice infected with lymphocytic choriomeningitis virus. Although immune complexes have been found in humans, viral antigens have been found in the complexes only in hepatitis B virus infections.

Antibodies may also enhance viral infection, as in dengue hemorrhagic fever. This disease is transmitted by mosquitoes and is endemic in the Caribbean, Central and South America, Africa, and Southeast Asia, where billions of people are at risk. Primary infection with dengue virus is usually asymptomatic, but in some cases, an acute febrile illness with severe headache, back and limb pain, and rash can develop. Although the infection is normally self-limiting, and patients recover in 7 to 10 days, the disease is referred to as "breakbone fever," owing to extraordinary muscle and join pain. There are four viral serotypes, and antibodies to any one serotype do not protect against infection by another. When an individual who has antibodies to one serotype is infected by a different serotype, nonprotective antibodies bind virus particles and facilitate their uptake into normally nonsusceptible peripheral blood monocytes via binding to Fc receptors. Consequently,

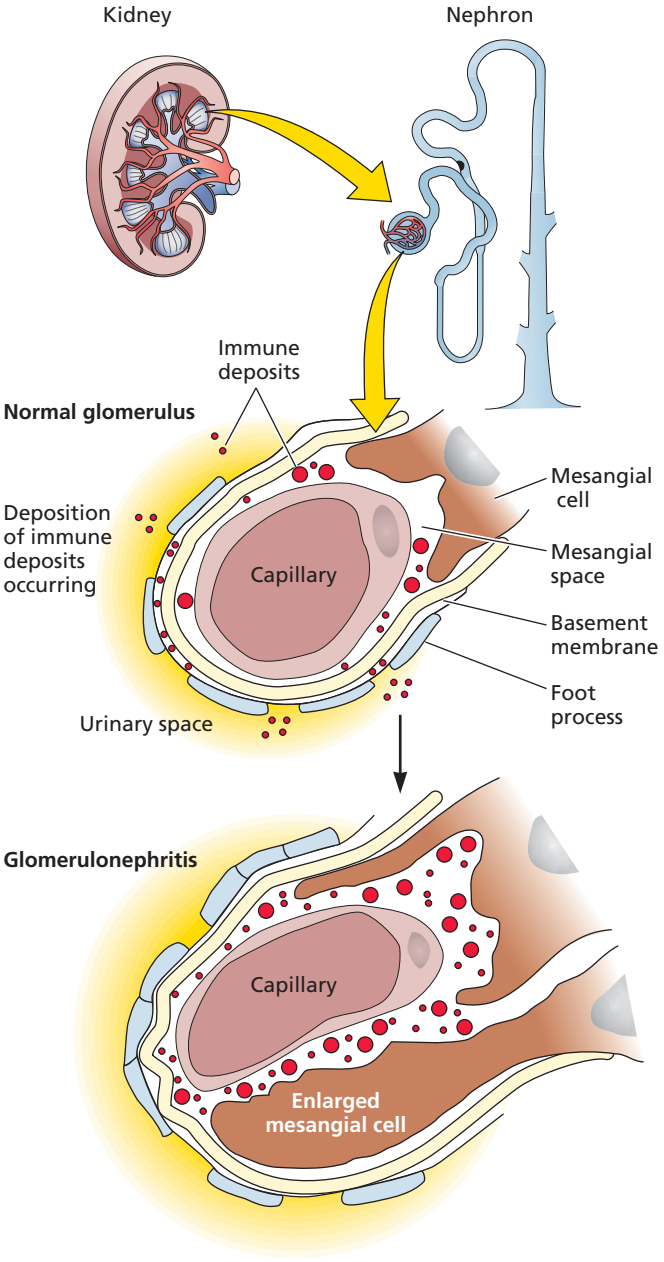


Figure 5.17 Deposition of immune complexes in the kidneys, leading to glomerulonephritis. **(Top)** Normal glomerulus and its location in the nephron and kidney. **(Middle)** Normal glomerulus. Red dots are immune complexes. The smaller complexes pass to the urine, and the larger ones are retained at the basement membrane. **(Bottom)** Glomerulonephritis. Complexes have been deposited in the mesangial space and around the endothelial cell. The function of the mesangial cell is to remove complexes from the kidney. In glomerulonephritis, the mesangial cells enlarge into the subepithelial space. This results in constriction of the glomerular capillary, and foot processes of the endothelial cells fuse. The basement membrane becomes leaky, filtering is blocked, and glomerular function becomes impaired, resulting in failure to produce urine. Adapted from C. A. Mims et al., *Mims' Pathogenesis of Infectious Disease* (Academic Press, Orlando, FL, 1995), with permission.

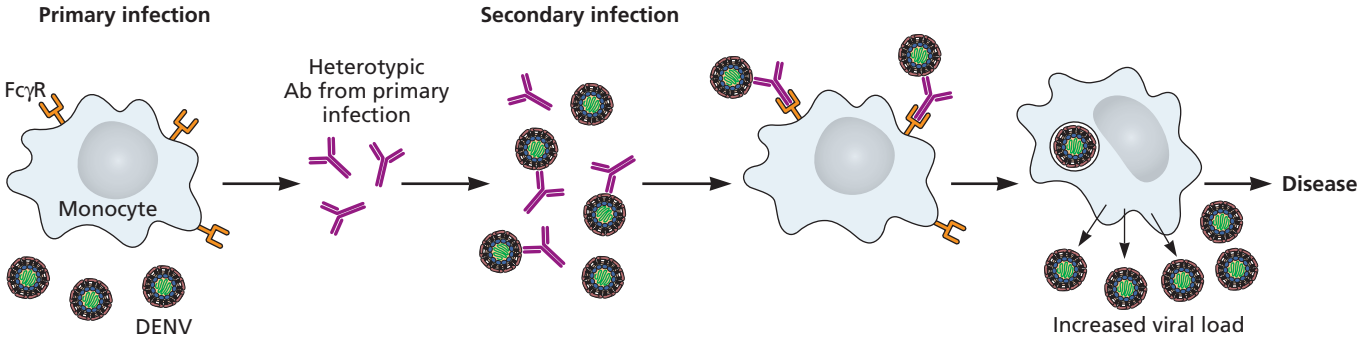


Figure 5.18 Model of antibody-dependent enhancement of dengue infection. Monocytes are not directly susceptible to dengue virus infection. However, when preexisting antibodies are present, a second exposure to dengue (for example, with a different serotype), allows for antibody-virus conjugates to bind to Fcγ receptors (FcγR) on circulating monocytes. Monocyte infection results in an increase in viral reproduction and a higher risk of severe dengue. DENV, dengue virus; Ab, antibody.

the infected monocytes contribute to an elevated viral load, and produce proinflammatory cytokines, which in turn stimulate T cells to produce more cytokines. This vicious cycle triggers the plasma leakage and hemorrhaging that are characteristic of dengue hemorrhagic fever (Fig. 5.18). In these instances, there may be so much internal bleeding that the often-fatal dengue shock syndrome results. Dengue hemorrhagic fever is generally rare, occurring in approximately 1 in 14,000 primary infections. However, after infection with a dengue virus of another serotype, the incidence of hemorrhagic fever increases dramatically to 1 in 90.

Systemic Inflammatory Response Syndrome (SIRS). An important tenet of immune defense is that virus reproduction induces a rapid, specific, and integrated host response to contain the infection. Typically, the scale of this response is appropriate to the pathogen, and once the pathogen is eliminated, inflammation is suppressed or limited. Precisely how this threshold is determined is not fully understood, but if it is breached too rapidly, or if the immune response is not proportional to the infection, the large-scale production and systemic release of inflammatory cytokines and stress mediators can overwhelm an infected host. Such a disastrous outcome can occur if the host is naive and has not coevolved with the invading virus (zoonotic infections; see Chapter 10), or if the host is very young, malnourished, or otherwise compromised. This type of pathogenesis is called the **systemic inflammatory response syndrome** (SIRS), and is sometimes referred to as a "cytokine storm." The lethal effects of the 1918 influenza virus have been attributed by some to this response, as well as those of human infections with Ebola and Marburg viruses. When this uncontrolled and systemic inflammation is induced by pathogens, it is referred to as **sepsis**, although noninfectious causes of SIRS, including trauma, burns, and anaphylaxis, also exist.

Heterologous T cell immunity. Much of Chapter 4 was dedicated to extolling the precise, antigen-driven induction of the adaptive immune response and resulting memory cells, but it turns out that memory cells are not always as specific as once thought. The first insight that this may be the case was the clinical observation that common infections can run surprisingly different courses in different individuals. Many variables may contribute to differential responses, but from experiments with genetically identical mice, it became clear that the history of previous encounters with pathogens can dictate the outcome of a new infection. The phenomenon is called **heterologous T cell immunity**: memory T cells specific for a particular virus epitope can be resurrected during infection with a completely unrelated virus. At first glance, it may seem advantageous for the host to quickly turn on potent immune effectors, but the consequences of activating T cells that are not tailored to the "new" pathogen may induce an inappropriate or poorly coordinated response. When mice are immunized against one of several viruses and then challenged with a panel of other viruses, the animals show partial, but not necessarily reciprocal, protection to the heterologous infection. Challenge with the arenavirus, lymphocytic choriomeningitis virus, provided substantial protection against the poxvirus, vaccinia virus, but not vice versa. For other virus pairs such as murine cytomegalovirus and vaccinia, the protection was partially reciprocal. The significance of these findings to human infections is emerging. For example, patients experiencing Epstein-Barr virus-induced mononucleosis may have a strong T cell response to a particular influenza virus epitope rather than the typical response to an immunodominant Epstein-Barr virus epitope. In these individuals, it appears that Epstein-Barr virus infection activated memory T cells that were produced by a previous exposure to influenza virus. These individuals had a different course of mononucleosis, often more severe, than did those with no previous

exposure to influenza virus. Heterologous T cell immunity is a variation of a concept known as "**original antigenic sin**." In this scenario, a primary infection by a virus (for example), induces a protective host response against the immunodominant viral antigens, leading to resolution. If that same individual is challenged later in life with an altered virus in which that immunodominant epitope is replaced by a different epitope, the host will still make the primary response to the former (now subdominant) epitope. This weaker host response would lead to either inefficient or delayed clearance, and attendant pathogenesis.

T cell cross-reactivities among heterologous viruses are more frequent than commonly expected, but not yet well understood. Our limited knowledge about immune redundancy following pathogen exposure may be due to our heavy use of mouse models as surrogates for human infections. One of the limitations of working with mouse models is that, generally, mice are infected with a single virus, parameters of interest are examined, and the mice are killed. As a result, most experimental mice have no "immune histories" to other infections. The important principle that is emerging from more sophisticated polymicrobial studies in animal models, which are aimed to mimic more closely human virus encounters, is that prior infections can affect the defense against pathogens that have not yet been encountered, sometimes in dangerous ways.

Superantigens "Short Circuit" the Immune System

Some viral proteins are extremely powerful T cell mitogens known as **superantigens**. These proteins interact with the stalk of the Vβ chain of the T cell receptor, rather than with the antigen-binding pocket as in typical MHC-T cell receptor interactions. As approximately 2 to 20% of **all** T cells produce the particular Vβ chain that binds the superantigen, these viral proteins short circuit the interaction of MHC class II-peptide complex and the T cell. As a consequence, rather than activating a small, specific subset of T cells (only 0.001 to 0.01% of T cells usually respond to a given antigen), **all** subsets of T cells producing the Vβ chain to which the superantigen binds are activated and proliferate, regardless of specificity.

All known superantigens are microbial products, and many are produced after viral infection. The best-understood viral superantigen is encoded in the U3 region of the mouse mammary tumor virus long terminal repeat. This retrovirus is transmitted efficiently from mother to offspring via milk. However, the virus reproduces poorly in most tissues in the mother. Neonates can become infected upon ingestion of milk. When B cells in the neonatal small intestine epithelium are infected, the viral superantigen is produced and recognized by T cells carrying the appropriate T cell receptor Vβ chain. Consequently, extraordinarily large numbers of T cells are activated, producing growth factors and other molecules that stimulate proliferation of the infected B cells. These cells then carry the virus to the mammary gland, enhancing transmission to the progeny of these mice, and increasing the risk for tumor formation (Fig. 5.19). Infection of mice with mutants harboring a deletion of the superantigen gene results in limited viral reproduction and minimal transmission to offspring via milk.

Mechanisms Mediated by Free Radicals

Two free radicals, superoxide (O_2^-) and nitric oxide, are produced during the inflammatory response and are probably critical effectors of virus-induced pathology. Superoxide is produced by the enzyme xanthine oxidase, present in phagocytes. The production of O_2^- is significantly increased in hypoxic cells and tissues, for example, in the lungs of mice infected with influenza virus or cytomegalovirus. Inhibition of xanthine oxidase protects mice from virus-induced death.

Nitric oxide is abundant in virus-infected tissues during inflammation as part of the innate immune response (Chapter 3). This compound inhibits the production of many viruses in cultured cells and in animal models. It acts within the cell to limit viral reproduction, but the molecular sites of action are not well understood. Nitric oxide is produced by three different IFN-inducible isoforms of nitric oxide synthase. Although low concentrations of nitric oxide have a protective effect, high concentrations or prolonged exposure can contribute to pathogenesis. While nitric oxide is relatively inert, it reacts rapidly with O_2^- to form peroxynitrite ($ONOO^-$), which is much more reactive than either molecule and may be responsible for cytotoxic effects on cells.

Immunosuppression Induced by Viral Infection

Virus-mediated suppression of immune defenses can range from a mild and rather specific attenuation to a marked global inhibition of the response. Immunosuppression by viral infection was first observed over 100 years ago when patients were unable to respond to a skin test for tuberculosis during and after measles infection. However, progress in understanding the phenomenon was slow until the human immunodeficiency virus epidemic was under way (Chapter 7).

While it is well known that human immunodeficiency virus is immunosuppressive, severe immune suppression can also result from infection by other human viruses such as rubella and measles. For example, the vast majority of the tens of thousands of measles virus-induced childhood deaths each year in Third World countries is due to opportunistic infections that arise during transient immunosuppression caused by this virus.

The ability of measles virus to cripple the entire immune response while infecting a very small proportion of cells of the immune system (only about 2% of total T cells are infected at the peak of viremia) was a long-standing mystery.

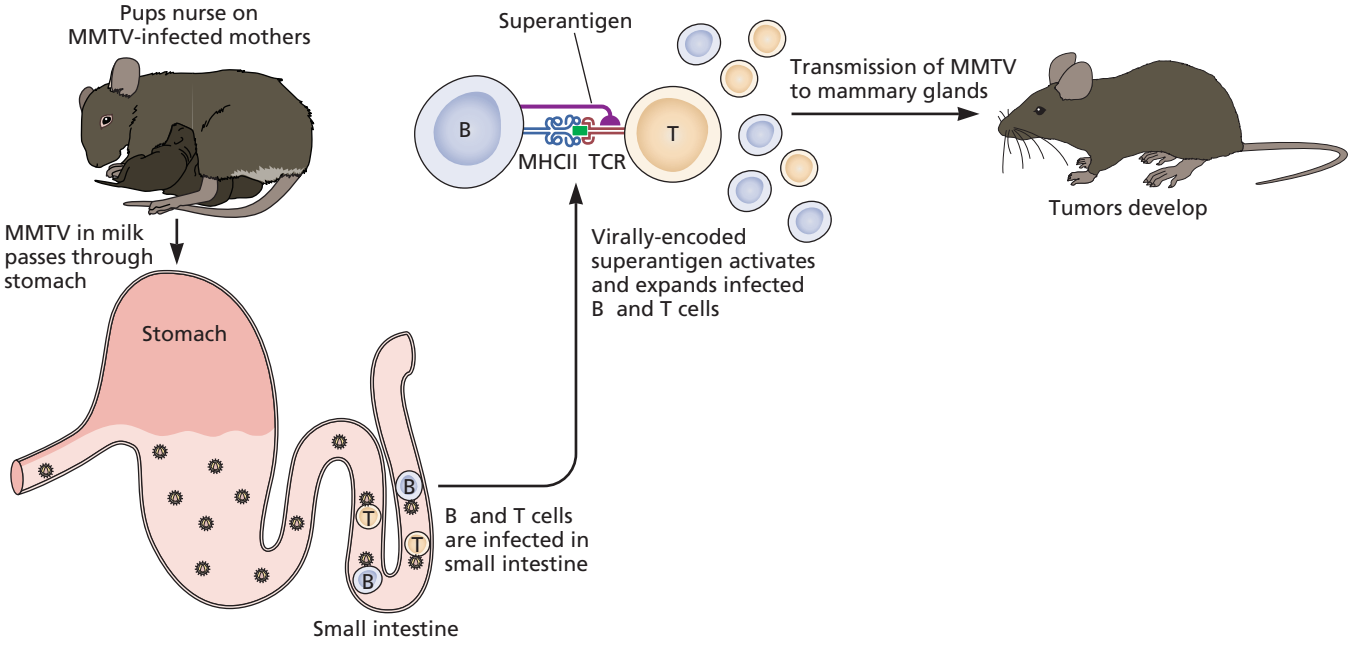


Figure 5.19 Infectious cycle of mouse mammary tumor virus. This retrovirus is produced in the mammary glands of infected female mice and is transmitted to newborn pups through the mother's milk. The ingested virus infects B and T cells in the gut-associated lymphoid tissues. The infected B cells express a superantigen that activates T cells non-specifically, providing more target cells for infection. In the mammary gland, hormonal stimulation during pregnancy and lactation dramatically increases mouse mammary tumor virus (MMTV) reproduction, and can lead to insertional mutagenesis of proto-oncogenes and the development of mammary tumors.

It is now appreciated that measles virus infection of macrophages and dendritic cells may be critical. One of the first bits of evidence to suggest a role of dendritic cell impairment was the observation that infection of these cells resulted in reduced expression of the cytokine, IL-12. This cytokine is important for skewing the immune response toward a T_h1 profile that favors clearance of virus infections. Low IL-12 directs the T cell response toward a T_h2 profile, which promotes induction of the humoral (antibody) response. When IL-12 production by dendritic cells is reduced, the cytokine microenvironment is not conducive to a cytolytic response, and T cells cannot proliferate in response to interaction with infected dendritic cells (Fig. 5.20). In measles virus-infected macaques, decreased IL-12 and increased IL-4 (a marker of a T_h2 response) is observed, and the concentration of IL-12 is also greatly reduced in the blood of measles virus-infected humans.

In separate studies, it was shown that infection of cells with the related paramyxovirus, Hendra virus, can limit the induction of an innate response by restricting nuclear access of critical signal transducers. When interferons bind to cell receptors, Stat1 is rapidly phosphorylated and homodimerizes, exposing a nuclear localization signal that allows the protein to enter nuclei and bind to interferon response elements within promoters of interferon-inducible genes (Chapter 4). Remarkably, infection with Hendra virus precludes nuclear localization of phosphorylated Stat1 (and Stat2), and thereby impedes the efficient induction of interferon genes (Fig. 5.21).

Other mechanisms that have been proposed to account for measles virus-induced transient immunosuppression include impaired development of infected dendritic cell precursors and decreased proliferation of infected T and B lymphocytes because of cell cycle arrest. These findings parallel clinical observations in humans, in which measles virus infection in immunosuppressed individuals is associated with profound reductions in the number of circulating white blood cells, and recovery from immunosuppression is directly correlated with the rate of synthesis of new cells from the bone marrow. This observation may explain why young children recover faster from infection than older children or adults. The diversity of the various mechanisms induced by this relatively simple virus (encoding only 9 proteins) underscores the evolutionary pressures that have been selected to frustrate host immunity.

Oncogenesis

Over 20% of all human cancers are associated with virus infection, and for some cancers, including liver cancer and cervical cancer, viruses are the major cause. Moreover, the history of cancer biology is, in great part, the history of

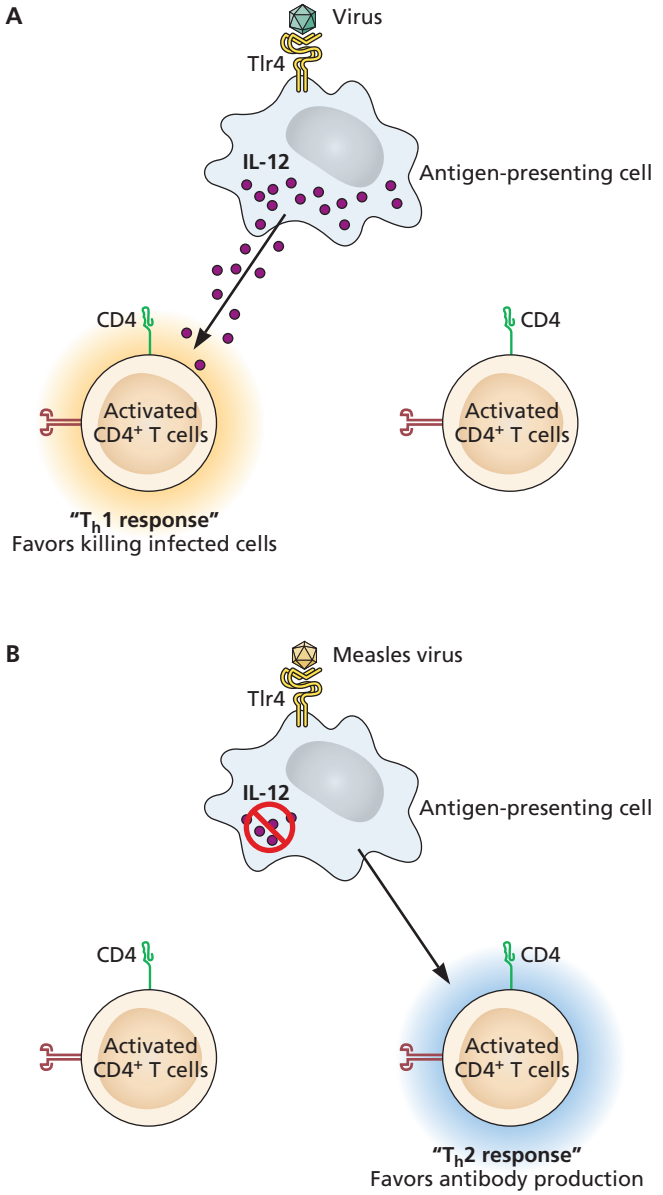


Figure 5.20 Measles virus infection of antigen-presenting cells blocks IL-12 production. One of the proposed ways by which measles virus induces global suppression is that infection of a small proportion of monocytes blocks the synthesis of a critical cytokine, IL-12. Normally, IL-12 is made in response to viral infections and skews the resulting T cell response toward a primarily T_h1-like profile. When IL-12 is blocked by measles virus, a T_h2 cytokine profile predominates. Consequently, although the host is making an aggressive response, it is not the optimal response to eliminate an intracellular viral infection. Adapted from C.L. Karp et al. *Science* **273:**228–231, 1996, with permission.

virology: discovery of tumor suppressors and oncogenes occurred as a result of studying DNA and RNA tumor viruses. Consequently, an entire chapter is dedicated to the various mechanisms by which transformation and oncogenesis occurs pursuant to viral infection (Chapter 6).

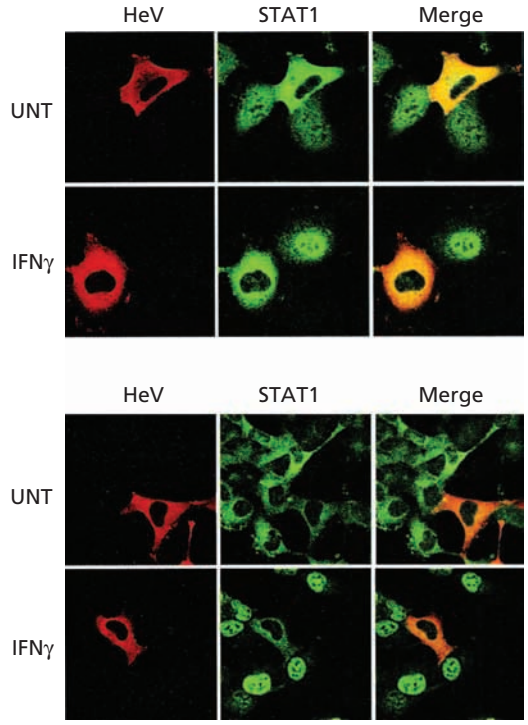


Figure 5.21 Hendra virus infection restricts nuclear localization of activated Stat molecules. An additional way by which paramyxoviruses can induce immunosuppression is by binding to signal transduction molecules that are needed to induce an antiviral response, and either retaining them in the cytoplasm or triggering their degradation.

Molecular Mimicry

Autoimmunity is caused by an immune response directed against host tissues (often described as "breaking immune tolerance"). One can envision multiple scenarios by which viral infections can trigger autoimmunity. Cytolytic virus reproduction leads to the release, and subsequent recognition, of self-antigens that are normally sequestered from the immune system. Additionally, cytokines, or virus-antibody complexes that modulate the activity of proteases in antigen-presenting cells, might cause the unmasking of self-antigens. For example, cytokines produced during infection may stimulate inappropriate surface accumulation of cellular membrane proteins that are recognized by host defenses. Another possibility is that, during virus particle assembly, host proteins that are not normally exposed to the immune system are packaged in particles that may be recognized as foreign upon entry into a different cell type. While these processes, in principle, could occur, to date none have been formally proven to be an etiological cause of human autoimmunity.

An additional hypothesis, termed "molecular mimicry," proposes cross-reactivity between a particular viral and host epitope, and is based on two observations. The first is that humans possess many putative autoreactive T cells that rarely cause disease because they are not appropriately activated via

BOX 5.16

EXPERIMENT

Viral infections promote or protect against autoimmune disease

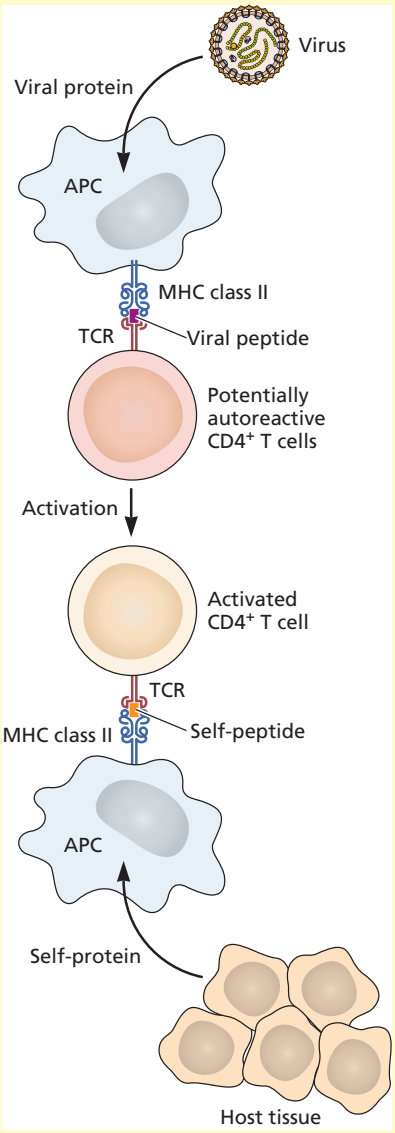
Transgenic mice that synthesize proteins of lymphocytic choriomeningitis virus in β cells of the pancreas or oligodendrocytes within the brain have been developed. Synthesis of these viral proteins has no consequence; the mice are healthy. The viral transgene products are present in the mouse throughout development, and therefore are perceived by the host defense as a self-antigen.

Infection of these transgenic animals with lymphocytic choriomeningitis virus stimulates an immune response in which the self-antigen is recognized, leading to insulin-dependent diabetes mellitus (when the protein is made in the pancreas) or central nervous system demyelinating disease (when made in the brain).

Fujinami R. 2001. Viruses and autoimmune disease—two sides of the same coin? *Trends Microbiol* **9:**377–381.

von Herrath M, Oldstone M. 1996. Virus-induced autoimmune disease. *Curr Opin Immunol* **8:**878–885.

Holmes S, Friese MA, Siebold C, Jones EY, Bell J,. Fugger L. 2005. Multiple sclerosis: MHC associations and therapeutic implications. *Exp Rev Mol Med* **7:**1–17.



A potentially autoreactive T cell, possessing T cell receptors that recognize both a foreign (viral) peptide and a self-peptide, is activated by a virus-derived peptide. Abbreviations: APC, antigen-presenting cell; MHC, major histocompatibility complex; TCR, T cell receptor. Adapted from S. Holmes, M. A. Friese, C. Siebold, E. Y. Jones, J. Bell and L. Fugger. *Exp Rev Molec Med* **7:** 1-17, 2005, with permission.

costimulation (Chapter 4). The second observation is that viral and host proteins can share antigenic determinants. The hypothesis is that infection leads to the activation of immune cells specific for a viral epitope; if this epitope is presented on host cells (derived from a cellular protein, and thus occurring by chance), the activated immune response may target such cells, even if they are not infected. Although many peptide sequences are shared among viral and host proteins, direct evidence for this hypothesis has been difficult to obtain. One reason for the difficulty is the long interval between events that trigger human autoimmune diseases and the onset of clinical symptoms. To circumvent this problem, transgenic mouse models, in which the products of foreign genes are expressed as self antigens, were established. Such model systems allowed proof-of-concept studies, which showed that this process can occur (Box 5.16). Although human parallels have yet to be ascribed definitively to molecular mimicry, some diseases, including multiple sclerosis and the neuropathology resulting from human immunodeficiency virus infection, have been proposed to be due to this process.

Perspectives

Patterns of viral infection are most likely established in the first minutes to hours after the initial inoculation. It appears that two distinct strategies of viral propagation have emerged during evolution: one produces large numbers of progeny (high reproductive output or *r*-replication; acute infections) and one results in a lower reproductive output, but better competition for resources (*K*-replication; persistent infections). Acute infections occur primarily because host defenses are modulated (passively or actively), at least for a short time. Such infections may progress beyond physical, intrinsic, and innate defenses only to be blocked and cleared by the adaptive immune response. Large numbers of new hosts are required to sustain the acute pattern of infection, as immune memory (or sometimes host death) limits the duration of a virus in a particular host.

Persistent infections result when essentially all defenses, including the adaptive immune system, are ineffective, often for long periods. Ineffective does not always mean nonfunctional. For example, in some persistent infections, such as those with hepatitis B virus, the low rate of viral reproduction is equal to the rate of immune elimination. This particular persistent infection pattern can be characterized as "smoldering," as it continues for very long periods in the face of active host defenses. While persisting viruses do not need to constantly hop from one infected individual to another, success is ensured only if there is a mechanism for periodic production of virus particles and their transmission to new hosts.

The existence of only two primary patterns of infection confront us with several questions. A particular pattern can be a defining characteristic of a virus family (e.g., influenza virus always produces an acute infection; herpes infections are forever). But this raises the fascinating philosophical question of why one particular pattern has been selected over another for these viruses. We know that acute and persistent infections are determined by properties of both host and virus. The patterns are not mutually exclusive, as some infections exhibit both acute and persistent phases. Some of the answers are discussed in Chapter 10, where we point out that viral populations emerge and prosper as a consequence of selection pressures. The selective advantages or disadvantages of rapid or limited reproduction manifest themselves quickly. Those host and viral genomes that can adapt survive to carry on the relationship for another day.

The role of pathogenesis as a selective force in the establishment and maintenance of viral infections is a subject of much research and debate. One hypothesis is that successful patterns result in symbiosis, neither helping nor harming the host. In this context, as suggested by Lewis Thomas, pathogenesis is an aberration of symbiosis, an overstepping of boundaries. Benign symbiosis is a recipe for stability, but many apparently successful viral infections are far from stable. Accordingly, another hypothesis posits that pathogenesis is a necessary survival feature of the viral population, and is selected during evolution of the relationship. Some individuals may be harmed in the short run to achieve long-term survival of the virus population. This discussion continues in Chapter 10.

Selection works in unexpected ways. In laboratory situations, adaptation to *r*-selection conditions (low MOI, rapid growth) yields viral populations that are less fit when exposed to *K*-selection conditions (high MOI, reduced growth) and *vice versa*. This fact may be obscured by the stability of one pattern compared to the other. Moreover, we are only beginning to appreciate how coincident infections may influence each other: infections with one virus can change the outcome of completely unrelated infections by systemic or local immunosuppression, by accidental induction of previous memory responses, or by recruitment of powerful immune responses to the "wrong" tissue. We have much to learn about how these viruses affect our lives, but remember that viruses are not "bad" *per se*. The pressure is for a virus to reproduce, not to debilitate its host: a powerful selective force.

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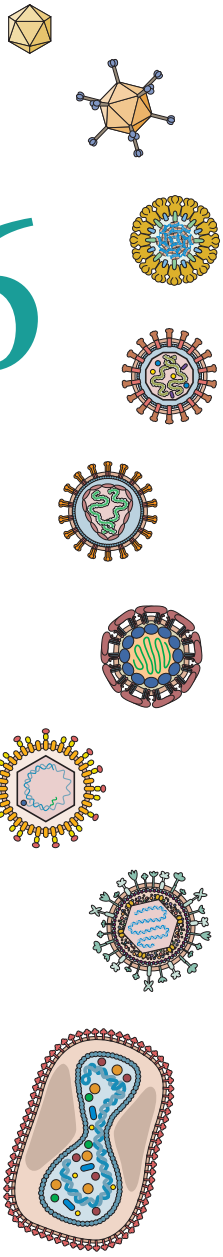
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6 Cellular Transformation and Oncogenesis

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- Properties of Transformed Cells
- Control of Cell Proliferation

Oncogenic Viruses
- Discovery of Oncogenic Viruses
- Viral Genetic Information in Transformed Cells
- The Origin and Nature of Viral Transforming Genes
- Functions of Viral Transforming Proteins

Activation of Cellular Signal Transduction Pathways by Viral Transforming Proteins
- Viral Signaling Molecules Acquired from the Cell
- Alteration of the Production or Activity of Cellular Signal Transduction Proteins

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Other Mechanisms of Transformation and Oncogenesis by Human Tumor Viruses
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- Oncogenesis by Hepatitis Viruses

Perspectives

References

LINKS FOR CHAPTER 6

⏭ *Video: Interview with Dr. Michael Bishop*
http://bit.ly/Virology_Bishop

⏭ *Movie 6.1: Mitosis in HeLa cells*
http://bit.ly/Virology_V2_Movie6-1

⏭ *Moore tumor viruses*
http://bit.ly/Virology_Twiv160

Cause and effect, means and ends, seed and fruit, cannot be severed; for the effect already blooms in the cause, the end pre-exists in the means, the fruit in the seed.

RALPH WALDO EMERSON

Introduction

Cancer is a leading cause of death in developed countries: about 8.2 million individuals succumb each year worldwide. Consequently, efforts to understand and control this deadly disease have long been high priorities for public health institutions. Our general understanding of the mechanisms of **oncogenesis**, the development of cancer, as well as of normal cell growth, has improved enormously since the latter part of the 20th century. This progress can be traced in large part to efforts to elucidate how members of several virus families cause cancer in animals. In fact, as we discuss in this chapter, study of oncogenic viruses has led to a detailed understanding of the molecular basis of this disease.

It is now clear that cancer (defined in Box 6.1) is a genetic disease: it results from the growth of successive populations of cells in which mutations and/or epigenetic modifications of genes and their associated nucleosomes have accumulated (Box 6.2). These changes affect various steps in the regulatory pathways that control cell communication, growth, and division, and lead to uncontrolled cell proliferation, increasing tissue disorganization, and ultimately cancer. One or more of these genetic changes may be inherited (Box 6.2), or they may arise as a consequence of endogenous DNA damage and exposure to environmental carcinogens or infectious agents, including viruses. It is estimated that viruses are a contributing factor in ~20% of all human cancers. For some, such as liver and cervical cancer, they are the major cause. However, it is important to understand that the induction of malignancy generally is **not** a requirement for the propagation of oncogenic viruses. A singular exception is described in a later section ("Discovery of Oncogenic Viruses"). In all other instances, this unfortunate outcome for the host is a side effect of either infection or the host's response to the presence of the virus. From this perspective, viruses can be thought of as cofactors, or unwitting initiators of oncogenesis.

Understanding the development of cancer ultimately depends on knowledge of how individual cells normally behave within an animal. As described in Chapters 1 to 4, analysis of viral pathogenesis must encompass a consideration of the organism as a whole, especially the body's immune defenses. However, elucidation of how members of several virus families cause cancer in animals began with studies of cultured cells in the laboratory. In particular, early investigators noticed that the growth properties and morphologies of some normal cells in culture could be changed upon infection with certain viruses. We therefore describe such cells as being **transformed**. The advantages of these cell culture systems are many: the molecular virologist can focus attention on particular cell types or specific viral genes and can readily distinguish effects specific to the virus. In many cases, cells transformed by viruses in culture can form tumors when implanted in animals. But it is important to realize that transformed cultures are **not** tumors. The major benefit of cell culture systems is that they allow researchers to study the molecular events that establish an oncogenic potential in virus-infected cells. Such studies were of great importance: they led to the identification of viral and cellular oncogenes and elucidation of the molecular circuits that control cell proliferation.

Properties of Transformed Cells

Cellular Transformation

The proliferation of cells in the body is a strictly regulated process. In a young animal, total cell multiplication exceeds cell death as the animal grows to maturity. In an adult, the processes of cell multiplication and death are carefully balanced. For some cells, high rates of proliferation are required to maintain this balance. For example, human intestinal cells and white blood cells have half-lives of only a few days and need to be replaced rapidly. On the other hand, red blood cells live for

PRINCIPLES *Cellular transformation and oncogenesis*

- Members of DNA and RNA virus families cause or contribute to ~20% of human cancers.
- Cancer is a disease of unregulated cell division, which can be the result of inherited mutations; exposure to environmental carcinogens; or infection with pathogens, including viruses.
- Immortalization, transformation, and oncogenesis are distinct states, but are part of a continuum.
- Transformed cells are distinguished from normal cells by their immortality, loss of contact inhibition, and often production of their own growth factors.
- With few exceptions, transformation is not required for viral reproduction.
- Retroviruses can either encode oncogenes (once derived from host genes) or integrate into the cellular genome and activate adjacent cellular proto-oncogenes.
- Small transforming DNA viruses encode proteins that bind to specific cellular proteins, notably the tumor suppressors Rb and p53, to promote cell cycle progression and block checkpoints.
- Proteins encoded by transforming viruses also can prevent cell death, block immune recognition, and promote blood vessel formation.
- Some viruses associated with human cancers do not transform cells directly, but rather induce a chronic immune response that, with time, results in tissue damage and the emergence of malignant cells.

BOX 6.1

TERMINOLOGY

Some cancer terms

Benign: An adjective used to describe a growth that does not infiltrate into surrounding tissues; opposite of malignant

Cancer: A malignant tumor; a growth that is not encapsulated and that infiltrates into surrounding tissues, replacing normal with abnormal cells; it is spread by the lymphatic vessels to other parts of the body; death is caused by destruction of organs to a degree incompatible with their function, by extreme debility and anemia, or by hemorrhage

Carcinogenesis: The multistage process by which a cancer develops

Carcinoma: A cancer of epithelial tissue

Endothelioma: A cancer of endothelial cells

Fibroblast: A cell derived from connective tissue

Fibropapilloma: A solid tumor of cells of the connective tissue

Hepatocellular carcinoma: A cancer of liver cells

Leukemia: A cancer of white blood cells

Lymphoma: A cancer of lymphoid tissue

Malignant: An adjective applied to any disease of a progressive and fatal nature; opposite of benign

Neoplasm: An abnormal new growth, i.e., a cancer

Oncogenic: Causing a tumor

Retinoblastoma: A cancer of cells of the retina

Sarcoma: A cancer of fibroblasts

Tumor: A swelling, caused by abnormal growth of tissue, not resulting from inflammation; may be benign or malignant

BOX 6.2

BACKGROUND

Genetic alterations associated with the development of colon carcinoma

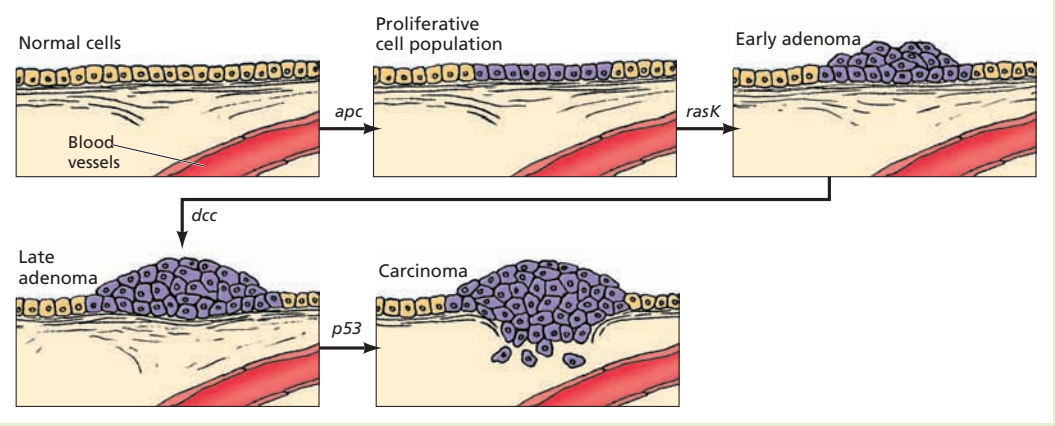
Colorectal cancer is the third-most-common cancer worldwide and the leading cause of cancer-associated deaths. The clinical stages in the development of this cancer are particularly well defined. Furthermore, as shown in the figure, several genes that are frequently mutated to allow progression from one stage to the next have been identified. The early adenomas or polyps that form initially are benign lesions. Their conversion to malignant metastatic carcinomas correlates with the acquisition of additional, loss-of-function mutations in the *p53* and *dcc* (deleted in colon carcinoma) tumor suppressor genes. Inherited mutations can greatly increase the risk that an individual will develop colon carcinoma. For example, patients with familial adenomatous polyposis can inherit defects in the *apc* (adenomatous polyposis coli) gene that result in the development of hundreds of adenomatous polyps. The large increase in the **number** of these benign lesions increases the chance that some will progress to malignant carcinomas. In contrast, patients with hereditary nonpolyposis colorectal cancer develop polyps at the same rate as the general population. However, their polyps develop into carcinomas more frequently, because these patients inherit defects in mismatch repair genes, resulting in a higher mutation rate that promotes oncogenesis. Consequently, the likelihood that an individual polyp will develop into a malignant lesion increases from 5 to 70%.

The genes shown in the figure were identified by classical methods in human genetics. More recently, mutations associated with colorectal cancer and many other types of human tumors have been catalogued by high-throughput sequencing of tumor genomes and protein-coding sequences (exomes). Such studies have confirmed that most tumors are caused by the accumulation of sequential mutations over a long period. They also establish that these mutations alter the function or production of components of a limited number of signal transduction pathways that govern cell proliferation and survival, determination of cell fate, and maintenance of genome integrity. In the case of colorectal cancer, somatic mutations detected by high-throughput sequencing methods in at least 80% of the patients examined are in genes that encode components of the mitogen-activated protein kinase, the Wnt/Apc, and p53 signaling pathways. Additional inherited mutations associated with predisposition to colorectal cancer have also been identified, notably in the gene that encodes the proofreading exonuclease of DNA polymerase δ (described in Volume I, Chapter 9).

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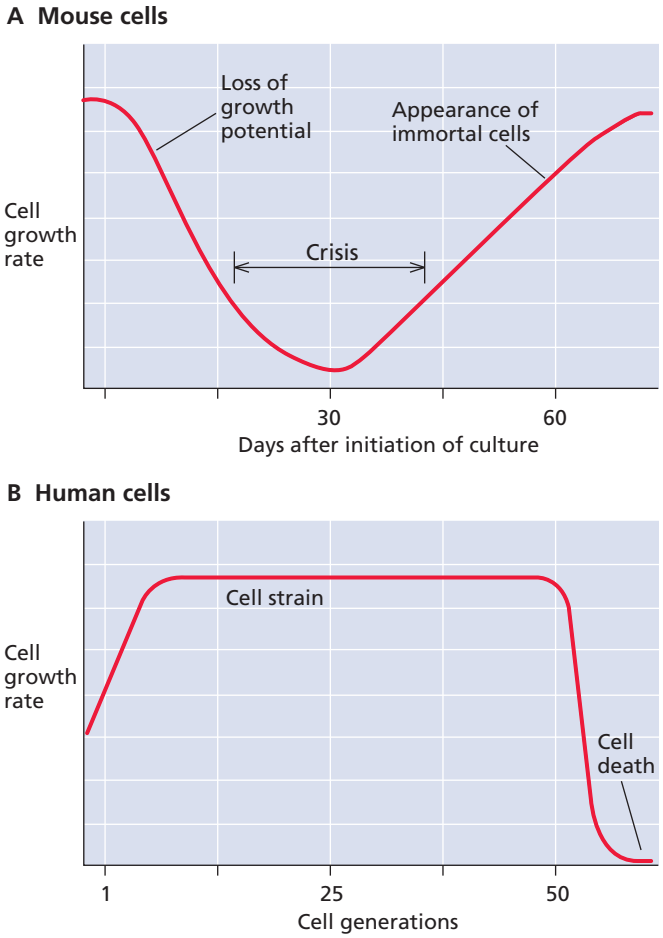


Figure 6.1 Stages in the establishment of a cell culture. **(A)** Mouse or other rodent cells. When mouse embryo cells are placed in culture, most cells die before healthy growing cells emerge. As these cells are maintained in culture, they begin to lose growth potential and most cells die (the culture goes into crisis). Very rarely, cells do not die but continue growth and division until their progeny overgrow the culture. These cells constitute a cell line, which will grow indefinitely if it is appropriately diluted and fed with nutrients: the cells are immortal. **(B)** Human cells. When an initial explant is made (e.g., from foreskin), some cells die and others (mainly fibroblasts) start to grow; overall, the growth rate increases. If the surviving cells are diluted regularly, the cell strain proliferates at a constant rate for about 50 cell generations, after which growth and division begin to decrease. Eventually, all the cells die.

over 100 days, and healthy neuronal cells rarely die. Occasionally, this carefully regulated process breaks down, and a particular cell begins to grow and divide even though the body has sufficient numbers of its type; such a cell behaves as if it were immortal. Acquisition of **immortality** is generally acknowledged to be an early step in oncogenesis. An immortalized cell may acquire one or more additional genetic changes to give rise to a clone of cells that is able to expand, ultimately forming a mass called a **tumor**. Some tumors are **benign**; they do not enter neighboring tissue and are not life-threatening. Other tumor cells grow and divide indefinitely to form invasive **malignant** cancers that damage and impair the normal function of organs and tissues. Some cells in a malignant tumor may acquire additional genetic changes that confer the ability to escape the boundary of the mass, to invade surrounding tissue, and to be disseminated to other parts of the body, where the cells take up residence. There they continue to grow and divide, giving rise to secondary tumors called **metastases**, which cause the most serious and life-threatening disease.

Many studies of the molecular biology of oncogenic animal viruses employed primary cultures of normal cells, for example, rat or mouse embryo fibroblasts. Such primary cells, like their normal counterparts in the animal, have a finite capacity to grow and divide in culture. Cells from some animal species, such as rodents, undergo a spontaneous transformation when maintained in culture. Immortalized cells appear after a "crisis" period in which the great majority of the cells die (Fig. 6.1A). As these surviving cells are otherwise normal, and do not induce tumors when introduced into animals, they can be used to identify viral gene products needed for steps in oncogenesis subsequent to immortalization. For reasons that are not fully understood, human and simian cells rarely undergo spontaneous transformation to immortality when passaged in culture (Fig. 6.1B). In fact, established lines of human cells generally can be derived only from tumors, or following exposure of primary cells to chemical carcinogens or to oncogenic RNA or DNA viruses (or their transforming genes). The realization that transformed cells share a number of common properties, regardless of how they were obtained, provided a major impetus for the investigation of viral transformation.

Properties That Distinguish Transformed from Normal Cells

The definitive characteristic of transformed cells is independence from the signals or conditions that normally control DNA replication and cell division. This property is illustrated by the list of growth parameters and behaviors provided in Table 6.1. As noted above, transformed cells are immortal: they can grow and divide indefinitely, provided that they are diluted regularly into fresh medium. Production

Table 6.1 Growth parameters and behavior of transformed cells

Immortal: can grow indefinitely
Reduced requirement for serum growth factors
Loss of capacity for growth arrest upon nutrient deprivation
Growth to high saturation densities
Loss of contact inhibition (can grow over one another or normal cells)
Altered morphology (appear rounded and refractile)
Anchorage independence (can grow in soft agar)
Tumorigenic

of **telomerase**, the enzyme that maintains telomeric DNA at the ends of chromosomes, is necessary for immortalization. In addition, transformed cells typically exhibit a reduced requirement for the growth factors present in serum. Some transformed cells actually produce their own growth factors and the cognate receptors, providing themselves **autocrine growth stimulation**. Normal cells cease to grow and enter a quiescent state (called G_0, described in "Control of Cell Proliferation" below) when essential nutrient concentrations drop below a threshold value. Transformed cells are deficient in this capacity, and some may even kill themselves by trying to continue to grow in an inadequate environment.

Transformed cells grow to high densities. This characteristic is manifested by the cells piling up and over each other. They also grow on top of untransformed cells, forming visually identifiable clumps called **foci** (Fig. 6.2). Transformed cells behave in this manner because they have lost **contact inhibition**, a response in which normal cells cease proliferation when they sense the presence of their neighbors. Unlike normal cells, many transformed cells have also lost the need for a surface on which to adhere, and we describe them as being **anchorage independent**. Some anchorage-independent cells form isolated colonies in semisolid media (e.g., 0.6% agar). This property correlates well with the ability to form tumors in animals and is often used as an experimental surrogate for malignancy. Transformed cells also **look** different from normal cells; they are more rounded, with fewer processes, and as a result many appear more refractile when observed under a microscope (Fig. 6.2).

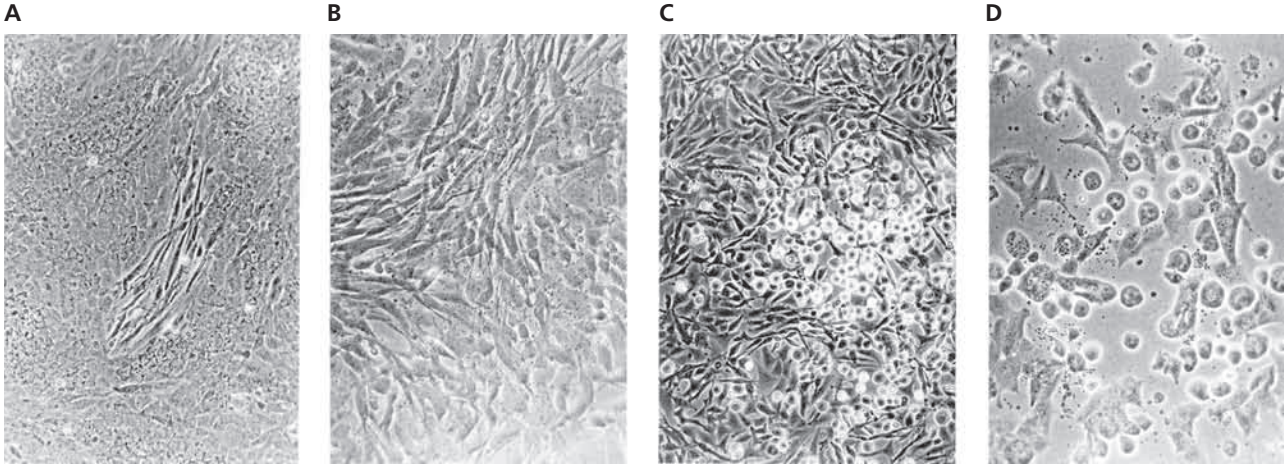
There are other ways in which transformed cells can be distinguished from their normal counterparts. These properties include metabolic differences and characteristic changes in cell surface and cytoskeletal components. However, the list in Table 6.1 comprises the standard criteria used to judge whether cells have been transformed.

Control of Cell Proliferation

Sensing the Environment

Because proliferation of cells in an organism is strictly regulated to maintain tissue or organ integrity and normal physiology, normal cells possess elaborate pathways that receive and process growth-stimulatory or growth-inhibitory signals transmitted by other cells in the tissue or organism. Much of what we know about these pathways comes from study of the cellular genes transduced or activated by oncogenic retroviruses. Signaling often begins with the secretion of a growth factor by a specific type of cell. The growth factor may enter the circulatory system, as is the case for many hormones, or may simply diffuse through the spaces around cells in a tissue. Growth factors bind to the external portion of specific receptor molecules on the surface of the same or other types of cell. Alternatively, signaling can be initiated by binding of a receptor on one cell to a specific protein (or proteins) present on the surface of another cell or to components of the extracellular matrix (Volume I, Chapter 5). The binding of the ligand triggers a change, often via oligomerization of receptor molecules, which is transmitted to the cytoplasmic portion of the receptor. In the case illustrated in Fig. 6.3, the cytoplasmic domain of the receptor possesses protein tyrosine kinase activity, and interaction with the growth factor ligand triggers autophosphorylation. This modification sets off a **signal transduction cascade**, a chain of sequential physical interactions among, and biochemical modifications of,

Figure 6.2 Foci formed by avian cells transformed with two strains of Rous sarcoma virus. Differences in morphology are due to genetic differences in the transduced *src* oncogene. **(A)** A focus of infected cells with fusiform morphology shown on a background of flattened, contact-inhibited, uninfected cells. **(B)** Higher magnification of a fusiform focus showing lack of contact inhibition of the transformed cells. **(C)** A focus of highly refractile infected cells with rounded morphology and reduced adherence. **(D)** Higher magnification of rounded infected cells, showing tightly adherent normal cells in the background. Courtesy of P. Vogt, The Scripps Research Institute.



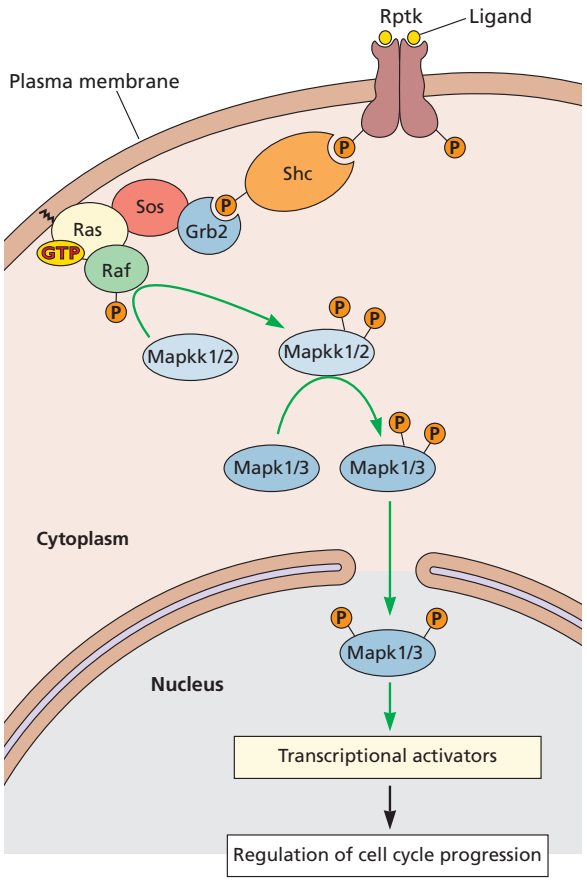


Figure 6.3 The mitogen-activated protein kinase signal transduction pathway. In the pathway shown, signal transduction is initiated by binding of a ligand to the extracellular domain of a receptor protein tyrosine kinase (Rptk), for example, the receptors for epidermal growth factor or platelet-derived growth factor. Binding of ligand (yellow circles) induces receptor dimerization and autophosphorylation of tyrosine residues in the cytoplasmic domain. Adapter proteins like Shc and the Grb2 component of the Grb2-Sos complex are recruited to the membrane by binding to these phosphotyrosine-containing sequences (or to a substrate phosphorylated by the activated receptor), along with Ras. Sos is the guanine nucleotide exchange protein for the small guanine nucleotide-binding protein Ras and stimulates exchange of GDP for GTP bound to Ras. The GTP-bound form of Ras binds to members of the Raf family of serine/threonine protein kinases. Raf then becomes autophosphorylated and initiates the mitogen-activated protein kinase (Mapk) cascade. The pathway shown contains dual-specificity Map kinase kinases (Mapkk1/2) and Mapk1/3. Phosphorylated Mapk1/3 molecules can enter the nucleus, where they modify and activate transcriptional regulators. These kinases can also regulate transcription indirectly, by effects on other protein kinases. Human cells contain multiple Mapkks and Mapks, and the pathway can be activated via plasma membrane receptors that respond to ligands such as inflammatory cytokines. Signal transduction cascades can also include enzymes that produce small molecules (e.g., cyclic AMP [cAMP] and certain lipids) that act as diffusible second messengers in the signal relay. Changes in ion flux across the plasma membrane, or in membranes of the endoplasmic reticulum, may also contribute to transmission of signals. Relay of the signal can terminate at cytoplasmic sites to alter metabolism or cell morphology and adhesion, but signaling to transcriptional regulators, as indicated, is common. To terminate signaling, ligand-bound receptor tyrosine kinases are internalized, GTPase-activating proteins induce hydrolysis of GTP bound to small G proteins like Ras, and protein phosphatases catalyze the hydrolysis of phosphate groups on signaling proteins.

membrane-bound and cytoplasmic proteins (Fig. 6.3). Ultimately, the behavior of the cell is altered.

Many signaling cascades culminate in the modification of transcriptional activators or repressors, and thereby alter the expression of specific cellular genes. The products of these genes either allow the cell to progress through another cell division cycle or cause the cell to stop growing, to differentiate, or to die, whichever response is appropriate to the situation. Errors in the signaling pathways that regulate these decisions can lead to transformation. The molecular features that transmit information are readily reversed, or short-lived, so that signal transduction pathways can be reset once the initiating cue is no longer present. Alterations that impair such mechanisms of termination of signal transmission can also contribute to transformation and oncogenesis.

Integration of Mitogenic and Growth-Promoting Signals

Prior to division, cells must increase in size and mass as they duplicate their components in preparation for the division that produces two daughter cells. Consequently, signals that induce cell proliferation also lead to the metabolic changes required to promote and sustain cell growth. Not surprisingly, the mechanisms that regulate growth of normal cells are integrated with those that lead to cell proliferation in response to mitogenic signals. The small G protein Ras and the protein kinase Akt are important components of the networks that achieve such integration: their activation leads to not only increased production of proteins that drive progression through the cell cycle (e.g., D-type cyclins), but also stimulation of translation and regulation of the production or activity of many metabolic enzymes (Fig. 6.4).

Regulation of the Cell Cycle

The capacity of cells to grow and divide is controlled by a molecular timer. The timer comprises an assembly of proteins that integrate stimulatory and inhibitory signals received by, or produced within, the cell. Eukaryotic cells do not divide until all their chromosomes have been duplicated and are precisely organized for segregation into daughter cells. Nor are DNA synthesis and chromosome duplication initiated until the previous cell division is complete, or unless the extra- and intracellular environments are appropriate. Consequently, the molecular timer controls a tightly ordered **cell cycle** comprising intervals, or phases, devoted to specific processes.

The duration of the phases in the cell cycle shown in Fig. 6.5 is typical of those of many mammalian cells growing actively in culture. However, there is considerable variation

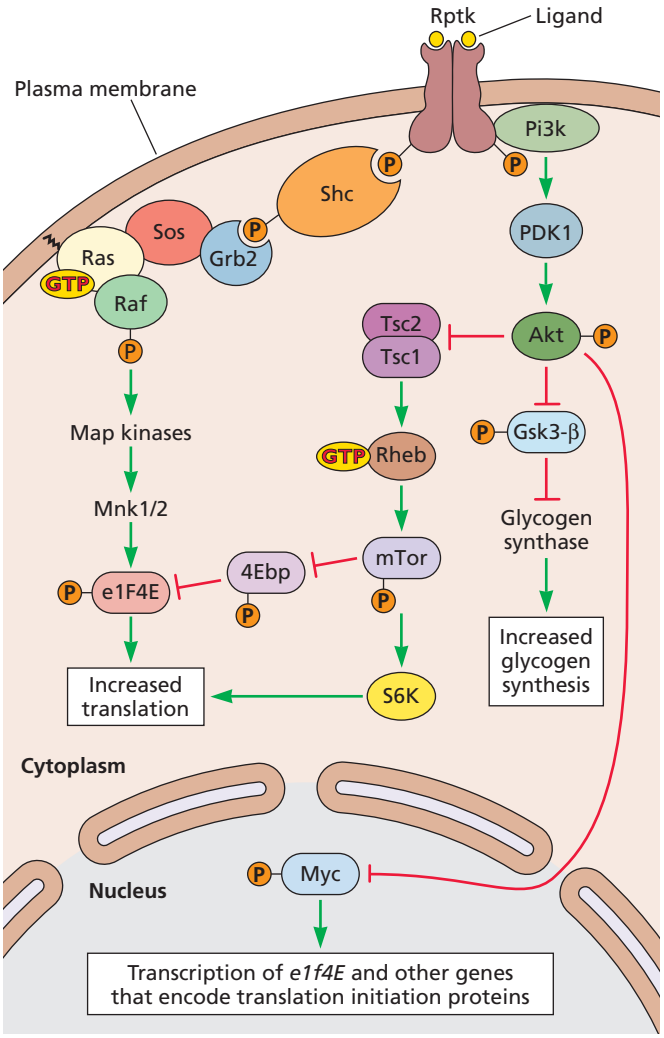


Figure 6.4 Some signaling pathways that promote cell growth. Upon activation, in this example by signaling initiated by binding of its ligand to a receptor protein tyrosine kinase (Rptk), signaling via Ras and the Map kinase cascade activates Map kinase-interacting serine/threonine protein kinases (Mnk1/2), which phosphorylate and activate the translation initiation protein eIF4E. The activity of this initiation protein is also increased when signaling from the Rptk via phosphatidylinositol 3-kinase (Pi3k) and 3-phosphoinositide-dependent protein kinase 1 (PDK1) stimulates the protein kinase Akt. The action of this kinase inhibits the tuberous sclerosis complex (Tsc1/2) and activates the small G protein Rheb (Ras homology enriched in brain) and mTor (mammalian target of rapamycin). Phosphorylation of the inhibitory eIF4E-binding protein (4Ebp) by mTor suppresses its ability to inactivate eIF4E. The transcription of the genes encoding eIF4E and other translation initiation proteins is stimulated via effects on the transcriptional activator Myc. Akt-dependent phosphorylation of ribosomal protein S6 kinase (S6K) increases the rate of translation elongation. These mechanisms increase the availability and activity of proteins crucial for protein synthesis and allow cells to provide proteins at a rate that sustains cells growth. Signaling from Akt also regulates metabolism via phosphorylation and inactivation of glycogen synthase kinase (Gsk3-β) and as a result of effects of activated mTor on lipid metabolism.

in the length of the cell cycle, largely because of differences in the **gap phases (G_1 and G_2)**. For example, early embryonic cells of animals dispense with G_1 and G_2, do not increase in mass, and move immediately from the **DNA synthesis phase (S)** to **mitosis (M)** and again from M to S. Consequently, they possess extremely short cycles of 10 to 60 min. At the other extreme are cells that have ceased growth and division. The variability in duration of this specialized **resting state**, termed **G_0**, accounts for the large differences in the rates at which cells in multicellular organisms proliferate. As discussed in Volume I, Chapter 9, viruses can reproduce successfully in cells that spend all or most of their lives in G_0, a state that has been likened to "cell cycle sleep." In many cases, synthesis of viral proteins in such resting or slowly cycling cells induces them to reenter the cell cycle and grow and divide rapidly. To describe the mechanisms by which these viral proteins induce such abnormal activity, we first introduce the molecular mechanisms that control passage through the cell cycle.

The Cell Cycle Engine

The orderly progression of eukaryotic cells through periods of growth, chromosome duplication, and nuclear and cell division is driven by intricate regulatory circuits. The elucidation of these circuits must be considered a tour de force of contemporary biology. The first experimental hint that cells contain proteins that control transitions from one phase of the cell cycle to another came more than 40 years ago. Nuclei of slime mold (*Physarum polycephalum*) cells in early G_2 were found to enter mitosis immediately following fusion with cells in late G_2 or M. This crucial observation led to the conclusion that the latter cells must contain a mitosis-promoting factor. Subsequently, similar experiments with mammalian cells in culture identified an analogous S-phase-promoting factor. The convergence of many observations eventually led to the identification of the highly conserved components of the cell cycle engine (Fig. 6.6A).

Mitosis-promoting factor proved to be an unusual protein kinase: its catalytic subunit is activated by the binding of an unstable regulatory subunit. Furthermore, the concentration of the regulatory subunit was found to oscillate reproducibly during each and every cell cycle. The regulatory subunit was therefore given the descriptive name **cyclin**, and the associated protein kinase was termed **cyclin-dependent kinase (Cdk)**. Similar proteins were implicated in cell cycle control in the yeast *Saccharomyces cerevisiae*, and it soon became clear that all eukaryotic cells contain multiple cyclins and Cdks, which operate in specific combinations to control progression through the cell cycle. The cyclins are related in sequence to one another, and they share such properties as activation of cyclin-dependent kinases and controlled destruction by the proteasome.

Various mammalian cyclin-Cdk complexes accumulate during the successive phases in the cell cycle (Fig. 6.6A).

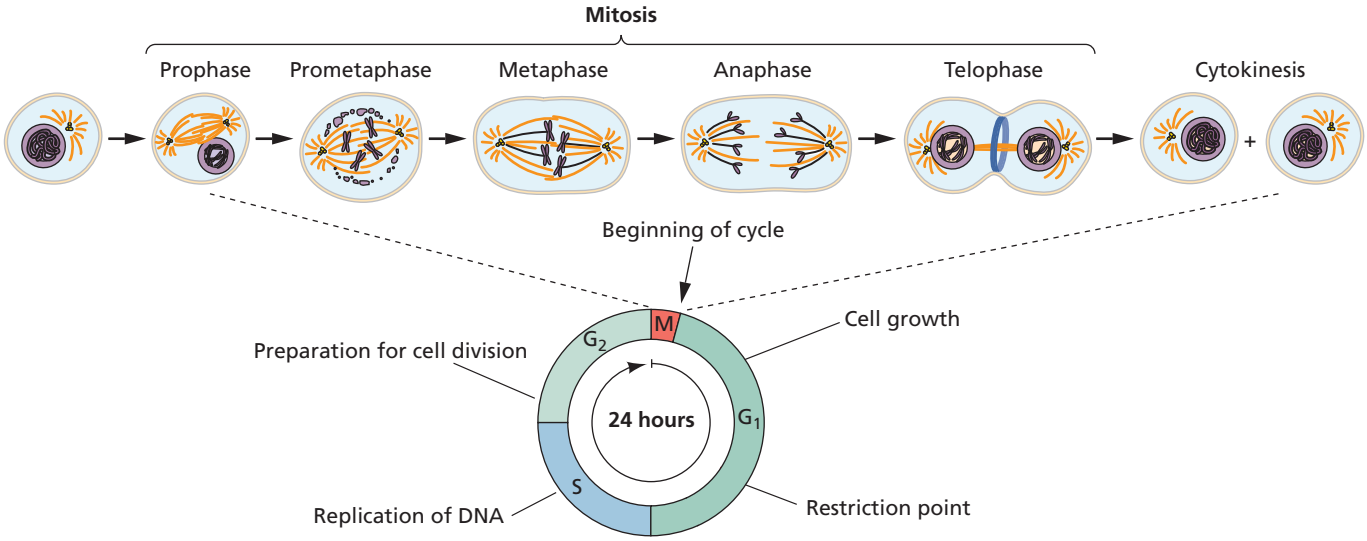
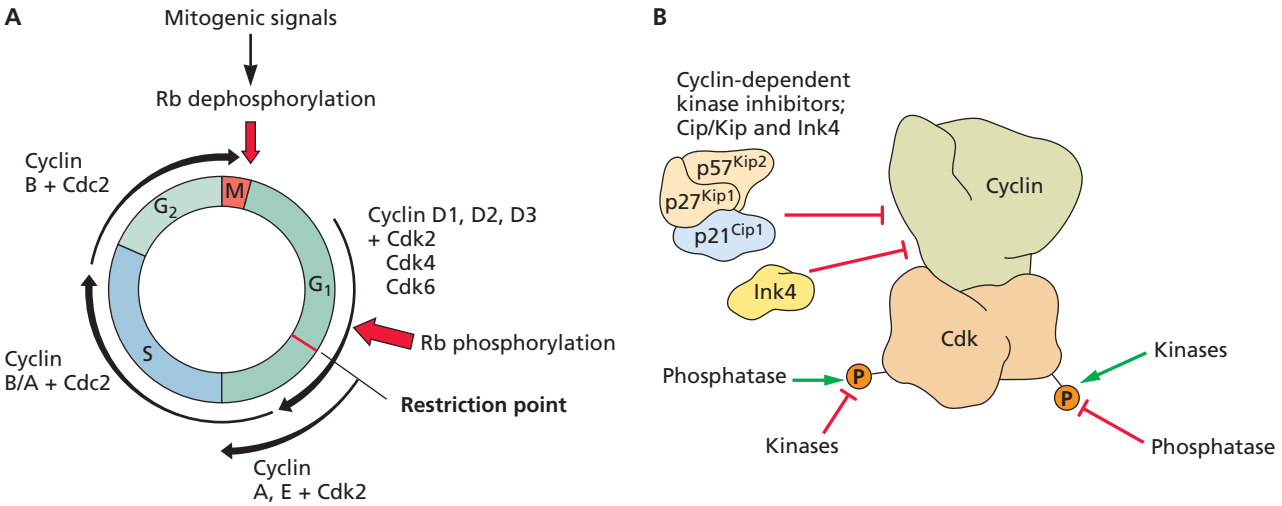


Figure 6.5 The phases of a eukaryotic cell cycle. The most obvious phase morphologically, and hence the first to be identified, is mitosis, or M phase, the process of nuclear division that precedes cell division. During this period, the nuclear envelope breaks down. Duplicated chromosomes become condensed and aligned on the mitotic spindle and are segregated to opposite poles of the cell, where nuclei re-form upon chromosome decondensation (top). The end of M phase is marked by cytokinesis, the process by which the cell divides in two. Despite this remarkable reorganization and redistribution of cellular components, M phase occupies only a short period within the cell cycle. During the long interphase from one mitosis to the next, cells grow continuously. Interphase was divided into three parts with the recognition that DNA synthesis takes place only during a specific period, the synthetic or S phase, which begins at about the middle of interphase. The other two periods, which appeared as "gaps" between defined processes, are designated the G_1 and G_2 (for gap) phases. Movie 6.1 (http://bit.ly/Virology_V2_Movie6-1) shows mitosis in HeLa cells that synthesize the microtubule-forming protein tubulin fused to enhanced green fluorescent protein to label the spindles and histone H2B fused to the red fluorescent protein mCherry to label chromosomes. Courtesy of Tim Yen, Fox Chase Cancer Center.

Figure 6.6 The mammalian cyclin-Cdk cell cycle engine. (A) The phases of the cell cycle are denoted on the circle. The progressive accumulation of specific cyclin-Cdks is represented by the broadening arrows, with the arrowheads marking the time of abrupt disappearance. **(B)** The production, accumulation, and activities of both cyclins and cyclin-dependent kinases are regulated by numerous mechanisms. Activating and inhibitory reactions are indicated by green arrows and red bars, respectively. Activation of the kinases can require not only binding to the appropriate cyclin, but also phosphorylation at specific sites and removal of phosphate groups at others. The activities of the kinases are also controlled by association with members of two families of cyclin-dependent kinase-inhibitory proteins, which control the activities of only G_1 (Ink4 proteins) or all (Cip/Kip proteins) cyclin-Cdks. Both types of inhibitor play crucial roles in cell cycle control. For example, the high concentration of $p27^{Kip1}$ characteristic of quiescent cells falls as they enter G_1, and inhibition of synthesis of this protein prevents cells from becoming quiescent.



A critical feature is that the individual cyclin-Cdks, the active protein kinases, accumulate in successive waves. The concentration of each increases gradually during a specific period in the cycle, but decreases abruptly as the cyclin subunit is degraded. In mammalian cells, proteolysis is important in resetting the concentrations of individual cyclins at specific points in the cycle, but production of cyclin mRNAs is also regulated. The orderly activation and inactivation of specific kinases govern passage through the cell cycle. For example, cyclin E synthesis is rate limiting for the transition from G_1 to S phase in mammalian cells, and cyclin E-Cdk2 accumulates during late G_1. Soon after cells have entered S phase, cyclin E rapidly disappears from the cell; its task is completed until a new cycle begins.

While the oscillating waves of active Cdk accumulation and destruction are thought of as the ratchet that advances the cell cycle timer, it is important not to interpret this metaphor too literally. The orderly and reproducible sequence of DNA replication, chromosome segregation, and cell division is not determined solely by the oscillating concentrations of individual cyclin-Cdks. Rather, the cyclin-Cdk cycle serves as a device for integrating numerous signals from the exterior and interior of the cell into appropriate responses. The regulatory circuits that feed into and from the cycle are both many and complex (e.g., Fig. 6.6B). These regulatory signals ensure that the cell increases in mass and divides **only** when the environment is propitious or, in multicellular organisms, when the timing is correct. Many signal transduction pathways that convey information about the local environment or the global state of the organism therefore converge on the cyclin-Cdk integrators. In addition, various surveillance mechanisms monitor such internal parameters as DNA damage, problems with DNA replication, and proper assembly and function of the mitotic spindle. Such mechanisms protect cells against potentially disastrous consequences of continuing a cell division cycle that could not be completed correctly. It is primarily these signaling and surveillance (checkpoint) mechanisms that are compromised during transformation by oncogenic viruses.

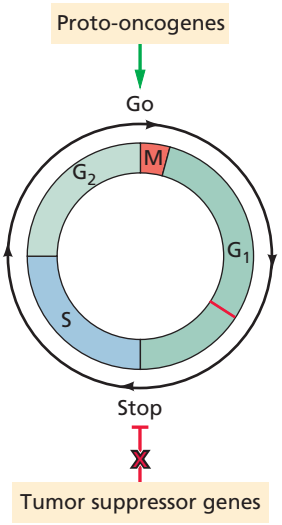


Figure 6.7 A genetic paradigm for cancer. The pace of the cell cycle can be modulated both positively and negatively by different sets of gene products. Cancer arises from a combination of dominant, gain-of-function mutations in proto-oncogenes and recessive, loss-of-function mutations in tumor suppressor genes, which encode proteins that block cell cycle progression at various points. The function of either type of gene product can be affected by oncogenic viruses.

Oncogenic Viruses

The study of the mechanisms of viral transformation and oncogenesis laid the foundation for our current understanding of cancer, for example, with the identification of oncogenes that are activated or captured by retroviruses (originally known as RNA tumor viruses) and viral proteins that inactivate tumor suppressor gene products (Fig. 6.7). Specific members of a number of different virus families, as well as an unusual, unclassified virus (Box 6.3), have been implicated in naturally occurring or experimentally induced cancers in animals (Table 6.2). It has been estimated that ~20% of all cases of human cancer are associated with infection by one of eight viruses: Epstein-Barr virus, hepatitis B virus, hepatitis C virus, human herpesvirus 8, human immunodeficiency virus type 1, human T-lymphotropic virus type 1, human papillomaviruses, and Merkel cell polyomavirus. In this section, we introduce oncogenic viruses and general features of their transforming interactions with host cells.

Discovery of Oncogenic Viruses

Retroviruses

Oncogenic viruses were discovered more than 100 years ago when Vilhelm Ellerman and Olaf Bang (1908) first showed that avian leukemia could be transmitted by filtered extracts (i.e., viruses) of leukemic cells or serum from infected birds.

Table 6.2 Oncogenic viruses and cancer

Family	Associated cancer(s)
RNA viruses	
Flaviviridae	
Hepatitis C virus	Hepatocellular carcinoma
Retroviridae	Hematopoietic cancers, sarcomas, and carcinomas
DNA viruses	
Adenoviridae	Various solid tumors
Hepadnaviridae	Hepatocellular carcinoma
Herpesviridae	Lymphomas, carcinomas, and sarcomas
Papillomaviridae	Papillomas and carcinomas
Polyomaviridae	Various solid tumors
Poxviridae	Myxomas and fibromas

BOX 6.3

EXPERIMENTS

A cancer virus with genomic features of both papillomaviruses and polyomaviruses

Efforts are under way to prevent the extinction of the western barred bandicoot, an endangered marsupial now found only on two islands in the UNESCO World Heritage Area of Shark Bay, Western Australia. Unfortunately, conservation has been hindered by a debilitating transmissible syndrome, in which wild and captive animals develop papillomas and carcinomas in several areas of the skin. The histological properties of the tumors suggested that a papillomavirus or a polyomavirus might contribute to development of the disease.

In fact, a previously unknown viral genome was discovered in tumor tissues from these animals by multiply primed amplification, cloning, and sequencing, and also by PCR with degenerate primers specific for papillomavirus DNA. This DNA genome exhibits features characteristic of both papillomaviruses and polyomaviruses and includes coding sequences related to those of both families. The papillomavirus-like and polyomavirus-like sequences were shown to be continuous with one another in the viral DNA genome. This property excludes the possibility that the tumor tissues were coinfected with a member of each family, as well as such artifacts as laboratory contamination of samples.

The origin of this unique virus, which was named bandicoot papillomatosis carcinomatosis virus type 1 (and a second, closely related virus isolated from a different bandicoot species), is not known. The virus might have arisen as a result of a recombination event between the genomes of a papillomavirus and a polyomavirus. Alternatively, it might represent the first known member of a new virus family that evolved from a common ancestor of the *Papillomaviridae* and *Polyomaviridae*. Regardless, the viral genome has been detected in 100% of bandicoots with papillomatosis and carcinomatosis syndrome, implicating the virus as a necessary factor in the development of this disease.

Woolford L, Rector A, Van Ranst M, Ducki A, Bennett MD, Nicholls PK, Warren KS, Swan RA, Wilcox GE, O'Hara AJ. 2007. A novel virus detected in papillomas and carcinomas of the endangered western barred bandicoot (*Perameles bougainville*) exhibits genomic features of both the *Papillomaviridae* and *Polyomaviridae*. *J Virol* **81:**13280–13290.

Perameles bougainville. From J. Gould, *Mammals of Australia*, vol 1 (J. Gould, London, United Kingdom, 1863).



Because leukemia was not recognized as cancer in those days, the significance of this discovery was not generally appreciated. Shortly thereafter (in 1911), Peyton Rous demonstrated that solid tumors could be produced in chickens by using cell extracts from a transplantable sarcoma that had appeared spontaneously. Despite the viral etiology of this disease, the cancer viruses of chickens were thought to be oddities until similar murine malignancies, as well as mouse mammary tumors, were found to be associated with infection by viruses. These oncogenic viruses all proved to be members of the retrovirus family. We now know that retroviruses are endemic in many species, including mice and chickens. When a chicken embryo is infected with avian leukosis virus, immune tolerance is established. In rare cases, tumors arise by a mechanism discussed below.

Early researchers classified the oncogenic retroviruses into two groups depending on the rapidity with which they caused cancer (Table 6.3). The first group comprises rare, rapidly transforming **transducing oncogenic retroviruses**. These are all highly carcinogenic agents that cause malignancies in nearly 100% of infected animals in a matter of days. They were later discovered to have the ability to transform susceptible cells in culture. The second class, **nontransducing oncogenic retroviruses**, includes less carcinogenic agents. Not all animals infected with these viruses develop tumors, which appear only weeks or months after infection. In the late 1980s, a third type of oncogenic retrovirus, a **long-latency retrovirus**, was identified in humans: tumorigenesis is very rare and occurs months or even years after infection.

Infection by each group of oncogenic retroviruses induces tumors by a distinct mechanism. The long-latency retroviruses encode transforming proteins with no cellular counterparts. As their name implies, the genomes of transducing retroviruses contain cellular genes that become **oncogenes** (genes encoding proteins that cause transformation or tumorigenesis) when expressed in the viral context. The virally transduced versions of these cellular genes are called **v-oncogenes**, and their normal cellular counterparts are called **c-oncogenes**

Table 6.3 The oncogenic retroviruses

Property or characteristic	Transducing viruses	Nontransducing viruses	Nontransducing, long-latency viruses
Example	Rous sarcoma virus	Avian leukosis virus	Human T-lymphotropic virus type 1
Efficiency of tumor formation	High (ca. 100% of infected animals)	High to intermediate	Very low (<5%)
Tumor latency	Short (days)	Intermediate (weeks, months)	Long (months, years)
Infecting viral genome	Viral-cellular recombinant; normally replication defective	Intact; replication competent	Intact; replication competent
Oncogenic element	Cell-derived oncogene carried in viral genome	Cellular oncogene activated in situ by a provirus	Virus-encoded regulatory protein controlling transcription?
Mechanism	Oncogene transduction	*cis*-acting provirus	*trans*-acting protein?
Ability to transform cells in culture	Yes	No	No

or **proto-oncogenes**. The genomes of the nontransducing retroviruses do not encode cell-derived oncogenes. Rather, the transcription of proto-oncogenes is activated inappropriately as a consequence of the nearby integration of a provirus in the host cell genome. In either situation, the oncogene products ordinarily play no role in the reproductive cycle of the retroviruses themselves. With the notable exception of the reproductive cycle of certain epsilonretroviruses (Box 6.4), the oncogenic potential of retroviruses is an accident of their infectious cycles. Nevertheless, the study of v-oncogenes and proto-oncogenes that are affected by retroviruses has been of great importance in advancing our understanding of the origins of cancer.

BOX 6.4

DISCUSSION

Walleye dermal sarcoma virus, a retrovirus with a unique transmission cycle

Episilonretrovirus is the latest genus to be recognized in the family *Retroviridae*. This genus includes retroviruses that infect fish, producing a proliferative disease first identified in walleyes collected in Oneida Lake in New York State in 1969. The genome of the best studied of these viruses, walleye dermal sarcoma virus, includes the conserved *gag*, *pol*, and *env* genes and three open reading frames, designated *orf a*, *orf b*, and *orf c*, which encode accessory proteins.

The most fascinating properties of this virus are its **seasonal reproductive cycle** and its ability to induce both **tumor formation and regression**. Naïve walleyes are infected at the time of spawning, when these fish congregate and the concentration of virus particles in the water is high. The newly infected fish are disease free until the fall, when skin tumors begin to form. The tumors contain ~1 provirus per cell and continue to increase in size through the winter, but only the *orf a* and *orf b* genes are expressed and there is no new virus production. The Orf A protein, called rv-cyclin, probably functions as an ortholog of cellular cyclin C, whereas production of Orf B leads to activation of specific signaling pathways. This protein has the capacity to transform cells *in vitro*. These two viral proteins are thought to work together to promote formation of dermal sarcomas.

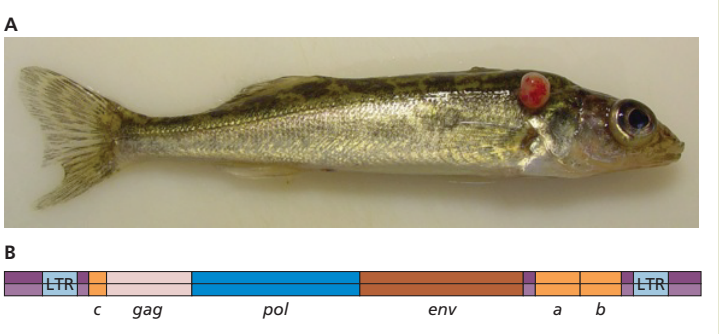
With the coming of spring, most likely triggered by change in water temperature, the proviral expression pattern and tumor fate change dramatically. The conserved viral genes are now expressed along with *orf c*. Tumor regression is initiated by the oncolytic Orf C protein in conjunction with the production of 10 to 50 progeny virus particles per cell. The tumors, along with large numbers of new infectious virus particles, are then shed into the water at the next spawning, just in time to initiate a new round of virus infection. Amazingly, the fish develop tumors for only one season and remain tumor free for the rest of their lives, suggesting that immune responses may also participate in tumor regression.

Although oncogenesis is an accidental occurrence with most other retroviruses, walleye dermal sarcoma virus is the exception to this rule, as both tumor production and regression are essential for the successful completion of its reproductive cycle.

Rovnak J, Quackenbush SL. 2010. Walleye dermal sarcoma virus: molecular biology and oncogenesis. *Viruses* **2:**1984–1999.

Walker R. 1969. Virus associated with epidermal hyperplasia in fish. *Natl Cancer Inst Monogr* **31:**195–207.

(A) A walleye salmon carrying a tumor experimentally induced by walleye dermal sarcoma virus. Courtesy of Sandra Quackenbush, Colorado State University. **(B)** Organization of the walleye dermal sarcoma virus genome showing the positions of the genes (*orf a*, *orf b*, and *orf c*) that encode accessory proteins.



Oncogenic DNA Viruses

The first DNA virus to be associated with oncogenesis was the papillomavirus that causes warts (papillomas) in cottontail rabbits; this virus was isolated by Richard Shope in 1933. The lack of cell culture systems for papillomaviruses initially precluded their use as experimental models for oncogenesis. Other viruses, in particular, polyomaviruses, such as simian virus 40, and human adenoviruses, proved much more tractable and soon dominated early studies of transformation and tumorigenesis by DNA viruses. It is important to note that neither simian virus 40 nor adenoviruses are associated with oncogenesis in their natural hosts. However, it was shown soon after their discovery that these viruses can induce tumors in rodents and transform cultured mammalian cells. The possibility that simian virus 40 could have contributed to human cancers continues to be the subject of much debate (Box 6.5). Reproduction of these viruses destroys permissive primate host cells within a few days of infection. In contrast, rodent cells are nonpermissive for viral reproduction or support only limited replication. Consequently, some infected cells survive infection and in rare cases become transformed.

The transforming genes of polyomaviruses and adenoviruses **are** necessary for viral reproduction. However, cellular transformation is a collateral consequence of the activities of the viral transforming proteins. These proteins contribute to transformation by altering the activities of cellular gene products.

BOX 6.5

DISCUSSION

Has simian virus 40 contributed to human cancer?

In 1960, simian virus 40 (SV40) was discovered in the African green monkey kidney cells used to produce poliovirus vaccines; within 2 years it was shown to be tumorigenic in hamsters. These were observations of great concern, because it was realized that many batches of the vaccines contained quite high concentrations of infectious SV40. It has been estimated that 98 million people in the United States, and many more worldwide, were exposed to potentially contaminated poliovirus vaccines before screening to ensure preparation of SV40-free vaccines was introduced in 1963. Ironically, monkey cells had been adopted for poliovirus vaccine production because of the concern that human cells might contain then unknown human cancer viruses!

Epidemiological studies initiated in the 1960s and 1970s monitored thousands of vaccine recipients for up to 20 years, with no evidence for increased cancer risk. The populations studied in this way included more than 1,000 children inoculated with SV40-containing poliovirus vaccine. This group was of particular importance as experimental infection of newborn hamsters with SV40 leads to tumor development in all, while animals older at the time of infection are more resistant to tumor development. The initial alarm raised by the tumorigenicity of SV40 in rodents therefore appeared to be laid to rest. Subsequent reports that the DNA of this virus is present in human tumors, including osteosarcomas and mesotheliomas analogous to those induced by SV40 in hamsters, have led some researchers to reconsider the contribution of this monkey virus to human cancer.

SV40 DNA could also be detected in normal tissue samples from mesothelioma patients and in tumors from individuals who did not receive potentially contaminated vaccine. These observations could indicate that factors in addition to SV40 (notably exposure to asbestos) lead to development of mesothelioma and that SV40 infection of humans is, in fact, quite common. However, careful analysis of the SV40 sequences detected in mesotheliomas (and other tissues) by PCR established that they arose from widespread viral DNA-containing laboratory plasmids that contain an engineered gap that is not present in the viral genome (see the figure). This observation undermined the conclusion of several studies based on PCR. Research and debate continue, for example, with a report in 2012 of an increase in the prevalence of serum antibodies that detect capsid proteins of SV40 (but not of closely related human polyomaviruses) from 15% in healthy individuals to 26% in age-matched mesothelioma patients.

This long-running debate highlights the difficulties of proving the association of a particular virus with human cancer (and the dangers of exquisitely sensitive molecular detection methods like PCR).

Garcea RL, Imperiale MJ. 2003. Simian virus 40 infection of humans. *J Virol* **77:**5039–5045.

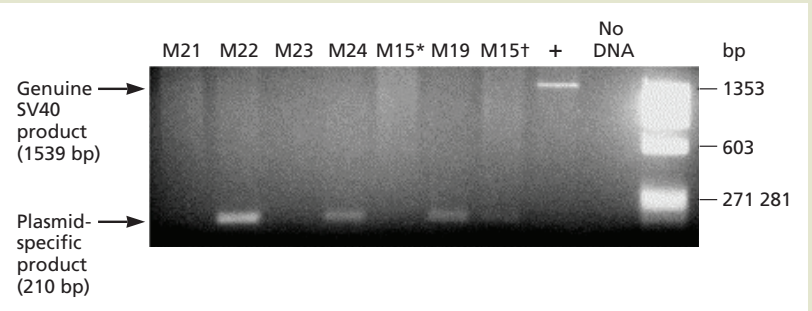
Jasani B, Cristaudo A, Emri SA, Gazdar AF, Gibbs A, Krynska B, Miller C, Mutti L, Radu C, Tognon M, Procopio A. 2001. Association of SV40 with human tumours. *Semin Cancer Biol* **11:**49–61.

López-Ríos F, Illei PB, Rusch V, Ladanyi M. 2004. Evidence against a role for SV40 infection in human mesotheliomas and high risk of false-positive PCR results owing to presence of SV40 sequences in common laboratory plasmids. *Lancet* **364:**1157–1166.

Mazzoni E, Corallini A, Cristaudo A, Taronna A, Tassi G, Manfrini M, Comar M, Bovenzi M, Guaschino R, Vaniglia F, Magnani C, Casali F, Rezza G, Barbanti-Brodano G, Martini F, Tognon MG. 2012. High prevalence of serum antibodies reacting with simian virus 40 capsid protein mimotopes in patients affected by malignant pleural mesothelioma. *Proc Natl Acad Sci U S A* **109:**18066–18071.

Poulin DL, DeCaprio JA. 2006. Is there a role for SV40 in human cancer? *J Clin Oncol* **24:**4356–4365.

The results of PCR analysis of DNA samples from human mesotheliomas (designated M plus a number) and an SV40-transformed mouse cell line positive control (1) with a primer pair that distinguishes artificially joined SV40 DNA sequences present in common laboratory plasmids (Plasmid-specific product) from the SV40 genome (Genuine SV40 product). The right lane shows double-stranded DNA markers, with the lengths indicated. Adapted from F. López-Ríos et al., *Lancet* **364:**1157–1160, 2004, with permission.



In some cases, such cellular proteins are encoded by the **same** proto-oncogenes transduced or otherwise affected by retroviruses. This important discovery, initially made in studies of the middle T protein (mT) of mouse polyomavirus in the early 1980s, provided the first indication that retroviruses and DNA viruses can transform cells by related mechanisms. Investigation of the biochemical properties of proteins encoded in other transforming genes of these DNA viruses led to equally important insights, notably the characterization of cellular proteins that can block cell cycle progression, the products of **tumor suppressor genes**.

It has been appreciated that herpesviruses can promote the development of tumors in humans and other animals since the discovery in 1966 of Epstein-Barr virus in cells derived from Burkitt's lymphoma. Infection of susceptible cells in culture by members of this family results in immortalization or induction of typical transformed phenotypes. Infection with poxviruses can induce cell proliferation that may be prolonged or rapidly followed by cell death, depending on the virus. Indeed, some members of this family, such as Shope fibroma virus, are associated with tumors of the skin. However, poxviruses do not transform cells in culture, in part because they are highly cytotoxic. The large sizes of their genomes initially presented a major impediment to analysis of the transforming properties of these viruses. It is now clear that herpesviral gene products generally alter cell growth and proliferation by mechanisms related to those responsible for transformation by the smaller DNA viruses or retroviruses. However, the genomes of some of these large DNA viruses also encode micro-RNAs (miRNAs) that contribute to transformation (described in Volume I, Chapter 10).

Contemporary Identification of Oncogenic Viruses

Oncogenic viruses associated with human disease continue to be isolated with some regularity. One discovered in 1994 was a previously unknown member of the family *Herpesviridae*, human herpesvirus 8, which was isolated from tumor cells of patients with Kaposi's sarcoma. Its genome, like those of transducing retroviruses, contains homologs of cellular proto-oncogenes. More recently (in 2008), a polyomavirus associated with a rare form of skin cancer was discovered (Box 6.6). Perhaps an even greater surprise was the realization

BOX 6.6

DISCUSSION

A polyomavirus that contributes to development of Merkel cell carcinoma in humans

Mouse polyomavirus and simian virus 40 have been important models for studies of oncogenesis and transformation (see the text). Two human members of this family, BK and JC polyomaviruses, were discovered in 1971. These viruses commonly establish persistent infections, and can be pathogenic in immunosuppressed patients (Appendix, Fig. 20). Eight other polyomaviruses were detected subsequently in human tissues. One, with a genome distantly related to those of other primate polyomaviruses, was detected in tumors from patients with Merkel cell carcinoma, a rare but rapidly metastasizing skin cancer.

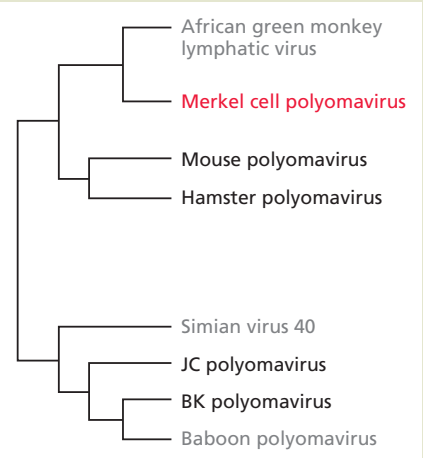
Viral DNA sequences initially were identified in tumor tissue by a method based on high-throughput sequencing. Among the unassigned sequences, one from the tumor exhibited significant homology to African green monkey lymphotropic polyomavirus and BK polyomavirus T antigen-coding sequences (see the figure). The 3′ end of this cDNA was shown to include sequences of the human receptor tyrosine phosphatase type G, suggesting that viral DNA sequences were integrated in the genome of tumor cells. Integration of the viral genome was subsequently confirmed by several methods, including Southern blotting. The organization of the viral genome is that typical of polyomaviruses, and includes sequences homologous to the transforming gene products, large and small T antigens, of animal members of the family.

The genome of this virus, which was called Merkel cell polyomavirus, is present in the majority of Merkel cell carcinomas, but the virus has generally not been detected in healthy surrounding tissues or other types of tumors. The pattern of viral DNA integration in the tissues examined indicated that the tumors were monoclonal in origin, implying that viral DNA integration preceded proliferation of the cells. Furthermore, the tumors, but not nearby healthy cells, synthesize T antigen(s), and inhibition of production of these viral proteins by RNA interference in Merkel cell carcinoma-derived cells in culture induces growth arrest or apoptosis. These observations establish that Merkel cell polyomavirus early gene products are required to maintain the oncogenic phenotype of these cells. They therefore provide strong support for causal association between virus infection and the development of Merkel cell carcinoma.

Feng H, Shuda M, Chang Y, Moore PS. 2008. Clonal integration of a polyomavirus in Merkel cell carcinoma. *Science* **319:**1096–1100.

Houben R, Shuda M, Weinkam R, Schrama D, Feng H, Chang Y, Moore PS, Becker JC. 2010. Merkel cell polyomavirus-infected Merkel cell carcinoma cells require expression of viral T antigens. *J Virol* **84:**7064–7072.

The evolutionary relationship of Merkel cell carcinoma polyomavirus to some other mammalian polyomaviruses is shown schematically. Adapted from R. P. Viscidi and K. V. Slak, *Science* **319:**1049–1050, 2008, with permission.



that RNA viruses other than retroviruses can be associated with cancer: hepatitis C virus, a (+) strand RNA virus belonging to the family *Flaviviridae* identified in 1989, is associated with a high risk for hepatocellular carcinoma.

Common Properties of Oncogenic Viruses

Although they are members of different families (Table 6.2), the majority of oncogenic viruses share several general features. In all cases that have been analyzed, transformation is observed to be a single-hit process (defined in Volume I, Chapter 2), in the sense that infection of a susceptible cell with a single virus particle is sufficient to cause transformation. In addition, all or part of the viral genome is usually retained in the transformed cell. With few exceptions, cellular transformation is accompanied by the continuous expression of specific viral genes. On the other hand, transformed cells need not and (except in the case of some retroviruses) **do not** produce infectious virus particles. Most importantly, transforming proteins alter cell proliferation by a limited repertoire of molecular mechanisms.

Viral Genetic Information in Transformed Cells

State of Viral DNA

Cells transformed by oncogenic viruses generally retain viral DNA in their nuclei. These DNA sequences correspond to all or part of the infecting DNA genome, or the proviral DNA made in retrovirus-infected cells. Viral DNA sequences are maintained by one of two mechanisms: they can be integrated into the cellular genome or persist as autonomously replicating episomes.

Integration of retroviral DNA by the viral enzyme integrase is an essential step in the viral reproductive cycle (Volume I, Chapter 7). Although there are some virus-specific biases, integration can occur at many sites in cellular DNA, but the reaction preserves a fixed order of viral genes and control sequences in the provirus (see Volume I, Fig. 7.15). When the provirus carries a v-oncogene, the site at which it is integrated into the cellular genome is of no importance (provided that viral transcription is unimpeded). In contrast, integration of proviral DNA within specific regions of the cellular genome is a hallmark of the induction of tumors by nontransducing retroviruses.

The proviral sequences present in every cell of a tumor induced by nontransducing retroviruses are found in the same chromosomal location, an indication that the tumor arose from a single transformed cell. Such tumors are, therefore, **monoclonal**. The proviruses in the tumor cells have usually lost some or most of the proviral sequences, but at least one long terminal repeat (LTR) containing the transcriptional control region is always present. Viral transcription signals, but not protein-coding sequences, are therefore required for transformation by nontransducing retroviruses. The significance of these properties became apparent when it was discovered that in several tumors proviruses were integrated in the vicinity of some of the same cellular oncogenes that are captured by transducing retroviruses. Because integration of retroviral DNA into the host genome can take place at many sites, there is a limited probability that integration will occur in the vicinity of an oncogene. The long latency for tumor induction by these viruses can be explained in part by the need for multiple cycles of replication and integration.

Integration of viral DNA sequences is not a prerequisite for successful propagation of **any** oncogenic DNA viruses. Nevertheless, integration is the rule in adenovirus- or polyomavirus-transformed cells. Such integration is the result of rare recombination reactions (catalyzed by cellular enzymes) between viral and host DNA sequences with minimal homology. Integration can therefore occur at essentially random sites in the cellular genome. The great majority of cells transformed by these viruses retain only partial copies of the viral genome. The genomic sequences integrated can vary considerably among independent lines of cells transformed by the same virus, but a common, minimal set of genes is always present. The low probability that viral DNA will become integrated into the cellular genome, and the fact that only a fraction of these recombination reactions will maintain the integrity of viral transforming genes, are major factors contributing to the low efficiencies of transformation by these viruses.

A second mechanism by which viral DNA can persist in transformed cells is as a stable, extrachromosomal episome (Volume I, Box 1.7). Such episomal viral genomes are a characteristic feature of B cells immortalized by Epstein-Barr virus, and they can also be found in cells transformed by papillomaviruses. The viral episomes are maintained at concentrations of tens to hundreds of copies per cell, by both replication of the viral genome in concert with cellular DNA synthesis and orderly segregation of viral DNA to daughter cells (described in Volume I, Chapter 9). Consequently, transformation depends on the viral proteins necessary for the survival of viral episomes, as well as those that modulate cell growth and proliferation directly.

Identification and Properties of Viral Transforming Genes

Transforming genes of oncogenic viruses have been identified by classical genetic methods, characterization of the viral genes present and expressed in transformed cell lines, and analysis of the transforming activity of viral DNA fragments directly introduced into cells (Box 6.7). For example, analysis of transformation by temperature-sensitive mutants of mouse polyomavirus established as early as 1965 that the viral early transcription unit is necessary and sufficient to initiate and maintain transformation of cells in culture. Of even greater value were mutants of retroviruses, in particular two mutants of Rous sarcoma virus isolated in the early 1970s. The genome

BOX 6.7

BACKGROUND

Multiple lines of evidence identified the transforming proteins of the polyomavirus simian virus 40

Early gene products are necessary and sufficient to initiate transformation.

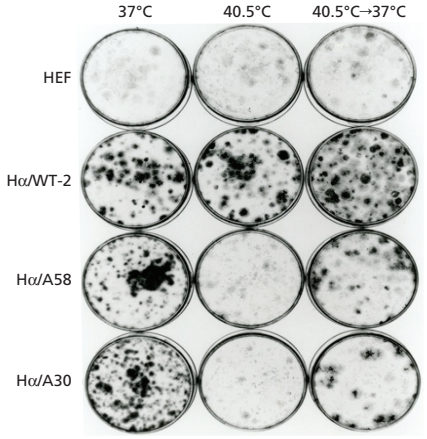
1. Viruses carrying temperature-sensitive mutations in the early transcription unit (*ts*A mutants), but no other region of the genome, fail to transform at a nonpermissive temperature.
2. Simian virus 40 DNA fragments containing only the early transcription unit transform cells in culture; DNA fragments containing other regions of the genome exhibit no activity.

Early gene products are necessary to maintain expression of the transformed phenotype.

1. Many lines of cells transformed by simian virus 40 *ts*A mutants at a permissive temperature revert to a normal phenotype when shifted to a nonpermissive temperature, and vice versa (see figure).
2. Integration of viral DNA sequences disrupts the late region of the viral genome but not the early transcription unit, and early gene products are synthesized in all transformed cell lines.

Both LT and sT contribute to transformation.

1. Simian virus 40 mutants carrying deletions of sequences that are expressed only in sT (Volume I, Appendix, Fig. 23) fail to transform rat cells to anchorage-independent growth.
2. Introduction and expression of LT complementary DNA are sufficient for induction of transformation, but expression of sT can increase efficiency (especially at low LT concentrations), is necessary for expression of specific phenotypes in specific cells, and is required for transformation of resting cells.



Hamster embryonic fibroblasts (HEF) or cells derived by transformation with wild-type simian virus 40 (Hα/WT-2) or with *ts*A mutants (Hα/A58 and Hα/A30) were plated at the temperatures indicated at the top or shifted to the lower temperature after 6 days. Cells were stained 11 days after seeding. In contrast to the wild-type transformed cells, those transformed by *ts*A mutants fail to form colonies at the higher (nonpermissive) temperature. However, this transformed phenotype is exhibited upon shift down to the lower, permissive temperature; that is, it is reversible. Adapted from J. S. Brugge and J. S. Butel, *J Virol* **15:**619–635, 1975. Courtesy of J. Butel, Baylor College of Medicine.

of one mutant carried a spontaneous deletion of ~20% of the viral genome. This mutant could no longer transform the cells it infected, but it could still reproduce. The second mutant was temperature sensitive for transformation, but the virus could reproduce at temperatures both permissive and nonpermissive for transformation. These properties of the mutants showed unequivocally that cellular transformation and viral reproduction are distinct processes. More importantly, the deletion mutant allowed preparation of the first nucleic acid probe specific for a v-oncogene, v-*src* (Box 6.8) (see the interview with Dr. Michael Bishop: http://bit.ly/Virology_Bishop). This *src*-specific probe was found to hybridize to cellular DNA, providing the first conclusive evidence that v-oncogenes are of cellular and **not** viral origin. This finding, for which J. Michael Bishop and Harold Varmus received the 1989 Nobel Prize in physiology or medicine, had far-reaching significance, because it immediately suggested that such cellular genes might become oncogenes by means other than viral transduction.

The presence of cellular oncogenes in their genomes turned out to be the definitive characteristic of transducing retroviruses (Fig. 6.8). As noted earlier, the acquisition of these cellular sequences is a very rare event. In addition, with the exception of Rous sarcoma virus, the transducing retroviruses are replication defective, having lost all or most of the viral coding sequences during oncogene capture. Such defective transducing viruses can, however, be propagated in mixed infections with replication-competent "helper" viruses, which provide all the proteins necessary for assembly of viral particles.

Viral and cellular protein-coding sequences are fused in many v-oncogenes (Fig. 6.8). The presence of viral sequences can enhance the efficiency of translation of the oncogene mRNA, stabilize the protein, or determine its location in the cell. Unregulated expression or overexpression of the cellular sequence from the viral promoter is sufficient to cause transformation by some v-oncogenes (e.g., *myc* and *mos*). However, in most cases, the captured oncogenes have undergone additional changes that contribute to their transforming potential. These alterations, which include nucleotide changes, truncations at either or both ends, or other rearrangements, affect the normal function of the gene product.

Transformation of primary cells by DNA viruses typically requires the products of two or more viral genes (Table 6.4). The majority of these genes exhibit some ability to alter the properties of the cells in which they are expressed in the absence of

BOX 6.8

TRAILBLAZER

Preparation of the first oncogene probe

In the early 1970s, modern molecular biology was already in full bloom, but some techniques that are currently commonplace, such as PCR amplification of specific genes, had not yet been invented. It was, however, possible at that time to make cDNA copies of RNA with retroviral reverse transcriptase and to separate double-stranded (hybridized) from single-stranded (nonhybridized) nucleic acids. The existence of two genetically related viral genomes, one that contained a transforming gene (Rous sarcoma virus [RSV]) and a deletion mutant (tdRSV) that was replication competent but nontransforming, made it possible to isolate a radioactively labeled probe for the transforming gene, *src*, by exploiting the available techniques, in a strategy known as **subtractive hybridization**.

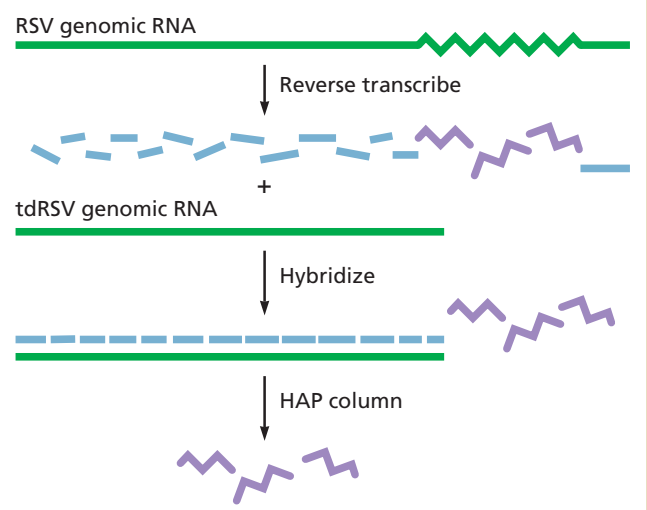
Complementary (−) strand DNA was prepared by reverse transcription of the (+) strand RSV genome and then hybridized to genomic RNA of the tdRSV mutant. The nonhybridizing DNA (purple) was separated from the double-stranded hybrids by hydoxylapatite chromatography (see the figure). This radioactive DNA was then used as a probe to search for corresponding genetic material in a variety of cells.

Hybridization to chicken genomic DNA and the DNA of other avian species immediately suggested that the *src* sequences and, by inference, other retroviral oncogenes had been captured from the host cells infected by the virus. The observation that *src*-related sequences are conserved among cells from widely different species in the animal kingdom suggested that the proteins they encode play a central role in cell growth and division and that their malfunction could explain the origin of cancers that arise independently of retroviral infection.

Spector DH, Varmus HE, Bishop JM. 1978. Nucleotide sequences related to the transforming gene of avian sarcoma viruses are present in DNA of uninfected vertebrates. *Proc Natl Acad Sci U S A* **75:**4102–4106.

Stehelin D, Varmus HE, Bishop JM. 1976. DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature* **260:**170–173.

Genomic RNAs are shown in green and the cDNA products of reverse transcription of common sequences and the unique sequence present on the RSV genome in blue and purple. HAP, hydroxylapatite.



other viral proteins. However, some are required only for the induction of specific transformed phenotypes or only under certain conditions (e.g., simian virus 40 small T antigen [sT]), and several exhibit no activity on their own (Table 6.4). A classic example of the latter phenomenon is provided by the adenoviral E1B gene: this gene, together with the E1A gene, was initially shown to be essential for transformation of rodent cells in culture, but it possesses no intrinsic ability to induce **any** transformed phenotype. This apparent paradox has been resolved with elucidation of the molecular functions of the viral gene products: E1A gene products induce apoptosis, but E1B proteins suppress this response and allow cells that synthesize E1A proteins to survive and display transformed phenotypes.

The Origin and Nature of Viral Transforming Genes

Two classes of viral oncogenes can be distinguished on the basis of their similarity to cellular sequences. The oncogenes of transducing retroviruses and certain herpesviruses (e.g., human herpesvirus 8) are so closely related to cellular genes that it is clear that they were captured relatively recently (since the divergence of primates). Such acquisition must be a result of recombination between viral and cellular nucleic acids, a process that has been documented for transducing retroviruses. Retrovirus particles contain some cellular RNAs, and rare recombination reactions during reverse transcription can give rise to transducing retroviruses. The limiting factor appears to be the frequency with which cellular mRNA molecules are encapsidated. Two mechanisms can increase the likelihood of encapsidation, and consequently increase the frequency of gene capture (Fig. 6.9). Both mechanisms depend on integration of a provirus in or near a cellular gene and incorporation of the cellular sequences into a transcript initiated within the LTR. The final step is a recombination reaction(s) between largely nonhomologous sequences in this chimeric transcript and a wild-type viral genome when both are incorporated into a viral particle.

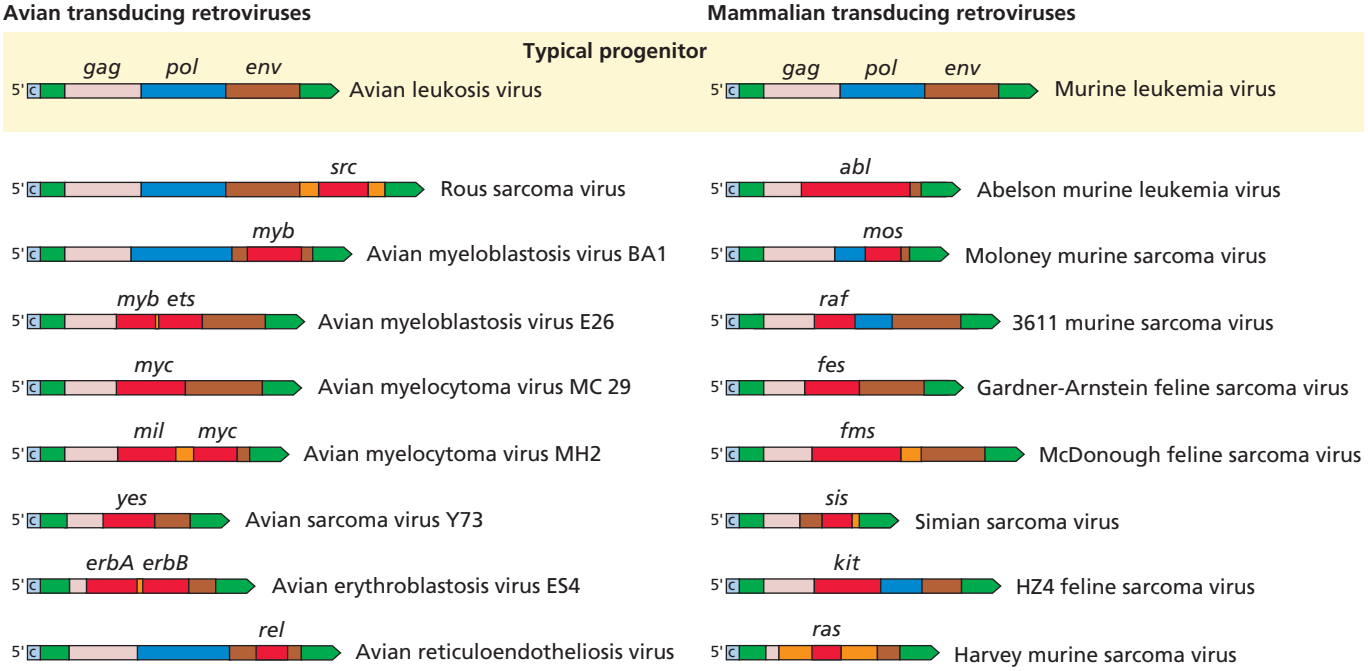


Figure 6.8 Genome maps of avian and mammalian transducing retroviruses. Avian leukosis virus (e.g., Rous-associated virus) and murine leukemia virus are prototypical retroviruses. Their genomes contain the three major coding regions: *gag* (pink), *pol* (blue), and *env* (brown). In Rous sarcoma virus, the oncogene *src* is added to the complete viral genome. In all other avian and mammalian transducing retroviruses, some of the viral coding information is replaced by cell-derived oncogene sequences (red). Consequently, such transducing viruses are defective in replication. The majority of the transducing retroviruses carry a single v-oncogene in their genomes, but some include more than one (e.g., *erbA* and *erbB* in avian erythroblastosis virus ES4). In such cases, one is sufficient for transformation, while the second accelerates this process. In some cases, additional cellular DNA sequences (orange) were also captured in the viral genome. Adapted from T. Benjamin and P. Vogt, p. 317–367, *in* B. N. Fields et al. (ed.), *Fields Virology*, 2nd ed. (Raven Press, New York, NY, 1990), with permission.

Table 6.4 Some transforming gene products of adenoviruses, papillomaviruses, and polyomaviruses

Virus	Gene product	Activities
Adenoviridae		
Human adenovirus type 2	E1A: 243R and 289R	Cooperate with E1B proteins to transform primary cells; not sufficient for establishment of transformed cell lines
	E1B: 55 kDa and 19 kDa	Necessary for E1A-dependent transformation of primary and established cells; counter apoptosis by different mechanisms
Papillomaviridae		
Human papillomavirus types 16 and 18	E6	Required for efficient immortalization of primary human fibroblasts and keratinocytes
	E7	Cooperates with E6 to transform primary rodent cells; required for efficient immortalization of primary human fibroblasts or keratinocytes
Polyomaviridae		
Polyomavirus	LT	Immortalizes primary cells; required to induce but not to maintain transformation of primary cells
	mT	Transforms established cell lines; required to both induce and maintain transformation of primary cells
Simian virus 40	LT	Immortalizes primary cells; required to induce and maintain transformation of primary and established cells
	sT	Required under many conditions, depending on LT concentration, genetic background of recipient cells, and transformation assay

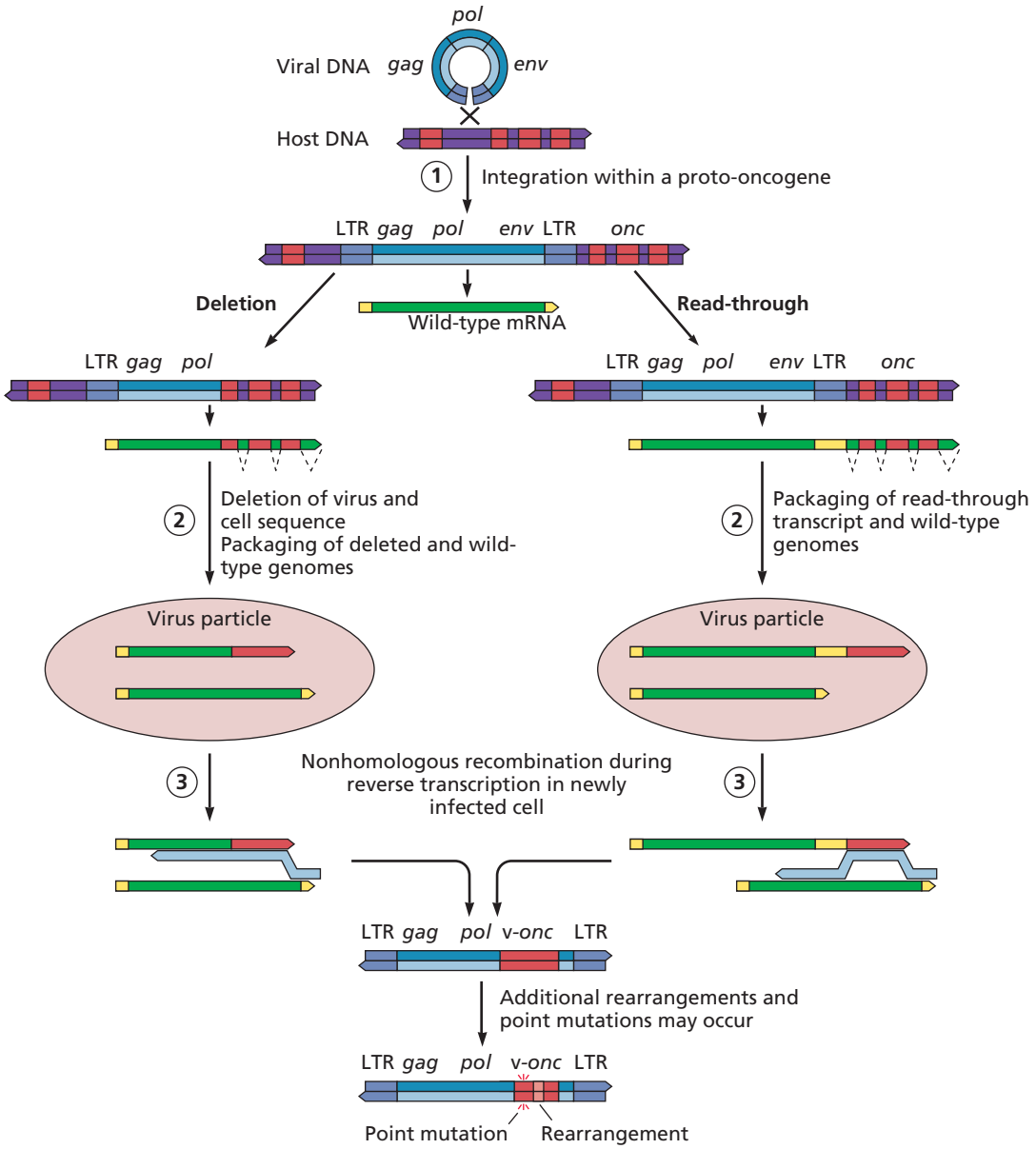


Figure 6.9 Possible mechanisms for oncogene capture by retroviruses. The first step in each of two mechanisms shown is integration of a provirus in or near a cellular gene (*onc*). The deletion mechanism (left) requires removal of the right end of the provirus, thereby linking cellular sequences to the strong viral transcriptional control region in the left LTR. The first recombination step in this mechanism therefore takes place at the DNA level. It leads to synthesis of a chimeric RNA, in which viral sequences from the left end of the provirus are joined to cellular sequences. Chimeric RNA molecules that include the viral packaging signal can be incorporated efficiently into viral particles with a wild-type genome produced from another provirus in the same cell. A second recombination reaction, during reverse transcription (as described in Volume I, Chapter 7), is then required to add right-end viral sequences to the recombinant. At a minimum, these right-end sequences must include signals for subsequent integration of the recombinant viral DNA into the genome of the newly infected host cell, from which the transduced gene is then expressed. The read-through mechanism (right) does not require a chromosomal deletion. Viral transcription does not always terminate at the 3′ end of the proviral DNA, but continues to produce transcripts containing cellular sequences. Such chimeric transcripts can then be incorporated into virus particles together with the normal viral transcript. The cellular sequences can then be captured by recombination during reverse transcription, as indicated. Important additional mutations and rearrangements probably occur during subsequent virus replication. Adapted from J. M. Coffin et al. (ed.), *Retroviruses* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1997), with permission.

Many of the cellular proto-oncogenes from which v-oncogenes are derived have been highly conserved throughout evolution: vertebrate examples often have homologs in yeast. The products of such genes must therefore fulfill functions that are indispensable for a wide variety of eukaryotic cells. Furthermore, as single copies of v-oncogenes are sufficient to transform cells, their functions must override those of the resident, cognate proto-oncogenes. Accordingly, v-oncogenes function as **dominant** transforming genes.

Members of the second class of viral oncogenes, such as adenovirus E1A and polyomavirus LT, are not obviously related to cellular genes. However, the products of these genes may contain short amino acid sequences also present in cellular proteins (for example, see Fig. 6.20). The precise origins of such oncogenes remain shrouded in mystery (see Chapter 10).

Functions of Viral Transforming Proteins

Many approaches have been employed to determine the functions of viral oncogene products. In some cases, the sequence of a viral transforming gene can immediately suggest the function of its protein product. For example, the genomes of certain herpesviruses and poxviruses include coding sequences that are closely related to cellular genes that encode growth factors, cytokines, and their receptors. Or the protein may contain amino acid motifs characteristic of particular biochemical activities, such as tyrosine phosphorylation or sequence-specific DNA binding. In other cases, notably many retroviral v-oncogene products, it has been possible to identify important biochemical properties, such as enzymatic activity, binding to a hormone or growth factor, or sequence-specific binding to nucleic acids (Table 6.5).

The breakthrough to understanding transformation by small oncogenic DNA viruses came with mutational analyses that correlated the transforming activities of viral gene products with binding to specific cellular proteins, notably tumor suppressors. The first such interaction identified was between sequences of adenoviral E1A proteins that are necessary for transformation and the cellular retinoblastoma tumor suppressor protein, Rb. The similar relationship between Rb binding and the transforming activities of simian virus 40 large T antigen (LT) and the E7 protein of oncogenic human papillomaviruses rapidly established the general importance of interaction with Rb in transformation. Interaction of transforming proteins of these small DNA viruses with a second cellular tumor suppressor, the p53 protein, is also required for oncogenesis. These observations established the importance of inactivation of tumor suppressors (Fig. 6.7) in transformation by these DNA viruses. A second common feature is that their transforming proteins affect multiple cellular proteins and pathways (Fig. 6.10).

Viral transforming proteins exhibit great diversity in all their properties, from primary amino acid sequence to biochemical activity. They also differ in the number and nature of the cellular pathways they alter. Despite such variation, these viral proteins induce continuous cell proliferation, the definitive characteristic of transformation, by related mechanisms. Indeed, the best characterized fall into one of only two classes, permanent activation of cellular signal transduction cascades or disruption of the circuits that regulate cell cycle progression.

Table 6.5 Functional classes of oncogenes transduced by retroviruses[a]

Transduced oncogene[b]	Function of cellular homolog
Growth factors	
sis	Platelet-derived growth factor
Receptor protein tyrosine kinases	
erbB	Epithelial growth factor receptor
Kit	Hematopoietic receptor; product of the mouse W locus
Hormone receptors	
erbA	Thyroid hormone receptor
G proteins	
H-*ras*, K-*ras*	GTPases
Adapter protein	
Crk	Signal transduction
Nonreceptor tyrosine kinases	
src, *abl*	Signal transduction
Serine/threonine kinases	
Mos	Required for germ cell maturation
Akt	Signal transduction
Nuclear proteins	
jun, *fos*	Transcriptional regulator (AP-1 complex)
Myc	Transcriptional regulator

[a]Adapted from T. Benjamin and P. Vogt, *in* B. N. Fields et al. (ed.), *Fields Virology*, 3rd ed. (Lippincott-Raven Publishers, Philadelphia, PA, 1996).

[b]Only some representative examples are listed.

Activation of Cellular Signal Transduction Pathways by Viral Transforming Proteins

The products of transforming genes of both RNA and DNA viruses can alter cellular signal transduction cascades. The consequence is permanent activation of pathways that promote cell growth and proliferation. However, as discussed in subsequent sections, these viral proteins can intervene at various points in these pathways, and they operate in several different ways.

Viral Signaling Molecules Acquired from the Cell

The Transduced Cellular Genes of Acutely Transforming Retroviruses

The v-*src* paradigm. The protein product of v-*src* was the first retroviral transforming protein to be identified,

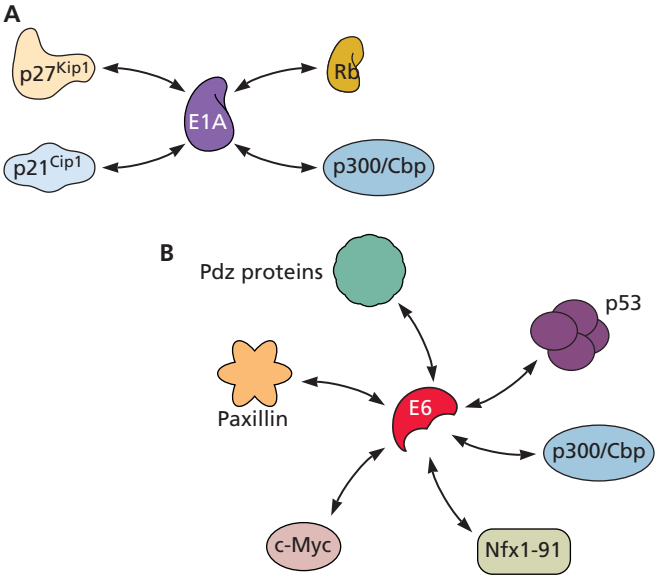


Figure 6.10 Schematic illustration of interactions of DNA virus transforming proteins with multiple cellular proteins. (A) The association of adenoviral E1A proteins with Rb family tumor suppressors, the histone acetyltransferases p300/Cbp, and the cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p21^{Cip1}$ has been implicated in transformation: E1A protein substitutions that impair these interactions reduce or eliminate transforming activity. **(B)** The human papillomavirus type 16 or 18 E6 protein also interacts with p300/Cbp and tumor suppressors, in this case the p53 protein, as well as several proteins that contain the Pdz domain and are localized at cell junctions (e.g., Dlg1 [discs large 1]) or are phosphatases (e.g., Ptpn3 [tyrosine-protein phosphatase nonreceptor type 3]). In addition, it associates with the transcriptional regulators c-Myc and Nfx1-91 to repress transcription of the gene encoding the protein component of telomerase. These interactions have been implicated in increased production of telomerase in cells synthesizing the E6 protein. In some cases, including p53 and Nfx1-91, the complex includes the cellular ubiquitin ligase E6-Ap, and the other cellular proteins are targeted for proteasomal degradation. Degradation of Pdz domain-containing proteins is also induced by the viral E6 protein.

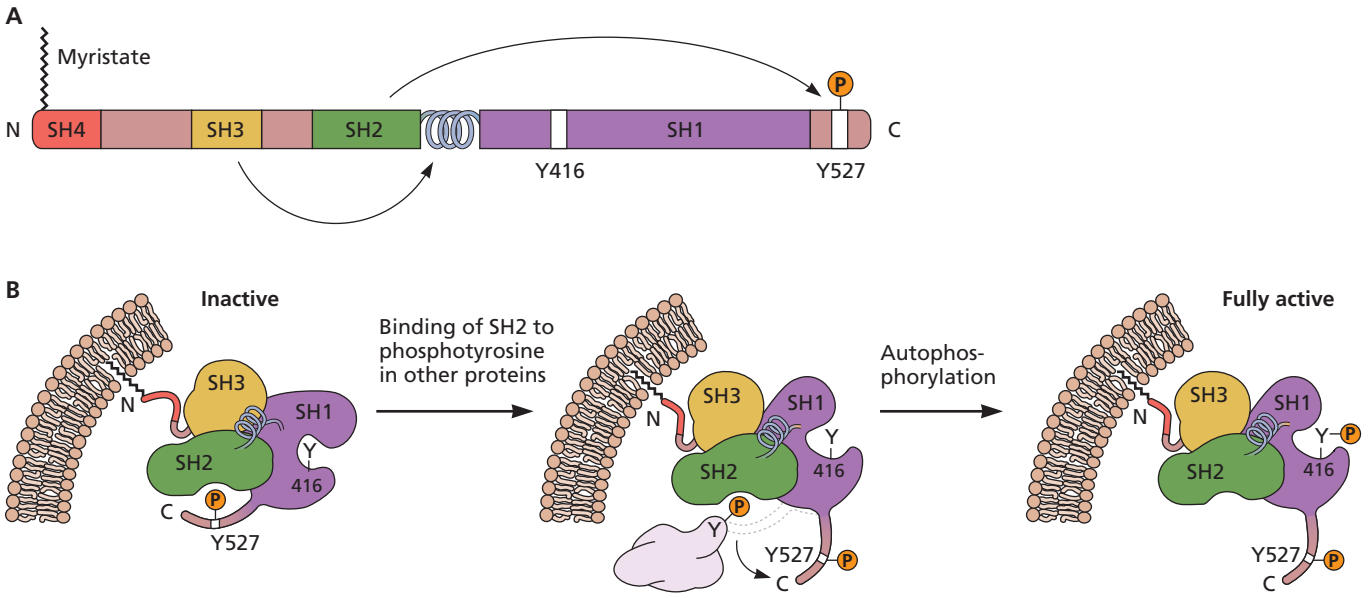
when serum from rabbits bearing tumors induced by Rous sarcoma virus was shown to immunoprecipitate a 60-kDa phosphoprotein. This v-Src protein was soon found to possess protein tyrosine kinase activity, a property that provided the first clue that phosphorylation of cellular proteins can be critical to oncogenesis. The discovery of this protein tyrosine kinase led to the identification of a large number of other proteins with similar enzymatic activity and important roles in cellular signaling.

The Src protein contains a tyrosine kinase domain (SH1, for Src homology region 1) and two domains that mediate protein-protein interactions (Fig. 6.11A). The first, the SH2 domain, binds to phosphotyrosine-containing sequences, whereas the SH3 domain has affinity for proline-rich sequences. A fourth Src domain (SH4) includes the N-terminal myristoylation signal that directs Src to the plasma membrane. All four domains are required for Src transforming activity.

The Src kinase phosphorylates itself at specific tyrosine residues. These modifications regulate its enzymatic activity: phosphorylation of Y416 in the kinase domain activates the enzyme, whereas phosphorylation of Y527 in the C-terminal segment inhibits activity. The crystal structures of cellular Src and another member of the Src family revealed the importance of the SH2 and SH3 domains in such regulation. For example, exchange of the intramolecular interaction of SH2 with Y527 for binding of the SH2 domains to phosphotyrosine-containing motifs in **other** proteins initiates conformational changes that activate the kinase (Fig. 6.11B). This autoregulatory mechanism explains earlier findings that transduction and overproduction of the normal Src protein do not lead to cellular transformation, and that the constitutive oncogenic activity of v-*src* requires loss or mutation of the Y527 codon.

Soon after its kinase activity was first discovered, v-Src was shown to localize to focal adhesions, the areas where cells make contact with the extracellular matrix. This observation led to identification of a second protein tyrosine kinase enriched in these areas as a protein that exhibits increased tyrosine phosphorylation in v-Src-transformed cells. This focal adhesion kinase (Fak) and Src family proteins turned out to be crucial components of a signal transduction cascade (normally controlled by cell adhesion) that modulates the properties of the actin cytoskeleton, and hence cell shape and adhesion. It also signals to the Ras/Map kinase pathway that controls cell proliferation (Fig. 6.12). The constitutive activity of v-Src can therefore account for the morphological and growth properties of cells transformed by this oncogene product.

Other transduced oncogenes. The transduced oncogenes of retroviruses are homologs of cellular genes that encode many components of signal transduction cascades, from the external signaling molecules (e.g., v-Sis) and their receptors (v-ErbB and v-Kit) to the nuclear proteins at the end of the relay (v-Fos and v-Myc) (Table 6.5). It therefore seems likely that any positively acting protein in such a cascade has the potential to act as a transforming protein. The oncogenic potential of such transduced oncogenes is realized by two nonexclusive mechanisms: genetic alterations that lead to constitutive protein activity and inappropriate production, or overproduction, of the protein. The former mechanism applies to most of the retroviral oncogenes (Table 6.5). For example, like other small, guanine nucleotide-binding proteins, Ras normally cycles between a conformation that is active (GTP bound) and one that is inactive (GDP bound).



See link: http://www.rcsb.org/pdb/explore/jmol.do?structureId=2SRC&bionumber=1

Figure 6.11 Organization and regulation of the c-Src tyrosine kinase. (A) The functional domains of the protein. The SH4 domain contains the site for addition of the myristate chain that anchors the protein in the cell membrane. The SH2 and SH3 domains mediate protein-protein interactions by binding to phosphotyrosine-containing and proline-rich sequences, respectively. Both domains are found in other proteins that participate in signal transduction pathways. One transforming protein, Crk, is made up of only an SH2 and an SH3 domain: it functions as an adapter, bringing together other proteins in a signal transduction pathway (Table 6.5). Arrows represent intramolecular interactions observed in the repressed-state crystal structures of Src. **(B)** The interactions and their reversal. When Y527 is phosphorylated, the C-terminal region of c-Src in which this residue lies is bound to the SH2 domain. This interaction brings a polyproline helix located between the SH2 and SH1 domains into contact with the SH3 domain, as illustrated at the top (see http://www.rcsb.org/pdb/explore/explore.do?structureId=2src). Binding of SH3 to the helix deforms the kinase domain, accounting for the inactivity of the Y527-phosphorylated form of the protein. Such intramolecular associations maintain the kinase domain (SH1) in an inactive conformation. A conformational change that activates the kinase can be induced as shown, as well as by binding of the SH3 domain to proline-rich sequences in other proteins and probably by dephosphorylation of Y527 (see Fig. 6.16). Once released from the autoinhibited state in this way, Y416 in the kinase domain is autophosphorylated, a modification that stabilizes the active conformation of the SH1 domain. The v-Src protein is not subject to such autoinhibition, because the sequence encoding the C-terminal regulatory region of c-Src was deleted during transduction of the cellular gene.

Such cycling is under the control of GTPase-activating and guanine nucleotide exchange proteins. The latter proteins (e.g., Sos [Fig. 6.3]) stimulate the release of GDP once bound GTP has been hydrolyzed. However, v-Ras proteins fail to hydrolyze GTP efficiently and therefore persist in the active, GTP-bound conformation that relays signals to downstream pathways, such as the Map kinase cascade (Fig. 6.3). Such constitutive activity is the result of mutations that lead to substitution of specific, single amino acids in the protein (at residues 12, 13, or 61) and render the protein refractory to the GTPase-activating protein. Analogous mutations are common in certain human tumors, such as colorectal cancers (Box 6.2), and were the first discrete genetic changes in a proto-oncogene linked to neoplastic disease in humans.

Less commonly, over- or misexpression of the transduced oncogene is sufficient to disrupt normal cell behavior. This type of mechanism is best characterized for *myc*. In normal cells, the expression of this gene is tightly regulated, such that the c-Myc protein is made only during a short period in the G_1 phase of the cell cycle, and is not synthesized when cells withdraw from the cycle or differentiate. The production of even small quantities of Myc or Myc-fusion proteins specified by retroviruses, such as the avian myelocytoma virus MH2 (Fig. 6.8), at an inappropriate time results in cellular transformation.

Viral Homologs of Cellular Genes

The genomes of some larger DNA viruses also contain coding sequences that are clearly related to cellular genes that encode signal transduction molecules. Human herpesvirus 8, a gammaherpesvirus related to Epstein-Barr virus, has been strongly implicated in the etiology of Kaposi's sarcoma, a malignancy common in acquired immunodeficiency syndrome (AIDS) patients, and primary effusion lymphoma (see Chapter 7). Its structural proteins and viral enzymes are closely related to those of other herpesviruses. The genome also contains several homologs of cellular genes that encode signaling proteins, which are clustered in regions interspersed among blocks of genes common to all

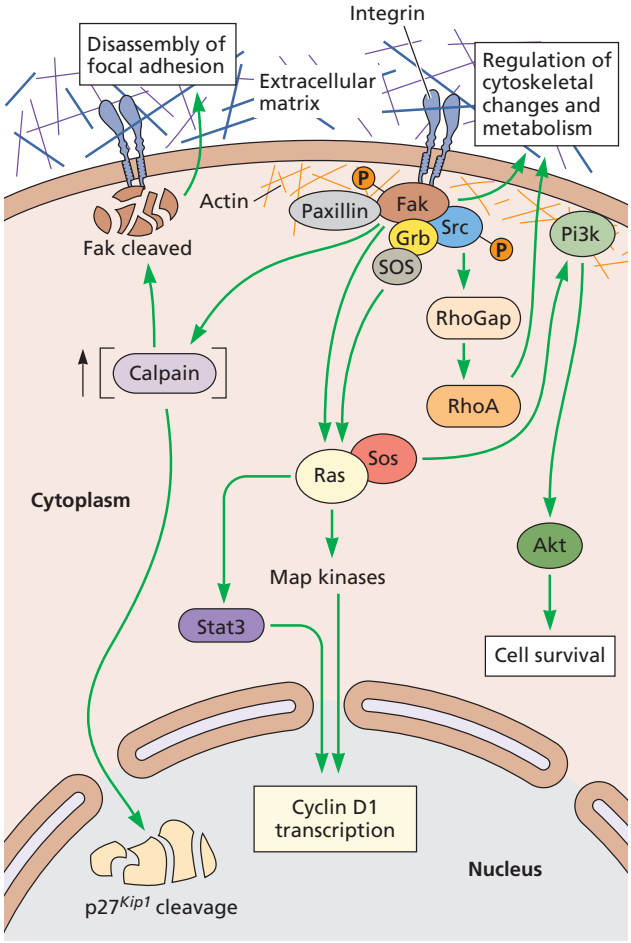


Figure 6.12 Regulation of cell proliferation and adhesion by Src. Both c-Src and v-Src are localized to focal adhesions, where they are associated with focal adhesion kinase (Fak) and adapter proteins, such as Grb2 and paxillin. These protein assemblies normally maintain contacts between the extracellular matrix via integrins and the actin cytoskeleton of the cell. When the Src tyrosine kinase is active, Fak is phosphorylated at specific sites and cleaved into several fragments by the protease calpain. These changes result in disruption of focal adhesions and account for the changes in morphology and motility of v-Src-transformed cells. Calpain-mediated proteolysis of Fak results, at least in part, from increased translation of calpain mRNA induced by v-Src. Another substrate of this protease is the cyclin-dependent kinase inhibitor $p27^{Kip1}$. As shown, v-Src also induces transcription of genes, including the cyclin D1 gene, via Ras and the Map kinase cascade and the transcriptional activator Stat3. These responses to v-Src result in cell proliferation.

herpesviruses (Fig. 6.13). Among the best characterized is the v-*gpcr* gene, which specifies a guanine nucleotide-binding protein-coupled receptor that is most closely related to a cellular receptor for CXC chemokines. The v-*gpcr* gene induces morphological transformation when introduced into mouse fibroblasts or endothelial cells in culture, and formation of tumors that resemble Kaposi's sarcoma in transgenic mice. Cellular chemokine receptors bind chemokines released at sites of inflammation to activate signal transduction. In contrast, v-Gpcr is fully active in the absence of any ligand and can trigger signaling via several cellular pathways to promote cell survival (phosphatidylinositol 3-kinase [Pi3k]/protein kinase B [Akt]) and to activate transcription of cellular genes (via activator protein 1 [Ap-1] and nuclear factor κb [Nf-κb]) (Fig. 6.13B). These genes include several that encode secreted cytokines and growth factors, such as interleukin (IL)-6 and vascular endothelial growth factor (Vegf). These secreted proteins and viral orthologs (for example, vIL-6) are thought to cooperate to induce sustained proliferation of latently infected cells and angiogenesis, the proliferation of new blood vessels (Fig. 6.13B). This characteristic feature of Kaposi's sarcoma is essential for tumor progression.

Alteration of the Production or Activity of Cellular Signal Transduction Proteins

Insertional Activation by Nontransducing Retroviruses

Most tumors induced by nontransducing retroviruses arise as a result of increased transcription of cellular genes located in the vicinity of integrated proviruses. This mechanism of oncogenesis is known as **insertional activation**. It has been implicated in a leukemia-like disease developed by patients participating in a gene therapy trial (Box 6.9). As in the case of the transducing retroviruses, Rous sarcoma-derived avian viruses played a seminal role in delineating the mechanisms of insertional activation. The original stocks of viruses isolated by Peyton Rous included replication-competent leukosis viruses. These viruses do not carry an oncogene, but in young chickens they induce B cell tumors that originate in the bursa of Fabricius, the major lymphoid organ of these birds. A provirus was found integrated in the vicinity of the cellular *myc* gene in each of these tumors. Although the exact integration site varied from tumor to tumor, many integration sites lay in the intron between exon 1 (a noncoding exon) and exon 2 (Fig. 6.14). However, in some tumors proviruses were located upstream or downstream of the cellular *myc* gene. In this avian system, inappropriate synthesis of the normal cellular Myc protein is associated with lymphomagenesis; no changes in the protein are required. Analysis of the sites of proviral DNA integration and the gene products formed in these tumors provided the first evidence for two types of insertional activation: promoter insertion and enhancer insertion (Fig. 6.15).

The first mechanism, **promoter insertion**, results in production of a chimeric RNA in which sequences transcribed from the proviral LTR are linked to cellular proto-oncogene sequences. If transcription originates from the left-end LTR, some viral coding sequences may be included. However, transcription from the right-end LTR seems to be more common, and in these cases the proviral left-end LTR has usually been deleted. Proviral integration often occurs within the cellular

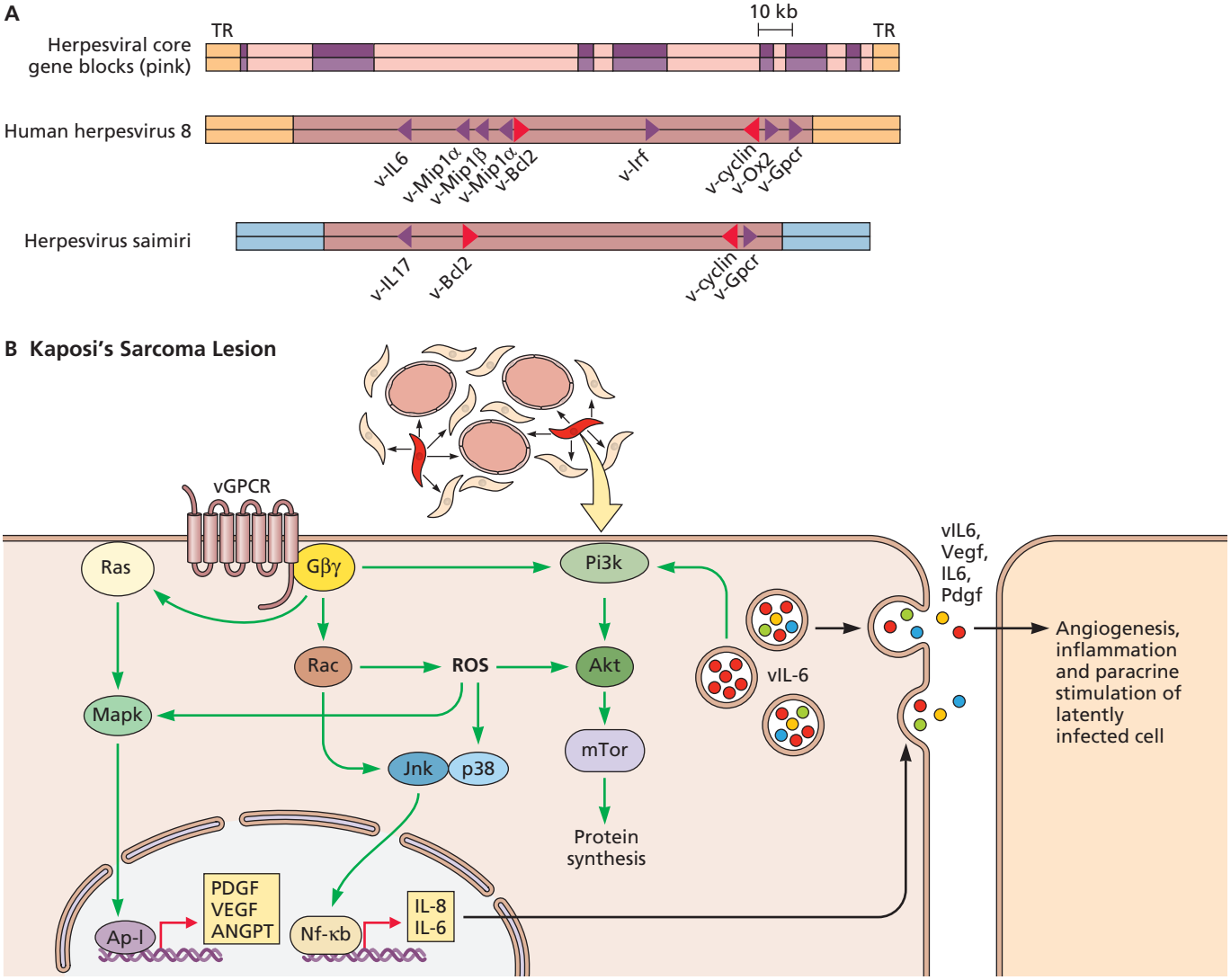


Figure 6.13 Model of paracrine oncogenesis by human herpesvirus 8 gene products. (A) The genomes of human herpesvirus 8 and herpesvirus saimiri contain homologs of various cellular genes. The two viral genomes are shown in orientations that align genes conserved among herpesviruses, the core gene blocks shown at the top. The conserved genes encode proteins needed for virus reproduction and assembly. Homologs of cellular genes (arrowheads) are interspersed among the core gene blocks. Those shown in purple are related to cellular chemokines (v-IL-6, v-IL-17, and macrophage inflammatory factor [v-Mip1α or 1β]), chemokine receptors (v-Gpcr; see the text), or other signaling molecules (interferon-responsive protein [v-Irf] and an N-Cam family transmembrane protein that participates in intercellular signaling [v-Ox2]). The human herpesvirus 8 v-IL-6 protein blocks the action of interferon and can also induce proliferation of B cells. Viral genes shown in red are related to cellular genes that encode proteins that regulate cell proliferation or apoptosis, cyclin D [v-cyclin; see the text] and Bcl-2 [v-Bcl2]. **(B)** As indicated at the top, Kaposi's sarcomas contain human herpesvirus 8-infected cells in which viral lytic genes are expressed (red), as well as latently infected cells (tan) and blood vessels. The former cells produce vGPCR, the product of an early gene, as well as vIL-6, but the latter do not. Rather, latently infected cells synthesize viral proteins that promote survival (vFLIP) or proliferation (v-cyclin). As lytic infection is cytotoxic, a paracrine model for oncogenesis has been proposed. In this model (below), vGPCR made in lytically infected cells triggers signaling via Ras and the β and γ subunits of a trimeric G protein (Gβγ) via Mapk, Pi3k, and Jnk (cJun N-terminal kinase) pathways to stimulate expression of cellular genes that encode cytokines (IL-6, IL-8) and angiogenic growth factors (Vegf and platelet-derived growth factor [Pdgf]). In cooperation with vIL-6 (and other virokines), which are also secreted from lytically infected cells, these cellular proteins act upon neighboring cells (paracrine stimulation) to maintain proliferation of latently infected cells and induce angiogenesis. This model is consistent with the increased incidence of Kaposi's sarcoma in immunosuppressed patients, when lytically infected cells cannot be removed by T cells, and the finding that antiviral drugs that inhibit human herpesvirus 8 reproduction can prevent development of Kaposi's sarcomas in AIDS patients.

BOX 6.9

WARNING

Inadvertent insertional activation of a cellular gene during gene transfer

Retroviruses have long been considered likely to be valuable vectors for gene therapy. One reason is that integration of the retroviral vector into the host genome results in permanent delivery of the potentially therapeutic gene to all infected cells and their descendants. However, an outcome detected in one of the first clinical trials indicates that this property is a double-edged sword.

A French trial was examining the potential of gene therapy using a vector based on mouse Moloney leukemia virus to treat children with severe combined immunodeficiency (SCID). This disease is caused by mutation in a single gene on the X chromosome, and the only therapies available are associated with severe, often fatal, side effects. A trial with 10 children with the disease, who were given gene transfer as early as possible, initially appeared to be very successful: in most cases, the immune system was restored without side effects. But, early in 2002, one patient was found to have developed a T cell leukemia-like disease. The overproliferating T cells were monoclonal: all carried a provirus integrated into the same site on chromosome 11, near a gene (*Lmo2*) that is expressed abnormally in a form of childhood acute lymphoblastic leukemia.

The monoclonal origin of the T cells that proliferated in this child indicates that proviral insertion contributed to the development of the disease. It initially seemed likely that other factors also did so: a predisposition to childhood cancers was evident in other members of the child's family. However, other children participating in the same trial or a similar trial in the United Kingdom were later diagnosed with leukemia associated with insertion of the provirus in the same chromosome 11 site (see the figure). This unfortunate outcome temporarily halted these and numerous other clinical trials of gene transfer using retroviral vectors in the United States and Europe.

Subsequent follow-up studies showed that while acute leukemia developed in four patients, three were treated successfully by chemotherapy. Furthermore, seven patients, including three survivors of leukemia, had sustained immune reconstitution: all were able to live in nonprotected environments, controlling microorganisms successfully, and are developing normally. These results demonstrate the therapeutic potential of gene therapy.

Since these first trials, Moloney leukemia virus vectors that lack the enhancers and/or are not targeted to promoters in the cellular genome have been developed. Vectors for gene transfer have also been derived from the lentivirus simian immunodeficiency virus, which shows no preference for integration into promoter regions. When pseudotyped with the envelope G protein from vesicular stomatitis virus, this lentivirus vector infects almost all types of cells. Numerous trials with these vectors are currently ongoing in the United States and elsewhere.

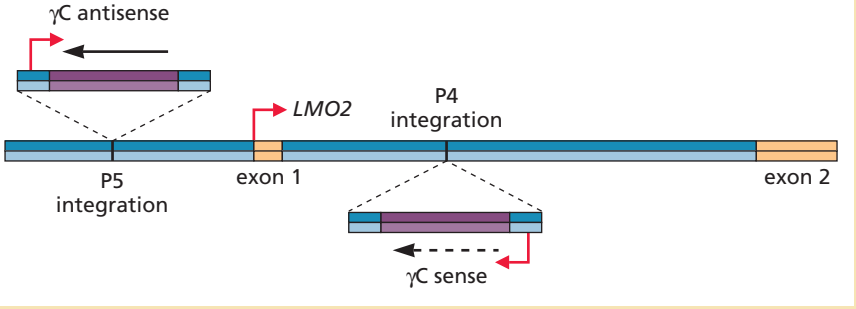
See http://www.genetherapynet.com/clinicaltrialsgov.html for information about gene therapy and viral vectors.

Deichmann A, Hacein-Bey-Abina S, Schmidt M, Garrigue A, Brugman MH, Hu J, Glimm H, Gyapay G, Prum B, Fraser CC, Fischer N, Schwarzwaelder K, Siegler ML, de Ridder D, Pike-Overzet K, Howe SJ, Thrasher AJ, Wagemaker G, Abel U, Staal FJ, Delabesse E, Villeval JL, Aronow B, Hue C, Prinz C, Wissler M, Klanke C, Weissenbach J, Alexander I, Fischer A, von Kalle C, Cavazzana-Calvo M. 2007. Vector integration is nonrandom and clustered and influences the fate of lymphopoiesis in SCID-XI gene therapy. *J Clin Invest* **117:**2225–2232.

Hacein-Bey-Abina S, Hauer J, Lim A, Picard C, Wang GP, Berry CC, Martinache C, Rieux-Laucat F, Latour S, Belohradsky BH, Leiva L, Sorensen R, Debré M, Casanova JL, Blanche S, Durandy A, Bushman FD, Fischer A, Cavazzana-Calvo M. 2010. Efficacy of gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* **363:**355–364.

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Sites of the retroviral vectors for the γ subunit of the interleukin-2 receptor carrying the gene (γC) in the first two children in the French trial who developed T cell leukemia. Both proviruses integrated close to the promoter of the lmo2 gene. Adapted from S. Hacein-Bey-Abina et al., *Science* **302:**415–419, 2003, with permission.



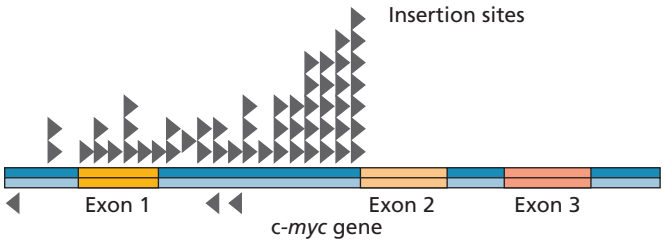


Figure 6.14 Insertional activation of c-*myc* by avian leukosis viruses. In avian cells derived from avian leukosis virus-induced B cell lymphomas, individual proviral integration sites are clustered as shown (arrowheads) within noncoding exon 1 and intron 1 of the *myc* gene. Most integrated proviruses are oriented in the direction of *myc* transcription (arrowheads pointing to the right). Adapted from J. Nevins and P. Vogt, p. 301–343, *in* B. N. Fields et al. (ed.), *Fields Virology*, 3rd ed. (Lippincott-Raven Publishers, Philadelphia, PA, 1996), with permission.

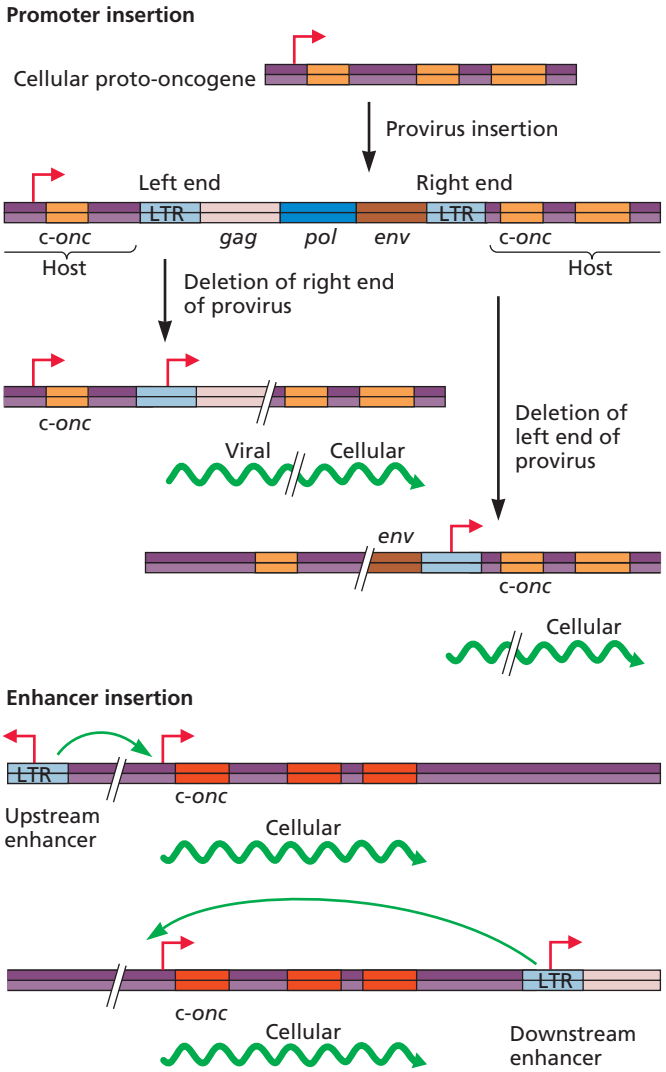


Figure 6.15 Mechanisms for insertional activation by nontransducing oncogenic retroviruses. During promoter insertion (top), the typical deletion of the proviral left-end LTR is probably important, because transcriptional read-through from the left-end promoter reduces transcription from the right-end LTR.

proto-oncogene, truncating cellular coding sequences and eliminating noncoding domains that may include negative regulatory sequences. Some chimeric transcripts formed in this way are analogous to the intermediates that give rise to oncogene capture by the transducing retroviruses (compare Fig. 6.15 and 6.9). Indeed, it has been possible to isolate newly generated, oncogene-transducing retroviruses from tumors arising as a result of promoter insertion.

In the second type of insertional activation, **enhancer insertion**, viral and cellular transcripts are not fused. Instead, activation of the cellular gene is mediated by the strong viral enhancers, which increase transcription from the cellular promoter (Fig. 6.15). Because enhancer activity is independent of orientation and can be exerted over long distances, the provirus need not be oriented in the same direction as the proto-oncogene, and may lie downstream of it.

Viral Proteins That Alter Cellular Signaling Pathways

Some viruses alter the growth and proliferation of infected cells by the action of viral signal transduction proteins that are not obviously related in sequence to cellular proteins. Some of these viral proteins operate by mechanisms well established in studies of cellular signaling cascades, but others function in different ways.

Constitutively active viral "receptors." The genomes of several gammaherpesviruses encode membrane proteins that initiate signal transduction. The best-understood example of this mechanism is provided by Epstein-Barr virus latent membrane protein 1 (LMP-1), one of several viral gene products implicated in immortalization of human B lymphocytes (Table 5.3) and synthesized in the majority of tumors associated with the virus. LMP-1 induces typical transformed phenotypes when synthesized in fibroblasts or epithelial cells in culture and induces lymphomas in transgenic mice. It is an integral plasma membrane protein that functions as a constitutively active receptor. In the absence of any ligand, LMP-1 oligomerizes to form patches in the cellular membrane and binds to the same intracellular adapter proteins as the active, ligand-bound form of members of the tumor necrosis factor receptor family (Fig. 6.16). This viral protein induces release of Nf-κb from association with cytoplasmic inhibitors by multiple mechanisms, and activates a second transcriptional regulator, Ap-1, as well as signaling via the lipid kinase Pi3k and protein kinase Akt (Fig. 6.16). Activation of these pathways accounts for the increased expression of most of the cellular genes that is observed in LMP-1-producing cells, and the alterations in the properties of these cells. These changes include increased production of certain cell adhesion molecules and cell proliferation.

It has long been known that LMP-1 is not synthesized in all cells within tumors associated with Epstein-Barr virus. Remarkably, recent studies suggest that such cells would nevertheless be influenced by LMP-1 secreted in exosomes from cells in which the viral protein is made efficiently, a previously unrecognized form of "bystander effect" (Box 6.10).

Viral adapter proteins. Members of both the *Polyomaviridae* and the *Herpesviridae* encode proteins that permanently activate signal transduction pathways as a result of binding to Src family tyrosine kinases. This mechanism was first encountered in studies of the mouse polyomavirus mT protein, a viral early gene product with no counterpart in the genome of the related polyomavirus simian virus 40 (Fig. 6.17A). This pro-

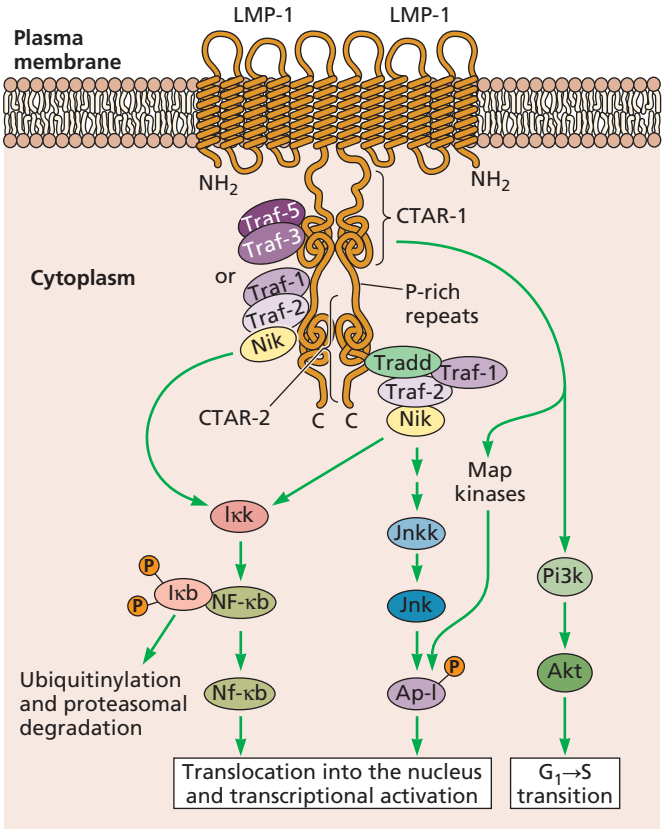


Figure 6.16 Constitutive signaling by Epstein-Barr virus latent membrane protein 1. LMP-1, which possesses six membrane-spanning segments but no large extracellular domain, oligomerizes in the absence of ligand, a property represented by the LMP-1 dimer depicted. When localized to the plasma membrane, the C-terminal segment of LMP-1 to which these proteins bind is sufficient for both immortalization of B cells and activation of cellular transcriptional regulators. The long cytoplasmic C-terminal domain of the viral protein contains three segments implicated in the activation of signaling, designated C-terminal activation regions (CTARs) 1 and 2, and proline (P)-rich repeats. As shown, multiple members of the tumor necrosis factor receptor-associated protein family (Trafs) bind to CTAR-1. Binding of Trafs leads to activation of the protein kinase Nik and Iκ-kinase (Iκk), and ultimately of Nf-κb, via induction of release of Nf-κb from association with its cytoplasmic inhibitors. The same pathway is activated in uninfected cells by binding of tumor necrosis factor to its receptor. The CTAR-2 domain of LMP-1 is responsible for activation of Ap-1 via the Jun N-terminal kinase (Jnk) pathway. The first reaction appears to be indirect association of this region of LMP-1 with Trafs via a second cellular protein (Tradd), a reaction that may lead to activation of both Nf-κb and Ap-1, as shown. The Traf-binding domain of CTAR-1 also induces activation of signaling via Pi3k and the protein kinase Akt, and of the Map kinase cascade. These responses to LMP-1 are required for transformation of rat fibroblasts.

tein can transform established rodent cell lines (Table 6.4) and induce endotheliomas (Box 6.1) when overproduced in transgenic animals.

mT becomes inserted into cellular membranes by means of a C-terminal transmembrane domain, and associates with cellular signaling proteins en route to the plasma membrane via the secretory pathway. An N-terminal mT sequence that is also present in sT (Fig 6.17A) becomes bound to cellular protein phosphatase 2A (Pp2A) in the cytoplasm, an interaction that is necessary for subsequent interaction with c-Src in the endoplasmic reticulum. This requirement ensures that the phosphatase is brought into close association with c-Src. When c-Src is bound to mT, its catalytic activity is increased by an order of magnitude, because autoinhibition of c-Src kinase activity (Fig 6.9) is reversed (Fig 6.17B).

It was initially surprising that mT-transformed cells do not contain elevated levels of phosphotyrosine, despite activation of c-Src family kinases. It is now clear that mT itself is a critical substrate of the cellular enzyme: phosphorylation of specific mT tyrosine residues by activated c-Src allows a number of cellular proteins that contain phosphotyrosine-binding domains to bind to mT (Fig. 6.17B). When bound to mT, these signaling proteins are phosphorylated by the activated c-Src kinase to trigger signal transduction, for example, by activation of Ras and the Map kinase pathway. Consequently, mT both bypasses the normal mechanism by which the kinase activity of c-Src is regulated, and also serves as a virus-specific adapter, bringing together cellular signal transduction proteins when they would not normally be associated.

Alteration of the Activities of Cellular Signal Transduction Molecules

Activation of plasma membrane receptors. Many signal transduction cascades are initiated by binding of external growth factors to the extracellular portions of cell surface receptor tyrosine kinases. Ligand-bound receptors are internalized rapidly (within 10 to 15 min) by endocytosis. Following acidification of the endosomes, the ligand is released and all but a small fraction of the receptor molecules are usually degraded. As a result, the initial signal is short-lived. The E5 protein of papillomaviruses that cause fibropapillomas, such as bovine papillomavirus type 1, interferes with the mechanisms that control the function of this class of receptor.

This E5 protein, a hydrophobic molecule of only 44 amino acids, efficiently transforms mammalian fibroblasts in culture in the absence of any other viral proteins (Table 6.4). This activity depends on binding to platelet-derived growth factor receptor β (Pdgfr-β). The E5 protein is a dimer that accumulates in host cell membranes, where it induces ligand-independent dimerization of the receptor, and hence activation of its tyrosine kinase and downstream signaling relays (Fig. 6.3). The E5 protein binds stably and with high specificity to the transmembrane domain and an adjacent internal segment of the receptor, in contrast to the natural ligand, which binds to the extracellular domain. This mechanism is likely to be important in the oncogenicity of the virus in its natural hosts: in bovine tumors, the E5 protein

BOX 6.10

DISCUSSION

Transformation by remote control?

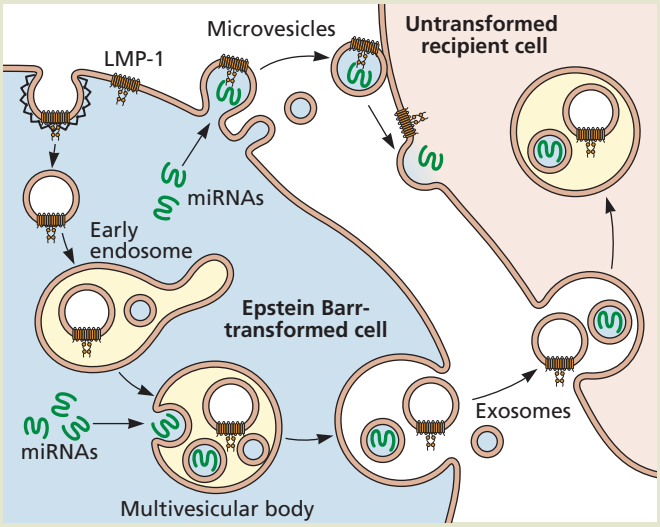
Epstein-Barr virus contributes to the development of several cancers of B lymphocytes and epithelial cells, including nasopharyngeal carcinoma. The viral genome is present in all such tumors, which are monoclonal in origin, but expression of the gene that encodes the transforming LMP-1 protein is variable and often difficult to detect in tumor samples. Nevertheless, this viral protein may stimulate the proliferation of infected cells in which it is not made by an unusual mechanism, transfer from cells that **do** produce LMP-1 via exosomes.

Exosomes are small (40 to 100 nm in diameter) vesicles that are secreted by many types of cell and permit intercellular communication. They form initially as intraluminal vesicles by inward budding of the membranes of multivesicular bodies, in which they accumulate prior to release by fusion of these bodies with the plasma membrane (see the figure). Exosomes have been implicated in several normal processes, including antigen presentation, maturation of sperm, and communication among neurons, as well as in transformation and tumorigenesis. They are thought to exert their effects by both interaction with target cells and direct transfer of cargo into cells following fusion with the plasma membrane. Exosomes can transfer not only numerous soluble and membrane proteins but also RNAs (mRNAs and miRNAs) from one cell to another.

Exosomes carrying the viral LMP-1 protein in their membranes are secreted from Epstein-Barr virus-transformed epithelial and B cells in culture, and have been observed in sera from nasopharyngeal carcinoma patients. The mechanism by which LMP-1, which normally resides in the plasma membrane, is recruited to exosomes is not well understood. However, the fusion of such exosomes with uninfected cells has been reported to stimulate signal transduction pathways that promote cell proliferation and survival, for example, signaling via Map kinases and Akt. Furthermore, these LMP-1-containing vesicles appear to be enriched in other signaling molecules and to contain viral miRNAs. These properties suggest that intercellular transfer of LMP-1 (and perhaps other molecules) via exosomes could contribute to viral transformation and tumorigenesis.

Meckes DG, Jr, Shair KH, Marquitz AR, Kung CP, Edwards RH, Raab-Traub N. 2010. Human tumor virus utilizes exosomes for intercellular communication. *Proc Natl Acad Sci U S A* **107:**20370–20375.

Exosomes formed in Epstein-Barr virus-infected cells carry the viral LMP-1 protein and have the potential to transfer this protein, and internal cargo such as viral miRNAs, to recipient cells.



and Pdgfr-β are colocalized, and both the receptor and downstream signaling pathways are activated.

Nontransducing retroviruses can also activate cell surface receptors, because these cellular gene products may be altered by provirus integration. In certain chicken lines, Rous-associated virus 1 induces erythroblastosis instead of lymphomas (Box 6.1). These tumors contain intact, nondefective proviruses integrated in the cellular *erbB* gene, which encodes the cell surface receptor for epidermal growth factor. The proviral integrations are clustered in a region that encodes the extracellular portion of this receptor, and readthrough transcription produces chimeric RNAs (Fig. 6.15). The proteins synthesized from these RNAs are truncated growth factor receptors that lack the ligand-binding domain and produce a constitutive mitogenic signal. The v-*erbB* gene captured by transducing retroviruses encodes a protein with a similar truncation.

Inhibition of protein phosphatase 2A. In preceding sections, we have discussed transformation by viral gene products as a result of permanent or prolonged activation of signal transduction pathways that control cell proliferation. In normal cells, such signaling is a transient process, because the molecular components are reset once they have transmitted the signal. Inhibition of the reactions that terminate signaling therefore can also contribute to transformation, a mechanism

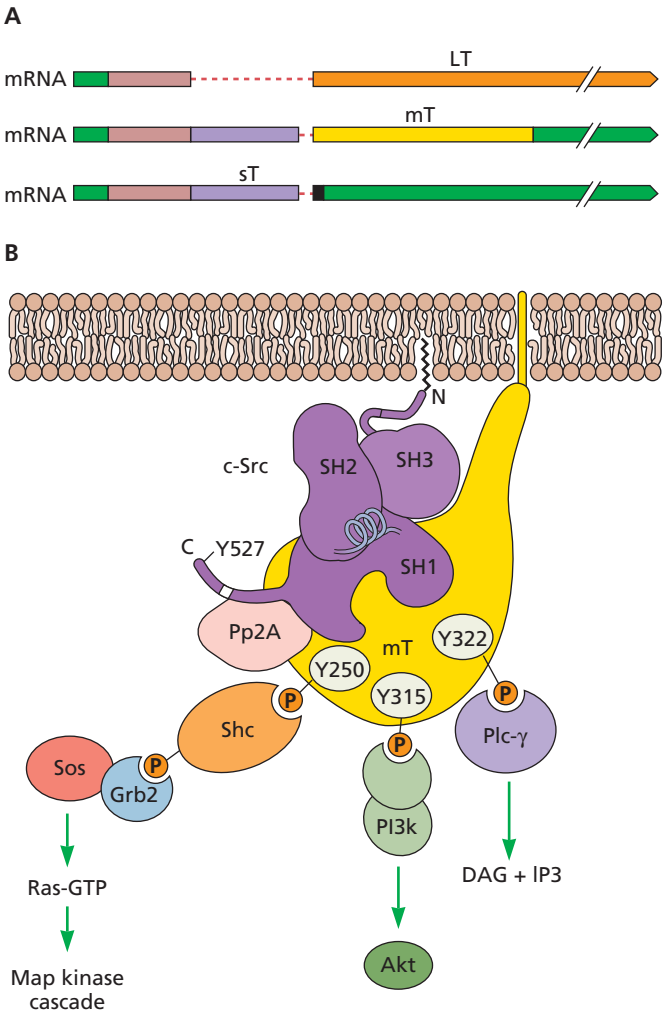


Figure 6.17 Polyomavirus mT protein, a virus-specific adapter. **(A)** The mouse polyomavirus early protein-coding sequences are shown as boxes within the mRNAs from which the proteins are synthesized. The mRNAs are drawn as arrows, in which the arrowheads indicate the site of polyadenylation and the dashed pink lines indicate the introns removed during RNA splicing. The three proteins produced from these mRNAs, LT, mT, and sT, share an N-terminal sequence but carry unique C-terminal sequences as a result of alternative splicing of early transcripts. **(B)** mT binds to c-Src (or the related tyrosine kinases c-Yes or c-Fyn) at cellular membranes and to Pp2A). As a result of formation of the ternary complex, c-Src is trapped in the active conformation and Y527 is unphosphorylated: mT sequesters the Y527-containing segment of c-Src for dephosphorylation of the tyrosine residue by Pp2A, thereby stabilizing the active conformation of the enzyme. Consequently, mT-bound Src is catalytically active and phosphorylates specific tyrosines in mT. These phosphorylated residues are then bound by cellular proteins that contain phosphotyrosine-binding motifs, such as Shc, phospholipase C-γ (Plc-γ) (an enzyme that catalyzes synthesis of lipid second messengers), and Pi3k. These proteins can then be phosphorylated by Src and activated. The lipids produced upon activation of Plc-γ act as second messengers, relaying signals to various pathways, while Pi3k activates signaling via the protein kinase Akt. In all cases, substitutions that disrupt binding of the cellular protein to mT impair the transforming activity of the viral protein.

exemplified by the sT protein of polyomaviruses such as simian virus 40.

This protein is not necessary for transformation of many cell types, but can stimulate transformation by simian virus 40 LT and is required for the transformation of resting cells. In both infected and transformed cells, the sT protein binds to protein phosphatase 2A, a widespread, abundant serine/threonine protein phosphatase. This protein is a heterotrimer, composed of a core enzyme comprising a scaffolding and a catalytic subunit bound to one of a substantial number of regulatory subunits. sT binds via two domains to the scaffolding subunit of the core enzyme to block access of substrates to the active site in the catalytic subunit and binding of regulatory subunits (Fig. 6.18A). One important consequence of sT binding is failure of the phosphatase to inactivate Map kinases, a process normally accomplished by the dephosphorylation of serine/threonine or tyrosine residues (Fig. 6.18B). Consequently, sT increases the activity of sequence-specific transcriptional activators that are substrates of Map kinases. The increased activities of these transcriptional stimulators lead to synthesis of G_1-phase and S-phase cyclins, thereby circumventing the need for growth factors or other mitogens during transformation by simian virus 40.

Disruption of Cell Cycle Control Pathways by Viral Transforming Proteins

One end point of many signal transduction pathways is the transcription of genes coding for proteins that regulate cell cycle progression and the metabolic activity of the cell. Consequently, **permanent activation** of such pathways by viral proteins, by any of the mechanisms described in the previous section, can result in an increased rate of cell growth and division or in proliferation of cells that would normally be in the resting state. Other viral proteins intervene directly in the intricate circuits by which cell cycle progression is mediated and regulated.

Abrogation of Restriction Point Control Exerted by the Rb Protein

The Restriction Point in Mammalian Cells

In mammalian cells, passage through G_1 into S and reentry into the cell cycle from G_0 depend on extracellular signals that regulate proliferation, termed **mitogens**. Late in G_1, cells that respond to such external cues become committed to enter S and to divide and complete the cell cycle; during this period, they are refractory to mitogens. Cells that have entered this state are said to have passed the G_1 **restriction point** (Fig. 6.6). Normal cells respond to mitogenic signals by mobilization of the G_1 Cdks that contain D-type cyclins. Expression of genes that encode one or more of these cyclins is induced by such signals via the Ras/Map kinase pathways

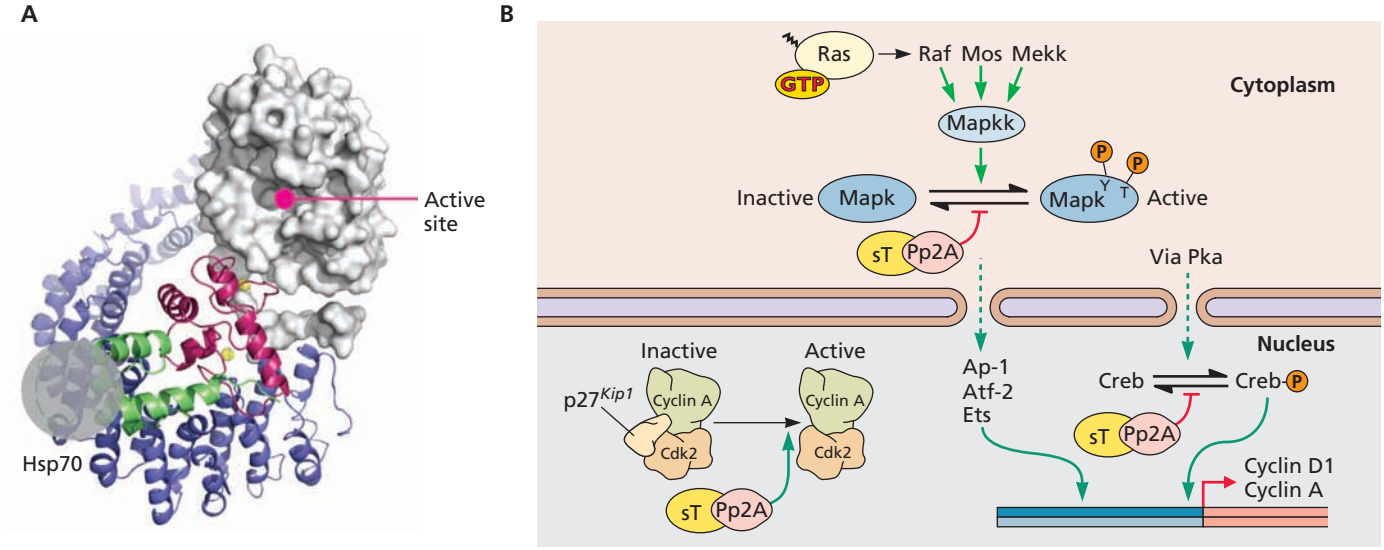


Figure 6.18 Inhibition of protein phosphatase 2A by simian virus 40 small T antigen. (A) Model of small T antigen bound to the core enzyme, which comprises a catalytic (gray, shown as a surface model) and a scaffolding (blue) subunit, with the small T J and unique C-terminal domains shown in green and magenta, respectively. This model was derived by superimposing X-ray crystal structures of a complete Pp2A (scaffolding, regulatory, and catalytic subunits) and of the sT-scaffolding subunit complex. The viral protein binds to the scaffolding subunit in place of the regulatory subunit and likely makes contact with the catalytic subunit via its J domain, which is necessary for efficient inhibition of the catalytic activity of Pp2A. Adapted from U. S. Cho et al., *PLoS Biol* **5:**e202, 2007, with permission. Courtesy of W. Xu, University of Washington, Seattle. **(B)** Inhibition of the activity of Pp2A by sT results in activation of cellular transcriptional regulators via the Map kinase pathway (e.g., activator protein 1 [Ap-1] and activating transcription factor 2 [Atf-2]). In addition, dephosphorylation of activated cAMP response element binding protein (Creb) within the nucleus is inhibited. Production of sT within cells stimulates cyclin D1 and cyclin A transcription. Binding of sT to Pp2A also induces a large increase in the activity of cyclin A-dependent Cdk2, concomitant with inhibition of dephosphorylation of the cyclin-Cdk inhibitor $p27^{Kip1}$ (Fig. 6.6) and degradation of this protein.

(Fig. 6.19A). When such stimulation is continuous, Cdk activity appears at mid-G_1 and increases to a maximum near the G_1-to-S-phase transition. Such activity must be maintained until the restriction point has been passed, but then becomes dispensable. This property implies that the kinase activity of the cyclin D-dependent Cdks is necessary for exit from G_1. The best-characterized substrates of these kinases are the Rb protein and the related p107 and p130 proteins. The Rb protein controls the activity of members of the E2f family of sequence-specific transcriptional regulators (described in Volume I, Chapter 8).

Hypophosphorylated Rb present at the beginning of G_1 binds to specific members of the E2f family. These complexes inhibit transcription of E2f-responsive genes (Fig. 6.19B). The Rb protein is phosphorylated at numerous sites by G_1 cyclin-Cdks. Phosphorylated Rb can no longer bind to E2f, which therefore becomes available to activate transcription from E2f-responsive promoters. These promoters include those of the genes encoding the kinase Cdk2, the cyclins that associate with this kinase, and E2f proteins themselves. The initial release of E2fs from association with Rb therefore triggers a positive feedback loop that augments both Rb phosphorylation and release of E2fs. The result is a rapid increase in the concentrations of E2fs and cyclin E-Cdk2. In this way, cell cycle progression becomes independent of the mitogens necessary for the production of cyclin D-Cdks. These regulatory circuits account well for passage through the restriction point and commitment of a cell to divide. Nevertheless, there is accumulating evidence for functional redundancy among the Rb family proteins and mechanisms for detecting mitogenic stimuli that do not operate via D-type cyclins.

The E2f proteins that accumulate upon Rb phosphorylation also stimulate transcription of genes that encode proteins needed for DNA synthesis (Volume I, Chapter 9), allowing genome replication to take place in S phase. The cyclin A-Cdk2 produced in response to E2f phosphorylates and thereby inhibits the ubiquitin ligase that marks cyclin B for proteasomal degradation throughout much of the cell cycle (Fig. 6.19B). Consequently, cyclin B, which is required for entry into mitosis, accumulates as S phase progresses. Phosphorylation of the Rb protein therefore ensures not only passage through the restriction point and entry into S phase, but also the coordination of these processes with later

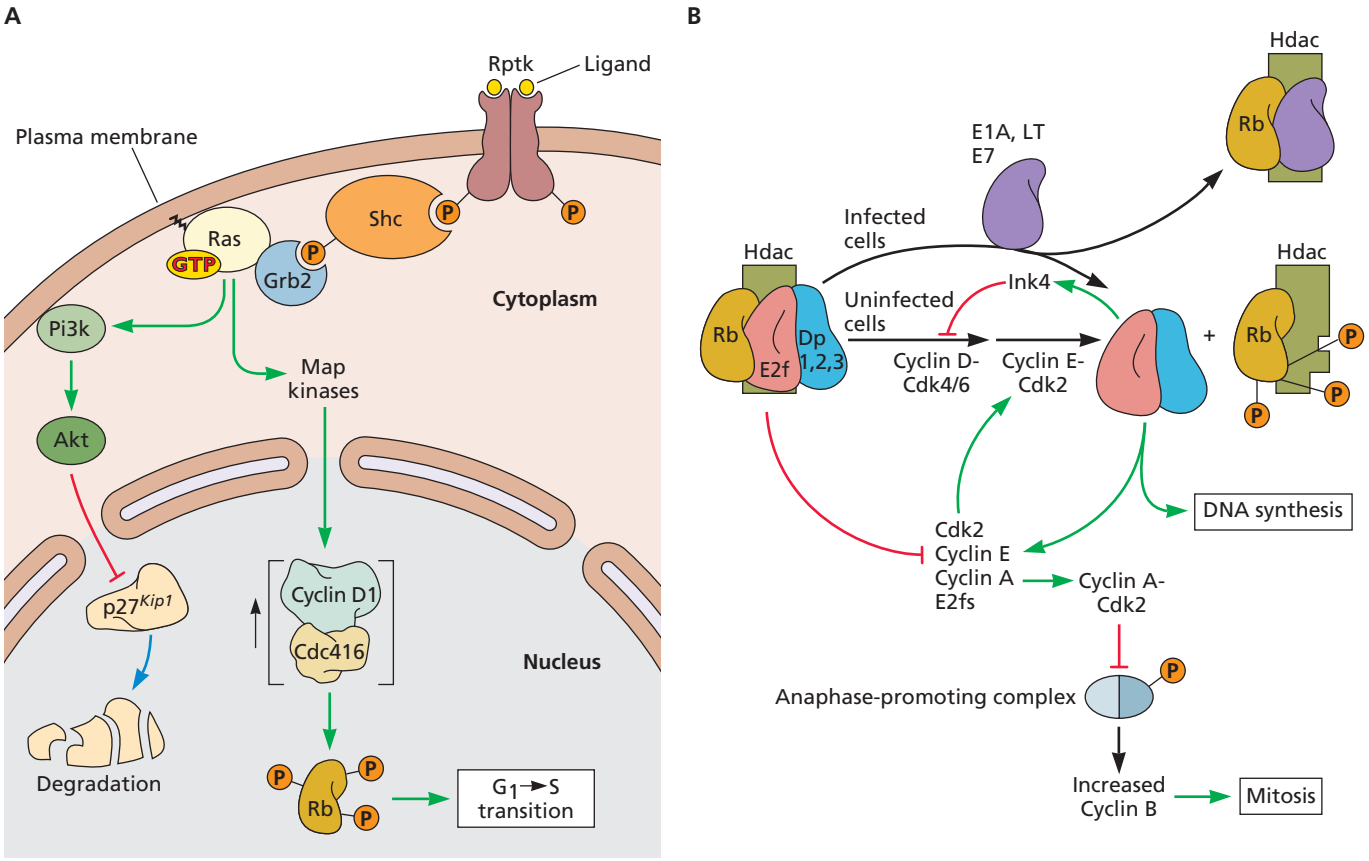


Figure 6.19 Passage through the restriction point in mammalian cells. (A) Mitogenic activation of cell cycle progression is initiated by binding of a growth factor to its cognate receptor protein (Rptk). Signaling via Ras and Map kinase cascades leads to increased transcription of the cyclin D1 gene and accumulation of cyclin D1-Cdc4/6 in the nucleus. Activation of this G_1 cyclin is facilitated by the degradation of its inhibitor $p27^{Kip1}$, which is induced by signaling via Pi3k and the protein kinase Akt. The active G1 cyclin phosphorylates the negative regulator of cell cycle progression Rb (and the related p107 and p130 proteins). Many lines of evidence indicate that cyclin D-dependent Cdks initiate the transition through the restriction point by phosphorylation of Rb. For example, inhibition of cyclin D synthesis or function prevents entry into S phase in Rb-containing cells, but this cyclin is not required in Rb-negative cells. **(B)** When cells enter G_1, hypophosphorylated Rb is bound to transcriptional regulators of the E2f family, which are heterodimers of an E2f and a Dp protein. The E2f-Rb complex represses transcription when bound to E2f recognition sites in promoters, because Rb is a transcriptional repressor. This function requires the binding of histone deacetylases (Hdacs). Phosphorylation of Rb by cyclin D- and cyclin E-dependent Cdks disrupts the binding of Rb to E2fs. Rb is phosphorylated at many sites by both cyclin D-Cdk4/6 and cyclin E-Cdk2. The latter cyclin, which appears in mid to late G_1 (Fig. 6.6A), is required for entry into S phase. Its modification of Rb depends on the prior action of cyclin D-Cdk4/6. Free E2f-Dp heterodimers activate transcription from E2f-responsive promoters, including those of the genes encoding cyclins E and A, Cdk2, and E2f proteins themselves, to establish a positive autoregulatory loop. The positive feedback loop for activation of cyclin E-dependent kinases and E2fs late in G_1 is subject to several checks and balances imposed by inhibitory proteins (Fig. 6.6B). These inhibitory proteins therefore must be inactivated or destroyed to allow progression into S phase (see panel A). The synthesis of at least one member of the Ink4 family of cyclin-Cdk inhibitors is also induced in response to free E2f. It is therefore thought that the accumulation of this inhibitor establishes a feedback loop that blocks the activity of the cyclin D-Cdks and hence the ability of cells to respond to mitogens, a characteristic property of cells that have passed the G_1 restriction point. Although E2f proteins are the best-characterized targets of Rb, the latter protein can also bind to numerous other proteins that mediate or stimulate transcription, as well as to regulatory proteins such as the Abl tyrosine kinase (Table 6.5). These interactions can lead to activation or repression of transcription and, at least in some cases, have been implicated in inhibition of cell cycle progression by Rb.

events in the cell cycle. Indeed, the results of genome-wide approaches to identify genes regulated by E2f family members suggest that E2fs contribute broadly to orderly progression through the cell cycle.

Viral Proteins Inhibit Negative Regulation by Rb and Related Proteins

The products of transforming genes of several DNA viruses bypass the sophisticated circuits that impose restriction point control, and hence the dependence on environmental cues for passage into S phase. The adenoviral E1A proteins, simian virus 40 LT, and the E7 proteins of oncogenic human papillomavirus (types 16 and 18) can induce DNA synthesis and cell proliferation. All three viral proteins make contacts with the two noncontiguous regions by which hypophosphorylated Rb associates with E2f family members (regions A and B in Fig. 6.20A) to disrupt Rb-E2f complexes. As a result, they induce transcription of E2f-dependent genes and inappropriate entry of cells into S phase (Fig. 6.19B), when cellular proteins needed for replication and transcription of viral genomes are synthesized.

The adenoviral E1A, papillomaviral E7, and simian virus 40 LT proteins share a sequence motif necessary for binding Rb (Fig. 6.20A). Nevertheless, they induce removal of Rb from its association with E2f by different mechanisms. The E1A CR1 segment (Fig. 6.21) is structurally similar to the Rb-binding site of E2Fs, and the viral protein competes efficiently for Rb. In contrast, dismantling of Rb-E2f complexes by LT appears to be an active process: the N-terminal J domain of LT, which recruits the cellular, ATP-dependent chaperone Hsc70, and ATP are also required (Fig. 6.20B). The E7 proteins interact with not only Rb but also a cellular cullin 2-containing E3 ubiquitin ligase to target Rb for degradation by the proteasome.

The Rb protein is the founding member of a small family of related gene products, which includes the proteins p107 and p130. The latter two proteins were discovered by virtue of their interaction with adenoviral E1A proteins (Fig. 6.21), but they also bind to both LT and E7 proteins. The Rb, p107, and p130 proteins bind preferentially to different members of the E2f family during different phases of the cell cycle. For example, hypophosphorylated Rb binds primarily to E2f-1, E2f-2, or E2f-3 during the G_0 and G_1 phases, and p130 binds E2f-4 and E2f-5 in G_0. Binding of p130 to these E2f family members appears to be critical for maintaining cells in the quiescent state, and such complexes predominate in mammalian cells in G_0. Their disruption by adenoviral, papillomaviral, or polyomaviral transforming proteins is thought to allow such cells to reenter the cycle, in part via stimulation of the transcription of genes encoding both the E2f proteins and the cyclin-dependent kinase (Cdk2) needed for entry into S phase.

Production of Virus-Specific Cyclins

Human herpesvirus 8 and its close relative herpesvirus saimiri encode functional cyclins. The cyclin gene of human herpesvirus 8, designated v-*cyclin*, has 31% identity and 53% similarity to the human gene that encodes cyclin D2. Its product binds predominantly to and activates Cdk6, which then phosphorylates the Rb protein. The viral cyclin also alters the substrate specificity of the kinase: the v-*cyclin*–Cdk6 complex phosphorylates proteins normally recognized by cyclin-bound Cdk2, but not by cyclin D-Cdk6. These targets include the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and the replication proteins Cdc6 and Orc1 (see Volume I, Fig. 9.25). Furthermore, neither the Cip/Kip nor the Ink4 inhibitors of cell cycle progression and cyclin-Cdks (Fig. 6.6) bind well to the v-cyclin. Synthesis of the viral cyclin can therefore overcome the G_1 arrest imposed when either type of inhibitory protein is made, and can induce cell cycle progression in quiescent cells and initiation of DNA replication. The specific advantages conferred by production of the viral cyclin during the infectious cycle have not been identified. However, it would be surprising if v-cyclin does not contribute to the oncogenicity of these herpesviruses in their natural hosts, as synthesis of this protein in B cells of transgenic mice results in B cell lymphoma.

The epsilonretroviruses encode an accessory gene (*orf a*), which specifies a protein with a cyclin fold called rv-cyclin (Box 6.4). The best studied rv-cyclin, that of walleye dermal sarcoma virus, includes a cyclin box and a C-terminal transcriptional activation domain. The viral protein accumulates in the nucleus of infected cells, and its location and physical association with transcriptional regulators are consistent with a function in transcriptional control. The viral rv-cyclin can promote cell cycle progression when produced in G_1 cyclin-deficient yeast cells. This property, and interaction of rv-cyclin with Cdk3, suggests that this viral protein functions as an ortholog of cellular cyclin C to promote proliferation and oncogenesis in the piscine host, but the mechanistic details have yet to be elucidated.

Inactivation of Cyclin-Dependent Kinase Inhibitors

The production of viral cyclins in infected cells appears to be a property of only certain herpesviruses and the epsilonretroviruses, but other DNA viruses encode proteins that facilitate cell cycle progression by inactivating specific inhibitors of Cdks (Fig. 6.22). One example is the E7 protein of human papillomavirus type 16, which binds to the $p21^{Cip1}$ protein and inactivates it. This member of the cellular Cip/Kip family inhibits G_1 cyclin-Cdk complexes (Fig. 6.6). The increase in intranuclear concentrations of p53 triggered by unscheduled inactivation of the Rb protein (see next section) results in accumulation of $p21^{Cip1}$. The ability of the E7 protein to inactivate both Rb and $p21^{Cip1}$ is necessary to induce differentiated human epithelial cells to enter S phase.

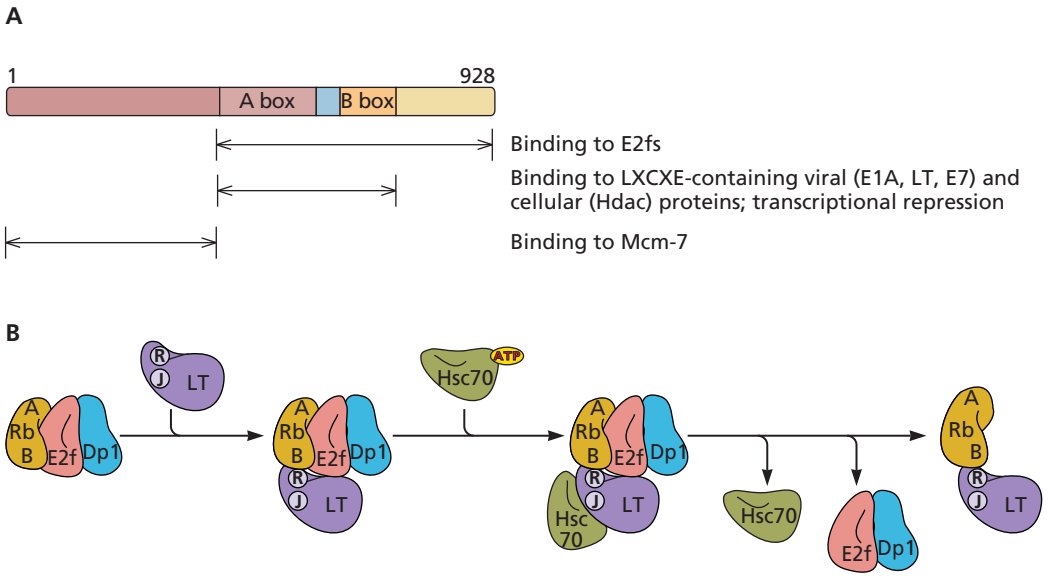


Figure 6.20 Model for active dismantling of the Rb-E2f complex by simian virus 40 LT. (A) Functional domains of the human Rb protein are shown to scale. The A- and B-box regions form the so-called pocket domain, which is necessary for binding of Rb to both E2fs and the viral proteins described in the text. The similarity of p107 and p130 to Rb is most pronounced in the A and B sequences, and the residues in Rb that contact the common binding motif of the viral proteins are invariant among the other family members. This segment is also sufficient to repress transcription when fused to a heterologous DNA-binding domain, and it is required for binding to histone deacetylases (Hdacs). Like the viral Rb-binding proteins, Hdacs contain the motif LXCXE within the region that binds to Rb. The N-terminal segment of the protein, which is also important for suppression of cell proliferation, binds to human Mcm-7, a component of a chromatin-bound complex required for DNA replication and control of initiation of DNA synthesis. **(B)** The LT protein binds to the Rb A- and B-box domains via the sequence that contains the LXCXE motif, designated R. The adjacent, N-terminal J domain of LT is not necessary for binding to Rb, but is required for induction of cell cycle progression. It has been proposed that the J domain recruits the cellular chaperone Hsc70. The chaperone then acts to release E2f-Dp1 heterodimers from their association with Rb, by a mechanism that is thought to depend on ATP-dependent conformational change.

Figure 6.21 Organization of the larger adenoviral E1A protein. Regions of the protein are shown to scale. Those designated CR1 to CR3 are conserved in the E1A proteins of human adenoviruses. The CR3 region, most of which is absent from the smaller E1A protein because of alternative splicing, is not necessary for transformation. The locations of the Rb-binding motif and of the regions required for binding to the other cellular proteins discussed in the text are indicated.

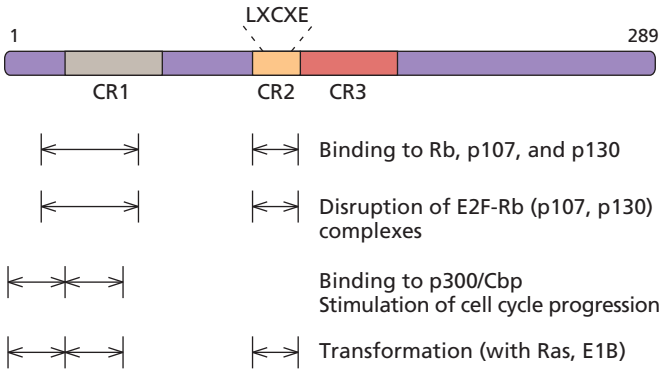
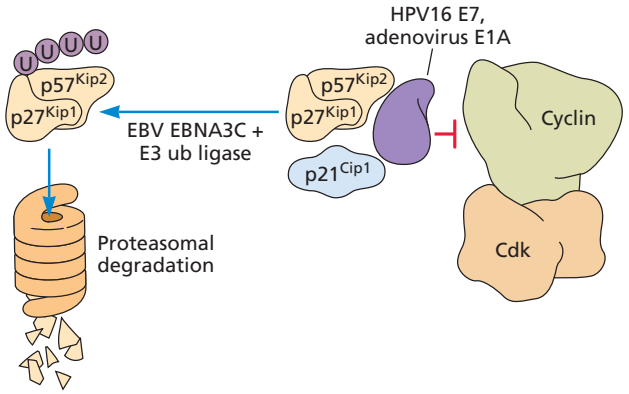


Figure 6.22 Inactivation of cyclin-dependent kinase inhibitors by viral proteins. The human papillomavirus (HPV) 16 E7 and adenovirus E1A proteins bind to and inactivate $p21^{Cip1}$ and, in the case of E1A proteins, also $p27^{Kip1}$. The viral proteins interact with the regions of the inhibitors that mediate association with G1 cyclins. They also block activation of transcription of the $p21^{Cip1}$ gene in response to DNA damage as a result of sequestration of the transcriptional coactivators p300 and Cpb. Rather than simply blocking the inhibitor-cyclin interaction, the Epstein-Barr virus (EBV) EBNA3C protein, which is necessary for transformation of B cells in culture, recruits a cellular E3 ubiquitin ligase that marks $p27^{Kip1}$ for degradation by the proteasome. This viral protein inactivates Rb in the same manner.



Transformed Cells Must Grow and Survive

The rapid proliferation of cells transformed by viral proteins depends on high rates of metabolism and growth during each cell cycle. It seems likely that any viral oncogene product that results in activation of Ras (or Akt) promotes cell growth (Fig. 6.4), as well as proliferation. How viral transforming proteins that impinge directly on the nuclear circuits that govern cell cycle progression accelerate cell growth is less clear. However, the actions of many of these proteins lead to changes in the transcription of numerous cellular genes, responses that might increase the concentrations of biosynthetic and other metabolic enzymes.

Mechanisms That Permit Survival of Transformed Cells

As discussed in Chapter 3, metazoan cells can undergo programmed cell death (apoptosis). This program is essential during development and serves as a powerful antiviral defense of last resort. Apoptosis can be activated not only by external cues, but also by intracellular events, notably damage to the genome or unscheduled DNA synthesis. Consequently, viral transforming proteins that induce cells to enter S phase and proliferate when they would not normally do so will also promote the apoptotic response. This potentially fatal side effect is foiled by a variety of mechanisms that allow survival of infected and transformed cells.

Viral Inhibitors of the Apoptotic Cascade

Many viral genomes encode mimics of cellular proteins that hold apoptosis in check (see Table 3.3). Such viral inhibitors of apoptosis can contribute to transformation. For example, the human adenovirus E1B 19-kDa protein, one of the first viral homologs of cellular antiapoptotic proteins to be identified, allows survival, and hence transformation, of rodent cells that also contain the proliferation-promoting viral E1A protein (Table 6.4).

Integration of Inhibition of Apoptosis with Stimulation of Proliferation

Cells must continually interpret the numerous internal and external signals that impinge upon them to execute an appropriate response. Not all the mechanisms that integrate the many types of information that cells receive have been elucidated. However, it is well established that signal transduction cascades that induce cell proliferation can simultaneously promote cell survival by blocking the apoptotic response. For example, signaling via the small G protein Ras results in activation of not only the cyclin-dependent kinases that drive the G_1-to-S-phase transition (Fig. 6.19A), but also Pi3k and the protein kinase Akt. Akt induces transcriptional and post-transcriptional mechanisms that inhibit the production and activity of proapoptotic proteins, such as Bad and Bim, and stimulate synthesis of inhibitors of apoptosis, including Bcl-2 (Fig. 6.23). Consequently, any viral transforming protein that elicits activation of Akt will also induce protection against apoptosis. Such proteins include the many retroviral gene products that function in the receptor protein tyrosine kinase pathway (Fig. 6.3; Table 6.5), v-Src (Fig. 6.12), and Epstein-Barr virus LMP-1 (Fig. 6.16).

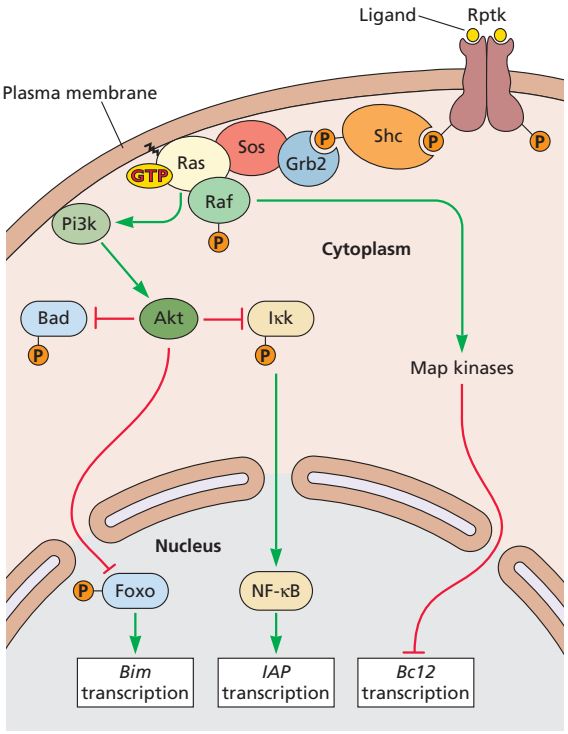


Figure 6.23 Signaling pathways that facilitate cell survival. Activation of Ras promotes cell survival by inhibition of synthesis or activity of proapoptotic proteins and by stimulation of production of inhibitors of programmed cell death. Substrates of activated Akt include the proapoptotic protein Bad and the transcriptional regulator Foxo, which are inactivated by phosphorylation. Akt also phosphorylates the inhibitor of Nf-κb (Iκk) to promote transcription of genes that encode other inhibitors of apoptosis. As shown in Fig. 6.19A, signaling via Ras and the Map kinase cascade induces cell proliferation. The Pi3k/Akt pathway also promotes proliferation, for example, by phosphorylation and inactivation of cyclin-dependent kinase inhibitors. Consequently, these (and other) signaling networks integrate cell proliferation and survival.

Inactivation of the Cellular Tumor Suppressor p53

Transformation by several DNA viruses requires inactivation of a second tumor suppressor, the p53 protein, first identified by virtue of its binding to simian virus 40 LT. This protein is a critical component of regulatory circuits that determine the response of cells to damage to their genomes, as well as to low concentrations of nucleic acid precursors, hypoxia, and other forms of stress. Its importance in the appropriate response to

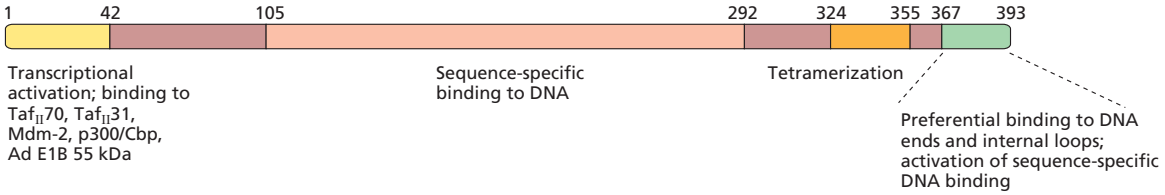
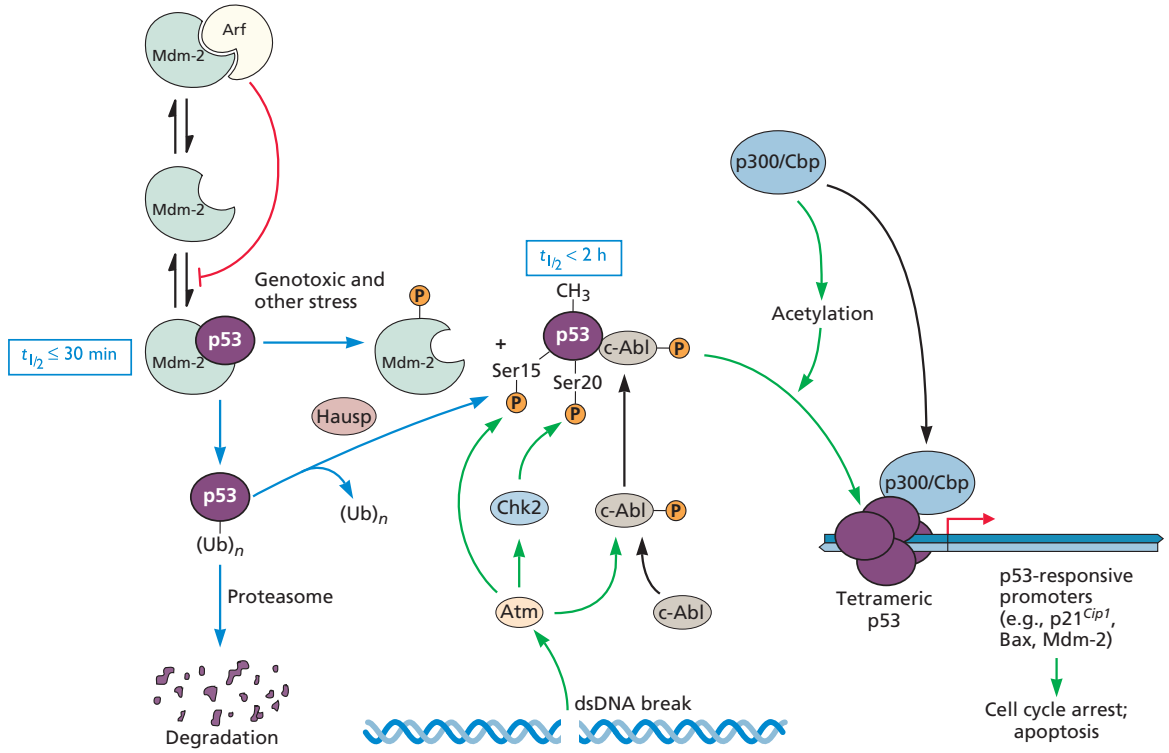


Figure 6.24 The human p53 protein. The functional domains of the protein are shown to scale. TafII70 and TafII31 are TATA-binding protein-associated proteins present in transcription initiation protein IID (TfIId) (Volume I, Chapter 8); other proteins are defined in the text.

such damage or stress is emphasized by the fact that *p53* is the most frequently mutated gene in human tumors.

The accumulation and activity of p53 are tightly regulated. The intracellular concentration of p53 is normally very low, because the protein is targeted for proteasomal degradation, for example, by binding of the Mdm-2 protein (Fig. 6.24 and 6.25). However, DNA damage, such as double-strand breaks produced by γ-irradiation, the collapse of replication forks, or the accumulation of DNA repair intermediates following UV irradiation, leads to the stabilization of p53 and a substantial increase in its concentration. The rate of translation of the protein may

Figure 6.25 Regulation of the stability and activity of the p53 protein. Under normal conditions (left), cells contain only low concentrations of p53. This protein is unstable, turning over with a half-life of minutes, because it is targeted for proteasomal degradation by the Mdm-2 protein. Mdm-2 is a p53-specific E3 ubiquitin ligase that catalyzes polyubiquitinylation of p53, the signal that allows recognition by the proteasome, to maintain inactive p53 at low concentrations. The availability and activity of Mdm-2 are also regulated, for example, by Arf proteins encoded by the *ink4a/arf* tumor suppressor gene and by stimulation of Mdm-2 transcription by the p53 protein itself. Signaling pathways initiated in response to damage to the genome or other forms of stress lead to posttranslational modification and stabilization of p53. Such posttranscriptional regulation is thought to allow a very rapid response to conditions that could be lethal to the cell. As illustrated with pathways operating in response to DNA damage (double-strand [ds] breaks caused by ionizing radiation), p53 is stabilized in multiple ways. These mechanisms include phosphorylation of p53 at specific serines by Atm (see the text) and checkpoint kinase 2 (Chk2), binding to the c-Abl tyrosine kinase, sequestration of the Mdm-2 protein by Arf, and deubiquitinylation of p53 (in the presence of Mdm-2) by the herpesvirus-associated ubiquitin-specific protease (Hausp). Multiple mechanisms, including various modifications within the C-terminal domain (e.g., acetylation), also stimulate the sequence-specific DNA-binding activity of p53 or its association with the transcriptional coactivators p300/Cbp, and hence transcription from p53-responsive promoters.



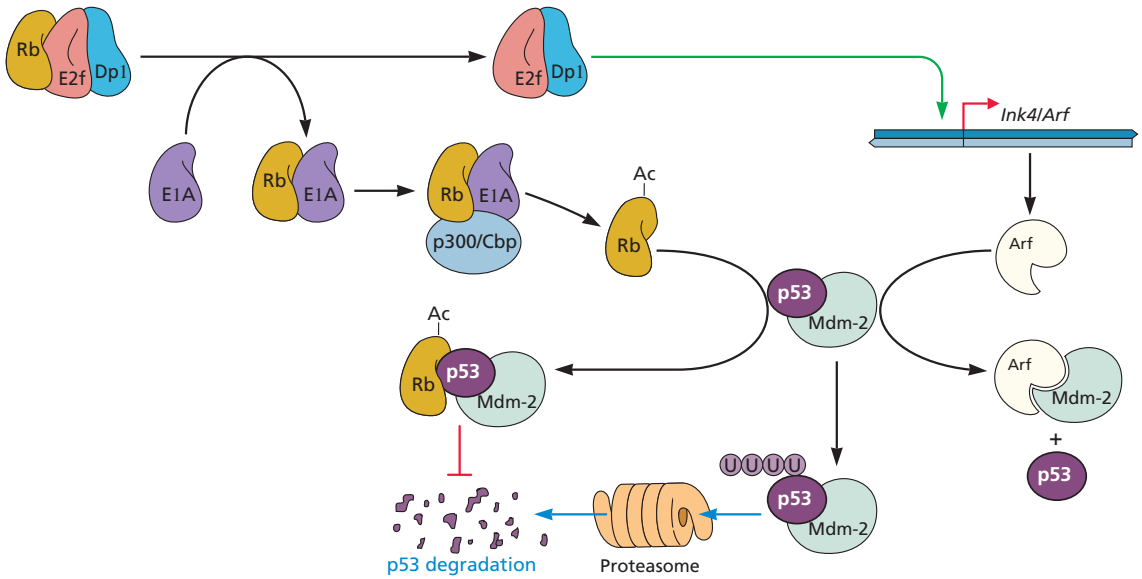
also increase. Various proteins that appear to be important for stabilization of p53 have been identified, including the product of a human gene called *Atm* (ataxia telangiectasia mutated), which recognizes potentially genotoxic DNA damage. Cells lacking the Atm protein do not accumulate the p53 protein and fail to arrest at the G_1/S boundary in response to DNA damage.

The p53 protein is a sequence-specific transcriptional regulator containing an N-terminal activation domain and a central DNA-binding domain (Fig. 6.24). This protein also operates in the cytoplasm, where it binds to proteins associated with mitochondria that inhibit apoptosis to induce release of proapoptotic effectors, such as Bax and Bak. The intracellular location, stability, and activities of p53, such as binding to DNA and stimulation or repression of transcription of particular sets of p53-responsive genes, are regulated by both multiple types of posttranslational modification of numerous residues and the constellation of associated proteins. The very large repertoire of such mechanisms provides the means to integrate the multiple signals that are monitored to ensure that this potent protein alters cell physiology only under extreme conditions.

In response to damage to the genome, or other inducing conditions, p53 prevents further cell proliferation by eliciting G_1/S arrest, apoptosis, or senescence (irreversible G_1 arrest). One important component of the pathway that leads to cell cycle arrest is the p53-dependent stimulation of transcription of the gene that encodes $p21^{Cip1}$, the G_1 cyclin-dependent kinase inhibitor (Fig. 6.6). The p53 protein promotes apoptosis both directly, by interaction with mitochondrial proteins that block this response, and indirectly, by stimulation of transcription of genes that encode proapoptotic proteins, such as Apaf-1 and Bax. It also impairs mechanisms that promote cell survival by increasing transcription of genes that encode inhibitors of certain signaling pathways. For example, increased production of the protein Pten leads to impaired signaling via Pi3k to Akt (Fig. 6.23), as Pten is a phosphatase that dephosphorylates phosphoinositides. The ability of p53 to repress transcription of genes for antiapoptotic proteins, such as *survivin*, may also be important. Whether p53 promotes cell cycle arrest, apoptosis, or senescence is determined by numerous parameters, including the cell type, the nature of extracellular stimuli, and the concentration of the p53 protein itself. However, the apoptotic response prevails in many cell types under many circumstances, in particular following expression of viral oncogenes that induce entry into S phase.

Viral proteins inactivate p53. The genomes of many viruses encode proteins that interact with p53. However, the mechanisms by which the functions of this critical cellular regulator can be circumvented are best understood for the small DNA tumor viruses. As we have seen, transforming proteins of these viruses induce release of E2f family members from association with Rb to promote cell cycle progression. Stabilization of p53 appears to be an inevitable consequence: E2f activates transcription from the promoter of the *Ink4/Arf* gene, which encodes a negative regulator of Mdm-2 (Fig. 6.26).

Figure 6.26 Stabilization of p53 by viral transforming proteins that bind to Rb. As described previously, binding of the adenoviral E1A (or polyomavirus LT or human papillomavirus E7) proteins to Rb allows transcription of E2f-responsive genes. This large set includes the *ink4/arf* gene, and Arf therefore accumulates. Binding of Arf to Mdm-2 sequesters this ubiquitin ligase, and hence leads to accumulation of the p53 protein. The E1A proteins also stabilize p53 via p300/Cbp-mediated acetylation of Rb. Acetylated Rb forms a ternary complex with p53 and Mdm-2 and blocks p53 degradation. The N-terminal transcriptional activation domain of p53 remains blocked by Mdm-2, but in this form p53 can repress transcription and promote apoptosis.



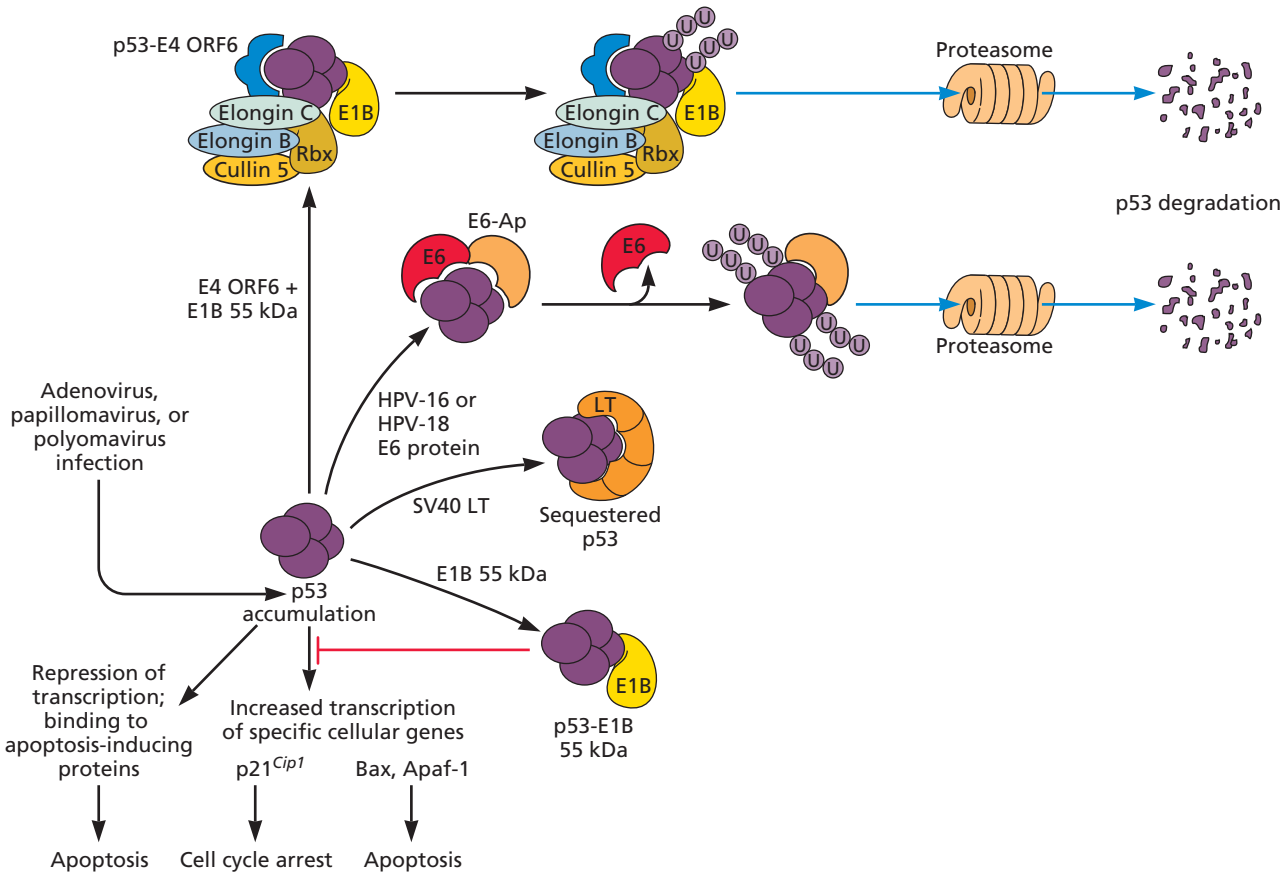
Viral proteins block p53 function in different ways (Fig. 6.27). The human papillomavirus type 16 or 18 E6 proteins bind to both p53 and a cellular E3 ubiquitin protein ligase (the E6-associated protein), and thereby target p53 for proteasome-mediated destruction. In conjunction with the viral E4 Orf6 protein, the adenoviral E1B 55-kDa protein also induces increased turnover of p53, but by directing it to a different E3 ubiquitin ligase. In contrast, simian virus 40 LT actually stabilizes the p53 protein, but sequesters this cellular regulator in inactive complexes.

Among the enzymes that acetylate p53 to increase its stability and activity are the histone acetyltransferases p300 and the closely related transcriptional activator cellular cAMP response element binding protein (Creb)-binding protein (Cbp). The former protein was first identified by virtue of its binding to adenoviral E1A proteins. This interaction with the cellular histone acetyltransferases blocks acetylation of p53, as does that of the human papillomavirus type 16 E6 protein, to limit activation of the tumor suppressor.

Tumorigenesis Requires Additional Changes in the Properties of Transformed Cells

The mechanisms described in the preceding sections account for the sustained proliferation and survival of cells transformed by viral oncogenes. However, they are not necessar-

Figure 6.27 Inactivation of the p53 protein by adenoviral, papillomaviral, and polyomaviral proteins. The synthesis of transforming proteins in infected or transformed cells leads to accumulation of p53 (Fig. 6.26). Each of these viral genomes encodes proteins that interfere with the normal function of this critical cellular regulator. Binding of simian virus 40 LT to p53, an interaction that is facilitated by sT, sequesters the cellular protein in inactive complexes. The E1B 55-kDa and E4 Orf6 proteins assemble with the cellular cullin 5, elongins B and C, and Rbx proteins to form a virus-specific E3 ubiquitin ligase. This enzyme ubiquitinylates p53 and marks it for proteasomal degradation. The E6 proteins of human papillomavirus types 16 and 18 bind to p53 via the cellular E6-associated protein (E6-Ap). The latter protein is a ubiquitin protein ligase that polyubiquitinylates p53 in the presence of the viral E6 protein, targeting p53 for degradation by the proteasome. The adenoviral E1B 55-kDa protein can also bind to the N-terminal activation domain of p53 to convert p53 from an activator to a repressor of transcription. This function of the E1B 55-kDa protein correlates with its ability to transform rodent cells in cooperation with E1A proteins. In transformed rodent cells, the E1B 55-kDa protein also induces relocalization of p53 from the nucleus to a perinuclear, cytoplasmic body. The results of mutational analyses have correlated the changes in concentration or activity in p53 induced by these viral proteins with their transforming activities.



ily sufficient for the induction of tumors or other types of cancer: tumorigenesis generally also requires the ability of transformed cells to survive in the face of immune defenses. In some cases, induction of the growth of new blood vessels (angiogenesis) is also necessary (see "Viral Homologs of Cellular Genes" above).

Inhibition of Immune Defenses

The crucial contribution of mechanisms that protect transformed cells from immune defenses is illustrated by the properties of rodent cells transformed by oncogenic or nononcogenic human adenoviruses (Box 6.11). As discussed in Chapters 3 and 4, mechanisms that render infected cells refractory to immune defenses are important for the ability of many viruses to reproduce in immunocompetent animals. How such mechanisms facilitate the survival of transformed cells and oncogenesis is best understood for herpesviruses associated with human cancers: Epstein-Barr virus and human herpesvirus 8.

Epstein-Barr virus is associated with Burkitt's lymphoma (a B cell lymphoma) and nasopharyngeal carcinoma. Although LMP-1 is the only viral gene product that can transform cells in culture, other viral proteins are made in such tumor cells. These products include Epstein-Barr virus nuclear antigen 1 (EBNA-1), which is necessary for replication and maintenance of the episomal viral genome (Volume I, Chapter 9). This protein also contains a sequence that inhibits presentation of EBNA-1 epitopes by major histocompatibility complex (MHC) class I proteins. Consequently, tumor cells cannot be detected so readily by T cells of the adaptive immune system. Similarly, several of the human herpesvirus 8 genes that have been implicated in transformation or tumorigenicity encode proteins that inhibit innate or adaptive immune responses. For example, the viral cytokine v-IL-6, which is a B cell mitogen, also blocks the action of interferon by inhibiting phosphorylation of substrates of the interferon receptor, such as Stat2. In addition, the vFLIP protein, which can enhance the tumorigenicity of murine B cells, inhibits killing by natural killer cells.

Other Mechanisms of Transformation and Oncogenesis by Human Tumor Viruses

The mechanisms by which some viruses associated with human cancers transform cells and contribute to tumor development cannot be subsumed within the general paradigms discussed in the preceding sections. Our current understanding of the development of these neoplastic diseases is described in this section.

Nontransducing Oncogenic Retroviruses: Tumorigenesis with Very Long Latency

The prototype for the nontransducing oncogenic retroviruses with complex genomes is human T-lymphotropic virus type 1, which is associated with adult T cell leukemia (ATL). This

BOX 6.11

BACKGROUND

Escape from immune surveillance and the oncogenicity of adenovirus-transformed cells

One of the earliest classifications of human adenovirus serotypes was based on the ability of the viruses to induce tumors in laboratory animals (see the table). Rodent cells transformed with the viral E1A and E1B genes in culture exhibit the tumorigenicity characteristic of the virus: cells transformed by the adenovirus type 12 genes form tumors efficiently when inoculated into syngeneic, immunocompetent animals, whereas cells transformed by adenovirus type 5 DNA induce tumors only in immunocompromised animals, such as nude mice. This difference was exploited to map the ability of transformed cells to form tumors efficiently in normal animals to a small region of the E1A gene, unique to adenovirus type 12 (and other highly oncogenic adenoviruses). The tumorigenicity of transformed cells was also correlated with repression of transcription of MHC class I genes: the adenovirus type 12 E1A proteins, but not those of adenovirus type 5, inhibit transcription of MHC class I genes by stimulating the binding of a translational repressor and histone deacetylases to the MHC class I enhancers.

The inhibition of MHC class I transcription induced by adenovirus type 12 E1A proteins results in reduced protein concentration on the cell surface, and hence in impaired presentation of antigens to cells of the immune system. Adenovirus type 12-transformed cells therefore escape immune surveillance and destruction, whereas those transformed by adenovirus type 5 do not.

Yewdell JW, Bennink JR, Eager KB, Ricciardi RP. 1998. CTL recognition of adenovirus-transformed cells infected with influenza virus: lysis by anti-influenza CTL parallels adenovirus-12-induced suppression of class I MHC molecules. *Virology* **162:** 236–238.

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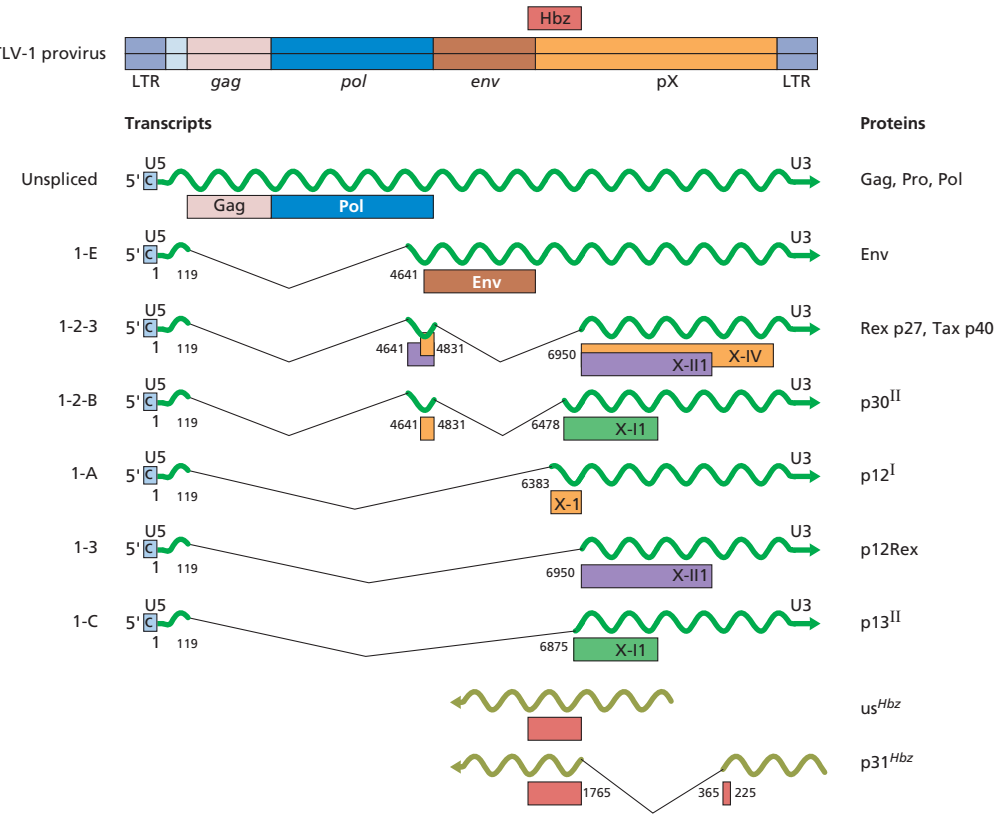
Classification of human adenoviruses on the basis of oncogenicity

Subgroup	Representative serotypes	Oncogenicity in animals
A	12, 18, 31	High: induce tumors rapidly and efficiently
B	3, 7, 21	Low: induce tumors in only a fraction of infected animals, with a long latent period
C	1, 2, 5	None

disease was first described in Japan in 1977, and has since been found in other parts of the world, including the Caribbean and areas of South America and Africa. The virus entered the human population as a zoonosis from infected primates some 30,000 to 40,000 years ago. The human virus was isolated in 1980 and is now classified as a deltaretrovirus (Volume I, Appendix, Fig. 29).

Human T-lymphotropic virus is transmitted via the same routes as human immunodeficiency virus: during sexual intercourse, by intravenous drug use and blood transfusions, and from mother to child. Infection is usually asymptomatic, but can progress to ATL in about 5% of infected individuals over a period of 30 to 50 years (see Chapter 5). Although some progress has been made using stem cell transplantation and antiviral drugs, there is no effective treatment for the disease, which is usually fatal within a year of diagnosis. The mechanism(s) by which the virus induces malignancies is still uncertain, but some of the features of ATL are consistent with a role for a viral regulatory protein. A provirus is found at the same site in all leukemic cells from a given case of ATL, indicating clonal origin, but there are **no** preferred chromosomal locations for these integrations. Activation or inactivation of a specific cellular gene is not, therefore, a likely mechanism of transformation. As the genome of human T-lymphotropic virus type 1 does not contain **any** cell-derived nucleic acid, some viral sequences must be responsible for this activity. The search for such sequences rapidly focused on the region denoted X, which encodes a number of regulatory and accessory proteins (Fig. 6.28). One of the best studied among these is the multifunctional transcriptional activator Tax. This protein is required for efficient proviral gene expression, and it also regulates the expression and function of a number of cellular genes and proteins that regulate T cell physiology, a feature consistent with its desig-

Figure 6.28 Transcription map of human T-lymphotropic virus type 1 (HTLV-1) proviral DNA showing gene-coding regions and their functions. Transcription of all but the *Hbz* gene is initiated between the unique 3′ (U3) and R regions in the 5′ LTR. Dotted lines indicate spliced introns. The *Hbz* gene is transcribed either from multiple Sp1-promoted initiation sites in the unique 5′ (U5) and R regions of the 3′′ LTR, which produce spliced mRNA transcripts (*sHbz*), or from an initiation site within the *Tax* gene to form the unspliced transcript (*usHbz*). Translation of the spliced and unspliced mRNAs produces two proteins of 206 and 209 amino acids, respectively, both containing three functional domains. However, the latter protein has a very short half-life, and only products of the spliced transcript can be detected readily in ATL cells. Adapted from P. Kannian and P. L. Green, *Viruses* **2:**2037–2077, 2010, with permission.



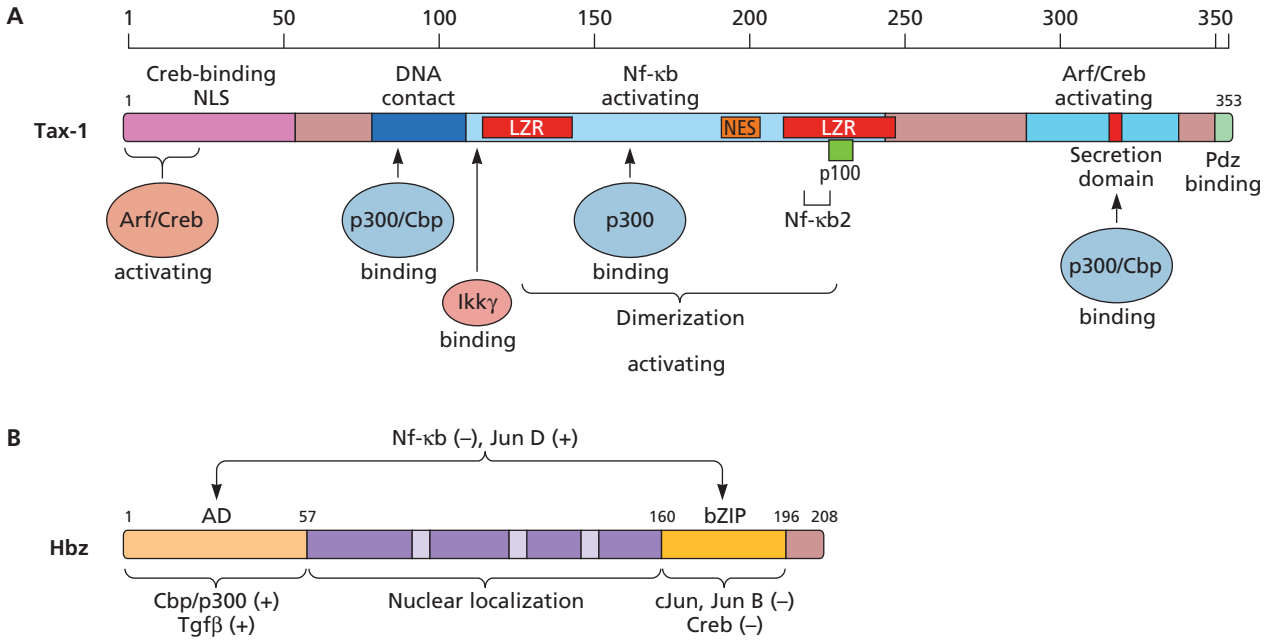
nation as a viral oncoprotein. It stimulates transcription indirectly, by interaction with Creb and by activation of Nf-κb (Fig. 6.29).

The latter effect leads to inhibition of apoptosis in infected T cells and enhanced transcription of a number of genes that encode cytokines, their receptors, and other regulatory proteins. Tax interactions with particular cellular proteins have also been shown to promote cell cycle progression and block cellular DNA repair, resulting in genome instability and evolution of malignant clones. Given the numerous and important functions attributed to this viral protein, it was surprising that *Tax* gene expression could be detected in the leukemic cells of only some 60% of ATL patients. This finding suggested that Tax might be required for the initiation of oncogenesis, but not for its maintenance. This notion was confirmed following the discovery and analysis of the Hbz open reading frame in the X region of the proviral DNA genome (Fig. 6.28).

Hbz was first identified as a Creb-binding protein that inhibits Tax-mediated transcription from the human T-lymphotropic virus type 1 LTR. Large quantities of the potent immunogen Tax are synthesized and even secreted by these cells. As Hbz is not very immunogenic, its inhibition of Tax reduces the host's immune response to ATL cells. Most importantly, Hbz is detected in all ATL cells, and although antagonistic to some Tax functions or activities, it also activates some of the same signaling and proliferation pathways that are required for tumor maintenance (Fig. 6.29). Hbz inhibition of the Nf-κB pathway also enhances cell proliferation and progression of disease by blocking Tax-induced senescence and by reducing host innate and inflammatory immune responses. In addition, *Hbz* RNA itself has been found to promote T cell proliferation.

Although Tax and Hbz are clearly major players in the "yin and yang" of initiation and maintenance of oncogenesis by human T-lymphotropic virus type 1, other accessory genes encoded in the X region are likely to contribute to viral pathogenesis. The products of these genes, p12/p8, p13, and p30, are dispensable for viral replication and transformation of cells in culture, but they are required for efficient

Figure 6.29 Domains and interactions of the human T-lymphotropic virus type 1 Tax and Hbz proteins. **(A)** Tax-1 protein. The protein includes two leucine zipper domains (LZR) and nuclear localization (NLS) and nuclear export (NES) sequences. As indicated, Tax interacts with numerous cellular proteins to regulate transcription. The Tax response element in the U3 region of the viral LTR is a 21-bp triple repeat, containing an octamer motif that is homologous to the binding site for Creb. Tax does not bind directly to the TRE, or the numerous cellular promoters that contain cAMP response element, but interacts with the basic leucine zipper domain of Creb, thereby enhancing its dimerization and its affinity to these response elements. Coactivators such as Cbp/p300 are also recruited to these complexes, inducing the chromatin remodeling that facilitates transcription. **(B)** The Hbz protein has three major domains: an N-terminal activating domain (AD), a C-terminal basic zipper domain (bZIP), and a central domain with three basic regions that include nuclear localization signals. The (+) and (–) symbols indicate activation or inhibition, respectively, upon binding. For example, binding of the bZIP domain to Creb blocks Creb dimerization and hence stimulation of Tax-dependent transcription.



replication and persistence of the virus in rabbits and nonhuman primates. Because virus-induced oncogenic events occur a long time before ATL appears, it is difficult to sort out and evaluate the multiple effects of all of these viral proteins. Furthermore, the lack of a suitable animal model for the disease and the inefficiency of infection of T cells have represented significant challenges to research with the human lymphotropic viruses.

Oncogenesis by Hepatitis Viruses

Hepatitis B Virus

Hepatitis B virus is a member of the family *Hepadnaviridae*. The major site of reproduction for all hepadnaviruses is the hepatocyte, the major cell of the liver. Infections by these viruses can be acute (3 to 12 months) or lifelong. In humans, the frequency of persistent infection ranges from 0.1 to 25% of the population in different parts of the world. Most persistent infections are acquired neonatally or during the first year of life. Chronic infection of hepatocytes leads to their persistent destruction by the immune system and formation of fibrotic scars that obstruct the passage of blood (a life-threatening condition known as cirrhosis). Long-term carriers are also at high risk for developing hepatocellular carcinoma (Box 6.1), which generally leads to death within 5 years of diagnosis. As many as 1 million people die of hepatocellular carcinoma each year, despite the fact that a vaccine that prevents infection with this virus, actually the very first "anticancer" vaccine, has been available for several decades.

Sustained low-level liver damage is characteristic of persistent infection by hepatitis B viruses. Almost all such damage can be attributed to attack on infected hepatocytes by the host's immune system. The rate of hepatocyte proliferation must increase in such cases to compensate for cell loss. It is generally accepted that such an increased rate of proliferation over long periods is a major contributor to the development of both cirrhosis and liver cancer. In addition, the inflammation and phagocytosis that are integral to the immune response can result in high local concentrations of superoxides and free radicals. It is therefore possible that DNA damage and the resulting mutagenesis also contribute to hepadnavirus-induced hepatocellular carcinoma. Consequently, there is considerable incentive for developing new antiviral therapies to treat persistent hepatitis B virus infection. While several reverse transcriptase inhibitors are available for treatment, current antiviral therapies cannot cure infections.

The almost universal presence of integrated fragments of hepadnaviral DNA in tumor genomes suggests that this feature plays a role in oncogenesis. However, there is no known association of viral sequences with proto-oncogenes. Other studies suggest that viral proteins, such as the X protein or a truncated form of the envelope protein encoded by integrated viral DNA sequences, may contribute to carcinogenesis in humans. In cell culture systems, and by inference in the infected liver, the hepatitis B virus X protein stimulates transcription from many cellular genes (including proto-oncogenes), both by altering the DNA binding of cellular transcriptional regulators and by activation of signaling via Nf-κb and other pathways. However, the long time required for development of human liver cancer implies that several low-probability reactions must take place over an extended period. The relative importance of X or other viral proteins and the indirect effects of immune damage to the process remain to be determined.

Hepatitis C Virus

Hepatitis C virus is a (+) strand RNA virus in the family *Flaviviridae*. Its discovery in 1989 established the etiology of what had been known previously as non-A, non-B hepatitis, a disease contracted by a small fraction of transfusion recipients who developed acute and chronic hepatitis and, years later in some cases, liver cancer. Routine screening of the blood supply has since reduced this mode of infection. Approximately 75 to 85% of infected individuals develop a persistent infection. An estimated 170 million people are still chronically infected worldwide; among these, 1 to 5% will develop hepatocellular carcinoma. Although a small percentage, this still amounts to 1.3 million to 7.2 million cases and approximately a third of all liver cancer cases worldwide. Fortunately, owing to recent success in the development of potent antiviral therapies that can cure chronic infections (see below), it can be expected that the number of liver cancers diagnosed each year will soon begin to decline.

Like hepatitis B virus, hepatitis C virus is hepatotropic. Chronic infection of hepatocytes leads to their destruction by the immune system, resulting in formation of fibrotic scars that obstruct the passage of blood. Not all patients with such cirrhosis develop cancer, and genome-wide association studies have suggested that the genetic background of the host influences the course of infection. Some evidence indicates that certain viral proteins (capsid, envelope, and several nonstructural proteins) can block the normal response of hepatocytes to apoptotic signals, affect signal transduction, and increase the concentration of damaging reactive oxygen species. Deregulation of cellular miRNA production has also been associated with hepatitis C infection. The importance of these activities to oncogenesis has been difficult to test. Although chimpanzees are susceptible to hepatitis C (and hepatitis B) virus, infection has not been shown to cause hepatocellular carcinoma. In addition, current guidelines restrict the use of chimpanzees for research in the United States, and there is no good small-animal model for hepatitis C virus-mediated hepatocellular carcinogenesis. However, as with hepatitis B

virus, the indirect effects of immune-mediated inflammation and oxidative damage induced by infection are thought to be the major contributors to cirrhosis and cancer. Whether viral proteins have a modulating role is yet to be determined.

In contrast to hepatitis B, infection with hepatitis C is curable because the virus reproduces entirely within the cytoplasm of infected cells and its persistence depends on continuous genome amplification. Early on, interferon-based therapies led to the cure of chronic infections in about 50 to 70% of patients. The establishment of cell culture systems for hepatitis C infection has enabled detailed study of the viral reproduction cycle and also served as an important tool for the development of direct-acting antiviral therapies. Selective inhibitors of the viral serine protease (product of the *NS3-NS4A* gene), the viral polymerase (product of the *NS5B* gene), and the NS5A protein are very potent antivirals that, in combination with interferon, can cure ~90% of infected patients. Some of these inhibitors are already approved by the Food and Drug Administration, and others are expected to gain approval in the near future.

Perspectives

The discovery that viruses can cause cancer, initially made over a century ago, was the harbinger of the spectacular progress in understanding the molecular basis of transformation and oncogenesis that has occurred within the past 4 decades. Because tumor cells grow and divide when normal cells do not, elucidation of the mechanisms of transformation has inevitably been accompanied by the tracing of the intricate circuits that regulate cell proliferation in response to both external and internal signals. The remarkable discovery that the transforming gene of the retrovirus Rous sarcoma virus was a transduced cellular gene paved the way for identification of many cellular proto-oncogenes, and the elucidation of the signal transduction pathways in which the proteins encoded by them function. Indeed, in several cases, we can now describe in atomic detail the mechanisms by which mutations introduced into these genes during or following their capture into retroviral genomes lead to constitutive activation of signaling. These viral genes and their cellular counterparts that have acquired specific mutations in tumors are dominant oncogenes. In contrast, studies of a hereditary juvenile cancer in humans, retinoblastoma, had indicated that neoplastic disease can also develop following the loss of function of specific genes, which were therefore named tumor suppressor genes. Our current appreciation of the critical roles played by the products of such tumor suppressor genes in the control of cell cycle progression stems directly from studies of transforming proteins of adenoviruses, papillomaviruses, and polyomaviruses.

The initial cataloging of viral transforming genes and the properties of the proteins they encode suggested a bewildering variety of mechanisms of viral transformation. With the perspective provided by our present understanding of the circuits that control cell proliferation, we can now see that the great majority of these mechanisms fall into one of two general classes: viral transformation can be the result of either constitutive activation of signal transduction cascades or disruption of pathways that negatively regulate cell cycle progression. In both cases, viral proteins or transcriptional control signals override the finely tuned mechanisms that normally ensure that cells grow, duplicate their DNA, and divide only when external and internal conditions are propitious and, in many cases, also promote cell growth and survival.

Such an integrated view of the mechanisms by which viruses belonging to very different families can transform cells is intellectually satisfying. However, transformation of cells in culture is not necessarily accompanied by acquisition of the ability to form tumors in animals. This dissociation is evident in the etiology of some human cancers associated with viral infections. For example, infection by Epstein-Barr virus, which immortalizes human B cells in culture by mechanisms that we can describe in detail, is but one of several factors implicated in the development of Burkitt's lymphoma. A deeper appreciation of the parameters that determine a host's response to transformed cells will clearly be necessary if we are to understand the complex process of tumorigenesis.

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LINKS FOR CHAPTER 7

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- ⏭ *Movie 7.1: Molecular Model for Apobec3F Degradation by the Vif/Cbf-b Ubiquitin Ligase*
  http://bit.ly/Virology_V2_Movie7-1
- ⏭ *Movie 7.2: HIV particles in a virological synapse between mature dendritic cells and susceptible T cells.*
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- ⏭ *Does a gorilla shift in the woods?*
  http://bit.ly/Virology_Twiv327
- ⏭ *Joint United Nations Programme on HIV/AIDS*
  http://www.unaids.org
- ⏭ *United States Centers for Disease Control and Prevention*
  http://www.cdc.gov/hiv/

Nature is not human-hearted.

LAO TZU
Tao Te Ching

Introduction

Worldwide Impact of AIDS

Acquired immunodeficiency syndrome (AIDS) is the name given to the end-stage disease caused by infection with human immunodeficiency virus (HIV). By almost any criteria, HIV qualifies as one of the world's deadliest scourges. First recognized as a clinical entity in 1981, by 1992 AIDS had become the major cause of death in individuals 25 to 44 years of age in the United States. Although the rates of both infection and deaths have been reduced substantially since 2000, the current worldwide statistics are still staggering, with the low-income countries of Africa and parts of Asia being especially hard hit (Fig. 7.1). An end-of-year report from the United Nations' AIDS program estimated the number of new HIV infections in 2012 to be 2.3 million, bringing the total number of infected people worldwide to approximately 35.3 million. This number corresponds to almost 1 in every 100 adults aged 15 to 49 in the world's population. The availability of effective drugs to treat HIV infection has decreased the annual death toll in high-income countries. In some lower-income countries, up to 90% of infected individuals have access to such drugs, but in others only about 30% are being treated, and HIV/AIDS is still the leading cause of death in many of these regions. AIDS still kills more people than any other infectious disease (Table 11.4).

The emergence and spread of HIV was probably the consequence of a number of political, economic, and societal changes, including the breakdown of national borders, migration of large populations because of military conflicts and economic distress, and the ease and frequency of travel throughout the world. International efforts, including large investments from the U.S. President's Emergency Plan for AIDS Relief (known as PEPFAR) and the Global AIDS Program (GAP) that started in 2004, have focused on bringing funds and expertise to bear on the pandemic in Africa and elsewhere. While the task is enormous, much progress has been made in the past decade (see Fig. 9.22), fueling hopes that it will be possible to end the AIDS pandemic in the next decade.

Because of its medical importance, HIV has become the most intensely studied infectious agent. Research with the virus has not only contributed to our understanding of AIDS and related veterinary diseases, but has also provided new insights into principles of virology, cell biology, and immunology. This chapter describes the many facets of HIV-induced pathogenesis and what has been learned from their analysis. The complexities illustrate the enormous scope of the challenges faced by biomedical researchers and physicians in their efforts to control this agent, which strikes at the very heart of the body's defense systems.

HIV Is a Lentivirus

Discovery and Characterization

The first clue to the etiology of AIDS came in 1983 with the isolation of a retrovirus from the lymph node of a patient with lymphadenopathy at the Pasteur Institute in Paris. Although initially not fully appreciated, the significance of this finding became apparent in the following year with the isolation of a cytopathic, T cell-tropic retrovirus from combined blood cells of AIDS patients by researchers at the U.S. National Institutes of Health, and of a similar retrovirus from blood cells of an AIDS patient at the University of California, San Francisco. Although the National Institutes of Health isolate was later shown to originate from a sample received from the Pasteur Institute (Box 7.1), the virus isolated at the University of California, San Francisco, and subsequent isolates at the

PRINCIPLES ***HIV pathogenesis***

- The disease associated with human immunodeficiency virus (HIV) infection, AIDS, still kills more people than any other disease of viral origin.
- HIV is transmitted from person to person by sexual contact, blood exchange, or from mother to child.
- The course of HIV infection is characterized by three phases: an initial acute phase, a variable asymptomatic phase, and eventual end-stage disease.
- The major target of HIV infection is the CD4+ T cell. Both the CD4 receptor and chemokine coreceptors, which are required for viral entry, are displayed on the surface of these cells.
- Individuals with mutations in the genes encoding chemokine coreceptors are resistant to HIV infection.
- Accessory proteins of HIV contribute to pathogenesis via a common mechanism of action; all function as adapter proteins that disrupt the normal trafficking of particular cellular proteins and, in most cases, facilitate their degradation.
- The gut-associated lymphoid tissue (GALT), which contains ~40% of the body's lymphocytes, is a major site of HIV reproduction.
- The defining feature of HIV disease is impaired function of immune cells, and most AIDS patients succumb to opportunistic infections with microorganisms that are little threat to individuals with healthy immune systems.
- The inability to identify with certainty the cell types, number, and body compartments that comprise the total HIV latent reservoir is a serious barrier to devising strategies for complete clearance of the virus, and effective cure of the infection.

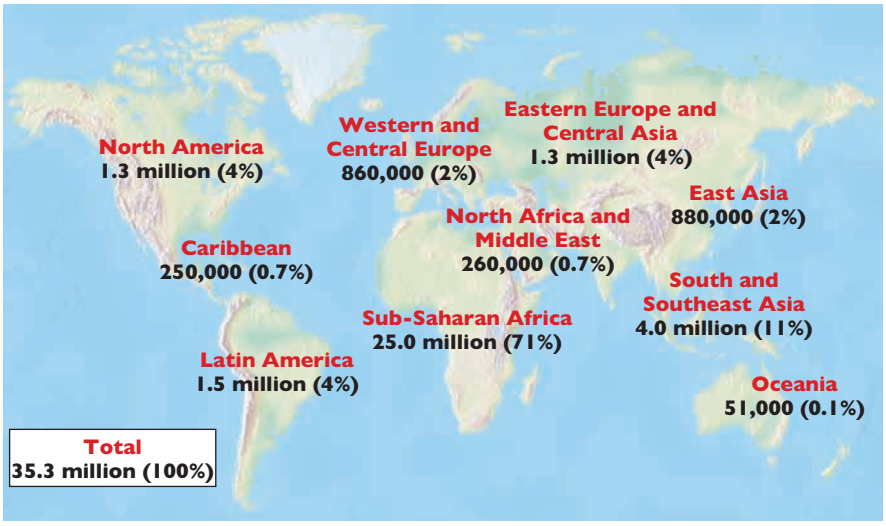


Figure 7.1 Estimated number of people living with HIV infection worldwide, 2012.
Data from the UNAIDS Report on Global AIDS Epidemic, 2013.

National Institutes of Health laboratory were unique. Electron microscopic examination revealed that these viruses were morphologically similar to a known group of retroviruses, the lentiviruses, and further characterization confirmed this relationship

Lentiviruses comprise a separate genus of the family *Retroviridae* (Table 7.1). The equine infectious anemia lentivirus was one of the first viruses to be identified. Discovered in 1904, this virus causes episodic autoimmune hemolytic anemia in horses. Lentiviruses of sheep and goats have also been known for many years. All these viruses are associated with long incubation periods and are therefore called **slow viruses** (Chapter 5). A distinct type of HIV that is prevalent in certain regions of West Africa was discovered in 1986, and given the name HIV-2. Individuals infected with HIV-2 also develop AIDS, but with a longer incubation period and lower morbidity.

Many independent isolates of both HIV-1 and HIV-2 have been characterized over the past two decades. Nucleotide sequence comparisons allow us to distinguish four distinct groups among HIV-1 isolates (Fig. 7.2). Group M includes most HIV-1 isolates, and is the cause of greater than 95% of HIV infections worldwide. Eleven distinct M subtypes are currently recognized (called **clade** A to K), each of which is prevalent in a different geographic area. For example, clade B is the most common subtype in North America and Europe. HIV-1 group O (for "outliers") also includes diverse subtypes but is relatively rare, and groups N and P have been identified only in a few individuals in Cameroon. Eight distinct groups of HIV-2 have also been identified. Of these, groups A and B (found in different parts of West Africa) account for most infections. Sequence analyses and identification of related nonhuman primate strains have indicated that these viruses entered humans by cross-species transmission on several occasions.

The African monkey and ape isolates are endemic to each of the species from which they were obtained and do not cause disease in their native hosts, most likely because of intrinsic defense mechanisms (Chapter 3). However, a fatal AIDS-like disease is caused by infection of Asian macaques with virus originating from the African sooty mangabey (SIV_{sm}). Close contact between sooty mangabeys and humans is common, as these animals are hunted for food and kept as pets. Such interactions, and the observation that several isolates of HIV-2 are nearly indistinguishable in nucleotide sequence from SIV_{sm}, support the hypothesis that HIVs emerged via interspecies transmission from nonhuman primates to humans. Other studies indicate that the M, N, and O HIV-1 groups arose via at least three independent transmissions from chimpanzees (Box 1.2). The strains of SIV_{cpz} from the chimpanzee *Pan troglodytes troglodytes* are closest in sequence to HIV-1, implicating this subspecies as the origin of the human virus (Box 1.2). Analyses of stored blood and tissue samples indicate that the common ancestor of group M viruses may have been transmitted to humans as early as 1900 (Box 7.2). In this chapter we use the abbreviation HIV to describe properties shared by HIV-1 and HIV-2, and specify the type when referring to one or the other.

As summarized in Table 7.1, lentiviruses cause immune deficiencies and disorders of the hematopoietic and central

BOX 7.1

DISCUSSION

Lessons from discovery of the AIDS virus(es)

The first AIDS virus was obtained from a patient with lymphadenopathy by Françoise Barré-Sinoussi in collaboration with Jean-Claude Chermann and Luc Montagnier at the Pasteur Institute (1983) and named LAV, for lymphadenopathy virus. The isolate, named Bru, grew only in primary cell cultures. We now know that Bru belonged to a class of slow-growing, low-titer viruses that are common in early-stage infection.

Between 20 July and 3 August 1983, Bru-infected cultures at the Pasteur Institute became contaminated with a second AIDS virus, called Lai, which had been isolated from a patient with full-blown AIDS, and which belonged to a class of viruses that grow well in cell culture. HIV-1 Lai rapidly overtook the cultures.

Unaware of this contamination, Pasteur scientists subsequently sent out virus samples from these cultures as "Bru" to several laboratories, including those of Robin Weiss in Britain and Malcolm Martin and Robert Gallo in the United States. Unlike earlier samples of Bru, this virus grew robustly in the laboratories to which it was distributed. Indeed, Lai was later discovered to have contaminated some AIDS patient "isolates" obtained by Weiss. In retrospect, such contamination is not surprising, as biological containment facilities were limited at the time, with the same incubators and hoods being used for maintaining HIV stocks and making new isolates.

Lai also contaminated cultures of blood cells combined from several AIDS patients in the Gallo laboratory at the National Institutes of Health. Because the properties of this virus were found to be different from those described for Bru, Gallo and coworkers reported the discovery of a second type of AIDS virus, which they believed to have originated from one of their AIDS patients. They called the virus HTLV-III, for human T cell lymphotrophic virus, believing it was unique, but probably related to the human T cell lymphotropic viruses I and II, which they had also been studying.

This second claim, a race to develop blood-screening tests, and the later revelation from DNA sequence analyses that the French and the Gallo viruses were one and the same (Lai) led to a much publicized scientific controversy in which patenting agencies, lawyers, businesses, and even governments were embroiled.

Simon Wain-Hobson and colleagues at the Pasteur Institute eventually sorted out the chain of events (in 1991) by comparing nucleotide sequences of stored samples of the original stocks of Bru and Lai. The controversy has since subsided, and the nomenclature was simplified in 1986, when the International Committee on Taxonomy of Viruses recommended that the current name, human immunodeficiency virus (HIV) replace LAV, HTLV-III, and ARV, a third name used by investigators in San Francisco who had obtained an independent isolate.

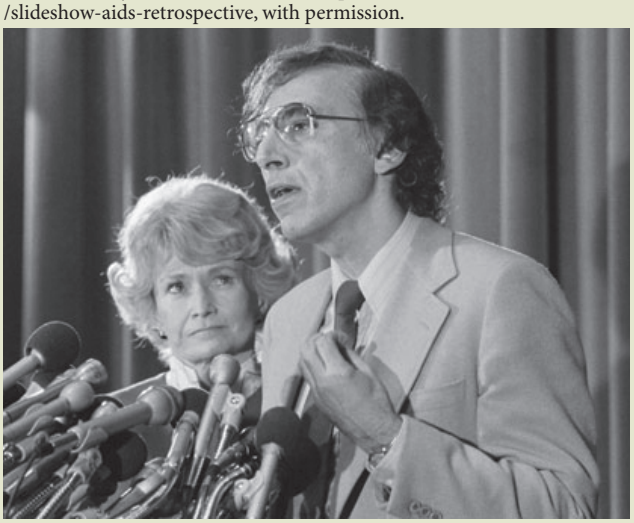
What remains from this story are three important lessons in virology: that contamination can be a real problem, that passage in the laboratory tends to select for viruses that reproduce rapidly, and that rigorous characterization (nowadays by genome sequencing) is a prudent safeguard against costly mistakes.

Goudsmit J. 2002. Lots of peanut shells but no elephant. A controversial account of the discovery of HIV. *Nature* **416:**125–126.

Wain-Hobson SJ, Vartanian P, Henry M, Chenciner N, Cheynier R, Delassus S, Martins LP, Sala M, Nugeyre MT, Guetard D, et al. 1991. LAV revisited: origins of the early HIV-1 isolates from Institut Pasteur. *Science* **252:**961–965.

Weiss R, Martin M. Personal communication.

Reagan HHS Secretary Margaret Heckler, shown with Robert Gallo. April 23, 1984. National Cancer Institute (NCI) researcher Robert Gallo reports isolation of an AIDS virus he calls HTLV-III. Later, it turns out to be LAV from a sample sent by the Montagnier lab, but not before HHS Secretary Margaret Heckler gives Gallo full credit. Heckler predicts a vaccine in 2 years. From WebMD http://www.webmd.com/hiv-aids/ss/slideshow-aids-retrospective, with permission.



nervous systems and, sometimes, arthritis and autoimmunity. Lentiviral genomes are relatively large. In addition to the three structural polyproteins Gag, Pol, and Env, common to all retroviruses, these genomes encode a number of additional **auxiliary proteins** (Fig. 7.3). Two HIV auxiliary proteins, Tat and Rev, perform regulatory functions that are essential for viral reproduction. The remaining four additional proteins of HIV-1, Nef, Vif, Vpr, and Vpu, are not essential for viral reproduction in most immortalized T cell lines and hence are known as **accessory proteins**. However, these proteins do modulate virus reproduction, and they are essential for efficient virus production *in vivo* and the ensuing pathogenesis.

Table 7.1 Lentiviruses[a]

Virus	Host infected	Primary cell type infected	Clinical disorder(s)
Equine infectious anemia virus	Horse	Macrophages	Cyclical infection in the first year, autoimmune hemolytic anemia, sometimes encephalopathy
Visna/maedi virus	Sheep	Macrophages	Encephalopathy/pneumonitis
Caprine arthritis-encephalitis virus	Goat	Macrophages	Immune deficiency, arthritis, encephalopathy
Bovine immunodeficiency virus	Cow	Macrophages	Lymphadenopathy, lymphocytosis
Feline immunodeficiency virus	Cat	T lymphocytes	Immune deficiency
Simian immunodeficiency virus	Primate	T lymphocytes	Immune deficiency and encephalopathy
Human immunodeficiency virus	Human	T lymphocytes	Immune deficiency and encephalopathy

[a]Adapted from Table 1.1 (p. 2) of J. A. Levy, *HIV and the Pathogenesis of AIDS,* 3rd ed. (ASM Press, Washington, DC, 2007).

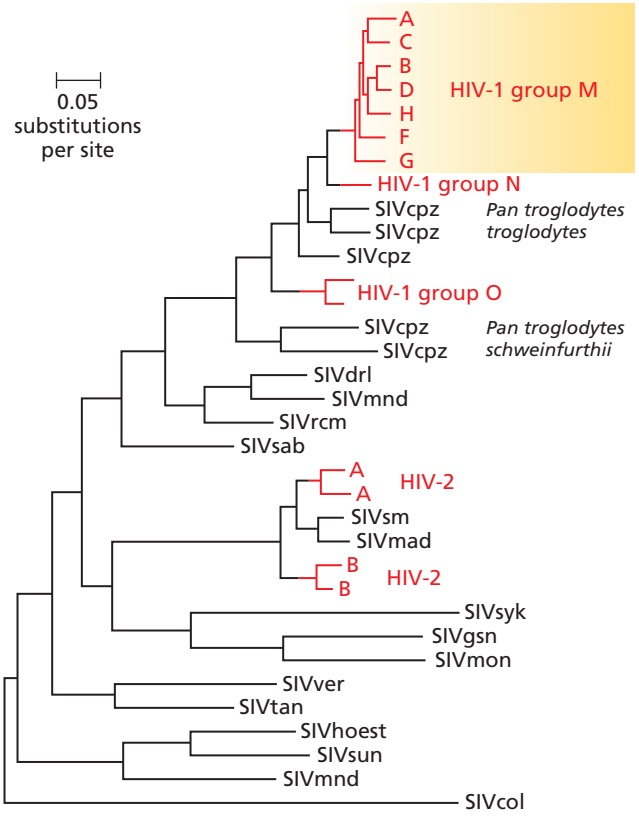


Figure 7.2 Phylogenetic relationships among primate lentiviruses. The tree was reconstructed by computational methods and alignment of 34 published nucleotide sequences of the retroviral *pol* genes, taken from GenBank. SIVcol signifies black and white colobus; SIVdrl, drill; SIVgsn, greater spot-nosed monkey; SIVlhoest, L'Hoest monkey; SIVmac, macaque; SIVmnd, mandrill; SIVmon, Campbell's mona monkey; SIVrcm, red-capped monkey; SIVsab, Sabaeus monkey; SIVsun, sun-tailed monkey; SIVsyk, Sykes' monkey; SIVtan, tantalus monkey; SIVver, vervet monkey; SIVcpz, chimpanzee. The species of transmission from chimps to humans, giving rise to HIV-1 groups M, N, and O, are identified. For clarity, only some subtypes of HIV-1 and HIV-2 are shown. From Figure 2 of A. Rambaut, D. Posada, K. A. Crandall, and C. Holmes, The causes and consequences of HIV evolution. *Nat Rev Genet* **5**:52–56, 2004, with permission.

Distinctive Features of the HIV Reproduction Cycle and the Functions of Auxiliary Proteins

Much of what we know about the function of the auxiliary proteins of HIV comes from studies of their effects on cells in culture, often produced transiently from plasmid expression vectors in the absence of other viral components (Volume I, Box 8.8). Although these methods are simple and sensitive, they do not necessarily reproduce the conditions of viral infection. Preparation and analysis of viral mutants have also been used to investigate the functions of these proteins in cell culture. However, as the hosts for these viruses are humans, it is difficult to evaluate the significance of many of the functions deduced from cell culture to pathology in the whole organism.

The Regulatory Proteins Tat and Rev

Tat (for transactivator of transcription) stimulates processive transcription. As in all retroviruses, expression of integrated HIV DNA is regulated by sequences in the transcriptional control region of the viral long terminal repeat (LTR), which are recognized by the cellular transcriptional machinery. The HIV LTR functions as a promoter in a variety of cell types, but its basal level is very low. As described in Volume I, Chapter 8 (Fig. 8.10), the LTR of HIV includes an enhancer sequence that binds a number of cell type-specific transcriptional activators, among them Nf-κb (Volume I, Fig. 8.11). The fact that Nf-κb enters the nucleus to promote T cell activation may explain why HIV reproduction requires T cell stimulation.

Just downstream of the site of initiation of transcription in the HIV LTR is a unique viral regulatory sequence, the *trans*-activating response element TAR (Fig. 7.4). TAR RNA forms a stable, bulged stem-loop structure that binds Tat together with a number of host proteins (Volume 1, Chapter 8, Fig. 8.13). The principal role of Tat is to stimulate processivity of transcription and thereby facilitate the elongation of viral RNA.

BOX 7.2

BACKGROUND

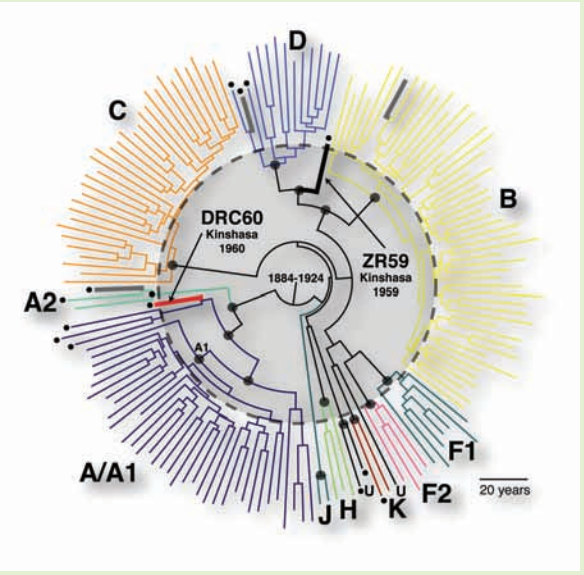
The earliest record of HIV-1 infection

For some time, the earliest record of HIV-1 infection came from a serum sample obtained in 1959 from a Bantu male in the city of Leopoldville, now known as Kinshasa, in the Democratic Republic of Congo (DRC). Phylogenetic analyses placed this viral sequence (ZR59) near the ancestral node of clades B and D. As this is not at the base of the M group, this group must have originated earlier, and back calculations suggested that the M group of viruses arose via cross-species transmission from a chimpanzee into the African population around 1930. Subsequent characterization of viral sequences in a paraffin-embedded lymph node biopsy specimen prepared in 1960 from another individual in Kinshasa (DRC60) led to a revision of that estimate. The ZR59 and DRC60 sequences differ by a degree (12%) seen in the most divergent strains within subtypes. Results from a variety of statistical analyses with these and additional archived samples indicate that the epidemic was well established by 1959/1960 and that the common ancestor was probably circulating as early as 1910. The initial transmission event could have occurred even earlier. Because the human strains shared a common ancestor with the chimp strains in about 1850, the period between ~1850 and ~1910 is the most likely window for the fateful first jump of what became the pandemic HIV/AIDS lineage. As the greatest diversity of group M subtypes has been found in Kinshasa, it seems likely that all of the early diversification of HIV-1 group M viruses occurred in the Leopoldville area, which was one of the largest urban centers at the time.

Sharp PM, Hahn BH. 2008. Prehistory of HIV. *Nature* **455:**605–606. (A personal account of the efforts to determine the routes of transmission from primates to humans can be found in an interview with Dr. Beatrice Hahn: http://bit.ly/Virology_Hahn)

Worobey M, Gemmel M, Teuwen DE, Haselkorn T, Kunstman K, Bunce M, Muyembe J-J, Kabongo J-MM, Kalengayi RM, Van Marck E, Gilbert MTP, Wolinsky SM. 2008. Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960. *Nature* **455:**661–665.

Branch lengths are depicted in unit time (years) and represent the median of those nodes that were present in at least 50% of the sampled trees. DRC60 (red), ZR59 (black), and the three control sequences from paraffin-embedded specimens from known AIDS patients (gray) are depicted in bold. Sequences sampled in the DRC are highlighted with a bullet at the tip. Nodes (sub-subtype and deeper) are marked with gray circles. DRC60 and the two control sequences from the DRC each form monophyletic clades with previously published sequences from the DRC, whereas the Canadian control sequence clusters, as expected, with subtype B sequences. Unclassified strains are labeled U. The dashed circle and shaded area show the extensive HIV-1 diversity in Kinshasa in the 1950s. From Fig. 2 of M. Worobey et al. *Nature* **455:**661–664, 2008, with permission.



Tat is released by infected cells; it can then be taken up by other cells and influence their function. Tat can act as a chemo-attractant for monocytes, basophils, and mast cells. It also induces synthesis of a variety of important proteins in the cells that it enters, and some of these can have a profound effect on virus spread and immune cell function. For example, in transient-expression assays Tat can stimulate the expression of genes encoding the CXCr4 and CCr5 coreceptors in target cells and also enhance the synthesis of a number of chemokines. The Tat protein is cytotoxic to some cultured cells and is neurotoxic when inoculated intracerebrally into mice.

Multiple splice sites and the function of Rev. In contrast to the oncogenic retroviruses with simpler genomes, the full-length HIV transcript contains numerous 5′ and 3′ splice sites. The regulatory proteins Tat and Rev (for regulator of expression of virion proteins) and the accessory protein Nef are synthesized early in infection from multiply spliced mRNAs (Volume 1, Appendix Fig. 29). As Tat then stimulates transcription, these mRNAs are found in abundance at this early time. However, accumulation of the Rev protein brings about a change in the pattern of mRNAs, leading to a temporal shift in viral gene expression.

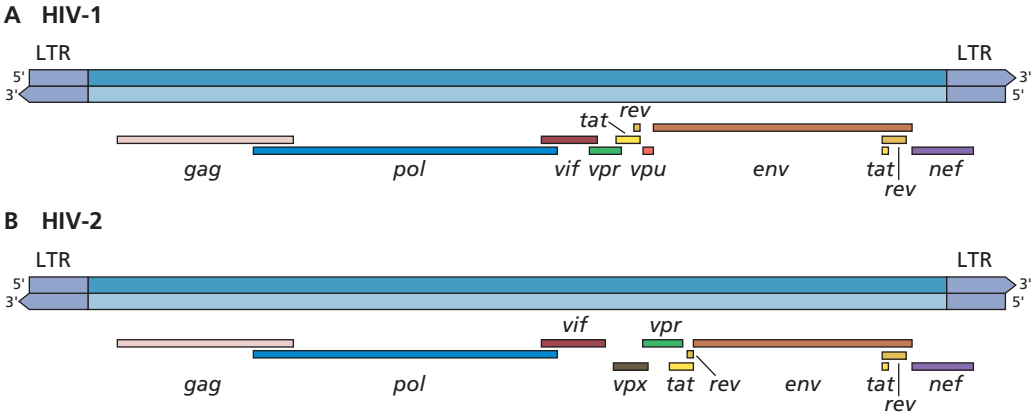
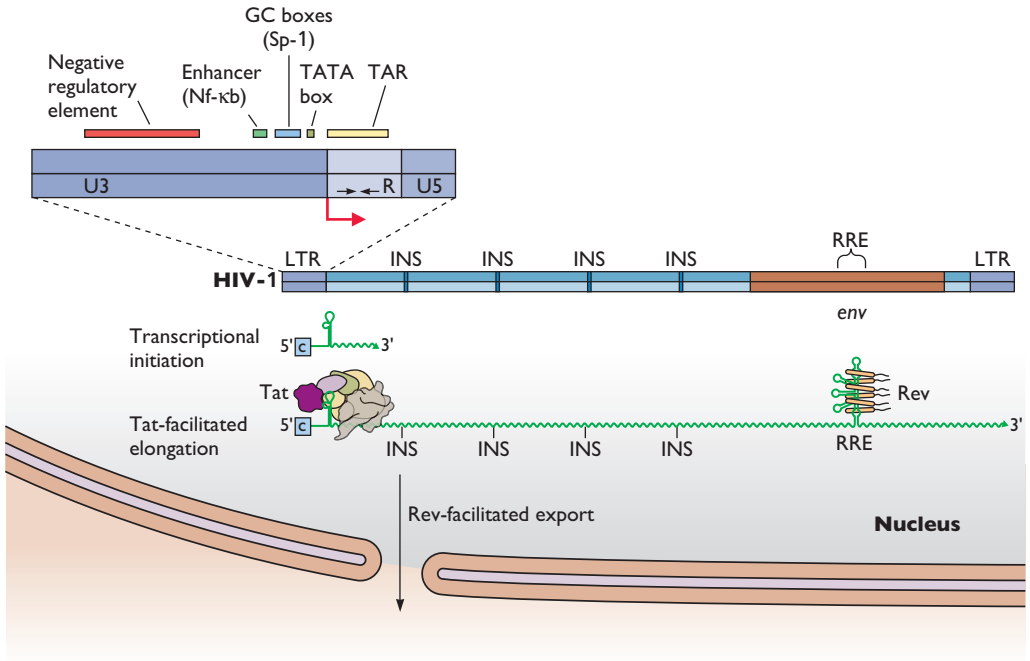


Figure 7.3 Organization of HIV-1 (A) and HIV-2 proviral DNA (B). Vertical positions of the colored bars denote each of the three different reading frames that encode viral proteins. The LTRs contain sequences necessary for transcriptional initiation and termination, reverse transcription, and integration.

Rev is an RNA-binding protein that recognizes a specific sequence within a structural element in the *env* region of the elongated transcript, called the **Rev-responsive element (RRE)** (Fig. 7.4). Rev mediates the nuclear export of any RRE-containing RNA by a mechanism discussed more fully in Volume I, Chapter 10 (Figs. 10.14 to 10.16). As the concentration of Rev increases, unspliced or singly spliced transcripts containing the RRE are exported from the nucleus. In this way, Rev promotes synthesis of the viral structural proteins and enzymes and ensures the availability of full-length

Figure 7.4 Mechanisms of Tat and Rev function. Some regulatory sequences in the HIV LTR are depicted in the expanded section at the top. The numbers refer to positions relative to the site of initiation of transcription. The opposing arrows in R represent a palindromic sequence that folds into a stem-loop structure (TAR) in the transcribed mRNA to which Tat binds (center). Tat recruits cellular proteins that are required for efficient elongation during HIV-1 RNA synthesis. The position of the RRE in the *env* transcript (with bound Rev dimers) and the *cis*-acting repressive sequences (instability sequences, INS) in the unspliced or singly spliced transcripts are also illustrated. Mutations in the A+U-rich INS increase the stability, nuclear export, and translation efficiency of the transcripts in the absence of Rev. Response to INS appears to be cell type-dependent, but the mechanisms by which they act, and exactly how Rev counteracts their effects, are not understood.



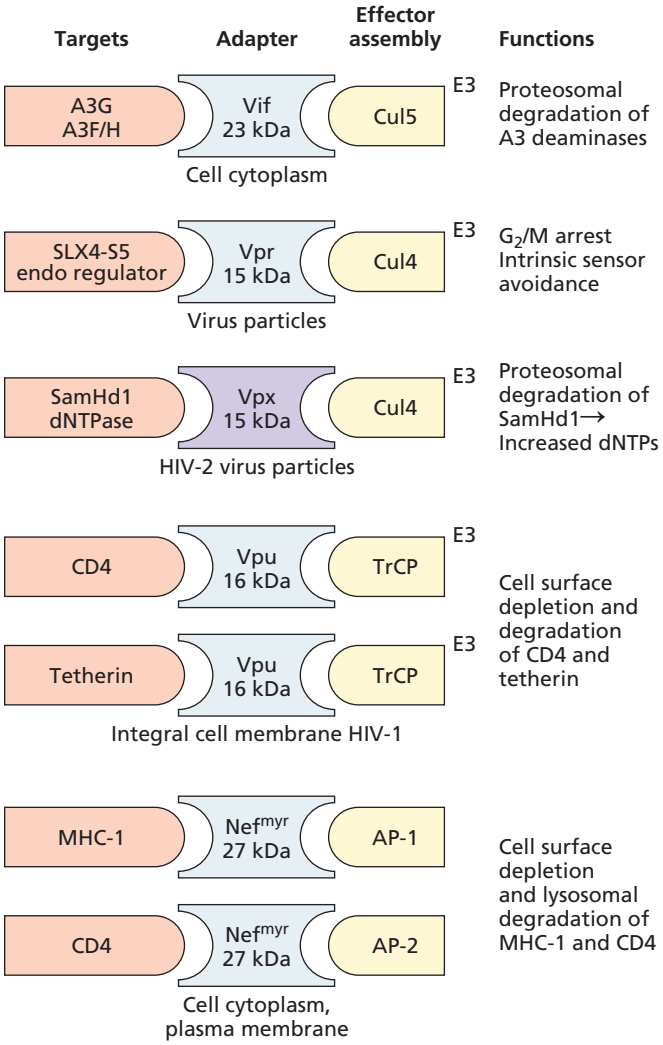


Figure 7.5 Adapter functions of HIV-1 accessory proteins. The major targets for the HIV accessory proteins, their locations, and the effector assemblies with which they interact are noted. Most effector assemblies function by mediating destruction of the targets via reactions in which the viral proteins are recycled: In a reaction that requires binding of the transcription regulator, cellular core binding factor beta (Cbfβ), **Vif** assembles with additional cellular proteins (Cul5, Elongins B and C, and Rbx1) in an E3 ubiquitin ligase that then targets Apobec proteins (A3G, F, and H) for ubiquitination and proteosomal degradation. While less potent than A3G, A3F and A3H have antiviral activities and are produced in abundance in lymphoid cells (Chapter 3). **Vpr** functions in a similar way, but interacts with a different member of the cullin family (Cul 4), in a damaged DNA-binding protein (Ddb1) and Cul-associated factor 1 (Dcaf1)-E3-ligase assembly. This interaction leads to dysregulation of cellular endonuclease activities, triggering a DNA damage response and cell cycle arrest. The **Vpx** protein of HIV-2 engages the same Cul 4-E3 complex as Vpr, but their targets are quite distinct: Vpx targets the dGTP-dependent deoxynucleoside triphosphohydrolase, SamHd1, for ubiquitination and proteosomal degradation. Phosphorylation of **Vpu** leads to recruitment of another multiprotein E3 ubiquitin ligase, Scf (which includes Cul1, Skp1, and Roc1) via interaction with the adapter, β-transducin repeat-containing protein (β-TrCP), and targets both CD4 and tetherin degradation. **Nef** binds to the cytoplasmic tail of CD4 and links this receptor to components of a clathrin-dependent trafficking pathway at the plasma membrane, leading to its internalization and delivery to lysosomes for degradation. Nef decreases cell surface expression of MHC class I molecules by a different pathway, mediating an interaction between the cytoplasmic domain of the MHC class I molecules and the clathrin adapter protein complex (AP-1).

genomic RNA for incorporation into progeny virus particles. The accessory proteins Vif, Vpr, and Vpu (for HIV-1) or Vif, Vpr, and Vpx (for HIV-2) are also produced later in infection from singly spliced mRNAs that are dependent on Rev for export to the cytoplasm (Volume I, Appendix Fig. 29).

The Accessory Proteins

While a very large number of seemingly disparate activities have been attributed to the HIV accessory proteins, recent studies have revealed a common mechanism for their action: all are **adapter proteins** that disrupt the normal trafficking of particular cellular proteins and, in most cases, lead to their destruction (Fig. 7.5). In this way, HIV accessory proteins function as antagonists of cellular intrinsic defense mechanisms that detect infection and counteract virus reproduction.

Vif protein. Vif (viral infectivity factor) is a 23-kDa protein that accumulates in the cytoplasm and at the plasma membrane of infected cells. Early studies showed that mutant virus particles lacking the *vif* gene were approximately 1,000 times less infectious than the wild type in certain $CD4^+$ T cell lines and peripheral blood lymphocytes and macrophages. It was discovered that production of Vif from a plasmid vector in susceptible host cells did not compensate for its absence in the cell that produced virus particles. Rather, Vif was needed at the time of virus assembly.

Vif is an RNA-binding protein and small quantities can be detected in HIV particles. Virus particles produced from *vif*-defective HIV genomes contain the normal complement of progeny RNA, and they are able to enter susceptible cells and to initiate reverse transcription, but full-length double-stranded viral DNA is not detected. These observations demonstrated that Vif is required in a step that is essential for completion of reverse transcription. The requirement for Vif is strikingly cell type-dependent, and experiments in which cells that are permissive for *vif* mutants were fused with cells that are nonpermissive established that the nonpermissive phenotype is dominant. The infectivity of virus particles produced in such heterokaryons was enhanced by Vif production. This observation suggested that Vif may suppress a host cell function that otherwise inhibits progeny virus infectivity.

All of these seemingly unusual properties were demystified with the discovery that Vif blocks the antiviral action of members of an RNA-binding family of cellular cytidine deaminases, called apolipoprotein B mRNA editing enzyme catalytic peptides 3 (Apobec3). In nonpermissive cells, these enzymes are incorporated into progeny virus particles via interactions with

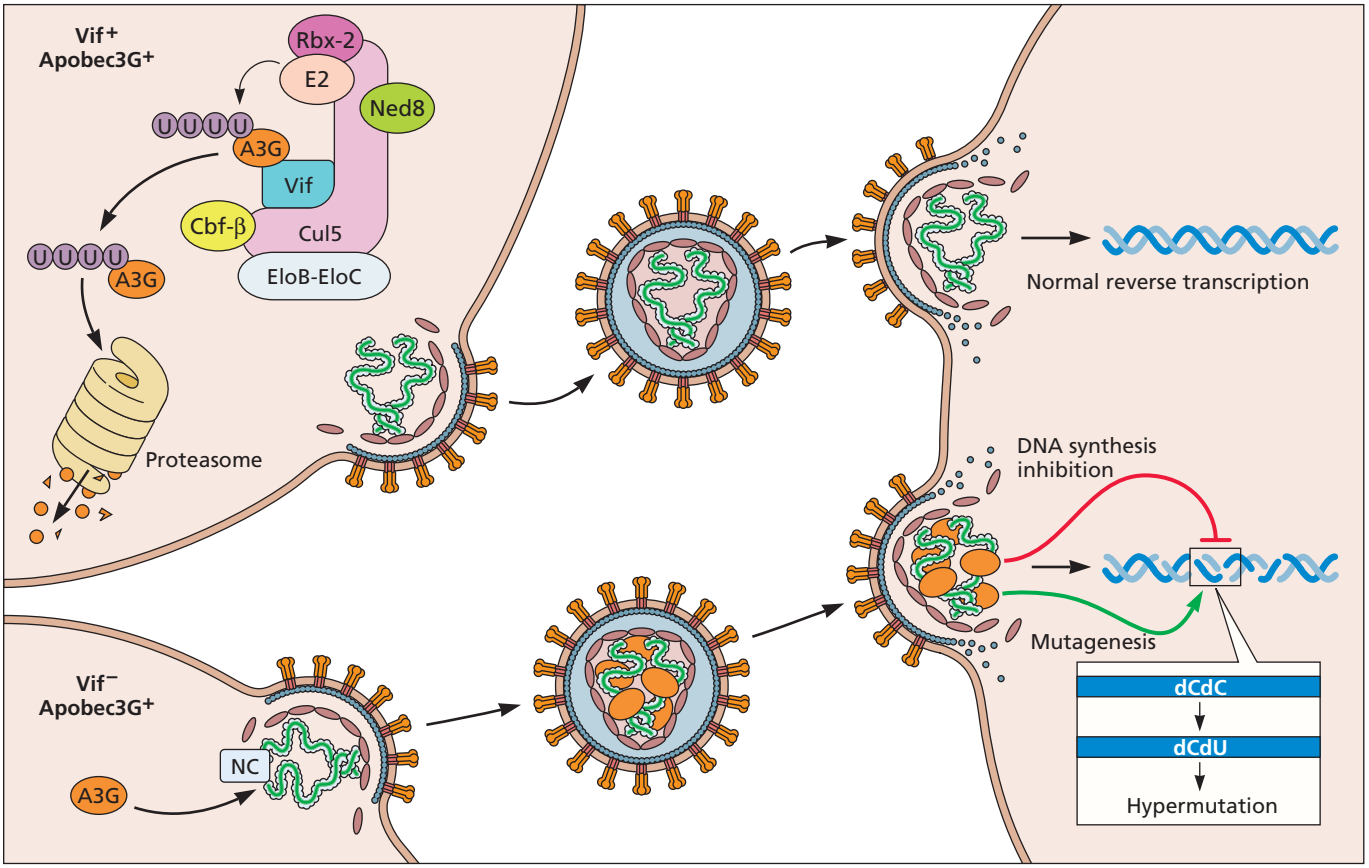


Figure 7.6 Mechanisms of action of Vif and Apobec3G. **(Top)** Vif counteracts the antiviral effects of Apobec3G (A3G) by mediating its polyubiquitination, which leads to proteosomal degradation. **(Bottom)** In the absence of Vif, A3G is incorporated into newly formed virus particles through interaction between viral RNA and NC protein. In the newly infected cell, reverse transcription is inhibited by A3G, and cytosines in the newly synthesized DNA are converted to uracil, causing hypermutation through eventual C to A transversions. Adapted from B. Cullen, *J Virol* **80:**1067–1076, 2006, with permission.

the viral RNA and possibly NC protein (Fig. 7.6). Apobec3G was the first family member to be identified as a Vif target. It was subsequently shown that Vif prevents its incorporation into virus particles by binding to the protein and inducing its depletion. In a reaction that requires binding of a transcriptional regulator, Vif assembles with additional cellular proteins to form an E3 ubiquitin ligase that recognizes Apobec3G as a substrate for polyubiquitination, a signal for its subsequent degradation in proteasomes (Fig. 7.7).

Apobec3G appears to inhibit virus reproduction in a number of ways. It has been proposed that binding to viral RNA may account, in part, for the deaminase-independent inhibition of reverse transcription in newly infected cells. The deaminase activity of Apobec3G leads to formation of deoxyuridine (dU), most frequently at preferred deoxycytidine (dC) sites in the first (−) strand of viral DNA to be synthesized by reverse transcriptase. Consequently, the (+) strand complement of the deaminated (−) strand will contain deoxyadenosine in place of the normal deoxyguanosine at such sites (Fig.7.6). Indeed, the frequency of G→A transitions is abnormally high in the genomes of *vif*-defective particles produced in nonpermissive cells, and incomplete protection from Apobec3 proteins by Vif may explain why such transitions are the most frequent point mutations in HIV genomes. It has been suggested that the Apobec3 proteins represent an ancient intrinsic cellular defense against retroviruses (see Chapter 3).

Vpr protein. The 15-kDa viral protein R, Vpr, derives its name from the early observation that it affects the **rapidity** with which the virus reproduces in, and destroys, T cells. Most T cell-adapted strains of HIV-1 carry mutations in *vpr*. The SIV and HIV-2 genomes include a second, related gene *vpx,* which is discussed below and appears to have arisen as a duplication of *vpr*. The other lentiviruses do not contain sequences related to *vpr* but do include small open reading frames that might encode proteins with similar functions.

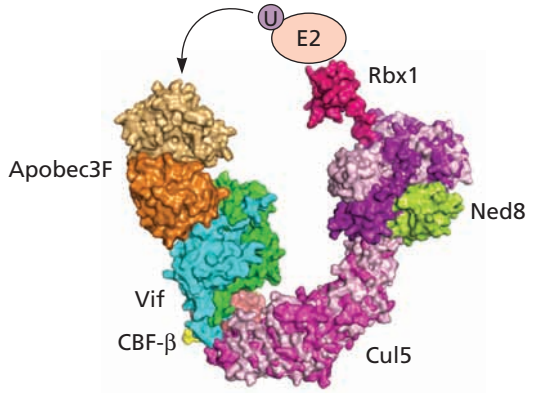


Figure 7.7. Molecular model for Apobec3F degradation by the Vif/Cbf-β ubiquitin ligase. Existing crystal structures of analogous proteins and complexes were used to model the Apobec3F degradation complex. In the model, Vif (green and blue) functions as a scaffold to assemble the E3 ligase by binding to Cbf-β (yellow and pink) and to Elo C and Cul5 (N-terminal domain). Activation of the E3 ligase is thought to occur by neddylation by Ned8 (lime green) of a lysine on the Cul5 (C-terminal domain). Subsequently, Rbx1 (magenta) adopts a conformation that facilitates polyubiquitination of Vif-bound Apobec3F (N-terminal domain, light orange and C-terminal domain, dark orange) via a cellular E2 ubiquitination enzyme. The model and Movie 7.1 was created by Drs. Nadine Shaban and Reuben Harris, University of Minnesota. For more details see J. S. Albin et al., A structural model of the APOBEC3F-Vif interaction informed by biological and computational studies. In preparation.

Vpr is incorporated into HIV-1 particles via specific interactions with a proline-rich domain at the C terminus of the Gag polyprotein. The host's uracil DNA glycosylase, Ung2, is then incorporated into particles by binding to Vpr. A substantial quantity, about 100 to 200 molecules of Vpr, is present in capsids. Its presence in virus particles is consistent with the observation that Vpr function is required at an early stage in the virus reproduction cycle.

Like Vif, Vpr functions as an adapter protein in an E3-ligase, but via interaction with a different member of the cullin family (Cul4, Fig. 7.5). Proteomic and biochemical studies have demonstrated that Vpr in this protein assembly facilitates recruitment of the cellular Slx4 structure-specific endonuclease regulator and untimely activation of the endonuclease activities that it controls. Such unleashed endonuclease activity could lead to the degradation of excess viral DNA, and it has been proposed that this feature may prevent detection by the intrinsic immune system (Chapter 3) and limit a defensive interferon response at early times after infection. Loss of endonuclease regulation at replication forks in the host DNA induces a damage response that may explain the G2/M arrest and apoptosis responses that are known to be triggered by Vpr. The possible advantage of preventing infected cells from entering mitosis is not apparent, especially as the requirement for Vpr function is most evident in HIV infection of macrophages, cells that do not divide. One idea is that the increased activity of the LTR promoters in the G_2 phase of the cell cycle may lead to enhanced virus production in the presence of Vpr.

In addition to cell cycle arrest and apoptosis, numerous other functions and interactions have been ascribed to this tiny protein, including modulation of the transcription of host and viral genes, maintenance of reverse transcriptase fidelity, recruitment of Ung2, and nuclear import of preintegration complexes in nondividing cells. Vpr has been shown to bind to nuclear pore proteins. Although these interactions are not essential for nuclear import, they may facilitate docking of the HIV-1 preintegration complex at the nuclear pore in preparation for import (Volume I, Chapter 5).

Vpx protein. Vpx is also packaged specifically via interaction with the Gag polyprotein. Vpx functions as an adapter that engages the same Cul4-E3 ubiquitin ligase as Vpr, but targets quite distinct proteins: Vpx recruits the dGTP-dependent deoxynucleoside triphosphohydrolase, SamHd1, for ubiquitination and proteosomal degradation (Fig. 7.5). SamHd1 blocks lentiviral DNA synthesis in myeloid cells by hydrolyzing cellular deoxynucleotide triphosphates to reduce concentrations to below those required for reverse transcription. The finding that Vpx can mediate degradation of this enzyme helped to explain why HIV-2 but not HIV-1 can propagate efficiently in macrophages or dendritic cells. Indeed, ectopic production of Vpx enhances HIV-1 infection in myeloid and $CD4^+$ T cells, as does RNA interference-mediated knockdown of SamHd1.

Vpu protein. This small, 16-kDa viral protein is unique to HIV-1 and the related SIV_{cpz} (Fig. 7.3), hence the name viral protein U. The predicted sequence of Vpu includes an N-terminal stretch of 27 hydrophobic amino acids comprising a membrane-spanning domain and a cytoplasmic domain containing two α-helices. Biochemical studies show that Vpu is an integral membrane protein that self-associates to form oligomers. In infected cells, the protein is located on all major membranes, but is concentrated mainly in the endoplasmic reticulum, the *trans*-Golgi network, and endosomes.

Synthesis of Vpu is required for the proper maturation and targeting of progeny virus particles and for their efficient release. In its absence, particles containing multiple cores are produced, and budding is targeted to multivesicular bodies rather than to the plasma membrane (Fig 7.8). Vpu also reduces the syncytium-mediated cytopathogenicity of HIV-1, perhaps because the inefficient release of virus particles prevents the accumulation of sufficient Env protein at the cell surface to promote cell fusion.

A major function of Vpu in the pandemic group M strains of HIV-1 is to block the activity of a cellular membrane protein initially called bone stromal antigen 2 (Bst-2), but now more commonly known by the more descriptive name, tetherin. Tetherin is a dimeric type II membrane protein with an N-terminal cytoplasmic tail, a transmembrane region, and a C-terminal glycophosphatidyl inositol membrane anchor.

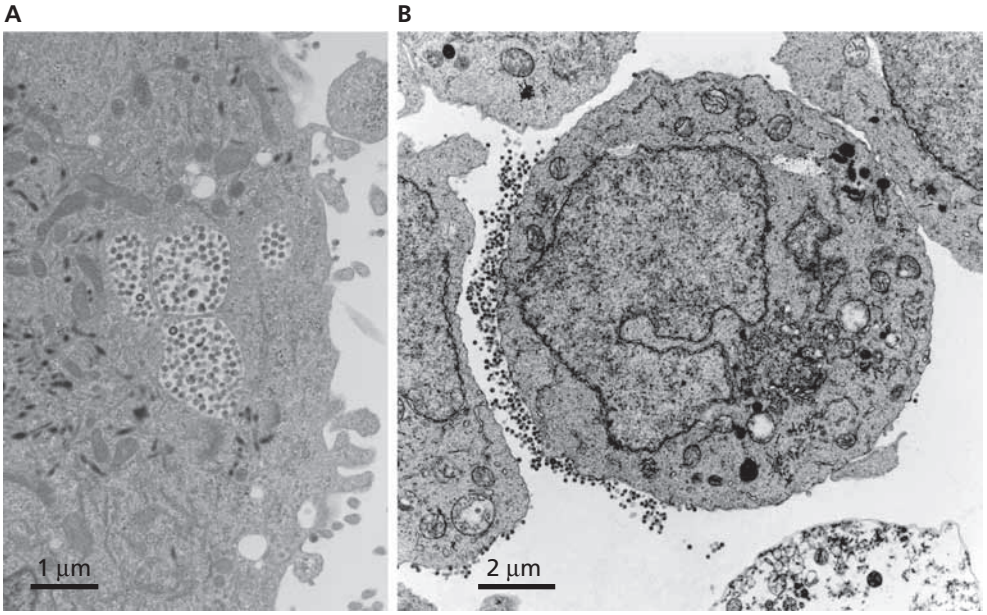


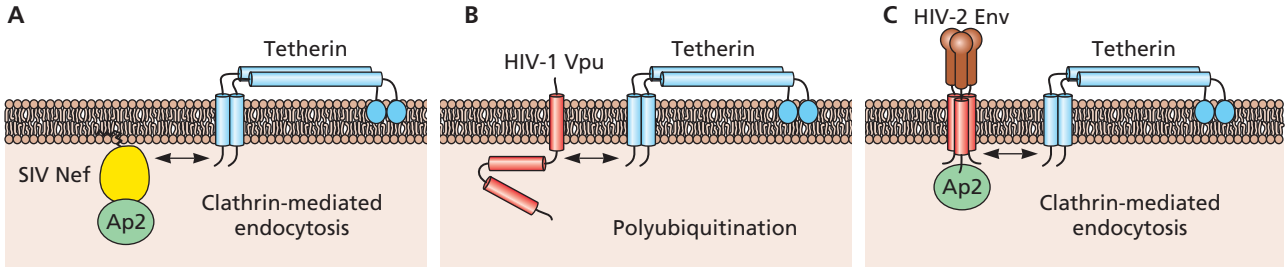
Figure 7.8 Human cells infected with an HIV-1 virus lacking Vpu. (A) Electron microscope image of a human macrophage showing intracellular accumulation of virus particles. **(B)** Electron microscope image of an infected H9 T cell showing accumulation of virus particles at the cell surface. Images courtesy of Drs. Jaang-Jiun Wang and Paul Spearman.

An interferon-inducible protein, tetherin restricts the propagation of enveloped viruses by inhibiting their release from infected cells (Fig. 7.9) and has been shown to act as a pattern-recognition receptor that induces Nf-κb-dependent proinflammatory gene expression in infected cells (Chapter 3).

Several residues in the transmembrane domain of Vpu interact directly with the transmembrane domain of tetherin. Vpu binds tetherin in the *trans*-Golgi network, inhibiting the transport of tetherin to the plasma membrane. Phosphorylation of the Vpr then leads to recruitment of another multiprotein E3 ubiquitin ligase, Scf, and the subsequent ubiquitinylation and degradation of tetherin in an endolysosomal pathway (Fig. 7.5). Vpu also facilitates the degradation of CD4: the viral protein traps newly formed CD4 receptor molecules in the endoplasmic reticulum, mediates ubiquitinylation by the Scf ubiquitin ligase, and entry of CD4 into the endoplasmic reticulum-associated proteasome degradation pathway. Reducing the quantity of CD4 at the cell surface limits superinfection by HIV-1. It also reduces loss of Env protein via CD4 binding, thereby enhancing production of infectious particles.

Oligomerization of the membrane-spanning domains of Vpu is the basis of yet another property of Vpu, namely formation of an ion-conducting channel known as a **viroporin**, similar to that of the influenza A virus protein M2. While cell membrane integrity is disrupted and permeability to small molecules is increased when Vpr is produced in *Escherichia coli* or cultured mammalian cells, the relevance of Vpr viroporin activity to HIV pathogenesis is still unclear. Such activity could certainly affect the function of internal membranes, which are the major sites of Vpr accumulation. It has also been proposed that virus particle release may be enhanced by changes in the membrane potential across the budding plasma membrane.

Figure 7.9 Antagonism by viral proteins. Illustration of ways in which simian immunodeficiency virus (SIV) Nef protein **(A)**, HIV-1 Vpu **(B)**, and HIV-2 Env **(C)** target the cellular protein tetherin. Protein interactions are indicated by the double-headed arrows; Ap2, clathrin adapter protein complex-2.



Genetic studies and experiments with a viroporin-specific inhibitor (BIT225) indicate that the ion channel and tetherin-antagonizing activities of the membrane-spanning domain of Vpu are independent of one another.

One might wonder: if tetherin antagonism is so important to HIV reproduction, why is Vpu only found in HIV-1? The answer is that different retroviral proteins have assumed this function during evolution (Fig. 7.9). The envelope protein of HIV-2 has evolved to include this function and the Nef proteins of several primate viruses are antagonists of the tetherin orthologs in their host species

Nef protein. Most laboratory strains of HIV-1, which have been adapted to grow well in T cell lines, contain deletions or other mutations in the *nef* gene. Restoration of *nef* reduces the efficiency of virus reproduction in these cells, hence the name "negative factor." Multiple functions have been attributed to Nef, and it is now clear that its importance may vary in different cell types.

Nef is translated from multiply spliced early transcripts. The 5′ end of Nef mRNA includes two initiation codons and, as both are utilized, two forms of Nef are produced in infected cells. The apparent size of these proteins can vary because of differences in posttranslational modification. Like Vpr, Nef is incorporated into virus particles via interaction with the Gag polyprotein. Nef molecules in virus particles appear to contribute to capsid disassembly following infection and may also enhance reverse transcription. The protein is myristoylated posttranslationally at its N terminus and thereby anchored to the inner surface of the plasma membrane.

Nef includes a protein-protein interaction domain (SH3) that mediates binding to components of intracellular signaling pathways, eliciting a program of gene expression similar to that observed after T cell activation. Such gene expression is thought to provide an optimal environment for viral reproduction. Among the best-studied, and clearly physiologically relevant activities of Nef is its downregulation of surface concentrations of CD4 and major histocompatibility complex (MHC) class I molecules (Fig. 7.5). The former activity is shared with Vpu. At the plasma membrane Nef binds to the cytoplasmic tail of CD4 and components of a clathrin-dependent trafficking pathway, leading to its internalization and delivery to lysosomes for degradation (Fig. 7.10, left). As with Vpu, the ensuing reduction in the number of CD4 molecules at the cell surface facilitates virus particle release and limits reinfection.

Nef decreases cell surface MHC class I molecules by a different pathway. It mediates interaction between the cytoplasmic domain of the MHC class I molecules and the clathrin adapter protein complex (AP-1) in the *trans*-Golgi network, prior to their transport to the cell surface (Fig. 7.10, right). The Nef-induced complex is retained in this Golgi compartment and MHC class I molecules are subsequently diverted to lysosomes for degradation. As a strong cytotoxic T lymphocyte (CTL) response against viral infection requires

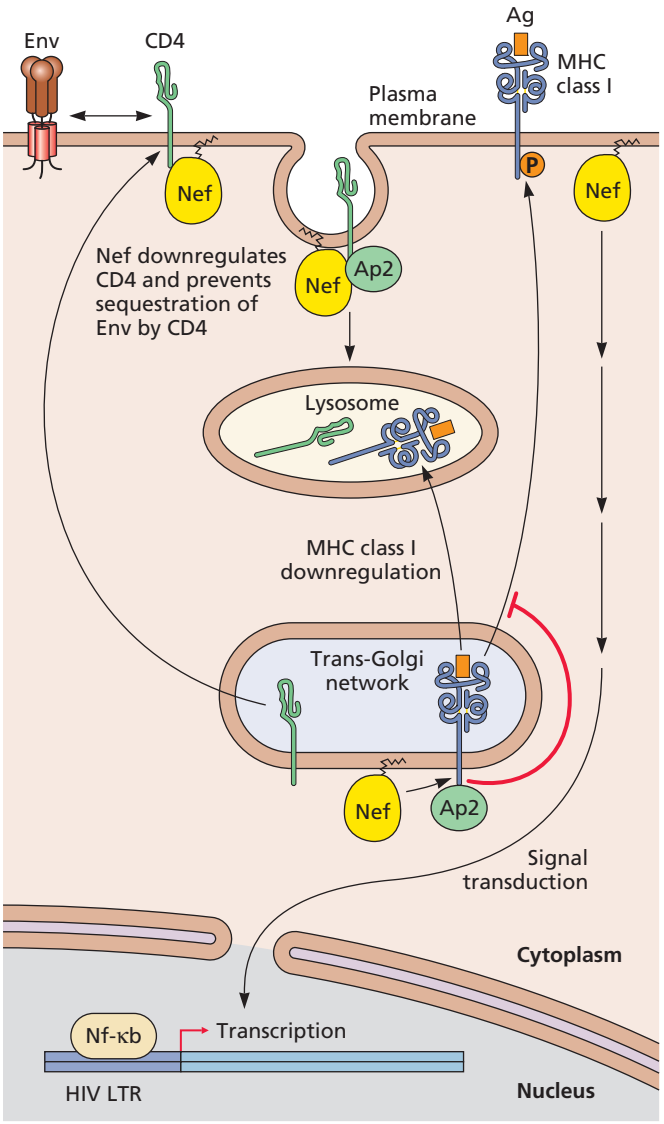


Figure 7.10 Intracellular functions attributed to Nef. Nef is myristoylated posttranslationally; the jagged protrusion represents myristic acid covalently linked to the glycine residue at position 2. Myristoylation enables Nef to attach to cell membranes, where it can interact with membrane-bound cellular proteins. Nef reduces the cell surface expression of CD4 by binding to sequences in the cytoplasmic domain of this receptor and enhancing clathrin-dependent endocytosis and the subsequent degradation of CD4 within lysosomes; Ap2, clathrin adapter protein complex-2. This activity reduces interaction of CD4 with surface Env proteins, thereby enhancing Env incorporation into budding virus particles. In contrast, MHC class I expression on the cell surface is reduced by Nef binding in the membrane of the *trans*-Golgi network. This interaction interferes with the normal vesicular sorting required for passage of the receptor to the cell surface, and MHC class I is directed to the lysosome for degradation. Nef also affects signal transduction by increasing the activity of the cellular transcriptional activator Nf-κb and perhaps other cellular transcription proteins.

recognition of viral epitopes presented by MHC class I molecules, this inhibitory activity of Nef allows infected cells to escape lysis by CTLs and is probably a major factor contributing to HIV-1 pathogenesis. Nef induces decreased concentrations of a number of other cell surface molecules including a component of the T cell receptor complex (CD3), the lymphocyte-specific protein tyrosine kinase (Lck), and the costimulatory molecule for T cell activation (CD28). These activities of Nef contribute to T cell activation and recognition of infected cells by cells of the immune system (Chapter 4).

Other activities ascribed to Nef also seem likely to contribute to pathogenesis in important ways. One example is Nef-mediated inhibition of endocytosis of the type II transmembrane lectin, DC-Sign (dendritic-cell-specific, Icam-3-grabbing nonintegrin), which binds to the HIV-1 envelope protein with high affinity. Such inhibition by Nef leads to **increased** concentration of this lectin on the surfaces of immature and mature dendritic cells. DC-Sign facilitates dendritic cell transmigration through the vascular and lymphoid endothelium, as well as the adhesion of these cells to T cells during antigen presentation. Studies with dendritic cells infected with HIV-1 have shown that such cells form many more clusters with activated primary T cells than dendritic cells infected with a *nef*− HIV-1 mutant. As dendritic cells can retain attached infectious virus particles for several days, the increase in surface accumulation of DC-Sign cell surface concentration induced by Nef may be important for both viral spread and transmission to T cells.

Although the initial cell culture experiments suggested a negative effect on virus production, subsequent experiments with animals showed that Nef augments HIV pathogenesis quite significantly. Rhesus macaques inoculated with a Nef-defective mutant of SIV had low virus titers in their blood during early stages of infection, and the later appearance of high titers was associated with reversion of the mutation. More importantly, adult macaques inoculated with a virus strain containing a deletion of *nef* did not progress to clinical disease and were, in fact, immune to subsequent challenge with wild-type virus. The observation that *nef* had been deleted in HIV-1 isolates from some individuals who remained asymptomatic for long periods also suggests that this viral protein can contribute to pathogenesis. Initial hopes that intentional deletion of *nef* might facilitate the development of a vaccine strain for humans were dashed when it was discovered that the humans infected with *nef* deletion mutants eventually developed AIDS.

The Viral Capsid Counters Intrinsic Defense Mechanisms

Following entry of HIV-1, capsid proteins remain associated with the reverse transcription machinery (Volume I, Chapter 7). This subviral structure moves through the cytoplasm to the nuclear pore via interaction with the host cell cytoskeletal fibers, as viral DNA is synthesized and an integration-competent nucleoprotein assembly is formed. Genetic and biochemical studies have identified two host proteins that bind to the HIV-1 capsid protein, Cpsf 6 (a cleavage and polyadenylation factor) and CypA (the peptidyl-prolyl isomerase cyclophilin A). Such binding imparts stability to the capsid structure and helps to suppress its premature disassembly by host proteins such as Trim5α and TrimCypA. The stabilized capsid structure also shields viral nucleic acids from detection by intrinsic immune sensors in the cytoplasm, such as double-stranded RNA helicase, Rig-I (viral RNA), and the cyclic GMP-AMP synthase, cGAs (viral DNA) (Chapter 3). The biological importance of these capsid protein interactions early in infection is emphasized by the finding that HIV-1 mutants with capsids that are either fragile, or abnormally stable, are replication defective.

Capsid protection of viral nucleic acids may account for the observation that there is little or no interferon response at early times after HIV infection. At late times, however, when large quantities of viral RNAs are produced, there is a robust interferon response. One interferon-induced human protein, Mx2, was found to be a potent inhibitor of HIV-1 reproduction in certain cell types, particularly macrophages. Mx2 also targets the HIV-1 capsid, inhibiting nuclear import of viral DNA by a mechanism yet to be discovered. Mx2 derives its name from its close sequence relationship to the myxovirus resistance 1 protein (Mx1), which is a broadly acting inhibitor of RNA and DNA viruses, including the orthomyxovirus influenza A virus (Chapter 3). However, these proteins have distinct activities as Mx1 is not an inhibitor of HIV-1, and Mx2 is ineffective against influenza A virus.

Cellular Targets

Attachment and entry into host cells depend on the interaction between viral proteins and cellular receptors (Volume I, Chapter 5). While the major receptor for the HIV envelope protein is the cell surface CD4 molecule, the envelope protein must also interact with a coreceptor to trigger fusion of the viral and cellular membranes and gain entry into the cytoplasm. The two major coreceptors for HIV-1 are the α- and β-chemokine receptors, CXCr4 and CCr5. Strains of HIV that bind to CXCr4 or CCr5 are commonly referred to as X4 and R5 strains, respectively (Fig. 7.11). For reasons that are still not completely understood, R5 viruses are transmitted preferentially during infection. X4 viruses predominate in the late stages, following extensive evolution of the virus population within an infected individual and concomitant with immune system breakdown (Box 10.5). The importance of these two chemokine receptors to HIV pathogenesis is demonstrated by two findings. People who carry a particular mutation in the gene encoding CCr5 produce a defective receptor protein and are resistant to HIV-1 infection. So too are individuals who carry a mutation in the gene for the ligand of CXCr4

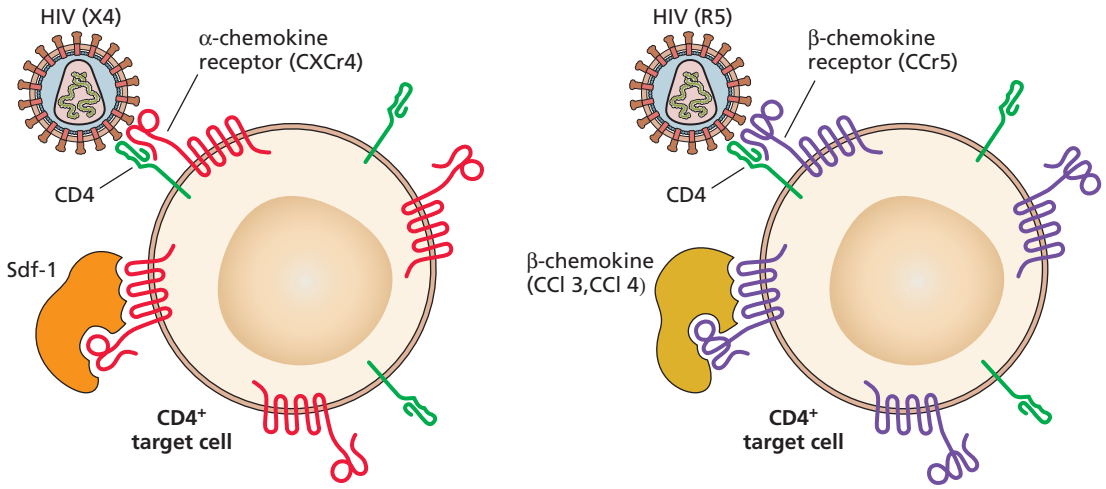


Figure 7.11 Coreceptors for HIV-1. CxCr4 is the coreceptor for HIV-1 variants that predominate during the late phase of infection; entry of such strains (denoted X4) is inhibited by the coreceptor's natural ligand, Sdf-1. CCr5 is the coreceptor for HIV-1 variants that predominate early in infection (denoted R5); their entry is inhibited by the coreceptor's natural ligands, CCl5 (Rantes), and the macrophage inflammatory proteins CCl3 and CCl4 (Mip-1α and Mip-1β). Primary T cells and monocytes can produce both coreceptors. Adapted from Fig. 3 of A. S. Fauci, *Nature* **384:**529–533, 1996, with permission.

(Table 3.7). The latter mutation may lead to increased availability of the ligand, which then blocks virus entry by competing for coreceptor binding. This idea is consistent with earlier observations that chemokine binding to the receptors inhibits the infectivity of specific strains of HIV in cell culture (Fig. 7.11). Cells of the hematopoietic lineage that bear CD4 and one or more of these chemokine receptors are the main targets of infection, and they produce the highest titers of progeny virus particles.

Several additional chemokine receptors have been identified as coreceptors for HIV and SIV in cell culture experiments, but their roles in natural infection remain to be determined. These additional coreceptors may allow the virus to enter a broader range of cells than first appreciated. Some are found on cells of the thymus gland and the brain, and some could play a role in infection in infancy or of cells in the central nervous system. It has also been proposed that binding to these additional coreceptors may trigger signals that affect virus reproduction in target cells, or that harm nonpermissive cells, producing a "bystander" effect.

Experiments in cell cultures have identified additional mechanisms by which HIV may enter cells. For example, the virus can be transmitted very efficiently through direct cell contact. In addition, cells may be infected by virus particles that are endocytosed after binding to cell surface galactosyl ceramide or to Fc receptors (as antibody-virus complexes). HIV can infect many different types of human cells in culture and has been found in small quantities in several tissues of the body. As discussed below, infection of these cells and tissues is likely to be relevant to HIV-1 pathogenesis.

Routes of Transmission

Even before HIV-1 was identified, epidemiologists had established the most likely routes of the agent's transmission to be sexual contact, blood exchange, and from mother to child. As might be anticipated, the efficiency of transmission is influenced greatly by the concentration of the virus particles in the body fluid to which an individual is exposed. Estimates of the percentage of infected cells and the concentration of HIV-1 in different body fluids indicate that highest quantities are observed in peripheral blood monocytes, in blood plasma, and in cerebrospinal fluid (Table 7.2), but semen and female genital secretions also appear to be important sources of the virus.

Other routes of transmission are relatively unimportant or nonexistent. Among these are nonsexual physical contact, exposure to saliva or urine from infected individuals, and exposure to blood-sucking insects. Fortunately, HIV-1 infectivity is reduced upon air-drying (by 90 to 99% within 24 h), by heating (56 to 60°C for 30 min), by exposure to standard germicides (such as 10% bleach or 70% alcohol), or by exposure to pH extremes (e.g., <6 or >10 for 10 min). This information and results from epidemiology studies have been used to establish safety regulations to prevent transmission in the public sector and the health care setting.

Modes of Transmission

Modes of HIV transmission vary in different geographic locations and among different populations within the same locations (Fig. 7.12). In the United States, the major overall route is via homosexual contact, specifically among men who have sex with men. Heterosexual contact is the predominant manner in

Table 7.2 Isolation of infectious HIV-1 from body fluids[a]

Fluid	Virus isolation[b]	Estimated quantity of virus[c]
Cell-free fluid		
Cerebrospinal fluid	21/40	10–10,000
Ear secretions	1/8	5–10
Feces	0/2	None detected
Milk	1/5	<1
Plasma	33/33	1–5,000[d]
Saliva	3/55	<1
Semen	5/15	10–50
Sweat	0/2	None detected
Tears	2/5	<1
Urine	1/5	<1
Vaginal-cervical	5/16	<1
Infected cells		
Bronchial fluid	3/24	Not determined
PBMC	89/92	0.001–1%[d]
Saliva	4/11	<0.01%
Semen	11/28	0.01–5%
Vaginal-cervical fluid	7/16	Not determined

[a]Adapted from Table 2.1 (p. 28) of Levy JA, *HIV and the Pathogenesis of AIDS,* 3rd ed. ASM Press, Washington, DC, 2007.

[b]Number of samples positive/number analyzed.

[c]For cell-free fluid, units are infectious particles per milliliter; for infected cells, units are percentages of total cells capable of releasing virus. Results from studies in the laboratory of J. A. Levy are presented.

[d]High levels associated with acute infection and advanced disease (~5×10^6 PBMCs/ml of blood).

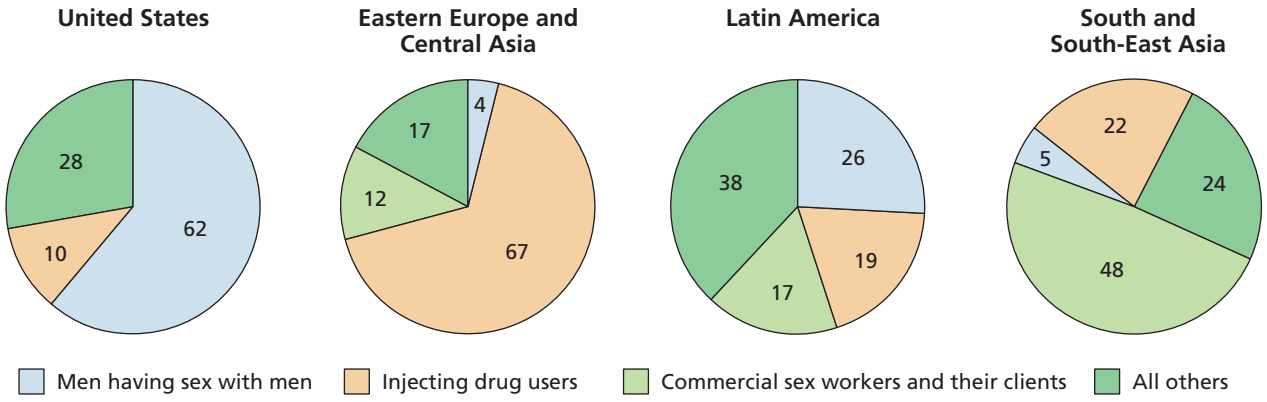
which the virus is transmitted in other parts of the world and also among females in the United States. Transmission of HIV from an infected to an uninfected person is generally characterized as being relatively inefficient. For example, the probability is reported to range from 0.005 to 0.0001 per heterosexual contact. However, a single interaction can be sufficient for transmission if the infected partner is highly viremic. The presence of other sexually transmitted diseases also increases the probability of transmission, presumably because infected inflammatory cells and mediators may be present in both seminal and vaginal fluids. The likelihood of transmission can also be increased by genital ulceration and consequent direct exposure to blood cells, and by antimicrobial peptides that may be produced by cells infected with other sexually transmitted viruses (Box 7.3). In both heterosexual and male homosexual contact, the recipient partner is the one most at risk.

Intravenous drug injection is a common mode of transmission throughout the world, owing to the widespread practice of sharing contaminated needles. Here again, the probability of transmission is a function of the frequency of exposure and the degree of viremia among a drug user's contacts. Of course, sexual partners of drug users are also at increased risk.

Until 1985, when routine HIV antibody testing of donated blood was established in the United States and other industrialized countries, individuals who received blood transfusions or certain blood products, such as clotting factors VIII and IX, were at high risk of becoming infected. Transfusion of a single unit (500 ml) of blood from an HIV-1-infected individual nearly always led to infection of the recipient. Appropriate heat treatment of clotting factor preparations and, more recently, *ex vivo* production of these proteins by modern biotechnology methods, have eliminated transmission from this source. Fortunately, other blood products, such as pooled immunoglobulin, albumin, and hepatitis B vaccine, were not implicated in HIV-1 transmission, presumably because their production methods include steps that destroy the virus.

Transmission of HIV from mother to child can occur across the placenta (5 to 10%) or, more frequently, at the time of delivery as a consequence of exposure to a contaminated genital tract (ca. 20%). The virus can also be transmitted from infected cells in the mother's milk during breast-feeding (ca.15%).

Figure 7.12 Modes of transmission of HIV in the United States and worldwide. Data for the United States includes adults and adolescents in the mainland and six dependent areas, and was compiled in 2011 by the U.S. Centers for Disease Control and Prevention. Data for the other areas of the world are from the World Heath Organization (WHO) AIDS Epidemic Update of December 2006.



BOX 7.3

BACKGROUND

Antimicrobial peptides induced by herpesvirus can enhance HIV-1 infection

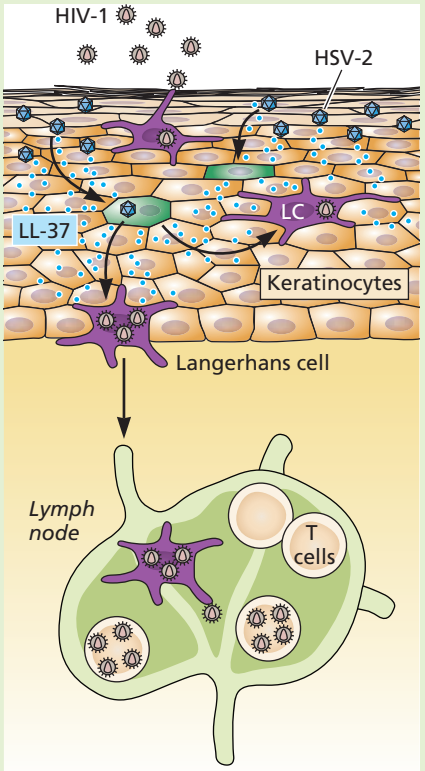
The risk of being infected with human immunodeficiency virus type 1 (HIV-1) is enhanced substantially in individuals with other sexually transmitted diseases. For example, infection with the genital herpes simplex virus type 2 doubles the risk of acquiring HIV-1. Explanations for this increased risk include direct inoculation of HIV-1 into the blood through herpesvirus-induced genital ulcers and the recruitment of inflammatory cells that are targets for infection by HIV-1. The results of infections carried out in skin explants and cultured cells suggest another mechanism for the enhancement of HIV-1 infection by herpes simplex virus type 2.

Langerhans (LC) and other dendritic cells patrol the mucosal epithelium, taking up and processing antigens and presenting them to T cells. Such dendritic cells are believed to be one of the first to encounter HIV-1 after sexual exposure (Fig. 7.13). Whether they can support HIV-1 reproduction in a natural infection is not yet clear. However, it was shown that LCs can be infected with HIV-1 in human skin explants, and coinfection with herpes simplex virus type 2 increased the number of HIV-1 infected LCs substantially. This observation could not be explained by infection of the same cell by both viruses because very few such doubly infected cells were observed. Furthermore, application of virus-free supernatant from herpesvirus-infected cultured cells led to an increase in the number of monocyte-derived, cultured LCs (mLCs) that were infected with HIV-1. These results suggested that the herpesvirus-infected cells produce one or more substances that can increase the efficiency of HIV-1 infection of LCs.

Human epithelial and epidermal cells are known to produce antimicrobial peptides such as defensins and cathelicidin. These are short, evolutionarily conserved peptides that inhibit the growth of bacteria, viruses, and fungi. Herpes simplex virus type 2-infected keratinocytes produce a number of such peptides, but the most important in the current context is LL-37. This peptide was shown to increase the concentration of the HIV-1 receptors, CD4 and CCr5, on the surface of LCs. Furthermore, removing LL-37 from the supernatant of herpesvirus-infected cells reduced its ability to stimulate HIV-1 infection of LCs.

It is unclear what, if any, advantage upregulation of these surface molecules on LCs might confer on herpes simplex virus type 2, and caution is needed when extrapolating results from cultured cells or even tissue explants, to a natural infection. Nevertheless, the proposed mechanism provides one plausible way in which HIV-1 infection may be enhanced by herpes simplex virus type 2. The proposal is supported by the observation that elevated levels of LL-37 correlate with HIV-1 infection in sex workers.

Ogawa Y, Kawamura T, Matsuzawa T, Aoki R, Gee P, Yamashita A, Moriishi K, Yamasaki K, Koyanagi Y, Blauvelt A, Shimada S. 2013. Antimicrobial peptide LL-37 produced by HSV-2-infected keratinocytes enhances HIV infection of Langerhans cells. *Cell Host Microbe* **13:**77–86.



Model for human genital herpes simplex virus 2 (HSV-2) stimulation of HIV-1 infection. Upon exposure to HSV-2, infected keratinocytes (green cells) release antimicrobial peptides, including LL-37 (blue dots). LL-37 stimulates the upregulation of HIV-1 receptors CD4 and CXCr5 in Langerhans cells (LCs). It is proposed that such upregulation augments the efficiency with which LCs disseminate HIV-1 to $CD4^+$ T cells in the local lymph nodes.

Without antiviral drug intervention, rates of transmission from an infected mother to a child range from as low as 11% to as high as 60%, depending on the severity of infection (concentration of virus particles present) in the mother and the prevalence of breast-feeding (frequency of the infant's exposure). Administration of antiviral drug therapy during pregnancy is a very effective measure for decreasing the frequency of transmission to newborns. Pediatric HIV infection is no longer a major public health problem in high-income countries where infected pregnant women are treated with combinations of anti-HIV drugs. However, even a single treatment with one antiviral drug early in labor can reduce the incidence significantly. In lower-income countries such treatment (with AZT or nevirapine) is becoming increasingly more common, and the number of HIV-infected infants is decreasing (Chapter 9 and Fig 9.22). Unfortunately, in some areas, testing facilities and drug treatment remained unavailable as recently as 2012, when an estimated 300,000 newborns were infected with HIV in lower-income countries where the AIDS burden is still high.

Mechanics of Spread

Except in cases of direct needle sticks or blood transfusion, HIV enters the body through mucosal surfaces, as do most viruses (Chapter 2). In the case of sexual transmission, a likely source is virus-infected cells, as they can be present in much larger numbers than free virus particles in vaginal or seminal

fluids. Results from various studies, including observations of SIV infection of macaques, indicate that partially activated $CD4^+$ cells of the genital mucosa in the recipient partner are the initial targets of infection (Fig. 7.13). Infection may be facilitated by interaction with antigen-presenting $CD4^+$ Langerhans or other dendritic cells in the vaginal and cervical epithelia, which can capture infectious virus particles and transport them to target $CD4^+$ T cells (Fig. 7.14).

Activated T cells, prevalent in genital lesions caused by infections, are also likely targets of the virus. Although the insertive partner is at relatively low risk for infection, transmission to the male can occur through cells in the lining of the urethral canal of the penis, presumably from infected cells in the cervix or the gastrointestinal mucosa of the infected partner. Uncircumcised males have a twofold-increased risk of infection, suggesting that the mucosal lining of the foreskin may be susceptible to infection. Both male and female hormones appear to facilitate HIV transmission by stimulating cell-cell contact (prostaglandins) or erosion of the vaginal lining (progestin).

The initial, localized infection is followed within days by migration of the virus via draining lymph nodes to the gut-associated lymphoid tissue (GALT), which contains ~40% of the body's lymphocytes, and, where susceptible, CCr5 $CD4^+$ T cells are abundant. The subsequent explosive production of virus in the GALT leads to acute viremia and widespread dissemination to lymphoid organs within 10 to 20 days. From this point, the infection runs its protracted course.

The Course of Infection

The Acute Phase

In the first few days after infection, virus particles are produced in large quantities by the activated lymphocytes in lymph nodes, sometimes causing the nodes to swell (lymphadenopathy) and/or producing flulike symptoms. Particles released into the blood can be detected by infectivity of appropriate cell cultures or by screening directly for viral RNA or proteins (Fig. 7.15). As many as 5×10^3 infectious particles or 1×10^7 viral RNA molecules (i.e., $\sim 5 \times 10^6$ particles) per ml of plasma can be found at this stage. During this time, some 30 to 60% of $CD4^+$ T cells in the gut are destroyed, either directly or as a result of bystander effects. The associated loss of mucosal integrity leads to the translocation of microbial products into the circulation and disruption of metabolic and digestive functions (Figure 7.16).

A percentage of quiescent memory T cells in the GALT can survive, because replication-competent proviruses cannot be transcribed in these cells. These cells then form a long-lived, latent viral reservoir. Interaction of such memory cells with their cognate antigens, sometimes many years after the initial HIV infection, will lead to their activation and subsequent transcription of the latent provirus. If antiviral treatments

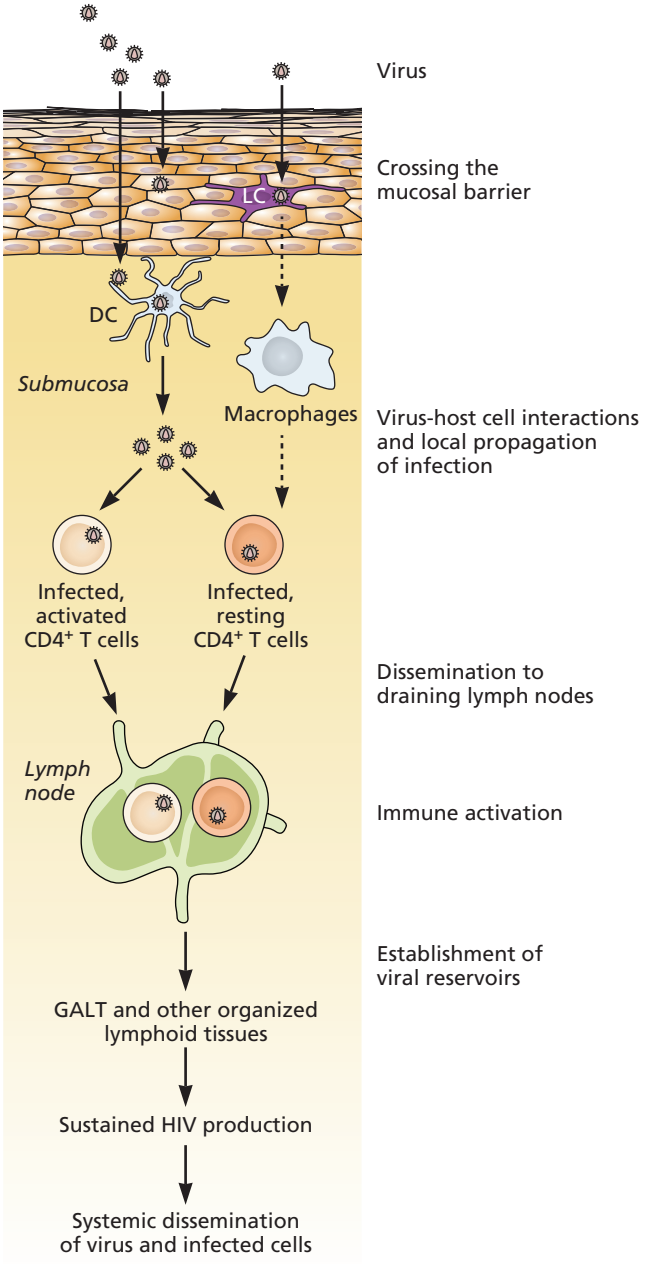


Figure 7.13 Mechanics of viral spread. Infection begins with transmission across a mucosal barrier either by a free virus particle (via transcytosis or following physical abrasion), an infected cell, or particles attached to dendritic (DC) or Langerhans (LC) cells. Virus propagation in partially activated $CD4^+$ T cells is followed by the transfer of virus particles to draining lymph nodes and to the gastrointestinal-associated lymphoid tissue (GALT) where massive propagation of the virus occurs (see Fig. 7.22). The virus is then disseminated to other lymphoid tissues, with the establishment of stable viral reservoirs and latently infected cells. Virus production and concomitant destruction of epithelial barriers in the GALT lead to sustained immune activation and continued dissemination of virus and infected cells.

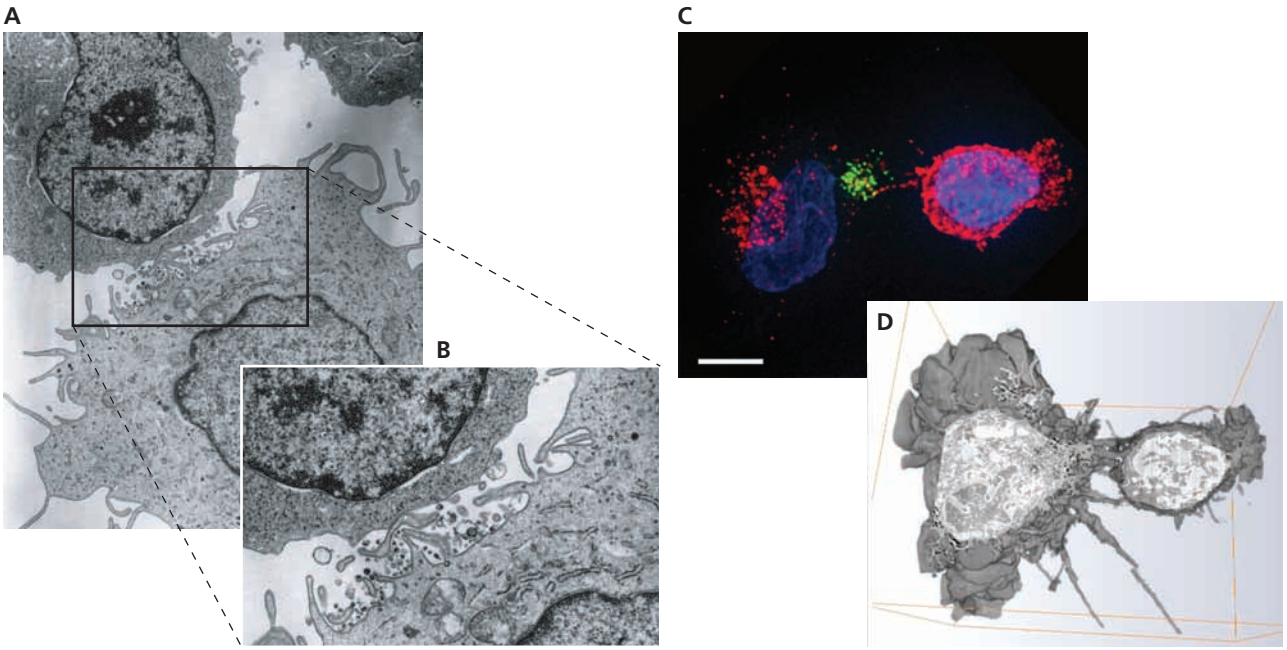


Figure 7.14 HIV particles in a virological synapse between mature dendritic cells and susceptible T cells. (A) Large numbers of HIV particles are concentrated at the mature dendritic–T cell junction (dendritic cell is at bottom right). **(B)** The inset shows a higher-magnification image of the boxed area. Electron micrographic images in A and B were produced by Clive Wells and provided through the courtesy of Li Wu, Medical College of Wisconsin. Reprinted from J.-H. Wang et al., *J Virol* **81:**8933–8943, 2007, with permission. **(C)** Fluorescence micrograph of a CD4 T cell (red) extending membranes into a pocket in a dendritic cell in which HIV-1 particles (green) are accumulated. Nuclei are depicted in blue, and the dendritic cell membrane stain is not shown for clarity. **(D)** Electron microscopic image of the cells in C. A midcell section is shown with half of the data rendered in volume (Voltex) view. The cells are displayed semitransparently to allow a view of the interior structures. These images indicate that virus particles can be stored within invaginated membrane "pockets" that remain contiguous with the surface of the dendritic cell. T cells appear to extend membranous projections into the pocket, where stored virus particle may be captured by binding to the CD4 receptor on the T cell. Images in C and D were prepared by K. Olszens and D. McDonald, and kindly provided by Dr. McDonald (Ohio State University). See also Movie 7.2.

have not been continued, the resulting progeny particles can initiate a new round of infection.

The initial peak of viremia is greatly curtailed within a few weeks after initial infection, as the susceptible T cell population is depleted and a cell-mediated (CTL) immune response is mounted. The number of CTLs increases before neutralizing antibodies can be detected. The inflammatory response that occurs upon primary infection stimulates the production of additional $CD4^+$ T cells, stemming the depletion of this population. Consequently, the $CD4^+$ T cell count returns to near normal levels, but these cells represent a new source of susceptible targets and their infection produces chronic immune stimulation.

During the period of acute infection, the virus population is relatively homogeneous. In most cases, the predominant virus in the infected individual is a minor variant in the population present in the source of the infection (Box 10.5). For example, in approximately 80% of infections resulting from heterosexual contact, the infection was found to originate from a single viral genome. The reason for this apparent genetic bottleneck, and selective transmission, is a topic of intense investigation, as it has great bearing on vaccine development.

The Asymptomatic Phase

By 3 to 4 months after infection, viremia is usually reduced to low levels (Fig. 7.15), with small bursts of virus particles appearing from time to time. It is known that the degree of viremia at this stage of infection, the so-called **virologic set point**, is a direct predictor of how fast the disease will progress in a particular individual: the higher the set point, the faster the progression. During this period, $CD4^+$ T cell numbers decrease at a steady rate, estimated to be approximately 60,000 cells/ml/year. Cytopathogenicity induced by the virus and apoptosis due to continued immune stimulation and inappropriate cytokine production seem likely explanations. In this protracted asymptomatic period, which can last for years, the CTL count remains slightly elevated, but virus reproduction continues at a low rate, mainly in the lymph

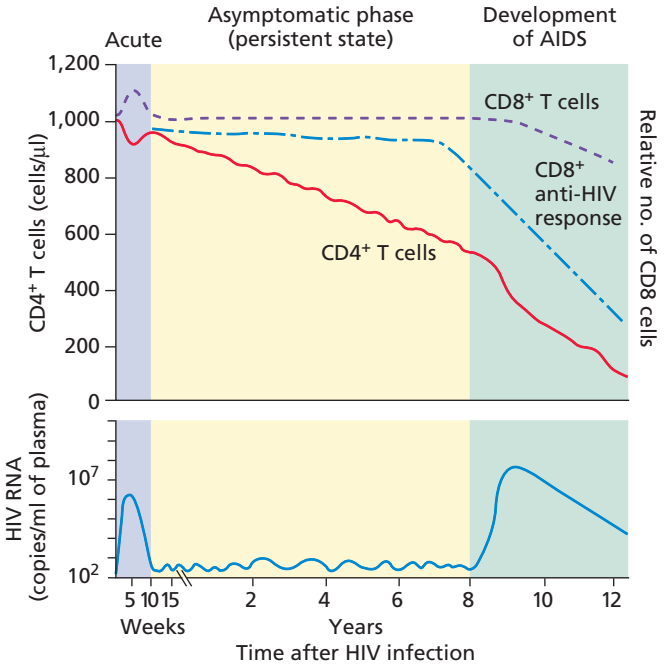


Figure 7.15 Schematic diagram of events occurring after HIV-1 infection. Adapted from Fig. 13.1 of J. A. Levy, *HIV and the Pathogenesis of AIDS*, 3rd ed. (ASM Press, Washington, DC, 2007).

nodes. In lymphoid tissues, a relatively large, stable pool of virus particles bound to the surface of follicular dendritic cells can be detected. Small numbers of infected T cells are also observed. During this phase of persistent infection, also known as **clinical latency**, only 1 in 300 to 400 infected cells in the lymph nodes may actually release virus particles. It is thought that, as in acute infection, virus propagation is suppressed at this stage by the action of antiviral CTLs. The number of these specific lymphocytes decreases toward the end of this stage. During the asymptomatic phase, viral genetic diversity is increased as a consequence of continuous, positive selection for mutants that can evade the host's immune responses (Chapter 4).

The Symptomatic Phase and AIDS

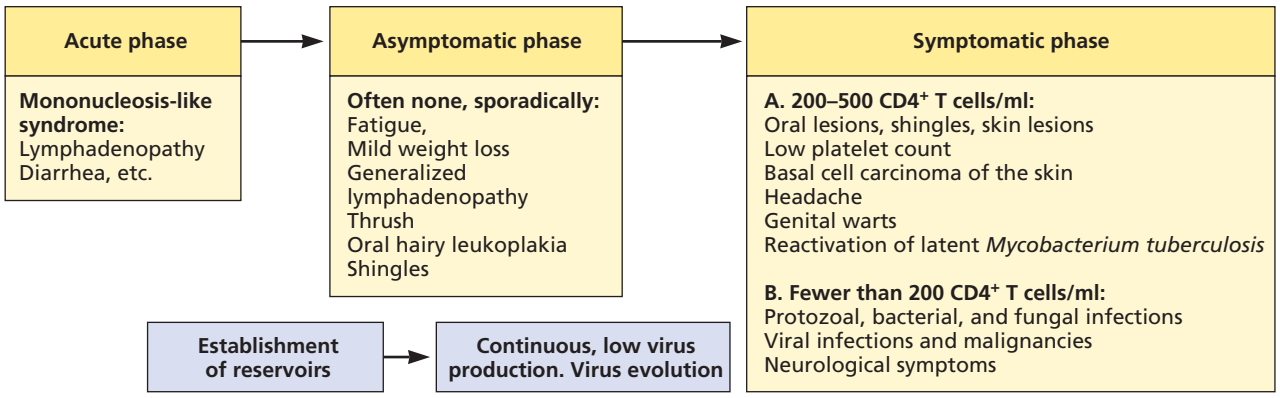
The end stage of disease, when the infected individual develops symptoms of AIDS, is characterized by vastly increased quantities of virus particles and a $CD4^+$ T cell count below 200 cells per ml (Fig. 7.15, Fig. 7.16). The total CTL count also decreases, probably because of the precipitous drop in the number specific for HIV. In the lymph nodes, virus reproduction increases, with concomitant destruction of lymphoid cells and of the normal architecture of lymphoid tissue. The cause of such lymph node degeneration is not clear; it may be the result of virus reproduction, indirect effects of chronic immune stimulation, or both. It has been proposed that chronic antigenic stimulation, which induces rapid turnover and differentiation in the various lymphocyte populations of an infected individual, ultimately culminates in progressive loss of their regenerative potential.

In this last stage, the virus population again becomes relatively homogeneous and specific for the CXCr4 receptor. Properties associated with increased virulence predominate, including an expanded cellular host range, ability of the virus to cause formation of syncytia, rapid reproduction kinetics, and $CD4^+$ T cell cytopathogenicity. Late-emerging virus also appears to be less sensitive to neutralizing antibodies and more readily recognized by antibodies that enhance infectivity. In some cases, strains with enhanced neurotropism or increased pathogenicity for other organ systems emerge. In some cases, these changes have been traced to specific mutations, for example, in the viral envelope gene or in a regulatory gene (e.g., *tat*).

Variability of Response to Infection

Studies of large cohorts of HIV-1-infected adults show that in the absence of antiviral therapy, approximately 10% progress to AIDS within the first 2 to 3 years of infection.

Figure 7.16 Pathological conditions associated with HIV-1 infection.



Over a period of 10 years, approximately 80% of untreated, infected adults will show evidence of disease progression and, of these, 50% will have developed AIDS. Of the remainder, 10 to 17% are AIDS free for over 20 years; a very small percentage of these individuals are completely free of symptoms, with no evidence of progression to disease. What are the parameters that contribute to such variability?

One is the degree to which an individual's immune system may be stimulated by infection with other pathogens. HIV-1 reproduces most efficiently in activated T cells, and it is known that virus concentrations increase when the immune system is activated by opportunistic infections with other microorganisms. Such activation can explain the fact that HIV-1 disease is generally more aggressive in sub-Saharan Africa, where chronic infection by parasites and other pathogens is frequent. As might be expected for an outbred population, variations in an individual's genetic makeup can modulate the immune response to infection and affect survival (Chapter 1). Genetic differences in chemokines or chemokine receptors and, probably, in any one of several components of the immune system can have an impact on the course of the disease. Genomic analyses have identified specific polymorphisms that are associated with variations in viral load in infected individuals, and, as might be expected, major histocompatibility alleles are among the genes affected.

Clearly, accumulation of mutations in the genomes of the virus also influences the course of the disease. As noted above, some long-term survivors of HIV-1 infection harbor viruses with deletions in the *nef* gene. Others appear to be infected with differently attenuated strains that produce low titers in cells in culture and have restricted cell tropism. The presence of virus-neutralizing antibodies, and the absence of virus-enhancing antibodies, are other characteristics of these infections. The fortunate individuals who are nonprogressors or long-term survivors of HIV-1 infection have been under intensive study: such investigations can provide a better understanding of the critical correlates of viral pathogenesis, and may suggest new strategies for effective prevention or therapy.

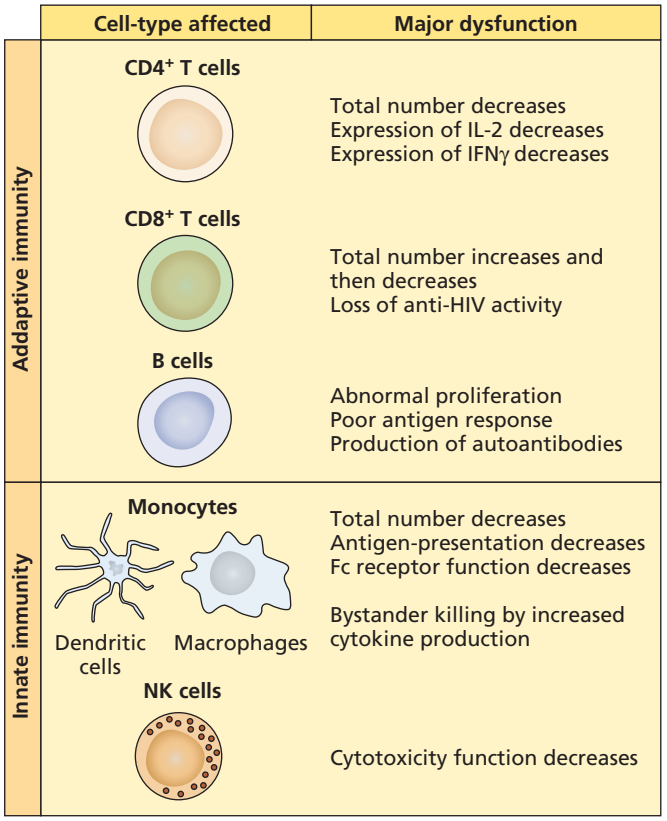
Origins of Cellular Immune Dysfunction

The defining feature of HIV disease is impaired immune cell function. This defect is eventually devastating because immune defenses are vital in the body's battle against the virus as well as other pathogens. At first, the immune system keeps the HIV infection in check. However, the virus is not eliminated, and the infection that persists in the asymptomatic stage leads to increasing dysfunction among immune cells. In the end, most AIDS patients actually succumb to opportunistic infections with microorganisms that are little threat to individuals with healthy immune systems. Impairment in immune cell function results from both direct attack of the virus on particular cell types and the responses of uninfected cells to viral gene products or specific proteins made in infected or stimulated cells.

$CD4^+$ T Lymphocytes

The major target of HIV, the $CD4^+$ T cell, is a critical regulator of the adaptive immune response (Chapter 4). Even before the profound depletion of these cells, which is a signal of end-stage disease, abnormalities in $CD4^+$ T cell function can be detected in the peripheral blood of HIV-infected individuals. These abnormalities include decreased ability to form colonies when grown in tissue culture, decreased expression of the cytokine interleukin-2 (IL-2) and its receptor, and reduced proliferative responses to various antigens (Fig. 7.17). The precise causes of these impairments are unknown. The MHC class II interactions required for antigen-specific responses in infected individuals may become less frequent, because the surface concentration of CD4 is reduced by some viral proteins in infected cells (SU, Vpu, and Nef). Furthermore, noninfectious virus particles and viral proteins (e.g., Tat or SU) shed from infected cells can bind

Figure 7.17 Immune cell dysfunction associated with AIDS.



to or enter uninfected $CD4^+$ T cells, triggering inappropriate (bystander) responses, such as the inhibition of synthesis of IL-2 and its receptor by SU. It is also possible that SU bound to CD4 on uninfected T cells interferes sterically with their interaction with MHC class II molecules. Finally, changes in cytokine production by HIV-1-infected macrophages can trigger programmed cell death in uninfected $CD4^+$ T cells.

Cytotoxic T Lymphocytes

The number of CTLs in the blood is abnormally high following the acute phase and decreases precipitously during the end stage of the disease (Fig. 7.17). The early increase may be the result of an imbalance brought about as the immune system attempts to achieve homeostasis of $CD8^+$ and $CD4^+$ cells while $CD4^+$ cells are being destroyed. The reduced numbers of anti-HIV-1 CTLs at late times can be explained in part by the direct infection and killing of their progenitors ("double positive" $CD4^+$ $CD8^+$ T cells). Additionally, and most importantly, because CTL proliferation and function depend on inductive signals from $CD4^+$ T cells (Fig. 4.7), the decline of the $CD4^+$ population also contributes to CTL dysfunction.

Monocytes and Macrophages

HIV-1-infected macrophages can be detected readily in tissues throughout the body of an infected individual. However, as only a small proportion of monocytes/macrophages in the blood are infected with the virus, it seems likely that the functional impairment seen in this population of cells is due to indirect effects. Monocyte/macrophage abnormalities include defects in chemotaxis, inability to promote T cell proliferation, and defects in Fc receptor function and complement-mediated clearance (Fig. 7.17). Some of these effects may be caused by exposure to the viral envelope protein.

B Cells

HIV-1-infected individuals initially produce abnormally large quantities of immunoglobulin G (IgG), IgA, and IgD. Such production is indicative of B cell dysfunction that may result from increased proliferation of cells of the lymph nodes. Binding of viral proteins (e.g., TM) induces polyclonal B cell activation, a property that might induce such proliferation. B cells isolated from infected individuals divide rapidly in culture without stimulation and are defective in their response to specific antigens or mitogens. The latter property could explain, in part, why infected individuals also show poor responses to primary and secondary immunization. The decline in $CD4^+$ T helper cell function (Fig. 4.7) may lead to a decrease in total number of B cells, sometimes seen in end-stage disease (Fig. 7.17). Finally, infection by Epstein-Barr virus and human cytomegalovirus, common in AIDS patients, may also contribute to abnormal B cell function.

Natural Killer Cells

Impairment of natural killer (NK) cell function is observed throughout the course of infection, becoming more severe during end-stage disease (Fig. 7.17). The reduction in NK cell function cripples the innate immune response to infection by HIV and other microorganisms. As NK cell cytotoxicity depends on IL-2, these abnormalities may be a consequence of impaired $CD4^+$ T cell function and the reduced production of this cytokine.

Autoimmunity

Because of the imbalance in the immune system, it is not surprising that immune disorders, such as a breakdown in the system's ability to distinguish self from nonself, accompany infection. In early studies, antibodies against platelets, T cells, and peripheral nerves were detected in AIDS patients. Subsequently, autoantibodies to a large number of normal cellular proteins have been found in infected individuals. The specific reason for the appearance of such antibodies is not clear, but their production might be stimulated in part by cellular proteins on the surface of viral particles or by viral proteins, regions of which may resemble cellular proteins (molecular mimicry) (Chapter 5).

Immune Responses to HIV

Innate Response

The rate of reproduction of HIV in the acute phase of infection is often reduced before induction of the adaptive immune response, suggesting that the innate immune system plays an early role in antiviral defense (Chapter 3). Recognition of viral components by dendritic cells is associated with the release of type I interferons and Tnf-α, robust induction of additional cytokines, and the inhibition of virus reproduction in infected cells. An array of additional cells, including phagocytes (e.g., macrophages) and cytolytic (e.g., NK) cells respond to this cytokine cascade and participate in the destruction of infected cells and the capture of viral antigens that can be presented to the adaptive immune system. The finding that dendritic cells from females produce larger quantities of IFN-α than do those from males may explain, in part, the fact that women generally show a lower viral set point than men.

The Cell-Mediated Response

Antigen-specific cellular immune responses include activities of CTLs and T-helper cells. The great majority of CTLs are $CD8^+$ and their general role in limiting or suppressing viral reproduction is discussed in Chapter 4. CTLs can also exert a suppressive effect by producing antiviral chemokines and small peptides called defensins that inhibit transcription of viral genes.

CTLs programmed to recognize virtually all HIV-1 proteins have been detected in infected individuals. There is a direct correlation among a good CTL response, low virus

load, and slower disease progression. Furthermore, a broadly reactive response appears to correlate with a less fulminant course of disease, and end-stage disease is characterized by a rapid drop in the number of anti-HIV-1 CTLs. Results of studies with severe combined immunodeficient (SCID) mice that have been reconstituted with human lymphoid cells show that adoptive transfer of human anti-HIV-1 CTLs provides some protection against subsequent challenge with the virus. These findings all demonstrate a significant role for CTLs in fighting HIV-1 disease.

Humoral Responses

Antibodies to the infecting strain of HIV-1 can be detected generally within 1 to 3 months after acute infection. These antibodies, which are secreted into the blood and are present on mucosal surfaces of the body, can be detected in various body fluids. This phenomenon has been exploited in the design of home kits for detecting anti-HIV antibodies in the blood or urine. Among the various isotypes, IgG1 antibodies are known to play a dominant role at all stages of infection, giving rise to an antibody-dependent cellular cytotoxicity response (called ADCC), complement-dependent cytotoxicity, and neutralizing and blocking responses (Fig. 7.18; Chapter 4).

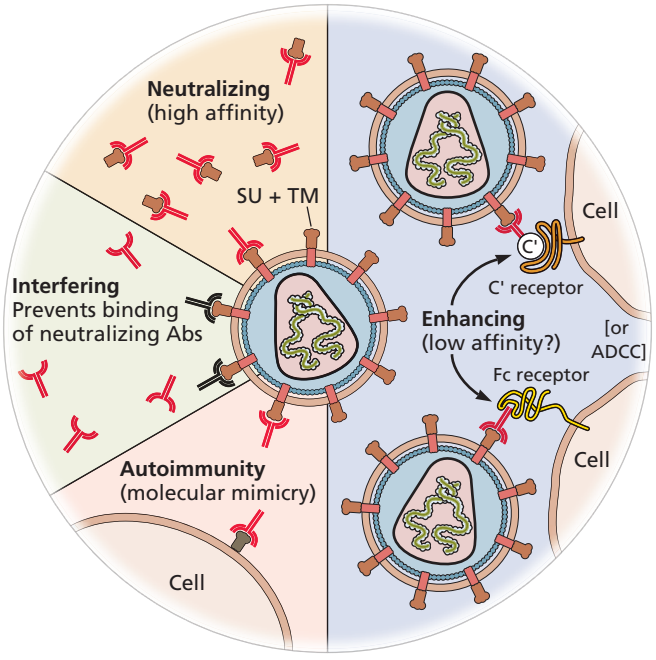
Neutralizing antibodies that can block viral infection of susceptible cells may contribute to limiting viral reproduction during the early, asymptomatic stage of infection. However, the titers of these antibodies are generally very low and, as such, may favor selection of resistant mutants. Indeed, many individuals produce antibodies that neutralize earlier virus isolates but not isolates present at the time of serum collection. Some studies show loss of neutralizing antibodies with progression to AIDS, but the clinical relevance of this change during the later stages of infection remains obscure.

Neutralizing antibodies generally bind to specific sites on the viral envelope protein (Fig. 7.18). Antibodies that bind to epitopes on the variable region 3 (V3) in HIV-1 SU can be detected early in infection. Due to the high sequence variation within the V3 loop (hence the name "variable"), such antibodies are usually strain specific. Consequently, despite its relatively strong antigenicity, V3 is not a good target for the development of vaccines or broadly specific antiviral drugs. Some neutralizing antibodies bind to conserved sites on SU or TM and hence can react with many strains of HIV-1; broad neutralizing activity against carbohydrate-containing regions of the viral envelope protein has also been detected.

Some antibodies (**interfering antibodies**) can bind to virus particles or infected cells and block interaction with neutralizing antibodies (Fig. 7.18, left). Others (**enhancing antibodies**) can actually facilitate infection by allowing particles coated with them to enter susceptible cells (Fig. 7.18, right). In complement-mediated antibody enhancement, the complement receptors Cr1, Cr2, and Cr3 provide a critical function in attaching such complexes to susceptible cells. In Fc-mediated enhancement, attachment is via Fc receptors that are abundant not only on monocytes/macrophages and NK cells but also on other human cell types. As HIV-1 has been shown to replicate in cells that lack CD4 but produce an Fc receptor, binding to the CD4 receptor is probably not required for Fc-mediated enhancement. It is noteworthy that the same cellular receptors (for complement and Fc) are implicated in infection enhancement and ADCC responses (Fig. 7.18, right). In the case of enhancement, the receptors allow antibody-coated virus particles to enter susceptible cells bearing such receptors. In the case of ADCC, such receptors on CTLs, NK cells, or monocytes/macrophages mediate the recognition for subsequent killing of antibody-coated infected cells.

As both neutralizing and enhancing antibodies recognize epitopes on SU and TM, it has been difficult to identify the features that specify the response. Indeed, polyclonal antibodies against SU possess both neutralizing and enhancing activities. The clinical importance of antibody-dependent

Figure 7.18 Antibody (Ab) responses to HIV infection. A summary of the various responses described in the text is presented. One idea is that the relative affinities of the antibodies may be critical determinants of the response. According to this hypothesis, high-affinity antibodies neutralize infectivity by binding tightly to SU, causing it to detach from the virus particles (**Top Left**); low-affinity antibodies bind to SU but not tightly enough to cause its detachment (**Right**). Conformational changes in SU that might occur as a consequence of such low-affinity binding would then facilitate viral entry. C′, complement; ADCC, antibody-dependent cellular cytotoxicity.



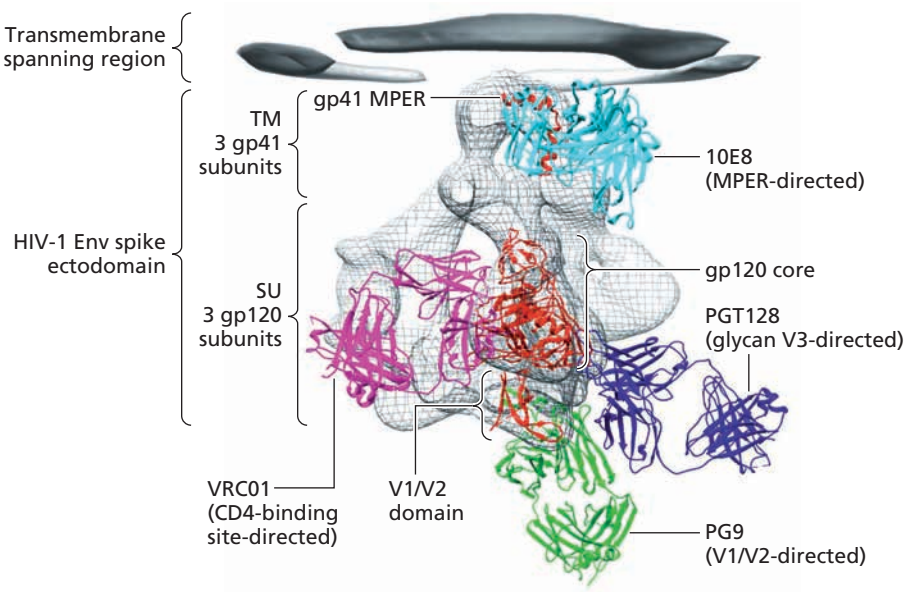
enhancement is uncertain, but the fact that circulating infectious virus-antibody complexes have been described suggests that this phenomenon may contribute to HIV-1 pathogenesis. In addition, infectivity-enhancing antibodies have been demonstrated in individuals who progress to disease. Results from studies of other lentiviral infections, as well as infections with other viruses (dengue viruses, coronaviruses, and others), have established a correlation between increased symptoms of disease and increased quantities of enhancing antibodies. These observations, together with the finding that enhancing antibodies have been found in individuals who generated immune memory to Env protein from a different HIV-1 strain, certainly complicate strategies for the development of an effective vaccine.

The discovery that strong **broadly neutralizing antibody** responses do develop in a subset of individuals after primary infection has stimulated efforts to develop an effective vaccine against HIV. The establishment of high-throughput assays has allowed the screening of sera from large numbers of HIV-infected individuals and identification of several potent and broadly neutralizing antibodies. Cocrystal structures of such antibodies and their viral envelope epitopes have been determined (Fig. 7.19). One type binds to a conserved site within the V2-V3 region of SU, and another to a conserved region in SU that interacts with the CD4 receptor. The TM portion of the HIV-1 envelope is also a target for some of these antibodies. It is hoped that these and other newly identified, broadly neutralizing human antibodies will be useful in the development of effective vaccination strategies. However, these high-affinity molecules appear only years after infection and an extended period of antigen exposure. Furthermore, extensive somatic mutation is apparently required to achieve their effective potency. Consequently, vaccination strategies will need to consider not only the nature/structure of the immunogen, but also the number of exposures required to reach the appropriate antibody affinity (see Chapter 8).

Summary: the Critical Balance

HIV-1 reproduction is controlled by what may be thought of as a finely balanced scale that can be tipped in either direction by a number of stimulatory or inhibitory host proteins. Among these, various cytokines have important but opposing

Figure 7.19 Structure of the HIV-1 envelope trimer and binding of broadly neutralizing human antibodies. An image of the viral spike obtained by cryo-electron microscopy (light gray) is shown with superimposed atomic-level ribbon models (red) for three portions of the HIV-1 envelope glycoprotein (Env): The membrane-proximal external region (MPER) of the transmembrane portion, TM (gp41), is toward the top of the image, the core of SU (gp120) with intact amino- and carboxyl termini is in the middle of the image, and the V1/V2 domain is toward the bottom. Antibodies that effectively neutralize HIV-1 target primarily four specific regions in Env: the MPER, which is bound by antibody 10E8 (cyan); the CD4-binding site on SU, which is targeted by antibody VRC01 (fuchsia); and two sites of *N*-linked glycosylation, one of which is in the V1/ V2 region at residue Asn160 and is bound by antibody PG9 (green), and the other which is a glycan V3 epitope that generally includes residue Asn332 and is bound by antibody PGT128 (blue). From Figure 1 in P. D. Kwong et al., Broadly neutralizing antibodies and the search for an HIV-1 vaccine: the end of the beginning. *Nat Rev Immunol* **13:**693–701, 2013, with permission.



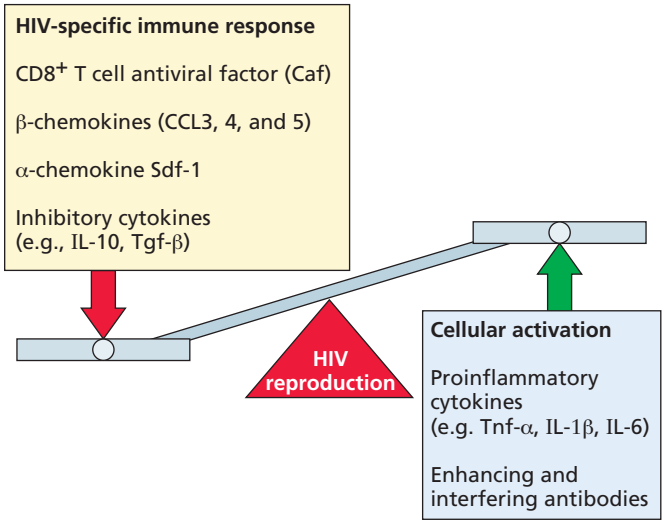


Figure 7.20 Control of HIV-1 reproduction and the progress of HIV-induced disease by the balance of host proteins. Cellular activation and proinflammatory cytokines stimulate viral reproduction. These stimulatory effects are counterbalanced by inhibitory proteins. Adapted from Fig. 4 of A. S. Fauci, *Nature* **384:**529–533, 1996, with permission.

effects. Immune responses and the production of specific chemokines can inhibit virus reproduction, whereas immune cell activation and certain antibodies can be stimulatory (Fig. 7.20). The challenge has been to identify practical interventions that tip the balance in the patient's favor.

Dynamics of HIV-1 Reproduction in AIDS Patients

The availability of potent drugs that block HIV-1 reproduction by inhibiting the activity of the viral enzymes reverse transcriptase and protease made it possible to measure the dynamics of virus production in humans. Clinical studies performed with patients near end stage, whose $CD4^+$ T cell counts are in decline, have revealed the magnitude of the battle between the virus and the immune system in HIV-1 disease. Within the first 2 weeks of treatment with a combination of these drugs, an exponential decline in viral RNA in the plasma was observed, followed by a second, slower decline. The initial drop represented clearance of free virus and loss of virus-producing $CD4^+$ lymphocytes from the blood. The most important contributor to the second drop is presumed to be the loss of longer-lived infected cells, such as tissue macrophages and dendritic cells, with a minor but lingering contribution from the clearance of latently infected, nonactivated T lymphocytes. The existence of the latter population, comprising infected $CD4^+$ T cells that have returned to a quiescent state (i.e., memory T cells), is the major barrier to eradication of the virus by treatment with antiviral drugs.

At steady state in the absence of drugs, the rate of virus production must equal the rate of virus clearance. Mathematical analyses of the data from clinical studies can therefore provide estimates of the rates of HIV-1 appearance in the blood and other compartments of the body, as well as the rate of loss of virus and virus-infected cells. The results are nothing less than astonishing. The minimal rate estimated for release into the blood is on the order of 10^{10} virus particles per day. This minimal number computes to approximately 1 cycle per infected cell per day. Continuous high-reproduction capacity is undoubtedly the principal engine that drives viral pathogenesis at this stage. Because of the high mutation rate of HIV-1, on average every possible change at every position in the genome is predicted to occur numerous times each day. It has therefore been estimated that the genetic diversity of HIV produced in a single infected individual can be greater than the worldwide diversity of influenza virus during a pandemic. This enormous variation and the continuous onslaught of infection must present a colossal challenge to the immune system.

More than 90% of the virus particles in the blood come from infected activated $CD4^+$ lymphocytes that have average half-lives of only ca. 1.1 days (Fig. 7.21). A smaller percentage, approximately 1 to 7%, comes from longer-lived cells in other compartments, with half-lives from 8.5 to 145 days. Consequently, even if *de novo* synthesis of virus could be blocked **completely** by drug treatment, it would take approximately 3 to 5 years before these longer-lived compartments were free of cells with the potential to produce virus. Sadly, this can be considered only a minimal estimate. Complete eradication will not be possible until proviruses are eliminated from long-lived quiescent memory T cells and cells residing in "sanctuary" compartments that are not readily accessible to drugs, such as the brain.

The other dramatic change that takes place in patients treated with antiviral drugs is a resurgence of the $CD4^+$ lymphocyte count in the blood. From the initial rates of recovery, it has been calculated that, during an ongoing infection, as many as 4×10^7 of these cells are replaced in the blood each day. Lymphocytes in the peripheral blood comprise a relatively small fraction (ca. 1/50) of the total in the body, and lymphocyte trafficking, homing, and recirculation are complicated processes. It is still uncertain whether such $CD4^+$ lymphocyte replacement following drug treatment represents new cells, or simply redistribution from other compartments. If one assumes that the increase in the circulation is proportional to the total, then as many as 2×10^9 new $CD4^+$ T cells are produced each day. This estimate is controversial because it seems to exceed significantly the normal proliferative capacity of these cells. However, some studies suggest that HIV-1-infected individuals who are treated with potent drug combinations do produce new $CD4^+$ T cells at rates higher

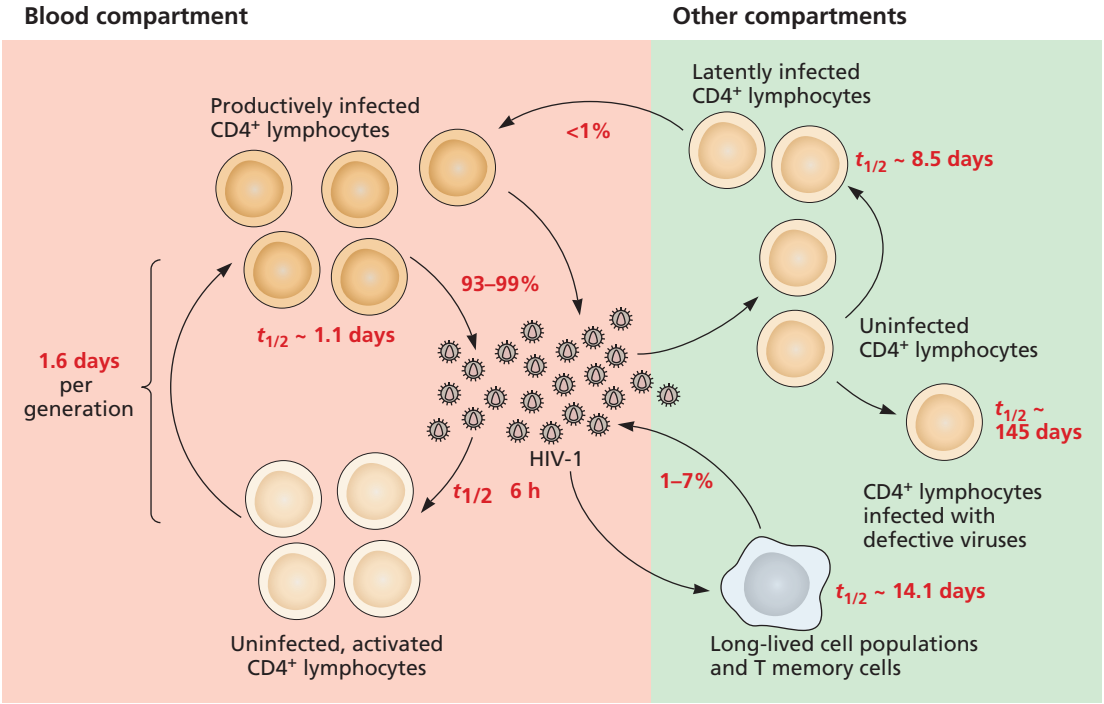


Figure 7.21 Summary of kinetics of HIV-1 production in the blood and other compartments. The percentages indicate the relative quantities of virus particles calculated to be produced in blood plasma by the various cell populations illustrated. The average time in days for 50% of the cells in each population to be destroyed or eliminated is indicated as the half-life ($t_{1/2}$). The average time in hours (h) for 50% of the particles to be eliminated from the plasma ($t_{1/2}$) is also shown. Adapted from Fig. 1 of D. D. Ho, *J Clin Investig* **99:**2565–2567, 1997, with permission.

than normal. Perhaps increased rates of both synthesis and redistribution contribute to the observed resurgence in CD4$^+$ T cells in the periphery.

Large-scale sequencing studies have established that subpopulations of cells in the blood of patients receiving antiviral therapy bear identical sites of HIV-1 provirus insertion. Furthermore, integrations into genes associated with cancer and cell cycle control are overrepresented in these subpopulations. These observations suggest that some latently infected cells may be driven to proliferate via insertional activation of growth-promoting genes by HIV proviruses.

Effects of HIV on Different Tissues and Organ Systems

Lymphoid Organs

Most of the critical steps in HIV pathogenesis occur not in the blood but in lymphoid tissues, which contain the majority of the body's lymphocytes (Chapter 4). Many take place within the first few weeks of infection and occur in tissues such as the GALT of the intestinal mucosa and lymph nodes. The function of these tissues is to retain invading microbes, such as HIV, and to present them to immunocompetent cells. Studies in primate model systems indicate that, within 2 weeks after infection, about 90% of CD4$^+$ T cells are depleted from the GALT, which contains the majority of all lymphocytes and macrophages in the body. Immune dysfunction in the intestine results in structural damage to the mucosa and breakdown of the epithelial barrier (Fig. 7.22). As a result, microbes and microbial antigens enter the bloodstream. Their passage through the now "leaky gut" stimulates release of inflammatory cytokines promoting sustained and systemic activation of the host response, which is a major source of the acquired immune deficiency. Intestinal mucosal tissues appear to be a primary site of HIV-1 persistence, even with antiviral drug treatment.

By 2 weeks after infection in primate models, virus is widely distributed in all lymphoid organs including lymph nodes, spleen, and thymus. Most HIV-1 particles in the lymph nodes of infected humans are trapped within the germinal centers that comprise networks of follicular dendritic cells with long interdigitating processes that surround lymphocytes. Follicular dendritic cells also trap antibodies and complement and present antigens to B cells. The subsequent activation of resident and recruited CD4$^+$ T cells makes them permissive for HIV-1 reproduction. Eventually, infection of macrophages is also observed. Lymph nodes appear to contain a much larger percentage of virus-infected cells than does the peripheral blood.

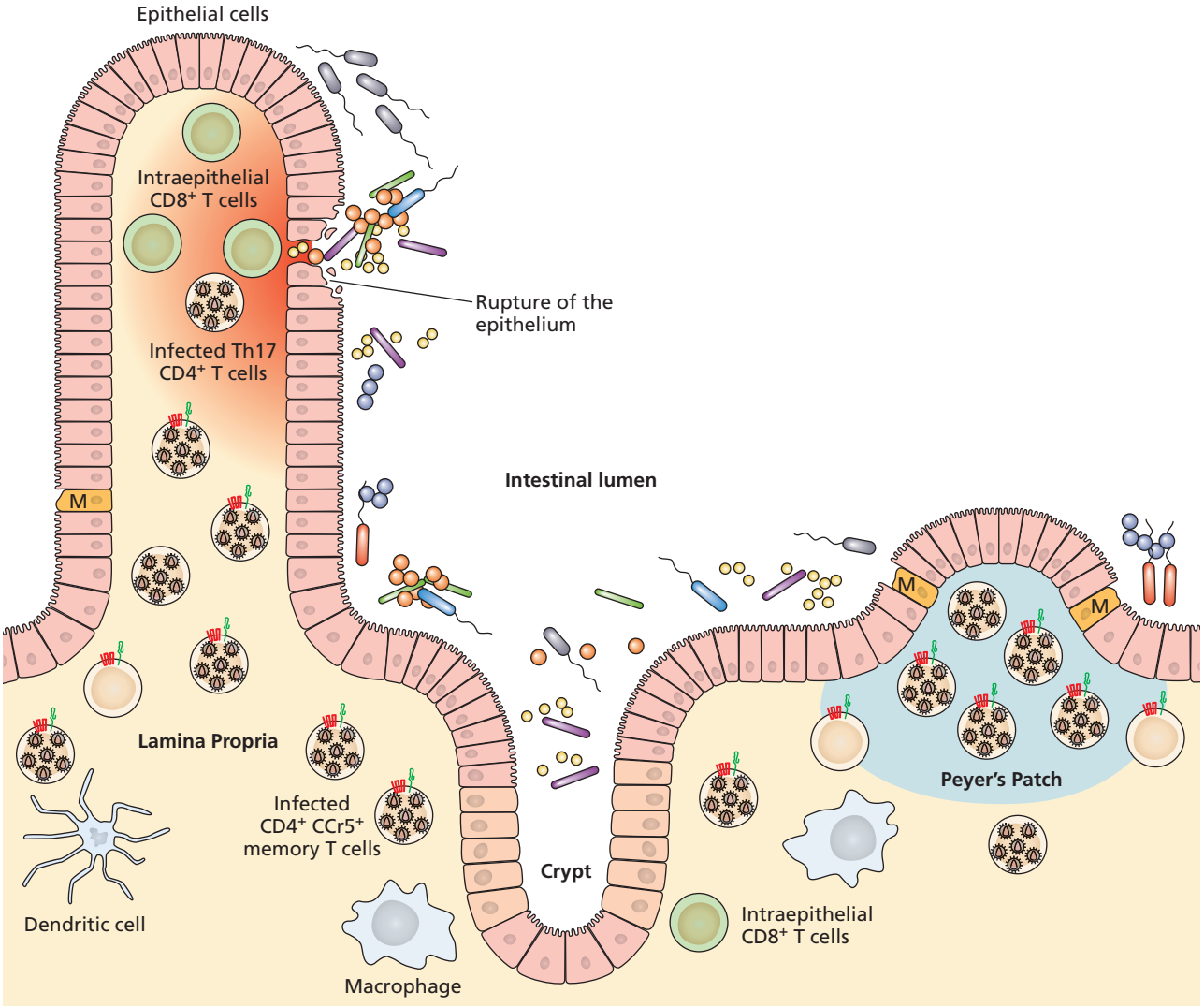


Figure 7.22. Effects of HIV-1 infection of the intestinal mucosa. The intestinal immune system is the largest immunological organ in the body. Terminally differentiated $CD4^+$ T memory cells in both the diffuse and organized (Peyer's patches) gastrointestinal-associated lymphoid tissue are massively depleted within just days after initial infection with HIV. T cells (Th17), which are essential for maintaining the integrity of the intestinal mucosa and defending against pathogens in the intestinal lumen, are also depleted. Epithelial cells are damaged in the wake of this T cell destruction, allowing entry of diverse luminal pathogens, sustained stimulation of the immune response, and persistent inflammation. Adapted from A. A. Lackner et al., HIV Pathogenesis: the host. Figure 2, p. 13. *Cold Spring Harb Perspect Med* **2:**a007005, 2012, doi: 10.1101/cshperspect.a007005, with permission. For an instructive, animated view of the GALT, see the following link: http://link.brightcove.com/services/player/bcpid1966016696001?bckey=AQ~~,AAAByWTdmvk~,YEX2I6TuT0mdQPquhJg1bWcq9Ufv7FQ_&bclid=0&bctid=2144234478001

Early in infection the germinal centers in lymph nodes appear to remain intact, although there is some proliferation of activated immune cells (Fig. 7.23). At the beginning of the asymptomatic stage, in addition to intestinal problems and diarrhea, infected individuals often have palpable lymphadenopathy at two or more sites as a result of follicular dendritic cell hyperplasia and capillary endothelial cell proliferation. Later, during an intermediate stage of disease ($CD4^+$ T cell counts of 200 to 500 per ml), the nodes begin to deteriorate, there is evidence of cell death, and the trapping efficiency of the follicular dendritic cells declines. At a more advanced stage of disease (<200 $CD4^+$ cells per ml), the architecture of the lymphoid tissue is almost completely destroyed and the follicular dendritic cells disappear (Fig.7.23).

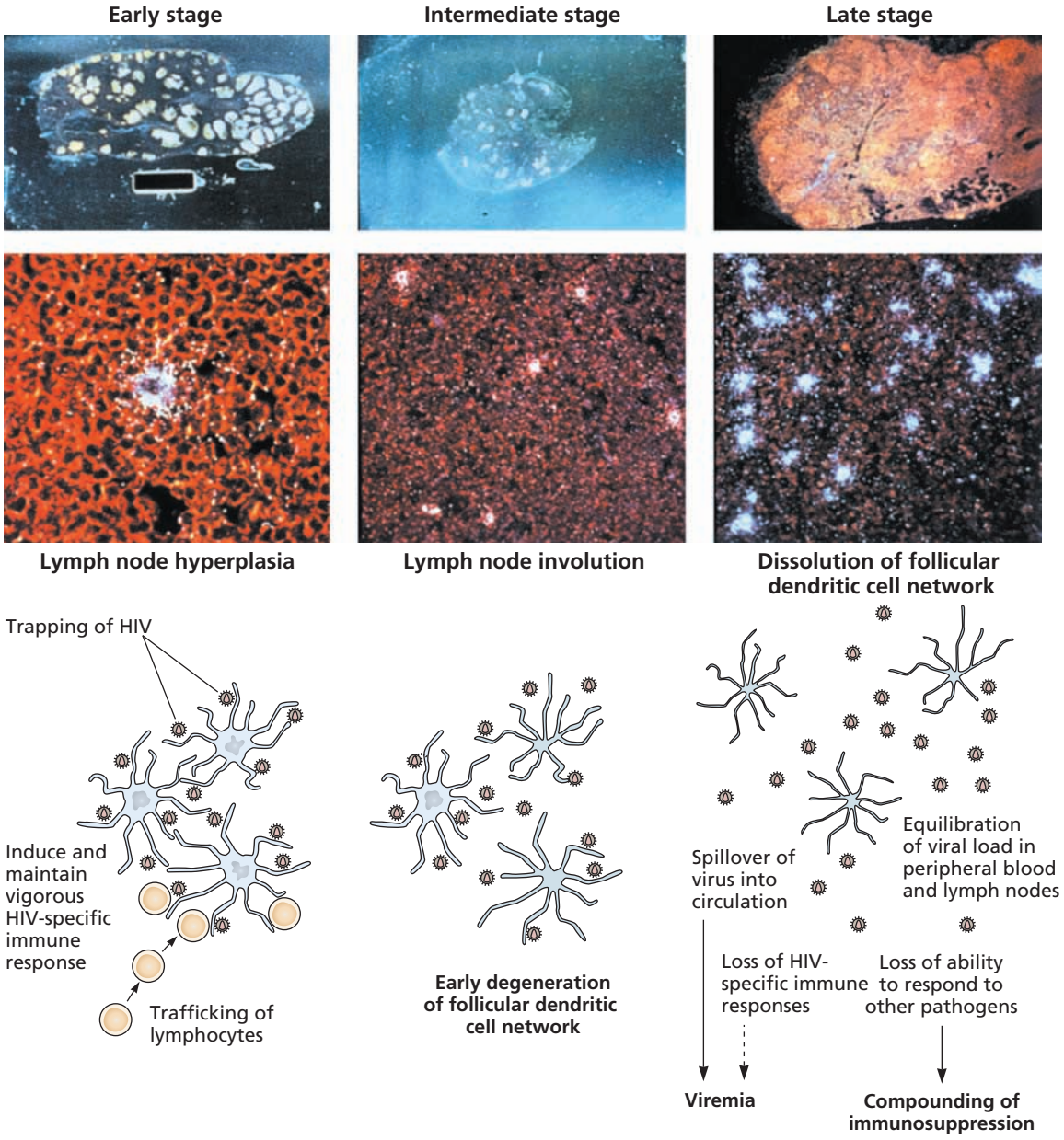


Figure 7.23 Effects of HIV-1 infection on lymphoid tissue. **(Top)** Shows changes in lymph node germinal centers, as determined by selective staining (above) and by the location of viral replication, as blue-white dots, in lymph node tissue (below). HIV-1 nucleic acid was detected by polymerase chain reaction (PCR). The examples illustrate conditions in the early and late stages of HIV infection when connective tissue replaces much of the normal cell population. Reprinted from J. A. Levy, *HIV and the Pathogenesis of AIDS*, 3rd ed. (ASM Press, Washington, DC, 1998). **(Bottom)** Illustrates events that take place in lymph node germinal centers during various stages of HIV-1 disease (see text). Adapted from Fig. 10 of A. S. Fauci and R. C. Desrosiers, p. 587–635. *In* J. M. Coffin et al. (ed), *The Retroviruses* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1998), with permission.

The Nervous System

HIV-1 can be detected in the spinal fluid of affected individuals early after infection. In the absence of antiviral therapy, nearly two-thirds of all HIV-1-infected individuals ultimately develop AIDS dementia. The disease progresses slowly over a period of up to 1 year, but mean survival time from the onset of severe symptoms is less than 6 months. Several AIDS-associated opportunistic infections can also produce neurological damage. A substantial proportion of patients on optimal antiviral therapy also exhibit neurological

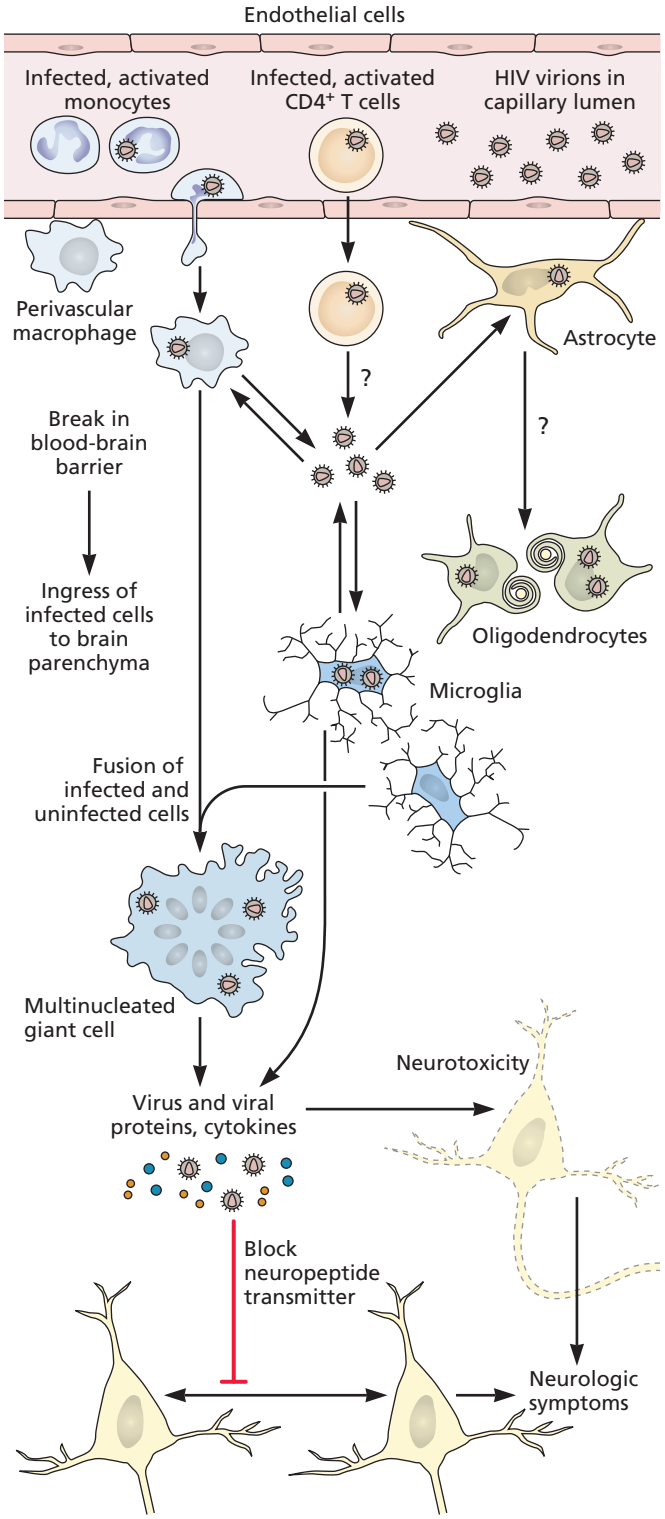


Figure 7.24 HIV-1 neuropathogenesis. It is proposed that infected activated macrophages or T cells may transfer HIV-1 across the blood-brain barrier, although infected CD4+ T cells may also enter via transcytosis. The infected cells can produce virus particles that then infect microglial cells. Microglial cell fusion results in the formation of multinucleated giant cells, which is a hallmark of HIV neuropathology. Astrocytes are affected by cytokines from infected cells, but are not thought to support viral propagation. Production of virus particles and proteins, and the release of various cytokines and other cellular products from infected cells, may lead to an interruption of neuronal cell-to-cell transmission by blocking the production of neurotropic factors. High concentrations of virus proteins (e.g., SU, TM, Tat, and Nef) and cytokines could lead to direct neurotoxicity through detrimental effects on the cell membrane.

abnormalities (called HIV-associated neurocognitive disorder, or simply HAND).

In general, brain-derived isolates of HIV-1 bind to the CCr5 receptor and reproduce effectively in cultured macrophages. This observation, and the fact that cells of the macrophage lineage in the brain (microglia) are routinely found to contain viral proteins and RNA, suggest that HIV-1-infected monocytes may be the main vehicle for virus transmission to the central nervous system. However, other potential sources including infected CD4+ T cells or even free virus particles have not been excluded (Fig 7.24). The entry of virus particles and infection of macrophages and microglial cells triggers an inflammatory response and, eventually, neuronal cell destruction. As HIV-1 does not appear to infect neurons, viral reproduction in these cells is unlikely to explain their loss. It is more probable that the release of toxic cellular products and viral proteins (e.g., SU, TM, or Tat) from infected macrophages or microglial cells is responsible for the damage to neurons as well as astrocytes (Fig. 7.25). There is ample evidence that these viral proteins could contribute to neuropathogenesis. Transgenic mice expressing HIV-1 *env* under the control of neuronal promoters show abnormalities in astrocytes and neuronal processes similar to those seen in HIV-1-infected individuals. It has also been proposed that the systemic activation of macrophages caused by microbial translocation through the leaky gut predisposes these cells to invade the perivascular spaces in the central nervous system.

The Gastrointestinal System

Infection of the GALT leads to disruption of gastrointestinal epithelia early after infection. The more advanced stages of HIV disease are often associated with severe damage to the gastrointestinal system. Diarrhea and chronic malabsorption, with consequent malnourishment and weight loss, are frequently observed. In Africa, this condition has been called "slim disease." In some cases, the disorders are associated with opportunistic infections with other microbial agents. Nevertheless, in cases where no opportunistic agent can be identified, HIV-1 itself is a likely primary cause of gastrointestinal pathogenesis.

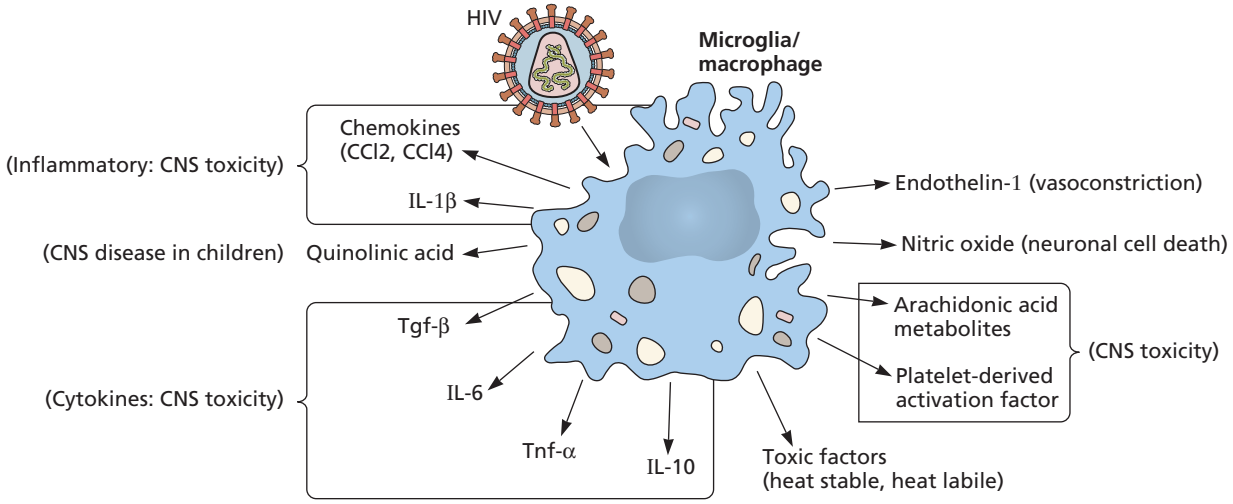


Figure 7.25 The role of infected microglia/macrophages in neural pathogenesis. After exposure to HIV or to viral envelope proteins, microglia/macrophages may be induced to produce cytokines and the macrophage inflammatory chemokines that can be toxic to cells of the central nervous system (CNS).

Other Organs and Tissues

HIV has been found in the lungs of patients with pneumonia, in the hearts of some with heart muscle dysfunction (cardiomyopathy), in the kidneys of some with renal injury, and in the joint fluid of patients with arthritis. It also has been identified in the adrenal glands of infected individuals. The contribution of the virus to these pathologies is not clearly understood. Opportunistic infection of the lungs is common; *Pneumocystis jiroveci*, a yeast-like fungus that is usually dormant in the host lung, causes pneumonia in approximately 50% of AIDS patients. Other microorganisms, most notably *Mycobacterium tuberculosis, Mycobacterium avium,* and human cytomegalovirus, may also cause pulmonary infections. The known effects of SU on membrane permeability or other toxic effects of viral proteins might explain some of the lung damage and the electrophysiological abnormalities associated with heart disease. Direct infection of endothelial cells or other cells in the kidneys has been proposed as a potential cause of tubular destruction. Deposition of antigen-antibody complexes could also account for some kidney damage.

HIV-1 is also found in the male and female genital tracts and in the breasts. There is evidence of genetic compartmentalization of the virus isolated from these locations, suggesting that virus may reproduce at these sites.

HIV and Cancer

HIV-1 infection leads to an increased incidence of neoplastic malignancies: some form of cancer eventually occurs in approximately 40% of untreated infected individuals. The mechanism of oncogenesis in this case is quite different from that of other retroviruses (Chapter 6). HIV-1 kills its major target cell, rather than promoting the immortalization and unrestrained proliferation typical of oncogenesis. HIV-associated malignancies arise from the indirect effects of deregulation of the host's immune system. Contributory factors probably include the absence of proper immune surveillance directed against other (oncogenic) viruses or transformed cells. High levels of cytokine production associated with HIV infection might induce inappropriate proliferation of uninfected cells and promote the generation of blood vessels (angiogenesis) in developing tumors. Indeed, cancers that develop in HIV-infected individuals generally are more aggressive than those in uninfected people. These malignancies can develop in a number of tissues and organs, but certain types, such as Kaposi's sarcoma and B cell lymphoma, are especially prevalent. It may be important that in these cases the neoplastic cells are derived from the immune system and the endothelial cells thought to give rise to Kaposi's sarcoma can act as accessory cells in lymphocyte activation. One reasonable hypothesis is that proliferation of endothelial cells, B cells, and the epithelial cells that give rise to carcinomas may be promoted by cytokines produced by immune cells. As discussed in this section, many malignancies that develop in HIV-1-infected individuals are associated with infection by oncogenic viruses.

Kaposi's Sarcoma

Kaposi's sarcoma was first described by the Hungarian physician Moritz Kaposi in 1872. It is a multifocal cancer in that the lesions contain many cell types; the dominant type is a called a spindle cell, thought to be of endothelial origin. The tumors contain infiltrating inflammatory cells and many newly formed blood vessels. Kaposi's sarcoma was typically found in older men from the Mediterranean region and Eastern

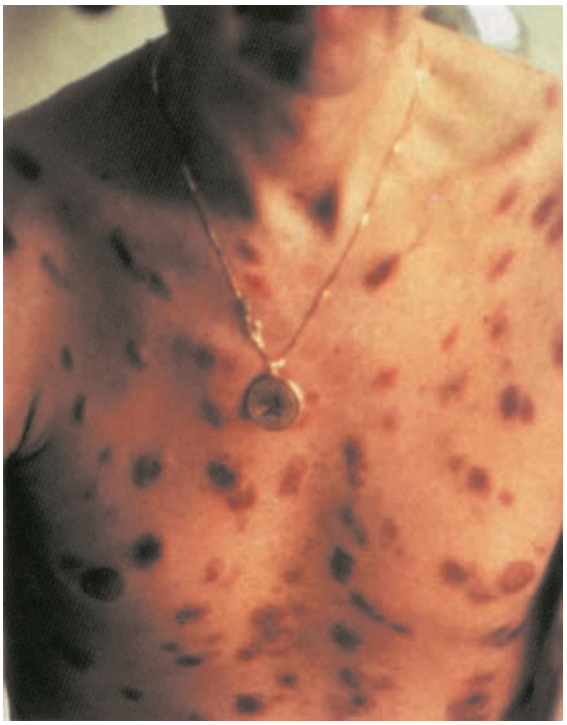


Figure 7.26 Kaposi's sarcoma in a young man infected with HIV-1. Note the distribution of the lesions, suggesting lymphatic involvement. Reprinted from Color Plate 19 of J. A. Levy, *HIV and the Pathogenesis of AIDS*, 3rd ed. (ASM Press, Washington, DC, 2007). Photo courtesy of P. Volberding.

Europe. In these areas, Kaposi's sarcoma normally appears in a nonaggressive (classical) form confined to the skin and extremities, and is rarely lethal. The classical form, as well as a more aggressive and sometimes lethal form, is found in sub-Saharan Africa, where there are more immunocompromised individuals. In HIV-1-infected men, Kaposi's sarcoma appears in the aggressive form, affecting both mucocutaneous and visceral areas. This disease occurs in about 20% of HIV-1-infected homosexual men (Fig. 7.26) but in only about 2% of HIV-1-infected women, transfusion-infected recipients, and blood product-infected hemophiliacs in the United States.

Spindle cell cultures established from Kaposi's sarcoma tumors are not fully transformed according to the criteria defined in Chapter 6, but they do produce a variety of proinflammatory and angiogenic proteins. It is thought that these products are responsible for recruiting other cell types in these tumors. Spindle cells from AIDS patients are also not infected with HIV-1, and the disease occurs in individuals who are not infected with the virus. Consequently, HIV cannot be the sole factor in the development of Kaposi's sarcoma. Epidemiologic studies suggested that another sexually transmitted virus was the inducing agent. Subsequently, a new member of the gammaherpesviruses, called human herpesvirus 8, that can infect spindle cells was found to be associated with Kaposi's sarcoma. The results of *in situ* hybridization studies show RNA transcripts from this virus in the vast majority of Kaposi's sarcoma lesions, irrespective of the presence or absence of HIV-1. Human herpesvirus 8 can also infect B cells and has been linked to certain AIDS-associated B cell lymphomas (Fig. 7.27).

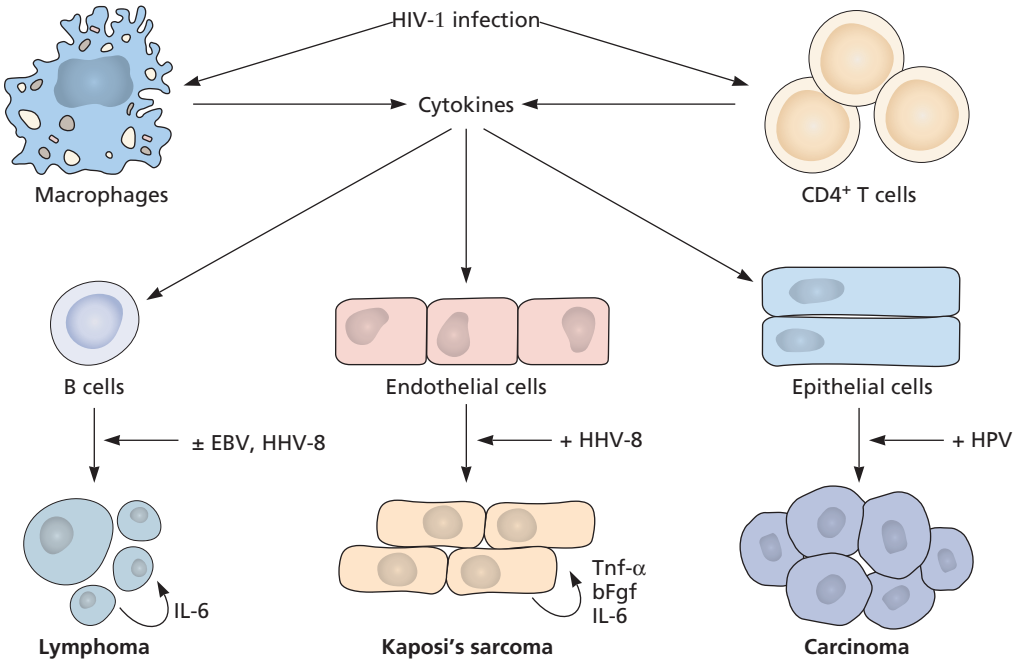


Figure 7.27 Induction of cancers in HIV-1 infection. Infection of macrophages, $CD4^+$ T cells, or other cells leads to the production of cytokines that can enhance the proliferation of certain other cells, such as B cells, endothelial cells, and epithelial cells. The enhanced proliferation of these cells, as a result of either cytokine production or subsequent viral infection, could lead to development of the malignancies noted. In some cases, such as B cell lymphomas and Kaposi's sarcoma, ongoing cytokine production by the tumor cells maintains the malignant state. Adapted from Fig. 12.12 of J. A. Levy, *HIV and the Pathogenesis of AIDS*, 3rd ed. (ASM Press, Washington, DC, 2007).

Antibodies to human herpesvirus 8 can be found in up to 6% of the general population, but in 25% of the population in areas of endemic classical infection, indicating widespread exposure. As very few people develop this disease, other parameters must be important in its etiology. The immune deficiency associated with HIV-1 is certainly one explanation for its prevalence in AIDS patients and synthesis of viral proteins, such as Tat, may be another. It has been reported that transgenic expression of Tat in mice causes a disease that resembles Kaposi's sarcoma. Although the human disease is certainly caused by herpesvirus 8, Tat contributes to the aggressive nature of this malignancy in AIDS patients by promoting the growth of spindle cells in the Kaposi's sarcoma lesions. In addition, the human herpesvirus 8 genome includes a number of open reading frames with sequence homology to genes encoding cellular proteins known to be important in growth control, cell signaling, and immunoregulation (Fig. 6.13).

It is notable that one of the first reports of AIDS in 1981 described seven patients with Kaposi's sarcoma. While HIV-1 was identified soon afterward, the discovery of human herpesvirus 8 did not occur until many years later, even though both viruses were present and contributed to the pathology in these first patients.

B Cell Lymphomas

B cell lymphomas are 60 to 100 times more common in AIDS patients than in the general population. The incidence is especially high among patients whose survival has been prolonged by anti-HIV-1 drugs. Tumors can be found in many locations, including lymph nodes, the intestine, the central nervous system, and the liver. B cell lymphomas in the peritoneal or other body cavities are almost always associated with human herpesvirus 8. Both polyclonal and monoclonal B cell lymphomas are found in the central nervous system, with monoclonal types being more common. Epstein-Barr virus is found in all AIDS-associated primary lymphomas in the brain. Why these two B cell lymphoma-inducing herpesviruses show such site specificity remains unknown. On the other hand, approximately 60% of the tumors outside the brain show no evidence of infection with either Epstein-Barr virus or human herpesvirus 8, indicating that B cell transformation in HIV-1-infected individuals does not require infection with such herpesviruses.

Lymphomas may arise because of the destruction of germinal centers in the lymphatic system. Lysis of antigen-presenting follicular dendritic cells would render B cells less sensitive to normal apoptotic processes, allowing them to live longer and to replicate. Epstein-Barr virus latent membrane protein 1 also inhibits apoptosis. Proliferation of B cells as a result of production of cytokines by macrophages or $CD4^+$ T cells, or even some viral proteins (e.g., SU or TM), may also play a role in this process. Uncontrolled proliferation, by whatever mechanism, could lead to the chromosomal changes required for cell transformation and malignancy.

Anogenital Carcinomas

Anogenital carcinomas are two to three times more frequent in HIV-1-infected individuals than in the general population. They are associated with human papillomavirus infections that are typically spread through sexual contact. The high-risk human serotypes 16 and 18 are associated with both cervical and anal carcinomas. Such cancers often arise in areas of squamous metaplasia near the glandular epithelium and reach more advanced stages in immunosuppressed individuals.

Prospects for Treatment and Prevention

Antiviral Drugs

The decrease in rate of new HIV infections worldwide is a consequence of more widespread availability of antiviral drugs, as well as improved methods for blood testing and screening and successful campaigns focused on behavioral changes to aid prevention. Treatment with highly effective antiviral drugs and drug combinations (Chapter 9) has reduced the problem of emerging resistant viruses, lowered the rates of transmission, and afforded HIV-infected individuals longer and more normal lives. While clearly a triumph, several areas of concern remain. Even though the overall life expectancy of treated individuals is approximately 10 years less than that of uninfected individuals, HIV-infected patients are now living longer than ever before. Complications in these patients include accelerated appearance of typical age-related ailments, such as cardiovascular disease, liver and renal failure, and neurocognitive dysfunction. Some pathology may be explained by incomplete recovery of the immune system following drug therapy: most individuals never achieve complete reconstitution. Secondary complications from chronic infections and reduced ability to suppress oncogenic viruses may account for the increased incidence of some types of cancer in these individuals. Other problems are connected to the antiviral drugs that must be taken constantly. For example, the risk of cardiovascular disease is compounded by effects of prolonged therapy with HIV reverse-transcriptase inhibitors on lipid metabolism. These considerations underscore the need for better drugs and treatment strategies.

Although at most times the virus is undetectable in treated patients, it is not gone: small, intermittent bursts of viremia occur. The hope for total "clearance," or at least a "functional cure" (in which no drug treatment would be necessary), was buoyed by the case of a particular AIDS patient (the "Berlin patient") who received a hematopoietic stem cell transplant from a donor with the CCr5Δ32 mutation (Box 7.4). The patient stopped taking antiretroviral drugs in 2007 and remains

BOX 7.4

BACKGROUND

The Berlin patient

Timothy Ray Brown, a native of Seattle, Washington, is the first, and as yet **sole,** individual to be cured of HIV.

While living in Berlin, Germany, in 1995, Brown was diagnosed with HIV and treated successfully with antiviral drugs for more than a decade. In 2007, he was diagnosed with acute myeloid leukemia and, after unsuccessful chemotherapy, discontinued antiviral drug therapy and underwent two stem cell transplantation procedures within a period of about a year. The second transplant followed a relapse of his leukemia and was preceded by a cytotoxic drug and whole-body irradiation regimen to ablate all or most of his leukemic and immune cells. Peripheral blood stem cells from the same donor were used for both transplant procedures. Most importantly, hoping to "kill two birds with one stone," Brown's Berlin physician screened 62 possible donors to identify an individual who carried a homozygous CCR5Δ32 mutation, which confers resistance to infection with CCr5-tropic HIV strains.

Despite enduring complications and undergoing two transplants, Brown's treatment was a success: he was cured both of his leukemia and HIV infection. Even though he had stopped taking antiviral drugs, there was no evidence of the virus in his blood following his treatment, and his immune system gradually rallied. Follow-up studies in 2011, including biopsies from his brain, gut, and other organs, showed no signs of viral RNA or DNA, and also provided evidence for the replacement of long-lived host cells with donor-derived cells. Recent studies also showed that Brown had no detectable CXCr4-tropic virus prior to transplantation. Brown remains HIV-1 free as of this writing (2015).

Although clearly somewhat of a "medical miracle," and by no means a practical road map for HIV treatment, the example of the "Berlin patient" has galvanized research efforts and continues to inspire hope that a simpler and more general cure for infection may someday be achieved.

Allers K, Hütter G, Hofmann J, Loddenkemper C, Rieger K, Thiel E, Schneider T. 2011. Evidence for the cure of HIV infection by CCR5 Δ32/Δ32 stem cell transplantation. *Blood* **117:**2791–2799.

Hütter G, Nowak D, Mossner M, Ganepola S, Müssig A, Allers K, Schneider T, Hofmann J, Kücherer C, Blau O, Blau IW, Hofmann WK, Thiel E. 2009. Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *New Engl J Med* 360**:** 692–698.

Berlin gate.



virus-free. Unfortunately, despite further efforts, this result has not been repeated: the virus reemerged consistently following transplants that were subsequently performed in other patients using a variety of strategies. Transplantation would certainly not be a practical treatment for the majority of HIV-1-infected individuals. Nevertheless, the successful Berlin case has encouraged efforts aimed at addressing the challenge of virus persistence and latency and at devising new gene therapy approaches.

Confronting the Problems of Persistence and Latency

Eradicating all traces of HIV from an infected individual is particularly challenging. Although small, the reservoir of long-lived, latently infected cells is established early in infection, can be replenished during short bursts of viremia and, in some cases, may be expanded by provirus-induced cell proliferation. The failure of early attempts to eliminate the reservoir by intensifying drug therapy is consistent with the notion that some of these cells may reside in drug-inaccessible sanctuaries, such as the brain. Most long-lived latently infected cells appear to have been derived from infected quiescent $CD4^+$ memory or activated T cells that survive infection long enough to revert to the resting memory state. A variety of mechanisms that inhibit proviral gene transcription in such cells have been described, including epigenetic suppression and deficiency of essential host transcriptional regulators, such as nuclear Nf-κb. Such knowledge underlies the treatment strategy known as "shock and kill," in which provirus expression is induced in latently infected cells, while virus infection of new cells is prevented by treatment with antiviral drugs and/or neutralizing antibodies. The first implementations of this strategy, in which patients were treated with IL-2 or FDA-approved epigenetic drugs, were not successful: although some increase in virus production could be detected after treatment, there was no apparent decrease in the size of the latent reservoir. However, research in this area continues with the expectation that a fuller appreciation of the cell types that comprise the latent reservoir and more detailed understanding of their biology may lead to more effective methods for their activation and elimination.

Gene Therapy Approaches

Modern biotechnology has provided a number of methods, including direct gene editing, by which $CD4^+$ T cells and hematopoietic stem cells may be modified to make them resistant to HIV-1 infection. The use of cells from a donor with the CCr5Δ32 mutation in the case of the Berlin patient inspired a variety of trial strategies in which $CD4^+$ T or hematopoietic stem cells are obtained from a patient, the CCr5 gene is either mutated or its expression blocked by RNA interference, and then the resulting virus-resistant cells are returned to the patient. In some cases genes that encode proteins that inhibit

HIV reproduction have also been added to such cells. Several clinical trials using these approaches have been initiated, and the initial results appear promising. Other gene therapy approaches include deletion of both CCr5 and CXCr4 coreceptors or infection of $CD4^+$ T cells or hematopoietic stem cells with viral vectors that encode genes for small peptides that block viral entry or that express a site-specific recombinase that has been tailored to excise the proviral LTR.

Immune System-Based Therapies

Because immunization seems to be protective against subsequent infection of rhesus macaques with simian immunodeficiency virus, treatment strategies that combine antiviral drug treatment with augmentation of HIV-specific immunity have been proposed. Thus far, interventions based on administration of various cytokines (IL-2, IL-7, IL-15) aimed at improvement in T cell function have not shown significant benefit, when administered singly. Another strategy is targeted at immune checkpoint molecules. Ligands for one such molecule, programmed cell death protein 1 (Pd-1), are produced widely in tissues and, when engaged, result in suppression of T cell function and return to an inactivated state. Inhibitors of the Pd-1 pathway restore T cell function. Clinical trials for the use of such inhibitors to augment host immune control in HIV-1-infected individuals have been initiated. It is possible that inhibitors of additional immune checkpoint molecules (Ctla-4 and others) may also be effective in this context.

The most potent immunological defense against viral infection is a vaccine, a topic discussed in more detail in Chapter 8. While HIV-1 vaccine development presents unique challenges, it is important to appreciate that an AIDS vaccine need not be 100% effective to be useful. With such a deadly disease, even partial protection that might spare 20 to 40% of potential victims would save millions of lives and reduce transmission significantly. In the absence of a vaccine, recent success in identifying potent broadly neutralizing antibody molecules suggests that such reagents could be generated *ex vivo* and used for passive immunization in certain situations. Broadly neutralizing antibodies may also be administered via viral vectors. Studies with primates and humanized mice have demonstrated that inoculation with viral vectors that produce such antibodies can provide long-lasting resistance to infection with simian immunodeficiency virus or HIV, respectively (Box 7.5). Other studies have shown that the affinity of these antibodies can be increased substantially by using recombinant DNA methods to produce bispecific molecules (called immunoadhesins) that can bind simultaneously to two separate epitopes on the HIV-1 envelope (e.g., in TM and SU). This strategy could improve both antibody recognition and viral neutralization activities, as the relatively low density of envelope protein on HIV-1 particles (ca. 7 to 17 spikes/particle) is thought to reduce the efficiency of bivalent binding by monospecific antibodies.

Antiviral Drug Prophylaxis

Postexposure prophylaxis (PEP)

It is well established that treating individuals with antiviral drugs within hours after exposure to HIV-1 (e.g., from needle sticks) reduces the risk of infection substantially. The efficacy of postexposure prophylaxis suggests that the first cells that are infected with HIV can be eliminated. Furthermore, treating infected individuals early, during acute infection, reduces the viral set point and the size of the latent reservoir and preserves immune function. The potential efficacy of early treatment was suggested by the case of the Mississippi baby who was infected *in utero* and started on aggressive antiviral treatment before she was 2 days old. The latent reservoir in this infant was so small that her body appeared to clear the virus while on drug treatment. Therapy was discontinued after 18 months. Although the virus reemerged 27 months later, the long delay suggested that the reservoir was indeed small and/or controlled for a relatively long time following such early treatment.

Preexposure prophylaxis (PrEP)

Infection with HIV-1 can be prevented in uninfected people who are at substantial risk of acquiring it by adherence to a regular regimen of antiviral drug treatment. The currently accepted regimen comprises daily ingestion of a single pill that contains a combination of antiviral drugs (e.g., tenofovir and emtricitabine). If an individual is then exposed to HIV, through sex activity or drug use, PrEP can keep the virus from establishing a permanent infection. According to the U.S. Centers for Disease Control and Prevention, "PrEP has been shown to reduce the frequency of HIV infection in people who are at high risk by up to 92%." Unfortunately, clinical trials of PrEP have had limited success, mainly because of lack of adherence to the treatment.

Perspectives

> *Pneumocystis* Pneumonia—Los Angeles. In the period October 1980–May 1981, 5 young men, all active homosexuals, were treated for biopsy-confirmed *Pneumocystis carinii* pneumonia at three different hospitals in Los Angeles, California. Two of the patients died....
>
> M. S. Gottlieb et al. (Centers for Disease Control)
> *Morb Mortal Wkly Rep* **30:**250–252, 1981

So began the first warning, soon echoed in large urban centers throughout the United States and Europe, where physicians were being confronted by a puzzling and ominous new disease that was killing young homosexual men. In a deceptively low-key editorial note with this report, it was observed that "*Pneumocystis* pneumonia is almost exclusively limited to severely immunosuppressed patients," that "the occurrence of the disease in these five previously healthy individuals is unusual," and that "the fact that these patients were all

BOX 7.5

EXPERIMENTS

Vector-derived immunoprophylaxis

The identification of broadly neutralizing human antibodies against HIV-1 has renewed vaccine development efforts. While many obstacles must be overcome before a vaccine is available, strategies for use of these reagents seem more immediately practical. One such strategy employs gene transfer technology with adenovirus-associated viral (AAV) vectors that encode neutralizing antibodies or engineered derivatives known as immunoadhesins.

Such studies, first with mice and then with Rhesus macaques, showed this approach to be quite promising. Long-lasting neutralizing activity was detected in the serum of animals injected intramuscularly with the AAV vectors. Furthermore, macaques injected with AAV vectors encoding antibodies that targeted simian immunodeficiency virus were protected against intravenous challenge with this virus. Six of nine animals that received the gene transfer were not infected, and none of the nine developed AIDS.

To test the efficacy of this approach for HIV-1, "humanized" mice that had been engrafted with human bone marrow, liver, and thymus tissue, were injected with AAV vectors encoding broadly neutralizing antibodies against HIV-1. After 4 weeks, high concentrations of the human IgG antibodies could be detected in the serum and even in vaginal washes of these animals. The mice were then challenged weekly with low concentrations of CXCr5 virus particles, administered vaginally to approximate the predominant mode of sexual infection in humans. The results showed that these mice were highly resistant to such challenge. Animals injected with a vector that encoded an optimized derivative of the SU binding antibody VRC01 (Fig. 7.19) were completely resistant to virus infection even after 21 weekly challenges, conditions in which all control animals became infected.

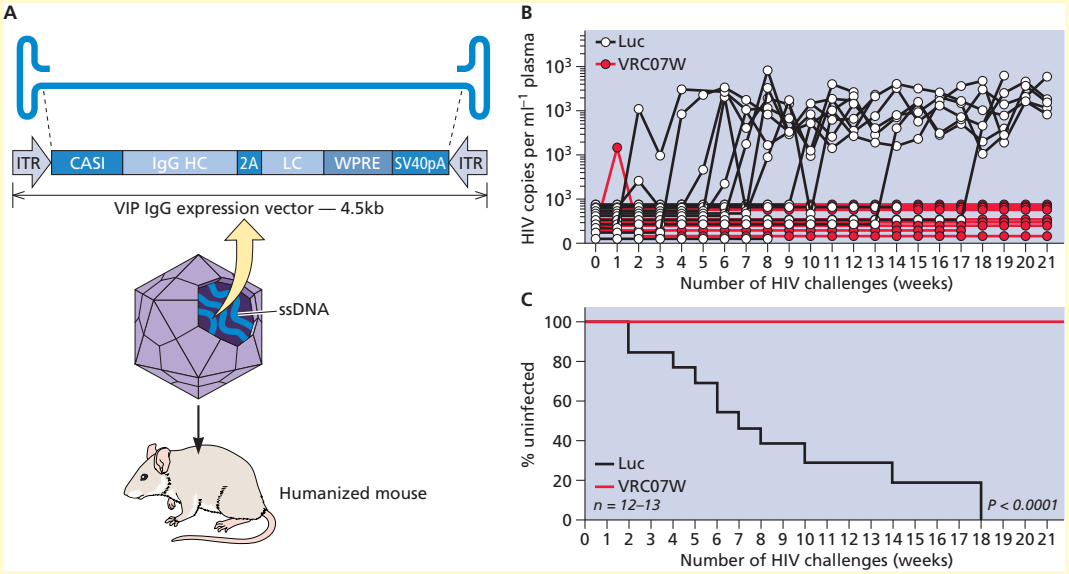
The investigators acknowledge that there are substantial anatomical differences between mice and people, and that the humanized mouse does not possess a fully functional human immune system. Nevertheless, this kind of immunoprophylaxis would seem to hold considerable promise as a strategy to reduce the probability of sexual transmission of the HIV between humans, especially in situations in which PrEP is impractical due to antiviral drug costs or lack of adherence to the prescribed regimen.

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Vector-derived immunoprophylaxis protects humanized mice from HIV-1 infection. (A) Experimental design. The optimized adenovirus-associated viral vector genome includes a promoter (CASI) that combines a cytomegalovirus enhancer and chicken β-actin promoter followed by a splice donor and splice acceptor flanking the ubiquitin enhancer region. Sequences encoding heavy (HC) and light chain (LC) V-regions from broadly neutralizing antibodies were inserted into the vector DNA, separated by a self-processing sequence (2A). A sequence for improved nuclear export of transcripts (WPRE), and the simian virus 40 late-polyadenylation signal (SV40pA), are also included to increase the efficiency of gene expression (Volume I, Chapter 10). ITR indicates the positions of the AAV inverted terminal repeats. Encapsidation by a protein from a rhesus macaque-derived subtype of the virus enhances the effectiveness of gene transfer by this vector. Humanized mice were injected in the gastrocnemius calf muscle with 1×10^{11} genome copies of the vector and challenged 4 weeks later with weekly vaginally administered HIV. **(B)** Viral load detected in plasma of vector-treated humanized mice following weekly intravaginal challenge with HIV-1. Limit of detection = 1000 copies ml^{-1}. **(C)** Fraction of uninfected vector-treated humanized mice over the course of repetitive intravaginal challenge. Black lines and open symbols show results with mice injected with a control vector that encodes a luciferase gene (Luc); Red lines and filled circles show results with mice injected with a vector encoding a potent, broadly neutralizing antibody directed against the CD4 binding site in SU (VRCO7W). Positive infection is defined by two consecutive viral load measurements above the limit of detection. From Figure 3 of A. B. Balazs et al., *Nat Med* **20:**296–300, 2014, with permission.



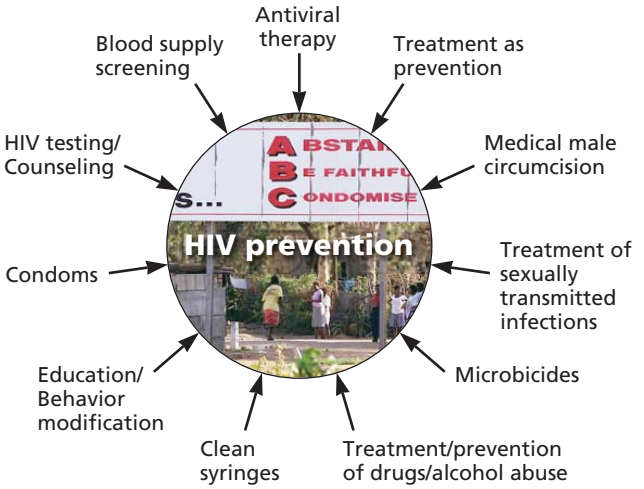


Figure 7.28. The multifaceted approach to prevention of infection with HIV.

homosexuals suggests an association between some aspect of a homosexual lifestyle or disease acquired through sexual contacts . . ." Soon the disease was to ravage this group and also the hemophiliac community, whose lives depended on blood products. This state of distress led to unprecedented social activism that demanded significant public investment for AIDS research and put patient advocates on scientific review panels for the first time.

Initial progress in AIDS research was impressive. The etiological agent of the disease was identified within 2 years, and screening assays to safeguard the blood supply were developed shortly thereafter. As a broad base of knowledge about retroviruses already existed, it seemed that treatment and a vaccine should soon be available. Unfortunately, more than 3 decades later, AIDS is still with us. Nevertheless, the prospect is much more upbeat today than it was in the last edition of this book. The use of potent drugs and drug combinations has reduced the rates of transmission and prolonged the lives of those infected with HIV throughout the globe. At the same time, a multifaceted approach has been applied to prevent new infections, including improved testing and counseling, and public education campaigns that discourage risky behavior (Fig. 7.28). While the two major challenges of finding a "cure" and developing a vaccine to prevent infection remain, there has been substantial progress in our understanding of the many aspects of HIV disease, from molecular to the epidemiological aspects. The continuously expanding knowledge base and technology toolbox have motivated renewed efforts to meet both of these challenges. The more optimistic among us can even envision the possibility of a world without AIDS in the future.

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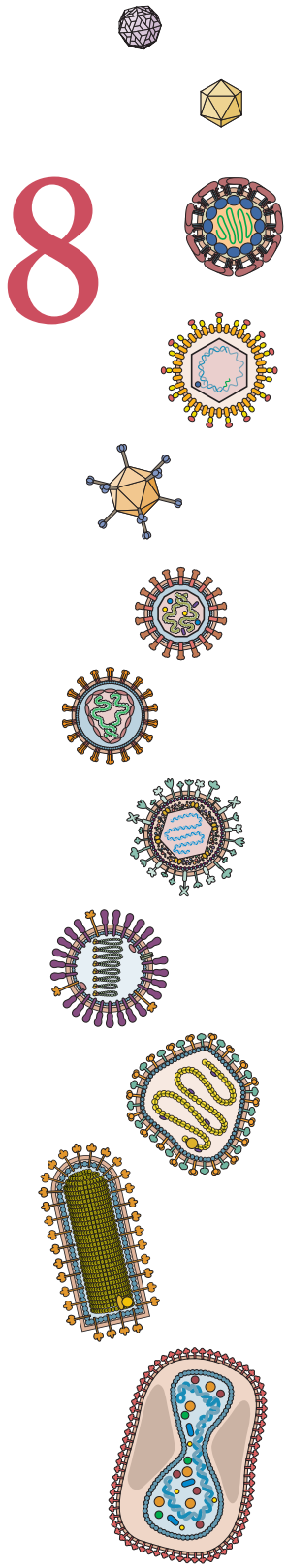
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8 Vaccines

Introduction

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LINKS FOR CHAPTER 8

⏭ ***Video: Interview with Dr. Gary Nabel***
http://bit.ly/Virology_Nabel

⏭ ***Ebola lite***
http://bit.ly/Virology_Twiv335

⏭ ***An unexpected benefit of inactivated poliovirus vaccine***
http://bit.ly/Virology_1-6-15

An ounce of prevention is worth a pound of cure.
BENJAMIN FRANKLIN

Introduction

Imagine that, while walking down your street, you encounter a new dog. You offer your hand in hope of a pleasant exchange, but you are greeted with snarls, bared teeth, and a menacing glare. When you meet this same dog the next time, it is most likely that you will walk past quietly and quickly, recalling your previous negative interaction. Recollection of a former encounter changes one's future response to that same stimulus. This simple example is the basis of immunological memory, described in Chapter 4: your immune system does more than "remember" a former pathogen; it responds to a second challenge differently from the first. Following an initial encounter with a pathogen, memory immune cells are established. Reexposure to that same pathogen reawakens these memory cells to control the secondary infection quickly and prevent subsequent disease. For many centuries, the illness that accompanied a primary infection was unavoidable, and for diseases such as smallpox, infected people often did not survive to face a second exposure. The goal of vaccination is to trigger an immune response more rapidly and with less harm than a natural infection: in essence, to avoid the disease that often accompanies the first exposure while enabling establishment of long-lasting immunological memory.

Vaccines against viral and bacterial pathogens prevent catastrophic losses of life in humans, other animals, and plants, and are considered among the greatest public health achievements (Fig. 8.1) (http://www.historyofvaccines.org/content/timelines/diseases-and-vaccines). But vaccines are not without their limitations and potential side effects. The history of vaccinology is therefore also the story of how the formulation and delivery of vaccines developed and improved to preserve efficacy while increasing safety and durability. In this chapter, we begin with a recounting of the fortuitous observations that catalyzed this field; highlight specific examples of how the use of vaccines has led to the eradication of devastating viruses; and discuss the differences among various vaccination strategies, comparing the benefits and challenges of each. (For more, see the interview with Dr. Gary Nabel: http://bit.ly/Virology_Nabel.)

The Origins of Vaccination

Smallpox: a Historical Perspective

Smallpox is the most destructive disease in history, and has probably been part of human existence since 10,000 BC or before. It has been estimated that infection by smallpox virus killed, crippled, or disfigured more than 1 in 20 of all humans who ever lived. In the 20th century alone, between 300 million and 500 million people died as a consequence of infection. As recently as 1967, there were >15 million cases worldwide, with 2 million deaths (note the high case-fatality ratio). Yet thanks to a worldwide vaccination campaign, a little more than a decade later the World Health Organization declared that smallpox had been eradicated. Smallpox remains the only infectious disease of humans for which this is true, and its eradication is surely among the greatest achievements of modern medicine.

While we correctly credit Edward Jenner with the development of the first vaccine, efforts to prevent infection and disease were made for many centuries before Jenner's contribution. Chinese and Indian physicians of the 11th century injected pus from smallpox lesions into healthy individuals, or blew a powder made from dried smallpox scabs into the nostrils of such individuals, with the hope of inducing mild disease that would provide lifelong protection (a process later called **variolation**). The word "variola," the name by which the disease was known for centuries, derives from the Latin

PRINCIPLES *Vaccines*

- Following an initial encounter with a pathogen, memory immune cells are established; reexposure to the same pathogen reawakens these memory cells to control the infection and prevent disease.
- The goal of vaccination is to trigger an immune response more rapidly and with less harm than a natural infection.
- Smallpox virus, which caused infections that killed, crippled, or disfigured more than 1 in 20 of all humans who ever lived, is the only human virus to be eradicated.
- Viral candidates for eradication must possess two essential features: the infectious cycle must take place in a single host, and infection (or vaccination) must induce lifelong immunity.
- Vaccination can be active (the host makes its own response to a viral preparation) or passive (components of the immune response are obtained from an appropriate donor or donors and injected directly into the patient).
- To be effective, a vaccine must induce protective immunity in a fraction of the population that is sufficient to impede person-to-person transmission, a concept called herd immunity.
- Active vaccination can occur by administration of virus preparations that have been inactivated or attenuated or by delivery of individual immunogenic proteins or recombinant DNA vectors that encode them.
- Inactivated virus particles or purified proteins often do not induce the same immune response as attenuated preparations, unless mixed with adjuvants that stimulate the early inflammatory response.
- The failure to develop a human immunodeficiency virus vaccine can be explained by both the biology of this virus and its interaction with the host immune system.

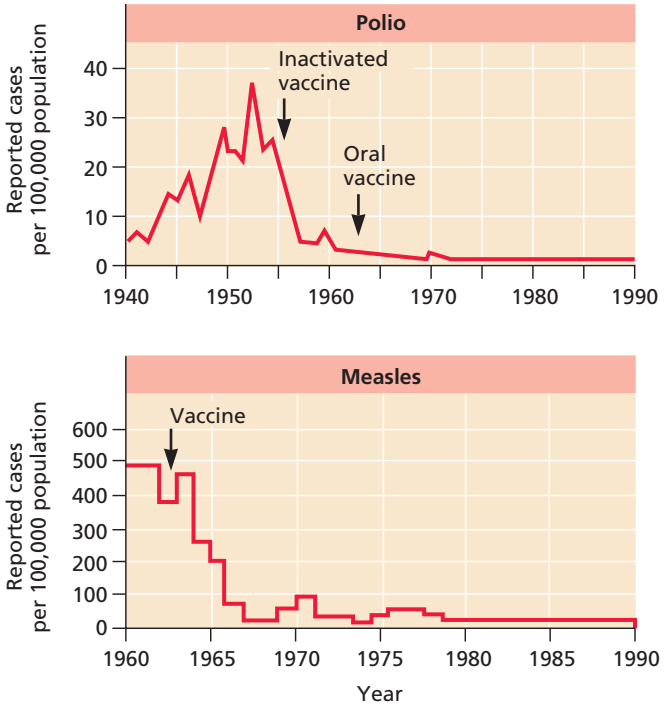


Figure 8.1 Profiles of successful vaccination campaigns. The number of reported cases of poliovirus (top) and measles virus (bottom) infection in the United States has been greatly reduced after massive vaccination programs. Adapted from C. A. Janeway, Jr., et al., *Immunobiology: the Immune System in Health and Disease* (Current Biology Limited, Garland Publishing Inc., New York, NY, 2001), with permission.



Figure 8.2 Irrational fears of the effects of vaccines. Some believed that vaccination using a virus that infected cows would cause cow-like features to appear in the recipient. The preponderance of data suggests this is not the case.

varius, meaning "mark on the skin." Variolation did confer a milder disease in some: the case-fatality ratio was ~10 times lower than in people infected directly. Nevertheless, this approach caused infections, not prevented them. Despite the dangers of variolation, little additional progress in controlling the disease was made for centuries, and a virtually unchanged protocol was widely in use in Europe in the 1800s. The process was always controversial and was banned in many countries. While part of the rationale for banning variolation was that people became infected, there was also a strong sense that this procedure was "sinful," interfering with God's intended plan for individuals. As we will see later in this chapter, cultural and religious beliefs continue to influence some individuals' willingness to be vaccinated.

The vaccination story begins with Edward Jenner (1749–1823), a country doctor and naturalist, who was well known at the time for a seminal paper titled "Observations on the Natural History of the Cuckoo." At first glance, Jenner seems an unlikely candidate to conceive of, and establish, the means by which natural infection by smallpox was eventually eradicated. However, he was a careful and thoughtful observer of both cuckoos and his patients, and one day overheard a dairymaid commenting that she would never get smallpox because she had been previously infected with cowpox. Jenner put this assertion to the test on May 14, 1796, when he injected fluid from a cowpox lesion on the finger of milkmaid Sarah Nelmes under the skin of James Phipps, a healthy 8-year-old boy. As expected, the boy developed a fever and a lesion typical of cowpox at the site of the injection. Two weeks later, Jenner then deliberately infected Phipps with smallpox. The young boy survived this potentially lethal challenge; needless to say, such an experiment would not be possible today.

Despite this promising result, the Royal Society in England rejected Jenner's paper, concerned that it was too anecdotal, leading Jenner to publish his work privately. While it is Jenner's name that is remembered, his colleague William Woodville, a prominent physician, was responsible for the first large-scale test that confirmed Jenner's observations. Introduction of the vaccine met with public skepticism and irrational fears (Fig. 8.2). Nevertheless, the smallpox vaccine was put into widespread use in 1800, and the disease was declared eradicated by the World Health Organization in 1979. Despite this monumental achievement, the specter of bioterrorism in the late 20th century has renewed interest in the virus and its vaccine (Box 8.1).

As is the case for many early discoveries, the scientific world was not prepared initially to exploit Jenner's approach and apply it to other pathogens. It took more than a century before the next practical vaccine for a viral disease appeared. Louis Pasteur, known for the germ theory and developing a technique to limit food spoilage due to microbes (pasteurization), prepared a rabies vaccine from the dehydrated spinal cord of an infected rabbit, and introduced the term **vaccination** (from *vacca*, Latin for "cow") in honor of Jenner's

BOX 8.1

BACKGROUND

The current U.S. smallpox vaccine

Prior to 2010, the vaccine stockpiled in the United States to protect civilian and military personnel against deliberate dissemination of smallpox virus was Dryvax, a freeze-dried, replication-competent vaccinia virus that was grown in calf lymph. Widespread distribution of this vaccine was discontinued by Wyeth in 1983, soon after the virus was declared eradicated. In 2008, the remaining stocks of Dryvax were destroyed by the Centers for Disease Control and Prevention and were replaced by a similar preparation, Sanofi-Pasteur's ACAM2000, which is prepared by infecting kidney epithelial cells in culture, rather than growing it in the skin of calves. A safer alternative was introduced in 2010 by Bavarian Nordic. This vaccine, Imvamune, is a nonreplicating strain that would eliminate the inherent risks of the replication-competent vaccine, which caused severe illness in 1 to 2% of recipients.

Development of a safer vaccine against smallpox is an important advance because the original vaccine caused rare but serious side effects, including severe skin reactions and central nervous system disorders. During the period in which every child in the United States was vaccinated, ~7 to 9 deaths per year were attributed to vaccination, with the highest risk occurring in infants. Inadvertent administration of the vaccine to immunodeficient individuals or to people with preexisting skin diseases resulted in a significantly larger number of adverse reactions. In 2002, following fears of bioterrorism in the wake of 9/11, the U.S. government announced that it would immunize military personnel and frontline civilian health care workers.

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A scar remaining following a successful vaccination against smallpox.



pioneering work. Even with Pasteur's success against rabies, other antiviral vaccines were slow to follow, largely because viruses were difficult to identify, propagate, and study. Consequently, the next vaccines (against yellow fever and influenza viruses) did not appear until the mid-1930s.

Large-Scale Vaccination Programs Can Be Dramatically Effective

Ideally, vaccination mobilizes the host immune system to prevent a pathogenic outcome upon reinfection. As progressively more individuals in a population become immunized, the transmission cycle of host-to-host spread in a population is disrupted. As illustrated by the eradication of smallpox and the steadily decreasing rates of some common viral diseases as a consequence of sustained vaccination efforts, vaccination provides a remarkably effective antiviral defense. The World Health Organization reported in 2012 that ~84% of all children received at least one dose of measles vaccine before their first birthday, a 12% increase since 2000. Increased vaccine coverage resulted in a 78% drop in measles deaths worldwide between 2000 and 2012. Such achievements require massive, coordinated efforts of public health workers, governments, local clinics, vaccine providers, and funding agencies. As an example of the magnitude of the effort, in a **single** day, the World Health Organization once administered 127 million poliovirus vaccines to children in more than 650,000 villages in India.

Eradicating a Viral Disease: Is It Possible?

Viruses have survived countless bouts of selection during evolution, so the objective to eliminate a virus from the planet may seem naïve and unattainable. However, since the proclamation from the Director General of the World Health Organization that smallpox was eradicated, no natural cases of smallpox have been reported. The debate continues: should existing laboratory stocks of smallpox be destroyed (Box 8.2)?

The second virus to be vanquished was rinderpest virus, which infects cattle, buffalo, and other hoofed animals. This morbillivirus is a relative of measles virus and can be transmitted by aerosol or through drinking contaminated water. Outbreaks would often devastate entire herds, with deaths approaching 100% in immunologically naïve populations. The vaccine, developed in 1962, resulted in global eradication in June 2011.

These successes have prompted anticipation that other devastating infections, including poliovirus and measles virus, may be next on the horizon for elimination. To have any rational hope of eradication, a viral candidate must possess two essential features: the infectious cycle must take place in a single host, and infection (or vaccination) must induce lifelong immunity. By definition, a vaccine that renders the host population immune to subsequent infection by a virus that can grow **only** in that host effectively eliminates the virus. In contrast, a virus with alternative host species in which to propagate cannot be eliminated by vaccination of a single host population; other means of blocking viral spread are required.

BOX 8.2

DISCUSSION

Should laboratory stocks of smallpox virus be destroyed?

Samples of smallpox still exist in carefully regulated and locked freezers in the United States and Russia, and there is much debate about whether these stocks should be destroyed or preserved for future, potential scientific research. Since the disease was eradicated in the late 1970s, the World Health Organization has, on several occasions, delayed destroying the virus to permit research on smallpox vaccines and treatments, particularly in light of concerns about bioterrorist attacks.

The important issues are the following:

- Should we destroy biodiversity and gene pools that are not well understood?
- Are stocks of smallpox virus necessary for development of new vaccines and antivirals?
- If we do move forward with "lab eradication," how do we ensure that all reserves have been destroyed?

While some argue that the presence of frozen smallpox samples leaves open the possibility for nefarious groups to amplify and use them to harm a nonvaccinated population, others, including most scientists, believe that these stocks would be of extraordinary value should the virus reemerge in the human population. These reserves would also be useful in the licensure of new antivirals and vaccines and for the development of accurate diagnostics that can distinguish smallpox from other poxvirus relatives.

Fenner F. 1996. History of smallpox, p 25–37. *In* Koprowski H, Oldstone MBA (ed), *Microbe Hunters, Then and Now*. Medi-Ed Press, Bloomington, IL.

Henderson DA, Fenner F. 2001. Recent events and observations pertaining to smallpox virus destruction in 2002. *Clin Infect Dis* **33:**1057–1059.

Credit: NASA.



Even when these requirements are fulfilled, global eradication can remain a formidable challenge. For example, widespread use of inexpensive, effective poliovirus vaccines has severely minimized this virus's impact on human health, and the vaccine is effective and inexpensive. Fewer than 100 cases of acute poliomyelitis were diagnosed in 2014. As the virus has no host other than humans, it should be possible to eliminate it by vaccinating a sufficient number of people and thereby ending the spread of the virus. Accordingly, the World Health Organization targeted the eradication of poliovirus by 2005 with a massive worldwide vaccination program. Sadly, the goal was not achieved, not because of lack of vaccine efficacy, but rather as a result of human variables that are difficult if not impossible to control, and which collectively limited complete coverage. Poverty, societal views of health care, lack of trust in physicians or the government, poor local health system infrastructure, and economic challenges often conspire to prevent vaccination of children in inner cities and in resource-poor countries. As a result, the virus remains endemic in Afghanistan, Nigeria, and Pakistan. Given that it may be impossible to eliminate all sources of the virus, poliovirus vaccination may be part of public health programs indefinitely (Box 8.3).

After poliovirus, measles virus is next on the World Health Organization's list for eradication. Measles virus is historically one of the world's leading infectious causes of childhood mortality, resulting in >100,000 deaths worldwide each year. Vaccination campaigns have reduced measles virus deaths by >75% since 2000, and the incidence of acute infections in the United States is steadily declining as a result of the efficacy of the vaccine (Fig. 8.3). However, unlike the polio vaccine, which can be given orally, the current measles vaccine requires two injections for maximal efficacy. This requirement alone imposes logistical and practical problems that will complicate global elimination of this virus. In addition, other virus properties of measles complicate its eradication. The infection is highly contagious, and the long period during which an individual is asymptomatic but shedding virus particles makes it difficult to identify and quarantine infected individuals. The reproduction number (R_0; see Chapter 10) of measles virus provides a measure of its contagious nature. R_0, the number of secondary infections produced by an infected person in a fully susceptible population, is estimated to be between 5 and 7 for smallpox virus, but for measles virus it is between 12 and 18. Amazingly, one person infected with measles infects 2 to 3 times as many susceptible people as does someone infected with smallpox. Whether a vaccination program can stay ahead of this pervasive foe remains to be determined.

National Programs for Eradication of Agriculturally Important Viral Diseases Differ Substantially from Global Programs

National vaccination and disease control programs are typically established for economically important livestock diseases. The goal is to keep a country free of a particular viral disease even though that disease may still be present in other countries. For example, the United States and Canada have been declared free of foot-and-mouth disease, but outbreaks still occur in parts of Europe, South America, and Asia. National programs can be successful only when augmented with broad governmental enforcement and border security,

BOX 8.3

DISCUSSION

The poliomyelitis eradication effort: should vaccine eradication be next?

The worldwide effort to eradicate poliomyelitis, launched in 1988 by the World Health Assembly (the decision-making body of the World Health Organization), remains stalled. The goal for eradication was set to occur in 2000, but setbacks necessitated shifting the target date forward to 2010. The current objective is for global eradication by 2018.

Enthusiasm was high during the initial years of the campaign; with the then-recent achievement of smallpox elimination, there was much optimism that the success could be duplicated for poliovirus. Indeed, the number of cases of the disease had been steadily falling, from a prevaccination estimate of 350,000 to <100 cases in 2014. The initial optimism has been replaced by doubt over whether eradication is realistic in light of the biological and political realities that have emerged in the course of the campaign.

The strategy to eradicate polio makes use of large-scale immunization campaigns with live attenuated poliovirus vaccine. These vaccine strains were known to revert to neurovirulence and cause vaccine-associated poliomyelitis. However, it was thought initially that vaccine-derived poliovirus strains do not circulate efficiently in the population, and that once wild-type poliovirus was eradicated, cessation of vaccination would eliminate vaccine-associated disease. Unfortunately, the 2000 outbreak of poliomyelitis in Hispaniola revealed this assumption to be incorrect. In this outbreak, 21 confirmed cases, all but 1 of which occurred in unvaccinated or incompletely vaccinated children, were reported. Subsequent analyses showed that the viruses responsible for the outbreak were derived from the Sabin poliovirus type 1 vaccine administered in 1998 and 1999. The neurovirulence and transmissibility of these viruses were indistinguishable from those of wild-type poliovirus type 1. Evidence of circulating vaccine-derived poliovirus was subsequently identified in Egypt and Nigeria. The previously underestimated threat of vaccine-derived polioviruses now makes the plan to cease vaccination unacceptable.

During the eradication campaign, regions are certified as free from wild-type polioviruses when the virus cannot be isolated for a 3-year period. As of 2015, poliovirus is endemic in only three countries: Afghanistan, Nigeria, and Pakistan. Since the last edition of this textbook, India has successfully transitioned to a "polio-free" country. Failure to eliminate transmission of wild polioviruses in these remaining countries is probably a consequence of insufficient vaccine coverage due to politics and war. For example, it is difficult to deliver polio vaccine to the border of Pakistan and Afghanistan, where skirmishes occur regularly and where health care workers are at great peril for kidnapping or murder. Even when the vaccine is administered, children continue to contract polio, probably as a result of poor sanitation, crowding, poverty, and infection with other microbes.

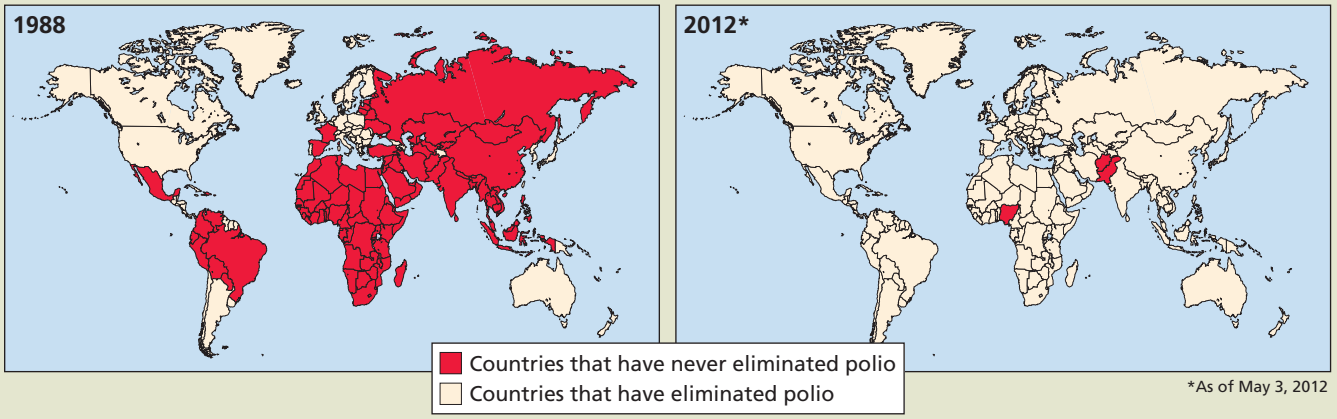
These considerations lend strength to the conclusion that polio eradication, followed by cessation of vaccination, is not a realistic goal, and that the program should be modified to ensure the protection of as many individuals as possible from poliomyelitis. The vaccine of choice would be one that does not revert to neurovirulence, such as the inactivated vaccine. However, this vaccine provides poor gut immunity compared to the attenuated vaccine, indicating that further improvements should be sought.

Chumakov K, Ehrenfeld E, Wimmer E, Agol VI. 2007. Vaccination against polio should not be stopped. *Nat Rev Microbiol* **5:**952–958.

Dove AW, Racaniello VR. 1997. The polio eradication effort: should vaccine eradication be next? *Science* **277:**779–780.

Minor PD. 2004. Polio eradication, cessation of vaccination and re-emergence of disease. *Nat Rev Microbiol* **2:**473–482.

Globally reported incidence of poliomyelitis in 1988 and 2012. The Americas, Western Pacific, European regions, and recently India have been declared poliomyelitis free by the World Health Organization. The number of cases has declined from an estimated 350,000 in 1988 to fewer than 100 cases in 2014. At the same time, the number of countries in which poliovirus is endemic has decreased from >125 to 3: Afghanistan, Nigeria, and Pakistan. Credit: Centers for Disease Control and Prevention.



as animals in the virus-free country are constantly at risk for exposure from sources elsewhere. The perpetual concern of accidental import of these agricultural viruses is why customs officials inquire if residents of disease-free countries were exposed to livestock when traveling abroad.

Countries in which the disease is still present must have other means of control to limit outbreaks. Surveillance and containment strategies must be mobilized quickly and aggressively to identify and stop the spread from localized outbreaks. A common practice is to slaughter every host

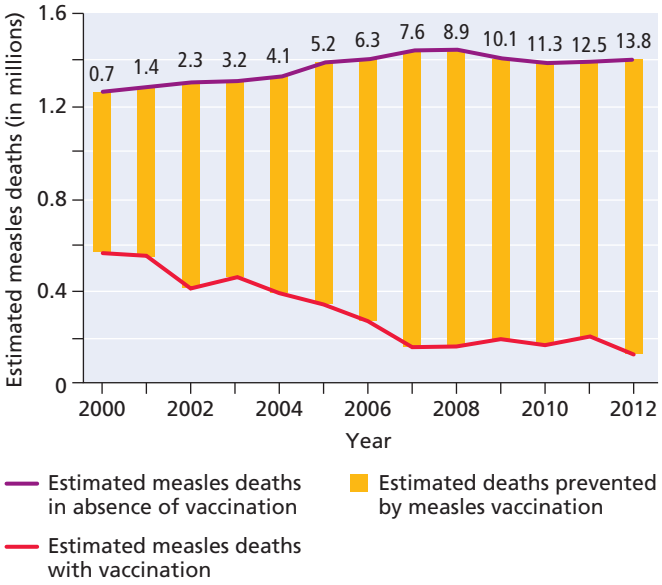


Figure 8.3 Decline in worldwide measles deaths due to vaccination. Estimated worldwide measles mortality and measles-induced deaths that were prevented by vaccination during 2000 to 2012 are shown. Compared with a scenario of no vaccination against measles, an estimated 13.8 million deaths were prevented by measles vaccination during this period.

animal in farms at increasing distances surrounding an outbreak site (the so-called "ring-slaughter" approach [Box 8.4]). Because acute infections spread rapidly from the outbreak site by many routes, and do so before identifiable symptoms are visible, the ring-slaughter containment often is breached unknowingly. As a result, preemptive slaughter of **all** animals on "at-risk" farms may be required. For example, South Korea faced a major foot-and-mouth disease outbreak on pig farms in 2010, in which >100 confirmed cases were identified. To halt the outbreak, >3 million animals (12% of the domestic pig population) were destroyed. Consequently, the devastating economic ramifications of such outbreaks have an impact even on those farms where no foot-and-mouth disease is present. Obviously, the faster an outbreak is identified, the more likely the success of containment efforts. Unfortunately, on-farm diagnostic tools that provide reliable identification of pathogens before symptoms are visible are not yet available. When developed, such tools need to be quick, easy, and accurate: false-positive identification of an outbreak could result in unnecessary sacrifice of many farm animals, with attendant economic loss.

Although prevention through vaccination is a powerful tool, and foot-and-mouth disease vaccines are available, other considerations limit the efficacy of this vaccine. Many serotypes for foot-and-mouth disease are currently in circulation, and vaccination against one serotype does not necessarily provide protection against others. Even strains within a given serotype may possess small sequence changes that invalidate the vaccine's efficacy. Moreover, standard blood tests to identify antibodies cannot distinguish between an infected animal and a vaccinated animal. Consequently, many farmers are reluctant to vaccinate for fear that their meat products will not be exportable to other markets.

Vaccine Basics

Immunization Can Be Active or Passive

Active immunization with attenuated or killed virus preparations or with purified viral proteins induces immunologically mediated resistance to infection or disease. In contrast, **passive immunization** introduces components of the immune response (e.g., antibodies or stimulated immune cells) obtained from an appropriate donor(s) directly into the patient. All neonates benefit from passive immunization fol-

BOX 8.4

BACKGROUND

Stopping epidemics in agricultural animals by culling and slaughter

Vaccination of agriculturally important animals such as cattle and pigs may not be cost-effective or may run afoul of government rules that block the shipping and sale of animals with antibodies to certain viruses. The 2001 foot-and-mouth disease epidemic in the United Kingdom provides a dramatic example of how viral disease is controlled when vaccination is not possible. The solution that stopped the epidemic was mass slaughter of **all animals** (infected or not) surrounding the affected areas and chemical decontamination of farms. It is estimated that >6 million animals were destroyed in less than a year before the spread of foot-and-mouth disease virus was contained, and similar mass killing of chickens and pigs was employed in South Korea in 2013 to prevent spread of influenza A virus and Nipah virus, respectively.

Animal slaughter is often the only action available to officials dealing with potential epidemic spread. For example, in recent years, millions of chickens in Hong Kong were killed to stop an influenza virus epidemic with potential to spread to humans. Influenza is not the only agricultural threat to poultry: chickens in California poultry farms were slaughtered in the 1970s to stem the spread of Newcastle disease virus.

Keeling MJ, Woolhouse ME, May RM, Davies G, Grenfell BT. 2003. Modelling vaccination strategies against foot-and-mouth disease. *Nature* **421:**136–142.

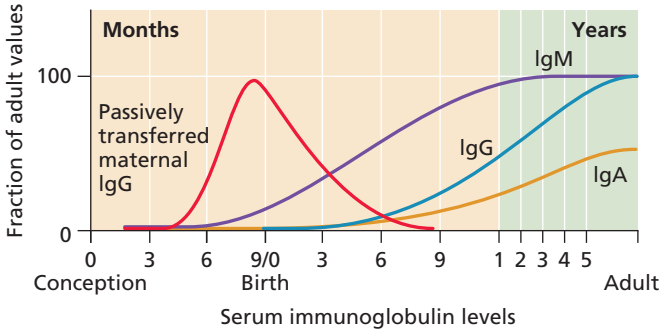
Kitching RP, Hutber AM, Thrusfield MV. 2005. A review of foot-and-mouth disease with special consideration for the clinical and epidemiological factors relevant to predictive modelling of the disease. *Vet J* **69:**197–209.

Woolhouse M, Donaldson A. 2001. Managing foot-and-mouth. *Nature* **410:**515–516.

lowing birth, as some of the mother's antibodies pass into the fetal bloodstream via the placenta to provide transient protection to the immunologically naïve newborn. Newborns who are breastfed are further protected by transfer of a particular type of antibody (IgA) in the antibody-rich colostrum. This protective effect can be detrimental if active immunization of infants is attempted too early, as maternal antibody may block a vaccine from stimulating immunity in the infant (Fig. 8.4). For this reason, most vaccines are not administered until 6 to 12 months postbirth.

Passive immunization is a preemptive response, usually adopted when a virus epidemic is suspected, because it provides immediate protection and does not require the host to mount an effective memory response. In 1997, consumption of contaminated fruit led to a widespread outbreak of hepatitis A virus infections in the United States. Pooled human antibodies (also called immunoglobulin) were administered in an attempt to block the spread of infection and reduce disease. This antibody cocktail contains the collective immunological experience of many individual infections and provides instant protection against some viruses. Passive immunization and immunotherapy are used for multiple virus infections, but the best-known instance is for rabies, in which a preparation of human immunoglobulin is delivered as soon as possible after a bite from a rabid animal to contain the virus before it can be disseminated. In addition, the standard procedure for smallpox vaccination with live vaccinia virus requires that so-called "vaccine immunoglobulin" be available should disseminated vaccinia occur in the vaccine recipient. When stimulated immune cells (e.g., T cells) are used, the process is called **adoptive transfer**; transfer of memory T cells may provide longer-lasting protection than antibody transfer, as the relatively short half-life of the antibody proteins limits sustained efficacy. Either way, passive immunization produces short-term effects, whereas active immunization can be lifelong.

Figure 8.4 Passive transfer of antibody from mother to infant. The fraction of the adult concentration of various antibody classes is plotted as a function of time, from conception to adulthood. Newborn babies have high levels of circulating IgG antibodies derived from the mother during gestation (passively transferred maternal IgG), enabling the baby to benefit from the broad immune experience of the mother. This passive protection falls to low levels at about 6 months of age as the baby's own immune response takes over. Total antibody concentrations are low from about 6 months to 1 year after birth, a property that may increase susceptibility to disease. Premature infants are particularly at risk for infections because the level of maternal IgG is lower and their immune system remains underdeveloped. The time course of production of various isoforms of antibody (IgG, IgM, and IgA) synthesized by the baby is indicated. Adapted from C. A. Janeway, Jr., et al., *Immunobiology: the Immune System in Health and Disease* (Current Biology Limited, Garland Publishing Inc., New York, NY, 2001), with permission.



Active Vaccination Strategies Stimulate Immune Memory

Vaccines work because they educate the host's immune system to recall the identity of a specific virus years after the initial encounter, a phenomenon called **immune memory** (Box 8.5). The resounding practical success of immunization in stimulating long-lived immune memory is among the greatest medical achievements.

Immune memory is maintained by dedicated T and B lymphocytes that remain after an infection has been resolved and most activated immune cells have died. These memory cells are able to respond quickly to a subsequent infection (Fig. 8.5). Antiviral vaccines establish immunity and memory without the pathogenic effects typical of the initial encounter with a virulent virus. Ideally, an effective vaccine is one that induces and maintains significant concentrations of memory cells in serum or at points of viral entry, such as mucosal surfaces and skin. When the pathogen to which they are specific returns, the memory B and T cells spring to action, unleashing an aggressive response that rapidly controls the pathogen before pathogenesis can ensue.

Protection from Infection or Protection from Disease?

It is important to consider that there are different possible outcomes following vaccine administration. In some cases, the antibody and memory T cells established by vaccination are maintained for long periods, and their mobilization will be sufficient to stop a subsequent infection before the virus can spread beyond the site of entry. Disease is prevented because the virus cannot reproduce or spread. In other cases, virus reproduction and spread may not be blocked immediately. Such infections can **only** be cleared by the coordinated action of vaccine-induced immune effectors **and** infection-induced immune responses (e.g., interferon production). In this case, disease may not be prevented, but its onset can be delayed or its severity lessened. In a third, less optimal outcome, the virus will not be eliminated because the host's response to the vaccine or to subsequent infection (or both) is inadequate. Consequently, disease is not prevented and vaccination may confer only a modest delay in the appearance of disease.

BOX 8.5

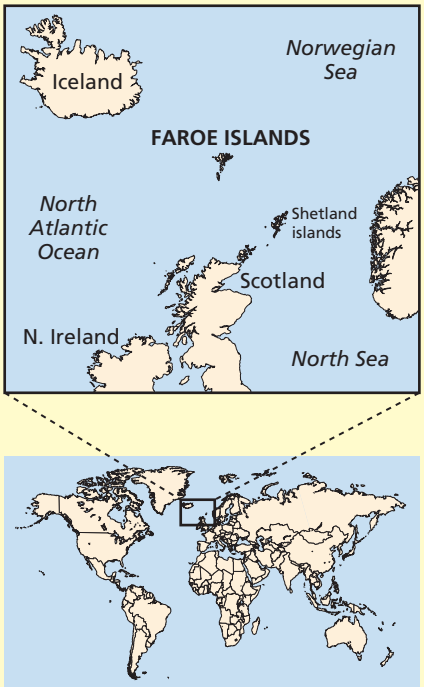
EXPERIMENTS

A natural "experiment" demonstrating immune memory

A striking example of immune memory is provided by a natural "experiment" in the 18th and 19th centuries on the Faroe Islands in the northern Atlantic Ocean. These islands were an ideal stopping point for cargo ships that transported goods between America and Europe. In 1781, measles, probably introduced by an infected sailor, infected many islanders and drastically reduced the islands' population. Subsequently, changes in shipping routes made the Faroes a less ideal stopping point, and for the next 65 years, the islands remained measles virus free and the surviving population flourished. In 1846, as the islands were revisited by these vessels, measles struck again, infecting >75% of the population with similar devastating results. In a personal and entertaining diary-like article titled "Observations Made during the Epidemic of Measles on the Faroe Islands in the Year 1846" (http://www.deltaomega.org/documents/PanumFaroeIslands.pdf), the Danish physician Peter Panum noted that none of those individuals who survived the 1781 epidemic became infected in 1846. As a perfect age-matched control, their peers who had **not** been infected earlier were ravaged by measles in this second outbreak. This natural experiment illustrates two important points: immune memory can last for decades, and it can be maintained without ongoing exposure to the virus.

Ahmed R, Gray D. 1996. Immunological memory and protective immunity: understanding their relation. *Science* **272:**54–60.

Panum PL. 1847. Observations made during the epidemic of measles on the Faroe Islands in the year 1846. *In Bibliothek for Laeger, Copenhagen, 3R.*, **1:**270–344.



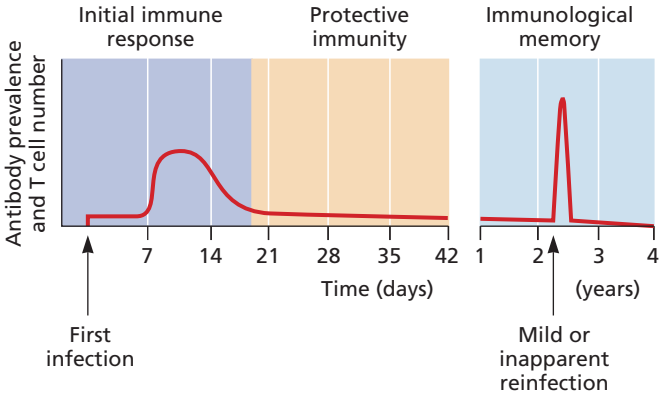


Figure 8.5 Antibody and effector T cells are the basis of protective immunity. The relative concentrations of antibody and T cells are shown as a function of time after first (primary) infection. Antibody levels and numbers of activated T cells decline after the primary viral infection is cleared. Reinfections at later times (years later), even if mild or inapparent, are marked by rapid and robust immune response because of the reanimation of a memory response. Adapted from C. A. Janeway, Jr., et al., *Immunobiology: the Immune System in Health and Disease* (Current Biology Limited, Garland Publishing Inc., New York, NY, 2001), with permission.

Vaccines Must Be Safe, Efficacious, and Practical

The two prerequisites for an effective vaccine are that it is safe and effective. Vaccines that are based on inactivated virus particles or immunogenic viral proteins must not contain infectious particles or viral nucleic acids, respectively. If a live vaccine is used, virulent revertants must be exceedingly rare. In addition, there can be no contamination of vaccines with other microbes introduced during production. These ideals may seem obvious, but given the potential for human error, absolute safety is impossible to guarantee. Furthermore, when rare side effects do appear, they are often identified only after millions of people have been vaccinated (Box 8.6). In addition, attenuated (replication-competent) vaccines have the potential to spread to individuals in a population who have not been vaccinated. The smallpox vaccine is not given to immunosuppressed individuals, because they cannot contain vaccine-associated infection caused by the live vaccine.

To be effective, a vaccine must induce protective immunity **in a significant fraction of the population**. Not every individual in the population need be immunized to stop viral spread, but the number must be sufficiently high to impede virus transmission. Person-to-person transmission stops when the probability of infection drops below a critical threshold. This effect has been called **herd immunity**. To appreciate the importance of this effect, one might consider the likely outcome in two hypothetical elementary schools: in elementary school A of some 500 students, all but 10 have been vaccinated against measles virus, whereas in school B, only half of the same number have been vaccinated. Most

BOX 8.6

DISCUSSION

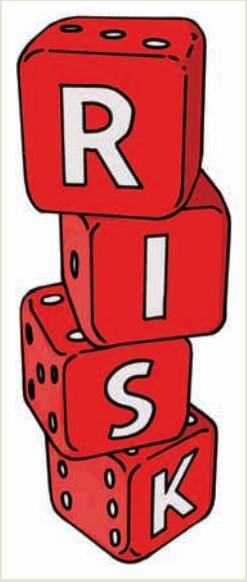
The public's view of risk-taking is a changing landscape

Whooping cough was a major lethal disease of children until the introduction of the DPT (diphtheria, pertussis, and tetanus) vaccine, which virtually eliminated the disease. Immunization resulted in frequent but mild side effects: ~20% of children experienced local pain and some tiredness. However, about 1 immunized child in 1,000 had more-severe side effects, including seizures and sustained high fever. Given that whooping cough was well known to be a child killer, these side effects were generally deemed acceptable. Because whooping cough is perceived as a "disease of the past," some parents now feel that the risk of immunization side effects is unacceptable and are electing not to vaccinate their children. This reluctance to vaccinate has resulted in the predictable presence of whooping cough victims in clinics and alarming increases in the frequency of this disorder in California, Michigan, and the United Kingdom, among other places. The risk posed by the vaccine has not changed, but in the face of reduced threat of natural disease, the perceived risk of vaccination is elevated.

A quote from a 2004 article on game theory defines the problem in succinct and powerful terms: "Voluntary vaccination policies for childhood diseases present parents with a subtle challenge: if a sufficient proportion of the population is already immune, either naturally or by vaccination, then even the slightest risk associated with vaccination will outweigh the risk from infection. As a result, individual self-interest might preclude complete eradication of a vaccine-preventable disease."

Bauch CT, Earn DJ. 2004. Vaccination and the theory of games. *Proc Natl Acad Sci U S A* **101:**13391–13394.

Johnson B. 2001. Understanding, assessing, and communicating topics related to risk in biomedical research facilities. *ABSA Anthology of Biosafety IV—Issues in Public Health*, chapter 10. http://www.absa.org/0100johnson.html.



would intuitively and correctly judge that an unvaccinated child is much less likely to become infected with measles virus in school A than in school B. This is true because the 490 vaccinated students in school A provide an immunological wall around the unvaccinated children. In school B, the wall is leakier, and consequently there are more opportunities for the virus to gain a foothold in this population and to be transmitted to those who have not been vaccinated.

The actual calculation for herd immunity is pathogen and population specific, but generally corresponds to 80 to 95% of the population acquiring vaccine-induced immunity to provide protection to all members of that community. The herd immunity threshold is calculated as $1 - 1/R_0$. Recall that R_0 is the number of nonimmune individuals that would get infected upon encounter with an actively infected individual. As this number increases (that is, as the virus is transmitted to more individuals), the value of $1/R_0$ decreases, and thus $1 - 1/R_0$ gets closer to 1, or 100%. For smallpox virus, the herd immunity threshold is 80 to 85%, while for measles virus (which has a high R_0), it is 93 to 95%. Subtle changes in herd immunity have direct implications for the risks of outbreaks of infection (Fig. 8.6). No vaccine is 100% effective in a population; consequently, the level of immunity is not equal to the number of people immunized. In fact, we know that when 80% of a population is immunized with measles vaccine, about 76% of the population is actually immune, clearly well below the 93 to 95% required to prevent measles virus from infecting this community. Obviously, achieving such high levels of immunity by vaccination is a daunting task. Moreover, if the virus remains in other populations or in alternative hosts, reinfection is always possible. In closed populations (e.g., military training camps or animal herds), high levels of immunity can be achieved by vaccination of all individuals, but larger or less-controlled populations in widespread areas present serious logistical challenges. In addition, public complacency or reluctance to be immunized is dangerous to any vaccine program (Box 8.6).

The protection provided by a vaccine must also be long-term, lasting many years. While some vaccines cannot provide lifelong immunity after a single administration, subsequent inoculations (booster shots) given after the initial dose can stimulate waning immunity. However, this practice may be impractical for administration to large populations and can pose serious record-keeping challenges in resource-poor areas. Mounting the "proper" immune response is also required. For example, primary infection by some viruses, such as poliovirus, can be blocked only when a robust antibody response is evoked by vaccination. On the other hand, a potent cellular immune response is required for protection against herpesviral disease. To maximize effectiveness, a vaccine must be tailored to elicit the same type of immune responses as the natural virus infection it is designed to prevent.

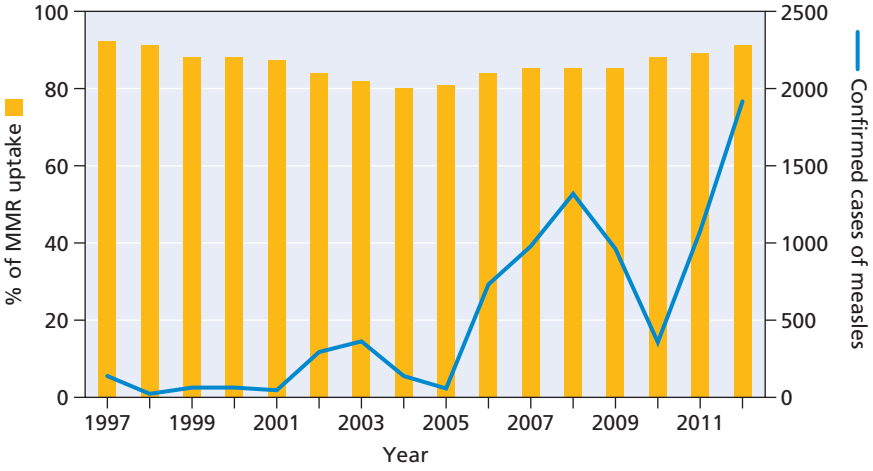


Figure 8.6 The correlation between herd immunity and the potential for outbreaks. Shown is the period between 1997 and 2012, with vaccination rates in Great Britain indicated on the left (gold bars expressed as a percentage of the population) and the number of reported cases of measles virus shown on the right (the solid blue line showing total number of cases). As the percentage of vaccinated individuals dips below ~90%, a corresponding rise in the number of acute cases is observed.

Outbred populations always have varied responses to vaccination. Some individuals exhibit a robust response, while others may not respond as well (a "poor take"). While many parameters influence such variability, the age and health of the recipient are major contributors. For example, the influenza virus vaccine available each year is far more effective in young adults than in the elderly. Weak immune responses to vaccination pose several problems. Obviously, protection against subsequent infection may be inadequate, but another concern is that upon such subsequent infection, viral reproduction will occur in the presence of weak immune effectors. Mutants that can escape the host's immune response can then be selected, and may spread in the immunized population. Indeed, vaccine "escape" mutants are well documented.

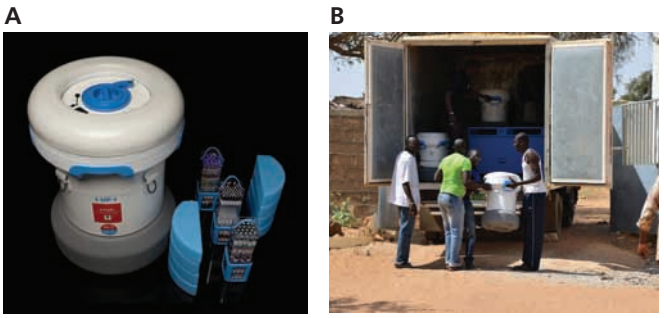
Once safety and efficacy are assured, other practical requirements including stability, ease of administration, and cost must be considered. If a vaccine can be stored at room temperature rather than refrigerated or frozen, it can be used where cold storage facilities are limited. One of the abiding challenges of measles virus eradication is that the vaccine, which is a live attenuated virus, must be kept cold from its synthesis to inoculation in the recipient host (the "**cold chain**"). Failure to keep the vaccine cold inactivates the attenuated virus and weakens its ability to induce immunity. In countries where electricity (and therefore, refrigeration) is not ubiquitous, this poses a substantial problem. But the science and engineering community loves a challenge: recently, the Gates Foundation supported the development of new thermoses that can keep vaccines frozen for more than a month without the need of any electrical source (Fig. 8.7). Continued collaborations with colleagues in the engineering field will be critical for the development of creative solutions to some of the practical challenges of worldwide vaccination (Box 8.7).

The route of administration and cost per dose are important considerations as well: when a vaccine can be administered orally rather than by injection, it will be more widely accepted. Similarly, the World Health Organization estimates that a vaccine must cost less than $1 per dose if its global use is to be meaningful. However, the research and development costs for a modern vaccine are in the range of hundreds of millions of dollars. Another, often prohibitive expense is covering the liability of the vaccine producer. Liability expenses can be astronomical in a litigious society and have forced many companies to abandon vaccine development completely. Unfortunately, there is an inherent conflict between providing a good return on investment to vaccine developers and supplying vaccines to people and government agencies with a limited ability to bear the cost. Nongovernmental organizations such as the Red Cross, the Global Vaccine Fund, and others have been instrumental in ensuring effective vaccine disbursal.

The Fundamental Challenge

Given the remarkable success of vaccines against smallpox, measles, and polioviruses, it might seem feasible to prepare vaccines that prevent all common viral diseases. Unfortunately, as described above, designing and producing an effective

Figure 8.7 Vaccine thermoses. Development of novel chambers that keep vaccines cold for extended periods without electricity may revolutionize the efficacy of delivery of some attenuated vaccines for which the cold chain must be maintained. Credit: Intellectual Ventures.



BOX 8.7

DISCUSSION

Development of new delivery vehicles for vaccines

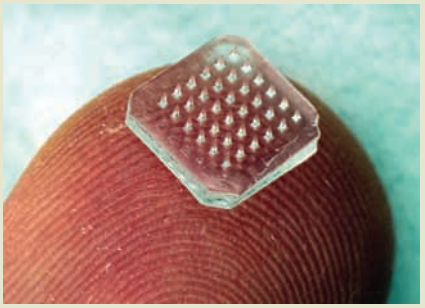
The need for trained professionals to administer traditional vaccines limits speedy distribution in some developing countries. A new patch that does not require any medical training to administer has been developed: this microneedle patch contains hundreds of microscopic needles that penetrate the outer epidermis and dissolve into the skin once the vaccine has been delivered. These patches can be self-administered, do not require costly and dangerous disposal of hypodermic needles, have enhanced stability, and may actually confer stronger protection than classical needle-based vaccination.

An additional recent development uses the sugars sucrose and trehalose to put vaccines into a kind of suspended animation, where stability can be maintained for >6 months, even if unrefrigerated. As noted in the text, freedom from the cold chain would almost certainly revolutionize vaccine administration in developing regions with poor or unreliable electricity. The sugar method, developed in the United Kingdom, allows the vaccine to gradually dry into a syrup, and ultimately a thin film, which can be rehydrated immediately before injection.

Alcock R, Cottingham MG, Rollier CS, Furze J, De Costa SD, Hanlon M, Spencer AJ, Honeycutt JD, Wyllie DH, Gilbert SC, Bregu M, Hill AV. 2010. Long-term thermostabilization of live poxviral and adenoviral vaccine vectors at supraphysiological temperatures in carbohydrate glass. *Sci Transl Med* **2:**19ra12. doi:10.1126/scitranslmed.3000490.

Norman JJ, Arya JM, McClain MA, Frew PM, Meltzer MI, Prausnitz MR. 2014. Microneedle patches: usability and acceptability for self-vaccination against influenza. *Vaccine* **32:**1856–1862.

A patch containing 36 dissolving microneedles. Credit: Jeong-Woo Lee, Georgia Tech.



vaccine are exceedingly difficult, and the enthusiasm for creating a new vaccine is dependent on medical need and the economic market. For example, during the 2014 Ebola outbreak, there was a massively accelerated effort to create a new vaccine, but it took a worldwide crisis to catalyze this initiative. Furthermore, despite considerable research progress, we cannot predict with confidence the efficacy or undesirable side effects of different vaccine preparations. Because we lack sufficiently detailed knowledge of the important mechanisms of immune protection against most viral infections, the optimal design of a vaccine is not always obvious. Questions such as "Is a neutralizing antibody response important?" or "Is a cytotoxic-T-lymphocyte response essential?" cannot be answered with certainty, even for the most common viral infections. In fact, only when a vaccine is effective (or more often, when it fails) can we learn what immune features constitute a protective response. To complicate the situation, even when an experienced vaccine manufacturer sets out to develop, test, and register a new vaccine, the process can take years and millions of dollars. For example, it took 22 years to develop and license a relatively straightforward hepatitis A virus vaccine. The fundamental challenge is to find ways to capitalize on the discoveries in molecular virology and medicine to expedite vaccine development.

The Science and Art of Making Vaccines

There are four basic approaches to produce vaccines (Fig. 8.8). Each uses components of the pathogenic virus that the vaccine is intended to target. A vaccine developer may produce large quantities of the virus of interest and chemically inactivate it (**inactivated vaccine**), attenuate the pathogenicity through laboratory manipulation (**replication-competent, attenuated vaccine**), produce individual proteins free of the viral nucleic acid (**subunit vaccine**), or molecularly clone all or portions of the viral genome for preparation of recombinant DNA vaccines (**recombinant vaccine**). The methods are designed to adhere to principles that would be understood by Pasteur (Box 8.8). The most common, commercially successful vaccines simply comprise attenuated or inactivated virus particles, though as we will see at the end of this chapter, cheaper, cleverer, and more effective strategies are in the pipeline (Box 8.9).

Inactivated or "Killed" Virus Vaccines

The inactivated poliovirus, influenza virus, hepatitis A virus, and rabies virus vaccines are examples of effective inactivated vaccines administered to humans (Table 8.1). Moreover, inactivated vaccines, such as those which prevent equine influenza virus and porcine circovirus infections, are widely used in veterinary medicine. To prepare such a vaccine, virulent virus particles are isolated and inactivated by chemical or physical procedures. These treatments eliminate the infectivity of the virus, but not its antigenicity (i.e., the ability to induce the desired immune response). Common methods to inactivate virions include treatment with formaldehyde or β-propriolactone, or extraction of enveloped virus particles with nonionic detergents. These vaccines are safe for immunodeficient individuals, as the treated viruses cannot reproduce. Immunization by inactivated vaccines, however, often requires the administration of multiple doses, as the first dose is generally insufficient to produce a protective response.

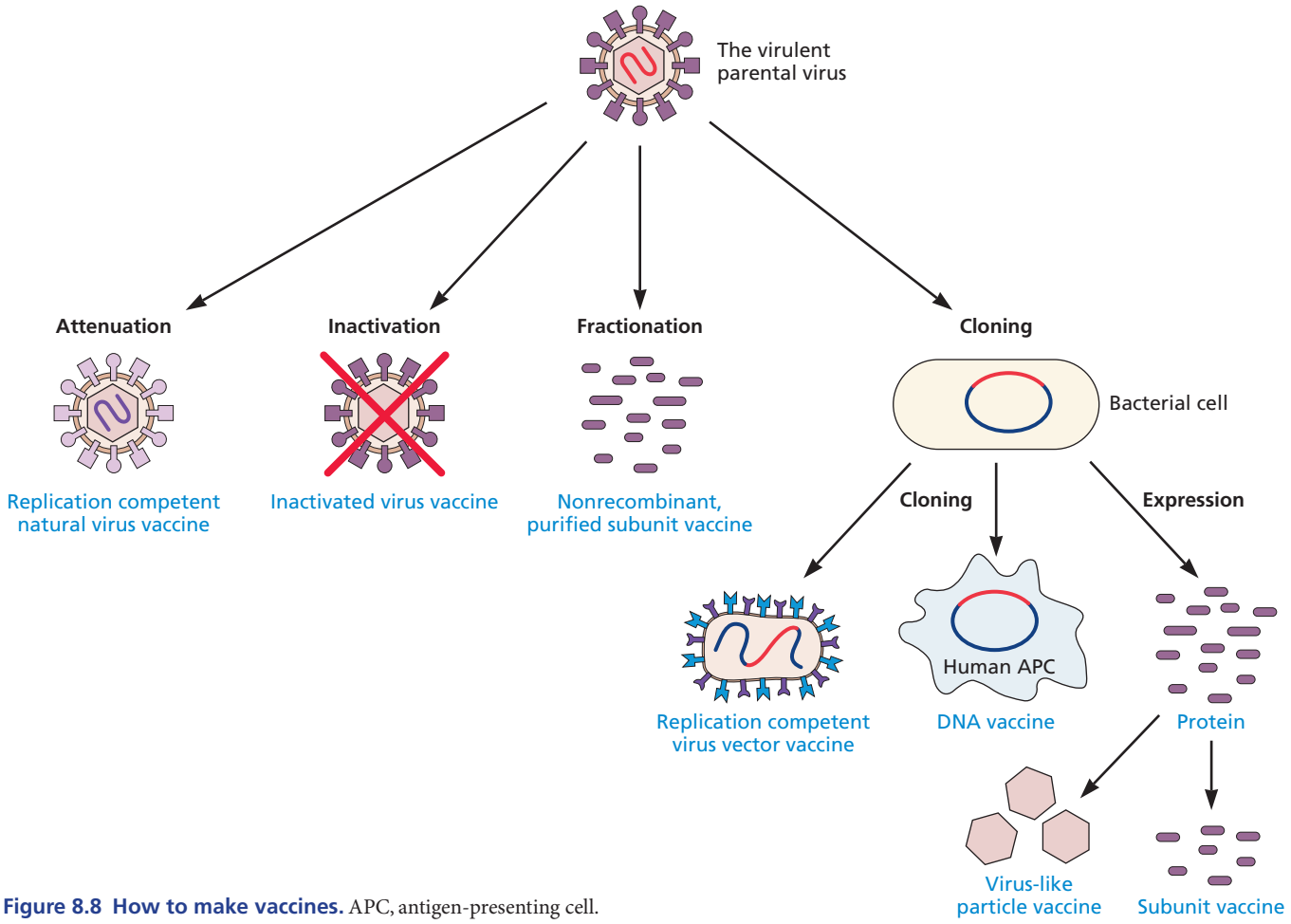


Figure 8.8 How to make vaccines. APC, antigen-presenting cell.

BOX 8.8

BACKGROUND

Most contemporary vaccines are based on old technology

In this age of modern biology, it seems ironic that the mutations in many of our common vaccine strains were not introduced by site-directed mutagenesis of genes known to be required for viral virulence, but rather were isolated by selection of mutants that could replicate in various cell types. The vaccines were produced with little bias for reduction of virulence *per se*.

Despite the old technology, the vaccines are relatively safe and remarkably effective. Consequently, their analysis has led to the identification of important attenuating mutations, as well as parameters that affect the protective immune response. The current vaccines not only provide protection from the intended virus, but are also the foundation for vaccines targeting other viruses. One example is the use of the yellow fever virus vaccine strain as a vehicle for West Nile and dengue viruses.



Painting of Louis Pasteur examining the dried spinal cord of an infected rabbit used to prepare an attenuated strain of rabies virus. Image courtesy of the Pasteur Institute (Photothèque/ Relations Presse et Communication externe, Institut Pasteur, Paris, France).

BOX 8.9

TERMINOLOGY

Live and let die

The authors of this textbook can be quite particular about word choice: for each edition, we sit around a table and read every word out loud. Even with the fourth edition, we still discovered long-standing inconsistencies and errors. While some of us are more aggrieved by the use of some words or phrases than others of us, we all concur that "live" and "dead" are misleading shorthands when appended to viruses. Alas, the vaccine community is less attentive to this issue, and so the terms "killed" and "live attenuated" vaccines are used generously in the discussion of vaccine types. Simply put: we will refer to such "killed" vaccines as "inactivated," and "live" vaccines will be termed "replication competent." We recognize that, while more accurate, it is also modestly more cumbersome: few would go to a James Bond movie called "Replication Competent and Let Inactivated." (See: http://www.imdb.com/title/tt0070328/ for the 007 classic that this Box is referring to.)

In principle, inactivated vaccines are very safe, but accidents can and do happen. In the 1950s, a manufacturer of Salk poliovirus vaccine, Cutter Laboratories, did not inactivate the virus completely, and >200 children developed disease as a result of vaccination (either by direct inoculation or contact with an infected child). Incomplete inactivation and contamination of vaccine stocks with potentially infectious viral nucleic acids have been singled out as major problems with this type of vaccine, though improved methods to detect residual infectious virus have reduced this risk substantially.

Administration of an inactivated vaccine annually is currently the most important measure for reducing influenza

Table 8.1 Viral vaccines licensed in the United States

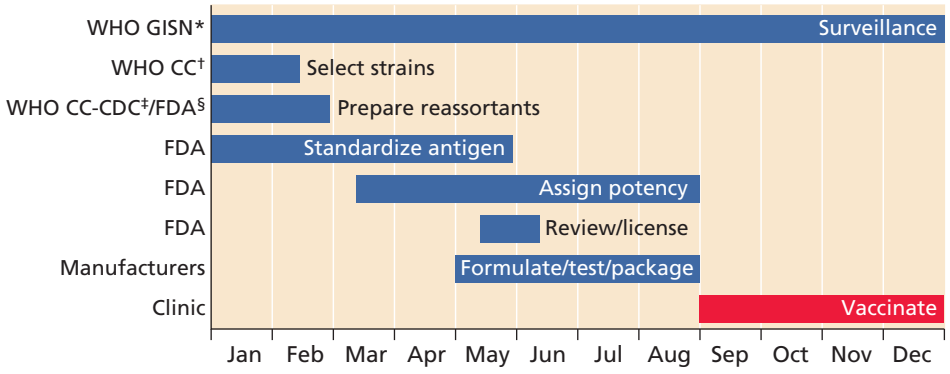
Disease or virus	Type of vaccine	Indications for use	Schedule
Adenovirus	Attenuated, oral	Military recruits	One dose
Hepatitis A	Inactivated whole virus	Travelers, other high-risk groups	0, 1, and 6 mo
Hepatitis B	Yeast-produced recombinant surface protein	Universal in children, exposure to blood, sexual promiscuity	0, 1, 6, and 12 mo
Influenza	Inactivated viral subunits	Elderly and other high-risk groups	One dose seasonally
	Recombinant proteins	Elderly; those with egg allergies	One dose seasonally
Influenza	Attenuated	Children 2–8 yr old, not previously vaccinated with influenza vaccine	Two doses at least 1 mo apart
		Children 2–8 yr old, previously vaccinated with influenza vaccine	One dose
		Children, adolescents, and adults 9–49 yr old (e.g., FluMist, FluBlo)	One dose
Japanese encephalitis	Inactivated whole virus	Travelers to or inhabitants of high-risk areas in Asia	0, 7, and 30 days
Measles	Attenuated	Universal vaccination of infants	12 mo of age; 2nd dose, 6 to 12 yr of age
Mumps	Attenuated	Universal vaccination of infants	Same as measles, given as MMR
Papilloma (human)	Yeast- or SF9-produced virus-like particles	Females 9–26 yr old	Three doses
Rotavirus	Reassortant	Healthy infants	2, 3, and 6 mo or 2 and 4 mo of age depending on vaccine
Rubella	Attenuated	Universal vaccination of infants	Same as measles, given as MMR
Polio (inactivated)	Inactivated whole viruses of types 1, 2, and 3	Changing: commonly used for immunosuppressed where live vaccine cannot be used	2, 4, and 12–18 mo of age, then 4 to 6 yr of age
Polio (attenuated)	Attenuated, oral mixture of types 1, 2, and 3	Universal vaccination; no longer used in United States	2, 4, and 6–18 mo of age
Rabies	Inactivated whole virus	Exposure to rabies, actual or prospective	0, 3, 7, 14, and 28 days postexposure
Smallpox	Vaccinia virus	Certain laboratory workers	One dose
Varicella	Attenuated	Universal vaccination of infants	12 to 18 mo of age
Varicella-zoster	Attenuated	Adults 60 yr old and older	One dose
Yellow fever	Attenuated	Travel to areas where infection is common	One dose every 10 yr

virus-induced morbidity and mortality. In the United States alone, influenza virus infections cause as many as 50,000 deaths every year and consume at least $12 billion in health care, although epidemics can cost as much as $150 billion. Each year, millions of citizens seeking to avoid infection receive their flu shot, which contains several strains of influenza virus that have been predicted to reach the United States in the next flu season. The magnitude of this undertaking is noteworthy: >150 million doses of inactivated vaccine must be manufactured every year. Typically, these vaccines are formalin-inactivated or detergent- or chemically disrupted virus particles. The viruses, which are mass-produced in embryonated chicken eggs, can be natural isolates or reassortant viruses constructed to contain the appropriate hemagglutinin (HA) or neuraminidase (NA) genes from the expected virulent strain.

Currently, a typical influenza vaccine dose is standardized to comprise 15 μg of each viral HA protein, but it contains other viral structural proteins as well. The efficacy of these vaccines varies considerably. They are reportedly 60 to 90% effective in protecting healthy children and adults younger than 65 years who are exposed to virus strains in the vaccine; they are less effective in the elderly, immunosuppressed individuals, and people with chronic illnesses. Protection against illness correlates with the concentration of antibodies that react with viral HA and NA proteins produced after vaccination. Immunization may also stimulate limited mucosal antibody synthesis and cytotoxic-T lymphocyte activities, but these responses vary widely.

The envelope proteins of influenza viruses change by antigenic drift and shift as the virus reproduces in various animal hosts around the world. Consequently, protection one year does not guarantee protection the next, which is why annual flu shots are recommended. More than 140 national influenza centers conduct year-round surveillance of influenza virus trends and types and relay this information to a number of World Health Organization agencies, including the Centers for Disease Control and Prevention in the United States, the National Institute for Medical Research in the United Kingdom, and the National Institute for Viral Disease Control and Prevention in China. The World Health Organization makes general recommendations about which influenza strains to include in the vaccine, but it is up to each country to make its own decision. Within the United States, this job falls to the Food and Drug Administration. Timing is critical, as the final decision for the virus composition in the vaccine must be made within the first few months of each year to allow sufficient time for production of the vaccine. Any delay or error in the process, from prediction to manufacture, has far-reaching consequences, given the millions of people who are vaccinated and expect safe protection (Fig. 8.9). Even if the vaccine contains the appropriate viral antigens and is made promptly and safely, inactivated influenza virus vaccines have the potential to cause side effects in some individuals who are allergic to the eggs in which the vaccine strains are grown. As an example of other problems, the H5N1 avian virus that first infected humans in Hong Kong in the 1990s was extraordinarily cytopathic to chicken embryos, making it difficult to propagate. Reassortants had to be constructed by placing the new H5N1 segments in less cytopathic viruses.

In addition to the antigens intended to stimulate protective immunity, inactivated vaccines also contain trace amounts of other ingredients that are introduced during creation of the vaccine or to improve safety and/or efficacy. These include residual egg proteins, residual antibiotics (present to prevent contamination by bacteria during the manufacturing process), preservatives, and stabilizers such as gelatin and sugars that maintain potency during transportation and storage. A vaccine that is propagated in cell culture avoids some of these concerns.



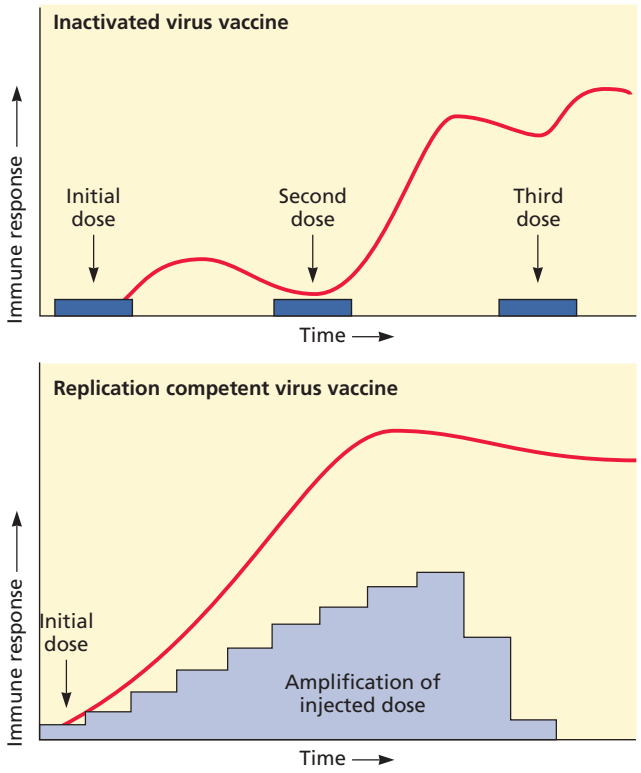
*World Health Organization Global Influenza Surveillance Network
†WHO Collaborating Centres
‡US Centers for Disease Control and Prevention
§US Food and Drug Administration

Figure 8.9 Annual timeline for creating an influenza virus vaccine. Data are collected from many surveillance centers by the World Health Organization, and plans are in place early in the calendar year to determine against which strains the annual influenza vaccine will be created. In spring and summer, the vaccine is mass-produced in time for vaccination in late fall, when the process begins again.

Attenuated Virus Vaccines

Replication-competent, attenuated vaccines are effective for at least two reasons. Progeny virus particles are generally restricted to tissues around the site of inoculation, and this focal restriction generally results in mild or inapparent disease (Fig. 8.10). However, the limited virus reproduction stimulates a potent and lasting immune response. Attenuated (less virulent) viruses are selected by growth in cells other than those of the normal host or by propagation at nonphysiological temperatures (Fig. 8.11). Mutants able to propagate under these conditions are isolated, purified, and subsequently tested for pathogenicity in appropriate models. Temperature-sensitive and cold-adapted mutants are often less pathogenic than the parental viruses because of reduced capacity for reproduction and spread in the warm-blooded host. In the case of viruses with segmented genomes (e.g., arenaviruses, orthomyxoviruses, bunyaviruses, and reoviruses), attenuated, reassortant viruses may be obtained after mixed infections with pathogenic and nonpathogenic viruses.

Figure 8.10 Comparison of the predicted immune responses to attenuated and inactivated viruses used in vaccine protocols. (**Top**) Immune responses plotted against time after injection of an inactivated virus vaccine (red curve). Three doses of inactivated virus particles were administered as indicated. (**Bottom**) Results after injection of a replication-competent, attenuated virus vaccine. A single dose was administered at the start of the experiment. The filled histogram (lavender-colored area) under the curve displays the titer of infectious attenuated virus. Redrawn from C. A. Mims et al., *Mims' Pathogenesis of Infectious Disease*, 4th ed. (Academic Press, Inc., Orlando, FL, 1995), with permission.



Replication-competent oral poliovirus vaccines in use today comprise three attenuated strains selected for reduced neurovirulence. Type 1 and 3 vaccine strains were isolated by passage of virulent viruses in different cells and tissues until mutants with reduced neurovirulence in laboratory animals were obtained (Fig. 8.12A). The type 2 component was derived from a naturally occurring attenuated isolate. The mutations responsible for the attenuation phenotypes of all three serotypes are shown in Fig. 8.12B.

The attenuated measles virus vaccine currently in use was derived from a virulent virus called the Edmonston strain, isolated in 1954 by John Enders. Attenuated virus particles were isolated following serial passage of this virus through various cell types. Even though this approach was undirected, the viruses that were isolated could propagate only poorly at body temperature and caused milder signs of infection in primates. As one would expect, the vaccine strain so derived harbors a number of mutations, including several that affect the viral attachment protein, hemagglutinin.

The attenuated varicella-zoster virus vaccine is currently the only licensed human herpesvirus vaccine. It has proven to be safe and effective in children and adults, providing significant protection against infection by varicella-zoster virus, which causes chickenpox. Because this virus establishes a latent infection in all unvaccinated infected hosts, even if the initial infection is resolved, the virus can be reactivated at later times in life, resulting in painful and often serious conditions (shingles and postherpetic neuralgia). Subsequently, a much more concentrated (by at least 14-fold) formulation of the vaccine was licensed for use in previously infected adults (>60 years of age) to protect against recurrent disease.

Live attenuated viruses are administered by injection (e.g., measles-mumps-rubella [MMR] and varicella-zoster vaccines), by mouth (e.g., poliovirus, rotavirus, and adenovirus vaccines), or by nasal spray (influenza virus vaccine). The highly effective Sabin poliovirus vaccine is given as drops to be swallowed, and enteric adenovirus vaccines are administered as virus-impregnated tablets. One virtue of the oral delivery method for enteric viruses is that it mimics the natural route of infection and, as such, has greater potential to induce an immune response similar to that of the natural infection. A second advantage is that it bypasses the traditional need for hypodermic needles, which creates undue anxiety in many young, and some adult, vaccine recipients.

Attenuated virus vaccines have some inherent risks. Despite reduced spread in the vaccinee, we know that in the case of poliovirus vaccination, some shedding of the vaccine strain occurs, and these virus particles then have the potential to infect unvaccinated individuals. In most cases,

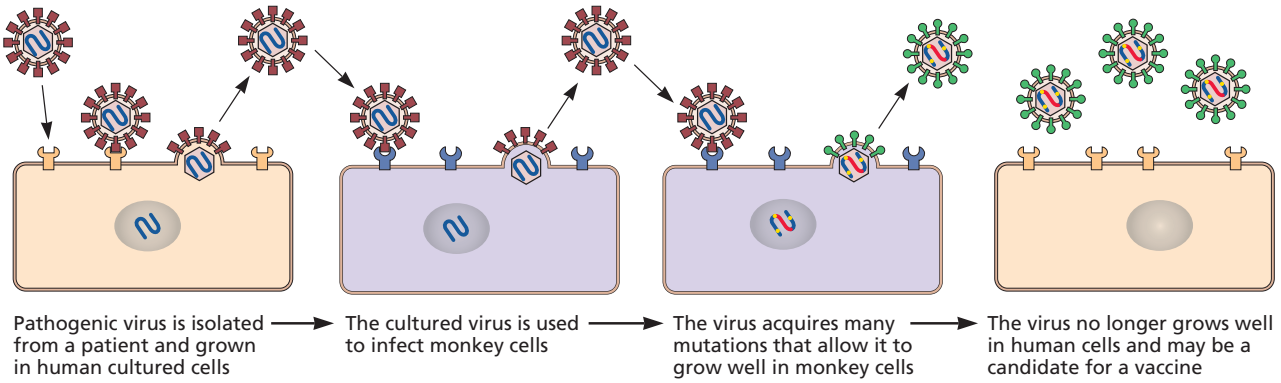


Figure 8.11 Viruses specific for humans may become attenuated by passage in nonhuman cell lines. The four panels show the process of producing an attenuated human virus by repeated transfers in cultured cells. The first panel depicts isolation of the virus from human cells (yellow). The second panel shows passage of the new virus in monkey cells (lavender). During the first few passages in nonhuman cells, virus yields may be low. Viruses that grow better can be selected by repeated passage, as shown in the third panel. These viruses usually have several mutations, facilitating growth in nonhuman cells. The last panel shows one outcome in which the monkey cell-adapted virus now no longer grows well in human cells. This virus may also be attenuated (have reduced ability to cause disease) after human infection. Such a virus may be a candidate for an attenuated vaccine if it induces immunity but not disease. Adapted from C. A. Janeway, Jr., et al., *Immunobiology: the Immune System in Health and Disease* (Current Biology Limited, Garland Publishing Inc., New York, NY, 2001), with permission.

this would be akin to "bystander vaccination," but given the high rate of mutation associated with RNA virus replication, reversion to virulence is expected. Shedding of a virulent revertant virus is one of the main obstacles to developing effective attenuated vaccines, and is formally equivalent to the emergence of drug-resistant mutants (see "Drug Resistance" in Chapter 9). While such revertants are a serious problem, considerable insight into virus biology and pathogenesis can be obtained by identifying the changes responsible for increased virulence (Fig. 8.12C). Moreover, when one considers that these vaccines are safe and afford lifelong protection for the majority of the recipients, some degree of public health risk may seem acceptable. How such risk is determined and tolerated within a community becomes more of a sociological and ethical discussion rather than a virological question (Box 8.10).

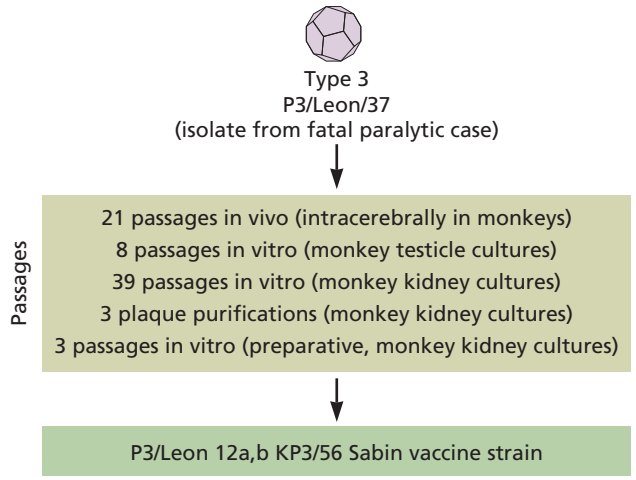
Ensuring purity and sterility of the product is a problem inherent in the production of biological reagents on a large scale. If the cultured cells used to propagate attenuated viruses are contaminated with unknown viruses, the vaccine may well contain these adventitious agents. Sensitive detection methods, such as polymerase chain reaction, have minimized this threat in most of today's vaccines, but in the 1950s, early batches of poliovirus vaccine were grown in monkey cells that were unknowingly infected with the polyomavirus simian virus 40. It is estimated that 10 million to 30 million individuals received one or more doses of simian virus 40 with their poliovirus vaccine, and many developed antibodies to simian virus 40 proteins. Some concern existed that rare tumors may be linked to this inadvertent infection, but this connection has since been discounted.

Alternatives to the classical empirical approach to attenuation can now be applied based on modern virological and recombinant DNA technology. For example, deletion mutations with exceedingly low probabilities of reversion can be created, though none yet exist (Fig. 8.13). In another approach that relies on genome segment reassortment of influenza viruses and reoviruses, genes encoding proteins that contribute to virulence are replaced with those from related but nonpathogenic viruses. No matter which technology is applied to achieve attenuation, the genetic engineer and the classical virologist must satisfy the same fundamental requirements: isolation or construction of an infectious agent with low pathogenic potential that is, nevertheless, capable of inducing a long-lived, protective immune response.

Subunit Vaccines

A vaccine may consist of only a subset of viral proteins, as demonstrated by the highly successful hepatitis B vaccine. Vaccines formulated with purified components of viruses, rather than the intact particles, are called subunit vaccines. Determining which viral proteins to include in a vaccine is accomplished by selecting those that are recognized by antibodies and cytotoxic T lymphocytes; this selection can be determined by assessing the immune responses of individuals who have recovered from the disease. Although the most obvious proteins would be those present on the virus surface, in fact, any viral protein could be a good immunological

A Derivation of Sabin type 3 attenuated poliovirus



B Determinants of attenuation in the Sabin vaccine strains

Virus	Mutation (location/nucleotide position)
P1/Sabin	5'-UTR nt 480 VP1 aa 1106 VP1 aa 1134 VP3 aa 3225 VP4 aa 4065
P2/Sabin	5'-UTR nt 481 VP1 aa 1143
P3/Sabin	5'-UTR nt 472 VP3 aa 3091

C Reversion of P3/Sabin

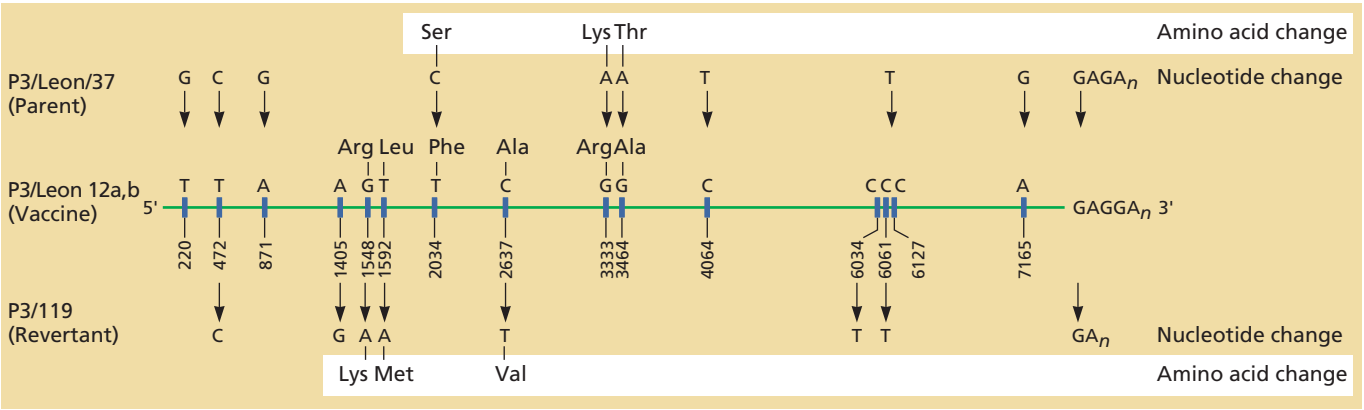


Figure 8.12 Replication-competent, attenuated Sabin oral poliovirus vaccine. (A) All three viral serotypes may cause poliomyelitis. Therefore, the Sabin vaccine is administered as a mixture of three different strains that are representatives of poliovirus serotypes 1, 2, and 3. Shown is the derivation of the type 3 vaccine strain, called P3/Sabin (the letter P means poliovirus). The parent of P3/Sabin is P3/Leon, a virus isolated from the spinal cord of an 11-year-old boy named Leon, who died of paralytic poliomyelitis in 1937 in Los Angeles. P3/Leon virus was passaged serially as indicated. At various intervals, viruses were cloned by limiting dilution, and the virulence of the virus was determined in monkeys. An attenuated strain was selected to be the final P3/Sabin strain included in the vaccine. **(B)** Determinants of attenuation in all three strains of the Sabin vaccine. The mutations responsible for the reduced neurovirulence of each serotype of the poliovirus vaccine are indicated (5′-UTR is the 5′ untranslated region; VP1 to VP4 are the viral structural proteins; aa, amino acid; nt, nucleotide). **(C)** Reversion of the P3/Sabin vaccine strain. Differences in the nucleotide sequences of the virulent P3/Leon strain and the attenuated P3/Sabin vaccine strain are shown above the depiction of the parental viral RNA (green) and of a virulent virus (P3/119) isolated from a case of vaccine-associated poliomyelitis (below). Note that "revertant" does not mean that the sequence of the virus "reverts" to the sequence of the pathogenic precursor; rather, changes in other residues enable the virus to regain its potential to reproduce at wild-type virus levels.

target. A further critical parameter is that the viral proteins must be recognized by most individuals: as humans are an outbred population, the specificities of immune recognition differ from person to person, though some antigens can be recognized by a large proportion of the human population.

Even fragments of viral proteins may be sufficient to induce protective immunity. Synthetic peptides of about 20 amino acids or more in length can induce specific antibody responses when chemically coupled to protein carriers that can be taken up, degraded, and presented by major histocompatibility complex class II proteins (discussed in Chapter 4). In principle, synthetic peptides should be the basis for an extremely safe, well-defined vaccine, in which reversion or contamination with infectious virus is impossible. To date, however, peptide vaccines have had little success, mainly because synthetic peptides are expensive to make in sufficient quantity, and the antibody response they elicit is often weak and short-lived. Furthermore, given the likely simplicity of

BOX 8.10

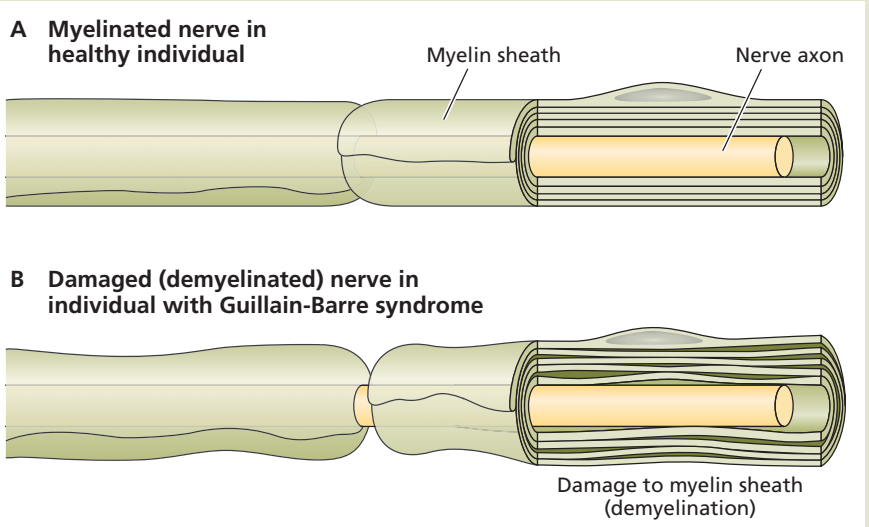
DISCUSSION

Vaccine risks

Because our knowledge about virulence is limited, it is difficult to predict how a replication-competent, attenuated virus will behave in individuals and in the population. The attenuating mutations may lead to unexpected diversions from the natural infection and expected host response: the attenuated virus may be eliminated from the vaccinated individual before it can induce a protective response; it may infect new tissues or cells in the host with unpredictable effects; or it may initiate atypical infections (e.g., slow or chronic infections) that can trigger immunopathological responses of unknown etiology, such as **Guillain-Barré syndrome**. While this syndrome is most typically associated with a bacterial infection (*Campylobacter jejuni*), human cytomegalovirus and influenza virus have also been implicated as potential causative agents. Bacterial or viral infections (or vaccinations) can trigger a host immune response that then may cross-react with proteins present in human peripheral nerves. This process, termed **molecular mimicry** (Chapter 5), results in autoimmunity that, in the case of Guillain-Barré syndrome, is characterized by rapidly progressing, symmetric weakness of the extremities. Vaccine side effects, whether real or not, often have a detrimental effect on public acceptance of national vaccine programs.

Given that all procedures have risks, a fascinating ethical question that we must address as a society is: "How much harm can we tolerate for the global good?" That is, if millions of people each year benefit from the protective effects of a vaccine (or some other medical intervention), how many "side effects" are we willing to tolerate? Questions like these—which, of course, have no single answer—lie at the heart of bioethics.

Destruction of myelin layer in the peripheral nerve processes of individuals with Guillain-Barré syndrome.



the host's "antipeptide" response (usually a single epitope is contained within the peptide), selection of escape mutants is highly probable.

Recombinant DNA Approaches to Subunit Vaccines

Recombinant DNA methods allow cloning of selected viral genes into nonpathogenic viruses, bacteria, yeasts, insect cells, or plant cells to produce the immunogenic protein(s). As only a portion of the viral genome is required for such production, there can be no contamination of the resulting vaccine with the original virus, solving a major safety problem inherent in inactivated virus vaccines. Viral proteins can be made inexpensively in large quantities by engineered organisms under conditions that simplify purification and quality control. For example, complications due to egg allergies after vaccination can be eliminated completely when influenza virus proteins are synthesized in *Escherichia coli*, insect cells, or yeasts. Baculoviruses, which infect insect cells in nature, can infect a large number of mammalian cell types in culture. Because this virus is nonpathogenic to humans and can be modified to express heterologous (and immunogenic) proteins from human viral pathogens, its use as a vaccine vector holds great promise.

Unfortunately, most candidate subunit vaccines fail because they do not induce an immune response sufficient to protect against infection, the gold standard of any vaccine. The immune repertoire evoked by an infectious virus infection may be only partially represented in a response to a subunit vaccine. In particular, purified protein antigens rarely stimulate the appearance of mucosal antibodies, particularly IgA. To date, the single exception of a successfully engineered subunit vaccine is Flublok, in which large quantities of the hemagglutinin protein of the three most prominent influenza viruses are synthesized from baculovirus vectors in a preservative- and egg-free cell culture system.

Virus-Like Particles

The capsid proteins of nonenveloped and of some enveloped virus particles may self-assemble into virus-like particles. These particles have capsid-like structures that are virtually identical to those in virus particles, but unlike authentic

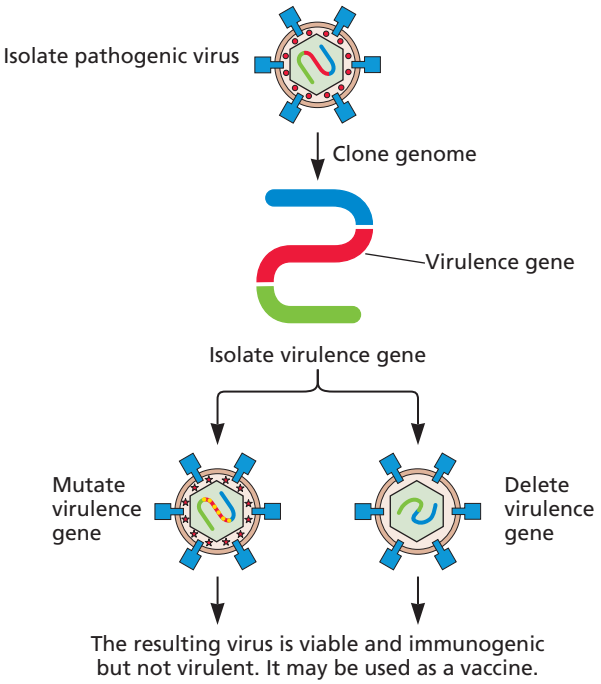


Figure 8.13 Construction of attenuated viruses by using recombinant DNA technology. Once the genome of a pathogenic virus is cloned in a suitable system, deletions, insertions, and point mutations can be introduced by standard recombinant DNA techniques. If the cloned genome is infectious or if mutations in plasmids can be transferred to infectious virus, it is possible to mutate viral genes systematically to find those required for producing disease. The virulence gene can then be isolated and mutated, and attenuated viruses can be constructed. Such viruses can be tested for their properties as effective vaccines. The mutations in such attenuated viruses may be point mutations (e.g., temperature-sensitive mutations) or deletions. Multiple point mutations or deletions are preferred to reduce or eliminate the probability of reversion to virulence. Adapted from C. A. Janeway, Jr., et al., *Immunobiology: the Immune System in Health and Disease* (Current Biology Limited, Garland Publishing Inc., New York, NY, 2001), with permission.

particles, these capsids are empty: they contain no genetic material and cannot propagate. Because the empty capsids retain most of the conformational epitopes not found on purified or unstructured proteins, virus-like particle vaccines often induce durable neutralizing antibodies and other protective responses after injection. Furthermore, as the particles are completely noninfectious, inactivation with formalin or other agents is not required. This feature affords at least two additional advantages: immunogenicity is not compromised (formalin and other alkylating chemicals often alter the conformation of epitopes in inactivated vaccines), and concerns about efficiency of inactivation are avoided. Virus-like particle vaccines have proven to be particularly attractive for viruses that are propagated poorly in cell culture.

The highly successful hepatitis B virus subunit vaccine comprises virus-like particles produced in yeast. This vaccine contains a single viral structural protein (the surface antigen) that assembles spontaneously into virus-like particles, whether made in yeast, *E. coli*, or cultured mammalian cells. Formation of particles is critical, as purified monomeric capsid protein does not induce a protective immune response. Typically, 10 to 20 μg of virus-like particles is administered in each of three doses over a 6-month period, and >95% of recipients develop antibody against the surface antigen. The hepatitis B vaccine was the first anticancer vaccine, as a portion of chronically infected individuals develop fatal liver cirrhosis and hepatocellular carcinoma.

The virus-like particle vaccine effective against papillomavirus infections is among the newest vaccines developed for humans. More than 80% of sexually active women will be infected with several serotypes of human papillomavirus during their lifetime. As a result, many will develop genital warts and/or cervical cancer. There are numerous serotypes of this virus, but serotypes 6, 11, 16, and 18 cause 70% of cervical cancers and 90% of genital warts. Men can develop anogenital warts as well, and both sexes may develop head and neck cancers as a consequence of oral sex with an infected individual (Chapter 2).

It had been known for some time that the human papillomavirus L1 capsid protein forms virus-like particles when synthesized in a variety of heterologous systems. These empty capsids proved to be exceptional inducers of a protective immune response. As a result, a quadrivalent, virus-like particle vaccine effective against the four major cancer-causing serotypes of the virus was formulated, and in 2006, the Food and Drug Administration approved this formulation as the first vaccine to be developed to prevent cervical cancer induced by a virus. As with any new vaccine, there were, and still are, attendant societal discussions about its use (Box 8.11).

DNA Vaccines

DNA vaccines, a variant of the subunit protein approach to immunization, consist simply of plasmids encoding viral genes that can be expressed in cells of the animal to be immunized. In the simplest case, the plasmid encodes only the immunogenic viral protein under the control of a strong eukaryotic promoter. The plasmid DNA, usually produced in bacteria, can be prepared free of contaminating protein and has no capacity to replicate in the vaccinated host, but can be the template for expression of the immunogenic protein. Remarkably, no adjuvants or special formulations are necessary to stimulate an immune response.

The main challenge when this technology was first described in 1992 was how to introduce the plasmids into host cells such that the cell's transcriptional and translational machinery would take over. Direct intramuscular delivery of the vaccine in an aqueous solution containing a few

BOX 8.11

DISCUSSION

Should men be encouraged to get the human papillomavirus vaccine?

The answer, simply, is yes. The vaccine is approved for use in males in several countries, including the United States. It has been shown to be effective for prevention of infection by those papillomavirus strains that can cause both genital warts and anal cancer. Beyond these direct benefits, immunization of males with the human papillomavirus vaccine (e.g., Gardasil) limits the male-to-female transmission of strains that are most typically associated with cervical cancer.

Unfortunately, not everyone supports this recommendation. Some believe that vaccination will give a false sense of security and promote promiscuity among young people, while others view the recommendations as an intrusion on parental rights. Unlike other vaccines that are mandated (as a requirement for entry into public school, for example), the papillomavirus vaccine remains elective. Consequently, individuals must make their own decisions about whether to vaccinate or not. What is not debatable is the value of vaccination, which provides protection both for the individual and for the community in which that individual resides.



micrograms of plasmid DNA was only moderately successful. A more effective delivery method uses a "gene gun" that literally shoots DNA-coated microspheres, inert particles coated with the DNA of interest, through the skin into dermal tissue. The goal of either approach is to ensure that plasmid DNA is engulfed by a macrophage or dendritic cell, such that the epitopes of the newly made viral protein are appropriately presented in the context of class I major histocompatibility complex molecules needed for T cell recognition and amplification. Both antibodies and cytotoxic T lymphocytes can be stimulated by DNA vaccination. The striking property of DNA vaccination is that a relatively low dose of DNA appears sufficient to induce long-lasting immune responses, and the cost of this approach is a fraction of that required to generate a protein-based vaccine. Moreover, the stability of DNA and its ability to withstand drying make this strategy particularly attractive for vaccine delivery in resource-poor areas where refrigeration is limited or unreliable.

It was subsequently shown that the method of inoculation could dictate the type of immune response that is generated by a DNA vaccine. A T_h1 response predominates after injection of an aqueous DNA solution into muscle. In contrast, after DNA immunization by gene gun, a T_h2 response predominates. Ensuring that the "correct" response is made following vaccination will be a key challenge as this vaccination technology moves forward.

Despite 30 years of study and enthusiasm that this vaccination strategy could be transformative for vaccine design, much remains to be investigated. Variations on this approach are showing promise. A technique called **gene shuffling**, which can be applied to produce diverse coding sequences, may have utility in DNA vaccine technology. Another variation on the single-gene DNA vaccine is a **genomic vaccine**, in which a library of all the genes of a particular pathogen is prepared in multiple DNA vaccine vectors. The entire plasmid mixture is injected into an animal. Such a vaccine has the potential to present every gene product of the pathogen to the immune system.

While DNA vaccines do not carry many of the risks of more traditional vaccines (such as reversions or the consequences of adjuvant use), other concerns about their safety must be addressed. Some possible dangers include unintentional triggering of autoimmune responses to the plasmid DNA (including the synthesis of anti-DNA antibodies) and induction of immune tolerance to the protein produced. However, an intramuscular DNA vaccine to prevent West Nile virus infection of horses has been approved, and there are promising leads for human immunodeficiency virus and hepatitis C virus DNA vaccines.

Attenuated Viral Vectors and Foreign Gene Expression

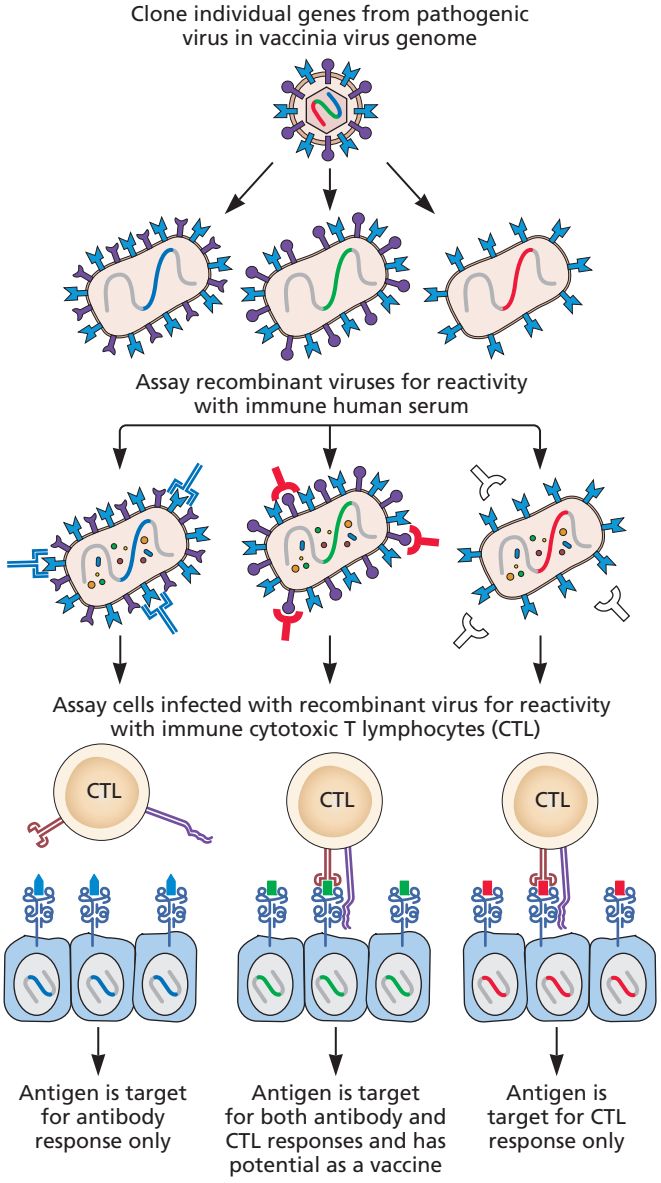
Genes from a pathogenic virus can be inserted into a nonpathogenic viral vector to produce viral proteins that can immunize a host against the pathogenic virus. In principle, the vector provides the benefits of a viral infection with respect to stimulating an immune response to the exogenous proteins, but without the attendant pathogenesis associated with a virulent virus. However, any replicating viral vector has the potential to produce pathogenic side effects, particularly if injected directly into organs or the bloodstream. The immune response to such hybrid viruses is not always predictable, particularly in more vulnerable populations (children, the elderly, and immunocompromised individuals).

Poxviruses, such as vaccinia virus, often are used as vaccine vectors. A wide variety of systems are available for the construction of vaccinia virus recombinants that cannot replicate in mammalian cells, but that allow the efficient synthesis of cloned gene products that retain their immunogenicity. Such vectors can accommodate >25 kb of new genetic

information. Vaccinia virus recombinants can also be used to dissect the immune response to a given protein from a pathogenic virus. This application is illustrated in Fig. 8.14. Other poxviruses, including raccoonpox, canarypox, and fowlpox viruses, are possible alternatives because they are able to infect, but not propagate in, humans. Moreover, these viruses can be used serially: the individual can be inoculated with a different vector expressing the same antigens, overcoming the limitations of making an immune response to the vector itself.

The successful use of an oral rabies vaccine for wild animals in Europe and the United States demonstrates that recombinant vaccinia virus vaccines have considerable potential. Recombinant vaccinia virus genomes encoding the major envelope protein of rabies virus yield virus particles that are formulated in edible pellets to be spread in the wild. The pellets are designed to attract the particular animal to be immunized (for example, foxes or raccoons). The animal eats the pellet, is infected by the recombinant virus, and becomes vaccinated. While effective, this clever approach must be applied with care. As vaccinia virus infection of humans is associated with rare but serious side effects, inadvertent human infection by these wildlife vaccines poses a risk (Box 8.12).

Figure 8.14 Use of recombinant vaccinia viruses to identify and analyze T and B cell epitopes from other viral pathogens. As illustrated, it is possible to determine if a particular viral protein contains a B cell epitope (binds antibodies), a T cell epitope (recognized by cytotoxic T lymphocytes), or both. Subsequent site-directed mutational analysis of the viral genes enables precise localization of these epitopes on the viral protein.



Vaccine Technology: Delivery and Improving Antigenicity

Adjuvants Stimulate an Immune Response

Charles Janeway once revealed what he called "the immunologist's dirty little secret": inactivated virus particles or purified proteins often do not induce the same immune response

BOX 8.12

DISCUSSION

Accidental infections "in the wild"

While inadvertent infection from a vaccine bait may seem unlikely, a case report in 2009 demonstrates how easily accidental infection can occur. In this case, a woman was picking blueberries in a rural area of Pennsylvania where oral rabies vaccine baits were distributed. Her dog picked up the bait in his mouth and punctured the pouch containing the vaccine with his teeth. The woman removed the bait from the dog's mouth, and the vaccine dripped onto her hand, which was scratched by thorns from the berry bush. Moreover, the woman was taking immunosuppressive drugs at the time. By four days after exposure, she noticed red blisters around her hand typical of classical vaccinia virus infection. The patient eventually recovered, but this example reminds us that "unlikely" is not the same as "impossible."

Rabies vaccine bait. Credit: CDC/U.S. Department of Agriculture Wildlife Services.



as replication-competent, attenuated preparations, unless mixed with a substance that stimulates the early inflammatory response. Such immunostimulants are called **adjuvants**. Their development has been largely empirical, although as our understanding of the various regulators of immune responses increases, more-specific and -powerful adjuvants are being discovered and employed. Vaccine researchers can optimize a vaccine by using different combinations of adjuvant and immunogen to induce a protective immune response.

Adjuvants act by stimulating early intrinsic and innate defense signals, which then shape subsequent adaptive responses. These immunostimulators function in at least three distinct ways: by presenting antigens as particles, by sequestering antigen at the site of inoculation, and by directly stimulating the intrinsic and innate immune responses. The latter occurs when adjuvants mimic or induce cellular damage or alter homeostasis (sometimes called "danger" signals), or when they engage intrinsic cellular defense receptors.

Adjuvants vary in composition, from complex mixtures of killed mycobacteria and mineral oil (complete Freund's adjuvant) to lipid vesicles or mixtures of aluminum salts. Some adjuvants, like alum (microparticulate aluminum hydroxide gel), are widely used for human vaccines such as the papillomavirus, hepatitis A, and hepatitis B vaccines. Others, such as complete Freund's adjuvant, are used only in research. This adjuvant is extremely potent, but causes extensive tissue damage and toxicity. Two of the active components in Freund's adjuvant have been identified as muramyl dipeptide and lipid A, both potent activators of the inflammatory response. We now understand that the strong adjuvant effects of this complex cocktail are, at least in part, a result of the presence in an emulsion of mycobacterial DNA that activates the Toll-like receptor 9 (Tlr9) pathogen recognition protein. Less toxic derivatives, along with saponins and linear polymers of clustered hydrophobic and hydrophilic monomers, are promising and far safer alternatives.

Delivery and Formulation

Delivery by injection has many disadvantages, and therefore improvement of the administration of vaccines is an important goal of manufacturers. In some cases, alternative delivery methods require unique formulations. At present, vaccines are delivered by a limited number of methods, including the traditional hypodermic needle injection, oral administration, and the "air gun" injection of liquid vaccines under high pressure through the dead layers of skin to reach dendritic cells. Other methods under consideration include new emulsions, artificial particles, and direct injection of fine powders through the skin. Oral delivery of vaccines can be effective in stimulating IgA antibodies at mucosal surfaces of the intestine and in inducing a more systemic response. Genetically engineered edible plants that synthesize immunogenic viral proteins represent an attractive approach to designing potent and cost-effective oral vaccines. Transgenic plants expressing viral antigens can be developed, or plant viruses with genomes encoding immunogenic proteins can be used to infect food plants. Early experiments are promising: when such a plant is eaten, antibodies to the viral structural protein can be demonstrated in the animal's serum. Oral vaccination, by whatever methodology, is not always possible, because the enzymes of the oral cavity, coupled with the high acidity of the alimentary tract, destroy many vaccines.

Immunotherapy

Vaccination of patients who are already infected with viruses that cause persistent infections, or that are reactivated from latency by an immune response, presents special problems. One approach to resolve an established infection is via immunotherapy. **Immunotherapy** is a strategy to provide the already-infected host with antiviral cytokines, antibodies, or lymphocytes over and above those provided by the normal immune response. Immunotherapy can be administered by introduction of purified compounds or of a gene encoding the immunotherapeutic molecule. An attenuated virus or a DNA vaccine can be modified to synthesize cytokines that stimulate a desired immune response. If an attenuated vaccine is used, care must be taken, as it is possible that the intended immune response will have unexpected effects, such as increased virulence, persistence, and/or pathogenesis of the vaccine strain.

It is also possible to isolate lymphocytes from patients, infect these cells with a defective virus vector (e.g., a retrovirus) encoding an immunoregulatory molecule, and then infuse the transduced cells back into the patient. If these cells survive and synthesize the transduced protein, the patient's immune response may be boosted. When stem cells are transduced, a long-term effect may be achieved, as these cells will continue to divide in the transfused patient, producing daughter cells that propagate the transgene.

Immunotherapy with cytokines can also be effective. For example, the cytokine interferon α is approved in the United States for treatment of chronic hepatitis caused by hepatitis B and C viruses. Its effect on chronic hepatitis B virus infection is remarkable: as many as 50% of treated patients have no detectable infection after treatment. However, similar treatment of hepatitis C virus-infected patients has been less successful, for reasons that are not clear. Limitations of interferon therapy (and probably cytokine therapy in general) are that the biological activity of the therapeutic interferon is not sustained for a prolonged time, side effects are significant and often so extreme that patients elect to stop therapy, and treatment is expensive.

Immunomodulating agents, including interferon, cytokines that stimulate the T_h1 response (e.g., interleukin-2), and certain immune cell-attracting chemokines, are being

studied individually and in combinations for their ability to reduce virus load and to moderate complications of persistent infections caused by papillomavirus and human immunodeficiency virus. Cytokines that stimulate natural killer cells (e.g., interleukin-12 and interferon γ) may hold promise as well.

The Quest for an AIDS Vaccine

In 1984, several years after human immunodeficiency virus was identified, officials in the U.S. government predicted that an AIDS vaccine would be available within 3 years. Despite 30 years of intensive work by laboratories across the world, substantial recent progress in identifying new epitopes, and developing strategies to overcome epitope diversity, a vaccine that protects against AIDS is still not on the horizon. Inaccurate predictions are nothing new in vaccine development: 3 years after poliovirus was isolated in 1908, Simon Flexner of the Rockefeller Institute confidently announced that a vaccine would be prepared in 6 months. Almost 50 years of research on basic poliovirus biology was necessary to provide the knowledge of pathogenesis and immunity that allowed the development of effective poliomyelitis vaccines (Table 8.2). The prevalence of human immunodeficiency virus throughout the world and the destruction that it causes to its victims and families make the development of an AIDS vaccine the new holy grail for vaccinologists.

The past 30 years has been a roller coaster of promise and disappointment. It was appreciated even in the 1990s that human immunodeficiency virus would be a formidable challenge for vaccine development. The lack of success can be explained by both the biology of this virus and its interaction with the host immune system. Although a vigorous immune response is induced after infection, the virus is not cleared. Infection and death of the very cells that coordinate an effective adaptive immune response, the $CD4^+$ T lymphocytes, frustrate a coordinated host response. Equally frustrating is the relationship of the virus with its host cell: lentivirus reproduction requires integration of a DNA copy of the viral genome into the cellular genome. In some cells, a latent infection can be established in which the integrated proviral DNA produces no proteins, and the infection thus remains invisible to immune recognition. A third obstacle to vaccine development is the high mutation frequency of the virus: the host may make a suitable response to a particular epitope, but when the epitope is altered by mutations in its coding sequence, this response will be rendered useless. Consequently, while most vaccines are intended to mimic the natural host response to infection, to be effective, a vaccine against human immunodeficiency virus must improve on that response.

In addition to these technical challenges, complicated social, ethical, and political issues arise when vaccines for viruses in which humans are the only hosts are to be tested. For example, should at-risk groups, including gay men or sex workers, be preferentially selected as candidates for vaccination tests? On a larger scale, significant political issues related to mutual trust arise when vaccines are to be tested in resource-poor countries. In northern Nigeria in 2003, the political and religious leaders of three states called on parents not to vaccinate their children with the "Western" poliovirus vaccine, stating that the vaccine could be contaminated with antifertility agents or cancer-causing compounds. Such scare tactics are effective: Nigeria remains one of only three countries in which poliovirus is still endemic.

At a minimum, we can make a short list of our expectations for a human immunodeficiency virus vaccine. Of course it must be effective and safe. The protection should last for many years and protect against as many of the diverse human immunodeficiency virus strains as possible. The vaccine should not be so complicated that it cannot be produced on a large scale at a reasonable price. It should be stable, with a significantly long shelf life, so that it can be distributed, stored, and delivered when needed, especially in underdeveloped regions of the world where human immunodeficiency virus infections are prevalent. As the window of opportunity to block a primary infection, integration, and dissemination within a host is very short, the vaccine must act quickly and result in complete protection before the virus can go into hiding.

Formidable Challenges and Promising Leads

In the early days of human immunodeficiency virus vaccine research, a number of approaches, including the use of inactivated virus particles, or subunit vaccines based on single viral

Table 8.2 When can we expect an HIV vaccine?

Viral vaccine	Yr when etiologic agent was discovered	Yr when vaccine was developed in the United States	No. of yr elapsed
Polio	1908	1955	47
Measles	1953	1983	30
Hepatitis B	1965	1981	16
Rotavirus	1970	1998	28
Hepatitis A	1973	1995	22
HIV	1983	None yet	>30

BOX 8.13

DISCUSSION

National vaccine programs depend on public acceptance of their value

The measles-mumps-rubella (MMR) vaccine, a cocktail of three attenuated virus strains, has proven to be remarkably effective in reducing the incidence of these highly contagious and serious diseases. The economic benefit in the United States alone from use of the MMR vaccine has been estimated to exceed $5 billion per year.

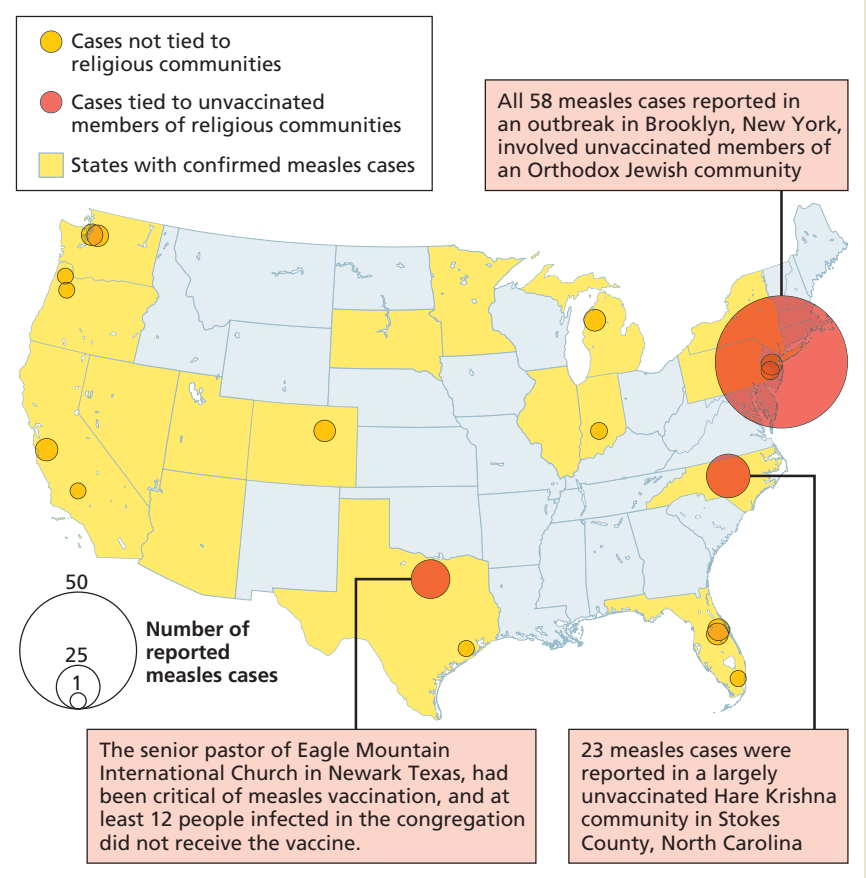
In 1998, a publication in the prestigious medical journal *The Lancet* raised the specter that vaccines may contribute to the development of childhood autistic spectrum disorders and the associated colitis that affects many children with these disorders. Because the rates of diagnosis of childhood autism were increasing without a defined etiological basis, this report was quickly embraced by news media, raising parental concerns about vaccine safety.

The controversial report triggered more than a dozen retrospective and prospective epidemiological studies across the world, all of which concluded that vaccination was **not** linked to the escalation in autism diagnoses. The Centers for Disease Control and Prevention, the American Academy of Pediatrics, the Institute of Medicine, the National Institutes of Health, and other global health organizations have unambiguously and repeatedly affirmed the vaccine's safety.

Moreover, further investigation into the *Lancet* report revealed conflicts of interest, scientific inconsistencies, and ethically questionable practices in the research methodology used in this paper. As a result, all but the lead author, Andrew Wakefield, removed their names from the manuscript, which was partially retracted in 2004 and fully retracted in 2010. Wakefield, a practicing physician in the United Kingdom, was found guilty of professional misconduct by the General Medical Council and was struck from the Medical Register.

Nevertheless, as a consequence of this misinformation, the measles immunization rate has fallen significantly in resource-rich countries that had previously had high levels of vaccine coverage and negligible cases of infection. In many communities, rates of protection have dipped below that required to maintain herd immunity, and predictably, measles virus infections are now appearing for the first time since the 1970s (see the figure). A measles outbreak in 2015 that began in Disneyland in California brought the risks of not vaccinating into clear focus, and began to change the conversation from parental choice to public obligation.

What can be learned from this sad chapter in vaccine history? First, it is heartening that theories proposed in the literature are subjected to repeated testing and revision by other groups: the rapid mobilization of many laboratories (at considerable effort and expense) provided an analytical and controlled counterpoint to the *Lancet* study. More sobering, however, is that, even when the data are unambiguous, the public response cannot be predicted. There remains a critical need to improve the lines of communication between scientists and the lay community.



proteins, as well as passive immunization, were tested with no success. In particular, subunit vaccines, although capable of inducing strong antibody responses, were markedly inefficient in eliciting a cytotoxic-T-lymphocyte response. Furthermore, attenuated human immunodeficiency virus type 1 vaccines, modeled after the successful poliovirus vaccine, presented not only difficult scientific challenges but also ethical problems: the risks associated with injecting thousands of healthy, uninfected volunteers with an infectious (attenuated) virus were simply too great.

In 2013, the field of human immunodeficiency virus vaccine research suffered a major and highly publicized setback with the failure of a trial designed to test the hypothesis that high levels of $CD8^+$ T cells could protect against transmission. Despite strong T cell responses and appreciable antibody levels in the vaccinees, there was no added protection against infection. But hopes were rekindled when it was shown that a cytomegalovirus vector that expressed simian immunodeficiency virus antigens could eliminate infection in half of the infected rhesus macaques. The search is now on to develop a similar strategy for use in humans. To date, the only trial in humans that showed any efficacy was one done in Thailand using a canarypox vector encoding the viral glycoprotein gp120, which provided protection for a third of the vaccinees.

Given the high mutation frequency, many believe that a vaccine will need to induce broadly neutralizing antibodies that can inhibit multiple viral strains to be efficacious. Technological advances have enabled the isolation of many such antibodies from infected patients (see Chapter 7), and this success has generated newfound enthusiasm that a vaccine to prevent infection and AIDS may, at last, be possible.

Perspectives

Historic successes with vaccines for smallpox, measles, polio, and other viral infections, combined with promising new formulations such as the virus-like particle preparations that protect against hepatitis B virus and papillomavirus infections, have transformed modern medicine. However, our lack of progress with other viruses, including the failure as yet to develop a human immunodeficiency virus vaccine, reminds us that viral infection and immunity are intricate and poorly understood processes. Differences in a host's immune response to individual viruses, and the difficulty in developing safe and efficacious vaccines without a complete grasp of how immunity "works" in each case, pose significant roadblocks to progress. We have learned that intervening in any complex host-parasite interaction often produces unanticipated effects. Indeed, we frequently find out how little we know when vaccines are tested in the real world: formulations that worked in the lab fail in the field.

Sadly, even when vaccines are available, people may refuse to accept vaccines, and societies may not use or be able to pay for them. These issues, which are more about public perception and education, determine how effective a vaccine campaign will be (Box 8.13). In some cases, individuals or certain communities may not be able to pay for vaccines. As viruses do not respect country boundaries, this is a problem that international organizations must address.

It is noteworthy, and somewhat humbling, that essentially all successful vaccines on the market today were developed empirically: think back to the high-risk experiment that Jenner attempted, and how this was based more on a hunch than on actual scientific evidence. As we learn more about the molecular mechanisms of antiviral immune defenses and the epidemiology of infections, we anticipate that the development of future generations of vaccines will be based on scientifically sound principles, rather than conversations overheard between milkmaids.

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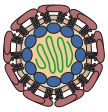
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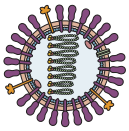
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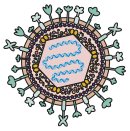
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9 Antiviral Drugs









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LINKS FOR CHAPTER 9

⏭ *Video: Interview with Dr. Benhur Lee*
http://bit.ly/Virology_Lee

⏭ *Draco's potion*
http://bit.ly/Virology_Twiv146

⏭ *Hacking aphid behavior*
http://bit.ly/Virology_Twiv70

⏭ *Combination antiviral therapy for hepatitis C*
http://bit.ly/Virology_10-14-14

Though the doctors treated him, let his blood, and gave him medications to drink, he nevertheless recovered.

LEO TOLSTOY
War and Peace

Introduction

Public health measures and vaccines can control some viral infections effectively. For those that cannot, we must rely on antiviral drugs. Unfortunately, despite more than 50 years of research, our armamentarium of such drugs remains surprisingly small. As will be described in this chapter, this paucity reflects the many challenges that must be met in drug development. However, when available, antivirals can have a major impact on human health. Because of their medical importance, most of our antiviral drugs are directed against infections with human immunodeficiency virus and herpesviruses. In these cases, literally millions of lives have been saved by use of antiviral drugs.

One major limitation in antiviral drug development is the requirement for a high degree of **safety**. This restriction can be difficult to surmount because of the dependence of viruses on cellular functions: a compound that blocks a pathway that is critical for the virus can also have deleterious effects on the host cell. Another requirement is that antiviral compounds must be extremely **potent**: even modest reproduction in the presence of an inhibitor provides the opportunity for resistant mutants to prosper. Achieving sufficient potency to block viral reproduction completely is remarkably difficult. Other limitations can be imposed by the difficulty in propagating some medically important viruses in the laboratory (e.g., hepatitis B virus and papillomaviruses) and the lack of small-animal models that faithfully reproduce infection in humans (such as measles and hepatitis C viruses). Lack of rapid diagnostic reagents has also hampered the development and marketing of antiviral drugs to treat many acute viral diseases, even when the effective therapies are available.

Historical Perspective

The first large-scale effort to find antiviral compounds began in the early 1950s with a search for inhibitors of smallpox virus reproduction. At that time, virology was in its infancy and smallpox was a worldwide scourge. Drug companies expanded efforts in the 1960s and 1970s, spurred on by increased knowledge and understanding of the viral etiology of common diseases, as well as by remarkable progress in the discovery of antibiotics to treat bacterial infections. The companies launched massive screening programs to find chemicals with antiviral activities. Despite much effort, there was relatively little success. One notable exception was amantadine (Symmetrel), approved in the late 1960s by the U.S. Food and Drug Administration (FDA) for treatment of influenza A virus infections. These antiviral discovery programs comprised **blind screening**, in which random chemicals and natural-product mixtures were tested for their ability to block the reproduction of a variety of viruses in cell culture systems. Candidate inhibitors were then tested in various cell and animal models for safety and efficacy. Promising molecules, called "leads," were modified systematically to reduce toxicity, increase solubility and bioavailability, or improve biological half-life. As a consequence, thousands of molecules

PRINCIPLES *Antiviral drugs*

- Antiviral compounds must be extremely potent to be effective: even modest viral reproduction in the presence of an inhibitor provides the opportunity for resistant mutants to prosper.
- Most antiviral compounds in clinical use target viral enzymes, such as proteases, and nucleic acid-synthesizing proteins.
- New drug design is focusing on blocking such viral functions as entry and uncoating, and the activities of viral regulatory proteins and RNA molecules.
- Whole-genome sequencing and methods to block gene expression make it possible to test for the requirement of every host gene in the reproduction of most viruses, further expanding the potential targets for antiviral drug design.
- Sophisticated computational methods have been developed to identify drug leads by "virtual screening," iterative docking of each chemical into a chosen site in a protein target.
- It is common for thousands of leads to yield but one promising drug candidate.
- Once a drug candidate is identified, clinical studies are needed to determine whether the compound gets to the right place in the body and at the appropriate concentration, persists in the body long enough to be effective, and is well tolerated and not toxic.
- More promising drug candidates are discarded because of toxicity and safety concerns than for any other reason.
- It often takes 5 to 10 years after identification of a candidate to get the drug to market.
- Emergence of drug-resistant mutants is of special concern during the extended period of therapy required for viruses that establish chronic infections. Combining two or more drugs with distinct targets circumvents the appearance of cells resistant to one treatment or the other.
- Potent drugs and drug combinations are now available to inhibit the reproduction of several human viruses, including herpesviruses, hepatitis B and C viruses, and human immunodeficiency virus.

were often made and screened before a specific antiviral compound was tested in humans. The mechanism by which these compounds inhibited the virus was often unknown. For example, the mechanism of action of amantadine, blocking the viral ion channel protein M2 and inhibiting uncoating, was not deduced until the early 1990s, almost 30 years after its discovery.

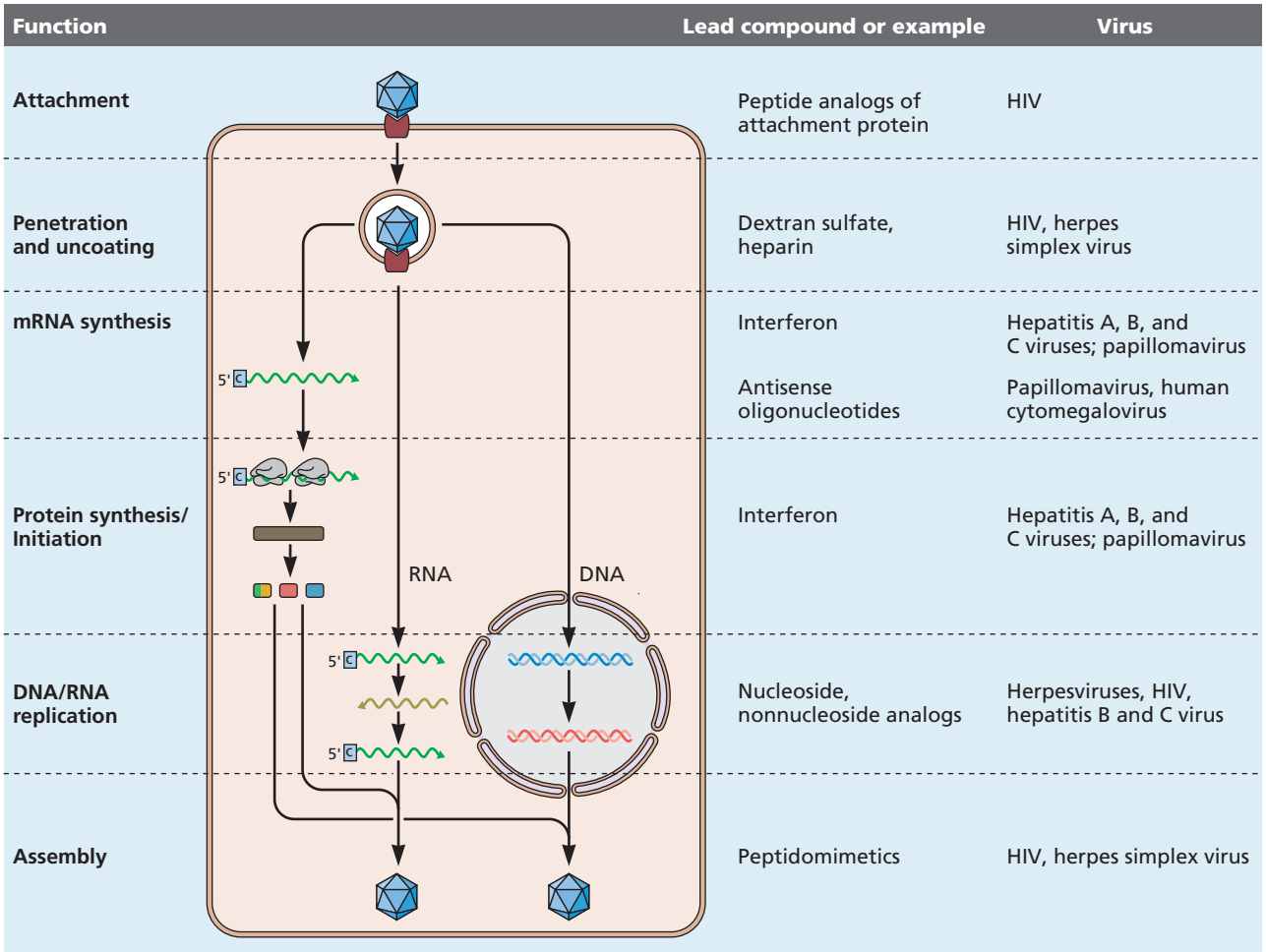
Discovering Antiviral Compounds

With the advent of modern molecular virology and recombinant DNA technology, the random, blind-screening procedures described above were all but discarded. Instead, viral genes essential for reproduction were cloned and expressed in genetically tractable organisms, and their products purified and analyzed in molecular and atomic detail. The life cycles of many viruses are known, allowing identification of numerous targets for intervention (Fig. 9.1). Inhibitors of critical processes can be found, even for viruses that cannot be propagated in cultured cells. With the development of whole-genome sequencing and methods to block gene expression, it is now possible to test for the requirement of every host gene in the reproduction of most viruses, thereby expanding the potential targets for intervention. The practical challenge now is how to work from medical need to effective product.

The Lexicon of Antiviral Discovery

The antiviral discovery toolbox has expanded in the last decade to include many new devices and methods (Fig. 9.2). But lead compounds discovered using these approaches are only the starting point for the development of clinically useful drugs, i.e., compounds **approved** and licensed for use in humans. Many challenges remain after a drug candidate is identified. The developers must determine if the compound will get to the right place in the body and at the appropriate concentration (**bioavailability**), will persist in the body long enough to be effective (**pharmacokinetics**), and will be tolerated or toxic.

Figure 9.1 Knowledge of viral life cycles identifies general targets for antiviral drug discovery.



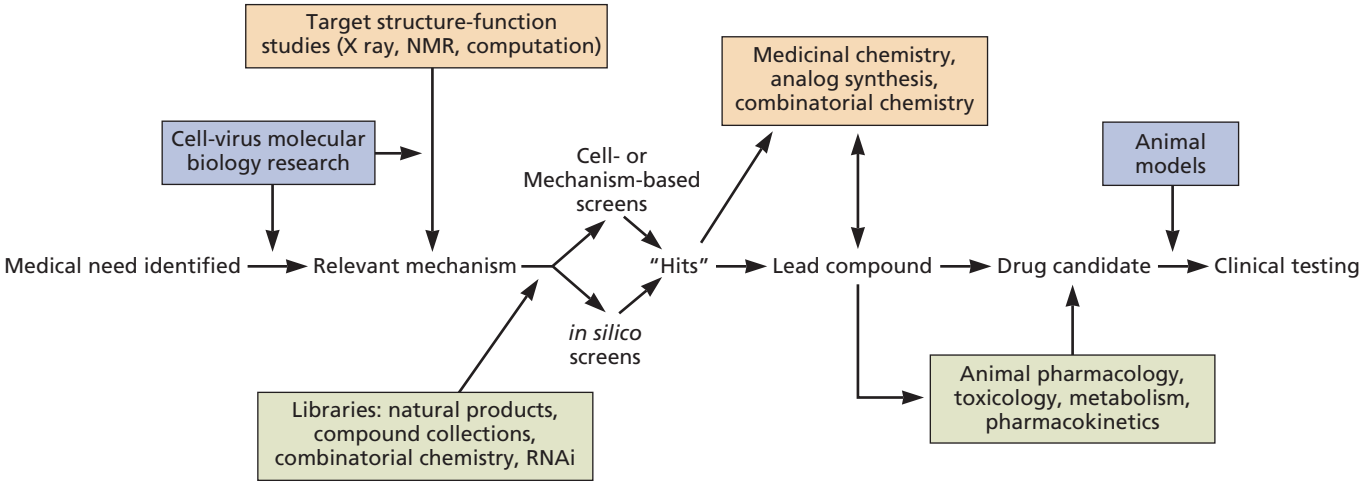


Figure 9.2 Path of drug discovery. The flow of information and action followed by modern drug discovery programs that ultimately yield compounds that can be tested clinically for efficacy is illustrated. NMR, nuclear magnetic resonance.

Screening for Antiviral Compounds

Genetics and Drug Discovery

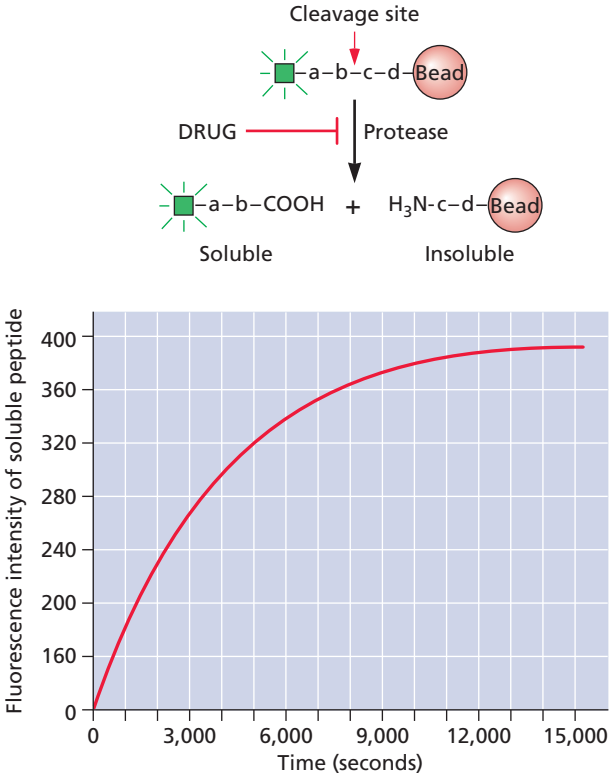
Viral targets. Modern antiviral discovery methods have focused on genes known to be essential for viral reproduction and the use of mechanism-based screens (Fig. 9.1). Essential viral functions are defined by genetics and informed by our knowledge of viral genomes. Viral genomes can be manipulated to determine if a particular gene product is a valid target by construction and analysis of a mutant in which the gene of interest is inactivated or deleted.

Host targets. In the past, identifying host gene products that are required for viral reproduction was often impossible. Modern technology has changed this situation dramatically. As sequences of the genomes of humans and common laboratory animals are now available, host proteins that are essential for efficient virus reproduction and pathogenesis can be identified using RNA interference (RNAi) technology, genetic manipulation through transgenic and knockout approaches, and assays that detect protein-protein interactions. Maraviroc, an antagonist of the CCr5 cellular coreceptor for human immunodeficiency virus type 1, is one example of an approved drug that targets a host protein that enables viral infection.

Mechanism-Based Screens

As the name "mechanism-based" implies, this type of screen seeks to identify compounds that affect the function of a known viral target. Enzymes, transcriptional activators, cell surface receptors, and ion channels are popular targets. Often this screening is carried out with purified protein in formats that facilitate automated assay of many samples. One example of a mechanism-based screen designed to identify inhibitors of a viral protease is shown in Fig. 9.3.

Figure 9.3 Mechanism-based screen for inhibitors of a viral protease. The substrate is a short peptide encoding the protease cleavage site. A fluorogenic molecule is covalently joined to the N terminus of the peptide, and the entire complex is attached via its C terminus to a polystyrene bead. When the peptide-bead suspension is exposed to active protease, the peptide is cleaved such that the fluorogenic N terminus is released into the soluble fraction, which can be quickly and cleanly separated from the insoluble beads containing the nonfluorogenic product as well as the fluorogenic unreacted substrate. Protease activity is assayed by the appearance of soluble fluorescent peptide as a function of time as shown.



Cell-Based Screens

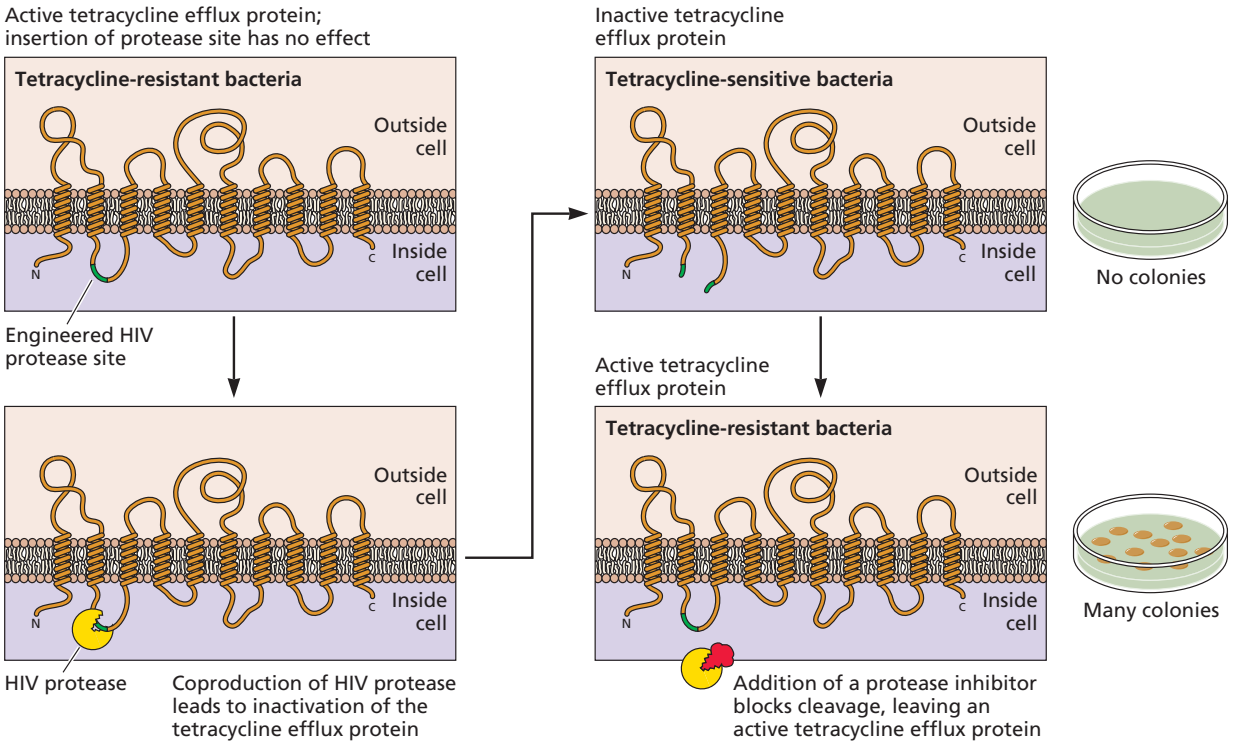
In cell-based assays, essential elements of the specific mechanism to be inhibited (e.g., a viral enzyme plus a readily assayable substrate) are engineered into an appropriate cell. An example of a bacteria-based screen with a convenient readout is shown in Fig. 9.4. Similar approaches work well in yeast or animal cells. In appropriate cell types, such assays can provide information not only about inhibition of the target reaction, but also about cytotoxicity and specificity. The use of several different reporter molecules may allow detection of more than one event at a time.

An interesting variation of the cell-based assay is the **minireplicon**. Certain viruses cannot be propagated readily in standard cultured cells (e.g., human papillomavirus) or are dangerous enough to require high biological containment (e.g., smallpox virus and the hemorrhagic fever viruses). The minireplicon system comprises a set of plasmids that separately carry genes that encode viral genome replication proteins, and an engineered viral genome segment marked with a reporter gene (i.e., the minireplicon); replication can be monitored by assaying for reporter gene expression. Inhibitors that block replication can be discovered, analyzed, and developed for therapeutic use. A subgenomic replicon system for human hepatitis C virus was used to great advantage for discovery of the first viral protease inhibitors capable of blocking reproduction of this virus.

High-Throughput Screens

High-throughput screens are mechanism- or cell-based screens that allow very large numbers of compounds to be tested in an automated fashion. It is not unusual for pharmaceutical companies to examine more than 10,000 compounds per assay per day, a rate inconceivable for early antiviral drug hunters. Compounds to be screened are typically arrayed in multiwell, plastic dishes. Robots then add samples of these compounds to other plastic dishes containing the cell-free or cell-based assay components, and after incubation, the signal created by the reporter gene (or other output) is read and recorded. Numerical data or images of cells or reactions can be captured, stored, and analyzed. Captured images are called **high-content screens** because they can examine more than one parameter simultaneously. For example, using antibodies, it is possible to monitor the import of transcriptional regulators from their site of cytoplasmic synthesis to their site

Figure 9.4 Cell-based screen for a viral protease inhibitor and a transcription regulatory protein. This cell-based assay for the protease of human immunodeficiency virus (HIV) uses the tetracycline resistance of genetically engineered bacteria as a readout. To facilitate uptake of small molecules that might inhibit the protease, a variety of *E. coli* strains that have reduced permeability barriers are available. It is important to include many controls and secondary assays to identify false-positive and false-negative results. This assay is described in more detail in R. H. Grafstrom et al., *Adv Exp Med Biol* **312:**25–40, 1992.



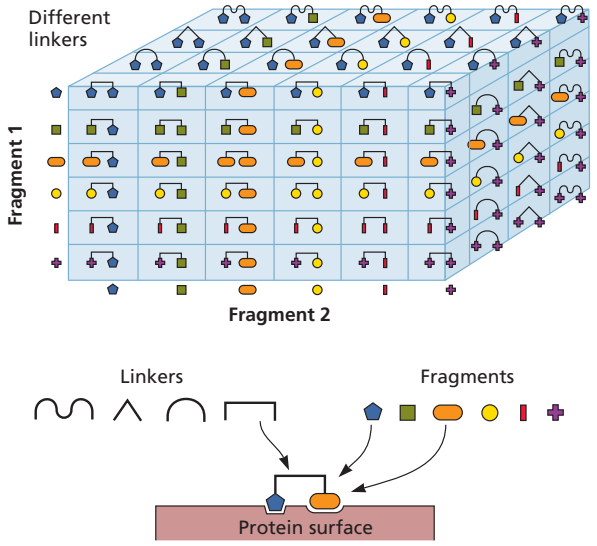
of action in the nucleus at the same time that changes in cell morphology or protein production are visualized.

Sources of Chemical Compounds Used in Screening

Many pharmaceutical and chemical companies maintain large libraries of chemical compounds. Usually, a sample of every compound synthesized by the company for any project is archived, and its history is stored in a database. Chemical libraries of half a million or more distinct compounds are not unusual for a large company. Other kinds of libraries containing natural products collected from all over the world, including "broths" from microbial fermentations, extracts of plants and marine animals, and perfusions of soils containing mixtures of unknown compounds, can be searched for components that may have antiviral activities. A small-molecule repository of >200,000 compounds is maintained by the U.S. National Institutes of Health for use by the scientific community, and libraries of small bioactive molecules are available from a number of private entities.

Another type of chemical library may be produced by **combinatorial chemistry**, a technology that provides unprecedented numbers of small, synthetic molecules for screening (Fig. 9.5). Before implementation of this technology, a medicinal chemist could reliably synthesize and characterize only about 50 compounds a year. Combinatorial chemistry can provide all possible combinations of a basic set of modular components, often on uniquely tagged microbeads or other chemical supports, such that active compounds in the mixtures can be traced, purified, and identified with relative ease. Making thousands of compounds in days is now routine.

Figure 9.5 Combinatorial chemistry and the building-block approach to chemical libraries. Small organic molecules predicted to bind to different pockets on the surfaces of proteins can be grouped into subsets of distinctive chemical structures (different colored symbols). With automated procedures, these chemical entities can be joined together by various chemical linkers (lines) to produce a large but defined library of small compounds. For example, if assembled pairwise with 10 linkers, a collection of 10,000 small molecules yields a library of 1 billion new combinations. These defined chemical libraries allow a detailed exploration of the binding surfaces of complex proteins. Adapted from P. J. Hajduk et al., *Science* **278:**497–499, 1997, with permission.

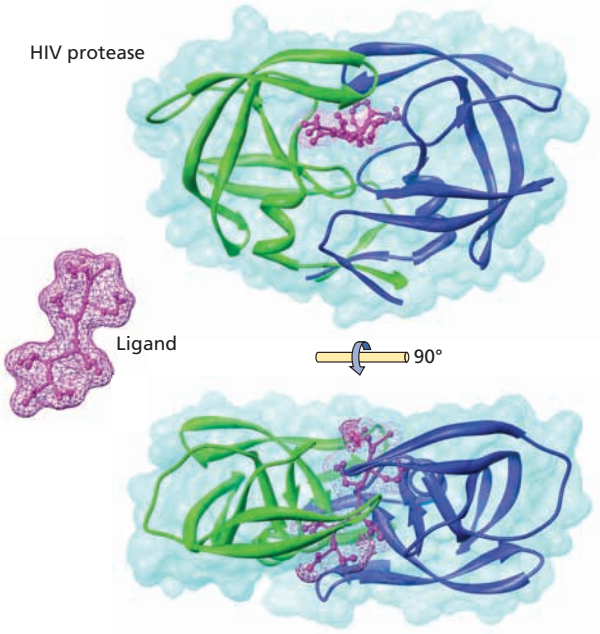


Computational Approaches to Drug Discovery

Structure-Assisted Drug Design

Structure-assisted design depends on knowing the atomic structure of the target molecule, usually obtained by X-ray crystallography. Computer programs, known and predicted mechanisms of enzyme action, fundamental chemistry, and personal insight all aid an investigator in the design of ligands that bind at a critical site and inhibit protein function. Currently, the atomic structures of tens of thousands of macromolecules, including important viral proteins, have been determined, many deposited in publicly accessible protein databases. Inhibitors of the human immunodeficiency virus protease may be the best example of successful antiviral agents that were designed from structure-assisted analyses (Fig. 9.6).

Figure 9.6 Structure of the human immunodeficiency virus (HIV) type 1 protease with the inhibitor saquinavir. The main chains of each monomer in the protease dimer are represented by blue and green ribbons inside the space-filling structure shown in pale blue mesh. A ball-and-stick model of saquinavir (Ligand) inside of a space-filling structure is shown in violet. The tight fit of the inhibitor in the active site of the protease is illustrated by the interlapping light blue and violet meshwork in the space-filling model. Two views, rotated by 90° (top and bottom), are shown. The images were prepared by J. Vondrasek and are reproduced from the protease database (http://xpdb.nist.gov/hivsdb).



Genome Sequencing and Other Advances Expose New Targets for Antiviral Drugs

Standard approaches to antiviral drug development have focused on viral enzymes as primary targets (e.g., proteases, replicases, and reverse transcriptase), and most compounds in clinical use are such **direct-acting antivirals**. However, as virus reproduction is dependent on numerous host functions, host cell components and the interaction of viral proteins with them also represent potential targets for antiviral drug development. Because host genes have substantially lower mutation rates than do viral genes, drug resistance should be less of a problem with such compounds. A number of new, high-throughput methods now exist for determining the function of individual viral proteins in the host cell, their interactions with viral and cellular proteins, and the consequences of these interactions for both virus and host.

High-density arrays of DNA fragments on a DNA chip the size of a microscope slide have been used to assess the expression of thousands of genes in an infected cell in a single experiment (**microarray analyses**). This technology enabled scientists to identify the changes in mRNA concentration in response to viral infection in various cells and tissues, but has been largely superseded by the application of high-throughput, next-generation RNA sequencing methods (RNA-seq). RNA-seq provides information on the changes in all types of RNA in a cell in response to viral infection. Such data can be used to identify cellular genes and pathways that may be targets for antiviral drug development. With RNAi technology, it is possible to reduce the concentration of a potential target protein in cultured cells and observe the effects on virus reproduction. Modern methods for host gene editing using sequence-targeted nucleases have made it possible to change or delete a particular gene in both cultured cells and model organisms, and examine the contribution of that gene to virus reproduction or pathogenesis.

Advances in protein separation techniques, mass spectrometry, and bioinformatics have revolutionized our capacity determine the total protein repertoire (the **proteome**) of virus samples, and host cells and tissues, with great sensitivity. Proteomic analyses have also made it possible to assess protein interactions on global scales and to discover critical nodes and previously unknown connections. Genome-wide protein interaction maps are available for model organisms such as budding yeasts, and partial maps have been constructed for many others, using a number of *in vitro* or cell-based assays. An example of the use of such methods is a systematic survey of the binding of cellular proteins to each of the human immunodeficiency virus type 1 proteins, produced from bacterial expression plasmids in human cell lines (Fig. 9.7). The results identified not only previously documented interactions, but also new connections with cellular proteins and pathways that are potential targets for future pharmacological intervention.

In Silico Drug Discovery via Virtual Screening

With all the advances in the "-omics" (genomics, transcriptomics, proteomics, and metabolomics), an enormous number of potential targets for drug discovery have been identified. Furthermore, as a result of advances in chemistry, the number of chemical structures that can be tested as antiviral compounds has increased dramatically. In fact, one estimate suggests that as many as 10^{64} chemicals can be made to test for activity against human protein targets. Despite the paucity of lead antiviral compounds that engage such targets, no one would seriously think of urging medicinal chemists and biologists to do random screening with all possible compounds. However, many researchers now are using computers to take on this daunting task.

Structural biologists have provided atomic-resolution models for numerous viral and cellular proteins, and homologs of the vast majority of possible enzymatic active sites are present in current protein structure databases. Sophisticated computational methods have been developed to identify drug leads by "**virtual screening**," iterative docking of each chemical into a chosen site in a protein target. When a virtual small molecule "fits" into a pocket, the molecule is obtained or synthesized and then tested in a mechanism- or cell-based assay. Modifications to improve activity are made, and the computational analysis and testing are reiterated (Box 9.1).

As noted for the protease of human immunodeficiency virus type 1 (Fig. 9.6), computational methods are also used to optimize the binding strength or selectivity of lead compounds obtained by other screening methods. Further technological advances, such as improved systems for homology modeling from known structures, and development of algorithms for predicting protein structures *de novo* from coding sequences are expected to increase the future effectiveness of *in silico* approaches. The paradigm of virtual screening has been called "genome-to-drug-to-lead," and it has the potential to reduce the formidable human resource requirements for chemistry and biology.

The Difference between "R" and "D"

Antiviral Drugs Are Expensive To Discover, Develop, and Bring to the Market

Even with modern methods, it is common for thousands of leads to yield but one promising candidate for further drug development (Fig. 9.8). Research and lead identification, the "R" of "R&D," represent only the beginning of the process of producing a drug for clinical use. The "D" of "R&D" is development, comprising all the steps necessary to take an antiviral lead compound through safety testing, scale-up of synthesis, formulation, pharmacokinetic studies, and clinical trials.

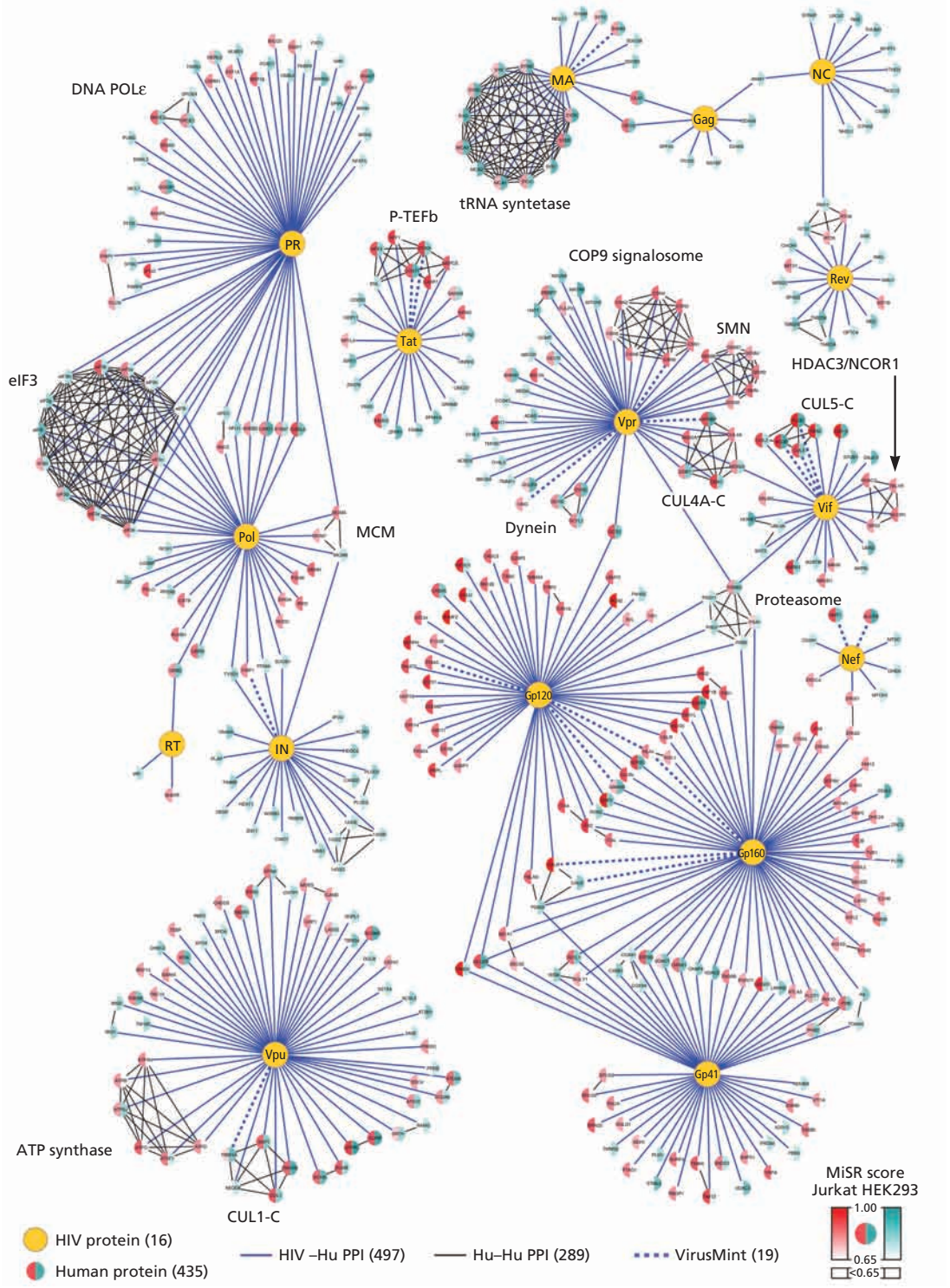


Figure 9.7 The human immunodeficiency virus (HIV) type 1 protein "interactome." The figure shows a network representation of 497 HIV-human interactions between 16 HIV proteins and 435 human proteins, connected by blue lines. Each node that represents a human protein is split into two colors; the intensity of each color represents a score for the relevant abundance of the protein and the reproducibility and specificity of the interaction in HEK293 (blue) or Jurkat (red) cultured cells. Black lines correspond to interactions between host proteins (289) that were obtained from publicly available databases; dashed lines correspond to links found in a virus interaction database. Reproduced from S. Jäger et al., *Nature* **481:**365–370, 2013, with permission.

BOX 9.1

EXPERIMENTS

An allosteric antiviral by in silico *design*

Retroviruses encode an enzyme, integrase, that catalyzes the specialized recombination reaction that joins viral and host DNAs, an essential step in the reproduction of these viruses (see Volume I, Chapter 6). The integrase protein of the human immunodeficiency virus type 1 is known to bind to the host cell transcriptional activator lens epithelium-derived growth factor (Ledgf/p75), which promotes this recombination reaction by tethering an integrase-viral DNA complex to host cell chromatin.

X-ray crystallographic analysis of the integrase-binding domain of Ledgf bound to the dimer interface of two isolated catalytic core domains (CCDs) of integrase revealed a well-defined binding pocket into which the end of an interhelical loop from the Ledgf domain was seen to extend (see panel A of the figure). On the basis of this structural information and other biochemical and genetic data, it was possible to define features (a pharmacophore) of a small molecule that would be optimal for binding in the CCD dimer pocket. Virtual, *in silico* screening of some 200,000 commercially available compounds that satisfied these features yielded several likely candidates. An *in vitro* assay for inhibition of the Ledgf-integrase interaction by these candidates identified 2-(quinolin-3-yl) acetic acid derivatives as lead compounds for further development. Analyses of structure-activity relationships led to the synthesis, by various groups of investigators, of structurally related inhibitors with ever-increasing potency when tested for antiviral activity in cell cultures. Related compounds are now being tested for use in the clinic (see panels B and C).

The inhibitory compounds, called LEDGINs or ALLINIs (for Ledgf- or allosteric integrase inhibitors), were found to be dual-acting both *in vitro* and in cell culture experiments: they not only impeded integration by blocking Ledgf tethering, but also inhibited enzymatic activity by preventing critical conformational dynamics of the integrase protein. Most surprising, however, was the discovery that their antiviral potency was determined primarily by their ability to block proper virus particle maturation, not by their effects on enzymatic activity. The compounds promote integrase multimerization, a reaction that, for as yet unknown reasons, hinders formation of the normal electron-dense progeny viral cores. Most importantly, this research demonstrated that allosteric sites on retroviral integrase are valid targets for antiviral drug discovery, and that *in silico* screening is a practical approach to identifying relevant lead compounds.

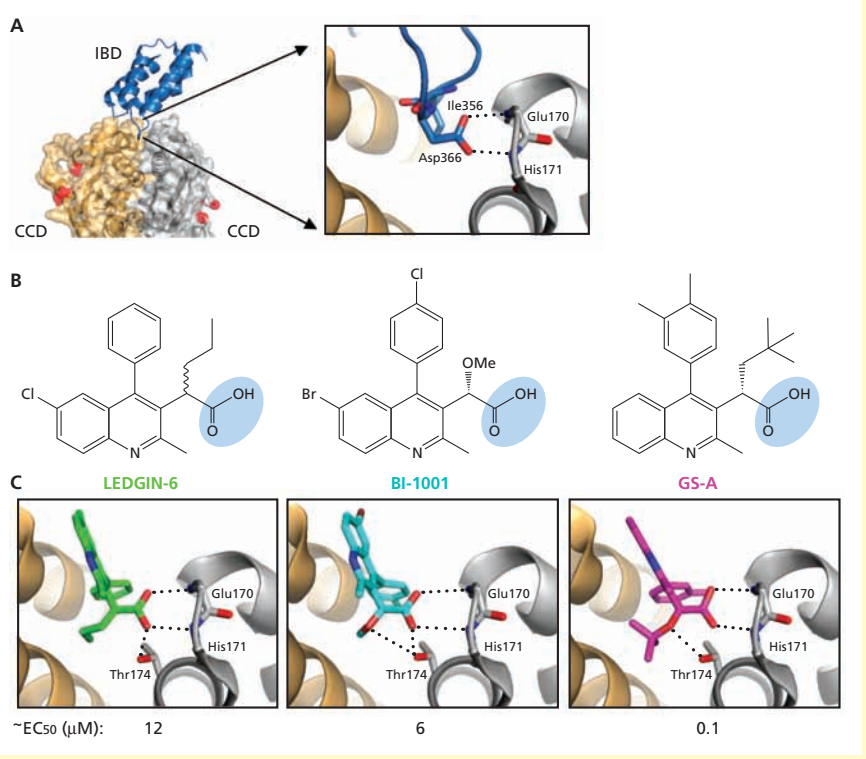
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ALLINI structures and binding mechanisms. (A) X-ray cocrystal structure of the human immunodeficiency virus type 1 (HIV-1) integrase (IN)-Ledgf/p75 complex (left; PDB code 2B4J) shows one IN-binding domain (IBD) molecule (blue) bound at the interface of two IN CCD monomers (gold and silver), with IN active-site residues colored red and the interhelical loop of Ledgf/p75 penetrating into the cavity at the dimer interface. The side chains of Ledgf/p75 contact residues Ile365 and Asp366, and main chain atoms of Glu170 and His171 of IN are shown as sticks, with oxygen and nitrogen atoms colored red and blue, respectively, and H bonds drawn as dotted lines (right). **(B)** Chemical structures of LEDGIN-6, BI-1001, and GS-A, with the carboxyl group that mimics Ledgf/p75 hot spot residue Asp366 highlighted. **(C)** Binding of LEDGIN-6 (left; PDB code 3LPU), BI-1001 (middle; PDB code 4DMN), and GS-A (right; PDB code 4E1M) at the HIV-1 IN CCD-CCD interface. Red and blue colors identify oxygen and nitrogen atoms, respectively, in the compounds, and in Glu170, His171, and Thr174 (for simplicity, only main chain atoms of these residues are included). H bonds between the drugs and IN are dotted lines. Approximate values for 50% inhibition of HIV-1 reproduction in cell culture (EC_{50}) are indicated at the bottom. Adapted from L. Krishnan and A. Engelman, *J Biol Chem* **287:**40858-40866, 2012, with permission.

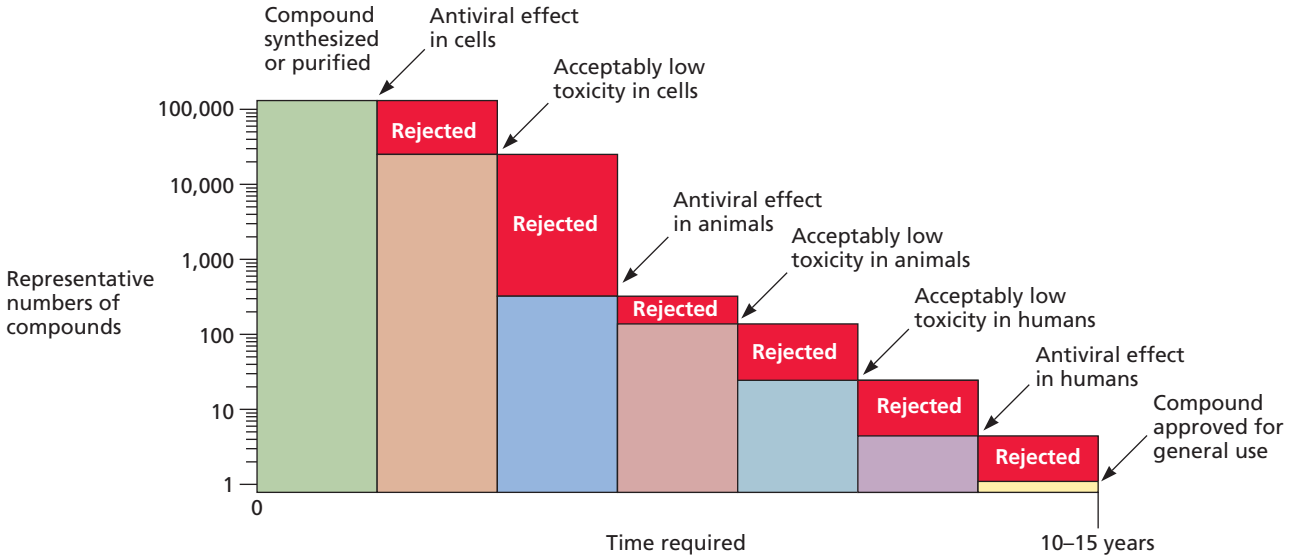


Figure 9.8 A descending staircase of drug discovery. Many compounds must be tested before a commercially viable antiviral drug will become available. The attrition rate is very high (red "rejected" label), as hundreds of thousands of chemicals are tested in multiple steps taking several years before one compound emerges as a drug. A few of the significant hurdles in the process and the extent of attrition at each step are illustrated.

With rare exceptions, it takes 5 to 10 years after the initial lead is identified to get a drug to the market. Decisions made by drug companies are influenced by these realities (Box 9.2).

According to an analysis in 2013 by *Forbes Magazine*, "a company hoping to get a single drug to market can expect to have spent $350 million before the medicine is available for sale . . . because so many drugs fail, large pharmaceutical companies that are working on dozens of drug projects at once spend an average of $5 billion per new medicine." Satisfying an unmet medical need, having no competitors, or being better than any competitive drug are all important issues for commercial development. The drug must also be relatively inexpensive to manufacture and easy to formulate and deliver (a pill to swallow is much preferred over injection, for example). Clinical testing must demonstrate that the drug is safe, effective, and has no serious side effects (Box 9.3). Finally, given the staggering costs noted above, the market for the drug must be large enough to ensure a profit.

Antiviral Drugs Must Be Safe

As in vaccine development, safety is the overriding concern of any company developing an antiviral drug. Toxicity to cultured cells and animals is the first indication that a compound may not be safe. More promising leads are discarded because of toxicity than for any other reason. Toxicity can be described in terms of the **cytotoxic index** (for cells) or the **therapeutic index** (for hosts). These indices are defined as the dose that inhibits virus reproduction divided by the dose that

BOX 9.2

DISCUSSION

New drugs, new mechanisms—no interest?



Two pharmaceutical companies independently discovered a new class of drug that inhibits herpes simplex virus reproduction. These compounds are targeted to the DNA helicase-primase, which is essential for viral genome replication. They represented the first new anti-herpes simplex virus drugs since acyclovir was developed in the 1970s. The helicase-primase inhibitors are more potent than acyclovir and its derivatives in animal models, and have remarkable potential.

However, neither company developed the inhibitors. The reason is that acyclovir is a safe, effective drug, and the expense of taking a new drug through clinical trials is enormous. Marketing strategy asserts that it is not cost-effective to compete with a proven drug. The reality is that companies must make choices about where to put their resources.

Crumpacker CS, Schaffer PA. 2002. New anti-HSV therapeutics target the helicase-primase complex. *Nat Med* **8**:327–328.

BOX 9.3

TERMINOLOGY

Clinical trials

Clinical trials are research studies in humans that test new ways to prevent, detect, diagnose, or treat a specific disease or condition. National and international regulations and policies have been developed to protect the rights, safety, and well-being of individuals who take part in clinical trials and to ensure that trials are conducted according to strict scientific and ethical principles. By taking part in clinical trials, participants can receive access to new treatments and help others by contributing to medical research.

Committees that are responsible for the protection of human subjects must approve all clinical trials. In the United States, this body is called the Institutional Review Board (IRB). Most IRBs are located in hospitals or other institutions in which the trial will be conducted, but approval by a central (independent/for profit) IRB may be acceptable for studies conducted at sites that do not have their own IRB.

Clinical trials are performed in a series of orderly, defined steps called "**phases**." The trials can vary in size and may involve a single site in one country or multiple sites in one or several countries, and it can take up to 10 or more years for a drug or treatment to be licensed for use in humans. The burden of paying for clinical trials (ultimately hundreds of millions of dollars) is borne by the sponsor, typically a pharmaceutical or biotech company, who designs the study in coordination with a panel of expert clinical investigators.

Phase I: The drug is tested in increasing doses with a small group of people (20 to 80). These trials are conducted mainly to evaluate the **safety** of chemical or biologic agents or other types of interventions. They help to determine the maximum dose that can be given safely (also known as the **maximum tolerated dose**), to assess whether an intervention causes harmful side effects, and to gain early evidence of effectiveness. Phase I trials may enroll people who have advanced disease that cannot be treated effectively with existing treatments or for which no treatment exists.

Phase II: These trials test the **effectiveness** of interventions in people who have a specific disease. They also continue to look at the safety of interventions. Phase II trials usually enroll fewer than 100 people but may include as many as 300. Although phase II trials can give an indication of whether or not an intervention works, they are almost never designed to show whether an intervention is better than standard therapy.

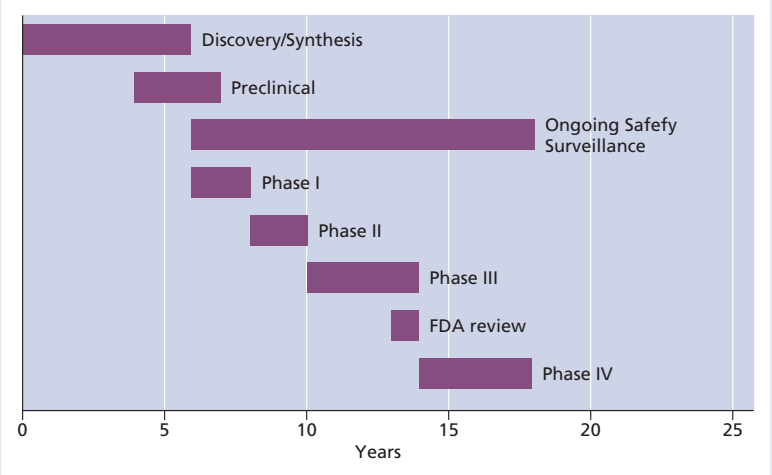
Phase III: These trials test the **effectiveness** of a new intervention or new use of an existing intervention. Phase III trials also examine how the **side effects** of the new intervention compare with those of the usual treatment. If the new intervention is more effective than the usual treatment and/or is easier to tolerate, it may become the new standard of care.

Phase III trials usually involve large groups of people (100 to several thousand), who are randomly assigned to one of two treatment groups, or "trial arms": a control group, in which everyone receives the usual treatment for their disease; or an investigational group, in which everyone receives the new intervention or new use of an existing intervention. Should a randomized clinical trial violate ethical standards (e.g., standard of care cannot be withdrawn), then an observational study of only a treated group is conducted.

Following successful completion of this phase, data are compiled in an application to the FDA for approval. If the drug is ruled "safe and effective," it is licensed for use in patients.

Phase IV: These trials further evaluate the effectiveness and long-term safety of drugs or other interventions. They usually take place after approval by the FDA for standard use, and are generally sponsored by drug companies. Several hundred to several thousand people may take part in a phase IV trial, which is also known as a postmarketing surveillance trial.

Approximate time frames from drug discovery to use in the clinic.



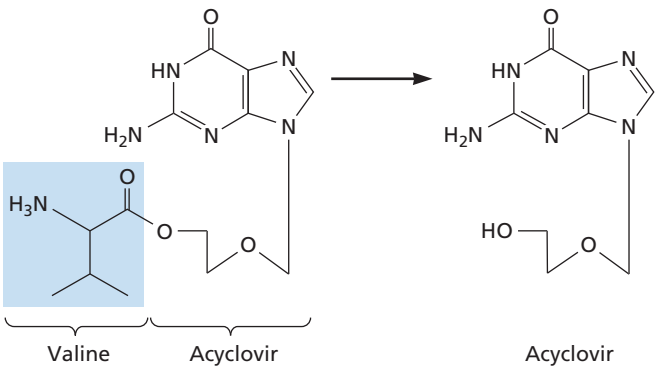
is toxic to cells or host. The lower the index, the better; indices of 1/1,000 or lower are preferred.

No human trial can be initiated without detailed safety studies in several animal species. Compounds that may be used in long-term treatment must be evaluated for toxicity, allergic effects, mutagenicity, and carcinogenicity. Safety overrides efficacy in most cases. On the other hand, when there are no other effective treatments, as in the early days of the acquired immunodeficiency syndrome (AIDS) pandemic, even drugs that caused some undesirable side effects can be licensed for human use (e.g., azidothymidine [AZT]).

Drug Formulation and Delivery

The science of formulation and delivery is an essential part of any antiviral discovery program. After administration, a drug must reach the proper place in a patient and remain at an effective concentration for a time sufficient to inhibit virus reproduction. A compound that cannot enter the bloodstream after ingestion is not likely to be effective. But satisfying this step may not be sufficient, as many compounds bind to albumin or other proteins in the blood and are thereby rendered ineffective. Other compounds may be inactivated as they pass through the liver and are cleared rapidly from the body. Such problems are generally discovered only by testing. Some insoluble compounds, or chemicals unable to enter the bloodstream after ingestion (poor bioavailability), can be modified by the addition of new side chains that may improve absorption from the intestine (Fig. 9.9). In addition, delivery vehicles such as liposomes, minipumps, skin patches, or slow-release capsules may improve bioavailability. Other desirable features include stability and cost-effective synthesis in large quantities. Literally tons of precursor materials are needed to manufacture commercial quantities of an antiviral drug.

Figure 9.9 Valacyclovir (Valtrex), an l-valyl ester derivative of acyclovir with improved oral bioavailability. Acyclovir is not taken up efficiently after oral ingestion. However, a derivative of acyclovir, valacyclovir, has as much as 5-fold-higher oral bioavailability than acyclovir, as determined by the amount in serum relative to the dose of drug given. The addition of a new side group to acyclovir allows increased passage of drug from the digestive tract to the circulation. These acyclovir derivatives are prodrugs, which are converted to acyclovir by cellular enzymes that cleave off the valine side chain. Adapted from D. R. Harper, *Molecular Virology* (Bios Scientific Publishers, Ltd., Oxford, United Kingdom, 1994), with permission.



Examples of Some Antiviral Drugs

The antiviral drugs currently approved for general use are surprisingly few, belong to a limited number of chemical classes, and, as noted previously, mostly target viral proteins. While many are safe and effective, some are marginally efficacious or have side effects that limit their use to cases in which there are no alternatives. Some antiviral drugs that are of historic interest and still in general use are described below. In what follows, generic names for the drugs are used with the best-known brand/trade names in parentheses (Box 9.4).

Approved Inhibitors of Viral Nucleic Acid Synthesis

Most approved antiviral drugs are nucleoside or nucleotide analogs, directed against viral proteins that catalyze nucleic acid synthesis, or are nonnucleoside inhibitors of these proteins.

Acyclovir and Ganciclovir—Herpesvirus Infections

Acyclovir (Zovirax) is an example of a specific, nontoxic drug that is highly effective against herpes simplex virus (genital and oral herpes) and, to some extent, varicella-zoster virus (chickenpox and shingles). It was initially synthesized in 1974, but it was not until the mid-1980s that its full potential as an antiherpesviral drug was realized. Acyclovir is a nucleoside analog related to guanosine, containing an acyclic sugar group (Fig. 9.10). It is a **prodrug**, a precursor of the active antiviral compound. Conversion to the drug requires the sequential activities of three kinases that produce a triphosphate derivative, the actual antiviral compound (Fig. 9.11A). Herpes simplex virus and varicella-zoster virus genomes encode an enzyme that normally phosphorylates thymidine to form thymidine monophosphate, but this kinase can also accept a wide range of other substrates, including acyclovir. Cellular enzymes cannot perform this first reaction, but they can synthesize the di- and triphosphates, the latter of which is then used as a substrate by the viral polymerase for incorporation into viral DNA. As acyclovir lacks the 3′-OH group of the sugar ring, the growing DNA chain is terminated upon its addition. The specificity of acyclovir for the herpesviruses depends therefore on the virally encoded thymidine kinase. Indeed, if this viral enzyme is synthesized in an uninfected cell and acyclovir is added, the cell will die because its DNA replication will also be blocked by the chain-terminating base analog. Such use of the viral enzyme is incorporated into several strategies for selective killing of tumor cells.

BOX 9.4

TERMINOLOGY

What's in a name?

Sorting out drug names can be mind-boggling. Many are tongue twisters and a challenge for the common mortal to pronounce. To make matters worse, every drug has more than one name. The definitions below are an attempt to provide some guidance to the novice.

Every drug has at least three names:

Chemical name: This is the scientific name, which based on the chemical composition and structure of the drug. It is the most specific and definitive name, but often too complicated to use for any but professional medicinal chemists or in scientific publications.

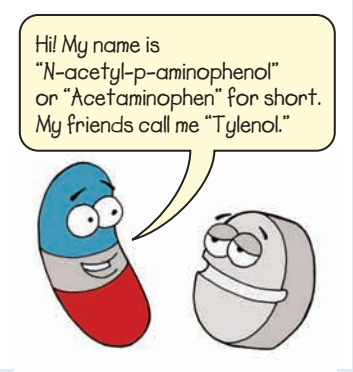
Generic name: The generic name, also known as the International Nonproprietary Name (INN), is assigned by a particular governing body, the FDA, in the United States. When the FDA approves a drug, it is given a generic name that is intended to be a shorthand derivative of the chemical name. Ergo: *N*-acetyl-*p*-aminophenol is generic **acetaminophen**. [Although it is anyone's guess how (2*R*,3*S*,4*R*,5*R*,8*R*,10*R*,11*R*,12*S*,13*S*,14*R*)-13-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadecan-15-one becomes generic **azithromycin**.] Letters are incorporated to refer to the action of the drug. For example, antiviral drugs all end in -vir, some monoclonal antibodies in -mab, and some antibiotics (as is azithromycin) end in -mycin.

Unfortunately, different countries may use different rules for their assignments, such that the same compound can have two or even more generic names. For example, generic acetaminophen in the United States is generic paracetamol in the United Kingdom. The latter name is used in other parts of Europe and Asia, which can be a source of confusion for world travelers.

Brand name: Also called a trade name, it is given to drugs by pharmaceutical companies. If more than one company markets a drug, the same chemical entity can have more than one name (at least 10 exist for acetaminophen worldwide), another potential source of confusion.

Brand naming is influenced strongly by the trademark system in the United States. Because almost all ordinary words are already taken, drug companies have to be quite creative in inventing entirely new names that can be identified with a registered trademark (®). These names are chosen with an eye to both customer appeal and loyalty. Some names may have Latin roots, such as the *pax* (for "peace") in Paxil, an antidepressant; or *vir* ("man") in Viagra. Drugs for women often include soft letters such as S, M, and L, as in Sarafem and Vivelle; and the letters X, Z, K, and N are often chosen to denote cutting-edge science, as in Zantac, Nexium, and Protonix. The intention here is that even if generic acetaminophen is available, the customer will still reach for Tylenol in the drug store.

Koven S. July 14, 2012. How are drugs named? Boston.com. http://www.boston.com/lifestyle/health/blog/inpractice/2012/07/how_are_drugs_named.html



Acyclovir remains the gold standard for treatment of herpes simplex and varicella-zoster virus infections, although other approved guanosine analogs, which show better oral absorption, have been approved for use in the clinic. **Ganciclovir (Cytovene)** is a derivative of acyclovir (Fig. 9.10) that was developed to treat human infections with the betaherpesvirus cytomegalovirus. The cytomegalovirus genome does not carry a thymidine kinase gene, but it does encode a protein kinase that can phosphorylate ganciclovir. Initial formulations of this drug given intravenously were quite toxic, and used only for life-threatening human cytomegalovirus infections in AIDS patients and immunosuppressed transplant recipients. Subsequently, an oral formulation that is much less toxic was developed and is effective for prophylaxis and long-term use for human cytomegalovirus infections.

AZT—Human Immunodeficiency Virus

Zidovudine; AZT (Retrovir) (Fig. 9.10), an analog of thymidine, was the first drug to be licensed for the treatment of AIDS. However, AZT was discovered initially in screens for antitumor cell compounds rather than for antiviral agents. The drug is phosphorylated by cellular enzymes and then incorporated into viral DNA, where, like acyclovir, it acts as a chain terminator (Fig. 9.11B). While phosphorylated AZT is not a good substrate for most cellular DNA polymerases, its selectivity depends mainly on the fact that retroviral reverse transcription takes place in the cytoplasm, where the drug appears first and in highest concentration. Because AZT monophosphate competes with TMP for the formation of nucleoside triphosphate, its presence causes depletion of the intracellular pool of TTP. Consequently, AZT has undesirable

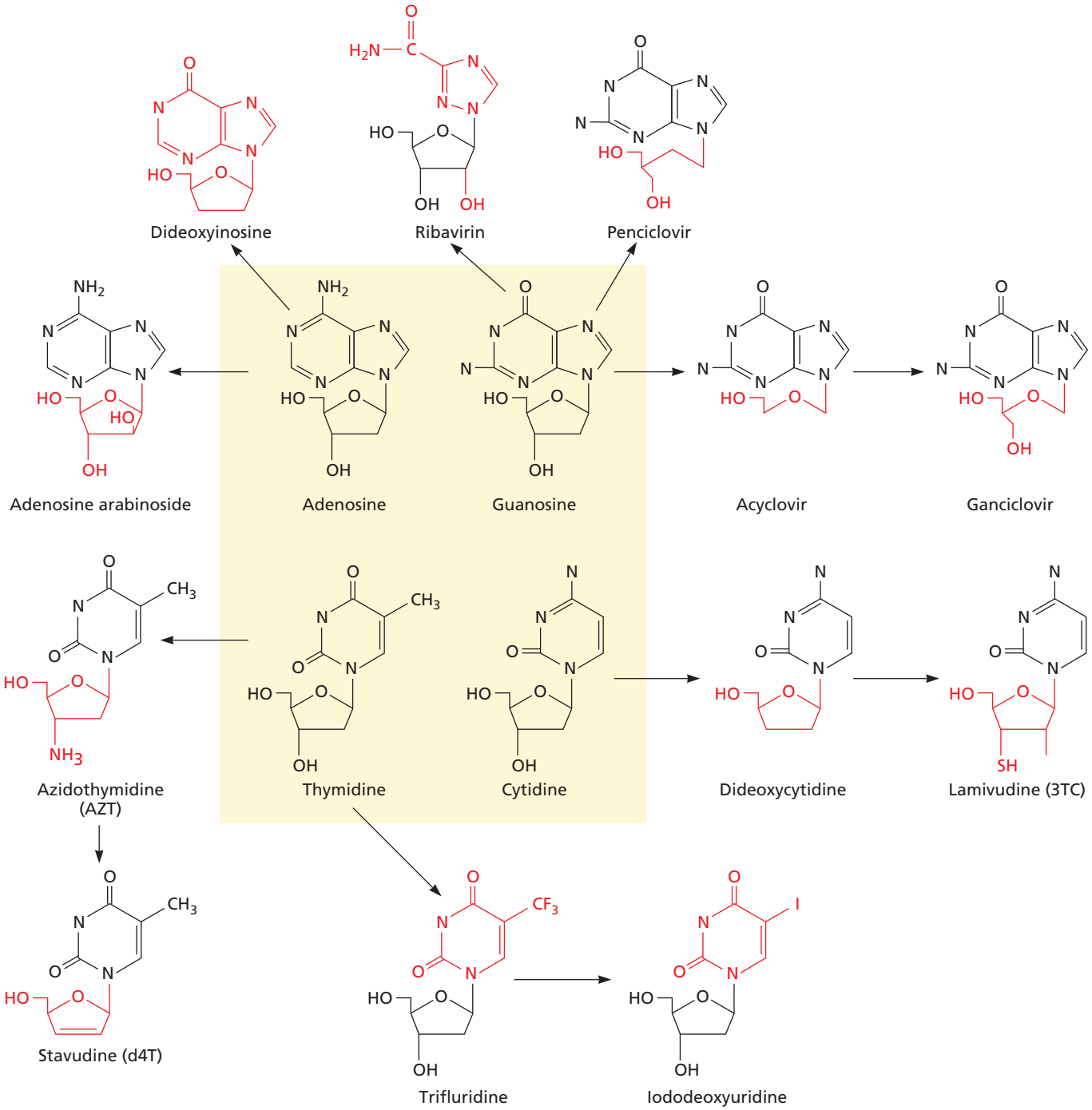


Figure 9.10 Many well-known antiviral compounds are nucleoside and nucleotide analogs. The four natural deoxynucleosides are highlighted in the yellow box. The chemical distinctions between the natural deoxynucleosides and antiviral drug analogs are highlighted in red. Arrows connect related drugs. Adapted from E. De Clercq, *Nat Rev Drug Discov* **1:**13–25, 2002, with permission.

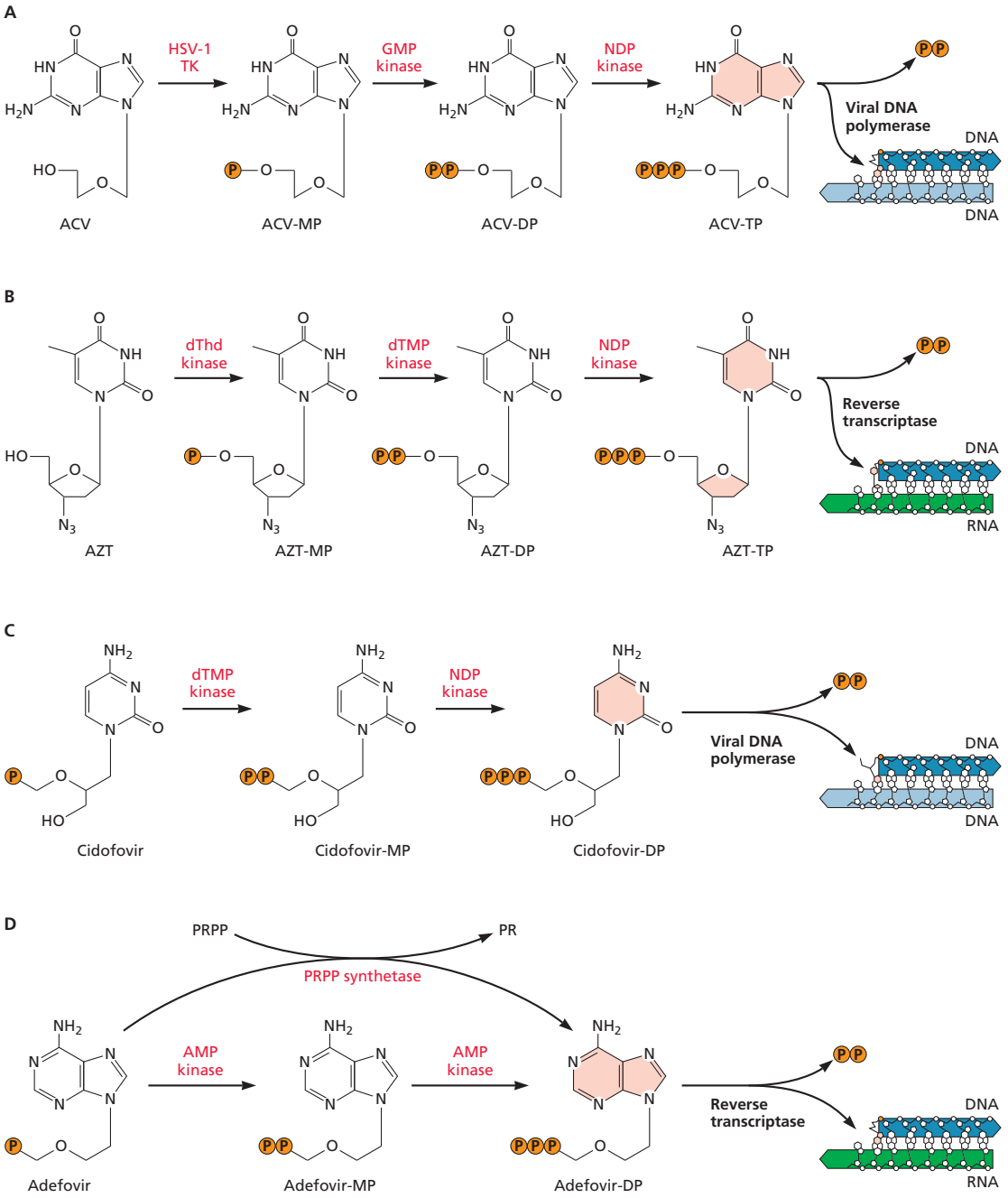


Figure 9.11 Chain termination by antiviral nucleos(t)ide analogs. (A) Acyclovir (ACV) is a prodrug that must be phosphorylated in the infected cell. The thymidine kinase of herpes simplex virus 1 (HSV-1 TK), but **not** the cellular kinase, adds one phosphate (orange circle labeled P) to the 5′ hydroxyl group of acyclovir. The monophosphate is a substrate for cellular enzymes that synthesize acyclovir triphosphate. The triphosphate compound is recognized by the viral DNA polymerase and incorporated into viral DNA. As acyclovir has no 3′ hydroxyl group, the growing DNA chain is terminated and viral genome replication ceases. **(B)** AZT targets the human immunodeficiency virus reverse transcriptase. The compound must be phosphorylated by cellular kinases in three steps to the triphosphate compound, which is incorporated into the viral DNA to block reverse transcription. **(C)** Cidofovir [*S*-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine] is an acyclic nucleotide analog. In contrast to acyclovir and AZT, cidofovir requires only two phosphorylations by cellular kinases to be converted to the active triphosphate chain terminator. **(D)** Adefovir [9-(2-phosphonylmethoxyethyl) adenine] is an acyclic nucleotide analog and also requires only two phosphorylations by cellular AMP kinases. Through the action of phosphoribosyl pyrophosphate (PRPP) synthetase, which forms the triphosphate from the monophosphate in one step, both cidofovir and adefovir bypass the nucleoside-kinase reaction that limits the activity of dideoxynucleoside analogs such as AZT. DP, diphosphate; dThd, (2′-deoxy)-thymidine; MP, monophosphate; NDP, nucleoside 5′-diphosphate; PR, 5-phosphoribose; TP, triphosphate. Adapted from E. De Clercq, *Nat Rev Drug Discov* **1**:13–25, 2002, with permission.

side effects when administered for long periods, including lactic acidosis (buildup of acid in the blood), liver problems, muscle weakness, and reduced numbers of red and white blood cells. The drug was used extensively in the early years of the AIDS pandemic, simply because there was nothing else available. Considerable effort has been devoted to discovering alternatives to AZT, and, as will be described in a later section, several nucleos(t)ide analogs that have better therapeutic value are now available and used for treatment of human immunodeficiency virus infections.

Lamivudine—Hepatitis B Virus

Lamivudine (Epivir) (Fig. 9.10), an orally delivered nucleoside analog, is also a prodrug that requires phosphorylation by cellular kinases to be incorporated into DNA, where it functions as a chain terminator. It is effective in blocking the reverse transcriptases of hepatitis B and human immunodeficiency viruses. Since the approval of lamivudine, a number of additional nucleoside analogs with improved properties have been developed for use in treatment of chronic hepatitis B virus infections. The latest of these, the human immunodeficiency virus reverse transcriptase nucleotide analog inhibitor tenofovir, was approved for treatment of hepatitis B in 2008, after its approval for treatment of AIDS.

Cidofovir—Broad-Spectrum Antiviral

Cidofovir (Vistide) (Fig. 9.11C) is an acyclic nucleoside phosphonate that can be considered a broad-spectrum drug, as it is active against various herpesvirus, papillomavirus, polyomavirus, adenovirus, and poxvirus infections. A prodrug, cidofovir, is converted to di- and triphosphate derivatives by host enzymes. The fully phosphorylated compound has a higher affinity for viral polymerases than host cell polymerases, a unique property of acyclic nucleotide analogs. For example, the binding affinity of cidofovir diphosphate to the DNA polymerase of human cytomegalovirus polymerase is 8 to 80 times higher than for any of the human DNA polymerases. Because the conversion of cidofovir to cidofovir monophosphate does not depend on a virus-induced thymidine kinase or a viral protein kinase, essentially all DNA viruses and retroviruses are susceptible. Related compounds are adefovir (Fig. 9.11D), approved for hepatitis B infections, and tenofovir, approved for HIV infections. These drugs have been formulated for intravenous, topical, and oral applications.

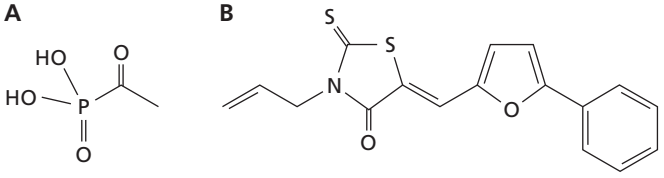
Ribavirin—RNA Virus Infections

Ribavirin (Virazole) (Fig. 9.10), one of the earliest antivirals, was synthesized in 1972 and purported to have broad-spectrum activity against many DNA and RNA viruses. However, the drug is relatively toxic, and its development and indications for use have been controversial. In fact, ribavirin is not licensed for general use in many countries. Despite its long history, its primary mechanism of action is not clear. Ribavirin monophosphate is a competitive inhibitor of cellular inosine monophosphate dehydrogenase, and such inhibition leads to reduced GTP pools in the cell, which may adversely affect the replication of some viral genomes. The drug also blocks initiation and elongation by viral RNA-dependent RNA polymerases and interferes with capping of mRNA. Finally, ribavirin is an RNA virus mutagen: once incorporated into a template, it pairs with C or U with equal efficiency. In some studies, its antiviral activity correlates directly with its mutagenic activity. Even with its unknown mechanism and its toxicity, ribavirin is used as an aerosol for treatment of infants suffering from respiratory syncytial virus infection, as well as for treatment of Lassa fever virus and hantavirus infections. Viramidine and levovirin are analogs of ribavirin that are in clinical development for treatment of hepatitis C virus infections.

Foscarnet—Viral DNA Synthesis Blocker

Foscarnet (Foscavir) is the only nonnucleoside DNA replication inhibitor of herpesviruses (Fig. 9.12A). The drug is a noncompetitive inhibitor that binds to the pyrophosphate-binding site in the catalytic center of herpesvirus DNA polymerase. Its ability to chelate one of the active-site metals while making other electrostatic interactions is proposed to trap the enzyme in a closed, inactive configuration (see Volume I, Box 6.2). Foscarnet also inhibits hepatitis B virus polymerase and the reverse transcriptase of human immunodeficiency virus, at concentrations that do not affect human DNA polymerases. The drug must be administered intravenously. As it causes kidney and bone toxicity, its use is recommended only for life-threatening infections for which other antiviral drugs are no longer effective.

Figure 9.12 Two nonnucleoside/nucleotide antiviral compounds. (A) Foscarnet is an FDA-approved drug that inhibits herpesviral DNA polymerase by binding noncompetitively to the pyrophosphate-binding site in the catalytic center. The drug also inhibits the activities of the reverse transcriptases of hepatitis B virus and human immunodeficiency virus. **(B)** LJ-001 is an aryl methyldiene rhodanine lead compound that intercalates into the lipid bilayers of enveloped viruses and blocks virus-cell membrane fusion.



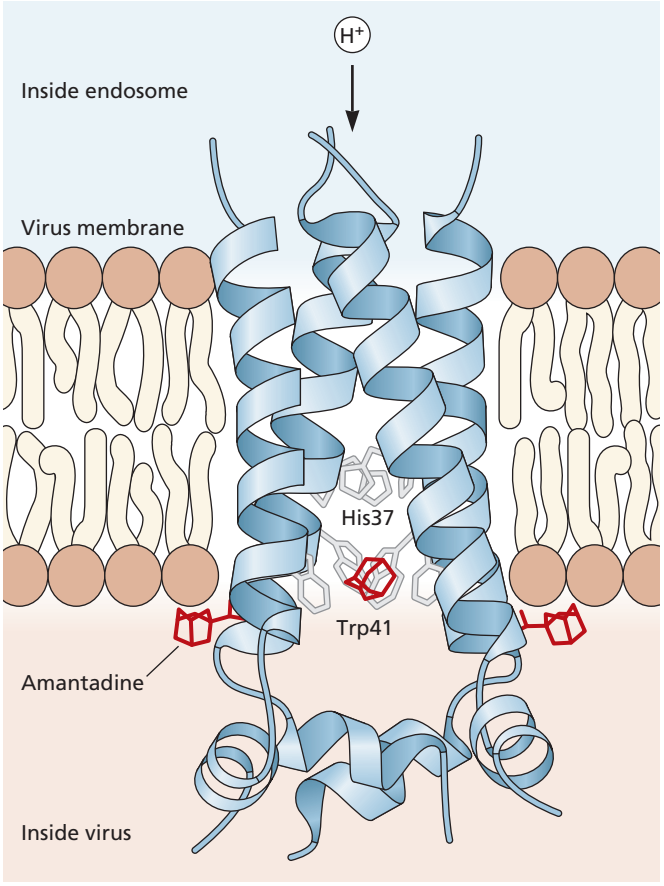
Foscarnet LJ-001

Approved Drugs That Are Not Inhibitors of Nucleic Acid Synthesis

Amantadine—Influenza A Virus Uncoating Inhibitor

Amantadine (Symmetrel) is a three-ringed symmetric amine that was developed by DuPont chemists almost 50 years ago. It was the first highly specific, potent antiviral drug effective against any virus. The target of the drug was shown to be the influenza A virus M2 protein, a tetrameric, transmembrane ion channel that transports protons (Fig. 9.13). Amantadine has no effect on influenza B viruses, as their genomes do not encode an M2 protein. Influenza A virus mutants resistant to amantadine, which arise after therapy, all have amino acid changes in the M2 transmembrane sequences predicted to form the ion channel. Amantadine blocks the channel, so that protons cannot enter the virion, effectively preventing the uncoating of influenza A virus (Volume I, Chapter 5). Unfortunately, during the 2008-2009 flu season, the U.S. Centers for Disease Control and Prevention found that 100% of seasonal H3N2 and the 2009 pandemic strain samples tested were resistant to amantadines, and these drugs are no longer recommended for the treatment of seasonal flu: the viral neuraminidase inhibitors oseltamivir and zanamivir are proposed as alternatives.

Figure 9.13 Interaction of amantadine with the transmembrane domain of the influenza A virus M2 ion channel. M2 protein is a tetramer with an aqueous pore in the middle of the four subunits. It is thought that at low concentrations (5 μM), amantadine (red) exerts its antiviral effect by blocking the M2 ion channel activity and preventing acidification of the virus particle. At higher concentrations, binding on the outside of the tetramer is thought to block proton entry allosterically. Acidification of the virus particle is required for release of viral nucleic acids (uncoating) in infected cells. For further detail see: J. R. Schnell and J. J. Chou, *Nature* **451:**591–595, 2008.



When treating infections with nonresistant strains, amantadine must be administered in the first 24 to 48 h and given at high doses for at least 10 days to have an impact on the clinical course of disease. The drug is most effective when susceptible patients are treated prophylactically, in anticipation of influenza virus infection. However, at high concentrations, side effects are common, particularly those affecting the central nervous system. On follow-up of these side effects, amantadine was found to be useful for relieving symptoms of Parkinson's disease in some patients. Today, more amantadine is sold for central nervous system disease than for antiviral treatment. Rimantadine, a methylated derivative, cannot cross the blood-brain barrier and therefore has fewer central nervous system side effects. For this reason, the drug often replaces amantadine in the treatment of influenza A virus infections.

Concentration dependence is an unusual property of amantadine. The drug has broad antiviral effects at high concentrations, but at low concentrations it is specific for influenza virus A. Analysis of resistant mutants provided insight into the apparently complex mechanism of action of amantadine. At concentrations of 100 mM or higher, the compound acts as a weak base and raises the pH of endosomes so that pH-dependent membrane fusion is blocked. Any virus with a pH-dependent fusion mechanism could be affected by high concentrations of amantadine. Resistant mutants of influenza A virus selected under these conditions in cultured cells harbor amino acid substitutions in hemagglutinin (HA) that destabilize the protein and enable fusion at higher pH. Influenza A virus mutants selected at concentrations of 5 mM or lower carried mutations in the M2 gene. These mutations affected amino acids in the membrane-spanning region of the M2 ion channel protein.

Zanamivir and Oseltamivir—Influenza Virus Neuraminidase Inhibitors

Zanamivir (Relenza) and **oseltamivir (Tamiflu)** are inhibitors of the neuraminidase enzyme synthesized by influenza A and B viruses (Box 9.5). Zanamivir is delivered via inhalation, while oseltamivir can be given orally. When used within 48 h of symptoms, the drugs reduce the median time to their alleviation by ~1 day compared to placebo. When used within 30 h of disease onset, the drugs reduce the duration of symptoms by ~3 days.

BOX 9.5

EXPERIMENTS

Inhibitors of influenza virus neuraminidase: development and impact

Influenza virus neuraminidase (NA) protein cleaves terminal sialic acid residues from glycoproteins, glycolipids, and oligosaccharides. It plays an important role in the spread of infection from cell to cell, because in cleaving sialic acid residues, the enzyme releases virus particles bound to the surfaces of infected cells and facilitates viral diffusion through respiratory tract mucus. Moreover, the enzyme can activate transforming growth factor β by removing sialic acid from the inactive protein. Because the activated growth factor can induce apoptosis, NA may influence the host response to viral infection.

NA is a tetramer of identical subunits, each of which consists of six four-stranded antiparallel sheets arranged like the blades of a propeller. The enzyme active site is a deep cavity lined by identical amino acids in all strains of influenza A and B viruses that have been characterized. Because of such invariance, compounds designed to fit in this cavity would be expected to inhibit the NA activity of all A and B strains of influenza virus, a highly desirable feature in an influenza antiviral drug. Moreover, as NA inhibitors are predicted to block spread, they may be effective in reducing the transmission of infection to other individuals.

Sialic acid fits into the active-site cleft such that there is an empty pocket near the hydroxyl at the 4-position on its sugar ring. On the basis of computer-assisted analysis, investigators predicted, correctly, that replacement of this hydroxyl group with either an amino or a guanidinyl group would fill the empty pocket and therefore increase the binding affinity by contacting one or more neighboring glutamic acid residues. The resulting "designer drug," zanamivir (Relenza), was licensed to GlaxoSmithKline in 1990 and approved by the U.S. FDA in 1999. Following their lead, Gilead Sciences developed the better-known neuraminidase inhibitor oseltamivir (Tamiflu), which was licensed to Hoffmann-La Roche and approved by the FDA that same year.

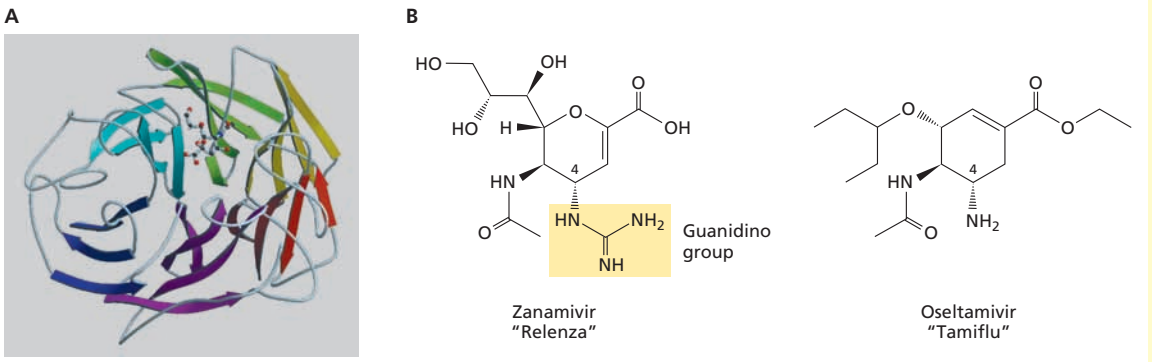
Both drugs are inhibitors of influenza A and B viruses. They do not inhibit other viral neuraminidases, an important requirement for safety and lack of potential side effects. The United States reportedly stockpiled $1.5 billion of oseltamivir prior to the global outbreak of H1N1 influenza in 2009, while the vaccine was being prepared. Many individuals established personal, preemptory "stockpiles" of the drug. However, given the limited reduction in duration of symptoms (8.4 to 25.1 h for adults; 12 to 47 h for children), the lack of reduction in hospitalizations and deaths, and the reported side effects (nausea, vomiting, headaches, and increased risk of renal and psychiatric syndromes), there remains uncertainty about whether the drug is worth taking by otherwise healthy flu patients.

Jefferson T, Jones M, Doshi P, Spencer EA, Onakpoya I, Heneghan CJ. 2014. Oseltamivir for influenza in adults and children: systematic review of clinical study reports and summary of regulatory comments *BMJ* **348:**g2545. doi:10.1136/bmj.g2545.

Varghese JN, Epa VC, Colman PM. 1995. Three-dimensional structure of the complex of 4-guanidino-Neu5Ac2en and influenza virus neuraminidase. *Protein Sci* **4:**1081–1087.

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Structure of influenza A virus NA and antiviral drugs. (A) Ribbon diagram of influenza A virus NA with α-sialic acid bound in the active site of the enzyme. The molecule is an N2 subtype from A/Tokyo/3/67. A monomer is viewed down the fourfold axis of an active tetramer. The C terminus is at the bottom right, near the subunit interface. The six β-sheets of the propeller fold are indicated in colors. Adapted from J. N. Varghese, p. 459–486, *in* P. Veerapandian (ed.), *Structure-Based Drug Design: Diseases, Targets, Techniques and Developments*, vol. 1 (Marcel Dekker, Inc., New York, NY, 1997), with permission. Courtesy of J. Varghese. **(B)** Chemical structures of two FDA-approved NA inhibitors.



Expanding Target Options for Antiviral Drug Development

As noted in an earlier section, technological advances have made it possible to identify both viral and host components that are required to complete the numerous steps in any virus life cycle successfully. Although viral nucleic acid synthesis inhibitors still predominate, it has now become practical to home in on other critical functions, viral protein activities, and viral host-interactions. (For additional comments on novel approaches see the interview with Dr. Benhur Lee: http://bit.ly/Virology_Lee)

Entry and Uncoating Inhibitors

The first step of the virus reproduction cycle has long been an attractive target, as virus-cell receptor interactions offer the promise of high specificity. Early enthusiasm for entry inhibitors came from experiments with monoclonal antibodies that blocked attachment or entry into cultured cells. Progress in the identification of potent or broadly neutralizing antibodies has promoted renewed interest in this approach. Passive immunization with these antibodies can often protect animals from challenge. One currently licensed antiviral monoclonal antibody, palivizumab, binds to the fusion protein of respiratory syncytial virus. This antibody is used to prevent infections of infants who are at high risk because of premature birth or medical problems such as congenital heart disease. There is considerable interest in developing neutralizing monoclonal antibodies against several other viruses including the human immunodeficiency virus, hepatitis C virus, and influenza viruses (Chapter 8). Monoclonal antibodies have also been valuable for identifying viral and host proteins required for entry and in elucidating their mechanisms of action. Moreover, their antiviral activities have suggested that small-molecule inhibitors of entry may be useful drug leads.

The binding sites of antibodies that block viral entry provide a starting point for screening chemical libraries or for design of small-molecule inhibitors. Optimally, the inhibitor should block viral entry but not interfere with the normal function of the cellular receptor. Because alternative receptors are available for some viruses (e.g., herpesviruses and human immunodeficiency virus), it may be necessary to block binding of a virus particle to more than one type of receptor for such treatment to be effective.

Membrane fusion, the usual process by which enveloped virus particles enter cells, is an attractive target for chemotherapeutic intervention because fusion mechanisms are conserved among enveloped viruses. To identify inhibitors of influenza virus fusion, a computer docking program was first used to predict which small molecules might bind into a pocket of the HA trimers and prevent a required low-pH-induced conformational change. From the molecules identified in this way, several benzoquinone- and hydroquinone-containing compounds were tested and found to prevent HA-mediated membrane fusion at low pH. One of these compounds inhibits influenza virus reproduction. Although it is not known how binding of the compound prevents fusion, it has been suggested that it blocks movements that juxtapose viral and cellular membranes (described in Volume I, Chapter 5). Several of these compounds are in clinical development.

The effectiveness of another type of virus-cell membrane fusion inhibitor depends on the fact that while cellular surface lipid bilayers are dynamic, viral envelopes are static. The inhibitor, a derivative of aryl methylene rhodanine (Fig. 9.12B), intercalates into both viral and cellular membranes, but can be removed rapidly from the cellular membrane by repair functions. As the viral envelope cannot be repaired, the inhibitor is retained and blocks the fusion step, most likely by affecting the fluidity/rigidity of the viral lipid bilayer. This broad-spectrum inhibitor was reported to block infection of cultured cells by numerous enveloped viruses but, consistent with its proposed mechanism of action, had no effect against nonenveloped viruses. Whether such inhibitors will be clinically useful awaits the results of testing in animal models and then in humans.

Microbicides

Considerable effort has been expended on development of **microbicides**, creams or ointments that contain compounds that either inactivate virus particles before they can attach and penetrate tissues, or enter cells and block virus reproduction. Particular attention has been focused on vaginal microbicides to prevent infection with sexually transmitted viruses. Formulations that incorporate acyclovir or tenofovir have been tested for prevention of transmission of herpes simplex viruses and human immunodeficiency virus, respectively. While investigations continue, there is as yet no approved microbicide for either of these viruses.

Viral Regulatory Proteins

Viral proteins that control transcription are often essential for virus reproduction and are prime targets for antiviral screens. Fomivirsen was the first licensed compound designed to inhibit the function of a viral regulatory protein. The drug is a phosphorothioate antisense oligonucleotide that is approved to treat retinitis caused by human cytomegalovirus, via direct injection into the vitreous of an infected eye. Inhibition depends on binding of the 21-nucleotide antisense molecule to the cytomegalovirus immediate-early 2 mRNA and hence preventing synthesis of this essential viral protein.

Regulatory RNA Molecules

Micro-RNAs (miRNAs), which induce degradation or inhibit translation of mRNA, are encoded in both host and viral genomes. One miRNA may regulate the expression of an entire network of genes. For example, the human miRNA miR-122, which is synthesized only in the liver, regulates

expression of >400 genes, including those that participate in cholesterol metabolism. When expression of miR-122 is inhibited, not only do levels of cholesterol in the circulation drop, but the liver is also protected from hepatitis C virus infection. miR-122 protects the viral (+) strand RNA from degradation by binding to two complementary sequences in the 5′ untranslated region (Volume I, Box 10.12). Antagonists of miR-122, such as antisense oligonucleotides, block virus reproduction with no harmful effect in animal models. This finding suggests that small RNA molecules may be valid targets for antiviral compounds.

Proteases and Nucleic Acid Synthesis and Processing Enzymes

Viral proteases have become among the most attractive targets for antiviral drug discovery. These enzymes are responsible for cleaving protein precursors to form functional units or to release structural components during or following particle assembly (maturational proteases). The requirement for proteases in the life cycle of several viruses makes them an excellent target for drug development. All herpesviruses encode a serine protease that is required for formation of nucleocapsids (Volume I, Chapter 13). Many features of these enzymes and their substrates are conserved among the members of the family *Herpesviridae*, and the X-ray crystal structure of the human cytomegalovirus protease has been solved. Interest in this enzyme as a drug target is based on the unusual serine protease fold and the mechanism of catalysis. Some success has been reported with a small molecule that inhibits the dimerization of the enzyme that is required for its activity.

As already noted, most presently approved drugs are nucleos(t)ide analogs that block viral replication by acting as chain terminators. However, we know from genetic analyses that DNA polymerase accessory proteins, such as those that promote processivity or bind to viral origins, are essential for viral genome replication and, consequently, are also attractive targets. The RNA-dependent RNA polymerases of RNA viruses appear to be unique to the virus world. Their varied activities, which include synthesis of primers, cap snatching, and recognition of RNA secondary structure in viral genomes (Volume I, Chapter 6), can be exploited for drug discovery. The unique helicases encoded by many RNA viruses are also promising targets.

Newly replicated, concatemeric herpesviral DNA is cleaved by viral enzymes into monomeric units during packaging and assembly. These processes, which are essential for herpesviral reproduction, are carried out by specific viral enzymes, which represent promising targets for antiviral drugs. For example, the compound 5-bromo-5,6-dichloro-1-β-D-ribofuranosyl benzimidazole binds to the human cytomegalovirus UL89 gene product, which is a component of the terminase complex responsible for cleaving and packaging replicated concatemeric DNA. Members of the UL89 gene family are highly conserved among all herpesviruses. This class of compounds may therefore be the basis for discovery of broad-based inhibitors of herpesvirus reproduction. Despite their promise, these compounds have not been developed for clinical use, primarily because the nucleoside analogs like acyclovir are so effective and safe.

Two Success Stories: Human Immunodeficiency and Hepatitis C Viruses

Recognition in the early 1980s that a retrovirus later named human immunodeficiency virus (HIV) was associated with the deadly AIDS disease galvanized the biomedical field. As the extent of the pandemic increased, the urgent need to develop effective therapies, together with pressure from effective political advocacy, led to unprecedented investment of both public and private resources in antiviral drug discovery. As of 2014, more than 20 antiretroviral drugs have been approved, with many more in clinical trials (Table 9.1). Although there are still more than **35 million** people living with HIV, the rate of new infections worldwide is declining as more people gain access to treatment. The impact of new drugs is especially striking in the United States and Europe, where for many patients AIDS has become not a death warrant, but rather a chronic disease.

A second virus of global impact, the (+) strand RNA flavivirus, hepatitis C virus (HCV), (Fig. 9.14), was discovered by tour de force screening of molecular clones from infected blood samples in 1989 (Box 11.6). An estimated **160 million to 185 million** people worldwide are infected with HCV, more than 4 times the number infected with HIV. Although HCV can be cleared in some individuals, the virus establishes a chronic infection in ~80% of those infected, and ~20% of these individuals develop liver cirrhosis within 20 to 30 years, with ~5% succumbing to fatal liver cancer. As with HIV, HCV is spread via exposure to virus-contaminated blood. Although progress in developing antivirals against HCV was impeded initially by the lack of a cell culture system for viral reproduction, substantial progress has been made in the last several years, based on lessons learned from experience with HIV drugs and the application of new technologies (Table 9.2). Drugs that can actually cure most patients entirely are already available, and many more are being developed.

With both HIV and HCV, the essential criteria for large-scale investment by the pharmaceutical industry are clearly satisfied: unmet medical needs; no (or insufficiently effective) existing/approved drugs; and, most assuredly, a market large enough to ensure a profit.

Knowledge of the single cell life cycles of these viruses has suggested many possible steps for antiviral drug intervention

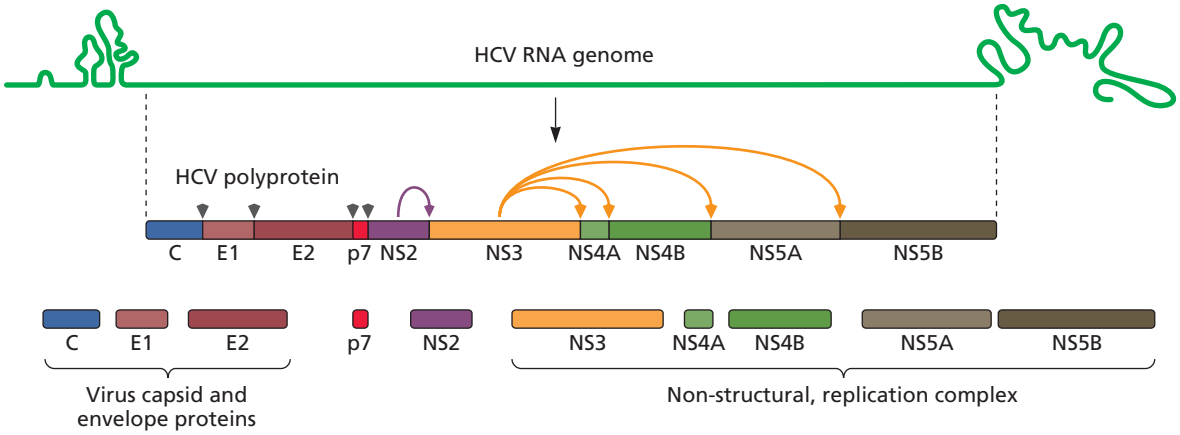


Figure 9.14 The hepatitis C virus polyprotein is cleaved by several proteases. Hepatitis C virus (HCV) is a human flavivirus with a (+) strand RNA genome. The viral proteins are encoded in one large open reading frame that is translated into a polyprotein. The polyprotein is processed by cellular and viral proteases to release the viral proteins. The cleavage sites for the viral proteases are indicated by arrows. The solid arrowheads show the cleavage sites for the host signal peptidase. The viral NS2 metalloprotease is an autoprotease comprising NS2 and the amino-terminal domain of NS3. The viral serine protease comprises the NS3 protein bound to an activator, NS4A. NS5B is the viral RNA polymerase. Viral proteins that are the targets of direct-acting antivirals in advanced clinical development are the protease NS3, the RNA-binding NS5A, and the polymerase NS5B (Fig. 9.15).

Table 9.1 Approved drugs targeted against HIV enzymes

Target	Generic name	Brandname	Manufacturer	Year
Reverse transcriptase	Zidovudine (AZT)	Retrovir	GlaxoSmithKline	1987
Nucleos(t)ide inhibitors	Didanosine (ddI)	Videx	Bristol-Myers Squibb	1991
	Zalcitabine (ddC)	Hivid	Hoffmann-La Roche	1992
	Stavudine (d4T)	Zerit	Bristol-Myers Squibb	1994
	Lamivudine (3TC)	Epivir	GlaxoSmithKline	1995
	Abacavir (ABC)	Ziagen	GlaxoSmithKline	1998
	Tenofovir (TDF)	Viread	Gilead Sciences	2001
	Emtricitabine (FTC)	Emtriva	Bristol-Myers Squibb	2003
Nonnucleoside inhibitors	Nevirapine (NVP)	Viramune	Roxane	1996
	Delavirdine (DLV)	Rescriptor	Pfizer	1997
	Efavirenz (EFV)	Sustiva	DuPont	1998
	Etravirine (ETR)	Intelence	Tibotec	2008
	Rilpivirine	Edurant	Tibotec	2011
Protease	Saquinavir (hard gel)	Invirase	Hoffmann-La Roche	1995
	Ritonavir	Norvir	Abbott	1996
	Indinavir	Crixivan	Merck	1996
	Nelfinavir	Viracept	Agouron	1997
	Amprenavir	Agenerase	GlaxoSmithKline	1999
	Lopinavir/ritonavir	Kaletra	Abbott	2000
	Atazanavir	Revataz	Bristol-Myers Squibb	2003
	Tipranavir	Aptivus	Boehringer Ingelheim	2005
	Darunavir	Prezista	Tibotec	2006
Integrase	Raltegravir	Isentress	Merck	2007
	Elvitegravir	Vitekta	Gilead Sciences	2012
	Dolutegravir	Tivicay	GlaxoSmithKline	2013
Combinations	TDF/FTC/EFV	Atripla	Bristol-Myers Squibb/ Gilead Sciences	2006
	TDF/FTC/rilpivirine	Complera	Gilead Sciences	2011
	TDF/FTC/elvitegravir + cobicistat	Stribild	Gilead Sciences	2012

Table 9.2 Examples of drugs targeted against HCV proteins

Target	Generic name	Brand name	Developer	Date approved/ Trial phase
Polymerase (NS5B)	Sofosbuvir	Sovaldi	Gilead Sciences	2013
Nucleoside	Mericitabine		Roche	II
Nonnucleoside	Deleobuvir		Boehringer Ingelheim	III
	ABT-333		Abbott	III
RNA binding (NS5A)	Ledipasvir		Gilead Scienes	III (filed)
	Daclatasvir		Bristol-Myers Squibb	III
	ABT-267		Abbott	III
Protease (NS3/4A)	Telaprevir	Incivek	Vertex/Johnson & Johnson	2011
	Boceprevir	Victrelis	Merck	2011
	Simeprevir	Olysio	Janssen/Tibotec/Medivir	2013
	Faldaprevir		Boehringer Ingelheim	III
	Vaniprevir		Merck	III
	Samatasvir		Idenix	II
Combinations	Sofosbuvir + ledipasvir		Gilead Sciences	III
	Faldaprevir + deleobuvir		Boehringer Ingelheim	III
	Simeprevir + samatasvir + TMC647055/r		Janssen	II
	ABT-450/r + ABT-267 and ABT-333		Abbott	II
	MK-8742 + MK-5172		Merck	II

(Fig. 9.15). However, while virus fusion and entry have been targeted successfully in the case of HIV, most drugs that are approved or close to approval are directed against the essential viral-encoded enzymes. In the following section, important examples are described individually, but in the clinic the drugs are administered mostly in combinations, as emergence of resistant mutants is a serious problem with both of these RNA viruses.

Inhibitors of Human Immunodeficiency Virus and Hepatitis C Virus Polymerases

Nucleoside and Nucleotide Analogs

Retroviruses are "defined" by their RNA- and DNA-dependent DNA polymerase, reverse transcriptase (Volume I, Chapter 7). The chain-terminating nucleoside AZT was the first inhibitor to be licensed, and was used extensively to treat AIDS before any other antivirals were available (Fig. 9.10). Since then, a number of nucleoside analog inhibitors with better pharmacological properties (such as once-a-day abacavir) have been approved for clinical use (Table 9.1). However, AZT is still used as a single drug (**monotherapy**) for prophylactic treatment of accidental needle sticks and, in some lower-income countries, for treatment of infected pregnant women, as it is relatively inexpensive and can reduce considerably the probability of delivering an HIV-infected baby.

The RNA polymerase of HCV (NS5B) presented several challenges for antiviral drug discovery: many nucleoside analogs initially appeared promising, demonstrating the feasibility of this approach, but it was not until December 2013 that the first inhibitor in this class, sofosbuvir, was approved for treatment of HCV infection, in combination with the standard-of-care regimen of interferon α and/or ribavirin. As with many of the nucleoside inhibitors, sofosbuvir is a prodrug that is converted in the cell to a chain-terminating substrate for HCV polymerase (Fig. 9.16). Treatment for 12 to 24 weeks with the approved regimen results in 60 to 90% cure rates, depending on the HCV genotype.

The success of this new drug generated great optimism but also some controversy, as its high price was likely to exclude access to many infected individuals who could benefit from it (Box 9.6). Agencies of the U.S. government have issued recommendations to test all individuals born between 1945 and 1965 for HCV infection: these "baby boomers" are 5 times more likely than others in the population to be infected because of increased drug use and sexual activity among young people during the 1960s and 1970s. It is estimated that detection and treatment of infected people in this group alone could save more than 120,000 lives.

Nonnucleoside Inhibitors

Nonnucleoside inhibitors of the HIV reverse transcriptase have been identified mainly by high-throughput screening.

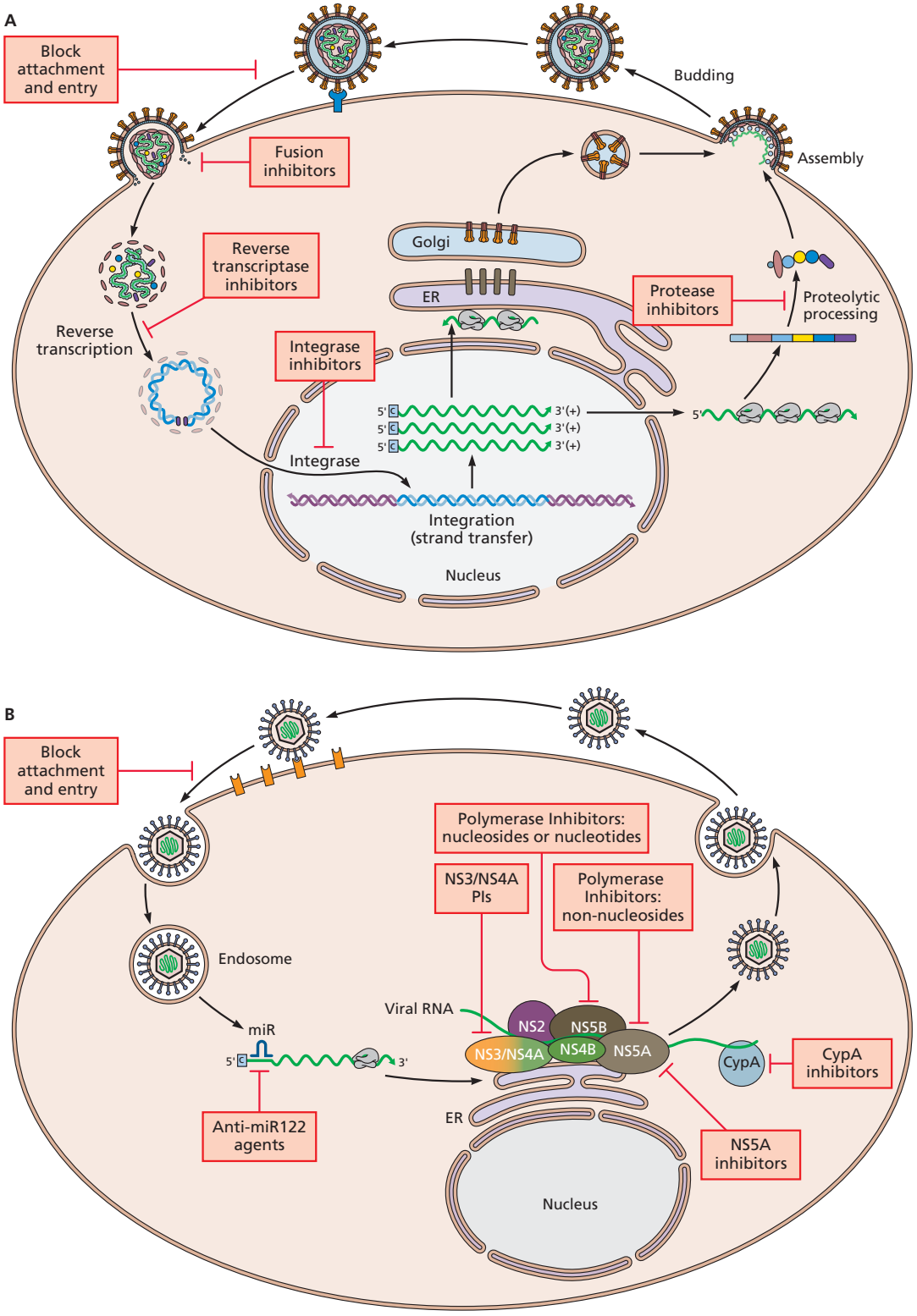


Figure 9.15 Steps in the reproduction of human immunodeficiency (HIV) and hepatitis C (HCV) viruses targeted by antiviral drugs. Steps in the life cycle of the viruses are illustrated, with important targets for antiviral development highlighted. **(A)** HIV; **(B)** HCV. ER, endoplasmic reticulum; PI, protease inhibitor.

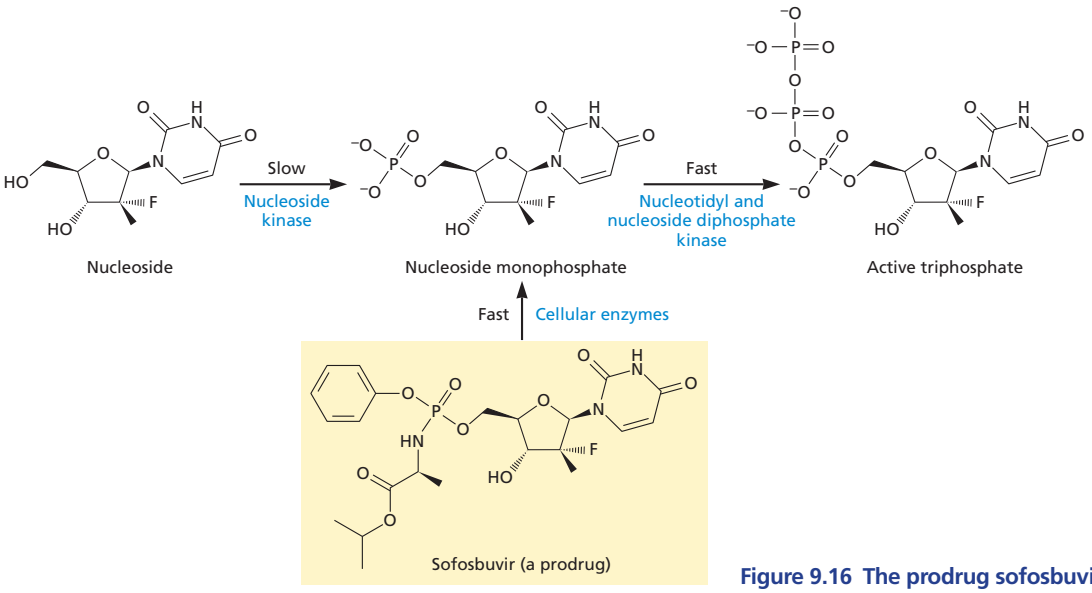


Figure 9.16 The prodrug sofosbuvir: structure and activation.

BOX 9.6

DISCUSSION

What price drugs?

In the United States, the FDA approves new drugs solely on the basis of safety and efficacy. Unlike similar agencies in some other countries (for example, the National Institute for Health and Care Excellence in the United Kingdom), there is no value assessment of the drug or treatment. Consequently, pharmaceutical companies in the United States are free to set their drug prices based mainly on what the market will bear. Nevertheless, even a jaded U.S. public reacted with "sticker shock" when Gilead Sciences announced that its just-approved anti-hepatitis C virus (HCV) drug sofosbuvir (Solvaldi) would be priced at $84,000 (plus the cost of necessary companion drugs) for a required 12-week course, and twice that for a 24-week course that would be needed to cure some patients.

According to a Gilead spokesman, the company considered the price to be fully justified: "We didn't really say, 'We want to charge $1,000 a pill.' . . . We're just looking at what we think was a fair price for the value that we're bringing into the health care system and to the patients."

Some medical specialists might agree, as it could cost up to $300,000 to treat patients with chronic HCV infection using less effective and less tolerable regimens. The potential benefit of a cure for patients with liver disease is clear, as the virus is the main reason that nearly 17,000 Americans are waiting for a liver transplant. The need for a well-tolerated, effective regimen is equally critical for people coinfected with human immunodeficiency virus (HIV) and HCV, because having both infections accelerates liver damage.

On the other hand, the high price will be a significant barrier to treatment access for others who could benefit, particularly those in limited- and fixed-budget programs, such as Medicare and Medicaid. Indeed, a panel of experts in San Francisco estimated that simply replacing current care of HCV-infected Californians with a Sovaldi-based regimen would raise drug expenditures in the state by $18 billion or more in a single year.

Gilead has agreed to help U.S. patients pay for Sovaldi if they can't afford it, or help patients look for drug coverage. In addition, the company will charge substantially less for a course of treatment in places such as India, Pakistan, Egypt, and China, where most people infected with HCV live. With deals announced early in 2014 of $2,000 for a 12-week course in India and $990 for the same in Egypt, U.S. citizens, government, and insurance companies may reasonably ask if they are being forced to subsidize the cost of the drug worldwide.

So what is a fair price for such a lifesaving drug? Gilead paid more than $11 billion in 2011 to acquire the smaller company that developed Sovaldi, and it is reasonable for it to seek to recoup that investment. On the other hand, Andrew Hill, of the Department of Pharmacology and Therapeutics at Liverpool University in the United Kingdom, and his colleagues have reported a conservative estimate of the manufacturing cost of a 12-week course of treatment with this drug to be on the order of $150 to $250 per person. Surely the answer to our question lies somewhere between this huge divide.

There are parallels between Sovaldi (and other new anti-HCV drugs in the pipeline) and the initially very pricey antivirals that were introduced ~20 years ago to treat HIV. In both cases, their use revolutionized the treatment of chronic, lethal infections that are major global health problems. But there are also important differences. Given the total number of people infected, HCV is actually a much larger public health threat than HIV. Furthermore, the new HCV antivirals can eliminate the virus completely, whereas anti-HIV drugs only suppress virus reproduction, so that they must be taken (and paid for) for life.

Callaway E. 2014. Hepatitis C drugs not reaching poor. *Nature* **508:**295–296.

The Editorial Board. March 15, 2014. How much should hepatitis C treatment cost? *New York Times*. http://nyti.ms/1fzwNQF.

Hill A, Khoo S, Fortunak J, Simmons B, Ford N. 2014. Minimum costs for producing hepatitis C direct-acting antivirals for use in large-scale treatment access programs in developing countries. *Clin Infect Dis* **58:**928–936.



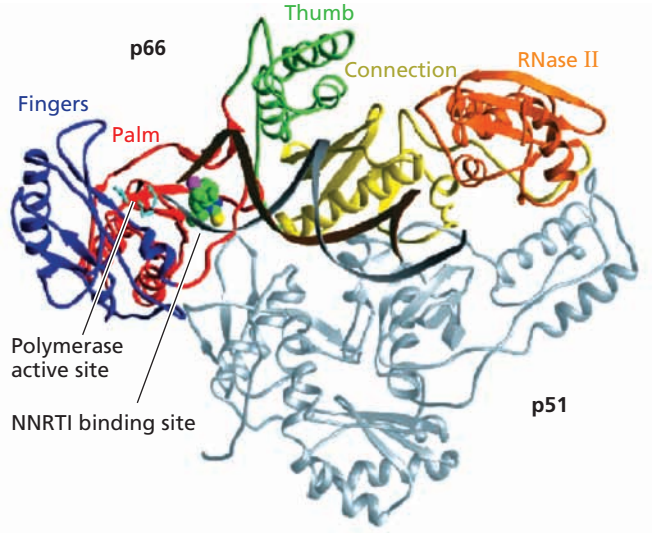


Figure 9.17 Structure of human immunodeficiency virus type 1 reverse transcriptase, highlighting the polymerase active site and the nonnucleoside reverse transcriptase inhibitor (NNRTI) binding site. The structure of the reverse transcriptase p66-p51 heterodimer is shown bound to a double-stranded DNA template-primer. Lines indicate the relative locations of the polymerase active site and the site at the bottom of the palm where NNRTIs are bound. Data from A. Jacobo-Molina et al., *Proc Natl Acad Sci U S A* **90:**6320–6324, 1993.

These compounds do not bind at the nucleotide-binding site of the enzyme, but in a hydrophobic pocket at the base of the palm subdomain (Fig. 9.17). Nevirapine was the first in this class of compounds, which are allosteric inhibitors of the enzyme. Although now used mainly in combination with other HIV antivirals, like AZT, nevirapine has proven to be valuable as a single-drug treatment for pregnant women before infant delivery. Indeed, a short course of the very affordable nevirapine is the preferred method for preventing transmission to newborns in lower-income countries.

Allosteric inhibitors of HCV polymerase have been identified in the same manner. The X-ray crystal structure of this protein has allowed optimization of drug binding to four distinct allosteric sites on the enzyme. Clinical trials of this class of compounds are ongoing, with several showing promise, especially for use in combination therapy.

Human Immunodeficiency Virus and Hepatitis C Virus Protease Inhibitors

HIV protease is encoded in the *pol* gene. During progeny particle maturation, the protease cleaves itself from the Gag-Pol precursor polyprotein and then cuts at seven additional sites in Gag-Pol to yield nine viral proteins, including the two other retroviral enzymes (Volume I, Appendix, Fig. 29). This small (only 99-amino-acid) aspartyl protease, which functions as a dimer, was the first HIV enzyme to be crystallized and studied at the atomic level (Fig. 9.6). The seven cleavage sites in Gag-Pol are similar but not identical. Mechanism-based screens benefited from the early discovery that the enzyme would cut short synthetic peptides that contained these sequences. Parameters of peptide binding and protease activity were determined by screening peptides that contained variations of the seven natural cleavage sites for their ability to be recognized and cut by the enzyme (Fig. 9.18). An additional boost to drug discovery was the similarity of this enzyme to another aspartyl protease, human renin, an enzyme implicated in hypertension. Indeed, the first inhibitor leads were peptide mimics (peptidomimetics) modeled after inhibitors of renin, and were developed into drugs such as saquinavir. Subsequent screens for mechanism-based and structure-based inhibitors designed *de novo* have yielded several powerful inhibitors of the protease and second-generation drugs, such as darunavir, for which many more viral mutations are needed to develop resistance.

The second HIV protease inhibitor to be approved, ritonavir, had an unexpected off-target effect that had important impact on the field: this compound was found to be an irreversible inhibitor of a detoxifying cytochrome P450 enzyme at only one-sixth the therapeutic dose for protease inhibition. Amazingly, this activity was found to improve the pharmacokinetic properties of other HIV protease inhibitors significantly. Ritonavir, or a derivative, is therefore included in many combination regimens at this lower dose as a "booster."

The HCV NS3/4A protein is a serine protease. Two HCV protease inhibitors, boceprevir and telaprevir (Fig. 9.19) were the first antivirals to be approved for treatment of chronic HCV infections. Although they are highly active against only one genotype, some efficacy against two others of the six known genotypes of HCV has been reported. Rapid development of resistance, which requires a substitution at only one site in the proteins, is a distinct disadvantage of these drugs. Two more recently developed HCV protease inhibitors, simeprevir and faldaprevir, have activity against several HCV genotypes and impose a higher genetic barrier for development of resistant mutants.

Human Immunodeficiency Virus Integrase Inhibitors

The HIV integrase is an excellent drug target, because it is a unique recombinase for which biochemical data and mechanism-based assays are available, thanks to earlier studies with avian retroviruses. Nevertheless, drug development lagged behind that targeted against the HIV reverse transcriptase and protease because of the difficulty in obtaining a crystal structure for an active tetramer of integrase, alone or bound to its DNA substrates. Despite this limitation,

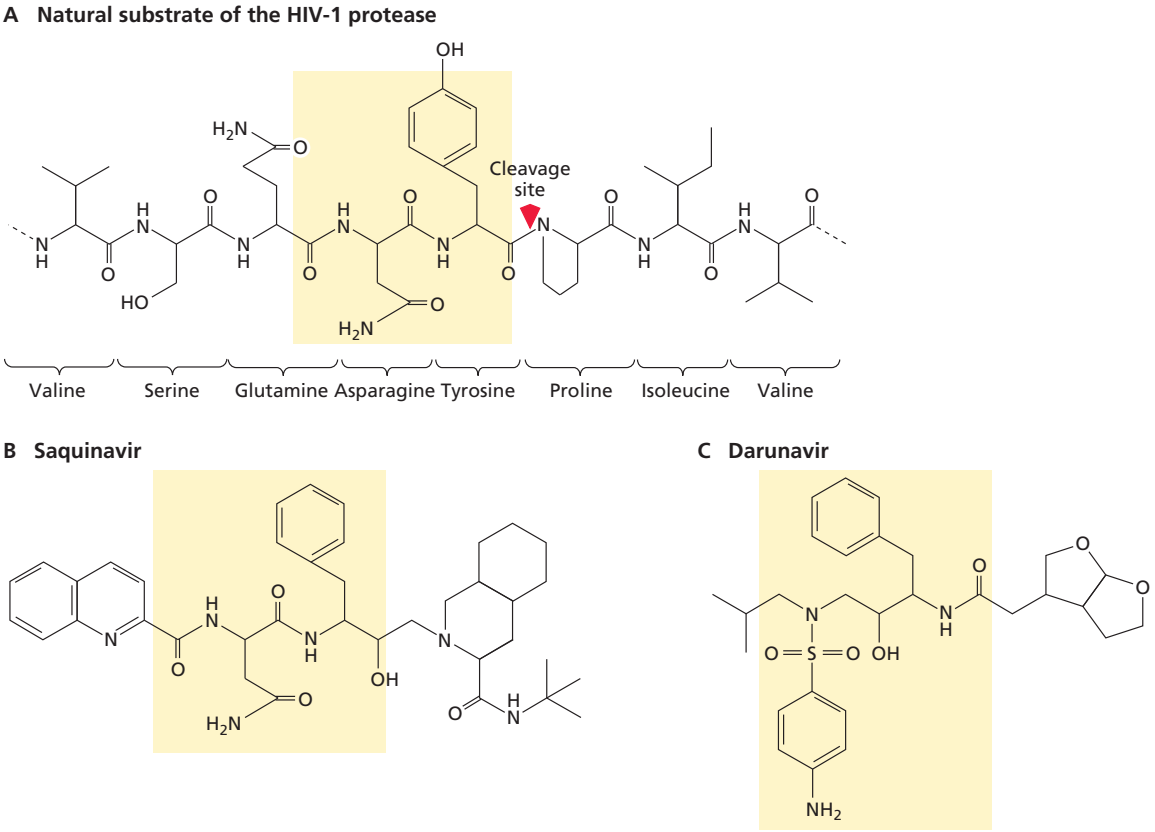
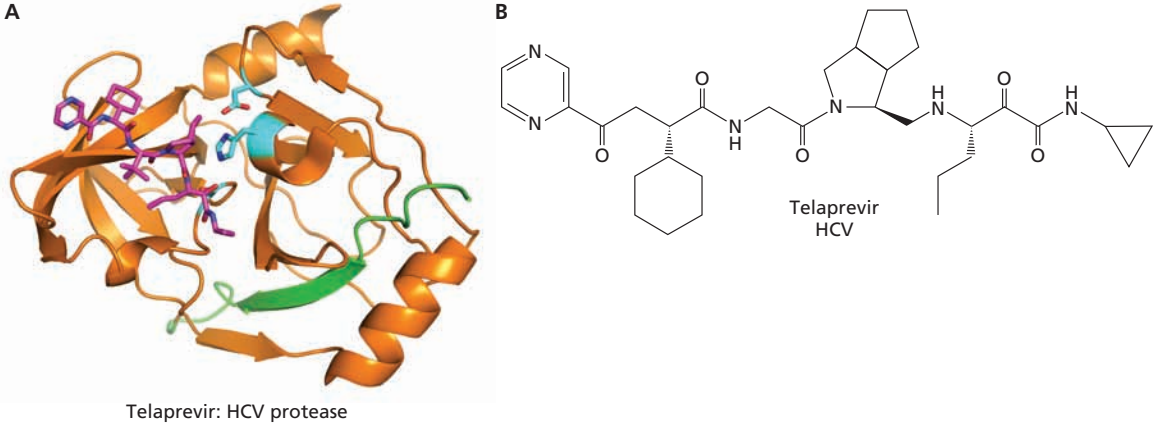


Figure 9.18 Comparison of one natural cleavage site for the human immunodeficiency virus type 1 protease with a peptidomimetic inhibitor and a second-generation compound. (A) The chemical structure of eight amino acids comprising one of the cleavage sites in the Gag-Pol polyprotein. The cleavage site between tyrosine and proline is indicated by a red arrow. **(B)** The chemical structure of an inhibitory peptide mimic, saquinavir. **(C)** A second-generation protease inhibitor, darunavir, for which more viral mutations are needed to acquire resistance. Regions of similarity are highlighted in yellow. Adapted from D. R. Harper, *Molecular Virology* (Bios Scientific Publishers, Ltd., Oxford, United Kingdom, 1994), with permission.

Figure 9.19 Structure of the hepatitis C virus protease NS3/4A and with a bound inhibitor (A) Crystal structure of Telaprevir bound to NS3-4A protease crystal structure (PDB code: 3SV6). The NS3 protease domain is shown in a gold-colored ribbon depiction. The active site triad residues Ser139, His57 and Asp81 are highlighted as sticks (with carbons colored cyan, oxygen in red, nitrogen in blue). The NS4A cofactor peptide segment is shown as in green ribbon. Telaprevir is shown as a stick diagram (with carbons colored magenta, oxygen in red, nitrogen in blue). Figure courtesy of Dr. G.R. Bhisetti, Biogen, Cambridge MA **(B)** Chemical structure of the FDA-approved protease inhibitor, Telaprivir.



successful application of a high-throughput assay that is specific for the second, joining step in the reaction (Volume I, Fig. 7.15 and Box 7.7) eventually led to the development of the first HIV integrase inhibitor, raltegravir, which was approved in 2007. Additional inhibitors of the same step in the reaction, elvitegravir and dolutegravir, were approved in 2012 and 2013, respectively. Solution of the crystal structure of the integrase of prototype foamy virus, with bound substrates and inhibitors, in 2010 provided the first clear picture of the mechanism of inhibition by these compounds. These drugs, called integrase strand transfer inhibitors, stabilize a viral DNA-protein intermediate while coordinating two catalytic magnesium ions bound to the three catalytic amino acids in the active site (Fig. 9.20). Allosteric inhibitors of HIV integrase, which bind at a specific dimer interface of the enzyme, are also being developed (Box 9.2).

A long-lasting analog of dolutegravir, which is suitable for monthly or quarterly clinical administration, has been shown to protect macaques from infection. These animal studies have suggested a promising approach to preexposure prophylaxis for humans at high risk for infection, such as partners of infected individuals.

Hepatitis C Virus Multifunctional Protein NS5A

The NS5A protein is an RNA-binding, phosphorylated protein that localizes to endoplasmic reticulum-derived membranes and functions in numerous steps of the virus life cycle, including genome replication and particle assembly. The mechanism by which the protein affects these processes, and causes hepatocyte apoptosis and carcinogenesis, is still unclear. Nevertheless, treatment with NS5A inhibitors, which are effective against all HCV genotypes, results in a rapid

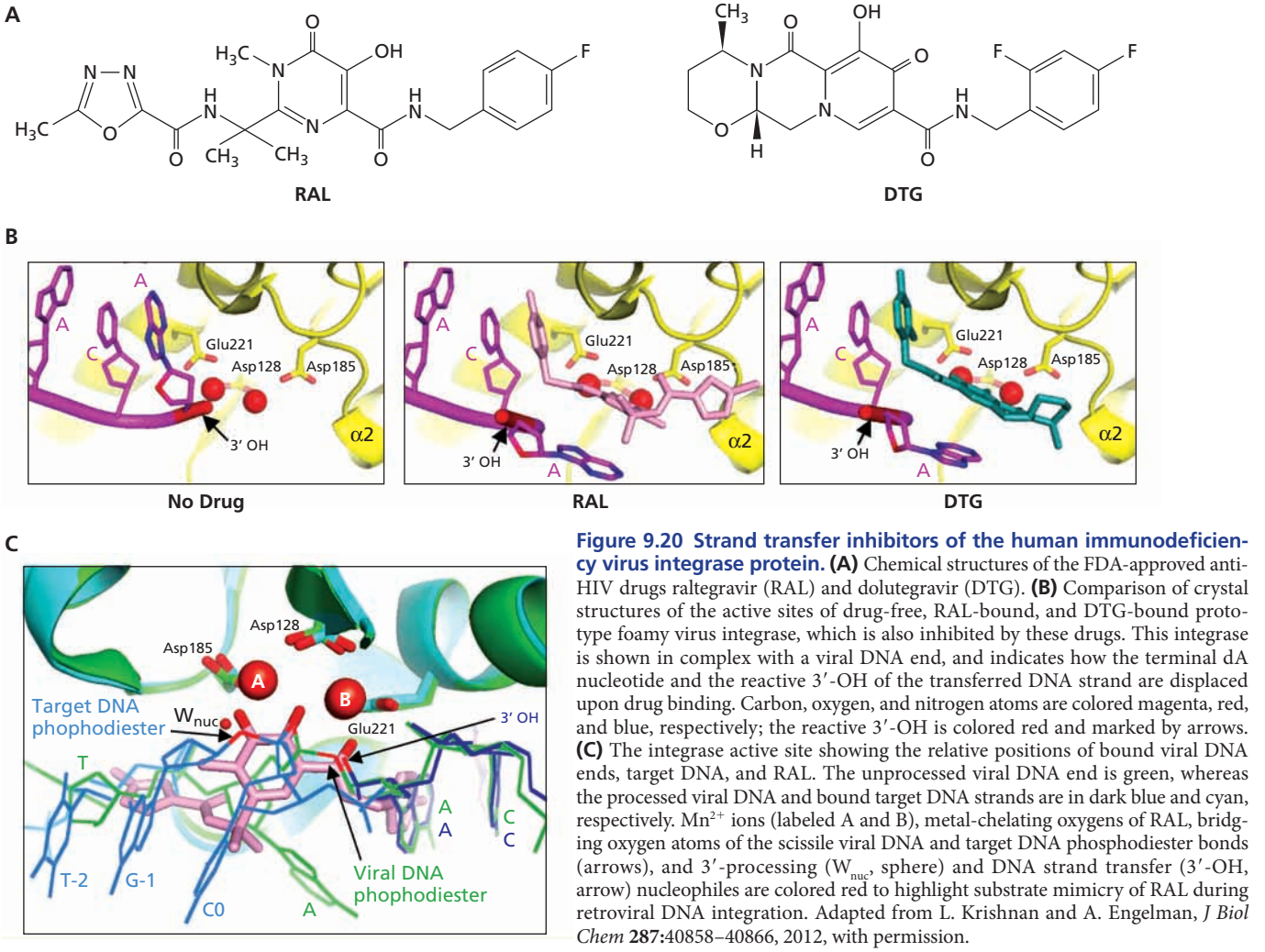


Figure 9.20 Strand transfer inhibitors of the human immunodeficiency virus integrase protein. (A) Chemical structures of the FDA-approved anti-HIV drugs raltegravir (RAL) and dolutegravir (DTG). **(B)** Comparison of crystal structures of the active sites of drug-free, RAL-bound, and DTG-bound prototype foamy virus integrase, which is also inhibited by these drugs. This integrase is shown in complex with a viral DNA end, and indicates how the terminal dA nucleotide and the reactive 3′-OH of the transferred DNA strand are displaced upon drug binding. Carbon, oxygen, and nitrogen atoms are colored magenta, red, and blue, respectively; the reactive 3′-OH is colored red and marked by arrows. **(C)** The integrase active site showing the relative positions of bound viral DNA ends, target DNA, and RAL. The unprocessed viral DNA end is green, whereas the processed viral DNA and bound target DNA strands are in dark blue and cyan, respectively. Mn^{2+} ions (labeled A and B), metal-chelating oxygens of RAL, bridging oxygen atoms of the scissile viral DNA and target DNA phosphodiester bonds (arrows), and 3′-processing (W_{nuc}, sphere) and DNA strand transfer (3′-OH, arrow) nucleophiles are colored red to highlight substrate mimicry of RAL during retroviral DNA integration. Adapted from L. Krishnan and A. Engelman, *J Biol Chem* **287:**40858–40866, 2012, with permission.

decline in viral load. Other favorable properties include once-daily, low-dose efficacy and resistance profiles that do not overlap with other HCV antivirals.

Inhibitors of Human Immunodeficiency Virus Fusion and Entry

Some neutralizing antibodies block viral attachment of the viral envelope protein (Env) to the CD4 receptor on host cells by binding to the third variable domain (the so-called V3 loop) of the SU component of Env. A variety of natural and synthetic molecules also interfere with V3 loop-dependent host cell attachment. These compounds include specific antibodies (Volume II, Fig. 7.19) and polysulfated or polyanionic compounds such as dextran sulfate and suramin. Identified early in the search for antiviral agents, these compounds were subsequently discarded as antivirals because of intolerable side effects such as anticoagulant activity. Although considerable effort was expended to develop other inhibitors of the SU-CD4 interaction, including production of a "soluble CD4" that would act as a competitive inhibitor of infection, no effective antiviral agents have been found using this strategy. This lack of success can be attributed, in part, to the high concentration of SU on the virus particle, as well as to the existence of alternative mechanisms for spread in an infected individual.

As often happens, the early research with failed Env protein inhibitors provided much insight into how virus particles enter cells, and has focused attention on other targets in the process. For example, it was curious that mutants resistant to neutralizing antibodies have clustered substitutions in the V3 loop, yet virus-cell fusion is not affected. The implication was that CD4-V3 interactions did not contribute to entry. As described in Volume I, Chapter 5, entry is a multistep process requiring that the target cells synthesize not only CD4, but also any one of several chemokine receptors, such as CCr5 or CXCr4. Chemokine receptors are attractive targets, because individuals homozygous for mutations in one such receptor (CCr5) are partially resistant to infection. The first drug targeting an HIV chemokine receptor, maraviroc, was approved in 2007. The drug is an allosteric modulator of CCr5 function and blocks binding of HIV SU. As HIV can use other coreceptors for entry, the tropism of virus in an individual patient must first be determined to decide if treatment can be effective.

We now know that the interaction of the SU V3 loop with the chemokine receptor exposes previously buried SU sequences that are required for membrane fusion and that these transiently exposed surfaces can be targeted by antiviral agents. A 36-amino-acid synthetic peptide, termed T20, derived from the second heptad repeat of SU, binds to the exposed grooves on the surface of a transient triple-stranded coiled-coil and perturbs the transition of SU into the conformation active for fusion (Volume I, Chapter 5). T20 (enfuvirtide), the first drug with this mode of action, was approved in 2003. It is difficult to develop a peptide as a drug: large-scale synthesis is expensive, and patients must actually prepare a peptide solution for injection. Nevertheless, enfuvirtide is remarkably effective in reducing HIV titers in the blood.

Drug Resistance

Potent drugs are now available to inhibit the reproduction of several human viruses, including herpesviruses and hepatitis B virus, as well as HIV and HCV. However, because viral reproduction is so efficient and is accompanied by moderate to high mutation frequencies, resistance to any antiviral drug must be anticipated. The emergence of drug-resistant mutants is of special concern during the extended therapy required for viruses that establish chronic infections.

Mutations appear only when the viral genome is replicated. If replication is blocked completely by an inhibitor, no new drug-resistant mutants can arise. Consequently, if an individual harboring a small number of viral genomes with no relevant preexisting mutations is given a sufficient concentration of drug to block all viral replication, the infection will be held in check. When the viral genome numbers are small, the infection may be cleared by the host's immune system before resistant mutants take over. If the drug concentration is insufficient to block virus reproduction entirely or if the same antiviral drug is given after the viral population has expanded, genomes that harbor mutations will survive and will continue to replicate and evolve (Fig. 9.21).

If resistance to an antiviral drug requires multiple mutations, the chance that all mutations preexist in a single genome is much lower than if only a single mutation is required. Minimizing the differences between the natural ligand of the target protein and the antiviral drug will decrease the probability of emergence of mutants that are able to distinguish between ligand and drug. But when replication is allowed in the presence of the inhibitor, resistant mutants will accumulate. If there are no alternative antivirals, drug-resistant mutants can be devastating for patients and the population at large.

Although the development of drug resistance is a discouraging certainty with all direct-acting antivirals, genetic and biochemical analyses of the phenomenon can provide powerful insight into drug mechanisms and may identify new strategies to reduce or circumvent resistance. For example, acyclovir-resistant mutants of herpes simplex virus arise spontaneously during viral genome replication and are selected after exposure to the drug. As might be expected, the majority of mutations that confer resistance are in the viral thymidine kinase gene and inactivate kinase function. However, a subset of mutations leading to acyclovir resistance are not in

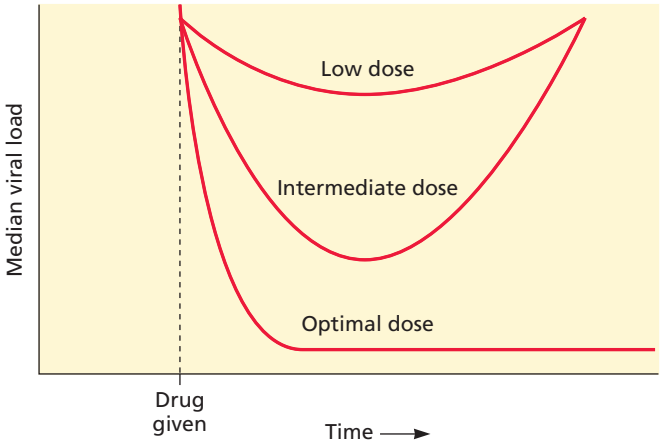


Figure 9.21 Viral load depends on the dose of antiviral drug. This relationship is illustrated by plotting median virus load in relative units on the *y* axis as a function of time after exposure to a drug on the *x* axis as indicated (Drug given). In the top curve (Low dose), the concentration of antiviral drug is insufficient to block virus reproduction, and the viral load is reduced only transiently, if at all. Virus mutants that are resistant to the drug may be enriched in this population following such treatment. In the middle curve (Intermediate dose), the concentration of antiviral drug is successful in lowering the viral load initially, indicating that some reproduction was blocked. In this example, the block was incomplete, and resistant mutants that can arise even during limited virus reproduction will eventually overwhelm the patient. In the bottom curve (Optimal dose), the concentration of the antiviral drug is high enough to block viral reproduction completely. As no new progeny viruses can be produced, the viral load drops dramatically, and the low level is maintained. Redrawn from J. H. Condra and E. A. Emini, *Sci Med* **4:**14–23, 1997, with permission.

this gene but, rather, in the viral DNA polymerase gene. The altered polymerases possess reduced ability to incorporate phosphorylated drug into DNA. Similar patterns of resistance have been reported when other nucleoside analog inhibitors are used against varicella-zoster virus.

Production of viral variants, drug-resistant mutants among them, is a hallmark of HIV infection, in which the initial, acute phase is followed by an asymptomatic period of clinical latency that lasts for years to decades (see Chapters 5 and 7). Although symptoms are kept in check by the patient's immune system, extensive viral reproduction and evolution continues throughout the asymptomatic period, until immune defenses finally break down and AIDS symptoms are manifested. Viral mutants that reproduced in the presence of AZT appeared almost immediately after the drug was approved for the treatment of AIDS. The genomes of the mutants were found to harbor single-base-pair changes at one of at least four sites in the reverse transcriptase gene. Reverse transcriptase enzymes bearing these substitutions no longer bound phosphorylated AZT, but they retained enzymatic activity. Mutants resistant to other nucleoside analogs, as well as to protease inhibitors, also arose with disheartening frequency when these drugs were administered as monotherapy. These drug-resistant mutants were transmitted to new hosts and threatened to undermine the entire antiviral effort.

Combination Therapy

Combining two or more drugs with distinct targets circumvents the appearance of cells resistant to one treatment or the other. In theory, if resistance to one drug occurs once in every 10^3 genomes, and resistance to a second occurs once in every 10^4, then the likelihood that a genome carrying both mutations will arise is the product of the two probabilities, or one in every 10^7.

Mutants resistant to different nucleoside analogs were often found to carry different amino acid substitutions in the HIV reverse transcriptase. Furthermore, in some cases a mutation conferring resistance to one inhibitor suppressed resistance to another (Table 9.3). Consequently, combinations of nucleoside analogs were tested with the expectation that double-resistance mutants would be rare, perhaps nonviable, or at least severely crippled. While initially promising, many such combinations failed, with mutants resistant to both drugs appearing after less than a year of therapy. The frequency of resistance to many pairwise combinations of nucleoside and nonnucleoside inhibitors was lower than that for any single drug, but not low enough. Experience with protease substrate analog inhibitors was similar; resistance to two inhibitors emerged almost as quickly as resistance to either one alone. As current protease inhibitors are all peptide mimics that bind to the substrate pocket of the enzyme, a change in residues lining this pocket can affect the binding of more than one inhibitor. It became clear from these experiences that treatment of a patient with one antiviral drug, or in some cases even two, at a time is of limited clinical value. Consequently, inclusion of three or more antivirals has become standard practice in treating HIV infection.

Combination therapy can be demanding for physician and patient. For example, if other infections are being treated, as they almost always are in AIDS patients, then many pills a day may be required. Other problems arise because storing and keeping track of different medications are daunting tasks for someone who is ill. To compound the problems, every drug has side effects, and some are severe. For example, the gastrointestinal problems that accompany many protease inhibitors are particularly stressful. Some side effects, such as changes in fat distribution, may appear only after months of continuous use of current antiprotease drugs (Box 9.7). Because of these problems, some patients simply do not take their medication. The most insidious failure lies in wait when the patient begins to feel better and stops taking the medication. Viral replication resumes when the inhibitors are removed.

Table 9.3 Unpredicted drug resistance and susceptibility patterns

Compound	Substitution conferring resistance	Drug sensitivity phenotypes (amino acid substitution)
Zidovudine	T215F in reverse transcriptase	Didanosine resistance (L74V) restores zidovudine susceptibility
		Lamivudine resistance (M184V) restores zidovudine susceptibility
		Nevirapine and loviride resistance (Y181C) restores zidovudine susceptibility
		Foscarnet resistance (W88G) restores zidovudine susceptibility
Amprenavir	M46I + I47V + I50V in protease	Saquinavir resistance (G48V + I50V + I84L) restores amprenavir susceptibility
	VX-479 in protease	Indinavir resistance (V32I, A71V) restores amprenavir resistance
Delavirdine	P236L in reverse transcriptase	Increased susceptibility to nevirapine; R82913 (TIBO derivative); and L-697,661 (pyridinone)
Foscarnet	E89K + L92I + S56A + Q161L, H208Y in reverse transcriptase	Increased susceptibility to zidovudine, nevirapine, and R82150 (TIBO)

BOX 9.7

EXPERIMENTS

Highly specific, designed inhibitors may have unpredicted activities

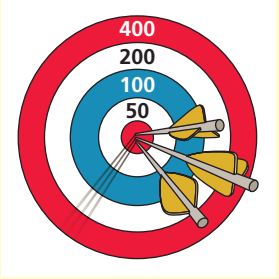
The discovery and development of structure-based inhibitors of HIV protease have been pronounced a triumph of rational drug design. Structural biology and molecular virology came together to provide the protease inhibitors that anchor today's highly active antiretroviral therapy. However, patients receiving some protease inhibitors responded in unexpected ways. For example, one study showed that the protease inhibitor ritonavir inhibits the chymotrypsin-like activity of the proteasome. As a result, the drug blocks the formation and subsequent presentation of peptides to cytotoxic T lymphocytes (CTLs) by major histocompatibility complex class I proteins. In another study, the saquinavir protease inhibitor was found to inhibit Zmpste24, a protease required for conversion of farnesyl-prelamin A to lamin A, a structural component of the nuclear lamina.

The challenge is to determine if such secondary activities help or hinder AIDS therapy. As discussed in Chapter 4, CTLs not only kill virus-infected cells, but also are responsible for significant immunopathology in persistent infections. Perhaps a drug like ritonavir can block such immunopathology. On the other hand, reduction in immunosurveillance by CTLs potentiates persistent infections. In this case, the secondary activity of such a drug may presage long-term problems. We now know that the HIV protease inhibitors ritonavir and saquinavir interfere with proteasome activity, while indinavir and nelfinavir do not. The inhibition of Zmpste24 by the saquinavir class of compounds may contribute to the observed debilitating partial lipodystrophy side effect (redistribution of adipose tissue from the face, arms, and legs to the trunk). Genetic data indicate that individuals with missense mutations in *LmnA*, the gene encoding prelamin A and lamin C, have a significant loss of adipose tissues.

These experiences show that it is important to monitor lymphocyte functions and accumulation of prelamin A in patients under treatment with different protease inhibitors. Furthermore, tailoring HIV protease inhibitors to limit their action to the intended target is an important goal. As noted by the investigators who found these surprising activities, the human genome carries ~400 genes encoding proteases. About 70 of these proteases are targets for new drugs, and the unexpected side effects of antiviral protease inhibitors may be useful in finding new therapies.

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Genome replication means mutation, and in such cases combination therapy may be ineffective if ever reinstated.

Because of these impediments to treatment compliance, there have been major efforts to develop drug combinations that can be taken less frequently and even together in a single pill for both HIV and HCV infections (Tables 9.1 and 9.2). A fixed-dose combination of two nucleos(t)ide inhibitors and a nonnucleoside inhibitor of HIV reverse transcriptase in a single pill (Atripla) that need be taken only once a day was approved in 2006. Development of this combination represented the first collaboration between two U.S. pharmaceutical companies to combine their patented anti-HIV drugs into one product. Atripla was followed in 2011 by Complera, comprising a similar cocktail, but with fewer side effects. Stribild, approved in 2012, contains four HIV inhibitors and is known as the "quad" pill. Stribild includes an integrase inhibitor, the two nucleos(t)ide analogs included in Atripla, and cobicistat. Cobicistat is a derivative of ritonavir that has no antiprotease activity but, like ritonavir, inhibits cytochrome P450, thereby increasing the effectiveness of the other three compounds. The clinical success of ever-improving combination therapy is truly remarkable and represents one of the high points in the battle against HIV. Similarly, once-a-day combinations are expected soon for the treatment of HCV.

Challenges Remaining

Infections with some viruses, such as HCV, can be cured. With aggressive use of potent antiviral drug combinations, reproduction of others, like hepatitis B virus and HIV, can be suppressed, but the infection **cannot** be cured. Even when HIV RNA has been undetectable in the bloodstream for years during drug therapy, virus appears again as soon as drug treatment is suspended. There have been isolated reports of cures, such as in one individual who received a bone marrow transplant from a donor carrying a mutation in the CCr5 cytokine receptor. However, there is no practical way at present to eliminate every last viral genome from the body of an HIV-infected individual. The current challenge is to devise workable strategies to rid an individual of all cells that contain proviruses or to remove established proviruses from all cells from which they may be activated. Some ideas that are being tested include activation of latent HIV proviruses with epigenetic drugs, followed by antiviral drug treatment, called "shock and kill." Cytokine therapy has yielded some promising results in inducing degradation of hepatitis B virus DNA in chronically infected hepatocytes.

Perspectives

The world's surprisingly small arsenal of antiviral drugs is directed against a subset of viral diseases. Few drugs are available for some of the most deadly established or emerging viral diseases, many of which are caused by RNA viruses. One formidable problem for delivery of antiviral drug therapy, even if available, is that many acute viral infections cannot be diagnosed accurately within sufficient time for effective intervention. Another arises from the fact that many debilitating viral infections affect people in the developing world, a population that lacks the means and possesses limited infrastructure for the delivery of antiviral drugs.

Persistent infections such as those caused by the human immunodeficiency virus, herpes simplex virus, and the hepatitis B virus present a special set of challenges. At present, these infections are controlled by drugs, but not cured. Often patients must take the drug, or more likely a combination of drugs, for the rest of their lives, a prospect that is both difficult and expensive. New approaches have been undertaken, and many promising lead compounds and therapies for treatment, and even cure, of persistent infections are being investigated. For example, in the future it may be possible to reduce viral load by antiviral drugs and then promote clearance of the remaining infection by treating with drugs that bolster immune responses. In most cases, however, selection of resistant mutants remains a problem for antiviral research and public health.

Despite the problems that remain, the successes in clinical development and distribution of increasingly effective antiviral drugs and drug combinations that target the human immunodeficiency virus and hepatitis C virus can be considered nothing less than a triumph, considering the millions of people whose lives have been saved not only in the United States and other high-income countries but also worldwide. A World Health Organization update in July 2013 reported an exponential increase in the number of people receiving anti-HIV therapy in lower- and middle-income countries since 2003 (Fig. 9.22). As a result, 4.2 million adult lives were saved and 800,000 infections of children were averted in these countries from 1996 to 2012. In addition, there were more than 700,000 fewer new HIV infections globally in 2011 than in 2001. Furthermore, prophylactic treatment against HIV infection for individuals at risk can be effective, and strategies for cures are being tested. Although much more needs to be done to end the AIDS pandemic, these results are most welcome news to the many millions worldwide who are infected with hepatitis C virus. In their cases, access to the new and expected antiviral therapies can promise a cure and elimination of the threat of fatal liver disease.

Based on experience gained from the successes with human immunodeficiency virus and hepatitis C virus, significant advances in technology, and increased understanding of virus biology and virus-host cell interactions, progress in developing drugs that block or even cure other viral infections should be more rapid in the future.

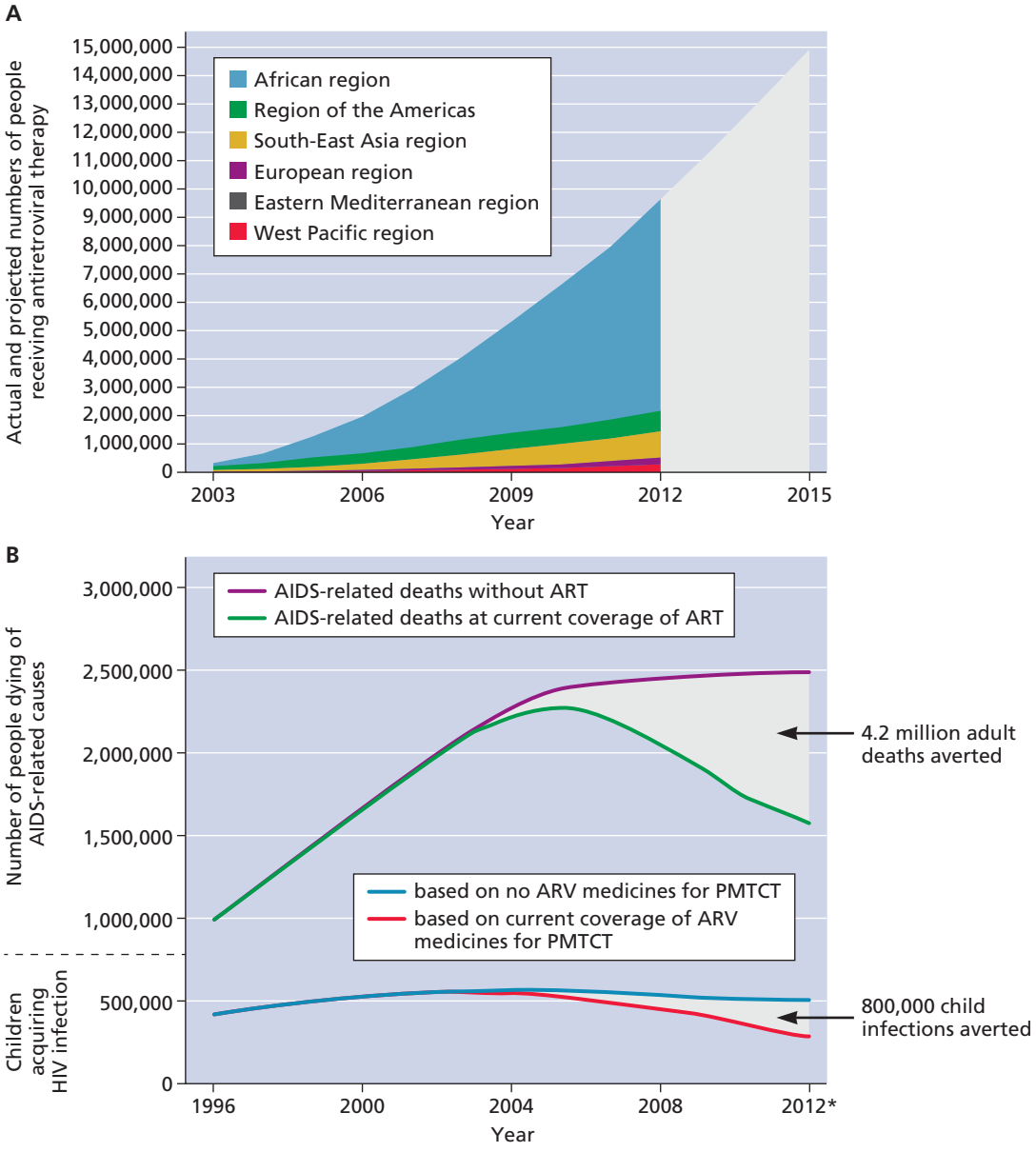


Figure 9.22 Anti-human immunodeficiency virus (HIV) therapy saves millions of lives. (A) Actual and projected numbers of people receiving antiretroviral therapy in lower- and middle-income countries. **(B)** Actual number of people dying from AIDS-related causes (top curves), and children acquiring HIV infection (bottom curves), in lower- and middle-income countries globally compared with a scenario of no antiretroviral therapy. ART, antiretroviral therapy; PMTCT, prevention of mother-to-child transmission; ARV, antiretroviral medication for prophylaxis in the third trimester of pregnancy (a regimen of twice-daily AZT, single-dose nevirapine at onset of labor, a combination of AZT + 3TC during delivery and 1 week postpartum, as well as infant prophylaxis for 1 week after birth). Adapted from World Health Organization, *Global Update on HIV Treatment 2013: Results, Impact and Opportunities* (World Health Organization, Geneva, Switzerland, 2013), with permission.

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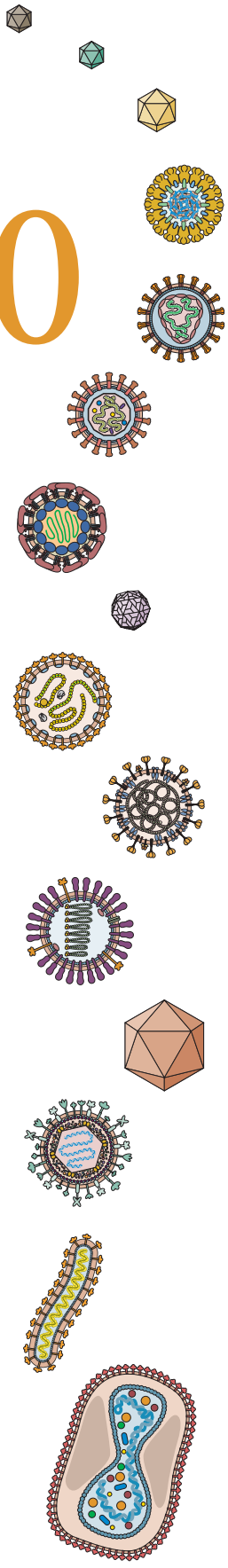
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10 Evolution

Virus Evolution

Classic Theory of Host-Parasite Interactions

How Do Virus Populations Evolve?

- Two General Survival Strategies Can Be Distinguished
- Large Numbers of Viral Progeny and Mutants Are Produced in Infected Cells
- The Quasispecies Concept
- Sequence Conservation in Changing Genomes
- Genetic Shift and Genetic Drift
- Fundamental Properties of Viruses That Constrain and Drive Evolution

The Origin of Viruses

Host-Virus Relationships Drive Evolution

- DNA Virus Relationships
- RNA Virus Relationships
- The Protovirus Hypothesis for Retroviruses

Lessons from Paleovirology

- Endogenous Retroviruses
- DNA Fossils Derived from Other RNA Viral Genomes
- Endogenous Sequences from DNA Viruses
- The Host-Virus "Arms Race"

Perspectives

References

LINKS FOR CHAPTER 10

- ⏭ *Video: Interview with Dr. Harmit Malik*
  http://bit.ly/Virology_Malik
- ⏭ *I want my MMTV*
  http://bit.ly/Virology_Twiv242
- ⏭ *Paleovirology with Michael Emerman*
  http://bit.ly/Virology_Twiv237
- ⏭ *Describing a viral quasispecies*
  http://bit.ly/Virology_4-16-15
- ⏭ *Viral genomes in 700 year old caribou scat*
  http://bit.ly/Virology_12-8-14
- ⏭ *A WORD on the constraints of influenza virus evolution*
  http://bit.ly/Virology_5-23-14

Anything produced by evolution is bound to be a bit of a mess.
SYDNEY BRENNER

We are not separate entities so much as interdependent, sharing our very cells (and DNA) with separate creatures. . .
LEWIS THOMAS
Lives of the Cell, 1974

Virus Evolution

The word "evolution" conjures up images of fossils, dusty rocks, and ancestral phylogenetic trees, covering eons. Thanks to the recent development of rapid sequencing methods, we can now discover fossils of ancient viruses, not in rocks, but in the DNA of living organisms. For currently circulating viruses, evolution is not only contemporary (and rapid), but also has profound effects on both viruses and their hosts: as host populations change or become resistant to infection, viruses that can overcome such changes are selected. Viral infections can also exert significant selective forces on the survival and evolution of host populations. In some ways, viral evolution can be thought of as the product of a continuing arms race in which both viral and host cell genes are selected in response to the pressures encountered during infection.

Classic Theory of Host-Parasite Interactions

Virus particles must spread from host to host to maintain a viable population. Spreading will occur if, on average, each infected host passes the agent to more than one new host before the host dies or clears the infection. The probability of such transmission is related to the size of the host population: infections can spread only if population density exceeds a minimal value.

These concepts have been incorporated into a comprehensive theory of host-parasite interactions that is well known in ecological circles, but not always appreciated among molecular virologists. This theory describes the parameters of viral infection in quantitative terms. The basic reproductive number for a virus population, $\boldsymbol{R_0}$ (pronounced **R-naught**), is defined as the number of secondary infections that can arise in a large population of susceptible hosts from a single infected individual during its life span. If $R_0 < 1$, it is impossible to sustain an epidemic; in fact, it may be possible to eradicate the disease. If $R_0 > 1$, an epidemic is possible, but random fluctuations in the number of transmissions in the early stages of infection in a susceptible population can lead to either extinction or explosion of the infection. If R_0 is much greater than 1 (Table 10.1), an epidemic is almost certain. The proportion of the susceptible population that must be vaccinated to prevent virus spread is calculated as $1 - 1/R_0$.

In the simplest model, $R_0 = \tau \cdot c \cdot d$, where τ is the probability of infection, given contact between an infected and uninfected host; c is the average rate of contact between them; and d is the duration of infectivity. The original host-parasite theory assumed well-mixed, homogeneous host populations in which each individual host has the same probability of becoming infected. Although the general concepts remain valid, additional parameters and constraints have been added to the mathematical models as more has been learned about population diversity and the dynamics of viral infections (Box 10.1; see also Fig. 5.2). For example, immune-resistant viral mutants with differences in virulence

PRINCIPLES *Evolution*

- Virus evolution is the product of continuing interaction between viral and host cell genes and selection for the most fit.
- Diversity of a virus population allows adaptation to environmental changes; remove diversity, and the population suffers.
- Viral diversity is generated by mutation, recombination, and reassortment of viral genes.
- Virus populations can be sustained by production of many progeny, better competition for resources, or both.
- Virus populations exist as dynamic distributions of nonidentical but related and interactive replicons, called **quasispecies**.
- Genomes of RNA viruses are replicated close to the error thresholds, i.e., the number of mutations within populations at which viruses can no longer be propagated. Such replication contrasts with that of DNA viruses, which generally proceeds with higher fidelity, and well below the error threshold.
- Mutations accumulate at every viral replication cycle. Virus populations cannot survive unless genomes that are free from harmful mutations and conserve beneficial mutations can be produced by reassortment or recombination. This principle is illustrated by **Muller's ratchet**.
- The origins of viruses remain puzzling, and three non-mutually-exclusive hypotheses have been proposed: reduction by loss of genes from a cell, accumulation of **cellular** components that gained independent reproduction capacity, and **independent** coevolution with cells from the origin of life.
- Although the primordial history of viruses cannot be known, evidence of virus-derived sequences in host genomes provides important insights into the nature and consequences of viral and host interaction over evolutionary time.
- The discovery of viruses with extremely large genomes that include coding capacities equal to or surpassing those of some prokaryotes, and the diversity of viral genomes revealed by metagenomic analyses, indicate that there is much more to learn about virus history and evolution.

Table 10.1 Reproductive numbers for some viruses

Virus	R_0[a]
Measles	12–18
Smallpox	5–7
Polio	5–7
Influenza	
2009 (H1N1)	1.47
1957, 1968 pandemics	1.8
1918 pandemic	2.4–5.4
Hemorrhagic fever (Ebola)	1.3–1.8[b]

[a]Values from Centers for Disease Control and Prevention website and literature.
[b]Source: G. Chowell et al., *J. Theor Biol* **229:**119–126, 2004.

and transmissibility can be selected, and some individuals (called super transmitters) can pass infection to others much more readily than the majority. We also now know that virus populations are more diverse than first imagined, and the constellation of possible host populations affects their evolution in ways not easily captured by mathematical equations. Consequently, although the calculations are useful indications of the thresholds that govern the spread of a virus in a population (i.e., they help to determine if a disease is likely to die out [$R_0 < 1$] or become endemic [$R_0 > 1$]), they cannot be used to compare possible outcomes in particular cases or for different diseases.

How Do Virus Populations Evolve?

A large, genetically variable host population dispersed in ever-changing environments may appear to present insurmountable barriers to the survival of viruses, yet viruses are plentiful and ubiquitous. The primary reason for this remarkable success is that virus populations display spectacular genetic diversity, manifested in the large collections of genome permutations that are present in a population at any given time. The sources of such diversity are **mutation**, **recombination**, and **reassortment** of viral genes. **Virus evolution** is driven by this diversity and selective pressures that promote survival of the most fit.

In most viral infections, thousands of progeny are produced after a single cycle of replication in one cell, and when genome copying is error prone, almost every new virus particle can differ from every other. Consequently, it is misleading to think of an individual particle as representing an average for that population. This great diversity of the virus population provides avenues for survival under varying conditions.

BOX 10.1

BACKGROUND

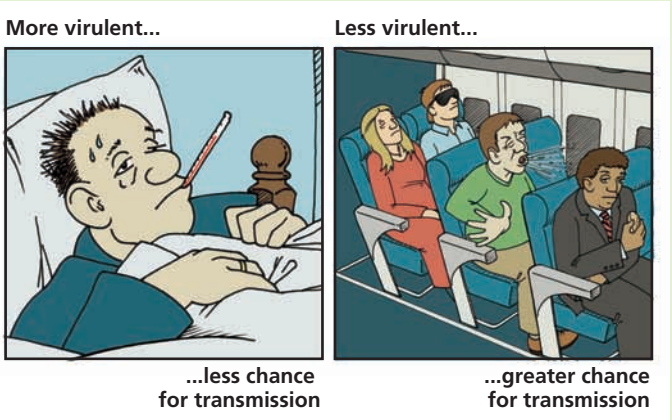
Virulence, selection, and evolution

Is virulence a positive or negative trait for selection? One idea is that increased virulence reduces transmissibility (hosts die faster, reducing exposure to uninfected hosts). Debilitating disease may actually reduce transmission because the infected individual may not interact with other susceptible hosts (see the figure).

If everything were simple, one might expect that all viruses would evolve to be maximally infectious and completely avirulent. A different view appears when real-life infections are studied. The interplay of contextual terms such as "severity of disease" and "transmissibility" is quite complicated. Indeed, for some diseases, and in some contexts, a strong case may be made that increased virulence actually increases R_0 and is strongly selected for in natural viral infections.

An instructive example of the complex relationship between virulence and transmissibility comes from the intentional release of myxoma virus in Australia to control the European rabbit population. Initially the virus was 99% lethal in rabbits, but within a few years both the virus and the rabbits evolved so that infection was 30% lethal. It is thought that moderate levels of virulence were selected because this was concomitant with production of sufficient amounts of virus particles to ensure spread in the rabbit population.

Boots M, Childs D, Reuman DC, Mealor M. 2009. Local interactions lead to pathogen-driven change to host population dynamics. *Curr Biol* **19:**1660–1664.
Fenner F. 1983. The Florey lecture, 1983. Biological control, as exemplified by smallpox eradication and myxomatosis. *Proc R Soc Lond B Biol Sci* **218:**259–285.
Weiss RA. 2002. Virulence and pathogenesis. *Trends Microbiol* **10:**314–317.



Every individual virion is a potential winner, and occasionally, the rarest genotype in a particular population will be the most common after a single selective event. As the famous biologist and science historian Stephen Jay Gould put it, the median is **not** the message when it comes to evolution.

Positive and negative selection of preexisting mutants in a population can occur at any step in a viral life cycle. The requirement to spread within an infected host, as well as between hosts, exposes virus particles to a variety of host antiviral defenses. In addition, host population density, social behavior, and health, represent but a few of the other forces that can affect the survival of virus populations.

Two General Survival Strategies Can Be Distinguished

Viral reproduction cycles typically produce large numbers of progeny particles, and in some cases, virus survival depends mainly on a very high reproductive output, the ***r*-replication strategy**. For other viruses, survival is compatible with a lower reproductive output but better competition for resources, the ***K*-replication strategy** (see Chapter 3). The notations *r* and *K* come from the following equation:

$$dN/dt = rN(1 - N/K)$$

where *r* is the intrinsic rate of increase (i.e., average rates of births minus deaths), *N* is the population size, and *K* is a measure of the resources available (see also Fig. 5.2).

The *r*-replication strategies are characterized by short reproductive cycles, and are effective when resources are in short supply. The *K*-replication strategies include the establishment of persistent or latent infections with little pathogenesis. In these cases, the viruses survive as long their hosts do. For some viruses (e.g., human immunodeficiency virus type 1), both strategies may be important for virus survival.

Large Numbers of Viral Progeny and Mutants Are Produced in Infected Cells

High reproduction rate (the *r*-replication strategy) is common among viruses. To illustrate the implications of this strategy, consider that a single cell infected with poliovirus yields ~10,000 virus particles in as little as 8 h. In theory, three or four cycles of reproduction at this rate could produce a sufficient number of particles to infect every cell in a human body. Such overreplication does not happen, for a variety of reasons, including a vigorous host defense and the fact that viruses can only reproduce in certain tissues or cell types. Nevertheless, this strategy is characteristic of many infections, including those of humans with human immunodeficiency virus type 1 and hepatitis B virus, in which high rates of particle production can continue for years. In the case of human immunodeficiency virus type 1, the time from release of thousands of virus particles from an infected T cell to infection and lysis of another T cell is estimated to be a mere 2.6 days during the later stages of infection (see Fig. 6.14). Mutations invariably accumulate during genome replication, although the *absolute* error rates for this process can be difficult to measure (Box 10.2).

RNA Virus Evolution

Most viral RNA genomes are replicated with considerably lower fidelity than those comprising DNA (see Volume I, Chapters 6 and 7). The average error frequencies reported for RNA genomes are about one misincorporation in 10^4 or 10^5 nucleotides polymerized, which is 1,000 to 10,000 times greater than the rate for a host genome. Given a typical RNA viral genome of 10 kb, a mutation frequency of 1 in 10^4 per template copied corresponds to an average of 1 mutation in *every* replicated genome. Not all viral RNA genomes have the same mutation rate: there is evidence that the replication machinery of viruses with larger RNA genomes operate with higher fidelity. One example is the 30,000-base human severe acute respiratory syndrome coronavirus; a proofreading exonuclease encoded in its genome may account for the lower mutation frequency. The rodent hantaviruses, members of the *Bunyaviridae*, appear to evolve very slowly, with mutation rates approaching those of double-stranded DNA viruses.

DNA Virus Evolution

The error rate for viral DNA replication is estimated to be from 10^{-6} to 10^{-8}, which is closer to the host rate than that for most RNA genomes described above. One reason for this difference is that many RNA polymerases lack error-correcting mechanisms, while most DNA polymerases can excise and replace misincorporated nucleotides (Volume I, Chapter 9). Experimental data indicate that replication of small, single-stranded DNA virus genomes (e.g., *Parvoviridae* and *Circoviridae*) is more error prone than is replication of the double-stranded DNA genomes of larger viruses.

Comparison of the number of mutations produced *per infected cell* shows an inverse relationship between genome size and mutation rate for both RNA and DNA viruses (Fig.10.1). These values can be somewhat higher than those estimated from the error rates of the respective polymerases as they include other sources of mutation, such as host enzyme-mediated base changes, additions or deletions (called RNA editing), or spontaneous damage of viral nucleic acids, e.g., via oxygen radicals or ionizing radiation. The lowest estimates determined for RNA viruses are close to the highest for DNA viruses. As illustrated in Fig. 10.1, the transition between them appears to be relatively smooth. The relationship between genome size and mutation rate suggests that extremes of mutation rate are selected against.

BOX 10.2

DISCUSSION

Error rates are difficult to quantify

Estimates of mutation rates must be viewed with caution. Determining the absolute error rate (measured as the number of misincorporations per nucleotide polymerized) for any nucleic acid polymerase is difficult, if not impossible. Estimates can vary substantially, depending on the experimental method by which they are assessed. For example, PCR technology is commonly used to sample viral genomes, but the polymerase used may itself introduce copying errors that must be factored into the analysis. Cloning viral sequences from a given population in a plasmid, followed by sequencing, can reduce this problem, but depending on the conditions, lethal mutations may be selected against.

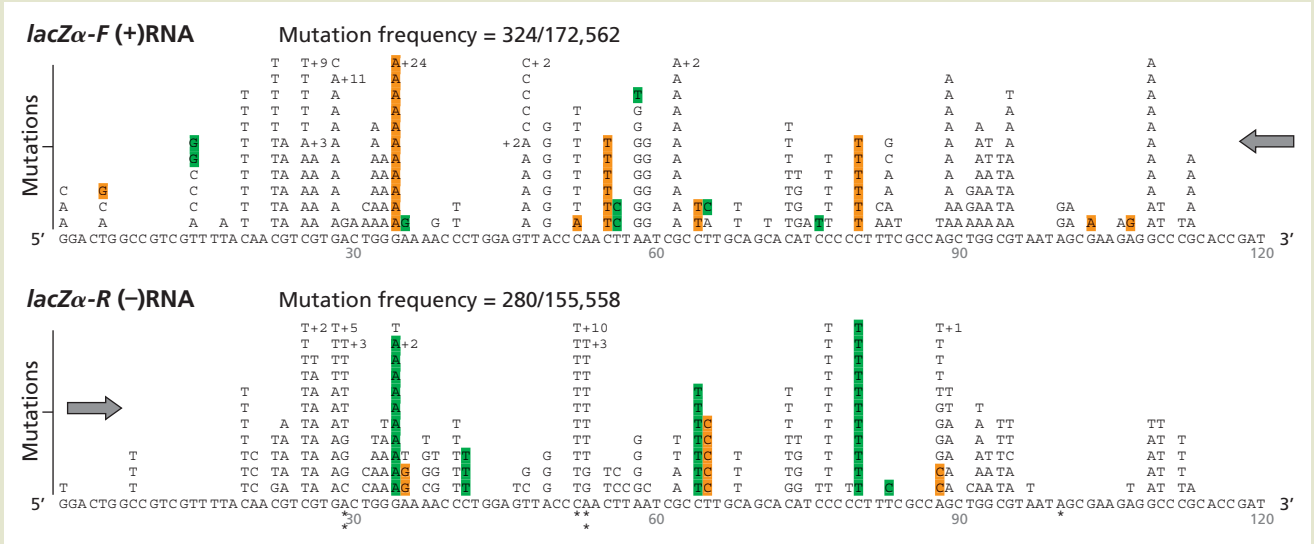
A popular method makes use of reporter genes (e.g., the *lacZ* gene, which encodes β-galactosidase). The reporter gene can be inserted into a viral genome such that enzyme-inactivating mutations can be scored simply by inspection of virus plaques. The mutation frequency for the viral genome is then extrapolated from that determined for the reporter gene. While this method is relatively simple, it can yield misleading data, because assay conditions and host editing functions can affect the results. Furthermore, errors of incorporation are not uniformly distributed as each genome is copied, and can be under- or overestimated depending on the particular polymerase and sequence analyzed. For example, when measured *in vitro* using the *lacZ* system, error rates for the human immunodeficiency virus type 1 reverse transcriptase are both extremely high and variable. However, despite the fact that both reverse transcriptase and the host cell RNA polymerase II can contribute to nucleotide misincorporations *in vivo*, the rate calculated with the same reporter in a single replication cycle of this virus is substantially lower and, at 1.4×10^{-5}, similar to the average rate measured for other retroviruses. Analysis of the recovered reporter genes shows that certain nucleotides are hot spots for mutation, as would be predicted for some loci in the viral genome.

Abram ME, Ferris AL, Shao W, Alvord WG, Hughes SH. 2010. Nature, position, and frequency of mutations made in a single cycle of HIV-1 replication. *J Virol* **84:**9864–9878.

Rezende LF, Prasad VR. 2004. Nucleotide-analog resistance mutations in HIV-1 reverse transcriptase and their influence on polymerase fidelity and viral mutation rates. *Int J Biochem Cell Biol* **36:**1716–1734.

Svarovskaia ES, Cheslock SR, Zhang WH, Hu WS, Pathak VK. 2003. Retroviral mutation rates and reverse transcriptase fidelity. *Front Biosci* **8:**d117–d134.

Single nucleotide substitutions detected in a human immunodeficiency virus type 1-encoded *lacZα* reporter. Numbers, types, and locations of the independent substitution errors are shown for both the forward orientation [(+) strand nucleotide sequence] and the reverse orientation [(−) strand nucleotide sequence] of the *lacZα* reporter. Opposing directional arrows indicate the actual sequence context and direction of minus-strand DNA synthesis during reverse transcription. The total length of the *lacZα* target sequence was defined as 174 nucleotides, representing codons 6 to 63 from GGA to the first TAA termination codon. Only the first 120 nucleotides, which incurred most of the substitutions, are shown here. Single substitution errors are shown as letters above the original wild-type template sequence, limited to 11 per position, with additional errors indicated by +n. Runs of three or more identical nucleotides are underlined. Misalignment/slippage of the primer or template strand that could result in a substitution error is highlighted in orange or green, respectively. Mutational hot spots for which there are significant differences in the forward and reverse *lacZα* orientations are indicated below the sequence by asterisks. Reprinted from M. E. Abram et al., *J Virol* **84:**9864–9878, 2010, with permission.



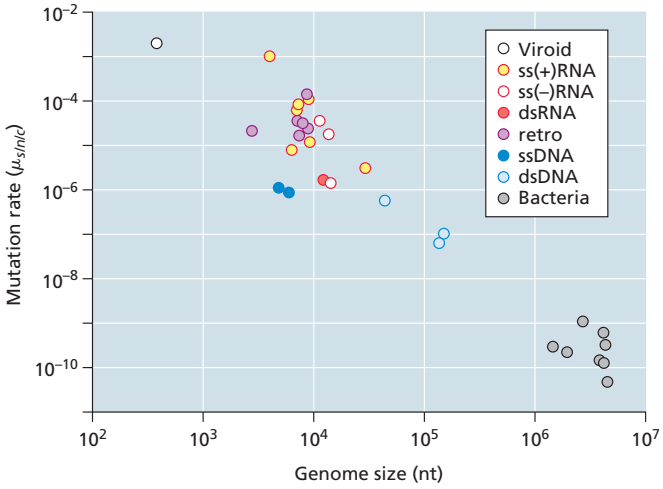


Figure 10.1 Relationship between mutation rate and genome size. Mutation rate is expressed as the number of substitutions per nucleotide per generation, defined as a single cell infection ($\mu_{s/n/c}$). ss, single-stranded; ds, double-stranded; retro, retroviruses; Bacteria includes points for *Bacillus*, *Deinococcus*, *Helicobacter*, *Sulfolobus*, enterobacteria, and mycobacteria. The white circle is a single reported rate for a viroid, expressed in substitutions per strand copied. Adapted from R. Sanjuán et al., *J Virol* **84:**9733–9748, 2010, with permission.

The Quasispecies Concept

A 1978 paper, which described a detailed analysis of an RNA bacteriophage population (phage Qβ), made the following, startling conclusion:

> A Qβ phage population is in a dynamic equilibrium, with viral mutants arising at a high rate on the one hand, and being strongly selected against on the other. The genome of Qβ phage cannot be described as a defined unique structure, but rather as a weighted average of a large number of different individual sequences.
>
> E. Domingo, D. Sabo, T. Taniguchi, and C. Weissmann, *Cell* **13:**735–744, 1978

This conclusion has since been validated for many virus populations. Indeed, we now understand that virus populations exist as dynamic distributions of nonidentical but related replicons, often called **quasispecies**, a concept developed by Manfred Eigen. A steady-state, equilibrium population of a given viral quasispecies must comprise vast numbers of particles. Indeed, such equilibria cannot be attained in the small populations typically found after isolated infections in nature or in the laboratory. In these cases, extreme fluctuations in genotype and phenotype are possible.

For a given RNA virus population, the genome sequences cluster around a consensus or average sequence, but virtually every genome can be different from every other. A rare genome with a particular mutation may survive a specific selection event, and this mutation will be found in all progeny genomes. However, any linked but unselected mutations in that genome will also be retained. Consequently, the product of selection after replication is a new, diverse population of genomes that share only the selected and closely linked mutations (Fig. 10.2).

The quasispecies theory predicts that a viral quasispecies is not simply a collection of diverse mutants, but rather a group of *interactive* variants that characterize the particular population. Diversity of the population, therefore, is critical for survival. It has been possible to test the idea that virus populations, **not** individual mutants, are the targets of selection by limiting diversity. Certain spontaneous mutants of human immunodeficiency virus type 1 that are resistant to the reverse transcriptase inhibitor lamivudine exhibit a 3.2-fold reduction in error frequency. However, this seemingly modest increase in fidelity was found to be associated with a significant reproductive **disadvantage** in infected individuals. As another example, poliovirus replication is notoriously error prone, producing a remarkably diverse population. Certain ribavirin-resistant poliovirus mutants have increased fidelity of ~6-fold, but such mutants were found to be **much less** pathogenic in animals than was the wild-type virus, and the reduced diversity led to attenuation and loss of neurotropism. Further studies showed that in a genetically diverse population, isolated viral mutants complement each other, consistent with the idea that it is the population, not the individual, that is evolving. In any case, as virus populations have maintained high mutation rates, we can infer that lower rates are neither advantageous nor selected in nature.

Another outcome of quasispecies dynamics is that viral mutants with low fitness can sometimes outcompete viruses with higher fitness if the low-fitness mutations are surrounded by beneficial ones. In other words, a population whose mutants have a similar mean fitness can outcompete a population with a lower average fitness that contains mutants with higher fitness. This situation has been called the **quasispecies effect**, or **survival of the flattest**. In contrast, in classic population genetics models, individual high-fitness variants are favored, a situation known as **survival of the fittest**.

Sequence Conservation in Changing Genomes

Despite high mutation rates, not all is in flux during viral genome replication. For example, the ***cis*-acting sequences** of RNA viruses change very little during propagation. These sequences include signals that are required for genome replication, messenger RNA (mRNA) synthesis, and genome packaging. They are often the binding sites for one or more viral or cellular proteins. Any genome with mutations in such sequences, or in the gene that encodes the corresponding viral binding protein, is likely to be less fit, or may not be replicated at all. Changes must occur in both interacting components for restoration of function. The tight, functional coupling of binding protein and target sequence is a marked constraint

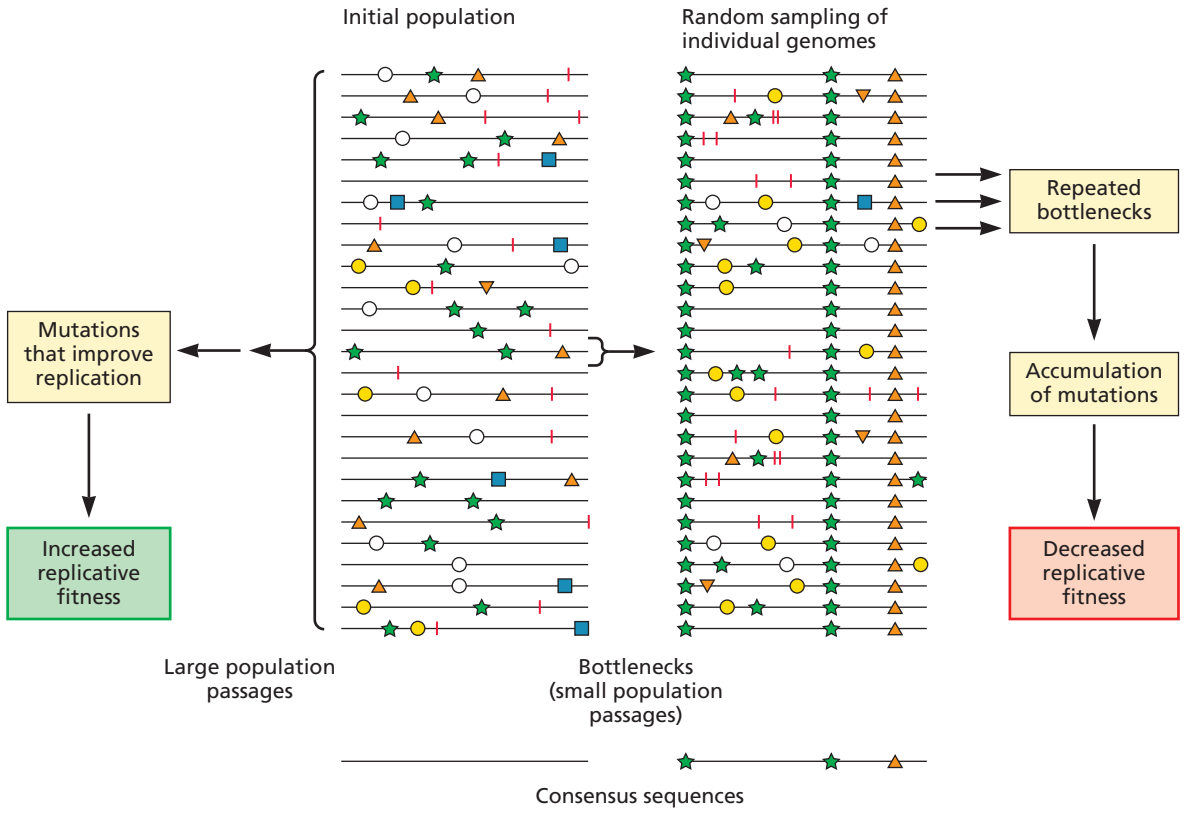


Figure 10.2 Viral quasispecies, population size, bottlenecks, and fitness. Genomes are indicated by the horizontal lines. Mutations are indicated by different symbols. When a viral genome is replicated, mutations accumulate in the progeny genomes. While every viral population exhibits genomic diversity, the extent of diversity depends on many parameters, including the frequency of mutation. (Left) A hypothetical population of genomes, in which each member contains a characteristic set of mutations. The consensus sequence for this population is shown as a single line at the bottom. Note that there are **no** mutations in the consensus sequence, despite their presence in most genomes in the population (almost every genome is different). (Right) A population of genomes that emerges after passage of one genome through one bottleneck. The consensus sequence for this population is shown as a single line at the bottom. Note that in this example, three mutations selected to survive the bottleneck are found in every member of the population, and these appear in the consensus sequence. If the large population is propagated without passage through bottlenecks (situation on the left), repeated passage enriches for mutant genomes that **improve replication and increase the fitness** of the population. Recent studies with plaque purified virus (a bottleneck; see text and Table 10.2), and using techniques that eliminate some artifacts of sequencing, found that for some RNA viruses (e.g., poliovirus), almost half of the particles at an early passage at low MOI had genomes with no mutations. As the population was passaged serially, the proportion of genomes with no mutations decreased and those containing multiple changes increased. If a viral population continues to be propagated through serial bottlenecks, mutations that result in **reduced fitness will accumulate**. Adapted from E. Domingo et al., p. 144, *in* E. Domingo et al. (ed.), *Origin and Evolution of Viruses* (Academic Press, Inc., San Diego, CA, 1999), with permission.

for evolution. In some instances, these sequences are stable enough to represent lineage markers for molecular phylogeny.

The Error Threshold

The capacity to sustain prodigious numbers of mutations is a powerful advantage for virus populations. Yet, at some point, selection and survival must balance genetic fidelity and mutation rate. Many mutations are detrimental, and if the mutation rate is high, their accumulation can lead to a phenomenon called **lethal mutagenesis** when the population is driven to extinction. Intuitively, mutation rates higher than one error in 1,000 incorporated nucleotides must challenge the very existence of the viral genome, as essential genetic information can be lost irreversibly. The **error threshold** is a mathematical parameter that measures the complexity of the information that must be maintained to ensure survival of the population. RNA viruses tend to evolve close to their error threshold, while DNA viruses have evolved to exist far below it. We can infer these properties from experiments with mutagens. After treatment of cells infected by an RNA virus (such as vesicular stomatitis virus or poliovirus) with a nucleoside analog such as 5-azacytidine, virus titers drop

dramatically, but the error frequency per surviving genome increases by only 2- to 3-fold at most. In contrast, a similar experiment performed with a DNA virus, such as herpes simplex virus or simian virus 40, results in a less precipitous drop in survival, but an increase of several orders of magnitude in single-site mutations among the survivors.

It is clear that many important biological parameters contribute to virus survival, including a complex property called **fitness**, the adaptability of an organism to its environment. Fitness depends on the context in which reproduction is examined and what outcome is measured. In the laboratory setting, it may be measured simply by comparison of reproduction rates or virus yields. However, fitness is far more difficult to measure under natural conditions, such as infection of organisms that live in large, interacting populations. Another essential parameter, equally difficult to measure, is the stability or predictability of the environment as it affects propagation of a virus. Host population dynamics and seasonal variation are but two examples of such complicated environmental parameters. Finally, given the diversity of any viral population, determining the fitness of one population versus that of another requires application of the mathematics of population genetics, a subject beyond the scope of this text.

Genetic Bottlenecks

Unlike lethal mutagenesis, which can lead to the extinction of large populations, the **genetic bottleneck** represents extreme selective pressure on small populations, which results in loss of diversity, accumulation of nonselected mutations, or both (Fig. 10.2). A simple experiment illustrating this principle can be performed readily in the laboratory (Table 10.2). Virus particles are isolated from a single, isolated plaque and used to infect fresh cells; the process is then repeated many times (**serial passage**). The perhaps surprising result is that, after about 20 or 30 cycles of single-plaque selection, the virus populations are barely able to propagate; they are markedly less fit than the original populations. The bottleneck is the consequence of restricting further viral reproduction to the progeny found in a single plaque, which contains only a few thousand virions—all derived from a single infected cell.

Table 10.2 Fitness decline compared to initial virus clone after passage through a bottleneck[a]

Virus	No. of bottleneck passages	% Decrease in fitness (avg)
ϕ6 (bacteriophage)	40	22
Vesicular stomatitis virus	20	18
Foot-and-mouth disease virus	30	60
Human immunodeficiency virus	15	94
MS2 (bacteriophage)	20	17

[a]Source: A. Moya et al., *Proc Natl Acad Sci U S A* **97**:6967–6973, 2000.

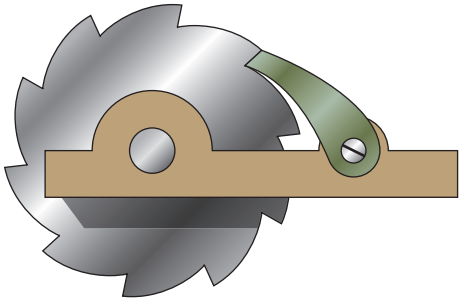


Figure 10.3 Muller's ratchet turns in only one direction. Mutations occur and accumulate at every replication cycle. Without recombination to recreate a genome that is free from harmful mutations, and conserve beneficial mutations, the population cannot survive.

The environment is constant, and the only apparent requirement is that the population of viruses obtained from a single plaque must be able to reproduce. Why does fitness plummet?

The answer lies in **Muller's ratchet** model, which explains why small, asexually reproducing populations decline in fitness over time if the mutation rate is high. The ratchet metaphor is fitting: a ratchet on a gear allows the gear to move forward, but not backward (Fig. 10.3). After each round of genome replication, mutations accumulate but are not removed. Each round of error-prone replication works like a ratchet, "clicking" relentlessly as mutations accumulate. Each mutation has the potential to erode the fitness of subsequent, limited populations. We have noted that the genomes of replicating RNA viruses accumulate many mutations and survive close to their error threshold. By restricting population growth to serial single founders (serial bottlenecks), so many mutations accumulate in **all** of the progeny that fitness decreases.

Simple studies such as the serial plaque transfer experiment show that Muller's ratchet can be avoided if a more diverse viral population is subjected to serial passage. One such study showed that pools of virus from 30 individual plaques were required in serial transfer to maintain the population's original fitness. This observation can be explained as follows: greater diversity in the population facilitates the construction of mutation-free genomes by recombination or reassortment, and hence removes or compensates for deleterious mutations. Such recombination or reassortment may be quite rare, but it imparts a powerful selective advantage in this experimental paradigm. Indeed, the progeny of such a rare virus will ultimately predominate in the population. The message is simple but powerful: the diversity of a virus population is important for its survival; remove diversity, and the population suffers.

As the particular bottleneck of single-plaque passage is obviously artificial, it is reasonable to ask: Does Muller's ratchet ever occur in nature? Infections by small virus populations include the tiny droplets of suspended virus particles

during transmission as an aerosol, activation of a latent virus from a limited population of cells, and the small volumes of inoculum introduced by insect bites. How virus populations pass through such natural bottlenecks and survive is not yet clear.

Genetic Shift and Genetic Drift

Selection of mutants that are resistant to elimination by antibodies or cytotoxic T lymphocytes is inevitable when successful virus reproduction occurs in an immunocompetent individual. This process of antigenic variation and its contribution to modulating the immune response is discussed in Chapter 5. The terms **genetic drift** and **genetic shift** describe distinct mechanisms for generation of diversity. Diversity that arises from genome replication errors and immune selection of single-site mutants (drift) is contrasted with diversity that results from recombination among genomes, or reassortment of genome segments (shift). Drift is possible every time a genome replicates, but shift is relatively rare. The episodic pandemics of influenza (Fig. 10.4) provided strong evidence for this conclusion. For example, there are only six established instances of genetic shift for the influenza virus hemagglutinin gene since 1889. However, the combination of frequent drift and infrequent shift, together with the availability of intermediate host species, contributes significantly to diversity in influenza virus populations (Box 10.3). When retrovirus infection results in integration of multiple proviral genomes in a single cell, genetic shift can occur via recombination if two different viral genomes are packaged in a progeny virus particle.

Exchange of Genetic Information

Genetic information is exchanged by recombination or by reassortment of genome segments (Volume I, Chapters 6, 7, and 9). In a single step, recombination can create new linkages of many mutations that may be essential for survival under selective pressures. As discussed above, this process allows the construction of viable genomes from debilitated ones. Recombination occurs when the polymerase that copies a viral genome changes templates (copy choice) during replication or when nucleic acid segments are broken and rejoined. The former mechanism is common among RNA viruses, whereas the latter is more typical of double-stranded DNA viruses. Reassortment of genomic segments takes place when cells are coinfected with segmented RNA viruses. This can be an important source of variation, as exemplified by reoviruses and orthomyxoviruses (Fig. 10.4 and Box 10.3).

Insertion of nonviral nucleic acid into a viral genome is also a well-documented phenomenon that can contribute to virus evolution. Incorporation of cellular sequences can lead to defective genomes, or to more-pathogenic viruses. Examples of such recombination include the appearance of a cytopathic

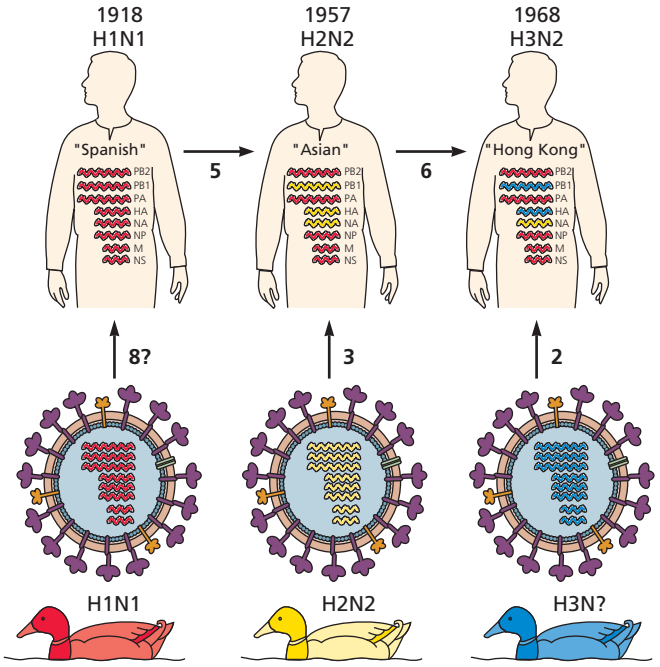


Figure 10.4 Appearance and transmission of distinct serotypes of influenza A virus in human pandemics in the 20th century. The major influenza pandemics are characterized by viral reassortants. The reassortants carried HA (H) and NA (N) genes that had not been in circulation in humans for some time, and consequently, immunity was low or nonexistent. With the introduction of each subtype, the world experienced an influenza pandemic characterized by a new combination of H and N. The viral genome segments are illustrated in three different colors, with each representing a particular viral genotype. Segments and gene products of the pandemic strains are indicated in each human silhouette. The numbers next to the arrows indicate how many segments of the viral genome are known to have been transmitted in each episode. Adapted from R. G. Webster and Y. Kawaoka, *Semin Virol* **5:**103–111, 1994, with permission.

virus in an otherwise nonpathogenic infection by the pestivirus bovine viral diarrhea virus (see Volume I, Chapter 6) or the sudden appearance of oncogenic retroviruses following infection with nononcogenic strains. The latter results from the acquisition of oncogenes from the host cell genome, and is characteristic of acutely transforming retroviruses such as Rous sarcoma virus (Chapter 7). Poxvirus and gammaherpesvirus genomes carry virulence genes with sequence homology to host immune defense genes, which must also have been acquired via genetic recombination. These genes are usually found near the ends of the genome. One explanation for this location is that the process of DNA packaging (gammaherpesviruses) or initiation of DNA replication (poxviruses) stimulates virus-host recombination when viral DNA is cleaved.

Information can be exchanged in a variety of unexpected ways during viral infections. For example, a host can be infected or coinfected by many different viruses during

BOX 10.3

BACKGROUND

Reassortment of influenza virus genome segments

Pandemic influenza strains result from shifts in H and N serotypes via exchange of the genome segments of mammalian and avian influenza viruses. Virologists have demonstrated that certain combinations of H and N are selected more strongly in avian hosts than in humans. An important observation was that both avian and human viruses replicate well in certain species such as pigs, no matter what the H-N composition. Indeed, the lining of the throats of pigs contains receptors for both human and avian influenza viruses, providing an environment in which both can flourish. As a result, the pig is a good host for mixed infection of avian and human viruses, in which reassortment of H and N segments can occur, creating new viruses that can reinfect the human population.

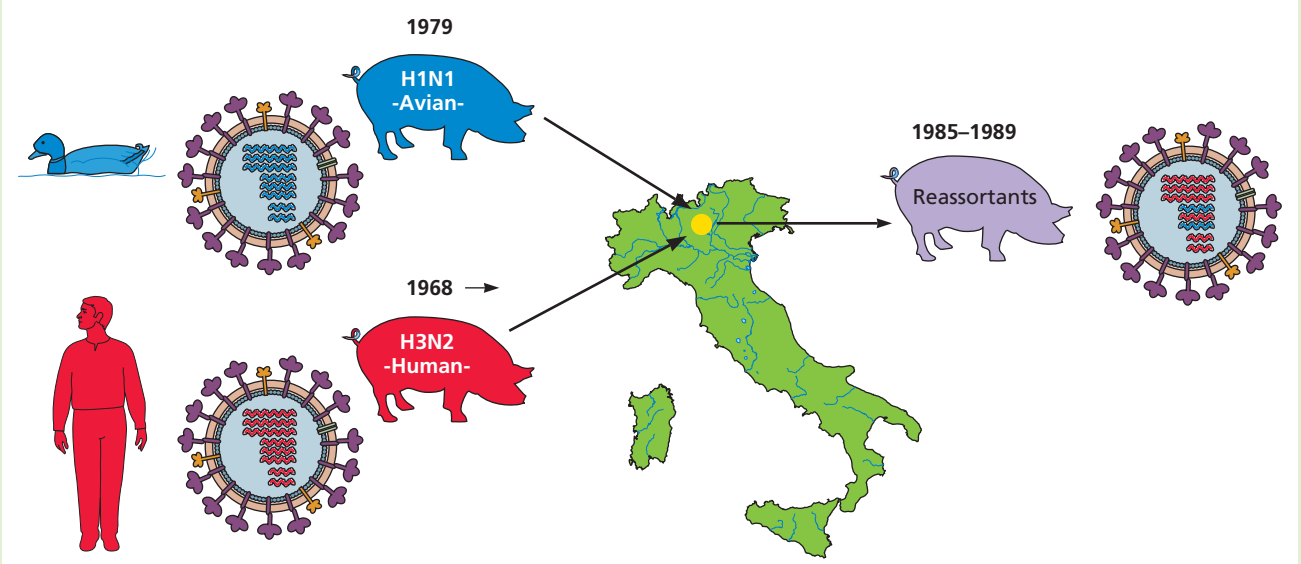
One might think that this combination of human, bird, and pig infections must be extremely rare. However, the dense human populations in Southeast Asia that come in daily contact with domesticated pigs, ducks, and fowl create conditions in which these interactions are likely to be frequent. Indeed, epidemiologists and virologists can show that the 1957 and 1968 pandemic influenza A virus strains (Fig. 10.4) originated in China and that the human H and N serotypes are circulating in wildfowl populations.

Studies of Italian pigs also provide evidence for reassortment between avian and human influenza viruses. The figure shows how the avian H1N1 viruses in European pigs reassorted with H3N2 human viruses. The color of the segments of the influenza genome indicates the origin of the avian and human viruses. The host of origin of the influenza virus genes was determined by sequencing and phylogenetic analysis. Results from these studies show that pigs can serve as an intermediate host in the emergence of new pandemic influenza viruses.

Reassortment among human, avian, and swine influenza virus strains led to the emergence of the 2009 H1N1 influenza pandemic. The U.S. Centers for Disease Control and Prevention has estimated that 43 million to 89 million people contracted H1N1 between April 2009 and April 2010, with 8,870 to 18,300 related deaths.

Peiris JS, Guan T, Markwell D, Ghose P, Webster RG, Shortridge KF. 2001. Cocirculation of avian H9N2 and contemporary "human" H3N2 influenza A viruses in pigs in southeastern China: potential for genetic reassortment? *J Virol* **75:**9679–9686.

New influenza A strains can emerge following reassortment of human and avian influenza viruses in pigs. Adapted from R. G. Webster and Y. Kawaoka, *Semin Virol* **5:**103–111, 1994, with permission.



its lifetime. In fact, serial and concurrent infections are commonplace and can have a major effect on virus evolution. In the simplest case, propagation of a viral quasispecies in an infected individual allows coinfection of single cells, phenotypic mixing, and genetic complementation. As a result, recessive mutations are not eliminated immediately, despite the haploid nature of most viral genomes. Of course, such coinfection also provides an opportunity for the physical exchange of genetic information (Box 10.4).

Two General Pathways for Virus Evolution

Because viruses are absolutely dependent on their hosts for their reproduction, viral evolution tends to take one of two general pathways. In one, viral populations coevolve with their hosts so that they share a common fate: as the host prospers, so does the viral population. However, given no other host, a serious roadblock exists: the entire virus population can be eliminated with potent antiviral measures (e.g., smallpox virus) or by extinction of the host. In the other pathway,

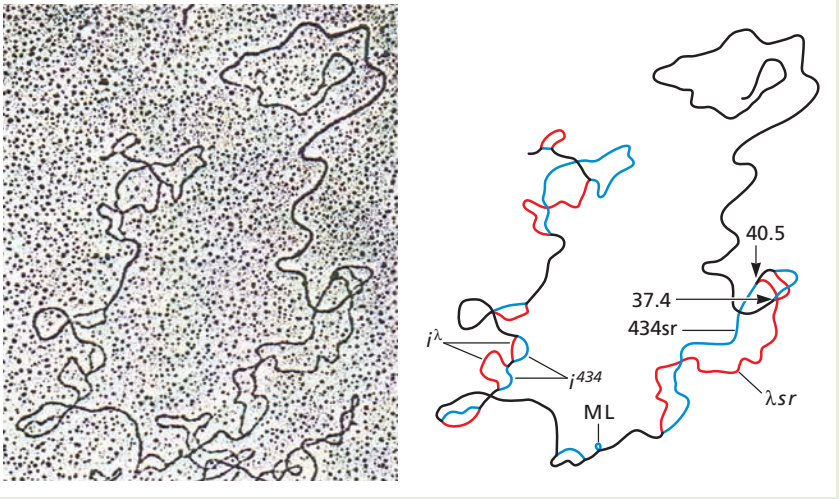
BOX 10.4

BACKGROUND

Evolution by nonhomologous recombination and horizontal gene transfer

In the early 1970s, scientists working with the bacteriophage lambda and related bacteriophages found that various pairs of viral DNA formed heteroduplexes when visualized in the electron microscope; as illustrated in the figure, homologous, double-stranded stretches were seen connected to single-stranded bubbles corresponding to nonhomologous stretches that cannot form base pairs. The images were striking, and showed that the genomes of this group of lambdoid phages were **mosaics**; that is, they contained blocks of genes that were shuffled by recombination during evolution. Further analyses of bacteriophages that had picked up host genes by nonhomologous recombination established that **horizontal gene transfer** among bacteria by bacteriophages was a central feature in the evolution of both. With large-scale genome sequencing, we now know that bacteriophage genomes have ancestral connections to viruses of the *Eukarya* and *Archaea*.

Murray NE, Gann A. 2007. What has phage lambda ever done for us? *Curr Biol* **17**:R305–R312.



A heteroduplex formed from DNA of bacteriophages lambda and 434. In the explanatory tracing, homologous double-stranded regions are shown as solid black lines and nonhomologous single-stranded regions are red (phage lambda) or blue (phage 434) lines. The numbers 37.4 and 40.5 mark the left termini of the first and second nonhomology loops, starting from the left end of the genetic maps. ML is a minute deletion. *sr*, silent genetic regions; *i*, immunity regions. Reprinted from M. Simon et al., p. 315, in A. D. Hershey (ed.), *The Bacteriophage Lambda* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1971), with permission.

virus populations occupy broader niches and infect multiple host species. When one species is compromised, the virus population can thrive in another. As discussed below, the first pathway is generally typical of DNA viruses, whereas the second is common for RNA viruses.

Fundamental Properties of Viruses That Constrain and Drive Evolution

The very characteristics that enable us to define and classify viruses are the primary barriers to major genetic change. Once a genome replication and expression strategy has evolved, there can be no turning back. For example, genomes that have suffered extreme alterations in the consensus do not survive selection. The nature of the genome is also fixed: DNA genomes cannot become RNA genomes, and vice versa. Furthermore, the solutions to replication or the decoding of viral information are limited, and because every step in viral reproduction requires interactions with host cell machinery, any change in a viral component without a compensating change in the cellular machinery may compromise viral propagation. Similarly, inappropriate synthesis, concentration, or location of a viral component is likely to be detrimental.

A second common constraint is the physical nature of the capsid required for transmission of the genome. Closed capsids have defined internal volumes that establish a limit on the size of the nucleic acids that may be packaged. Once the genes that encode the structural proteins of such capsids are selected, genome size is essentially fixed; only small duplications or acquisitions of sequences are possible without compensating deletion of other sequences. A final constraint is the requirement for balance (Box 10.5). All viral genomes encode products capable of modulating a broad spectrum of host defenses, including physical barriers to viral access and the vertebrate immune system. A mutant that is too efficient in bypassing host defenses will kill its host and suffer the same fate as one that does not reproduce efficiently enough: it will be eliminated. These general constraints define the viruses that we see today, as well as the further evolution of new ones.

Finite Strategies for Replication and Expression of Viral Genomes

We have described the seven viral genome replication strategies that are likely to represent all possible solutions, as well as the small number of strategies for expression of these

BOX 10.5

DISCUSSION

An unexpected constraint on evolution: selection for transmission and survival inside a host

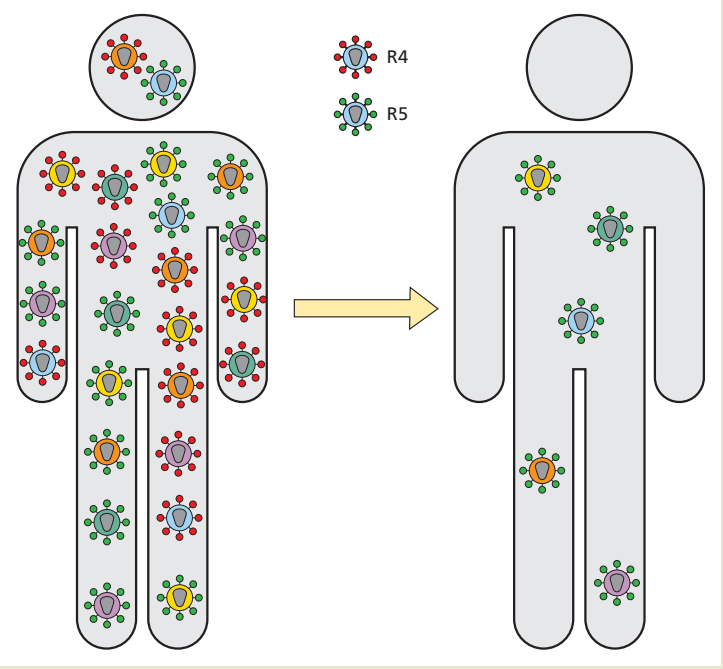
The human immunodeficiency virus type 1 particles that initiate infection of their human hosts appear to be underglycosylated and are characterized by use of the CCr5 coreceptor and requirement for large amounts of the CD4 receptor on their target cells, suggesting selection for reproduction in T cells. At the late stage of disease, the infected individual produces billions of virus particles that survive in the face of host defenses and antiviral therapy. Invariably these late-stage virions can infect an expanded range of targets that include not only mature T cells, but also CCr5-positive macrophages that display small amounts of the CD4 receptor, and naïve T cells that produce very little CCr5 but large amounts of the CXCr4 coreceptor. Importantly, diversity in this final virus population is a result of the evolution of viral envelope receptor determinants inside a single infected individual.

When virus particles are transmitted from individuals with late-stage disease to new hosts, the first viruses that can be detected in the new hosts have the same characteristics as those that initiated the infections in the donors, indicating that only a few of the diverse variants present at the late stage in the donor are passed on. The processes that select these variants from the infecting virus population are not well understood. However, one conclusion is clear: the virus particles that ultimately devastate the immune system after years of reproduction and selection within a host are not those most fit for infection of new hosts.

Joseph SB, Arrildt KT, Swanstrom AE, Schnell G, Lee B, Hoxie JA, Swanstrom R. 2014. Quantification of entry phenotypes of macrophage-tropic HIV-1 across a wide range of CD4 densities. *J Virol* **88:**1858–1869.

Ping LH, Joseph SB, Anderson JA, Abrahams MR, Salazar-Gonzalez JF, Kincer LP, Treurnicht FK, Arney L, Ojeda S, Zhang M, Keys J, Potter EL, Chu H, Moore P, Salazar MG, Iyer S, Jabara C, Kirchherr J, Mapanje C, Ngandu N, Seoighe C, Hoffman I, Gao F, Tang Y, Labranche C, Lee B, Saville A, Vermeulen M, Fiscus S, Morris L, Karim SA, Haynes BF, Shaw GM, Korber BT, Hahn BH, Cohen MS, Montefiori D, Williamson C, Swanstrom R, CAPRISA Acute Infection Study and the Center for HIV-AIDS Vaccine Immunology Consortium. 2013. Comparison of viral Env proteins from acute and chronic infections with subtype C human immunodeficiency virus type 1 identifies differences in glycosylation and CCR5 utilization and suggests a new strategy for immunogen design. *J Virol* **87:**7218–7233.

Bottleneck for transmission of human immunodeficiency virus type 1.



genomes (Volume I, Chapter 3). That the provenance of all viruses can be described by such a short list is remarkable. We have no clear understanding of how these replication and expression strategies have evolved, but some observations have been thought-provoking. For example, the replication complexes of different RNA virus families exhibit some fundamental similarities. Localization of genomes to membrane sites or to assembling capsids leads to the precise temporal and spatial organization of viral compartments, a property that is important for gene expression, replication, and particle assembly (Fig. 10.5). Are these overtly similar mechanisms products of convergent evolution and coincidence, or do they imply a common evolutionary origin for this abundant group of viral genomes? One notion is that similar mechanisms were selected because they sequestered viral nucleic acid from intrinsic defense proteins present in the cytoplasm, such as RigI/Mda5, Pkr, and Tlrs.

The Origin of Viruses

One cannot help but conclude that nothing looks quite like the world of viral genomes (the virosphere). Soon after their discovery, many speculated that viral genomes might be very ancient, even predecessors to cellular microbes. Consistent with this hypothesis, the genomes of viruses that infect hosts in all three domains of life (*Archaea*, *Bacteria*, and *Eukarya*) share structural and coding features. Hypotheses about the origins of viruses center around three nonexclusive ideas. One is that viruses originated **before cells**, >3.6 billion years ago,

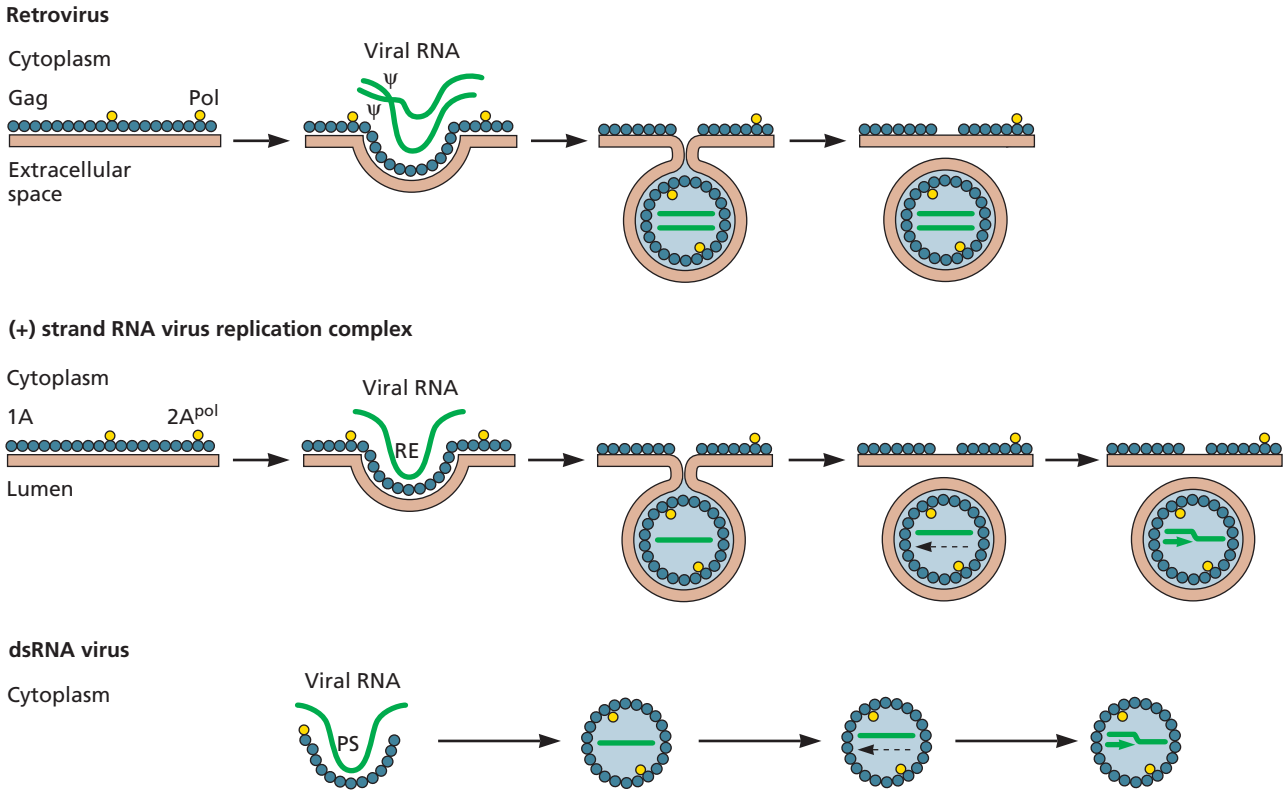


Figure 10.5 Parallels in replication of (+) strand and double-stranded RNA genomes. The mRNA templates of viruses with double-stranded RNA (dsRNA) and single (+) strand RNA genomes (including retroviruses) are sequestered in a multisubunit protein core that directs synthesis of the RNA or DNA intermediate from which more viral mRNA is made. Similarities in how the mRNA template and core proteins are assembled suggest that, despite a complete lack of genome sequence homology, all three virus groups may share evolutionary history. It is possible that this ancient replicative strategy provides RNA genomes with increased template specificity and retention of (−) strand products in the core or vesicle for template use. In addition, by sequestering RNA in vesicles or capsids, host defenses such as RNA interference, dsRNA-activated protein kinase, and RNase L are avoided. (Top) Retroviruses: specific sequences in the RNA genome (ψ) bind to Gag proteins that define the budding site. Gag proteins encapsidate two viral RNA genomes and reverse transcriptase within the plasma membrane. The final reaction for retroviruses is the release of an enveloped particle with two copies of the RNA genome and polymerase. (Middle) (+) strand RNA genomes: intracellular membrane vesicles form in response to a viral protein that binds to membranes and polymerase complexes, and viral RNA templates are recruited to these vesicles. In the case of (+) strand RNA viruses, the product is not an enveloped virion, but rather an involuted vesicle or the surface of a membrane vesicle where mRNA synthesis, (−) strand genome template synthesis, and (+) strand genome synthesis occur. (Bottom) dsRNA genomes: replication occurs in compartments formed by assembling capsid proteins that sequester single-stranded genome templates via specific protein-RNA interactions. In this case, the product is a capsid compartment within which mRNA synthesis and complementary strand genome replication occur. Blue circles are Gag proteins (retrovirus), 1A protein [(+) strand RNA virus], and the inner capsid protein (dsRNA virus). The polymerase proteins (Pol or $2A^{pol}$; yellow circles) are incorporated with Gag or 1A, respectively. The polymerase is part of the assembling capsid of dsRNA viruses. RNA genomes are indicated in green, and the binding sites for interaction with Gag, 1A, or capsid protein are ψ, RE, or PS, as indicated for each virus. Adapted from M. Schwartz et al., *Mol Cell* **9:**505–514, 2002, with permission.

and might have contributed to the structure of the first cells. A second hypothesis is that viruses arose **after the first cellular organisms**, acquiring the ability to replicate and become packaged in particles. The third hypothesis is that viruses are **derived from intracellular parasites** that have lost all but the most essential genes, those encoding products required for genome replication and maintenance. These hypotheses are certainly not mutually exclusive; for example, some viruses predate cells, while others arose after that time.

It is widely believed that the first genomes and enzymes comprised RNA, with the transition from RNA to DNA genomes made possible by the evolution of reverse transcriptase. However, this transition would have had to wait for the evolution of genes that encoded the machinery for synthesis of deoxyribonucleotides (e.g., ribonucleotide reductase and thymidylate synthases). Some have hypothesized that DNA genomes were a viral invention that was shared later with cells harboring RNA genomes (Box 10.6).

BOX 10.6

DISCUSSION

Hypothetical origins of cells and viruses

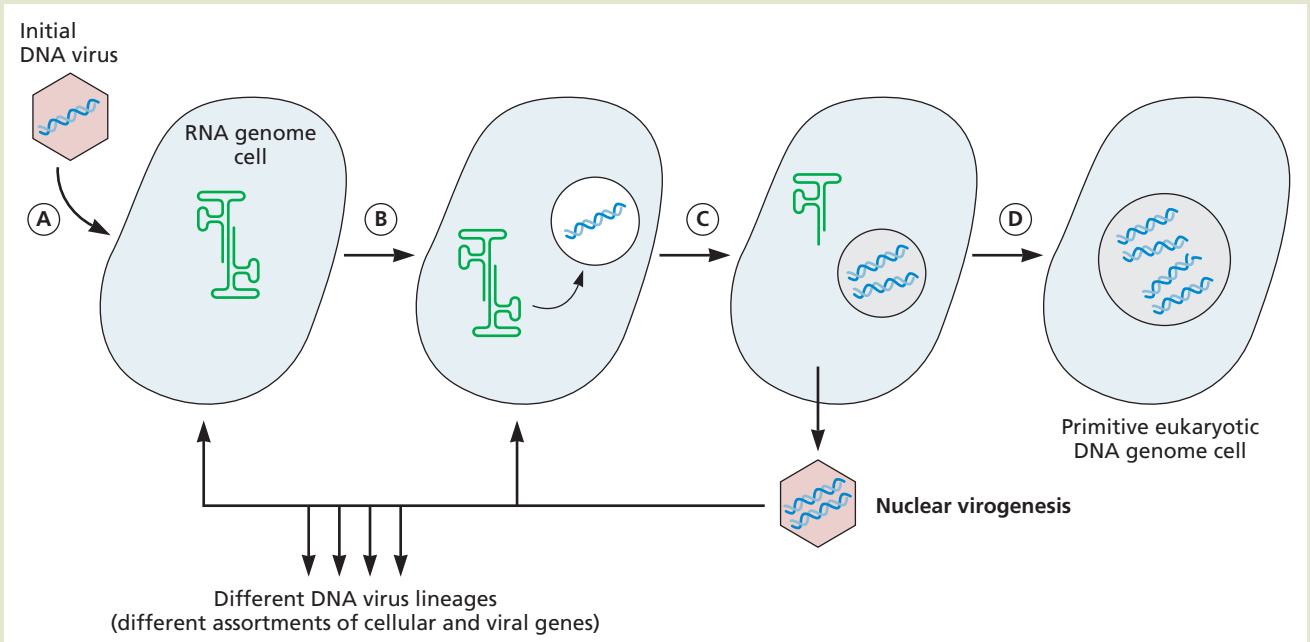
The coexistence of two distinct genomes in a common cell is thought to have driven major evolutionary leaps such as the acquisition of mitochondria by eukaryotic cells. Some evolutionary biologists have proposed that eukaryotic nuclei arose following fusion of the cells of primordial bacteria and archaea, as genes derived from both have been identified in eukaryotic genomes. Others have proposed that eukaryotic cells existed before bacteria and archaea, noting that nucleus-like structures are present in certain unusual soil and freshwater bacteria, the planctomycetes. In this scenario, a nucleus was retained in eukaryotes, but lost in most bacteria and archaea over evolutionary time. Both of these hypotheses are controversial, as many gaps in our knowledge of evolutionary relationships remain to be filled.

Because it is commonly believed that the very first genomes (and enzymes) comprised RNA, yet a third group of scientists have proposed that cellular DNA genomes arose from the infection of a primordial cell with an RNA genome by a DNA virus. In this scenario, it is hypothesized that viral DNA genomes arose via reverse transcription from a viral RNA genome, following the emergence of reverse transcriptase and enzymes required for the synthesis of desoxyribonucleotides, including ribonucleotide reductase and thymidylate synthetase. It is noted that many viruses with large DNA genomes encode both of the latter enzymes. However, how these enzymes might have evolved in viral genomes is unclear, and the possibility that they were acquired from some ancestral host cell cannot be excluded. Consequently, while the proposal of a viral origin of cellular genomes has promoted much lively discussion, this idea remains controversial. Ideas about the origin of viruses with RNA genomes are even more speculative. They could be escapees from an ancient RNA world, but their distribution among the archaea, eukaryotes, and bacteria is decidedly nonuniform.

Claverie JM. 2006. Viruses take center stage in cellular evolution. *Genome Biol* **7:**110. doi:10.1186/gb-2006-7-6-110.

Forterre P. 2005. The two ages of the RNA world, and the transition to the DNA world: a story of viruses and cells. *Biochimie* **87:**793–803.

Hypothetical origin of the eukaryotic cell nucleus. (A) An early DNA virus (perhaps a bacteriophage ancestor) infects a cell with an RNA genome. **(B)** The DNA virus is sequestered within a vesicle in the "cytoplasm" and replicates in this compartment. Cellular genes are recruited to the enlarging nucleus; new DNA chemistry provides selective advantages. **(C)** This unstable situation may produce altered viruses better adapted to infection of and cytoplasmic replication in cells with RNA genomes, as well as **(D)** the evolution of a stable eukaryotic cell with a nucleus and DNA replication machinery. Adapted from J.-M. Claverie, *Genome Biol* **7:**110–114, 2006, with permission.



Speculation about the origin of viral genomes was provoked anew by the discovery of giant DNA viruses with genomes of up to 2.5 Mbp, larger than some parasitic bacteria. Based on analyses of their DNA polymerases, these giants have been assigned to a group known as the "nucleocytoplasmic large DNA viruses," which includes herpesviruses and poxviruses (Fig. 10. 6). The genomes of all families in this group include genes normally found in host cells, for example, genes for proteins of the glycosylation machinery, nucleotide processing, DNA transcription, and even some translation components. The largest members in the group of nucleocytoplasmic DNA viruses, the megaviruses, pandoraviruses,

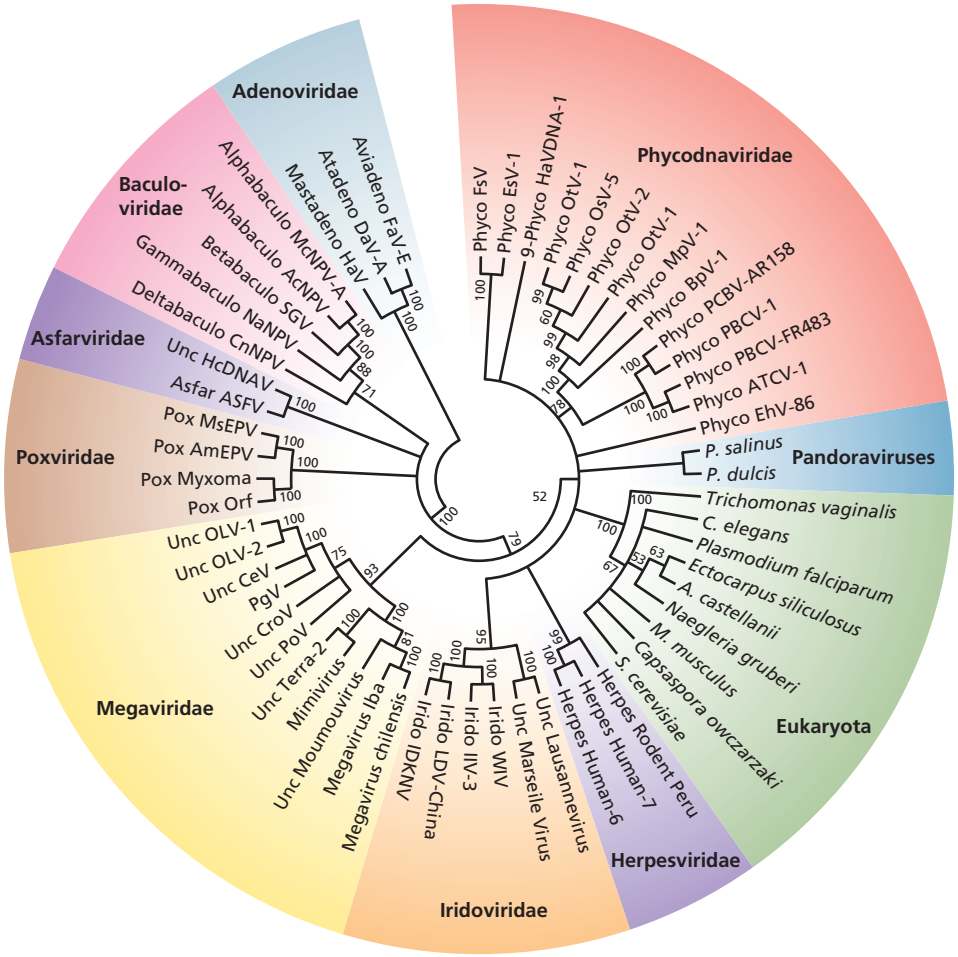


Figure 10.6 Phylogenetic analysis of the B-family DNA polymerase of nucleocytoplasmic large DNA viruses. A multiple alignment of 9 eukaryote and 50 viral DNA polymerase B sequences. Adapted from N. Philippe et al., *Science* **341:**281–286, 2013, with permission.

and an ancient iridovirus-related species, have unusual structural features or architecture (Box 10.7). A puzzling fact is that the majority of genes in the largest genomes have no obvious homology to sequences in the databases, a feature that is hard to reconcile with a gene reduction model. Some have proposed that these genes originated in a fourth domain of life that is either extinct or not yet discovered.

Genomes of many of the large DNA viruses have **sequence coherence**: they do not appear to be mosaics (i.e., the products of multiple recombination reactions between groups of genes [Box 10.4]). The homogeneity of genomes within a family and the lack of any obvious homology among families are difficult to explain using the model that they arose by the sequential acquisition of exogenous genes by a single, primordial precursor viral genome. However, technological advances in nucleic acid chemistry, sequencing, proteomics, and bioinformatic analyses have yielded data that are consistent with the idea that members of these families coexisted with the first primordial cells (Boxes 10.7 and 10.8).

We currently know the sequences of thousands of viral genomes (roughly equal numbers of RNA and DNA genomes are in the databases), and more are being added all the time. Furthermore, it is now possible to sample the ecosystem and determine the nature and diversity of viral genomes without having to propagate the viruses in the laboratory, for example by purifying particles from water samples and then sequencing all of the DNA released from them. This type of unbiased survey, called **metagenomic analysis**, has revealed remarkable diversity among known virus families. Even more amazing is the fact that the vast majority of viral sequences determined so far by these technologies represent previously **unknown** viral genomes. It may be that future analyses of this type will stimulate new or refined hypotheses about the origin of viruses and their place in the biosphere.

BOX 10.7

BACKGROUND

Discovery of virus giants: the largest known viral particles and genomes

An outbreak of pneumonia in Bradford, England, led to the isolation in 1992 of what was then the world's largest virus. Investigators attempted to isolate *Legionella*-like pathogens of amoebae from hospital cooling towers and recovered what appeared to be a small, Gram-positive bacterium. All attempts to identify it using universal bacterial 16S ribosomal RNA PCR amplification failed. Transmission electron microscopy of *Acanthamoeba polyphaga* infected with this agent revealed 400-nm icosahedral virus particles in the cytoplasm. Mature particles were surrounded by a profusion of fibers, the bases of which form an external protein capsid layer. The virus was named "mimivirus" because it mimicked a microbe.

Approximately 10 years later, this giant was eclipsed by discovery of two other pathogens of amoebae, one in marine sediment off the coast of central Chile and a second from a freshwater pond near Melbourne, Australia. These new giant virus particles exhibit no morphological or genomic resemblance to any previously defined virus families, and they have been proposed as the first members of a new genus, *Pandoravirus*. A third giant virus was discovered in 2014 in a sample from the frozen permafrost of Siberia, estimated to be >30,000 years old from carbon dating of associated late Pleistocene sediments. Amazingly, the virus was nevertheless still able to infect a cultured amoeba host. This ancient virus looks somewhat like pandoraviruses, but the replication cycle and genomic features are more like icosahedral DNA viruses. Another unique feature is the large fraction (21.2%) of the genome comprising multiple, regularly interspersed copies of 2-kb-long tandem arrays of a conserved 150-bp palindrome. A movie of the reproduction of this virus, called *Pithovirus sibericum*, in *Acanthamoeba castellanii*-infected cells can be found at http://www.pnas.org/content/suppl/2014/02/26/1320670111.DCSupplemental/sm01.mp4.

General features of these three giant viruses of amoebae are summarized below:

	Mimivirus	Pandoravirus	Pithovirus
Virion size	0.75 μm (diameter)	~1.0 μm (length)	1.5 μm (length)
Capsid shape	Icosahedral	Ovoid	Ovoid
Genome composition	AT-rich (>70%)	GC-rich (>61%)	AT-rich (64%)
Genome size (bp)	1.2×10^6	1.9×10^6–2.5×10^6	0.6×10^6
Genes (protein-coding)	911	2,556	467

Despite having a number of genes predicted to specify components of protein synthesis machinery, the giant viral genomes do not encode a complete translation system. As is typical for all viruses, they also undergo an eclipse period after entering their host cells. Similarities among the genomes of these viruses and some parasitic bacteria have prompted the suggestion that the viruses arose from a common cellular ancestor by gene loss. However, the presence of a large number of coding sequences with no homology to any genes in the current database (>50% of the total) implies that the putative cellular ancestor is now extinct or has not yet been discovered.

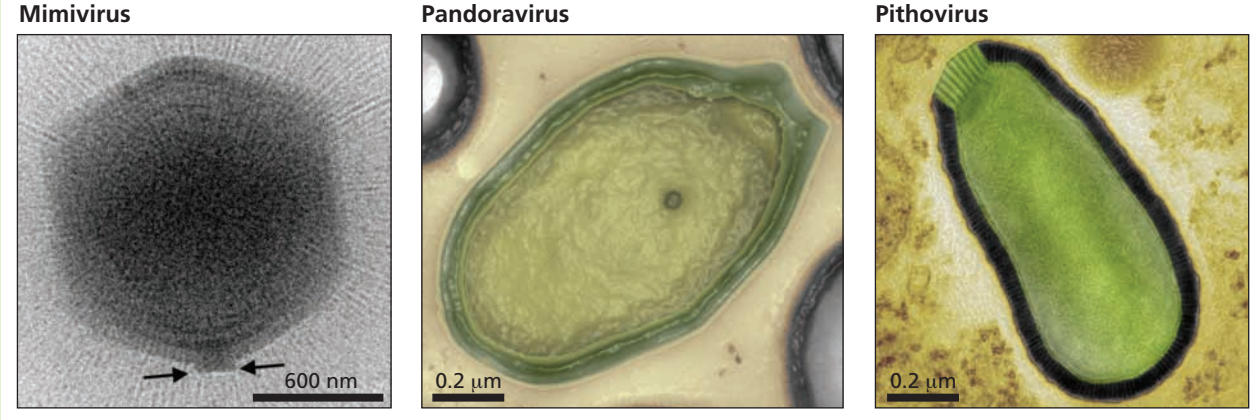
Legendre M, Bartoli J, Shmakova L, Jeudy S, Labadie K, Adrait A, Lescot M, Poirot O, Bertaux L, Bruley C, Couté Y, Rivkina E, Abergel C, Claverie JM. 2014. Thirty-thousand-year-old distant relative of giant icosahedral DNA viruses with a pandoravirus morphology. *Proc Natl Acad Sci U S A* **111:**4274–4279.

Philippe N, Legendre M, Doutre G, Couté Y, Poirot O, Lescot M, Arslan D, Seltzer V, Bertaux L, Bruley C, Garin J, Claverie JM, Abergel C. 2013. Pandoraviruses: amoeba viruses with genomes up to 2.5 Mb reaching that of parasitic eukaryotes. *Science* **341:**281–286.

Raoult D, Audic S, Robert C, Abergel C, Renesto P, Ogata H, La Scola B, Suzan M, Claverie JM. 2004. The 1.2-megabase genome sequence of Mimivirus. *Science* **306:**1344–1350.

Zauberman N, Mutsafi Y, Halevy DB, Shimoni E, Klein E, Xiao C, Sun S, Minsky A. 2008. Distinct DNA exit and packaging portals in the virus *Acanthamoeba polyphaga mimivirus*. *PLoS Biol* **6:**e114. doi:10.1371/journal.pbio.0060114.

Mimivirus, pandoravirus, and pithovirus particles. (Left) Cryo-electron micrograph showing the icosahedral capsid with copious attached fibers of mimivirus. Arrows mark the position of "stargate," the structure that allows release of the viral DNA following infection of a new host cell. Scale bar, 600 nm. Reprinted from Xiao C et al. *J Mol Biol* **353**:493–496, 2005, with permission. **(Center)** Electron micrograph of *Pandoravirus salinus*, the larger, Chilean isolate. Like mimivirus, pandoravirus particles are internalized via phagocytic vacuoles in host cells and the viral genome is later emptied into the cytoplasm through an apical pore (top, right corner) . Scale bar, 200 nm. **(Right)** Electron micrograph of *Pithovirus sibericum*. Shaped like the pandoravirus, this particle has a cork structure (top, left corner) that opens to allow viral contents to be emptied into the cytoplasm after fusion of an inner membrane with that of the phagocytic vesicles. Scale bar, 200 nm. *Pandoravirus* and *Pithovirus* images were kindly provided by Dr. Chantal Abergel, CNRS, Aix-Marseille Université.



BOX 10.8

DISCUSSION

Protein analyses point to primordial origins for the nucleocytoplasmic large DNA viruses

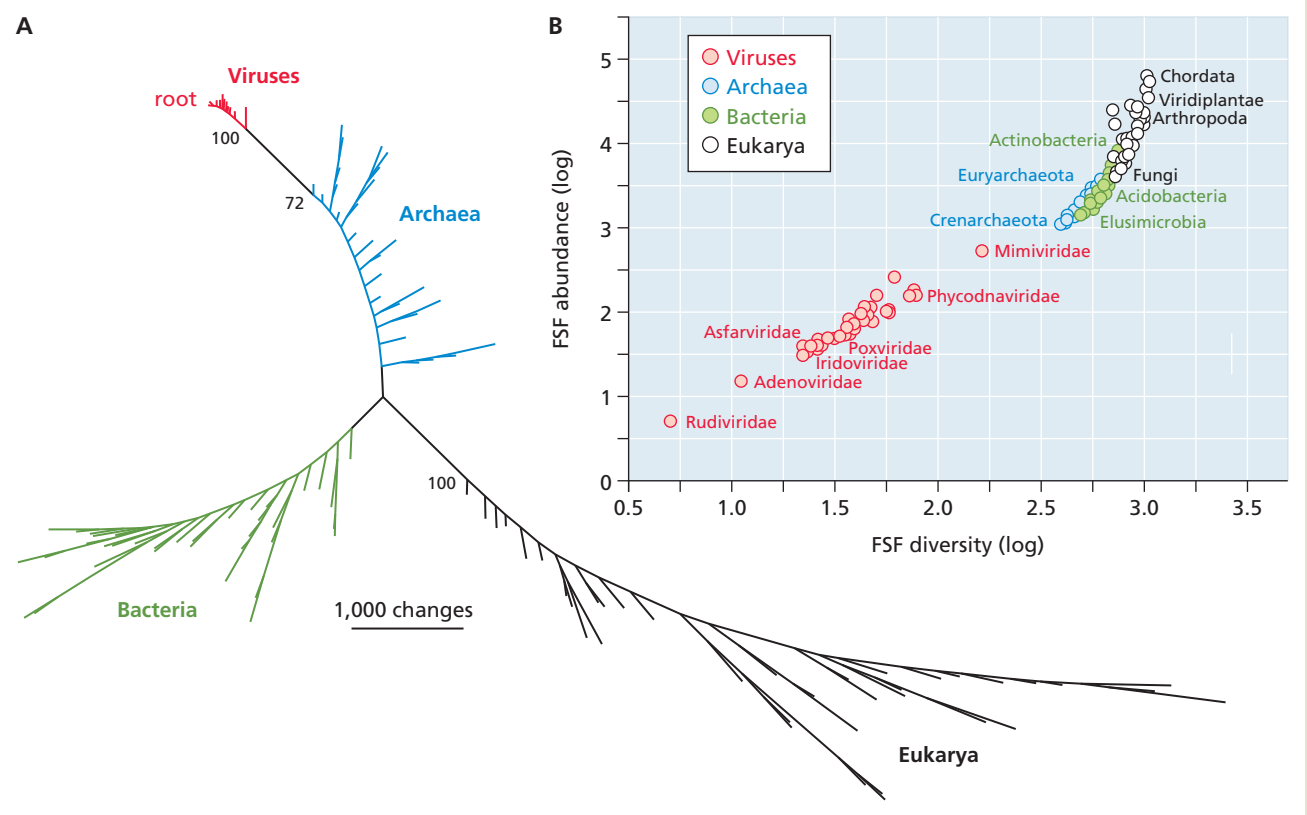
Protein domains that exhibit high amino acid sequence conservation (>30% identity) and the same major secondary structure (folds) can be grouped into families. Fold families that include members with similar function and are likely of common origin are defined as fold superfamilies (FSFs). The number of FSFs (~2,000) identified in the Structural Classification of Proteins website (version 1.75: http://scop.mrc-lmb.cam.ac.uk/scop/) is small in comparison to the approximately half-million protein sequence entries in the databases, indicating that a limited number of protein design units exist in nature. In multidomain proteins, such units are gained, lost, or rearranged during evolution.

Phylogenomic trees can be derived by assuming that the most abundant FSFs appeared earliest in evolution and have been reused as gene duplications and rearrangements increased proteomic repertoires. An analysis of FSFs in the proteomes of *Archaea*, *Bacteria*, *Eukarya*, and nucleocytoplasmic large DNA viruses showed that each forms a distinct group, with the viruses the most ancient and *Archaea* the second oldest. These large DNA viruses were also found to encode a number of FSFs with no current cellular representatives. It was therefore proposed that their primordial host predated or coexisted with the first living cells. A plot of abundance versus diversity of FSFs showed that the viruses have the simplest proteomes, and that structural diversity and organismal complexity are congruent. These and other results are consistent with the idea that the lack of diversity in these viral proteomes can be explained by a reductive evolutionary history in which, as with parasitic bacteria, nonessential genes have been lost.

Caetano-Anollés G, Nasir A. 2012. Benefits of using molecular structure and abundance in phylogenomic analysis. *Front Genet* **3:**172. doi:10.3389/fgene.2012.00172.

Nasir A, Kim KM, Caetano-Anolles G. 2012. Giant viruses coexisted with the cellular ancestors and represent a distinct supergroup along with superkingdoms Archaea, Bacteria and Eukarya. *BMC Evol Biol* **12:**156. doi:10.1186/1471-2148-12-156.

The universal tree of life and proteomic diversity. (A) An optimal most parsimonious phylogenomic tree describing the evolution of 200 proteomes (50 each from *Archaea*, *Bacteria*, *Eukarya*, and viruses) generated using the census of abundance of 1,739 protein domain FSFs. Terminal leaves of viruses, *Archaea*, *Eukarya*, and *Bacteria* are labeled in red, blue, black, and green, respectively. Numbers on the branches indicate bootstrap values. **(B)** FSF diversity (number of distinct FSFs in a proteome) plotted against FSF abundance (total number of FSFs that are encoded) for 200 proteomes. Major families/phyla/kingdoms are labeled. Reprinted from A. Nasir et al., *BMC Evol Biol* **12:**156, 2012, with permission.



Host-Virus Relationships Drive Evolution

Although the primordial history of viruses cannot be known, nucleic acid sequence analyses have identified many relationships among contemporary viruses and their host species, providing considerable insight into viral evolution.

DNA Virus Relationships

Papillomaviruses and Polyomaviruses

Coevolution with a host is a characteristic of small DNA viruses, the parvoviruses, polyomaviruses, and papillomaviruses. The evidence for coevolution comes from finding close association of a given viral DNA sequence with a particular host group. The linkage of host to virus was particularly striking when human papillomavirus types 16 and 18 were compared: the distribution of these viral genomes is congruent with the racial and geographic distribution of the human population. Another example of the same phenomenon is provided by JC virus, a ubiquitous human polyomavirus associated with a rare, fatal brain infection of oligodendrocytes. This virus exists as five or more genotypes identified in the United States, Africa, and parts of Europe and Asia. Recent polymerase chain reaction analyses of subtypes of JC and BK polyomaviruses indicate that they not only coevolved with humans, but also did so within specific human subgroups. Some studies have indicated that these viruses may provide convenient markers for human migrations in Asia and the Americas in prehistoric and modern times.

How can virus evolution be linked with specific human populations in a manner akin to vertical transmission of a host gene? We can begin to appreciate this perhaps counterintuitive phenomenon from the unusual biology of human papillomaviruses. Infection of the basal keratinocytes of adult skin leads to viral reproduction that is coordinated with cellular differentiation. The final step, assembly of progeny virus particles, occurs only as cells undergo terminal differentiation near the skin surface. Mothers infect newborns with high efficiency, because of close contact or reactivation of persistent virus during pregnancy or birth. The infection therefore appears to spread vertically, in preference to the more standard horizontal spread between hosts. This mode of transmission is the predominant mechanism for papillomaviruses and polyomaviruses. It stands in contrast to that observed for most acutely infecting viruses of humans, which are spread by aerosols, contaminated water, or food.

Herpesviruses

The three main subfamilies of the family *Herpesviridae* (*Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*) are readily distinguished by genome sequence analysis even though the original taxonomic separation of these families was based on general, often arbitrary, biological properties. Researchers have related the timescale of herpesviral genome evolution to that of the hosts. For most of these viruses, points of sequence divergence coincide with well-established points of host divergence. The conclusion is that an early herpesvirus infected an ancient host progenitor, and subsequent viruses developed by coevolution with their hosts. Consistent with this conclusion, the genomes of **all** members of the three major subfamilies that have been sequenced contain a core block of genes, often organized in similar clusters in the genome.

Our current best estimate is that the three major groups of herpesviruses arose ~180 million to 220 million years ago. This implies that the three subfamilies must have been in existence before mammals spread over the earth 60 million to 80 million years ago. Fish, oyster, and amphibian herpesviruses have virtually identical architecture but little or no sequence homology to the major subfamilies, and therefore must represent a very early branch of this ancient family.

RNA Virus Relationships

Relationships among RNA viruses can also be deduced from nucleotide sequence analyses, but the high rates at which mutations accumulate in these genomes impose some difficulties. Moreover, genomes of RNA viruses are often small and, in contrast to those of the large DNA viruses, contain few, if any, genes in common with a host that might be used to correlate virus and host evolution. Nevertheless, when nucleotide sequences of many (+) and (−) strand RNA viral genomes are compared, blocks of genes that encode proteins with similar functions can be identified. Common coding strategies can also be inferred. These groups are often called "supergroups" because the similarities suggest a common ancestry.

An obvious feature common to the sequences of many (−) strand RNA genomes is the limited number of genes that encode proteins (as few as 4 and not more than 13). These proteins can be placed in one of three functional classes: core proteins that interact with the RNA genome, envelope glycoproteins that are required for attachment and entry of virus particles, and a polymerase required for replication and mRNA synthesis (Fig. 10.7).

The (+) strand RNA viruses (excluding the retroviruses) are the largest and most diverse subdivision. The number of genes that encode proteins in their genomes ranges from 3 to more than 12 and, as with the (−) strand RNA viruses, the proteins can be divided into the same three groups by function, although in this case their organization is not necessarily colinear (Fig. 10.8). A unifying feature is that the RNA polymerase gene appears to be the most highly conserved, implying that it arose once in the evolution of these viruses. Consequently, these viruses are organized into three virus supergroups, based on similarities in their polymerases. As each of the supergroups contains members that infect a broad variety of animals and plants, an ancestor present before the

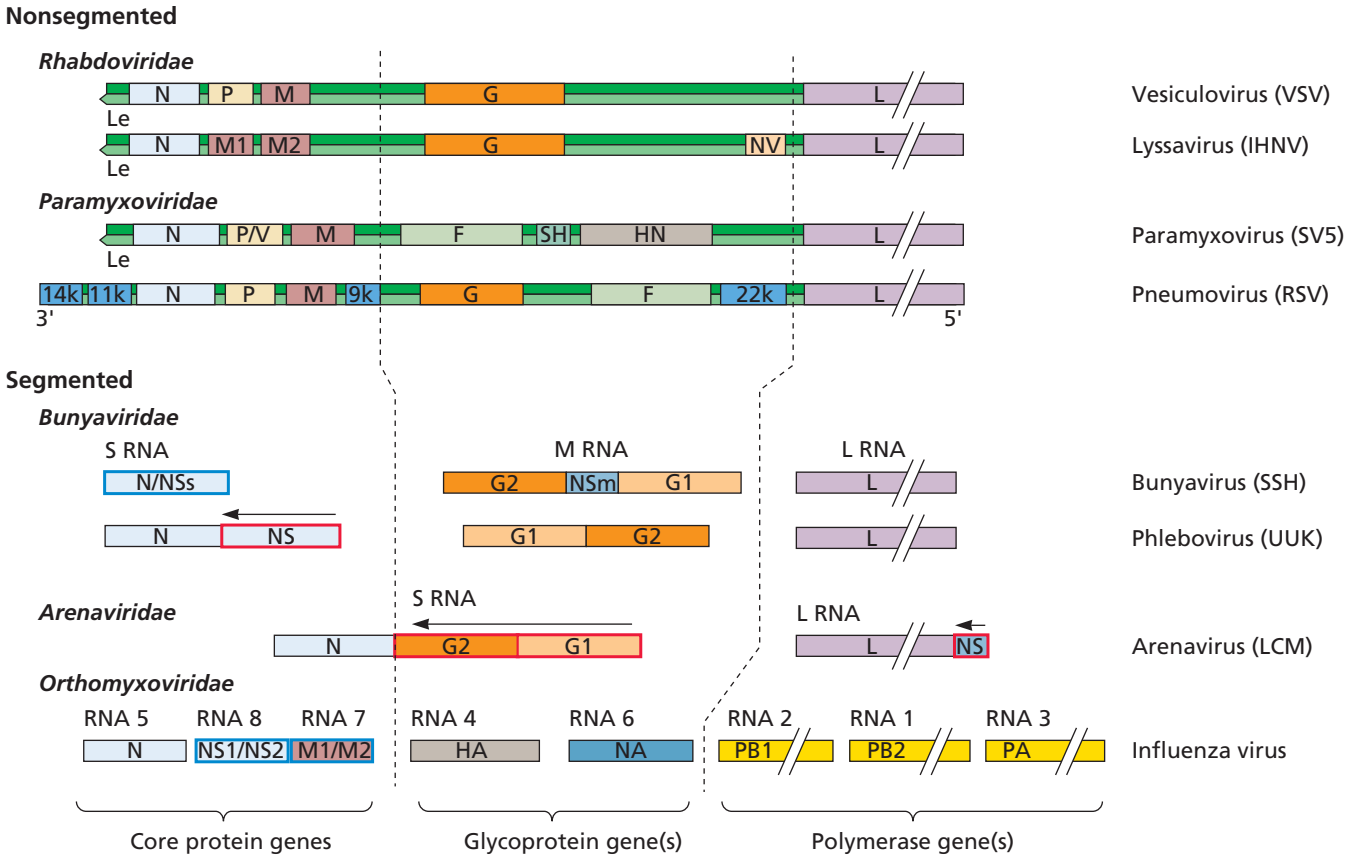


Figure 10.7 The genetic maps of selected (−) strand RNA viral genomes. Maps of the genes of *Rhabdoviridae*, *Paramyxoviridae*, *Bunyaviridae*, *Arenaviridae*, and *Orthomyxoviridae* are aligned to illustrate the similarity of gene products. The individual gene segments of the *Orthomyxoviridae* are arranged according to functional similarity to the two other groups of segmented viruses. Within a given genome, the genes are approximately to scale. For segmented genomes, blue-outlined genes are those that encode multiple proteins from different open reading frames. Red-outlined genes are expressed by the ambisense strategy, as indicated by the arrow. Virus abbreviations: VSV, vesicular stomatitis virus; IHNV, infectious hematopoietic necrosis virus; SV5, simian virus 5; RSV, respiratory syncytial virus; SSH, snowshoe hare virus; UUK, Uukuniemi virus; LCM, lymphocytic choriomeningitis virus. Le is a nontranslated leader sequence. Gene product abbreviations: N, nucleoprotein; P, phosphoprotein; M (M1 and M2), matrix proteins; G (G1 and G2), membrane glycoproteins; F, fusion glycoprotein; HN, hemagglutinin/neuraminidase glycoprotein; L, replicase; NA, neuraminidase glycoprotein; HA, hemagglutinin glycoprotein; NS (NV, SH, NSs, and NSm), nonstructural proteins; PB1, PB2, and PA, components of the influenza virus replicase. Figure derived from J. H. Strauss and E. G. Strauss, *Microbiol Rev* **58:**491–562, 1994, with permission.

separation of these kingdoms might have provided the primordial RNA polymerase gene. Alternatively, the ancestral (+) strand virus could have infected hosts in all branches of the tree of life.

As discussed above for herpesviruses, it has been possible to determine the timescale of RNA virus evolution by using host divergence times. A good example is the retrovirus simian foamy virus, for which there is a 30-million-year match between host and virus phylogenetic trees. The use of endogenous viral sequences to determine the timescale of viral evolution places foamy viruses in mammals for the past 100 million years (see "Lessons from Paleovirology" below).

Influenza Virus

Study of the ecology and biology of influenza has shown that the same virus population can infect many different species, and each host species imposes new selections for reproduction and spread of the infection. As a result, the influenza virus gene pool is immense, with a dynamic ebb and flow of genetic information as the virus is transmitted among many different animals. Large-scale sequencing has provided a view of the state of the viral gene pool at various points in time and space, during transmission from human to animal, animal to human, and human to human. In one analysis alone, a consortium of scientists sequenced >200 human influenza virus

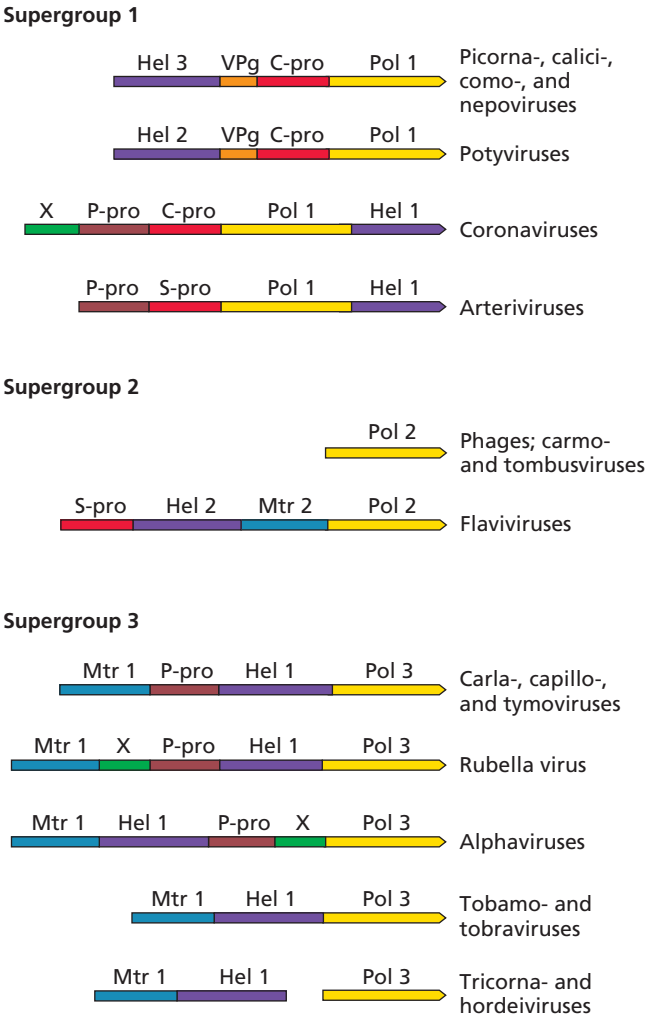


Figure 10.8 RNA virus genomes and evolution. Organization of (+) strand RNA genomes. The genomes of (+) strand RNA viruses comprise several genes for replicative functions that have been mixed and matched in selected combinations over time. These functions include a helicase (Hel, purple), a genome-linked protein (VPg, orange), a chymotrypsin-like protease (C- or S-pro, red), a polymerase (Pol, yellow), a papain-like protease (P-pro, brown), a methyltransferase (Mtr, dark blue), and a region of unknown function (X, green). Differences in the polymerase gene define the three supergroups. In this figure, the genes are not shown to scale and the structural proteins have been omitted for clarity. Derived from J. H. Strauss and E. G. Strauss, *Microbiol Rev* **58:**491–562, 1994, with permission.

genomes and collected almost 3 million bases of sequence. One salient finding was that a given influenza virus population in circulation contains multiple lineages at any time. In addition, alternative minor lineages exchange information with the dominant one. As selection pressures change, the numbers of distinct immune escape mutants rise and fall, as do the numbers of mutants with alterations in receptor-binding affinity.

Important clues to the epidemiology of influenza virus came from the sequencing and analysis of the genomes of >1,300 influenza A virus isolates from various geographic locations. It was clear that the viral genome changes by frequent gene reassortment and occasional bottlenecks of strong selection. More importantly, the study suggests that new antigenic subtypes have different dynamics but that all follow a classical "sink-source" model of viral ecology: in this model, antigenic variants emerge at intervals from a persisting reservoir in the tropics (the source) and spread to temperate regions, where they have only a transient existence before disappearing (the sink) (Fig. 10.9).

The Protovirus Hypothesis for Retroviruses

Hypotheses about the origin of retroviruses and their evolution may be easier to propose than for any other virus group, because of their distinctive association with their hosts. Upon infection, accompanying reverse transcriptase and integrase enzymes convert their RNA genomes into a DNA copy, which is then inserted into the host DNA (the provirus). Howard Temin, who shared the Nobel Prize for the discovery of reverse transcriptase, first proposed the "protovirus theory" for the origin of this virus family. This theory posits that a cellular reverse transcriptase-like enzyme copied segments of cellular RNA into DNA molecules that were then inserted into the genome to form retroelements. These DNA segments in turn acquired more sequences, including those encoding RNase H, integrase, regulatory sequences, and structural genes. This hypothesis predicts that evidence of such sequential acquisitions might exist in the genomes of mammals and other species. Indeed, many of the proposed intermediates are found in abundance, including long interspersed nuclear elements (LINEs), retrotransposons, and a variety of endogenous retroviruses (see Volume I, Chapter 7). As reverse transcriptase-encoding, mobile elements are present in bacterial, archaeal, and bacteriophage genomes, this function is likely to have arisen quite early in evolutionary history. Recent genomic and computational analyses indicate that the retroviral reverse transcriptase was likely derived from a retrotransposon precursor related to LINEs; RNase H, integrase, and the regulatory long terminal repeats (LTRs) were added later during evolution of retroviral genomes.

Lessons from Paleovirology

Traces of virus-derived sequences are present in all living species but historically have been considered to be mostly genomic "junk." In the last decade or so, the rapid expansion of nucleic acid sequence databases and new methods of genomic analyses have altered that perception, providing unique insights into the nature and consequences of viral and host interactions over evolutionary time.

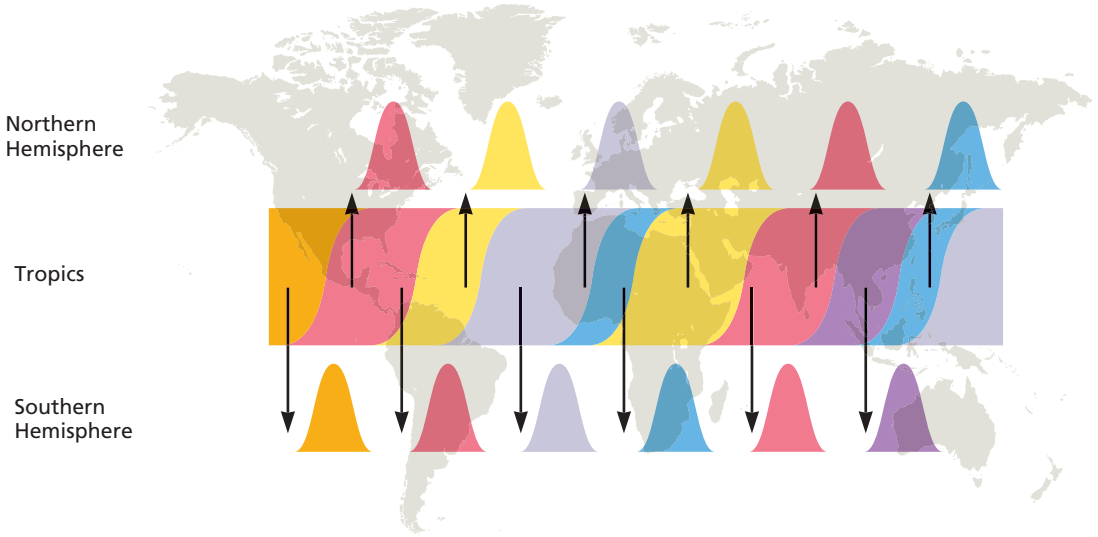


Figure 10.9 The genomic and epidemiological dynamics of human influenza A virus. Viral genetic and antigenic diversity (shown by different colors) is continuously generated in a reservoir, or "source" population, perhaps represented by the tropics, before being exported to "sink" populations in the Northern and Southern Hemispheres, as shown by the arrows. The continuous transmission of influenza A virus in the source population, and hence its larger effective population size, allows natural selection for antigenic diversity to proceed more efficiently than in the sink populations that are afflicted by major seasonal bottlenecks. Reprinted from A. Rambaut et al., *Nature* **453:**615–619, 2008, with permission.

Endogenous Retroviruses

We now know that endogenous retroviruses are abundant in vertebrate genomes; they account for 6 to 14% of all genomes analyzed to date. In humans, endogenous retroviruses comprise ~8% of genomic DNA, almost an order of magnitude greater than that encoding all of our proteins! Endogenous retroviral sequences originate from proviral DNA integrations into the genomes of the host's germ line cells that are passed on through subsequent generations. Consequently, a comparison of the insertion sites of endogenous retroviruses in present-day vertebrate species, and knowledge of the species' evolutionary relationships, can allow one to estimate when shared endogenous sequences were inserted into an ancestral host germ line. Such analyses have revealed that most human endogenous retroviruses (HERVs) are at least 10 million to 50 million years old and were circulating on the earth long before the emergence of *Homo sapiens*.

Most endogenous retroviral proviruses are defective, but some do retain an ability to replicate, and if their transcription is not repressed, they can reemerge as infectious agents. For example, the high incidence of spontaneous leukemogenesis in the well-studied AKR strain of mice has been traced to the production of replication-competent leukemic viruses that arise via recombination between the genomes of three different endogenous retroviruses. Of course, endogenous retroviruses can also serve as genetic reservoirs for recombination with exogenously infecting viruses. Although all of the endogenous retroviruses in the human genome are defective, scientists have managed to regenerate one of the "youngest" of these ancient viruses, which was circulating in ancestral species ~1 million years ago. They accomplished this feat by deriving a nondefective consensus sequence from several endogenous members of the family. Shades of *Frankenstein* (or maybe *Jurassic Park*), the resulting proviral clone, called HERV-K_{con}, forms ("raised from the dead") infectious particles when introduced into human cells!

Endogenous retroviruses can have profound effects on the evolution and function of their host's genome. For example, recombination between endogenous retroviral sequences integrated at different loci can account for several large-scale deletions, duplications, and other types of chromosomal reshuffling that occurred during the evolution of primate genomes. In humans, such recombination has contributed to the extensive duplication of gene blocks that comprise the major histocompatibility complex class I locus. The diversity that then arose in such duplications, as well as heterozygosity at this locus, confers a strong selective advantage on human populations in the battle against pathogenic agents. In addition, transcription factor-binding sites and regulatory sequences in the LTRs that flank endogenous retroviruses can modify the expression of neighboring host genes by providing alternate promoters and enhancers. In some cases, inserted LTRs may be responsive to tissue-specific regulators: expression of the human salivary amylase is controlled by

an endogenous LTR and may have enabled our ancestors to thrive on a starch diet. Finally, some endogenous retroviral proteins have been repurposed by their hosts to serve new functions. One example is the resistance to retroviral infection of certain mouse strains, which is conferred by the expression of endogenous sequences related to a retroviral capsid gene (called *Fv-1*). Similarly, retroviral Env proteins called syncytins have been coopted independently by various host species at least seven times during evolution. These fusogenic proteins are essential for formation of placental syncytiotrophoblasts, which allow passage of essential nutrients and may protect a fetus from the maternal immune system. Indeed, selection and fixation of this retroviral protein was a pivotal step in the evolution of placental mammals.

DNA Fossils Derived from Other RNA Viral Genomes

Although the existence of endogenous retroviruses in ancient evolutionary time has long been appreciated, until quite recently the ages of other RNA viruses remained a mystery. Approximations could be made only by comparison of currently circulating representatives and measurements of their rates of genetic change. It was not until databases for numerous viruses and vertebrate species became available that sequences related to other RNA viruses were discovered in host genomes. The first hints of such unexpected inheritance came from reports that sequences related to the flaviviruses and picornaviruses were incorporated into the genomes of plants and insects. These findings stimulated comprehensive bioinformatic analyses in which the sequences of all currently known viruses with RNA genomes were matched against the now extensive library of vertebrate genomes. The results revealed that as long as 30 million to 40 million years ago almost half of the vertebrate species analyzed had acquired sequences related to genes in two currently circulating single (−) strand RNA virus families, the filoviruses and the bornaviruses, deadly pathogens that cause lethal hemorrhagic fever and neurological disease, respectively (Fig. 10.10, left). Some of these sequence fossils could be traced back ~90 million years. Because host genomes are also subject to genetic drift, and sequences can change beyond recognition, this is close to the limit of such analyses. The conservation and current-day expression of some of these endogenous sequences suggest that they may have afforded a selective advantage in vertebrate populations at some time. Integration of sequences related to other single-strand RNA viruses was also found in the genomes of various insect species. Analysis of the nucleotides that flanked many of these viral fossils indicates that they were likely derived from viral mRNAs that were reverse-transcribed and integrated into the host genome by mobile LINEs (see Volume I, Fig 7.12). It is noteworthy that LINEs tend to be active in germ line cells, a necessary requirement for fixation.

The error rate for the replication of host DNA is much lower than the rates of the RNA-dependent RNA synthesis of currently circulating viruses. Consequently, the ability to detect ancient sequences that are related to particular currently circulating RNA virus families may be due partly to the fact that their genome sequences are relatively stable, as appears to be the case for the bornaviruses. It seems probable that LINEs have inserted DNA copies of the mRNAs of many other viruses into their host genomes over evolutionary time, but our ability to recognize such sequences has been clouded by the high rate of mutation in most present-day relatives.

Endogenous Sequences from DNA Viruses

A survey of vertebrate genomes for sequences related to DNA viruses revealed numerous examples of the integration of sequences related to members of two single (−) strand DNA viral families, the circoviruses and the parvoviruses. These were found broadly distributed among ~70% of the vertebrate species tested. Some of these insertions are >50 million years old, but others have occurred more recently (Fig. 10.10, right). Both of these virus families have tiny genomes that encode only two proteins, replicase and capsid. Host enzymes are recruited by the replicase to hairpin regions in the viral genomes where self-primed viral DNA synthesis is initiated. The parvovirus, adenovirus-associated virus is known to be able to insert its DNA into its host genome at sequences that are recognized by the viral replicase protein (Volume I, Chapter 9). It seems likely, therefore, that the germ line insertions that gave rise to the endogenous sequences result from the occasional copying of circovirus and parvovirus DNA at loci in their host genomes that resemble viral replication hairpin regions.

As contemporary hepadnavirus genomes are known to be incorporated randomly into host genomes via nonhomologous recombination, and a member of this virus family infects birds, it is not so surprising that sequences related to this double-strand DNA hepadnavirus genome have been detected in the zebra finch genome.

The Host-Virus "Arms Race"

The discovery of ancient viral fossil sequences paints a picture in which all current living forms have evolved in a virtual sea of viruses that are capable of rapid evolution. We can assume that host genes associated with antiviral defense evolved by mutation, with individuals who encoded ineffective alleles dying from infection and thereby being eliminated from the population. Virus populations with compensatory mutations would then emerge, exerting selective pressure back on the host in an ever-escalating, molecular "arms race." (For more insight into the arms race concept see the comments of Dr. Harmit Malik: http://bit.ly/Virology_Malik.) Constitutively expressed host cell genes encode antiviral proteins that function in a cell-autonomous manner. These proteins

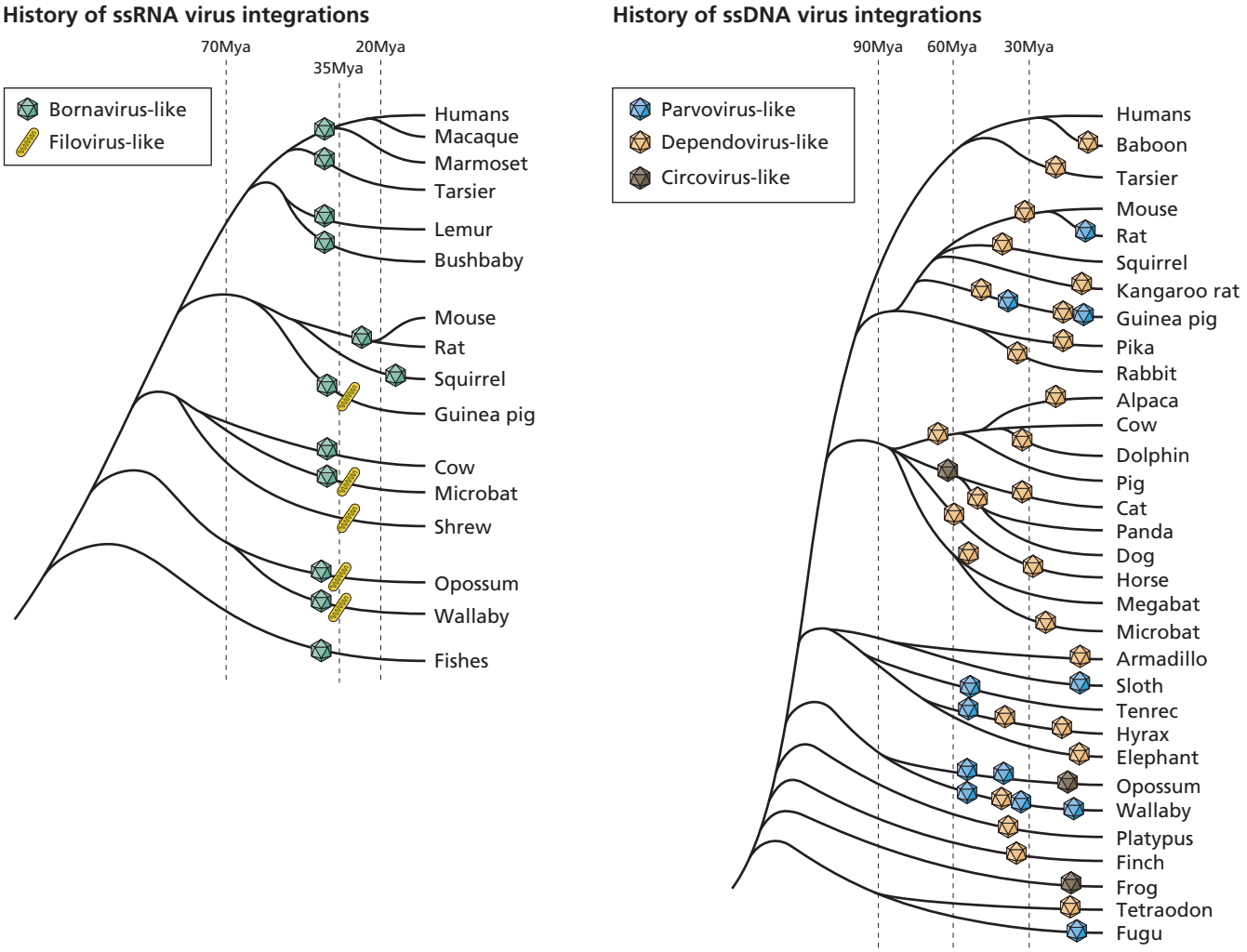


Figure 10.10 Integration of nonretroviral sequences into vertebrate genomes. (Left) Comparisons of sequences representing all known single-stranded (nonretroviral) RNA (ssRNA) genomes with the genomes of vertebrate species revealed that almost all of the nearly 80 integrations identified were related to only two viral families, the filoviruses and the bornaviruses. Based on signature landmarks, some, and perhaps all, of the endogenous virus-like DNA sequences appear to be LINE-facilitated integrations of viral mRNAs. The integrations occurred in a limited time frame, ~40 million years ago, coincident with the rapid evolutionary diversification of mammalian species. It is noteworthy that an analysis of pseudogene formation suggests that the predominant mammalian LINE-1 underwent a peak of retrotransposition activity around this time. The reason for finding mRNA copies of genes from only these viral families is unknown. (Right) Similar comparisons with ssDNA viral genomes also revealed a preference for integration of sequences from certain virus families, in this case the parvoviruses and circoviruses. However, these integrations occurred both throughout evolution, stretching back to >90 million years ago, and in the present time. It has been suggested that shared features of the replication strategies of these viruses may explain the broad history and high incidence of their integration. Times of the viral gene integrations are approximate. Adapted from V. A. Belyi et al., *PLoS Pathog* 6:e1001030, 2010, and V. A. Belyi et al., *J Virol* 84:12458–12462, 2010, with permission.

contribute to intrinsic cellular defenses by interacting directly with viral components and inhibiting the reproduction of infecting viruses, at various stages and by a variety of mechanisms (Chapter 3). They include, among others, the Apobec3 family of cytidine deaminases, which create mutations in viral DNAs; the tripartite motif (Trim) proteins that interact with the capsids of infecting retroviruses and block uncoating; and the membrane protein tetherin, which inhibits budding of a number of enveloped viruses from the cell surface. These proteins have been studied most intensely in the context of the primate immunodeficiency viruses that have altered capsid proteins, and the viral accessory protein Vpr, respectively, each of which provides a countering function (described in Chapter 6). Points of contact between the viral and host

BOX 10.9

EXPERIMENTS

Host-virus arms race and the transferrin receptor

Located at the cell surface, the dimeric transferrin receptor protein (TfR1) controls the uptake of iron—a "housekeeping" function essential for all living cells. The observation that this protein is also the receptor for a variety of viruses prompted an investigation into how opposing selective pressures, avoiding infection and maintaining iron uptake, might be balanced during evolution of the *TFR1* gene. By analysis of this gene in a number of evolutionarily related rodent species, it was found that, while most of the amino acids in the encoded proteins were conserved, several residues were quite variable, with dN/dS > 1, indicating a high probability of positive selection. Using the crystal structure of the ectodomain of human TfR1 as a model, it was noted that these residues are included in the known overlapping binding sites for arenaviruses and the retrovirus mouse mammary tumor virus, but separate from the transferrin-binding site. Furthermore, experiments showed that naturally occurring substitutions of these residues block virus entry while preserving iron uptake by both rodent and human TfR1.

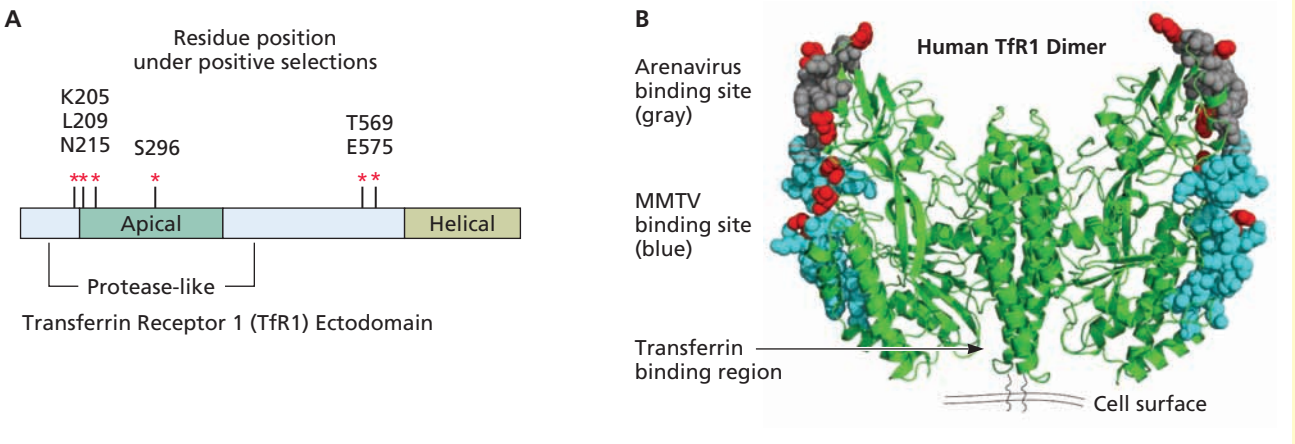
The TfR1 protein is also the receptor for parvoviruses that infect dogs and related species of carnivores. Residues that show evidence for positive selection in these receptors have also been mapped to the ectodomain in a region close to the mouse mammary tumor virus-binding site and distinct from the transferrin-binding site. These results indicate that by the repeated changing of codons in a few key places, in a cyclic game of rock-paper-scissors between the *TFR1* gene and the viral receptor-binding genes, virus entry can be blocked while iron uptake function is maintained in host cells.

Demogines A, Abraham J, Choe H, Farzan M, Sawyer SL. 2013. Dual host-virus arms races shape an essential housekeeping protein. *PLoS Biol* **11:**e1001571. doi:10.1371/journal.pbio.1001571.

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Identification of residues in the TfR1 protein that are under positive selection and in binding sites for two viral families. (A) Red stars represent the six rapidly evolving codon positions identified in rodent TfR1, mapped to a linear schematic of the TfR1 ectodomain. The amino acid encoded by human TfR1 at each of these positions is indicated. **(B)** Residue positions under positive selection are indicated in red on the structure of human TfR1 (PDB 1CX8). TfR1 is a homodimer, and the six sites of positive selection are indicated on the outer edge of each monomer. Known binding regions on TfR1 for the arenavirus Machupo virus GP protein and the retrovirus mouse mammary tumor virus (MMTV) Env protein are indicated in gray and blue, respectively, and the small region where they overlap is indicated with cross-hatching. The binding region for transferrin is indicated with a black arrow. Adapted from A. Demogines et al., *PLoS Biol* **11:**e1001571, 2013, with permission.



proteins that participate in such arms races can be identified by surveying orthologous genes in related host species for codons that exhibit a higher proportion of nucleotide substitutions that change an amino acid (called a nonsynonymous substitution, dN) than those that are silent (called synonymous, dS). A ratio of dN/dS > 1 indicates a high probability of positive selection. An example of the power of this approach, applied to viral-host receptor interactions in nonprimate species, is described in Box 10.9.

Perspectives

The relationships of viruses and their hosts are in constant flux. The combined perspectives of evolutionary biologists, ecologists, and epidemiologists are needed to decipher both the nature and consequences of these relationships. At present, the extent and significance of interplay between environment and genes, as well as the interactions of virus and host populations, are largely unknown. For viruses, rapid production of large numbers of progeny, tolerance to changes in host populations,

and a capacity to produce enormous genetic diversity provide the adaptive palette that ensures their survival (Box 10.10). Host survival in this competition has depended on the evolution of intrinsic, innate, and adaptive immune defense systems, which are capable of recognizing and then blocking reproduction of or destroying invading viruses.

Present-day hosts represent progeny of survivors of ancient infections. Until quite recently, we have been limited to considering virus and host evolution mainly in the context of the currently circulating populations, to which we have access. With the exception of the retroviruses, there had been no lasting record of other viral families to estimate how old they might be or how they may have changed over eons. As is now clear from the constantly expanding DNA fossil record, experience with numerous other viral families over vast stretches of evolutionary time is also recorded in the gene pools of host survivors.

Viral infections have far-reaching effects, ranging from shaping the host immune system in survivors to eliminating entire populations. However, given the ever-changing viral populations and the drastic modifications of the ecosystem that have accompanied the current human population explosion, we are hard-pressed to predict the future. The trajectory of evolution has long been a subject of deliberation, and both scientists and philosophers have pondered the parameters that may determine its paths. Virology provides a productive area for research in this area, with the caveat that outcomes cannot be judged as "good" or "bad." From the first principle that there is no goal but survival, we can deduce that evolution does not move a viral genome from "simple" to "complex" or along some trajectory aimed at "perfection." Change is effected by elimination of the ill-adapted of the moment, not by the prospect of building something better for some unknown future.

BOX 10.10

BACKGROUND

The world's supply of human immunodeficiency virus genomes provides remarkable opportunity for selection

Tens of millions of humans are infected by human immunodeficiency virus. Before the end stage of disease, each infected individual produces billions of viral genomes per day. As a result, $>10^{16}$ genomes are produced each day on the planet. Almost every genome has a mutation, and every infected human harbors viral genomes with multiple changes resulting from recombination and selection. Practically speaking, these large numbers provide an amazing pool of diversity. For example, thousands of times each day simply by chance, mutants arise that are resistant to **every combination** of the current >20 Food and Drug Administration-approved antiviral drugs or any others that might be developed in the future to treat AIDS.

Global view of HIV infection. More than 30 million people are living with HIV.



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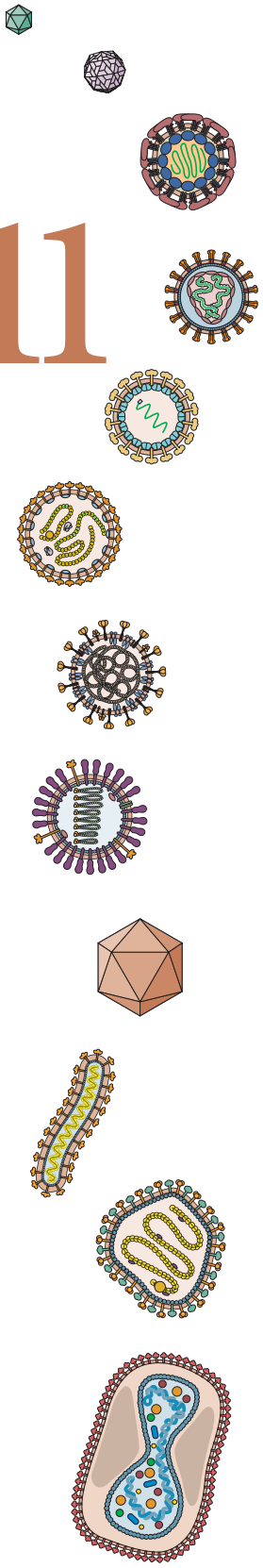
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11 Emergence

The Spectrum of Host-Virus Interactions
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- The Evolving Host-Virus Interaction
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- Emerging Viral Infections Illuminate Immediate Problems and Issues
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- Preventing Emerging Virus Infections

Perspectives

References

LINKS FOR CHAPTER 11

- *Video: Interview with Dr. Ian Lipkin*
  http://bit.ly/Virology_Lipkin
- *The real Batman, Linfa Wang*
  http://bit.ly/Virology_Twiv296
- *Heartland virus disease*
  http://bit.ly/Virology_3-28-14
- *MERS-coronavirus in dromedary camels*
  http://bit.ly/Virology_2-26-14
- *Influenza A viruses in bats*
  http://bit.ly/Virology_11-12-13
- *An epidemic of porcine diarrhea in North America*
  http://bit.ly/Virology_1-28-14

We live in a dancing matrix of viruses; they dart, rather like bees, from organism to organism, from plant to insect to mammal to me and back again, and into the sea, tugging along pieces of this genome, strings of genes from that, transplanting grafts of DNA, passing around heredity as though at a great party.

LEWIS THOMAS
Lives of the Cell, 1974

Humans have suffered for millions of years from infectious diseases. However, since the rise of agriculture (the past 11,000 years), new infectious agents have invaded human populations, primarily because these infections (e.g., measles and smallpox) can be sustained only in large, dense populations that were unknown before agriculture and commerce. The source of such emerging infectious agents is a popular topic of research, debate, and concern.

We define an **emerging virus** as the causative agent of a new or hitherto unrecognized infection. Occasionally, emerging infections are manifestations of expanded host range with an increase in disease that was not previously obvious. More generally, emerging infections of humans reflect transmission of a virus from a wild or domesticated animal, with attendant human disease (**zoonotic infections**). Occasionally, such cross-species infection will establish a new virus in a population (e.g., human immunodeficiency viruses moving from chimpanzees to humans). On the other hand, some cross-species infections, although not without consequence, cannot be sustained (e.g., Ebola and Marburg viruses moving from bats to humans).

While the term "emerging virus" became common in the popular press in the 1990s (usually with dire implications ["killer viruses on the loose"]), such infections are not new to virologists, epidemiologists, or public health officials but have long been recognized as an important manifestation of virus evolution. Parameters that drive such evolution include changes brought about by unprecedented human population growth and large-scale disturbances of ecosystems that result from human occupation of almost every corner of the planet (Fig. 11.1). In recent years, emerging infections have been detected with increasing frequency, thanks to advances in technology and better communication about disease outbreaks. Indeed, global communication has brought some emerging viral infections to center stage on the local news. Anyone with access to television, radio, the Internet, or newspapers has heard something about the AIDS virus, human immunodeficiency virus type 1, Ebola virus, and certainly H5N1 avian influenza virus. Examples of zoonotic infections and conditions that contributed to the emergence of particular viruses are provided in Table 11.1. Despite the variety of virus families and different geographic locations of these outbreaks, some common parameters do exist. These parameters define the rules of engagement for viruses and their potential hosts.

The Spectrum of Host-Virus Interactions

Four general types of interactions between hosts and viruses can be recognized: **stable**, **evolving**, **dead-end**, and **resistant** (Fig. 11.2). These four categories identify the extremes of

PRINCIPLES *Emergence*

- An emerging virus is defined as the causative agent of a new or previously unrecognized virus infection in a population.
- Zoonoses are infections of humans by viruses that preexist in stable relationships with nonhuman hosts. Most emerging viruses come from zoonotic infections.
- There are four general types of interaction between a virus and its host: stable, evolving, dead-end, and resistant.
  - Stable host-virus interactions are those in which both participants survive and reproduce.
  - The hallmarks of the evolving host-virus interaction are instability and unpredictability.
  - The dead-end interaction, in which the virus is not transmitted to other members of the new host species, is a frequent outcome of cross-species infection.
  - The **resistant host** interaction represents situations in which the host blocks infection completely.
- The vast majority of human encounters with viruses are uneventful because host cells are not susceptible or the body's defenses are so strong that potential invaders cannot initiate an infection.
- Outcomes of a virus-host interaction depend on many factors, including ecological, host, and viral parameters.
- The predominant parameters for spread of infection are the population density and the age and health of individuals in that population.
- Even when large numbers of susceptible individuals are inoculated with an equivalent quantity of a virulent virus, the results can be quite variable, and not everyone succumbs to disease.
- The rate of virus discovery has risen with the development of new technologies; it is now possible to detect and characterize unknown viruses with comparative ease and speed.
- Virus names are often misleading.
- Despite much new knowledge and advances in technologies to detect viruses, we cannot predict the next pandemic.

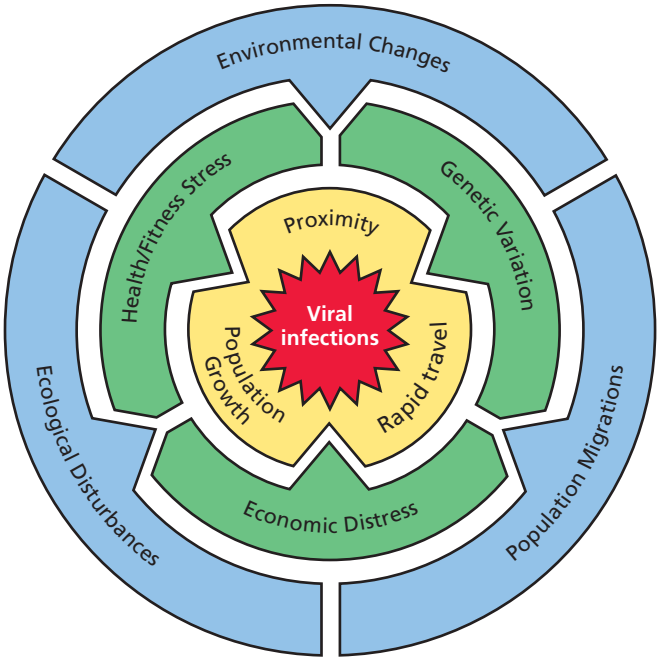


Figure 11.1 Multiple parameters converge to promote emerging viral infections. In the ecology of virus-host interactions, many interlocking and interconnected parameters are in play.

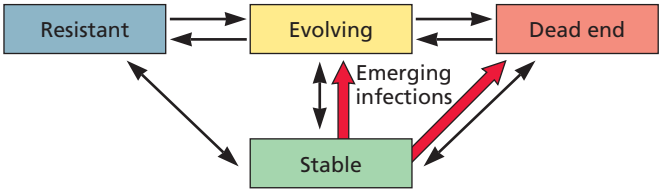


Figure 11.2 General categories of interactions between hosts and viruses. Four categories of host-virus interactions are indicated in the boxes. The **stable** interaction maintains the virus in the ecosystem. The **evolving** interaction describes the passage of a virus from "experienced" populations to naive populations in the same or other host species. The **dead-end** interaction represents one-way passage of a virus to different species. The host usually dies, or if it survives, the virus is not transmitted efficiently to the new host species. The **resistant host** interaction represents situations in which the host blocks infection completely. The arrows indicate possible transition from one category to another or the possible transformation of one into another. The red, filled arrows indicate the major pathways of zoonotic or other emerging viruses.

dynamic host-virus interactions, and their names emphasize the defining feature of each. The figure shows how relationships can shift from one category to another and illustrates the continuity of viral interactions in nature. It is important to note that these categories are meant to describe interactions among large populations and **not** single virus-host interactions. In this framework, emerging viral infections are defined as human infections that derive from stable host-virus relationships that preexist in nonhuman hosts, which serve as **reservoirs** for particular viruses.

Stable Interactions

Stable host-virus interactions are those in which both participants survive and reproduce. Such relationships are essential for the continued existence of the virus and may influence host survival as well. This state is optimal for a host-parasite interaction, but need be neither benign nor permanent in an outbred population. Infected individuals can become ill, recover, develop immunity, or die, yet in the long run, both virus and host populations survive. While this situation is often described as an equilibrium, the term is misleading because the interactions are dynamic and fragile, and are rarely reversible. Viral populations may become more or less

Table 11.1 Examples of viruses that cause zoonotic infections

Virus	Family	Drivers of emergence
Dengue virus	*Flaviviridae*	Urban population density; open water storage favors mosquito breeding (e.g., millions of used tires)
Ebola virus	*Filoviridae*	Human contact with unknown natural host (Africa); importation of monkeys in Europe and the United States
Hantaan virus	*Bunyaviridae*	Agriculture techniques: human-rodent contact
Hendra virus	*Paramyxoviridae*	Domestic horses to stable workers
Human immunodeficiency virus	*Retroviridae*	Hunting and butchering of infected primates (bushmeat trade)
Influenza virus	*Orthomyxoviridae*	Integrated pig-duck agriculture; mobile population
Junin virus	*Arenaviridae*	Agriculture techniques: human-rodent contact
Machupo virus	*Arenaviridae*	Agriculture techniques: human-rodent contact
Nipah virus	*Paramyxoviridae*	Proximity of fruit bats, the natural reservoir, favors transmission to pigs and then to humans
Rift Valley virus	*Bunyaviridae*	Dams, irrigation
Sin Nombre virus	*Bunyaviridae*	Natural increase of deer mice and subsequent human-rodent contact
West Nile virus	*Flaviviridae*	Birds ↔ mosquitoes ↔ humans; recent introduction into United States

virulent, if such a change enables them to be maintained in the population, while host mechanisms that attenuate the more debilitating effects of the viruses may be selected.

Some stable interactions are effectively permanent. This is the case when there is only one natural host, because a stable relationship is required for the survival of the virus population. Examples include measles virus, herpes simplex virus, human cytomegalovirus, and smallpox virus in humans, and simian cytomegalovirus, monkeypox virus, and simian immunodeficiency virus, which infect only certain species of monkeys. Stable interactions can also be sustained by infection of more than one host species with the same virus: influenza A virus, flaviviruses, and togaviruses are capable of propagating in a variety of species. Indeed, many members of these virus families replicate efficiently in some insects as well as in mammals and birds.

Establishment of a stable host-virus interaction is not necessarily the optimal solution for survival. The trajectory of evolution is unpredictable: what is successful today may be lethal at another time. Furthermore, if a virus population becomes completely dependent on one, and only one, host, it will have entered a potential bottleneck that may constrain its further evolution. If the host becomes extinct for whatever reason, so will the virus. For example, if humans disappeared, many virus populations, including poliovirus, measles virus, and several herpesviruses, would cease to exist. Eradication of natural smallpox virus was possible because humans are the only hosts and worldwide immunization was achieved.

The Evolving Host-Virus Interaction

The hallmarks of the evolving host-virus interaction (Fig. 11.2) are instability and unpredictability. These properties are to be expected, as selective forces are applied to both host and virus, and are magnified when host populations are small. An example of such an interaction is the introduction of measles viruses to natives of the Americas by Old World colonists and slave traders. Measles infections devastated the native populations. While less affected at the time, European populations had experienced the same horror when these viruses first spread from Asia, and only developed some resistance and immunity over time. Other opportunities to enter the evolving host-virus interaction may arise if the virus in a stable relationship acquires a new property that increases its virulence or spread, or if the host population suffers a far-reaching catastrophe that reduces resistance (e.g., famine or mass population changes during wars). The introduction of West Nile virus into the Western Hemisphere in 1999 provides a contemporary example of an evolving host-virus interaction in which a virus was introduced **accidentally** into a new geographic location (Box 11.1). A classic case of the **deliberate** release of a virus in a new geographic location is the attempt to use poxvirus infection to rid Australia of rabbits. The consequences of this "experiment" provide another example of an evolving virus-host interaction (Box 11.2).

The Dead-End Interaction

In a dead-end interaction (Fig. 11.2), the virus is not transmitted to other members of the new host species. Like the evolving host-virus interaction, it represents a departure from a stable relationship, often with lethal consequences. A dead-end interaction is a frequent outcome of cross-species infection. In many cases of such infection, the host is killed so quickly that there is little or no subsequent transmission of the virus to others. In other cases, the virus cannot be transmitted to other individuals of the same species.

The dead-end interaction is often observed with viruses carried by arthropods, such as ticks and mosquitoes, which cycle in the wild in a stable relationship with a vertebrate host. Occasionally the infected insect bites a new species (e.g., humans) and transmits the virus (Fig. 11.3 and 11.4). Even though the consequences to the infected individual may be severe, because the human is not part of the natural, stable host-virus relationship, these interactions have little, if any, effect on the evolution of the virus and its natural host. While generally accurate, such a view may be too simplistic in some cases; infection by a less virulent mutant or infection of a more resistant individual may be the first step in establishing a new host-virus interaction.

Infection of humans by the filoviruses, such as marburgviruses or ebolaviruses, is an example of one type of dead-end virus-host interaction. In these cases, disease onset is sudden, with 25 to 90% case-fatality ratios reported (Appendix, Fig. 5). Virus disseminates through the blood and reproduces in many organs, causing focal necrosis of the liver, kidneys, lymphatic organs, ovaries, and testes. Capillary leakage with massive hemorrhaging, shock, and acute respiratory disorders are observed in fatal cases. Patients usually die rapidly of intractable shock without evidence of an effective immune response. Even when recovery is under way, survivors do not have detectable neutralizing antibodies. The infection clearly overwhelms a particular individual, but does not spread widely because these viruses can be transmitted to other humans only by contact with infected blood and tissue. Consequently, human infections tend to cluster in local areas. Current evidence points to fruit bats as the reservoir species for Ebola virus in Central Africa. Humans are not the only dead-end hosts: gorillas are susceptible, and large numbers of these primates have died from Ebola virus infection.

Many animal models of disease might also be considered examples of dead-end interactions. Herpes simplex virus is a human virus, but when it is introduced into mice, rabbits, or guinea pigs in the laboratory, these animals become infected and show pathogenic effects that mimic some aspects of the human disease. However, in their natural environment, these

BOX 11.1

DISCUSSION

An evolving virus infection: the West Nile virus outbreak

In August 1999, six people were admitted to Flushing Hospital in Queens, NY, with similar symptoms of high fever, altered mental status, and headache. These people were discovered subsequently to be infected with West Nile virus. This Old World flavivirus was discovered in 1937 in the West Nile district of Uganda and had never before been isolated in the Western Hemisphere. The virus has now spread in the United States from the Atlantic to the Pacific, as well as both south and north as far as the Canadian provinces and territories.

The New York isolate of West Nile virus is nearly identical to a virus isolated in 1998 from a domestic goose in Israel during an outbreak of the disease. The close relationship between these two isolates suggests that the virus was brought to New York City from Israel in the summer of 1999. How it crossed the Atlantic will probably never be known for sure, but it might have been via an infected bird, mosquito, human, horse, or other vertebrate host. These events mark the first introduction in recent history of an Old World flavivirus into the New World. A fascinating, and yet unanswered, question is how the epidemic got started in New York City. The summer of 1999 was particularly hot and dry. Similar conditions spawn outbreaks of West Nile virus encephalitis in Africa, the Middle East, and the Mediterranean basin of Europe.

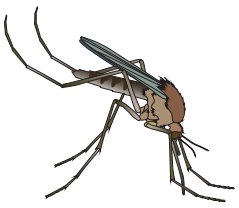
West Nile virus or virus-specific antibodies have been found in numerous species of birds. Crows and jays appear to be particularly sensitive, but many zoos have reported deaths of their exotic birds from West Nile virus infections. Humans and other animals acquire the virus from mosquito bites after the insect has fed on infected birds. Infected horses develop lethal encephalitis, of which hundreds of cases have been reported.

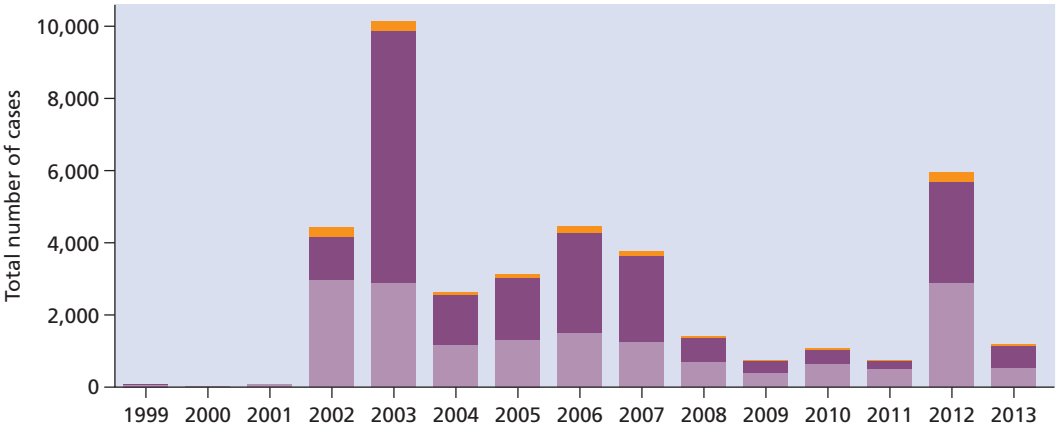
About 20% of infected humans experience flu-like symptoms when infected by West Nile virus; <1% of these individuals develop life-threatening neuroinvasive disease with meningitis-, encephalitis-, or poliomyelitis-like symptoms. However, the risk is higher in immune-compromised and elderly individuals. In the summers of 2002 and 2003, human infection reached epidemic status, causing encephalitis in hundreds of individuals. As the majority of infected people do not develop symptoms, the importance of screening the blood supply was appreciated early on. Since tests were developed in 2003, thousands of contaminated blood donations were removed from the blood supply, and infections attributable to blood transfusions are now quite rare.

By 2007, the North American epidemic seemed to be resolving, but the numbers reported to the U.S. Centers for Disease Control and Prevention (CDC) rose again in 2012 and 2013. Warmer winter temperatures that allow survival of more infected mosquitoes is a likely contributing factor. Sequencing of isolates did not reveal anything unique about the circulating 2012 strains relative to previous strains. Unfortunately, there is no treatment for West Nile disease beyond supportive care, and as yet, no vaccine exists for humans. Until we understand the complex ecology of this viral infection, the consequences for public health are difficult to predict.

FAQ: West Nile Virus, a publication of the American Academy of Microbiology, is available online: http://academy.asm.org/index.php/faq-series/793-faq-west-nile-virus-july-2013. For timely updates on West Nile virus, see http://www.cdc.gov/westnile/index.html

Data on West Nile virus cases, derived from CDC statistics, 1999 to 2013.





	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
Neuroinvasive disease cases	59	19	64	2,946	2,866	1,148	1,309	1,495	1,227	689	386	629	486	2,873	529
Non-neuroinvasive disease cases	3	2	2	1,210	6,996	1,391	1,691	2,774	2,403	667	334	392	226	2,801	606
Total cases	62	21	66	4,156	9,862	2,539	3,000	4,269	3,630	1,356	720	1,021	712	5,674	1,135
Deaths	7	2	10	284	261	100	119	177	124	44	32	57	43	286	44

BOX 11.2

EXPERIMENTS

A classic experiment in virus evolution: deliberate release of rabbitpox virus in Australia

In 1859, 24 European rabbits were introduced into Australia for sport, and lacking natural predators, the amorous bunnies went on to reproduce in plague proportions. In 1907, the longest unbroken fence in the world (1,139 miles long) was built to protect portions of the country from invading rabbits, who consumed all vegetation in their paths. Such desperate actions were to no avail. As a last resort, the rabbitpox virus, myxoma virus was released in Australia in the 1950s in an attempt to rid the continent of these pests. The natural hosts of myxoma virus are the cottontail rabbit, the brush rabbit of California, and the tropical forest rabbit of Central and South America. The virus is spread by mosquito vectors, and the natural hosts develop superficial warts on their ears. However, European rabbits, a distinct species, are killed rapidly by myxoma virus. In fact, infection is 90 to 99% fatal in these hosts!

In the first year, the virus was efficient in killing rabbits, with a 99.8% mortality rate. However, by the second year, the mortality dropped dramatically to 25%. In subsequent years, the rate of killing was lower than the reproductive rate of the rabbits, and hopes for 100% eradication were dashed. Careful epidemiological analysis of this artificial epidemic provided important information about the evolution of viruses and their hosts.

As expected, the infection spread rapidly during spring and summer, when mosquitoes are abundant, but slowly in winter. Given the large numbers of rabbits and virus particles, and the almost 100% lethal nature of the infection, attenuating mutations were selected quickly; within 3 years, less-virulent viruses appeared, and some infected rabbits were able to survive over the winter. The host-virus interaction observed was that predicted for an evolving host, coming to an equilibrium with the pathogen. A balance is struck: some infected rabbits die, but many survive.

The most obvious lesson from this experience was that the original idea to eliminate rabbits with a lethal viral infection was flawed. Powerful selective forces that could not be controlled or anticipated were at work. Surprisingly, more experiments in the virological control of rabbits are under way in Australia. One approach used a lethal rabbit calicivirus. As resistance has developed in the rabbit population (as might have been predicted), more-lethal strains are being tested for possible use. Another approach employs a genetically engineered myxoma virus designed to sterilize, but not kill, rabbits. The latter viruses encode a rabbit zona pellucida protein, and infected animals synthesize antibodies against their own eggs (so-called immunocontraception). Stay tuned ...

Cooke, B. 7 March 2012. Controlling rabbits: let's not get addicted to viral solutions. The Conversation. http://theconversation.com/controlling-rabbits-lets-not-get-addicted-to-viral-solutions-5701,

Rabbits and ever more rabbits!



animals contribute nothing to transmission or maintenance of the virus.

Common Sources of Animal-to-Human Transmission

Rodents play critical roles in the introduction of new viruses into human populations in areas where these animals are abundant. Most hemorrhagic disease viruses, including Lassa, Junin, and the Sin Nombre virus, are endemic in rodents, their natural hosts. The viruses establish a persistent infection, and the rodents show few, if any, ill effects. However, substantial numbers of virus particles are excreted in urine, saliva, and feces to maintain the virus in the rodent population. Humans become infected when they happen to come in contact with rodent excretions that contain infectious virus particles. Unfortunately, infection by such rodent viruses can cause lethal outbreaks in humans as dead-end hosts.

Bats are the natural hosts of several viruses that cause dead-end, zoonotic infections. Hendra virus and Nipah virus are known to have entered the human population via bats. The Old World fruit bats (genus *Pteropus*), commonly called flying foxes, are widely distributed in Southeast Asia, Australia, and the Indian subcontinent. Despite having high antibody titers against the deadly viruses, the animals exhibit no obvious disease (Box 11.3). High virulence in humans and our complete lack of therapeutic interventions require that these viruses be studied only under the highest biological and physical containment (biosafety level 4 [BSL4]). Accordingly, we know very little about their biology, ecology, and pathogenesis.

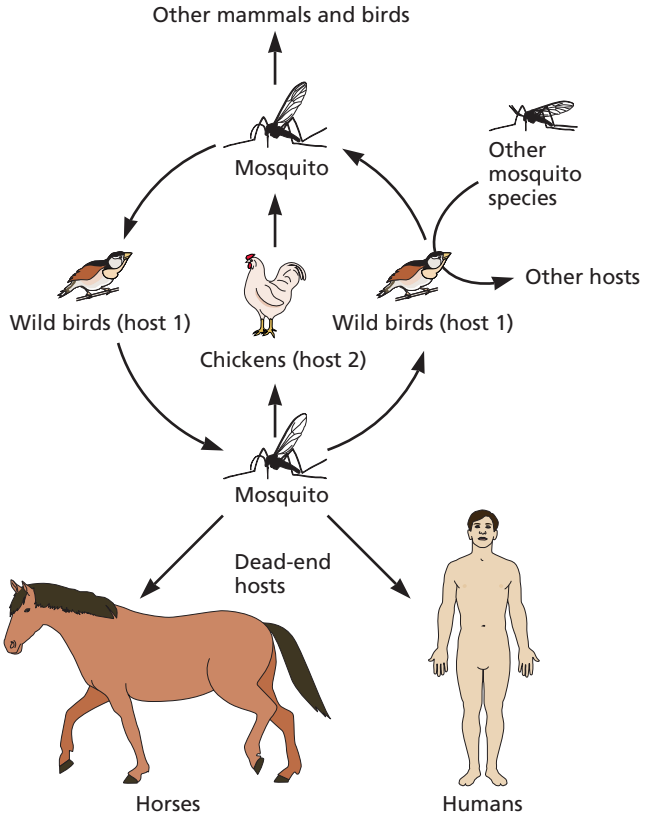


Figure 11.3 The dead-end host scenario as illustrated by a complex host-virus relationship. This illustration summarizes how multiple host species can maintain and transmit a virus (e.g., arthropod-transmitted dengue yellow fever viruses). In this example, the virus population is maintained in two different hosts (wild birds and domestic chickens) and is spread among individuals by a mosquito vector. The virus reproduces both in species of bird and in the mosquito. Disease is likely to be nonexistent or mild in these species, as these hosts have adapted to the infection. A third host (in this example, horses or humans) occasionally is infected when bitten by a mosquito that previously fed on an infected bird. Horses and humans are dead-end hosts and contribute little to the spread of the natural infection, but they may suffer from serious, life-threatening disease. Occasionally another species of biting insect (e.g., other mosquito species) can feed on an infected individual (bird, horse, or human) and then transmit the infection to another species not targeted by the original mosquito vector.

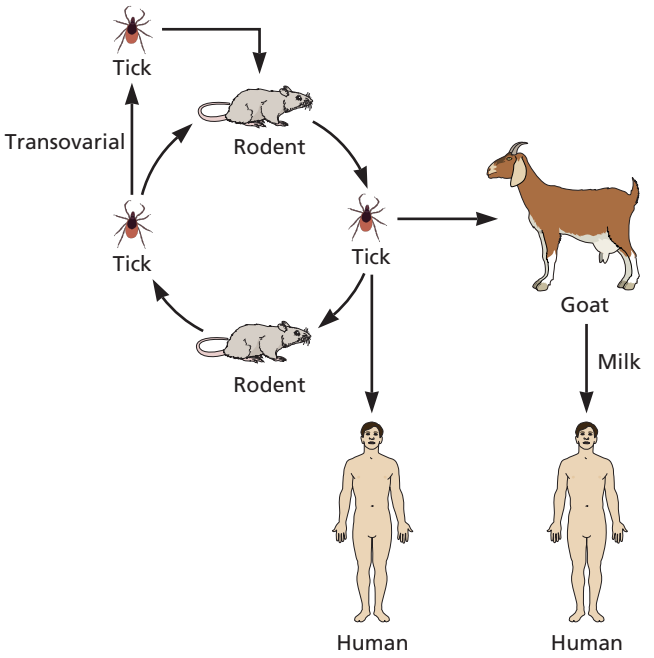


Figure 11.4 Replicative cycle of the central European tick-borne flavivirus may involve zoonotic infections. The European tick-borne flavivirus is maintained and spread by multiple host infections. Congenital transmission in the tick maintains the virus in the tick population as they feed upon rodents. The infected newborn ticks have adapted to the virus and thrive. The virus is transmitted from the tick to a variety of animals, including cows, goats, and humans. Humans can also be infected by drinking milk from an infected goat, sheep, or cow. As the virus cannot be transmitted from human to human, this zoonotic infection is another example of the dead-end host interaction.

The Resistant Host

All living things are exposed continuously to viruses of all types, yet the vast majority of these interactions are uneventful (Fig. 11.2). In some cases, there is no infection because host cells are not susceptible, not permissive, or the primary physical, intrinsic, and innate defenses are so strong that most potential invaders are diverted or destroyed upon contact. In other cases, organisms may become infected and produce some virus particles, but the virus is cleared rapidly without activation of the host's acquired immune system (Chapter 5). This outcome contrasts with an inapparent infection, in which an immune response is mounted but the individual exhibits no signs of disease.

The obstacles that limit the ability of a host to support viral reproduction need not be insurmountable. This is certainly the case in the practice of **xenotransplantation** (the use of animal organs in humans). Not only does such transplantation bypass physical and innate defenses by surgery, but the drugs used to block transplant rejection also suppress the immune response. Consequently, any virus particles or genomes in xenografts would have direct access to the once-resistant host in the absence of crucial antiviral defenses. As many of these viruses can infect human cells or have close, human-adapted relatives, the xenotransplantation patient represents a potential source of new viral diversity. Clearly this practice raises many medical and ethical issues.

Encountering New Hosts: Ecological Parameters

Establishment of all virus-host relationships depends on the concentrations of the participants and the probability of

BOX 11.3

DISCUSSION

What is it about bats?

Bats comprise a quarter of all mammalian species. There are almost 1,000 recognized bat species, which are grouped into two suborders: *Microchiroptera* (microbats, mostly insect eaters) and *Megachiroptera* (megabats, mostly fruit eaters). Bats are the only mammals capable of sustained flight and have much longer life spans than any other mammals of similar size; for example, ~2 years for a mouse versus almost 40 years for the little brown bat common to North America. These amazing animals have been around for more than 65 million years, since long before the appearance of *Homo sapiens*.

Bats are gregarious, social creatures, some existing in extremely large populations, likely to facilitate the transmission of viruses among individuals. We now know that bats are natural reservoirs that can sustain many viruses that are extremely pathogenic to humans, including Nipah, Hendra, rabies, Ebola, and SARS coronavirus. As illustrated in Fig. 10.10 such associations are likely to have occurred throughout evolutionary times, as sequences related to filoviruses and another deadly pathogen, Bornavirus, were integrated into the bat genome more than 40 million years ago.

Recent comparative analyses of their genomes have provided clues both to the long life span of bats and their ability to interact with numerous deadly pathogens without ill effect. In these studies, a large number of genes in the DNA damage checkpoint-DNA repair pathway of bats showed evidence of positive selection ($dN/dS > 1$). It was hypothesized that such selection may have been driven by the need to counteract a high production of DNA-damaging reactive oxygen species associated with adaptation to flight. A more efficient DNA damage repair system might contribute to longevity.

These genomic studies also revealed genetic changes that affect components shared by the DNA damage pathway and the innate immune system (i.e., Nf-κb signaling), as well as an absence of the genes that encode certain natural killer cell receptors and proteins required for formation of inflammasomes. These findings indicate that there are likely to be significant differences in immune function among bats and other mammals. Such differences might explain how bats can provide a stable reservoir for viruses that wreak havoc on other mammalian hosts.

Zhang G, Cowled C, Shi Z, Huang Z, Bishop-Lilly KA, Fang X, Wynne JW, Xiong Z, Baker ML, Zhao W, Tachedjian M, Zhu Y, Zhou P, Jiang X, Ng J, Yang L, Wu L, Xiao J, Feng Y, Chen Y, Sun X, Zhang Y, Marsh GA, Crameri G, Broder CC, Frey KG, Wang LF, Wang J. 2013. Comparative analysis of bat genomes provides insight into the evolution of flight and immunity. *Science* **339:**456–460.



All Pteropus species of megabats are considered flying foxes. *Pteropus vampyrus* is the Malayan flying fox (found in peninsular Malaysia), and is one of the species that carries Nipah virus. Photograph courtesy of Juliet Pulliam, Princeton University.

productive encounters. Many ecological and social parameters affect the transmission of infection to new hosts in natural populations (Tables 11.2 and 11.3). Living together and sharing resources facilitates inter- and intraspecies transmission. Droughts concentrate many species at water holes, which increases the probability for transmission; destruction of habitat forces new species interactions. Predators eat their prey and become unwitting "test tubes" for cross-species infection by viruses found in tissues of the prey.

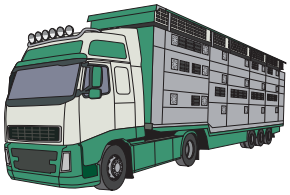
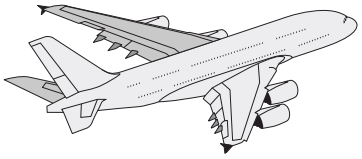
In contrast, rare chance encounters of viruses with new hosts may give rise to infections that are never seen, or at least never appreciated. These rare single-host infections may not be transmitted among humans for any number of reasons, including insufficient quantity of progeny virus shed, limited duration of shedding, and small numbers of new human hosts exposed to the infected individual. In addition, the progeny virus produced in the new host may not have the genetic repertoire to facilitate high levels of reproduction and transmission to other hosts.

Successful Encounters Require Access to Susceptible and Permissive Cells

Potential new hosts must have cells with accessible receptors that can engage ligands on virus particles. The influenza virus hemagglutinin protein has a high affinity for sialosaccharides found on the cell surfaces of many different host species. The linkage of the terminal sialic acid/galactose residues is an important determinant of tropism. Avian influenza virions bind sialic acid α(2,3)-galactose-terminated oligosaccharides, whereas the human influenza virus hemagglutinin proteins bind tightly to oligosaccharides carrying a terminal α(2,6)-linked sugar. Cells of the human respiratory tract do display α(2,3)-galactose-terminated oligosaccharides, but such cells lie deep in respiratory tissues. Conversely, sialic acid with terminal α(2,6) linkages is abundant in the more accessible regions of the upper respiratory tract. This anatomical fact appears to be a prime reason why humans cannot be infected easily with avian influenza viruses.

Nipah virus was first identified during an outbreak in swine and humans in Malaysia in 1998 and 1999. While Nipah virus

Table 11.2

Human actions
 Dams and water impoundments Irrigation Massive deforestation Rerouting of wildlife migration patterns Wildlife parks Long distance transport of livestock and birds
Air travel Uncontrolled urbanization Day care centers Hot tubs Air conditioning Millions of used tires 
 Blood transfusion Xenotransplantation Societal changes with regard to drug abuse and sex

infection of bats is apparently nonpathogenic, large quantities of virus particles are excreted in bat urine and feces. Two facts are relevant to the Malaysian outbreak: (i) pig farmers often plant mango and durian trees next to pig pens, and (ii) fruit bats are messy eaters. When pigs come in contact with partially eaten contaminated fruit, they suffer a respiratory disease, and spread virus particles into the environment efficiently by sneezing and from mucous secretions.

Table 11.3 Ecological and social parameters facilitate transmission of infection to new hosts

Transmission parameter	Action or example
Contact with bodily fluids of infected hosts	Hunting and consumption of wild game; intimate contact with infected animals in the wild, in farms, at zoos, or in the home
Sharing a resource with different species	Infected fruit bats, pigs, rodents, and humans share food or inhabit the same or nearby space
Being host to the same insect vector	Japanese encephalitis virus infection is spread by mosquitoes that feed on herons, people, and pigs
Encroachment by one species into the habitat of another	Humans enter the jungle and are bitten by mosquitoes that are part of an established host-virus interaction or cycle

Bats and pigs establish a one-way conduit for a zoonotic infection of humans in rural Indonesian communities, where humans often share accommodations with domestic swine. In addition, slaughterhouse workers are exposed to infected pigs. Remarkably, when Nipah virus infects humans, it causes encephalitis, not respiratory infection. While often lethal for the infected human (Nipah virus killed 105 of 265 infected people in the Malaysian outbreak mentioned above), the infection is contained in infected brain tissue and does not spread. Nipah virus can also infect the human upper respiratory tract, and these infections are spread among humans in close contact.

Population Density, Age, and Health Are Important Factors

The predominant parameters influencing the spread of infection are the population density and the age and health of individuals in that population. The importance of population density is illustrated by the fact that at least half a million people in a more or less confined urban setting are required to ensure a large enough annual supply of susceptible hosts to maintain measles virus in a human population (Chapter 1). When this large population of interacting hosts is not available, measles virus cannot be propagated.

Variables such as duration of immunity and the quantity of virus particles that are produced and shed from each individual have marked effects on spread of infection, as do opportunities for direct (e.g., via sexual contact) or indirect (e.g., contaminated water) exposure. The age distribution of any potential host population is also an important determinant for the spread of infection. For example, the very young and the very old are commonly more susceptible to a given virus than is the general population and, consequently, serve as sources of transmission. Predictably, prevention of infection in these groups tends to reduce the overall infection rate in the population at large. The distribution of poor and wealthy individuals in a population can also influence infection rates.

Experimental Analysis of Host-Virus Interactions

Consideration of the different types of host-virus interactions listed above highlights some of the difficulties in identifying the parameters that affect virus evolution and emergence in natural populations. Mathematical models are being developed to analyze the dynamics of this process and to predict the critical population size necessary to support the continual transmission of viruses. The goals of such modeling are to increase our understanding of the conditions that lead to the persistence of a virus population in its reservoir species and its spread to other hosts, and to test hypotheses for effective methods to prevent and control viral diseases. However, such models can be hard to evaluate simply because it is difficult to perform controlled experiments in nature.

Learning from Accidental Infections

Our understanding of the dynamics of a viral infection in a large outbred human population is rudimentary. But some insight has been gained from a limited number of accidental "experiments." Two classic examples are provided by hepatitis B virus and poliovirus infections. During World War II, large doses of infectious hepatitis B virus were accidentally introduced into ~45,000 soldiers when they were injected with a contaminated yellow fever vaccine. Only 900 (2%) developed clinical hepatitis, and <36 developed severe disease. Similarly, in 1955, 120,000 school-aged children were vaccinated with an improperly inactivated poliovirus vaccine. About half were protected by preexisting antibodies to poliovirus as a result of inapparent infections. Of the remainder, ~10 to 25% were infected by the vaccine virus, as determined by the appearance of antibodies. More than 60 cases of paralytic poliomyelitis were documented among these infected children, but the remainder escaped disease. These two experiments tell us that even when large numbers of individuals are inoculated with an equivalent amount of a virulent virus, the outcomes can be quite variable, and not everyone succumbs to disease. The finding that infection was relatively rare even in these cases, where large amounts of virus particles were administered to numerous individuals, suggests that special circumstances or conditions are required for natural cross-species infections to emerge.

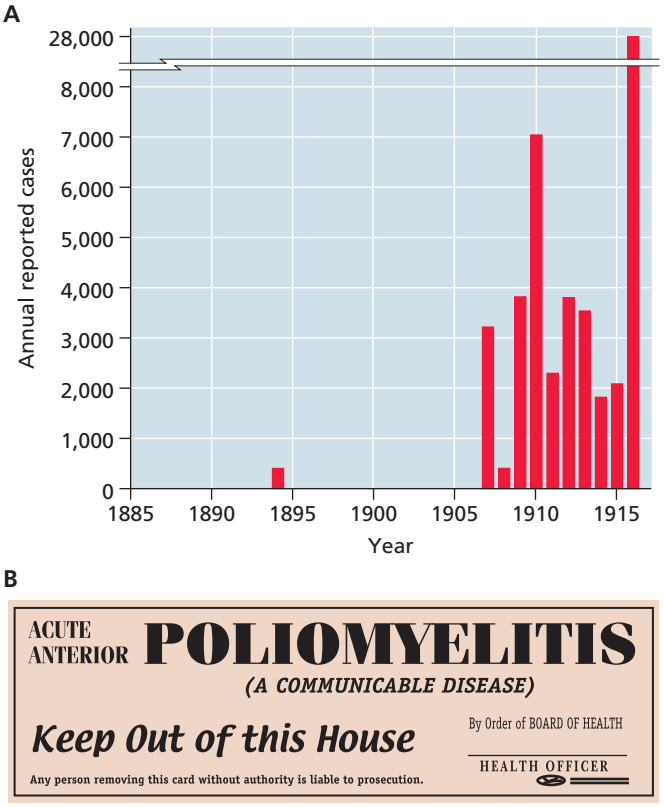


Figure 11.5 Poliovirus in the early 20th century. (A) The emergence of paralytic poliomyelitis in the United States, 1885 to 1915. From N. Nathanson, *ASM News* **63:**83–88, 1997, with permission. **(B)** Board of Health quarantine notice, San Francisco, CA, circa 1910.

Expanding Viral Niches: Some Well-Documented Examples

Poliomyelitis: Unexpected Consequences of Modern Sanitation

Host populations change with time, and each change can have unpredictable effects on virus evolution. An example is poliomyelitis, a disease caused by poliovirus infection. The disease is ancient, postulated by some to be present >4,000 years ago (see Volume I, Chapter 1). For centuries, the host-virus relationship was stable, and infection was endemic in the human population. Poliomyelitis epidemics were not reported, but we imagine that occasional outbreaks of disease occurred in scattered areas. This state of affairs changed radically in the first half of the 20th century, when large annual outbreaks of poliomyelitis were seen in Europe, North America, and Australia (Fig. 11.5). Retrospective analysis established that these outbreaks were not correlated with any substantial change in the viral genome.

Emergence of epidemic poliomyelitis can be explained by a change in human lifestyle: unprecedented urbanization and improvement in sanitation. Poliomyelitis is caused by an enteric virus that is spread by oral-fecal contact. As a consequence, endemic disease was characteristic of life in rural communities, which generally had poor sanitation and small populations. Because the virus circulated freely, most children were infected at an early age and developed antibodies to at least one of the serotypes. Maternal antibodies, which protect newborns, were also prevalent, as most mothers had experienced a poliovirus infection at least once. A salient point is that most infected children do not develop paralysis, the most visible symptom of poliomyelitis. Paralysis is a more frequent result when older individuals are infected. Even the most virulent strains of poliovirus cause 100 to 200 subclinical infections for every case of poliomyelitis. These inapparent infections in children provided a form of natural vaccination. As childhood disease and congenital malformations were not uncommon in rural populations, the few individuals who developed poliomyelitis were not seen as out of the ordinary. No one noticed endemic poliovirus.

During the 19th and 20th centuries, industrialization and urbanization changed the pattern of poliovirus transmission. Improved sanitation broke the normal pattern and effectively stopped natural vaccination, thereby increasing the pools of susceptible children. As a result, children tended to encounter the virus for the first time at a later age, without the protection of maternal antibodies, and were therefore at far greater risk for developing paralytic disease. Consequently, epidemic poliovirus infections emerged time and time again in communities across the world.

Smallpox and Measles: Exploration and Colonization

Explosive epidemic spread may occur when a virus enters a naive population (**the evolving host-virus interaction**) (Fig. 11.2). The results can be frightening, often devastating, as infections appear to "come out of the blue." Charles Darwin was aware of this phenomenon, as he wrote in *The Voyage of the Beagle*: "Wherever the European has trod, death seems to pursue the aboriginal."

Smallpox reached Europe from the Far East in AD 710 and attained epidemic proportions in the 18th century as populations grew and became concentrated. The effects on society are hard to imagine today, but as an example, at least five reigning monarchs died of smallpox. Smallpox virus continued its spread around the world when European colonists and slave traders moved to the Americas and Australia. This viral infection certainly changed the balance of human populations in the New World. The first recorded outbreak of smallpox in the Americas occurred among African slaves on the island of Hispaniola in 1518, and the virus spread rapidly through the Caribbean islands. This toehold of smallpox in the New World enabled the conquest of the Aztecs by European colonists. In 1520, smallpox reached the American mainland from Cuba. Within 2 years, 3.5 million Aztecs were dead, far more than could be accounted for by the bullets and swords of Hernán Cortez's small band of conquistadors. Smallpox spread like wildfire in the native population, which was highly interactive and of sufficient density for efficient virus transmission. Infection reached as far as the Incas in Peru before Francisco Pizarro made his initial invasion in 1533.

As is true in most smallpox epidemics, some Aztecs and Incas survived, but those who did were then devastated by measles virus, probably brought in by Cortez's and Pizarro's men. Conquest was accomplished by a one-two virological punch rather than by military prowess. Slave traders (who were most likely immune to infection) were populating Brazil with their infected human cargo at approximately the same time, with the same horrible result.

The devastation of indigenous peoples by these viruses was recapitulated in the colonization of North America and continued into the 20th century as contaminated explorers infected isolated groups of Alaskan Inuit and native populations in New Guinea, Africa, South America, and Australia.

Notable Zoonoses

Hantavirus Pulmonary Syndrome: Changing Climate and Animal Populations

A small but alarming epidemic of a highly lethal infectious disease appeared in the Four Corners area of New Mexico in the United States in 1993. Individuals who were in excellent health developed flu-like symptoms that were followed quickly by a variety of pulmonary disorders, including massive accumulation of fluid in the lungs, and death. Rapid action by local health officials and a prompt response by the Centers for Disease Control and Prevention were instrumental in discovering that these patients had low-level, cross-reacting antibodies to previously identified hantaviruses. These members of the family *Bunyaviridae* had been associated with renal diseases in Europe and Asia and were well known to be associated with viral hemorrhagic fever during the Korean War. Hantaviruses commonly infect rodents and are endemic in these populations around the world. PCR technology was used to determine that the patients were infected with a new hantavirus. Subsequently, field biologists found this virus in a rodent called the deer mouse (*Peromyscus maniculatus*), which is common in New Mexico. The virus, which was given the name Sin Nombre virus (no-name virus), is an example of an emerging virus, endemic in rodents, that causes severe problems when it crosses the species barrier and infects humans. Sin Nombre virus has since caused a few additional isolated outbreaks in North America.

Humans became infected with Sin Nombre virus most likely because of a dramatic increase in the deer mouse population. A higher-than-normal rainfall resulted in a bumper crop of piñon nuts, a favorite food for deer mice and local humans. Mouse populations increased in response, and contacts with humans inevitably increased as well. Hantavirus infection is asymptomatic in mice, but virus particles are excreted in large quantities in urine and droppings, where they are quite stable. Human contact with contaminated blankets or dust from floors or food storage areas provided ample opportunities for infection. Hantavirus syndrome is rare because humans are not the natural host, and apparently are not efficient vehicles for virus spread.

Severe Acute and Middle East Respiratory Syndromes (SARS and MERS): Two New Zoonotic Coronavirus Infections

A new human viral disease called severe acute respiratory syndrome (SARS) first appeared in humans in Guangdong Province in China in the fall of 2002. A doctor who treated these patients traveled to Hong Kong on February 21, 2003, and checked into a hotel. He became ill and died in the hospital the very next day. During his stay in the hotel, the virus was transmitted to 10 other residents, who subsequently flew to Singapore, Vietnam, Canada, and the United States before symptoms were evident. A major viral epidemic was spread by air travel. This small number of infected people efficiently transmitted the new SARS coronavirus to other individuals around the world, such that ~8,000 people in 29 countries became infected in less than a year. The case-fatality ratio was almost 1 in 10, a chilling statistic that activated health organizations worldwide. The scientific community mobilized with unprecedented speed and cooperation, and the causative agent was identified within only a few months.

It is now generally accepted that bats serve as the natural reservoir for the SARS coronavirus. Whether the virus was

transmitted directly from bats to humans or though an intermediate host is not yet known (Box 11.4). Amazingly, the epidemic never reached pandemic proportions, despite the existence of billions of susceptible hosts and widespread seeding of infected people around the world. After a frightening few months, SARS all but disappeared from the human population, although a few cases were reported subsequently.

Ten years later, reports from the Arabian Peninsula described the emergence of a new virus that caused severe pneumonia in infected humans, with an ~50% case-fatality ratio. Rapid sequencing of isolates identified the agent as another member of the family *Coronaviridae*, and the virus was given the name Middle East respiratory syndrome (MERS) coronavirus. As of this writing, the reservoir of the virus is believed to be dromedary camels. Although most cases of this disease have occurred in Saudi Arabia and countries nearby, some have been reported in different parts of Europe in people who had visited the Middle East. The total number of cases is small, but the virus has been seen to spread through close contact from infected individuals to others. This situation appears to be a contemporary example of an evolving host-virus interaction. (For more on such emerging viruses, check out the video interview with Dr. Ian Lipkin: http://bit.ly/Virology_Lipkin).

Acquired Immunodeficiency Syndrome (AIDS): Pandemic from a Zoonotic Infection

The emergence of human immunodeficiency virus type 1 can be traced to transmission from a chimpanzee to a human in West Central Africa (Box 1.2). There is strong evidence that humans are exposed to many zoonotic infections by the bushmeat trade in West Africa, which involves killing and consumption of wild animals, including chimpanzees, gorillas, other primates, and rodents. It is easy to imagine how

BOX 11.4

DISCUSSION

A SARS-like coronavirus from bats can infect human cells

The SARS pandemic of 2002–2003 is believed to have been caused by a bat coronavirus that first infected a civet and was then passed on to humans. The isolation of a new SARS-like coronavirus from bats suggests that the SARS coronavirus could have infected humans directly from bats.

A single colony of horseshoe bats (*Rhinolophus sinicus*) in Kunming, Yunnan Province, China, was sampled for coronavirus sequences over a 1-year period. Of a total of 117 anal swabs or fecal samples collected, 27 (23%) were positive. Using PCR amplification, seven different SARS-like coronavirus sequences were identified, including two new ones. The complete genome sequences were determined for the new coronaviruses, which showed a higher nucleotide sequence identity (95%) with the SARS coronavirus than had been previously observed among bat viruses.

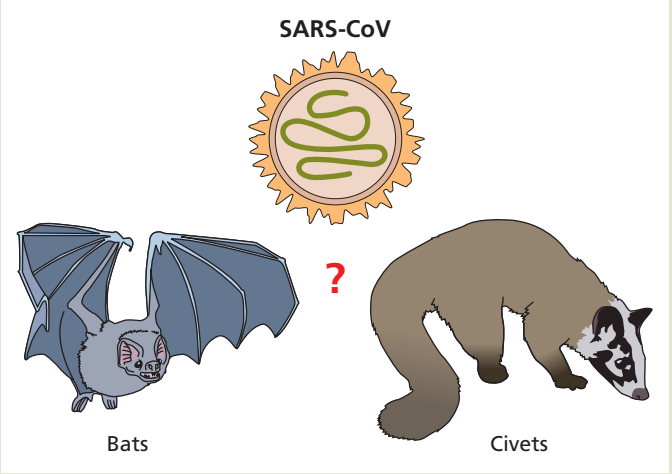
One of the new viruses was recovered by infecting monkey cell cultures with one of the positive bat samples. The recovered virus was able to infect human cells and for entry utilize human angiotensin-converting enzyme 2 (ACE2), which is the receptor for SARS coronavirus. Furthermore, infectivity of the recovered virus could be neutralized with sera collected from seven SARS patients.

The spike glycoprotein of SARS-like coronaviruses previously isolated from bats does not recognize the ACE2 receptor, and thus these viruses are unable to infect human cells. Because the SARS-like coronaviruses isolated from palm civets during the 2002–2003 outbreak have amino acid changes in the viral spike glycoprotein that improve its interaction with ACE2, these animals were believed to be an intermediate host for adaptation of the SARS virus to humans. However, the properties of the new bat SARS-like coronavirus suggest that humans might have been infected directly from bats.

This finding has important implications for public health: if SARS-like coronaviruses that can infect human cells are currently circulating in bats, they have the potential to cause another outbreak of disease. The investigators in this study believe that the diversity of bat coronaviruses is probably greater than previously suspected. They speculate that further surveillance may reveal a broad diversity of bat SARS-like coronaviruses that are able to use ACE2, some of which may have closer homology to the SARS virus than even their latest isolate.

Ge XY, Li JL, Yang XL, Chmura AA, Zhu G, Epstein JH, Mazet JK, Hu B, Zhang W, Peng C, Zhang YJ, Luo CM, Tan B, Wang N, Zhu Y, Crameri G, Zhang SY, Wang LF, Daszak P, Shi ZL. 2013. Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. *Nature* **28:**535–538.

Deciphering the source of human SARS coronavirus (SARS-CoV).



butchering an infected chimp could facilitate the transfer of a retrovirus particle from an animal's blood. It is sobering to consider that the progenitor of human immunodeficiency virus type 1 was on the path to extinction, because the population of wild chimpanzees had dropped to about 150,000 animals living in isolated troops. The new human host exceeds 7 billion individuals. A second, related human immunodeficiency virus subtype, called type 2, was acquired by zoonotic infection from another primate, the sooty mangabey.

Host Range Can Be Expanded by Mutation, Recombination, or Reassortment

Canine Parvoviruses: Cat-to-Dog Host Range Change by Two Mutations

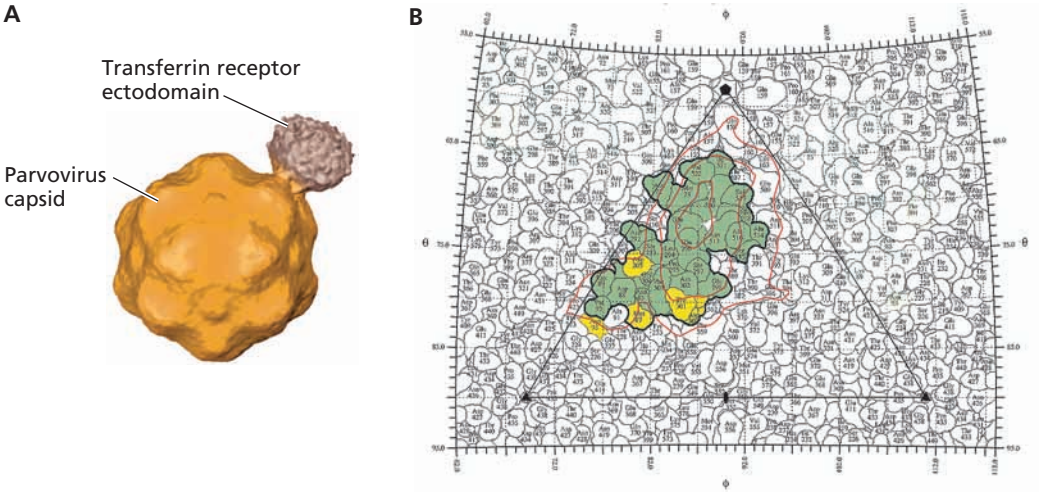
Canine parvovirus was identified in several countries in 1978 as the cause of a new enteric and myocardial disease in dogs. Canine parvovirus apparently evolved from the feline panleukopenia virus that infects cats, mink, and raccoons, but not dogs. However, the new canine virus did not reproduce in cats. Because canine parvovirus appeared less than 40 years ago, it has been possible to analyze dog and cat tissue collected in Europe in the early 1970s to search for the progenitor canine parvovirus. The ancestor of canine parvovirus began infecting dogs in Europe during the early 1970s, and within 8 years, it had spread to several other continents. The stability of the new virus and its efficient fecal-oral transmission were important factors in its emergence.

It is now clear that only two amino acid substitutions in the VP2 capsid protein were necessary to change the tropism from cats to dogs (Fig. 11.6). These critical amino acids are located on a raised region of the capsid that binds the host transferrin receptor, the protein used to establish infection. Feline panleukopenia virus particles bind only to the feline transferrin receptor, but the substitutions in VP2 allow the canine parvovirus particles to bind the canine transferrin receptors. The emergence of the canine parvovirus group provided an extraordinary opportunity to study virus-host adaptation and host-range shifts in the field.

Influenza Epidemics and Pandemics: Escaping the Immune Response by Reassortment

Influenza serves as the paradigm for the situation in which continued evolution of the virus in several host species is essential for its maintenance. The life cycle of influenza virus, while comparatively well understood at the molecular level, is remarkable for its complexity in nature (Box 10.3; Fig. 11.7). Sequencing data indicate that the H1N1 virus, which claimed >25 million human lives in the pandemic of 1918 (Table 11.4), is likely to be the ancestor of all current human influenza viruses, as well as some that are circulating in the world's swine populations. However, new influenza viruses constantly

Figure 11.6 The transferrin receptor mediates canine and feline parvovirus host range. The transferrin receptors for feline and canine parvoviruses have a large extracellular domain (ectodomain) that is a homodimer of a single protein (see Box 10.9). The binding of the canine parvovirus virion to the ectodomain is determined by combinations of amino acid residues on the surface of the capsid. **(A)** Cryo-electron microscopy was used to determine the structure of the purified ectodomain of the feline transferrin receptor bound to the canine parvovirus capsid. Only a small number of transferrin receptors bind to each capsid, and in the model, only one is shown. **(B)** The binding site (footprint) of the transferrin receptor, in a representation of the surface-exposed amino acids of the capsid, is colored green. One of the 60 asymmetric units of the icosahedral capsid is outlined. Residues that are known to affect binding of the canine transferrin receptor or the host range are indicated in yellow. Figures prepared by Susan Hafenstein and Colin Parrish, Cornell University. See S. Hafenstein et al., *Proc Natl Acad Sci U S A* **104:** 6585–6589, 2007.



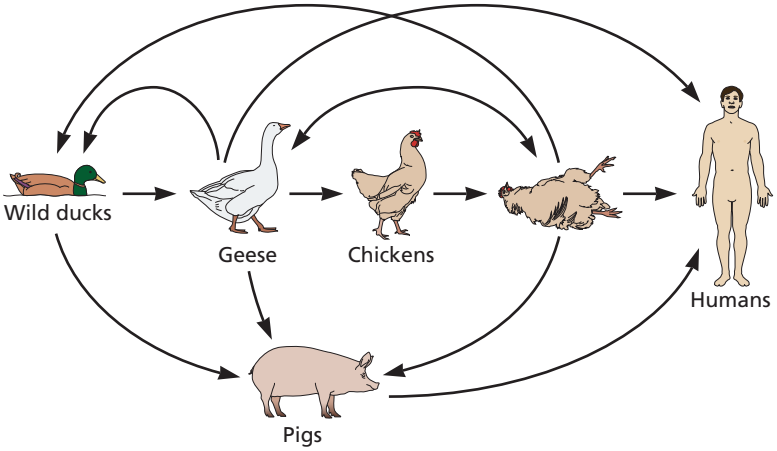


Figure 11.7 Emergence and transmission of H5N1 influenza virus. H5N1 influenza virus has its origins in wild waterfowl, where it is relatively nonpathogenic. Infection is thought to have spread to domestic ducks and chickens, and the virus evolved to be highly pathogenic in chickens. Transmitted back to ducks and geese, the viral genome underwent reassortment with the genomes of other influenza viruses of aquatic birds, resulting in a virus that could be transmitted directly to domestic chickens, humans, and pigs. Transmission was facilitated by mutations or changes in the viral PB2, HA, NA, and NS genes, which made the virus more pathogenic to domestic and wild waterfowl and humans. Spread to humans without need for an intervening "mixing host" is a particularly worrisome feature of this virus.

emerge from migratory populations of aquatic birds to infect humans, pigs, horses, domestic poultry, and aquatic mammals. In birds, influenza virus reproduces in the gastrointestinal tract and particles are excreted in large quantities, a most efficient virus distribution system. The widespread dispersal of virus particles in water, the facile changing of hosts, and the ease of genetic reassortment form a powerful engine for creation of new pathogenic strains.

Outbreaks of swine and avian influenza periodically devastate agricultural operations that produce these animals for food. Despite large-scale immunization programs, virulent strains of swine influenza virus continue to emerge in these animal hosts. The lethal consequences of direct transfer of a virulent avian H5N1 virus to humans were first documented in 1997. The World Health Organization recognizes the avian influenza virus subtypes H5, H7, and H9 as potential pandemic strains, because humans have no immunity to them. Attempts to predict critical changes that can affect the transmissibility of some of these subtypes to humans, using laboratory animal models, have generated considerable controversy worldwide (Box 11.5).

One surprising finding is that, in contrast to the genomes of human and other nonavian influenza viruses, the genome of the avian influenza virus has not changed much in more than 60 years. Although the avian viral genomes exhibit mutation and reassortment rates as high as those of human and swine influenza viruses, only sequences with neutral mutations are selected and maintained in the bird population. While virulent mutants do arise occasionally, birds infected with avian influenza viruses generally experience no overt pathogenesis. These properties indicate that influenza virus is in evolutionary stasis in birds. The avian hosts provide the stable reservoir for influenza virus gene sequences that emerge as recombinants capable of transspecies infection.

New Technologies Uncover Hitherto Unrecognized Viruses

In addition to being acquired from animals, emerging viral infections may simply be caused by previously unknown agents. The rate of virus discovery has risen with

Table 11.4 The 1918–1919 influenza pandemic: one of history's most deadly events[a]

Event	Estimated no. of deaths (millions)
Influenza pandemics (1918–1919)	20–50
Black Death (1348–1350)	20–25
AIDS pandemic (through 2013)	35
World War II (1937–1945)	
Military	15
Civilian	45
World War I (1914–1918)	
Military	10
Civilian	7

[a]Data from *The New York Times*, August 21, 1998, and from the World Health Organization.

BOX 11.5

DISCUSSION

Avian influenza viruses: scientific and societal implications of transmissibility experiments using animal models

Highly pathogenic variants of the H5N1 avian influenza virus, known commonly as "bird flu," moved from Asia to India to Europe, and also to Africa, in the space of only 10 years. Variants of H5N1 continue to evolve, and are moving around the world in wild birds, notably waterfowl, which are considered the natural reservoirs and sources of infection for other species. Highly pathogenic avian influenza viruses also become established in domestic fowl, which are kept at very high densities in farms and markets. Efforts to control these infections have led to the culling of millions of domestic birds in Asia and Europe. Humans have helped to spread the virus by transporting infected "exotic" birds by car, truck, and railroad. Although the virus has not yet reached the Americas, it is sobering to note that, after narcotics, live birds for the pet trade are the next most commonly smuggled items brought into the United States.

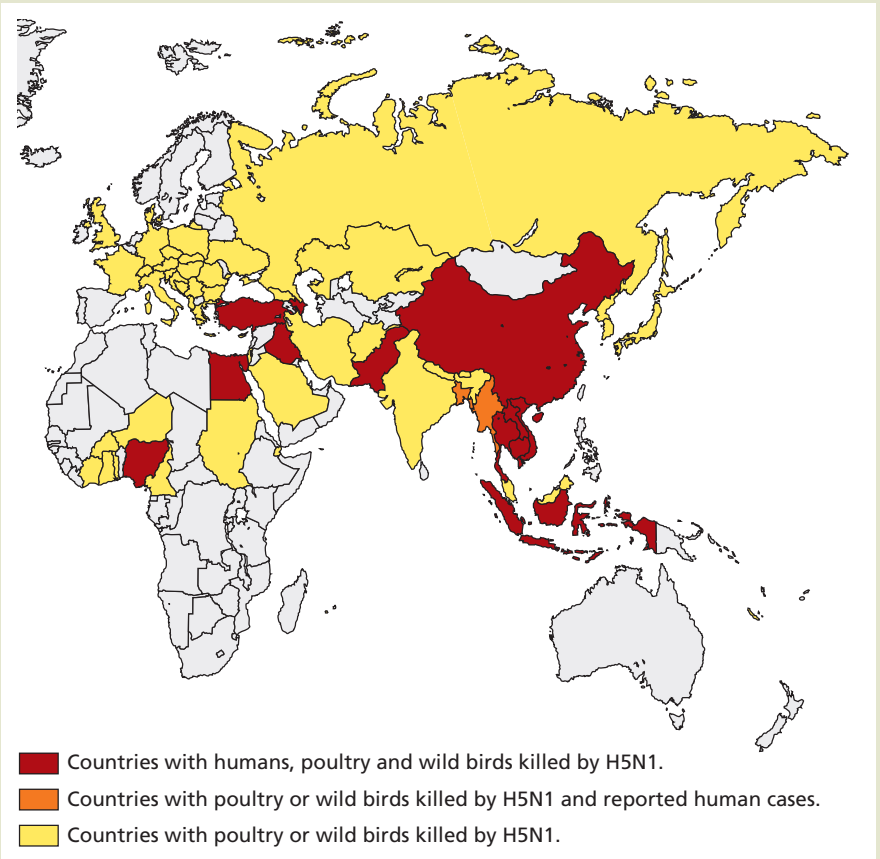
Transmission of this virus to humans is rare, requiring close contact with bodily fluids of infected birds. As of July 2013, the World Health Organization reported a total of only 630 confirmed human cases of H5N1 infection since 2003, with a 60% case-fatality ratio. Several species of cats can be infected with and transmit the H5N1 virus to other cats. Transmission of virus from humans to humans or from cats to humans has not been demonstrated.

In 2011, two groups of scientists, one in the Netherlands and one in the United States, reported results from experiments that used a combination of genetic engineering and animal models to show that a very few mutations, 4 in the hemagglutinin (HA) gene and 1 in the PB2 gene, are sufficient to create a variant of H5N1 that could spread among ferrets in the laboratory, without direct contact and loss of virulence. As ferrets are susceptible to many of the same viruses as humans, these so-called gain-of-function studies sparked international concern and heated controversy about the potential for the creation and intentional or unintentional release of a pandemic agent, or whether transmission in ferrets is even a valid model for human transmission.

Some scientists called for censoring experimental details in the publications from the two groups. The public concerns eventually prompted 39 leading influenza researchers, including the 2 who led the controversial studies, to impose a voluntary moratorium on research designed to increase the transmissibility of H5N1 viruses in mammals until the development of government policies on biosafety and biosecurity for such research. Following two separate reviews by the U.S. National Science Advisory Board for Biosecurity, which advises the Department of Health and Human Services, it was decided that the full versions of both studies should be published. Finally, in March 2012, the United States government issued a Policy for Institutional Oversight of Life Sciences Dual Use Research of Concern, defined as "research that is intended for benefit, but might easily be misapplied to do harm." International bodies, including the World Health Organization, are also expected to propose international guidelines for such research.

In an unprecedented action, 22 influenza researchers from the United States, United Kingdom, and China, including the 2 leaders of the H5N1 gain-of-function experiments, published a letter in August 2013 announcing, and justifying, their intentions to conduct similar experiments (under appropriate biosafety conditions) with a second avian influenza virus, H7N9, which began infecting humans in eastern China earlier that year. The H7N9 virus can infect ferrets and can be transmitted between them by close contact. It can also infect pigs, a common intermediate host for the evolution of viruses that can then circulate in humans, but is not transmitted between pigs. This virus is highly pathogenic in humans, but in contrast to H5N1, infection of domestic fowl is asymptomatic, so that virus spread is more difficult to track.

Supporters of research on influenza virus transmissibility note that there are both scientific and practical needs to increase our



Map of the global spread of H5N1 influenza virus.

understanding of the parameters of immunogenicity, virulence, and transmissibility of these viruses. They suggest further that the results could allow public health workers to monitor wild viruses for critical mutations. Health agencies could then advise manufacturers of drugs and vaccines to increase production appropriately, or they may impose stricter public health measures to prevent transmission. Detractors of such research note that it is unclear if laboratory experiments on transmissibility can ever recapitulate events that occur in nature, and therefore such experiments are unlikely to yield results of relevance to natural human pandemics. How the laboratory results relate to evolution in nature is not clear, as the fitness of a virus with the identified mutations, singularly or in combinations, is unknown. Other scientists, and some groups in the public sector, contend that the potential risks of such experiments outweigh any possible benefits. Given the yearly death toll of seasonal influenza and the potential catastrophic consequences of a future pandemic, the current consensus is that such work should continue, albeit with appropriate oversight and safeguards.

Fouchier RA, Kawaoka Y, Cardona C, Compans RW, García-Sastre A, Govorkova EA, Guan Y, Herfst S, Orenstein WA, Peiris JS, Perez DR, Richt JA, Russell C, Schultz-Cherry SL, Smith DJ, Steel J, Tompkins SM, Topham DJ, Treanor JJ, Tripp RA, Webby RJ, Webster RG. 2013. Gain-of-function experiments on H7N9. *Science* **341:**612–613.

Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, Munster VJ, Sorrell EM, Bestebroer TM, Burke DF, Smith DJ, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* **336:**1534–1541.

Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K, Zhong G, Hanson A, Katsura H, Watanabe S, Li C, Kawakami E, Yamada S, Kiso M, Suzuki Y, Maher EA, Neumann G, Kawaoka Y. 2012. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature* **486:**420–428.

Patterson AP, Tabak LA, Fauci AS, Collins FS, Howard S. 2013. Research funding. A framework for decisions about research with HPAI H5N1 viruses. *Science* **339:**1036–1037.

Russell CA, Fonville JM, Brown AE, Burke DF, Smith DL, James SL, Herfst S, van Boheemen S, Linster M, Schrauwen EJ, Katzelnick L, Mosterín A, Kuiken T, Maher E, Neumann G, Osterhaus AD, Kawaoka Y, Fouchier RA, Smith DJ. 2012. The potential for respiratory droplet-transmissible A/H5N1 infuenza virus to evolve in a mammalian host. *Science* **336:**1541–1547.

the development of new technologies, as illustrated for those that infect humans (Fig. 11.8). It is now possible to detect and characterize unknown viruses with comparative ease.

Hepatitis Viruses in the Human Blood Supply

One of the first examples of the power of the new technologies was the recognition of hepatitis C virus. With the development of specific diagnostic tests for hepatitis A and B viruses in the 1970s, it became clear that most cases of hepatitis that occur after blood transfusion are caused by other agents. Recombinant DNA technology was used in the late 1980s to identify one of the non-A, non-B hepatitis (NANBH) agents as a new virus, named hepatitis C virus (Box 11.6). The availability of the hepatitis C virus genome sequence made possible the development of diagnostic reagents that effectively eliminated the virus from the U.S. blood supply, substantially reducing the incidence of transfusion-derived NANBH.

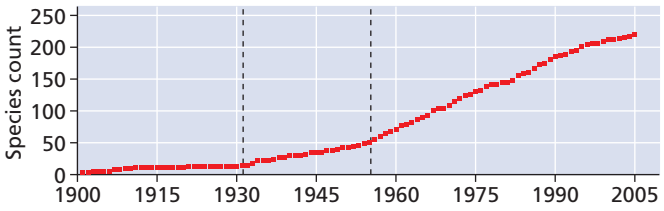
Hepatitis C virus was not the only previously hidden virus in the human blood supply; the same recombinant DNA technology permitted the discovery of several other viruses, including TT virus, a ubiquitous human circovirus of no known consequence.

A Revolution in Virus Discovery

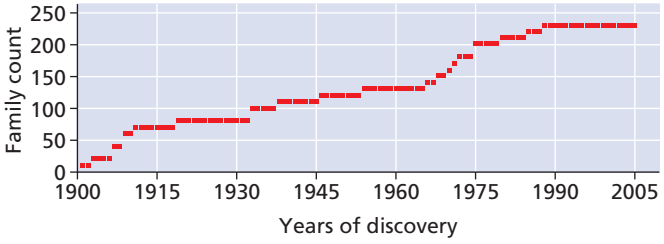
Newly discovered nucleic acid sequences can now be associated with diseases and characterized in the absence of standard virological techniques, in time frames measured in days rather than month or years. The etiological agent of SARS was identified after PCR amplification using a "virochip" that contained oligonucleotides from each of the known viral genomes. However, because of high sensitivity and the potential for contamination by adventitious viruses, special caution

Figure 11.8 Major developments in methods for virus discovery drive the identification of viruses that infect humans. **(A)** Discovery by species of virus. Vertical lines indicate significant upward breakpoints. **(B)** Discovery by virus family. **(C)** Technological advances through the 20th century to the present. Reprinted from M. Woolhouse et al., *Philos Trans R Soc Lond B Biol Sci* **367:**2864–2871, 2012, with permission.

A



B



C

Year	Technology
1890s	Filtration
1929	Complement fixation
1948	Tissue culture
1970s	Monoclonal antibodies
1985	Polymerase chain reaction (PCR)
2000s	High throughput sequencing

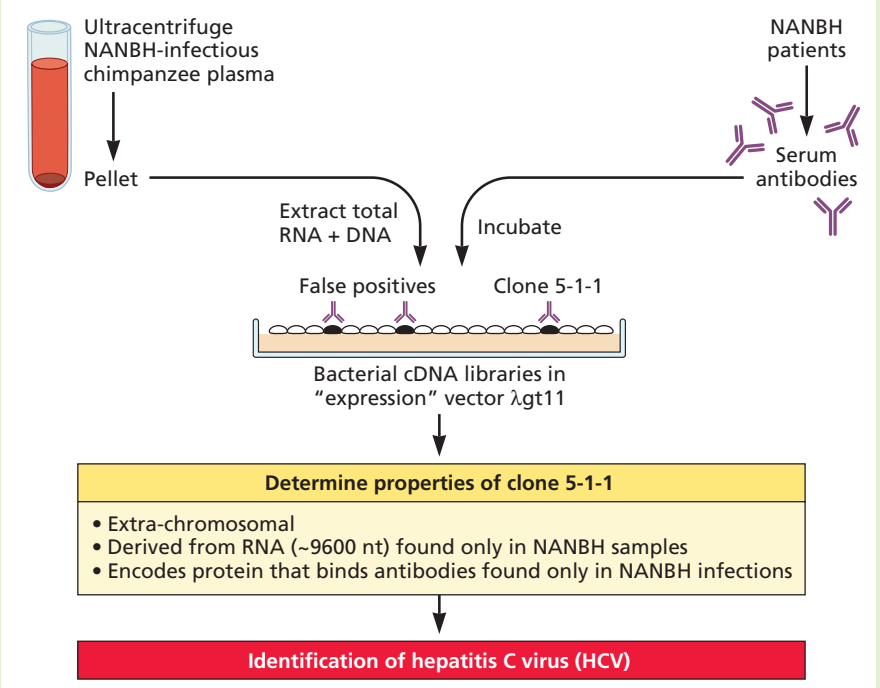
BOX 11.6

BACKGROUND

Discovery of hepatitis C virus, a triumph of persistence

The virus called non-A, non-B hepatitis (NANBH) virus was known to be contracted via blood transfusion. As it was refractory to laboratory culture, the identity of this virus remained elusive until Chiron Corporation, a California biotechnology company, isolated a DNA copy of a fragment of what was later identified as the hepatitis C virus genome from a chimpanzee with NANBH. Following nearly 6 years of intensive investigations, this remarkable feat was accomplished by screening a library of ~1,000,000 randomly primed complementary DNA clones made from RNA in the plasma of the infected chimp. The researchers tested the clones for their ability to produce proteins that were recognized by serum from a patient with chronic NANBH. A single small cDNA clone was positive in this assay and was found to be derived from an RNA molecule comprising a (+) strand genome of ~10,000 nucleotides with high homology to those of known flaviviruses.

Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244:**359–362.



Schematic for molecular isolation and identification of hepatitis C virus. Adapted from Q.-L. Choo et al., *Science* **244:**359–362, 1989, with permission.

is necessary when using any PCR-based or hybridization method. Without proper controls, these techniques have the potential to associate a particular virus with a disease incorrectly, confounding the deduction of etiology. One example was the misidentification of what later proved to be a contaminating recombinant mouse retrovirus (XMRV) as a "novel" human virus associated with prostate cancer and chronic fatigue syndrome. Similarly, a variety of studies have been published in which PCR identified human herpesviruses in the brains of patients who had died of Alzheimer's disease, implying a causal link between the disease and the virus. The latter conclusions are called into question because the same viruses can be found in the tissues of similarly aged patients who died of other causes. These examples illustrate the fact that availability of the new technologies does not circumvent the need to satisfy the modified Koch's postulates (Volume I, Box 1.4).

For virus discovery, virochip technology was superseded rapidly by the use of methods that apply high-throughput, next-generation sequencing technology and associated computational tools. For example, a new virus of major agricultural importance was discovered in 2011 by the use of metagenomic analysis of sequences in tissues obtained from diseased animals. The agent was named Schmallenburg virus after the German town from which the first positive tissue samples were obtained. The virus is transmitted by biting midges and has spread rapidly to farms across Europe and the United Kingdom. Infection of cattle, goats, or sheep is associated with fairly mild disease symptoms, but the fetuses of these animals are stillborn or malformed. The Schmallenburg virus has been identified as a member of the family *Bunyaviridae*. Following Koch's postulates, it has been shown that animals injected with purified virus particles do, indeed, develop the disease. Schmallenburg virus dissemination is now being monitored by PCR.

Perceptions and Possibilities

While emerging virus infections are well known to virologists, in recent years they have become the subject of widespread public interest and concern. Less than 40 years ago, many people were ready to close the book on infectious diseases. The public perception was that wonder drugs and vaccines had microbes fully under control. This optimistic view has now changed dramatically. Announcements of new and

destructive viruses and bacteria appear with increasing frequency. The reality of the human immunodeficiency virus pandemic and its effects at every level of society have attracted worldwide attention, while exotic viruses like Ebola virus capture front-page headlines. Movies and books bring viruses to the public consciousness more effectively and dramatically than ever before. After the events of September 11, 2001, concern that terrorists might use infectious agents was widespread (Box 11.7).

Virus Names Can Be Misleading

Much information is implied (inappropriately in some cases) when naming a virus by the host from which it was isolated. By using the name **human** immunodeficiency virus for the virus that causes AIDS, we give short shrift to its nonhuman origins. Canine parvovirus is clearly a feline virus that recently switched hosts. Similarly, canine distemper virus is not confined to dogs, but can cause disease in lions, seals, and dolphins. Well-known viruses can cause new diseases when they change hosts. Much is implied, and more is ignored, about the host-virus interaction when the virus is given a host-specific name.

BOX 11.7

DISCUSSION

Viral infections as agents of war and terror

Infectious agents have a documented capacity to cause harm, and can cause epidemics as well as pandemics. Well-known deadly viruses range from agents of universal scourges, such as the recently eradicated smallpox virus and the influenza viruses, to the less widely distributed, but no less deadly, hemorrhagic fever viruses. Any viral infection that can kill, maim, or debilitate humans, their crops, or their domesticated animals has the potential to be used as a biological weapon. Obviously, a biological attack need not cause mass destruction to be an instrument of terror, as was demonstrated by the far-reaching effects of the introduction of bacteria causing anthrax into the United States mail system. Society has only a limited set of responses to frightening outbreaks: vaccination, quarantine, and antimicrobial drugs. One example is the unintentional 1947 outbreak of smallpox in New York City, which originated from a single businessman who had acquired the disease in his travels. He died after infecting 12 others; to stop the epidemic, >6 million people were vaccinated within a month.

As with such natural outbreaks, potential bioterrorism threats pose serious problems with few clear solutions. Some argue that the resources devoted by governments to counterterrorism and research on category A pathogens would be better applied to research on public health or naturally occurring common diseases. Others are concerned that publication of research data on such pathogens could aid terrorists, and maintain that measures to control the publication of such information must be considered (Box 11.5). Practically speaking, public health officials view bioterrorism as a low-probability but high-impact event, much like major hurricanes or tsunamis. When such events occur, they are devastating. However, the hallmarks of these calamities are that they cannot be predicted with accuracy or easily prevented. Societies can only prepare by ensuring that remedial actions can be taken as quickly as possible.

All Viruses Are Important

It is not uncommon to consider disease-causing viruses important while deeming nonpathogenic viruses uninteresting and irrelevant. But as we have seen, a virus that is stable in one host may have devastating effects when it enters a different species. Conversely, a virus may be pathogenic in one species but not in another. Some viruses are even beneficial to their hosts or the environment (Volume I, Chapter 1). Misconceptions often arise from human-centered thinking, a belief that viruses causing human diseases are more important than those that infect mammals, birds, fish, or other hosts, forgetting that all life forms are interconnected. By focusing solely on viruses that can infect humans, we are blinded to the intertwined networks of interactions that comprise host-virus relationships.

What Next?

Can We Predict the Next Viral Pandemic?

It is now clear that some of the most serious threats to the human population come not come from the popularized, highly lethal filoviruses (e.g., Ebola virus), the hemorrhagic disease viruses (e.g., Lassa virus), or even some undiscovered virus lurking in the wild. Rather, the most dangerous viruses are likely to be the well-adapted, multihost, evolving viruses already in the human population. Influenza virus fits this description perfectly. Its yearly visits show no signs of diminishing; genes promoting pandemic spread and virulence are already circulating in the virus population, and the world is ever more prone to its dissemination. A pandemic of influenza on the scale of, or even greater than, that of the 1918–1919 outbreak is thought by many to be the next emerging disease most likely to affect humans (Table 11.4).

Traditional monitoring tools are used to detect the onset and gauge the severity of the yearly influenza outbreaks in various countries. Such monitoring depends, in large part, on networks of physicians who report cases of patients with flu-like symptoms. Traditional monitoring might also detect the emergence of some new infection, especially if suspicious symptoms appear at an unusual time of the year. Other methods attempt to predict the onset of influenza outbreaks from Internet queries and communication on social media. For example, Google-flu relies on data-mining records of flu-related search terms entered into its search engine, combined with computer modeling. In the United States, Google-flu estimates have matched those obtained from traditional methods by the Centers for Disease Control and Prevention, but Google can deliver the information several days faster. Another computational approach, intended to

inform influenza vaccine production, has been to incorporate genetic data on beneficial and deleterious mutations in the antibody-binding domains on the hemagglutinin proteins of circulating strains from previous years into a fitness-based model that will predict the frequency of descendent strains in the following year. A retrospective analysis indicates that the model can successfully predict the year-to-year evolution of individual influenza clades. Although not yet perfect, these nontraditional approaches represent a promising complementary strategy for the future, at least for the short-term prediction of potential epidemics.

Emerging Viral Infections Illuminate Immediate Problems and Issues

The AIDS pandemic and our experience with SARS illustrate the ease with which a new virus can enter the human population. The secondary infections that accompany AIDS have underscored the fragility of both the world's health care systems and the infrastructure of developing countries. Outbreaks of new exotic viruses, zoonoses, or well-known viruses that have invaded new geographic niches highlight the need for standardized methods of diagnosis, epidemiology, treatment, and control.

Humans Constantly Provide New Venues for Infection

Past experiences with poliovirus, measles virus, and smallpox virus demonstrate that viruses can cause illness and death on a catastrophic scale following a change in human behavior. Current technological advances and changing environmental and social behaviors continue to influence the spread of viruses (Table 11.2). Most of the contemporary opportunities for interaction between humans and viruses did not exist 50 years ago. Furthermore, the highly connected human population is larger than it has ever been, and is still growing (Fig. 11.9). As a consequence, humans are interacting among themselves and with the environment on a scale unprecedented in history.

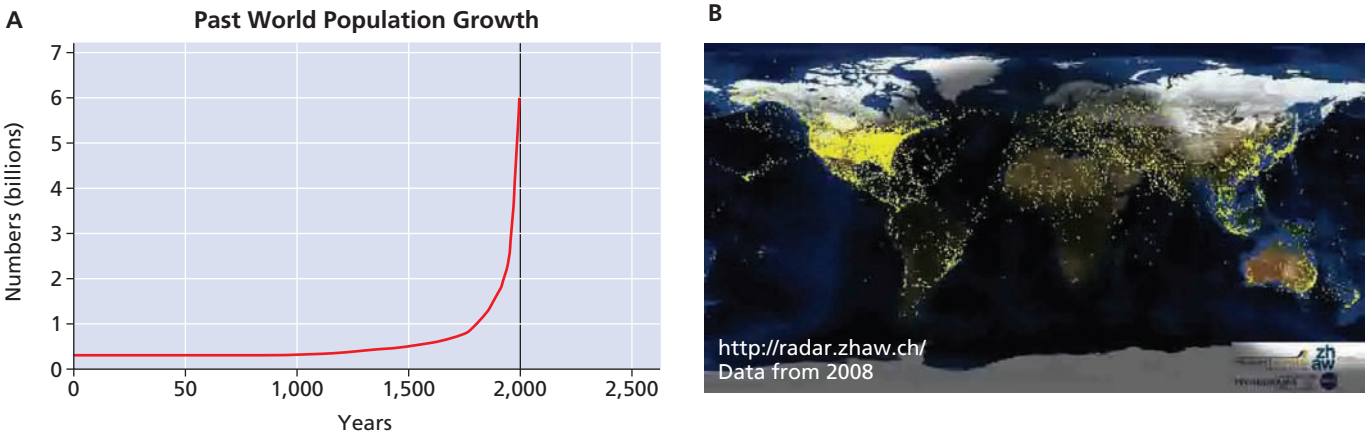
Many human activities have major effects upon the transmission of viruses by vectors, such as insects and rodents (Table 11.2). Population movements, the transport of livestock and birds, the construction and use of irrigation systems, and deforestation provide not only new contacts with mosquito and tick vectors, but also mechanisms for transport of infected hosts to new geographic areas.

Humans can also provide new habitats for viruses, as demonstrated with used tires. Several species of tropical mosquitoes (e.g., *Aedes* species) prefer to breed in small pockets of water that accumulate in tree trunks and flowers in the tropics. The used tire has provided a perfect mimic of this breeding ground, and as a consequence, the millions of used tires (almost all carrying a little puddle of water inside) accumulating around the world provide a new habitat for mosquitoes and their viruses. Furthermore, used tires are shipped all around the world for recycling, effectively transmitting mosquito larvae along with them. Insect vectors for viruses may be given the chance to establish new ranges thanks to such shipments.

Another contemporary example of the consequences of humans moving viruses to new hosts comes from transport of livestock. African swine fever virus, a member of the family *Iridoviridae*, causes a serious viral disease that is threatening the swine industries of both developing and industrialized countries. The African swine fever virus was spread from Africa to Portugal in 1957, to Spain in 1960, and to the Caribbean and South America in the 1960s and 1970s, via long-distance transport of livestock and their resident infected arthropods.

The construction of dams and irrigation systems can influence host-virus interactions through creation of vast areas of standing water. The 1987 outbreak of Rift Valley fever along

Figure 11.9 World population growth over the last 2 millennia. (A) The world population grew from 1.6 billion to 6.1 billion in the 20th century, and is expected to increase to 9.2 billion by 2050, with growth almost entirely in less-developed regions. Graph from the United Nations Population Division, used with permission. **(B)** A connected world: one day in global air traffic. Each dot represents one plane.



the Senegal River was associated with the new Diama Dam, which provided conditions ideal for mosquito propagation. Not only do water impoundments affect insects, but they also alter the population and migration patterns of waterfowl and other animals, including the viruses they carry, bringing together previously separated viruses and potential new hosts.

In industrialized countries, the increasing need for day care centers has led to new opportunities for viral transmission. In the United States, many millions of children are in day care centers for several hours a day, and the vast majority are under 3 years of age. As most parents can testify, respiratory and enteric infections are common, and these infections spread easily among other children, day care workers, and the family at home.

Finally, among the most important human activities likely to affect the emergence of viral disease are those that are causing climate change. Global warming is already having an impact on all living things; viruses are no exception. Warming temperatures and increased rainfall in certain areas have led to an upsurge in the incidence of insect vector-borne infections; the spread of dengue virus from the equatorial areas to which it was previously confined is one clear example. Indirect effects of climate change such as flooding, disruption of human and wildlife populations, reduced food supply, and economic distress can all increase opportunities for new virus-host relationships to be established. As global warming continues, new reports of emerging viruses can be expected.

Preventing Emerging Virus Infections

The modernization of society and the expanding human population have facilitated the spread of infection, selection of virus variants, and virus emergence. We cannot turn back the clock, but experience and acquired knowledge can provide some guidance for ameliorating actions in the future. In some cases, viral emergence can be blocked quite effectively, as illustrated for infections of humans by the highly pathogenic influenza strain H7N9—once wild bird markets were identified as the major route of infection (Fig. 11.10). Knowledge that camels are a likely source of the equally pathogenic MERS coronavirus should lead to methods for preventing future human infections.

Modern diagnostic techniques have made it possible, although not yet entirely practical, to estimate the total viral diversity in any, or all, animal species. For example, one study of almost 2,000 samples collected from a particular species of bat, the Indian flying fox (*Pteropus giganteus*), showed that these animals collectively harbor 58 different viruses from 7 known families, the majority of which had not been identified previously. Extrapolation from these results to all mammalian species suggests that at least ca. 320,000 different viruses are waiting to be discovered. Although that number seems daunting, it would be possible to screen viruses identified in common or suspected reservoir species for their

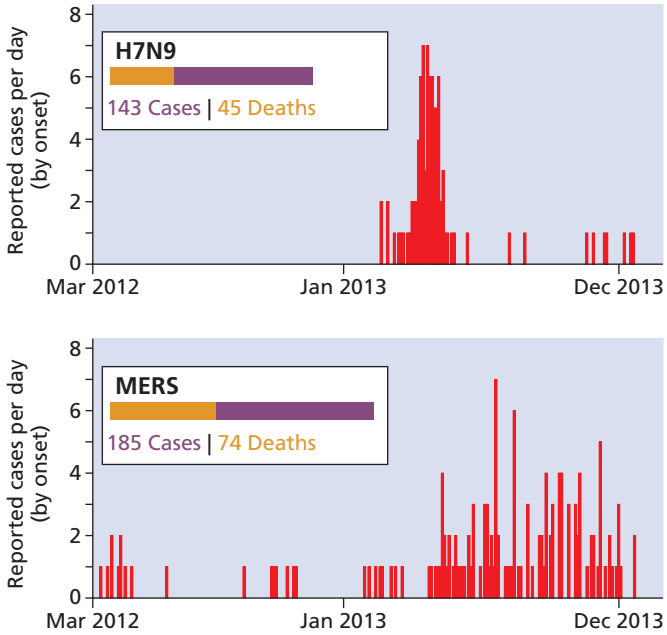


Figure 11.10 Knowledge of the reservoir species affects the outcome of infection of humans by emerging viruses. Adapted from Andrew Rambaut/WHO, with permission.

potential to bind to receptors on human cells (Box 11.4). Diagnostic reagents for such viruses could then be applied in disease cases of unknown etiology. Identification of the particular virus and animal reservoir could help to "nip" nascent zoonoses "in the bud" before they become more widespread public health problems.

Because of the potential for rapid spread of viruses via air travel and urban development, a system of global surveillance and early warning is required to alert primary care physicians and health care workers. Such efforts have begun, as illustrated in the recent SARS epidemic and MERS outbreaks. With modern technology, it is conceivable that all viral pathogens circulating in humans could be monitored. When a new (or old) viral disease is suspected, the agent could be identified and characterized and the information could be shared widely. These responses would be facilitated with the development of methods for rapid diagnosis in the field and ready access to early-warning databases for primary health care workers.

Perspectives

It has been estimated that upwards of 60% of human infectious diseases originate from animal reservoirs, and many of these zoonoses are caused by viruses. The relationships between viruses and their hosts are in constant flux, and numerous factors, most related to the modern human population explosion, have led to an increase in the adoption of new ecological niches or geographic zones by emerging viruses.

Given the ever-changing viral populations and drastic modifications of the earth's ecosystem, we are hard-pressed

to predict the future. Mathematical modeling, powerful new diagnostic tools, and increased efforts at surveillance in various governmental and international agencies, particularly of animal species known to present the greatest risk, should help to provide early warnings of potential emergence. Our ability to identify viral pathogens has increased enormously in the past decade. While it took a few weeks in 2003 to identify SARS coronavirus by nucleic hybridization on a "virochip," only a few days were needed in 2012 to identify the MERS coronavirus by next-generation sequencing and bioinformatic tools. A deeper understanding of the diversity of viruses in various species will point to areas or situations in which particular vigilance may be warranted for possible cross-species infections. In addition, computer simulations are improving our ability to track the potential spread of emerging viruses, a great advantage in cases where the isolation or treatment of infected individuals can prevent further transmission.

Open avenues of communication between scientists, health care workers, and veterinarians in all parts of the globe can help to minimize the spread of infections and enable the development of strategies to cope with the consequences. Experience tells us that future incidences of infection by newly emerging viruses are inevitable.

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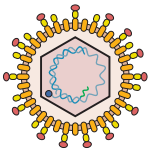
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12 Unusual Infectious Agents



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LINKS FOR CHAPTER 12

⏭ ***Darwin gets weird***
http://bit.ly/Virology_Twiv78

⏭ ***Wasting deer and the Hulk rabbit***
http://bit.ly/Virology_Twiv67

⏭ ***Is chronic wasting disease a threat to humans?***
http://bit.ly/Virology_3-11-15

Simple solutions seldom are.
ALFRED NORTH WHITEHEAD

Introduction

Genomes of nondefective viruses range in size from 2,400,000 bp of double-stranded DNA (*Pandoravirus salinus*) to 1,759 bp of single-stranded DNA (porcine circovirus). Are even smaller viral genomes possible? **Viroids**, **satellites**, and **prions** provide answers to these questions. The adjective "subviral" was coined, in part, because these agents did not fit into the standard taxonomy schemes for viruses. The Subviral RNA Database (http://subviral.med.uottawa.ca/cgi-bin/home.cgi) lists 2,923 nucleotide sequences for viroids and satellites. No prion sequences will be found in this database, because these infectious agents are, remarkably, devoid of nucleic acid.

Viroids

Viroids, the smallest known pathogens, are unencapsidated, circular, single-stranded RNA molecules that do not encode protein yet replicate autonomously when introduced into host plants. Potato spindle tuber viroid, discovered in 1971, is the prototype (Fig. 12.1); 29 other viroids ranging in length from 120 to 475 nucleotides have since been discovered. Viroids are known to infect only plants (Box 12.1); some cause economically important diseases of crop plants, while others appear to be benign, despite their widespread presence in the plant world. Two examples of economically important viroids are coconut cadang-cadang viroid (which causes a lethal infection of coconut palms) and apple scar skin viroid (which causes an infection that results in visually unappealing apples).

The 30 known viroids have been classified in two families. Members of the *Pospiviroidae*, named for potato spindle tuber viroid, have a rodlike secondary structure with small single-stranded regions, and a central conserved region (Fig. 12.1A), and replicate in the nucleus. The *Avsunviroidae*, named for avocado sunblotch viroid, have both rodlike and branched regions, but lack a central conserved region (Fig. 12.1B) and replicate in chloroplasts. In contrast to the *Pospiviroidae*, the latter RNA molecules are functional ribozymes, and this activity is essential for replication.

Viroids typically have a narrow host range that is implied by their names. However, host range expansion of some viroids has been observed. For example, potato spindle tuber viroid can infect avocado and tomato, and the weeds found in potato and hop fields can support the replication of both this viroid and hop stunt viroid.

After introduction into a plant, all viroids reproduce according to the following steps: import into a cellular organelle, replication, export out of the organelle, trafficking to adjacent cells, entry into the phloem (the plant vascular system that conducts nutrients downward from the leaves), long-distance movement to leaves and roots, and exit from phloem into new cells to repeat the cycle.

Replication

There is no evidence that viroids encode proteins or mRNA. Unlike viruses, which are parasites of the host translation machinery, **viroids are parasites of cellular transcription proteins:** they depend on cellular RNA polymerases for replication. Such polymerases normally recognize DNA templates, but can copy viroid RNAs.

In plants infected with members of the *Pospiviroidae*, viroid RNA is imported into the nucleus, probably by the nuclear import machinery. Plant DNA-dependent RNA polymerase II binds to the left terminal domain of potato spindle tuber viroid, suggesting that this structure serves as an origin of replication. In the nucleus the viroid is copied by a rolling circle mechanism that produces complementary linear, concatemeric, RNAs (Fig. 12.2). These products are copied again to produce concatemeric, linear RNA molecules, which are cleaved by RNase III. The linear, monomeric RNA molecules produced by cleavage of multimers have 5′-monophosphate and 3′-hydroxyl at their termini, groups required for ligation by DNA ligase I.

In plants infected with members of the *Avsunviroidae*, viroid RNA is imported into the chloroplast by an unknown import pathway, and complementary RNAs are produced by chloroplast DNA-dependent RNA polymerase. The circular RNA is then copied into a linear concatemeric RNA. After self-cleavage by ribozymes and ligation, the RNAs serve as templates for a round of concatemeric RNA synthesis,

PRINCIPLES ***Unusual infectious agents***

- Viroids and prions are the smallest known pathogenic agents.
- Viroids comprise only noncoding RNA that is replicated by enzymes of plant host cells.
- Satellites depend on helper viruses for their reproduction.
- Diseases caused by viroids and satellites appear to be the result of silencing of expression of host genes.
- Hepatitis delta satellite virus, which exacerbates the pathogenesis of hepatitis B virus, is a unique hybrid of a viroid and a satellite.
- Prions are infectious proteins that cause neurological diseases of protein misfolding (transmissible spongiform encephalopathies, TSEs).
- There are three ways to contract a TSE: sporadic, infectious, and familial.
- Humans have increased the prevalence of TSEs by feeding cattle the remains of diseased animals.
- TSEs are surprisingly prevalent in wild deer and elk populations in North America, and represent a potential source of transmission to hunters and agricultural animals.

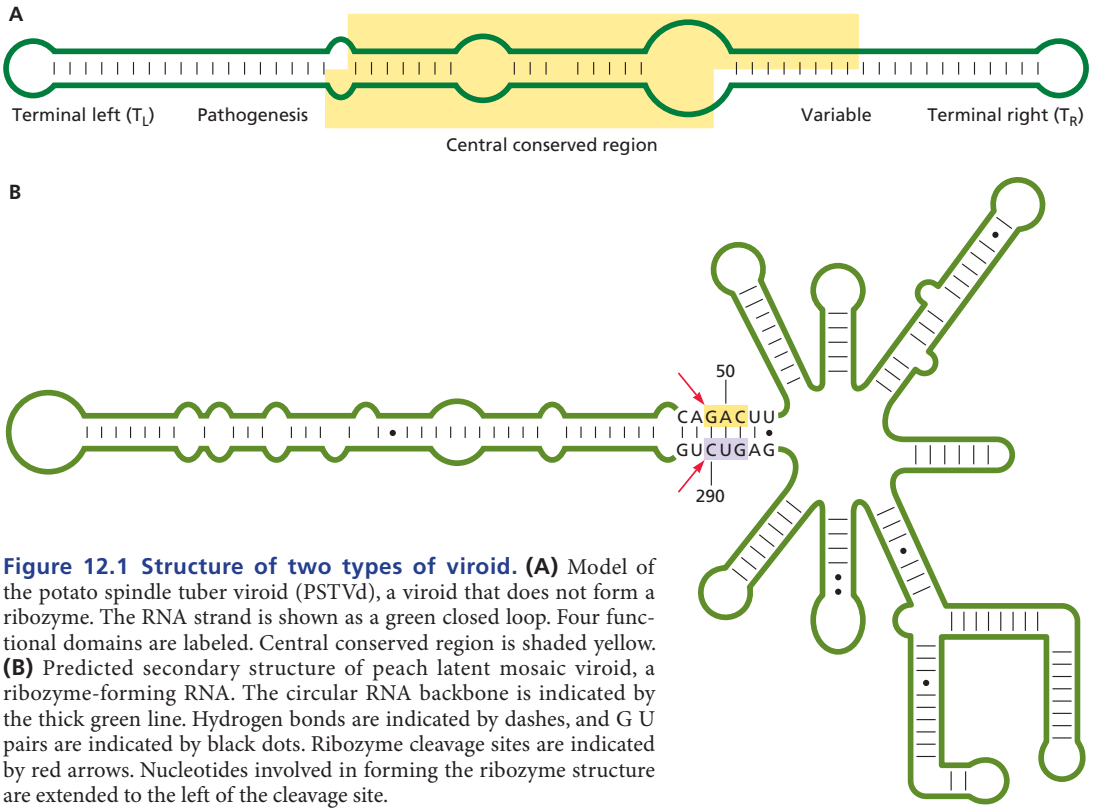


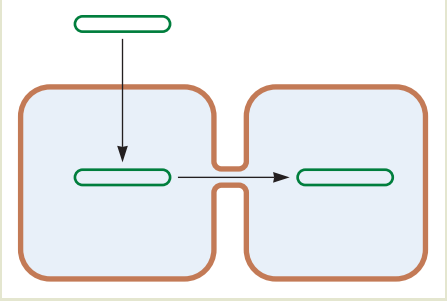
Figure 12.1 Structure of two types of viroid. (A) Model of the potato spindle tuber viroid (PSTVd), a viroid that does not form a ribozyme. The RNA strand is shown as a green closed loop. Four functional domains are labeled. Central conserved region is shaded yellow. **(B)** Predicted secondary structure of peach latent mosaic viroid, a ribozyme-forming RNA. The circular RNA backbone is indicated by the thick green line. Hydrogen bonds are indicated by dashes, and G U pairs are indicated by black dots. Ribozyme cleavage sites are indicated by red arrows. Nucleotides involved in forming the ribozyme structure are extended to the left of the cleavage site.

BOX 12.1

DISCUSSION

Why do viroids only infect plants?

Viroids infect plants cells after mechanical damage of the plant cell wall. Unlike plant viruses, there are no known animal vectors that transmit viroids from plant to plant. After genome replication in the plant cell, viroid RNA moves to the next cell by passage through plasmodesmata, the microchannels that connect neighboring plant cells (Chapter 12, Box 12.2). Animal cells do not have such connections, requiring that viruses travel from cell to cell either after release in the extracellular fluids, or by direct fusion with the membrane of a neighboring cell. Viruses may also travel from host to host in many ways, including aerosols and vectors, that are not available to viroids. Plants therefore seem well suited to serve as hosts for small, circular naked RNA molecules.



followed by cleavage and ligation to produce mature viroids. The self-ligating activity of viroids in the family *Avsunviroidae* is enhanced by a chloroplast tRNA ligase.

Replication of viroids therefore requires three enzymatic activities: RNA polymerase, RNase, and RNA ligase, and, for the former and the latter cases, the unusual situation of DNA enzymes working on RNA templates.

Sequence Diversity

Members of the family *Pospiviroidae* display little sequence diversity: the consensus sequence of different strains does not differ substantially. In contrast, members of the *Avsunviroidae* vary considerably. The difference is likely a consequence of the fidelity of the two RNA polymerases that carry out viroid replication. Two viroids that infect the same plant, the nuclear Chrysanthemum stunt viroid and the chloroplastic Chrysanthemum chlorotic mottle viroid have widely different mutation frequencies and provided the first experimental support of the "survival of the flattest" model of evolution (Box 12.2).

Movement

After replication, viroid progeny leave the nucleus or chloroplast and move to adjacent cells through plasmodesmata, and can travel systemically via the phloem. The results of mutational analyses demonstrate the requirement of specific

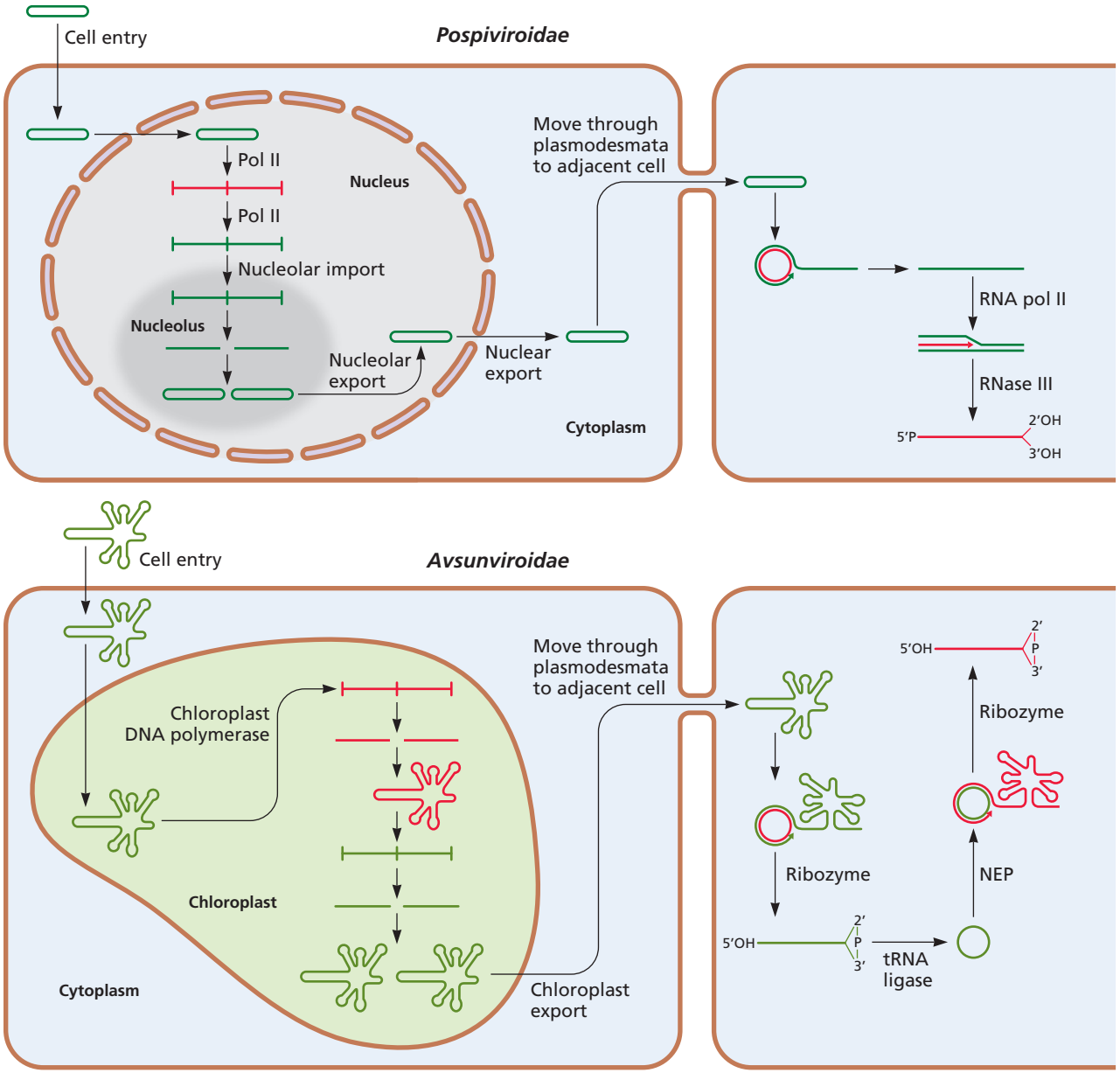


Figure 12.2 Replication of two different types of viroid in plants. (Top) Replication of *Pospiviroidae* in the nucleus. After entering the cell, circular viroid RNA is imported into the nucleus and copied by RNA polymerase II to form concatemeric RNAs. These are imported into the nucleolus where they are processed by cellular enzymes to genome-length RNAs and circularized before export and movement to the next cell through plasmodesmata (Pd). Right panel shows the details of viroid rolling-circle replication without showing cellular compartments. Complementary RNAs are red. **(Bottom)** Replication of *Avsunviroidae* in the chloroplast. After entering the cell, circular viroid RNA is imported into the chloroplast copied to form concatemeric RNAs. These are processed by the ribosome activity of the viroid. Right panel shows the details of rolling-circle replication without showing cellular compartments.

RNA loops and bulges for systemic transport within plants. Furthermore, a variety of host proteins that bind viroid RNA have been identified. These results have led to the hypothesis that trafficking of viroids within plants, from cell to cell or over longer distances, depends upon specific RNA sequences and structures that interact with cell proteins, including those that participate in movement. Some of these movement proteins also move viruses within plants (Volume I, Chapter 13). Viroids enter the pollen and ovule, from where they are transmitted to the seed. When the seed germinates, the new plant becomes infected. Viroids can also be transmitted among plants by contaminated farm machinery and insects.

BOX 12.2

DISCUSSION

Viroids and mutation rates

The quasispecies model of evolution predicts that, at a high mutation rate, populations with higher mutational robustness can displace those with a higher replicative capacity. This phenomenon has been called "survival of the flattest." The fitness associated with a sequence depends on the average fitness of its neighbors. In the survival of the flattest effect, a population in an area with neutral neighbors can out-compete another population with a higher fitness peak but surrounded by more deleterious neighbors (Figure). As predicted by the "survival of the fittest" paradigm, Chrysanthemum stunt viroid out-competed Chrysanthemum chlorotic mottle viroid because it is faster replicating and more genetically homogeneous. However, when mutation rates were increased by ultraviolet irradiation of infected plants, the opposite effect was observed: Chrysanthemum chlorotic mottle viroid won the race.

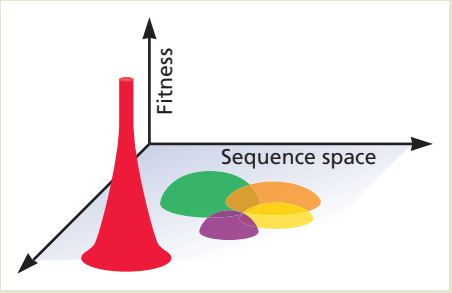


Illustration of a population with high fitness surrounded by deleterious neighbors (red) and one with lower fitness and neutral neighbors (multiple colors).

Codoñer FM, Daros JA, Solé RV, Elena SF. 2006. The fittest versus the flattest: experimental confirmation of the quasispecies effect with subviral pathogens. *PLoS Pathog* **2:**e136.

Pathogenesis

Symptoms of viroid infection in plants include stunting of growth, deformation of leaves and fruit, stem necrosis, and death. Because viroids do not produce mRNAs, it was first proposed that disease must be a consequence of viroid RNA binding to host proteins or nucleic acids. Consequently much effort was directed at identifying host proteins that interact with viroid RNAs. These studies also revealed that viroid infection causes extensive changes in the expression of many host genes. The results of mutational analyses showed that specific RNA sequences and structures are associated with pathogenesis (Fig. 12.1A). The discovery of RNA silencing in plants led to the hypothesis that small RNAs derived from viroid RNAs guide silencing of host genes, leading to induction of disease. For example, peach latent mosaic viroid small interfering RNAs that silence chloroplast heat shock protein 90 and lead to disease symptoms have been identified. The two small interfering RNAs that target heat shock protein 90 mRNA are derived from the less abundant complementary strand. These observations suggest that different disease patterns caused by viroids in their hosts might all have in common an origin in RNA silencing.

Our current understanding is that the disease-causing viroids were transferred from wild plants used for breeding modern crops. The widespread prevalence of these agents can be traced to the use of genetically identical plants (monoculture), worldwide distribution of breeding lines, and mechanical transmission by contaminated farm machinery. As a consequence, these unusual pathogens now occupy niches around the planet that never before were available to them.

Satellites

Satellites are subviral agents that differ from viroids because they depend on the presence of another virus (the **helper virus**) for their propagation. Two general classes of satellites can be distinguished. **Satellite viruses** are distinct particles that were discovered in preparations of their helper viruses. These particles contain nucleic acid genomes that encode a structural protein that encapsidates the satellite genome. **Satellite RNAs** do not encode capsid proteins, but are packaged by a protein encoded in the helper virus genome. Satellite genomes may be single-stranded RNA or DNA (Table 12.1), and are replicated by enzymes provided by the helper virus. The origin of satellites remains obscure, but they are **not** derived from the helper virus: their genomes have no homology to the helper. At least one satellite RNA, of cucumber mosaic virus, appears to have originated from repetitive DNA in the plant genome.

Table 12.1 Viroids and satellites

	Viroids		
Property	***Avsunviroidae***	***Pospiviroidae***	**Satellites**
Requires coinfection with helper virus	No	No	Yes
Encodes protein	No	No	Yes
Replication	By host RNA polymerase and viroid ribozyme	By host RNA polymerase and host RNase	By helper virus replication proteins

Satellite viruses may infect plants, animals, or bacteria. An example of a satellite virus is satellite tobacco necrosis virus, which encodes a capsid protein that forms a $t = 1$ icosahedral capsid that selectively packages the 1,260-nucleotide satellite RNA. The helper virus, tobacco necrosis virus, encodes an RNA polymerase that replicates its genome and that of the satellite. The tobacco necrosis virus 5 kb RNA genome is packaged in a $t = 3$ icosahedral capsid made only from viral subunits. The structures of satellite virus and helper virus need not always be similar: the capsid of satellite tobacco mosaic virus is icosahedral, while virus particles of its helper virus are helical rods.

Satellite RNAs do not encode a capsid protein and therefore require helper virus proteins for both genome encapsidation and replication. Satellite RNA genomes range in length from 220 to 1,500 nucleotides, and have been placed into one of three classes. Class 1 satellite RNAs are 800- to 1,500-nucleotide linear molecules with a single open reading frame encoding at least one nonstructural protein. Class 2 satellite RNAs are also linear, but less than 700 nucleotides long and do not encode protein. Class 3 satellite RNAs are 350- to 400-nucleotide-long circles without an open reading frame.

Replication

The linear genomes of satellite viruses and satellite RNAs are copied by helper virus enzymes, and the mechanisms of replication are presumably similar (Volume I, chapters 6, 9). Satellite viruses typically impair production of the helper virus. For example, satellite tobacco necrosis virus reduces the yield of tobacco necrosis virus to undectable levels, while adenovirus-associated virus decreases the yield of adenovirus by >90%. How helper virus RNA polymerases recognize satellite RNA genomes is not known, because they have no sequence or structural similarity with the genome of the helper virus. Low-level replication in the absence of a helper virus has been demonstrated for a satellite RNA of cucumber mosaic virus. Such replication, which occurs in the cell nucleus and likely requires a host enzyme, may be a mechanism for persistence of satellite RNA.

Circular satellite RNA genomes are replicated by a rolling-circle mechanism like that of viroids (Fig. 12.2), except that replication by the helper virus RNA polymerase takes place in the cytoplasm. This enzyme recognizes a sequence on the satellite encapsidated RNA genome and produces complementary concatemers (Fig. 12.3). Depending on the satellite RNA, this product may be the template for the synthesis of multimeric copies of the encapsidated strand, or it may be cleaved and circularized by a ribozyme, prior to production of multimeric copies. The latter are then cleaved and ligated by a ribozyme followed by packaging. In some cases, linear strands may be packaged, but these are circularized upon infection of a new cell.

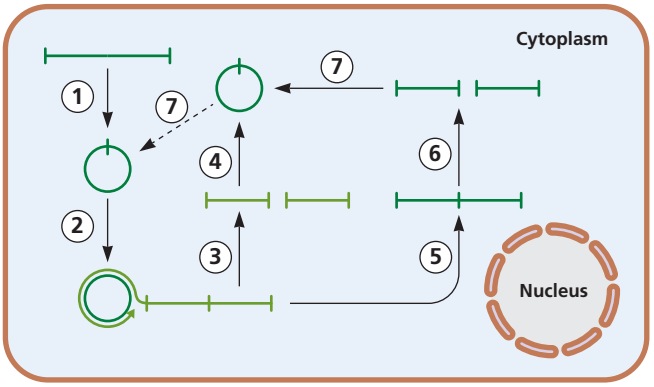


Figure 12.3 Replication of satellite RNA. Satellite RNA enters the plant cell and, if linear, is converted to circular RNA (1) which is then copied by a rolling-circle mechanism to produce concatemeric complementary copies (2). These are cleaved to form genome-length RNAs, possibly by ribozymes (3). Alternatively, the concatemeric RNAs might be copied (5) and then cleaved (6). Newly synthesized RNAs could then reenter the replicative pathway after circularization by a ribozyme or host enzyme.

Pathogenesis

In plants, satellites and satellite viruses may attenuate or exacerbate disease caused by the helper virus. Examples of disease include necrosis and systemic chlorosis, or reduced chlorophyll production leading to leaves that are pale, yellow, or yellow-white. Most satellite RNAs reduce the reproduction and yield of helper viruses, which leads to milder disease. The same satellite RNA may attenuate symptoms in one host, and cause greater disease in another. For example, disease caused by cucumber mosaic virus in tomatoes is much more severe in the presence of satellite cucumber mosaic virus RNA. However, in other hosts, the presence of the satellite RNA attenuates signs of disease symptoms. This effect may be due to the induction of a strong plant antiviral response mediated by RNA interference, resulting from high concentrations of satellite RNA.

The symptoms induced by satellite RNAs are thought to be a consequence of silencing of expression of host genes. For example, the Y-satellite RNA of cucumber mosaic virus causes systemic chlorosis in tobacco. This syndrome is caused by production of a small RNA from the Y-satellite RNA that has homology to a gene needed for chlorophyll biosynthesis. Production of this small RNA leads to degradation of the corresponding mRNA, resulting in bright yellow leaves. Consistent with this hypothesis, production of a potyvirus suppressor of silencing in tobacco plants reduces the severe yellowing caused by the cucumber mosaic virus and its satellite RNA.

Virophages or Satellites?

The giant DNA viruses including *Acanthamoeba polyphaga* mimivirus, *Cafeteria roenbergensis* virus, and others are associated with much smaller viruses (sputnik and mavirus,

respectively) that depend upon the larger viruses for reproduction. For example, sputnik virus can only replicate in cells infected with mimivirus, and does so within viral factories. Whether these are satellite viruses or something new (they have been called virophages) has been a matter of controversy.

Sputnik and others have similar relationships with their helper viruses as satellite viruses: they require their helper for their propagation, but their genomes are not derived from the helper, and they negatively impact helper reproduction. Others argue that the definition of satellite viruses as subviral agents cannot apply to these very large viruses. For example, sputnik virophage contains a circular double-stranded DNA genome of 18,343 bp encoding 21 proteins encased in a 75-nm $t = 27$ icosahedral capsid. Sputnik is dependent upon mimivirus not for DNA polymerase (it encodes its own), but probably for the transcriptional machinery of the helper virus. Those who favor the name virophage argue that dependence upon the cellular transcriptional machinery is a property of many autonomous viruses, the only difference is that Sputnik depends upon that provided by another virus. For example, the replication-defective adenovirus-associated viruses, which require adenovirus as a helper virus, are classified by the International Committee on the Taxonomy of Viruses both in the parvovirus family and as a satellite. It seems likely that a redefinition of what constitutes a satellite virus will be required to solve this disagreement.

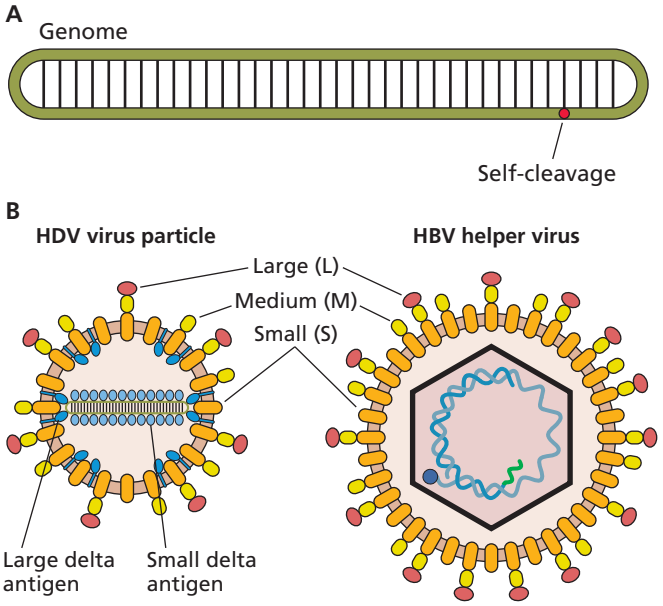


Figure 12.4 Genome and virus particle of hepatitis delta satellite virus. (A) Schematic of the circular (−) strand hepatitis delta virus RNA. Red dot indicates the ribozyme cleavage site. **(B)** Schematic of hepatitis delta satellite virus particle (left) and its helper virus, hepatitis B virus. The hepatitis delta RNA genome is encapsidated with the small and large delta antigens. The lipid envelope, derived from the host cell, contains the hepatitis B virus glycoproteins, comprising the large, medium, and small antigens.

Hepatitis Delta Satellite Virus

Most known satellites are associated with plant viruses, but hepatitis delta satellite virus is associated with a human helper virus, hepatitis B virus. This satellite virus was discovered in 1977 in the nucleus of hepatocytes from patients with severe hepatitis. It was thought to be another marker of hepatitis B virus infection and was therefore called delta antigen. Later, the antigen was found to be encoded in the genome of a separate, defective virus. The genome is 1.7 kb (the smallest of any known animal virus) of circular single-stranded RNA that is 70% base paired and folds upon itself in a tight rodlike structure (Fig. 12.4A). The RNA molecule is replicated by cellular RNA polymerase II, a process that requires the self-cleaving activity of a ribozyme that is formed by a part of the delta satellite virus RNA (Chapter 6, Fig. 6.24). These properties resemble those of viroid genomes. On the other hand, the genome encodes a protein (delta) that encapsidates the RNA, a property shared with satellite nucleic acids. The hepatitis delta satellite virus particle comprises the satellite nucleocapsid packaged within an envelope that contains the surface protein of the helper, hepatitis B virus (Fig. 12.4B).

Upon entry into a cell, the hepatitis delta satellite virus RNA moves to the nucleus where antigenomic RNA is produced. This molecule in turn serves as template for the synthesis of an mRNA that encodes delta protein. Two functionally distinct forms of the delta protein are made as a result of RNA editing (Fig. 6.24). Cellular adenosine deaminase changes an A in the delta antigen stop codon to inosine. Consequently, the stop codon becomes a tryptophan codon, extending the open reading frame by 19 amino acids. Small delta antigen is essential for viral RNA synthesis, regulating RNA editing, and is required for the accumulation of processed viral RNAs. It is also present in the virus particle, and assists in transport of the viral genome into the cell nucleus via an RNA binding domain and a nuclear localization signal. Large delta antigen is an inhibitor of viral replication, contains a nuclear export signal for transport of viral ribonucleoprotein from the nucleus to the cytoplasm, and is essential for the assembly of new virus particles. The latter activity is made possible by a cysteine that is four amino acids from the C terminus, which becomes farnesylated, allowing interaction of the proteins with membranes. It is remarkable that such a short C-terminal extension of the protein endows it with numerous new functions.

Infection with hepatitis delta satellite virus occurs only in individuals infected with hepatitis B virus: it is globally distributed, present in approximately 5% of the 350 million carriers of hepatitis B virus. Acute coinfections of the two viruses can be more severe than infection with hepatitis B virus alone, increasing the rate of liver failure. In chronic hepatitis B virus infections, hepatitis delta satellite virus aggravates preexisting

liver disease, and may lead to more rapid progression to cirrhosis and death than monoinfections. Why coinfection with both viruses leads to more serious outcomes is not known.

Prions and Transmissible Spongiform Encephalopathies

The question of whether infectious agents exist without genomes arose with the discovery and characterization of infectious agents associated with a group of diseases called **transmissible spongiform encephalopathies (TSEs)**. These diseases are rare, but always fatal, neurodegenerative disorders that afflict humans and other mammals (Table 12.2). They are characterized by long incubation periods, spongiform changes in the brain associated with loss of neurons, and the absence of host responses. We now know that TSEs are caused by infectious proteins called **prions**.

Scrapie

The first TSE recognized was scrapie, so called because infected sheep tend to scrape their bodies on fences so much that they rub themselves raw. A second characteristic symptom, skin tremors over the flanks, led to the French name for the disease, tremblant du mouton. Motor disturbances then manifest as a wavering gait, staring eyes, and paralysis of the hindquarters. There is no fever, but infected sheep lose weight and die, usually within 4 to 6 weeks of the first appearance of symptoms. Scrapie has been recognized as a disease of European sheep for more than 250 years. It is endemic in some countries, for example, the United Kingdom, where it affects 0.5 to 1% of the sheep population each year.

Physical Nature of the Scrapie Agent

Sheep farmers discovered that animals from affected herds could pass the disease to a scrapie-free herd, implicating an infectious agent. Infectivity from extracts of scrapie-affected sheep brains was shown to pass through filters with pores small enough to retain everything but viruses. As early as 1966, scrapie infectivity was shown to be considerably more resistant than that of most viruses to ultraviolet (UV) and ionizing radiation. Other TSE agents exhibit similar UV resistance. On the basis of this relative resistance to UV irradiation, some investigators argued that TSE agents are viruses well shielded from irradiation, whereas others claimed that TSE agents have little or no nucleic acids.

The infectivity of scrapie agents is also more resistant to chemicals, such as the combination of 3.7% formaldehyde and autoclaving routinely used to inactivate virus particles. While it is possible to reduce infectivity by 90 to 95% after several hours of such treatment, complete elimination is exceedingly difficult. This property has led to unfortunate human infections caused by sterilized surgical instruments.

Table 12.2 Some transmissible spongiform encephalopathies

TSE diseases of animals
Bovine spongiform encephalopathy (mad cow disease)
Chronic wasting disease (deer, elk)
Scrapie in sheep and goats
TSE diseases of humans
Creutzfeldt-Jakob disease
Variant Creutzfeldt-Jakob disease
Fatal familial insomnia
Gerstmann-Straussler-Scheinker syndrome
Kuru

Human TSEs

Several lines of evidence indicated that human spongiform encephalopathies might be caused by an infectious agent. Carleton Gajdusek and colleagues studied the disease **kuru**, found in the Fore people of New Guinea. This disease is characterized by cerebellar ataxia (defective motion or gait) without loss of cognitive functions. Kuru spread among women and children as a result of ritual cannibalism of the brains of deceased relatives. When cannibalism stopped in the late 1950s, kuru disappeared. Others observed that lesions in the brains of humans with kuru were similar to lesions in the brains of animals with scrapie. It was soon demonstrated that kuru and other human TSEs can be transmitted to chimpanzees and small laboratory animals.

Human spongiform encephalopathies are placed into three groups: infectious, familial or genetic, and sporadic, distinguished by how the disease is acquired initially. An infectious (or transmissible) spongiform encephalopathy is exemplified by kuru and the iatrogenic spread of disease to healthy individuals by transplantation of infected corneas, the use of purified hormones, or transfusion with blood from patients with the TSE Creutzfeldt-Jakob disease (CJD). Over 400 cases of iatrogenic Creutzfeldt-Jakob disease have been reported worldwide. The epidemic spread of bovine spongiform encephalopathy (mad cow disease, see below) among cattle in Britain can be ascribed to the practice of feeding processed animal by-products to cattle as a protein supplement. Similarly, the new human disease, variant CJD, arose after consumption of beef from diseased cattle. Sporadic CJD is a disease affecting one to five per million annually, usually late in life (with a peak at 68 years). As the name indicates, the disease appears with no warning or epidemiological indications. Kuru may have been originally established in the small population of Fore people in New Guinea when the brain of an individual with sporadic CJD was eaten. Familial spongiform encephalopathy is associated with an autosomal dominant mutation in the *prnp* gene (see below). Together familial and sporadic forms of prion disease account for ~99% of all cases. Diseases of all three classes can usually be transmitted experimentally or naturally by inoculation or ingestion of diseased tissue.

Hallmarks of TSE Pathogenesis

Clinical signs of infection commonly include cerebellar ataxia, memory loss, visual changes, dementia, and akinetic mutism, with death occurring after months or years. The infectious agent first accumulates in the lymphoreticular and secretory organs and then spreads to the nervous system. In model systems, spread of the disease from the site of inoculation to other organs and the brain requires dendritic and B cells. The disease agent then invades the peripheral nervous system and spreads from there to the spinal cord and brain. Once the infectious agent is in the central nervous system, the characteristic pathology includes severe astrocytosis, vacuolization (hence, the term spongiform), and loss of neurons. Occasionally, dense fibrils or aggregates (sometimes called plaques) can be detected in brain tissue at autopsy. There are no inflammatory, antibody, or cellular immune responses. The time course, degree, and site of cytopathology within the central nervous system are dependent upon the particular TSE agent and the genetic makeup of the host.

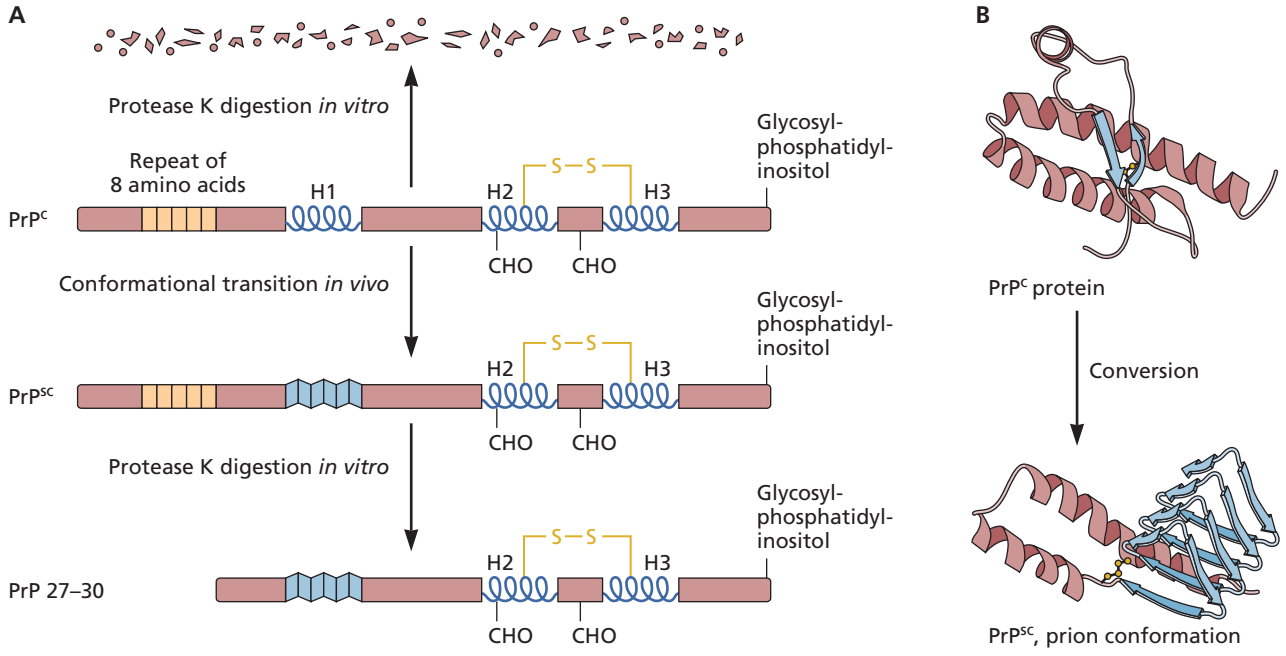
Prions and the *prnp* Gene

The unconventional physical attributes and slow infection pattern originally prompted many to argue that TSE agents are not viruses at all. In 1967, it was suggested that scrapie could be caused by a host protein, not by a nucleic acid-carrying virus. These ideas were among the first of the protein-only hypotheses to explain TSE.

An important breakthrough occurred in 1981, when characteristic fibrillar protein aggregates were visualized in infected brains. These aggregates could be concentrated by centrifugation and remained infectious. Stanley Prusiner and colleagues developed an improved bioassay, as well as a fractionation procedure that allowed the isolation of a protein with unusual properties from scrapie-infected tissue. This protein is insoluble and relatively resistant to proteases. He named the scrapie infectious agent a **prion**, from the words protein and infectious.

Prusiner's unconventional proposal was that an altered form of a normal cellular protein, called PrP^{C}, causes the fatal encephalopathy characteristic of scrapie. This controversial protein-only hypothesis caused a firestorm among those who study infectious disease. The hypothesis was that the essential pathogenic component **is** the host-encoded PrP^{C} protein with an altered conformation, called PrP^{Sc} ("PrP-scrapie"). Furthermore, PrP^{Sc} was proposed to have the property of converting normal PrP^{C} protein into more copies of the pathogenic form (Fig. 12.5). PrP^{C} and PrP^{Sc} can be differentiated by sensitivity to protease digestion: PrP^{C} is completely degraded

Figure 12.5 The conversion of nonpathogenic, α-helix-rich PrP^{c} protein to the β-sheet-rich conformation of PrP^{Sc}, the pathogenic prion. (A) PrP^{c} is the mature normal cellular protein. The precursor is 254 amino acids long with a signal sequence that is removed. Twenty-three amino acids of the carboxy terminus also are removed as the glycosylphosphatidylinositol (GPI) anchor is added. PrP^{Sc} is the β-sheet-rich, pathogenic prion. This conformation is relatively resistant to protease K digestion, in contrast to PrP^{c}, as indicated. This protease K-resistant PrP fragment of PrP^{Sc} is diagnostic of the prion protein. H1, H2, and H3 are helical regions of PrP^{c}. The yellow boxes indicate repeats of 8 amino acids [P(Q/H)GGGWGQ]. CHO indicates two N-linked carbohydrate chains. S–S indicates disulfide bonds. **(B)** Ribbon diagram of the PrP^{c} and PrP^{Sc} protein backbones with α-helices in red and β-sheets in blue. From P. Chien, J. Weissman, and A. DePace, *Annu Rev Biochem* **73:**617–656, 2004, with permission.



by proteinase K, while digestion of PrP^{Sc} produces a 27- to 30-kDa fragment. PrP^{C} has little β-sheet structure and high α-helical content, whereas PrP^{Sc} has high β-sheet structure and low α-helical content. In recognition of his work on prions, Prusiner was awarded the Nobel Prize in physiology or medicine in 1997.

Sequence analysis of this protein led to the identification of the *prnp* gene, which is highly conserved in the genomes of many mammals, including humans. Expression of this gene is now known to be essential for the pathogenesis of TSEs. The *prnp* gene encodes a 35-kDa membrane-associated neuronal glycoprotein, PrP^{C}. The function of this protein has been difficult to determine, because mice lacking both copies of the *prnp* gene develop normally and have few obvious defects. However, these mice are resistant to TSE infection, showing that PrP^{C} is essential for prion propagation. When $prnp^{-/-}$ mice are inoculated with PrP^{Sc} they develop antibodies that also recognize PrP^{C}, showing that the two forms of the protein share epitopes.

The discovery of the *prnp* gene has helped explain the basis of familial TSE diseases such as Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker disease, and fatal familial insomnia. Gerstmann-Straussler-Scheinker disease is associated with the change of PrP^{C} amino acid 102 from proline to leucine. Introduction of this amino acid change into mice gives rise to a spontaneous neurodegenerative disease characteristic of a TSE. Familial Creutzfeldt-Jakob disease may be associated with an insertion of 144 bp at codon 53, or changes at amino acids 129, 178, or 200. In fatal familial insomnia, adults develop a progressive sleep disorder and typically die within one year. In this disease, PrP^{Sc} is found only in the anteroventral and dorsal medial nuclei of the thalamus. Development of the disease is strongly linked to the D178N amino acid change and V129. When D178N is present with V129, the patients develop familial Creutzfeldt-Jakob disease, which is characterized by dementia; in this case, PrP^{Sc} is found throughout the brain. How the sequence of the protein affects pathogenesis is not known. Over 40 different mutations of the PrP gene have been identified. The familial prion diseases are fully penetrant. Both infectious and sporadic TSEs develop in the absence of mutations in the wild-type *prnp* gene (Fig. 12.6).

Although altered PrP proteins are produced early in human development, progress of neurological disease is generally delayed for decades. This observation has led to the suggestion that an event associated with aging is required for producing TSE. However, there is no evidence that any age-dependent process, such as mitochondrial DNA mutations, oxidative modifications of DNA and proteins, or proteasome malfunction, is responsible for TSE. Another possibility is that the accumulation of PrP^{Sc} in numbers sufficient to be self-sustaining is a time-consuming process. Not until the number of prions being produced reaches a threshold would infection continue unchecked, at which point neurological dysfunction could occur.

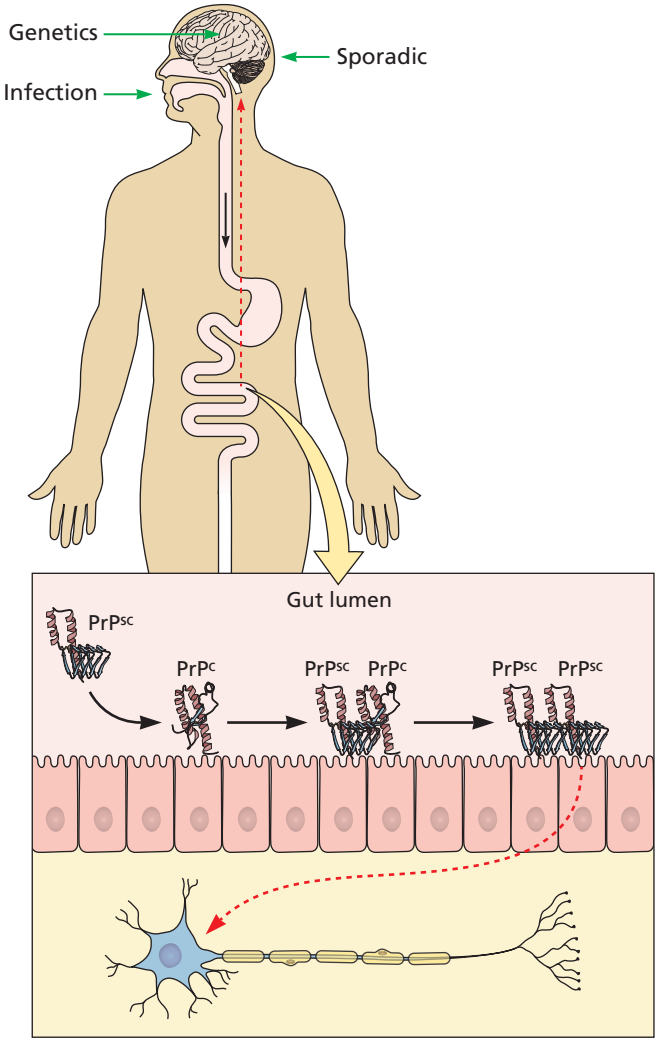


Figure 12.6 Human prion infections. Human prion diseases are spontaneous, genetic, or acquired by exposure to infectious materials. When prions are ingested, they move to the intestine and are taken up by mucosa and Peyer's patches. It is thought that ingested infectious prions convert PrP^{C} to PrP^{Sc}, which spreads to the central nervous system via enteric nerves (red line).

The rate of prion formation in an inoculated animal is influenced by many parameters. It is inversely related to the incubation time, and proportional to the quantity of PrP^{C} in the brain and in the inoculum. The PrP sequence also matters: prion propagation is faster when PrP^{C} and PrP^{Sc} are identical in sequence. The incubation time until development of disease in mice inoculated with Syrian hamster prions is >500 days. In contrast, the incubation time of mice transgenic for the Syrian hamster *prnp* gene is 70 to 75 days, the

same for hamsters inoculated with hamster PrP^{Sc}. Transgenic mice inoculated with Syrian hamster prions produce Syrian hamster, and not mouse, prions: in other words, they will only infect hamsters. When the same mice are inoculated with mouse prions, only mouse prions are produced.

Some prions have a distinct host range. For example, mouse-adapted scrapie prions (produced by serial passage of scrapie prions in mice) cannot propagate in hamsters, but hamster-adapted scrapie prions can propagate in mice. A single amino acid substitution in the hamster protein enables it to be converted efficiently by mouse PrP^{Sc} into hamster PrP^{Sc}. The barrier to interspecies transmission is therefore in the sequence of the PrP protein: the infecting PrP^{Sc} must match the PrP^{C} of the host. Bovine spongiform encephalopathy prions have an unusually broad host range, infecting a number of meat-eating animals, including domestic cats, wild cats, and humans, but not mice. The latter can be infected with bovine prions if they are made transgenic for the bovine *prnp* gene.

How prions propagate after PrP^{Sc} enters a cell remains obscure. PrP^{Sc} may act as a seed or template, recruiting PrP^{c} monomers into ordered polymers and altering their conformation. Despite widespread presence of PrP^{C}, formation of PrP^{Sc} is restricted to a few cell types (neurons, cornea, myocytes, follicular dendritic cells), suggesting that auxiliary molecules participate in the formation of PrP^{Sc}. Conversion of PrP^{C} to PrP^{Sc} has been achieved *in vitro* using purified proteins, but the efficiency of conversion is very low. Addition of glycosaminoglycans increases the conversion frequency, providing evidence for the role of auxiliary molecules in the formation of PrP^{Sc}. Increased efficiency of conversion of PrP^{C} to PrP^{Sc} has been achieved using a technique called protein misfolding cyclic amplification. In this method, crude brain homogenates containing PrP^{Sc} and PrP^{C} are mixed and incubated for 1 to 3 days with intermittent sonication, resulting in amplification of PrP^{Sc}. The cyclic sonications are thought to speed the polymerization reaction by fragmenting PrP^{Sc} polymers, increasing the concentration of seeds. By depleting different molecules from brain extracts it was found that RNA or phosphatidylethanolamine can facilitate the conversion of PrP^{C} to PrP^{Sc}. It seems likely that other cofactor molecules participate in this process. Addition of phosphatidylethanolamine to mouse PrP^{C} produced in *E. coli*, in the absence of PrP^{Sc}, leads to the production of infectious prions. This finding will facilitate studies on the mechanism of conversion, and could enable development of therapeutics or diagnostic tests.

Until recently, the presence of a prion could be detected definitively only by injection of organ homogenates into susceptible recipient species or by proteinase K digestion, procedures that cannot be done with living patients. This challenge has been overcome by using amplification procedures to detect Creutzfeldt-Jakob prions in nasal brushings and in urine (Box 12.3).

Prion Strains

Serial infections of mice and hamsters with infected sheep brain homogenates have led to the production of distinct strains of scrapie prion. Strains are distinguished by length of incubation time before the appearance of symptoms, brain pathologies, relative abundance of various glycoforms of PrP^{c}, and electrophoretic profiles of protease-resistant PrP^{Sc}. A striking finding is that different scrapie strains can be propagated in the same inbred line of mice, yet maintain their original phenotypes.

Prion strains do not differ in amino acid sequence, but rather in their glycosylation patterns, protease resistance, and conformation. Each of the distinctive pathogenic conformations is postulated to convert the normal PrP protein into a conformational image of itself. Recent evidence suggests that strain diversity may be a function of different compounds present during the formation of PrP^{Sc} molecules. In support of this hypothesis, it was found that when three different PrP^{Sc} strains are propagated *in vitro* in the presence of phosphatidylethanolamine, they were converted into a single novel strain.

These observations demonstrate that the properties of PrP^{Sc}, including its conformation, can override sequence differences between the infecting prion and host PrP^{Sc}. A mechanistic understanding of this process will require determining the structures of different PrP^{Sc} strains. Unfortunately to date no structure of PrP^{Sc} has been determined because the protein is insoluble.

Bovine Spongiform Encephalopathy

In the mid-1980s, a new disease appeared in cows in the United Kingdom: bovine spongiform encephalopathy, also called mad cow disease (Fig. 12.7). It is believed to have been transmitted to cows by feeding them meat and bone meal, a high-protein supplement prepared from the offal of sheep, cattle, pigs, and chicken. In the late 1970s the method of preparation of meat and bone meal was changed, resulting in material with a higher fat content. It is believed that this change allowed prions, from either a diseased sheep or cow, to retain infectivity and pass on to cattle. Before the disease was recognized in 1985, it was amplified by feeding cows the remains of infected bovine tissues. The incubation period for bovine spongiform encephalopathy is 5 years, but disease was not observed because most cattle are slaughtered between 2 and 3 years of age. Three years later, as the number of cases of mad cow disease increased, a ban on the use of meat and bone meal was put in place, a practice that together with culling infected cattle stopped the epidemic. Over 180,000 cattle, mostly dairy cows, died of bovine spongiform encephalopathy from 1986 to 2000.

Cases of variant Creutzfeldt-Jakob disease, a new TSE of humans, began to appear in 1994 in the United Kingdom. These were characterized by a lower mean age of the

BOX 12.3

EXPERIMENTS

Detection of Creutzfeldt-Jakob prions in nasal brushings and urine

The human prion disease, Creutzfeldt-Jakob, is diagnosed by a variety of criteria, including clinical features, electroencephalograms, and magnetic resonance imaging. Until recently, there was no noninvasive assay to detect PrP^{Sc}, the only specific marker for the disease. New diagnostic tests using nasal brushings or urine seem to fill this need.

These assays utilize two different methods for amplifying the quantity of prions *in vitro*. In real-time quaking-induced conversion, PrP^{C} (produced in *E. coli*) is mixed with a small quantity of PrP^{Sc}. The mixtures are subjected to cycles of shaking and rest at 42°C for 55 to 90 hours, a procedure that leads to the formation of amyloid fibrils that can be detected by fluorescence. The assay can detect femtograms of PrP^{Sc} in brain homogenates from humans with Creutzfeldt-Jakob disease. In protein misfolding cyclic amplification, samples are incubated for 30 minutes at 37 to 40°C, subjected to a pulse of sonication, and the cycle is then repeated 96 times. Prions are detected by Western blot analysis after treatment with proteinase K. This process can detect a single oligomeric PrP^{Sc}. Although done with protein, the assay resembles PCR in the use of templates to provide amplification of PrP^{Sc}.

Two noninvasive assays using these amplification approaches were developed. The first is a nasal-brushing procedure to sample the olfactory epithelium, where PrP^{Sc} is known to accumulate in patients with the disease. The real-time quaking-induced conversion assay was positive in 30 of 31 patients with Creutzfeldt-Jakob disease, and negative in 43 of 43 healthy controls (a sensitivity of 97%). Furthermore, nasal brushings gave stronger and faster positive results than cerebrospinal fluid in this assay. The high concentrations of PrP^{Sc} detected in nasal brushings suggest that prions can contaminate nasal discharge of patients with the disease, a possible source of iatrogenic transmission that has implications for infection control.

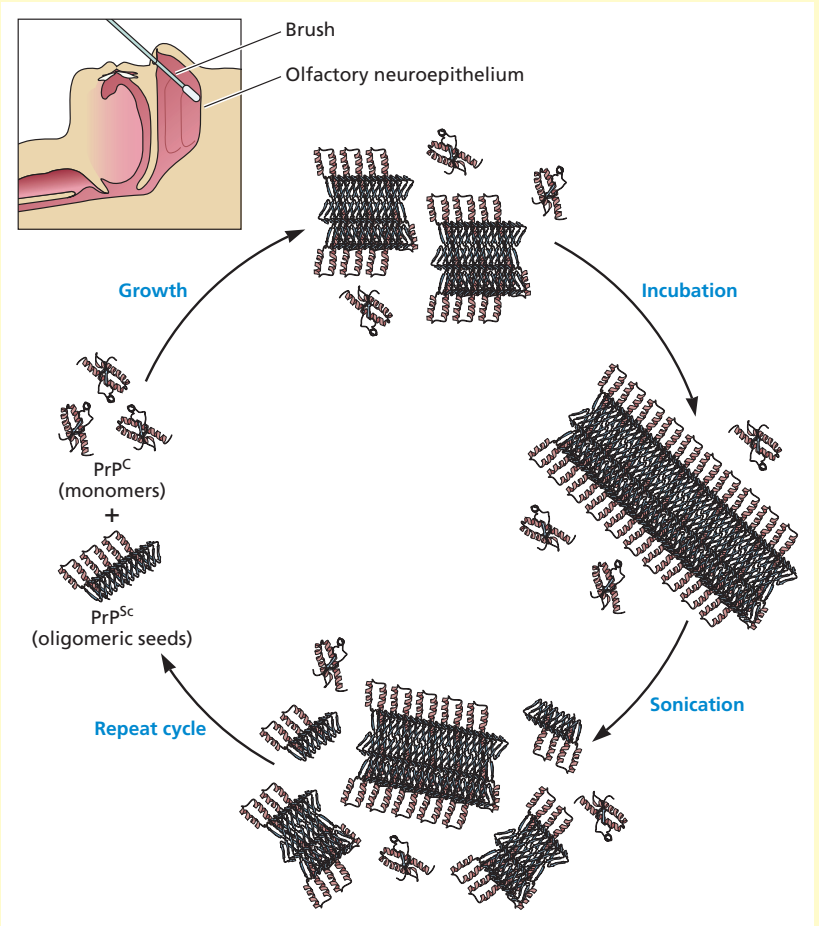
Protein misfolding cyclic amplification was used to assay for the presence of PrP^{Sc} in the urine of patients with variant Creutzfeldt-Jakob disease, which had been previously shown to contain prions. PrP^{Sc} was detected in 13 of 14 urine samples from patients with the disease, but not in 224 urine samples from healthy controls and patients with other neurological diseases, including other TSEs. The estimated concentration of PrP^{Sc} in urine was 40 to 100 oligomeric particles per ml.

Because Creutzfeldt-Jakob disease is so rare, any assay for the disease must have near-perfect specificity. A problem with both cycling assays is that PrP^{C} converts into oligomers and fibrils in the absence of PrP^{Sc}. Additional work is needed to address this problem. Nevertheless it is possible that these assays could one day lead to earlier diagnosis and treatments, if the latter become available.

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Detection of PrP^{Sc} by protein misfolding cyclic amplification. Inset, sampling of olfactory neuroepithelium. Samples (labeled oligomeric seeds) are mixed with PrP^{C} monomers and incubated to allow growth of the polymers. The mixture is sonicated to fragment the aggregates and increase the number of nuclei for prion replication. After 96 cycles, additional PrP^{C} substrate is added, and the samples are subjected to another cycle.



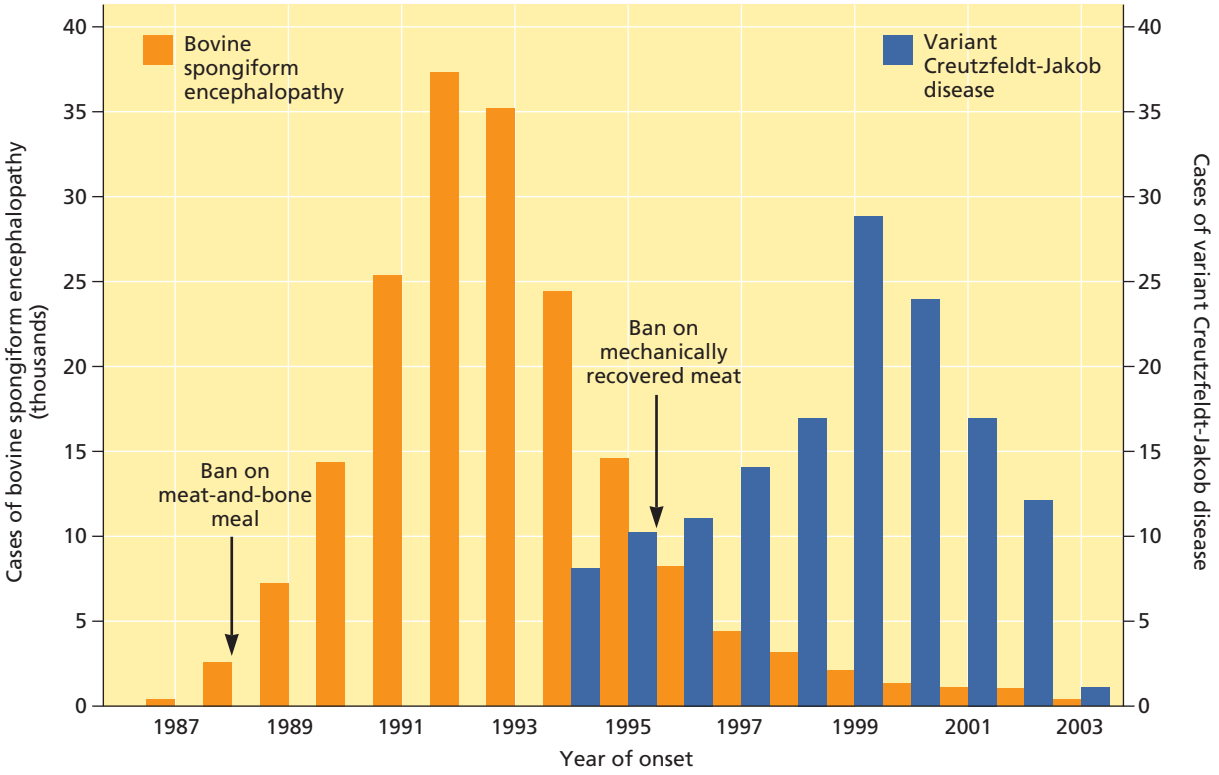


Figure 12.7 Time course of the reporting of bovine spongiform encephalopathy in cattle and variant Creutzfeldt-Jakob disease in humans in the United Kingdom over a period of 9 years. The peak of the bovine epidemic was in 1992, and the peak of the human disease was in 1999. The incidence of both is now rare. Data obtained from http://webarchive.nationalarchives.gov.uk/20090505194948/http://www.bseinquiry.gov.uk/report/index.htm

patients (26 years), longer duration of illness, and differences in other clinical and pathological characteristics. The results of epidemiological and experimental studies indicate that variant Creutzfeldt-Jakob disease is caused by prions transmitted by the consumption of cattle with bovine spongiform encephalopathy. As of 2011 there had been 177 cases of variant Creutzfeldt-Jakob disease in the United Kingdom, and 229 globally.

Bovine spongiform encephalopathy continues to be detected in cattle. As of April 2012, 4 cases had been identified in the United States and 19 in Canada. These cases may arise sporadically, or through consumption of contaminated feed. Because cattle are slaughtered before disease symptoms are evident, there is concern that variant Creutzfeldt-Jakob might increase as contaminated meat enters the food supply. These concerns are being addressed by imposing bans on animal protein-containing feed, and increased surveillance of cows for the disease, for which diagnostic tests are being developed.

Chronic Wasting Disease

Chronic wasting disease is a transmissible spongiform encephalopathy of cervids such as deer, elk, and moose. It is the only TSE known to occur in free-ranging animals. The disease has been reported in the United States, Canada, and South Korea (Fig. 12.8). In captive herds in the United States and Canada, up to 90% of mule deer and 60% of elk are infected, and the incidence in wild cervids is as high as 15%.

Mice have been used to understand whether chronic wasting disease prions might be transmitted to humans. Mice are not efficiently infected with chronic wasting disease prions unless they are made transgenic for the cervid *prnp* gene. Four different research groups have found that mice transgenic for the human *prnp* gene are not infected by chronic wasting disease prions. These findings suggest that such prions are not likely to be transmitted directly to humans. However, changing four amino acids in human *prnp* to the cervid sequence allows efficient infection of transgenic mice with cervid prions.

Another concern is that prions of chronic wasting disease could be transmitted to cows grazing in pastures contaminated by cervids. It is not known how the disease is spread among cervids, but transmission by grass contaminated with saliva and feces is one possibility. When deer are fed prions they excrete them in the feces before developing signs of infection, and prions can also be detected in deer saliva. In the laboratory, brain homogenates from infected deer



Figure 12.8 Chronic wasting disease in North America. CWD, chronic wasting disease. Figure courtesy of the Chronic Wasting Disease Alliance (www.cwd-info.org).

can transmit the disease to cows. Therefore, it is possible that contamination of grass could pass the agent on to cows, from where it could then enter the human food chain.

A further worry is that bovine spongiform encephalopathy (BSE) prions shed by cows in pastures might infect cervids, which would then become a reservoir of the agent. BSE prions do not infect mice that are transgenic for the cervid *prnp* gene. However, intracerebral inoculation of deer with BSE prions causes neurological disease, and the prions from these animals can infect mice that are transgenic for the cervid *prnp* gene. Therefore, caution must be used when using transgenic mice to predict the abilities of prions to cross species barriers.

No case of transmission of chronic wasting disease prions to deer hunters has yet been reported. Although the risk of human infection with chronic wasting disease prions appears to be low, hunters are advised not to shoot or consume an elk or deer that is acting abnormally or appears to be sick, to avoid the brain and spinal cord when field dressing game, and not to consume brain, spinal cord, eyes, spleen, or lymph nodes.

Treatment of Prion Diseases

There are currently no therapeutics available to slow or stop the neurodegeneration characteristic of transmissible spongiform encephalopathies, although symptoms may be mitigated by the drug L-dopa. A potential breakthrough came when researchers discovered that the antimalarial drug quinacrine blocked accumulation of infectious prions in cultured cells. Unfortunately human trials of quinacrine in patients with advanced Creutzfeldt-Jakob disease showed that the drug is not effective. This failure was suggested to be a consequence of poor penetration of the drug into the central nervous system. When quinacrine was given to genetically altered mice in which drugs can more easily penetrate the brain, PrP^{Sc} levels were depressed transiently but disease was not prevented. Monoclonal antibodies specific for PrP inhibit scrapie prion propagation in mice and delay the development of prion disease. Although delivery of antibodies into the central nervous system is not efficient, clinical trials to evaluate the efficacy of these molecules in treating prion disease are planned. Small molecules that bind to prion proteins, enhance their clearance, or cause dominant-negative inhibition of prion propagation have been identified, but none have yet been tested in humans.

Perspectives

We have discussed viroids, satellites, and prions together in this chapter because they are not accommodated by the classification schemes for viruses. The origin of satellites and viroids remains an enigma, but it has been proposed that they are relics from the RNA world, which is thought to have been populated only by noncoding RNA molecules that catalyzed their own synthesis. Both types of infectious agents have properties that make them candidates for survivors of the RNA world: small genome size (to avoid error catastrophe caused by error-prone replication), high G+C content (greater thermodynamic stability), circular genomes (to avoid the need for mechanisms to prevent loss of information at the ends of linear genomes), no protein content, and the presence of a ribozyme, a fingerprint of the RNA world. Today's viroids can no longer self-replicate, possibly having lost that function when they became parasites of plants. What began as a search for viruslike agents that cause disease in plants has led to new insights into the evolution of life.

Many intriguing questions about viroids and satellites remain, including the nature of plant defense mechanisms against these elements, how they enter and exit cellular organelles, the precise mode of their spread within plants, the role of host proteins in reproduction, and the mechanisms of pathogenesis. Perhaps the thorniest question is why satellites impair the reproduction of their helper viruses, rather than being mutually beneficial as might be anticipated. The intriguing possibility that satellite viruses provide a function to helper viruses remains unaddressed. Even more enigmatic is hepatitis delta virus, a hybrid of viroid and satellite with a mammalian helper virus. This satellite virus likely arose in the liver of a patient infected with hepatitis B virus, but the source might have been a plant viroid or satellite passing through the host's intestine. Given the very high rate of discovery of small RNAs, it seems likely that other elements similar to hepatitis delta virus will be identified.

If the reader does not believe that viroids and satellites are distinctive, then surely prions, infectious agents composed only of protein, must impress. Precisely how prions are formed from normal cell proteins, and how their structures provide strain differences are just two of many important unanswered questions. While TSEs are rare, they are uniformly fatal, and better methods of diagnosis and treatment are needed. Since prions were discovered it has become clear that protein misfolding is involved in a wide spectrum of neurodegenerative diseases. For example, the amyloid fibrils in Alzheimer's disease contain the amyloid-β peptide that is processed from the amyloid precursor protein; familial disease is caused by mutations in the gene for this protein. Mutations in the tau gene are responsible for heritable tauropathies including familial frontotemporal dementia and inherited progressive supranuclear palsy. Self-propagating tau aggregates pass from cell to cell. The prionlike spread of misfolded α-synuclein is believed to be associated with Parkinson's disease. In these cases there is good evidence that the causative protein, like PrP^{Sc}, adopts a conformation that becomes self-propagating.

Despite the contribution of prions in human neurological diseases, in other organisms such proteins are not pathogenic but rather impart diverse functions through templated conformational change of a normal cellular protein. Such prions have been described in fungi where they do not form infectious particles and do not spread from cell to cell. These proteins change conformation in response to an environmental stimulus and acquire a new, beneficial function. An example is the *Saccharomyces cerevisiae* Ure2p protein, which is a nitrogen catabolite repressor when cells are grown in the presence of a rich source of nitrogen. In the aggregated prion state, called [URE3], the protein allows growth on poor nitrogen sources. These findings prompt the question of whether the conversion of PrP^{C} to PrP^{Sc} once had a beneficial function that became pathogenic. If so, identifying that function, and how it was usurped, will be important for understanding the pathogenesis of transmissible spongiform encephalopathies.

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Website

http://www.prion.ucl.ac.uk/clinic-services/research/drug-treatments/
Prion therapies

APPENDIX

Diseases, Epidemiology, and Disease Mechanisms of Selected Animal Viruses Discussed in This Book

This appendix presents key facts about the pathogenesis of selected animal viruses that cause human disease. Information about each virus or virus group is presented in four sections. In the first section, the selected viruses and associated diseases are listed. The second section, "Epidemiology," outlines virus transmission, worldwide distribution, those at risk or risk factors, and vaccines or antiviral drugs currently available. The third section, "Pathogenesis," provides simple images that will enable the reader to visualize infection and the resulting pathogenesis, with green arrows indicating portals of viral entry and red arrows indicating sites of virus egress from the human body. Finally, in a section entitled "Human Infections," we provide an indication of the impact of the infection on the human population. These pages were designed to be made into slides for lectures or teaching, providing "snapshots" of the pathogenesis of specific viruses.

Adenoviruses

Virus

57 adenovirus serotypes that infect humans, classified into 6 subgroups

Disease

Respiratory diseases
- Upper tract infection
- Pharyngoconjunctival fever
- Pertussis-like disease
- Pneumonia

Other diseases
- Acute hemorrhagic cystitis
- Epidemic keratoconjunctivitis
- Gastroenteritis
- Myocarditis

Epidemiology

Transmission
- Aerosol, fecal matter, fomites
- Poorly sanitized swimming pools
- Ophthalmologic instruments (eye infections)

Distribution
- Worldwide
- No seasonal incidence

At risk or risk factors
- Children aged <14 years
- Day care centers, military camps, swimming clubs
- Immunosuppression

Vaccines or antiviral drugs
- Attenuated vaccine for serotypes 4 and 7 has been produced for the military
- Cidofovir

Pathogenesis

Virus infects mucoepithelial cells of respiratory and gastrointestinal tract, conjunctivae

Virus can persist in lymphoid tissue (tonsils, adenoids, and Peyer's patches)

Human Infections

>80% of humans are seropositive

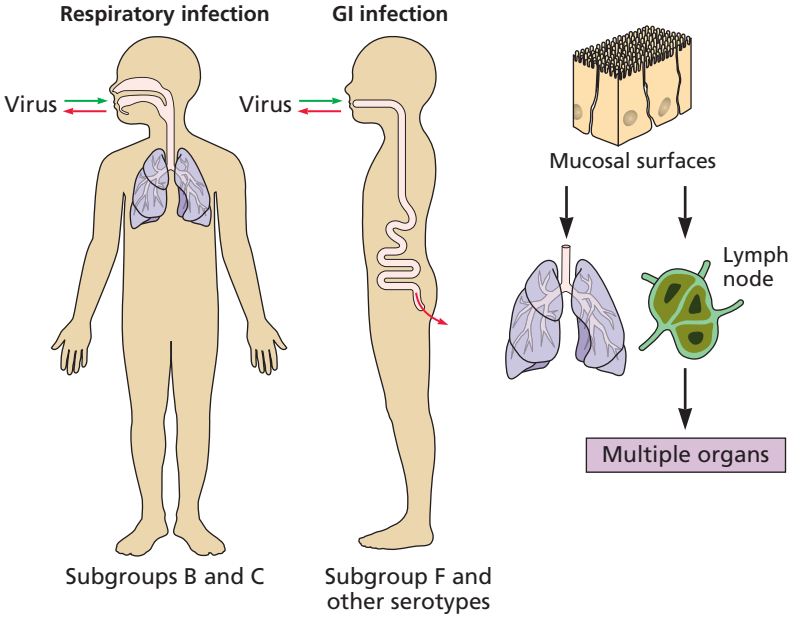


Figure 1

Arenaviruses

Virus	Disease
Lymphocytic choriomeningitis virus	Fever, muscle pain, meningitis
Lassa virus	Lassa hemorrhagic fever (severe systemic illness, increased vascular permeability, shock)
Junin virus	Argentine hemorrhagic fever similar to Lassa fever but more extensive bleeding

Epidemiology

Transmission
- Contact with infected rodents or their excreta

At risk or risk factors
- Lymphocytic choriomeningitis virus: contact with pet hamsters, areas with rodent infestation
- Other arenaviruses: proximity to rodents

Distribution
- Lymphocytic choriomeningitis virus: hamsters and house mice in Europe, Americas, Australia, possibly Asia
- Other arenaviruses: Africa, South America, United States
- No seasonal incidence

Vaccines or antiviral drugs
- No vaccines
- Antiviral drug: ribavirin

Pathogenesis

Persistent infection of rodents caused by neonatal infection and induction of immune tolerance

Viruses infect macrophages, which release mediators of cell and vascular damage

Tissue destruction caused by T cell immunopathology

Human Infections

Lassa fever: several thousand cases per year worldwide; none in U.S.

~50% seropositive in Africa

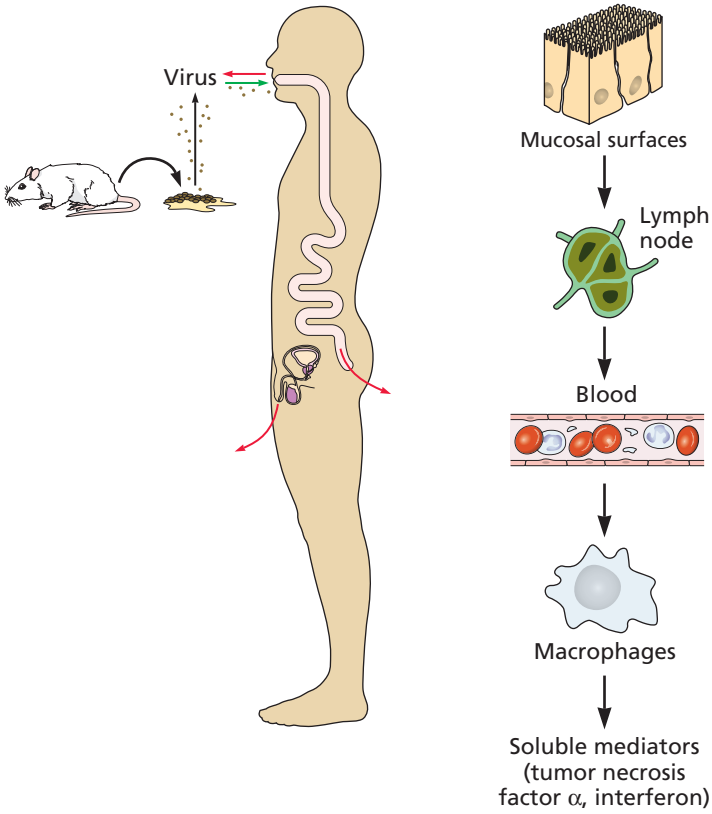


Figure 2

Bunyaviruses

Virus	Vector	Disease
Bunyavirus (>350 species) • Bunyamwera virus • California encephalitis virus • La Crosse virus • Oropouche virus	Mosquito	Febrile illness, encephalitis, rash
Hantavirus (22 species) • Hantaan virus	None	Hemorrhagic fever with renal syndrome, adult respiratory distress syndrome
• Sin Nombre virus	None	Hantavirus pulmonary syndrome, shock, pulmonary edema
Nairovirus (6 species) • Crimean-Congo hemorrhagic fever virus	Tick	Sandfly fever, hemorrhagic fever, encephalitis, conjunctivitis, myositis
Phlebovirus (9 species) • Rift Valley fever virus • Sandfly fever virus	Fly	Hemorrhagic fever

Epidemiology

Transmission
- Arthropod bite
- Contact with rodent excreta

Distribution of virus
- Depends on distribution of vector or rodents
- Disease more common in summer

At risk or risk factors
- People in area of vector, e.g., campers, forest rangers, woodspeople

Vaccines or antiviral drugs
- No vaccines
- No antivirals

Pathogenesis

Primary viremia, then secondary viremia leads to virus spread to target tissues, including central nervous system, various organs, and vascular endothelium

Human Infections

Hantavirus pulmonary syndrome in U.S.: ~650 cases (through 2013)

Sporadic, limited outbreaks worldwide, especially South America

Case fatality: 20–50%

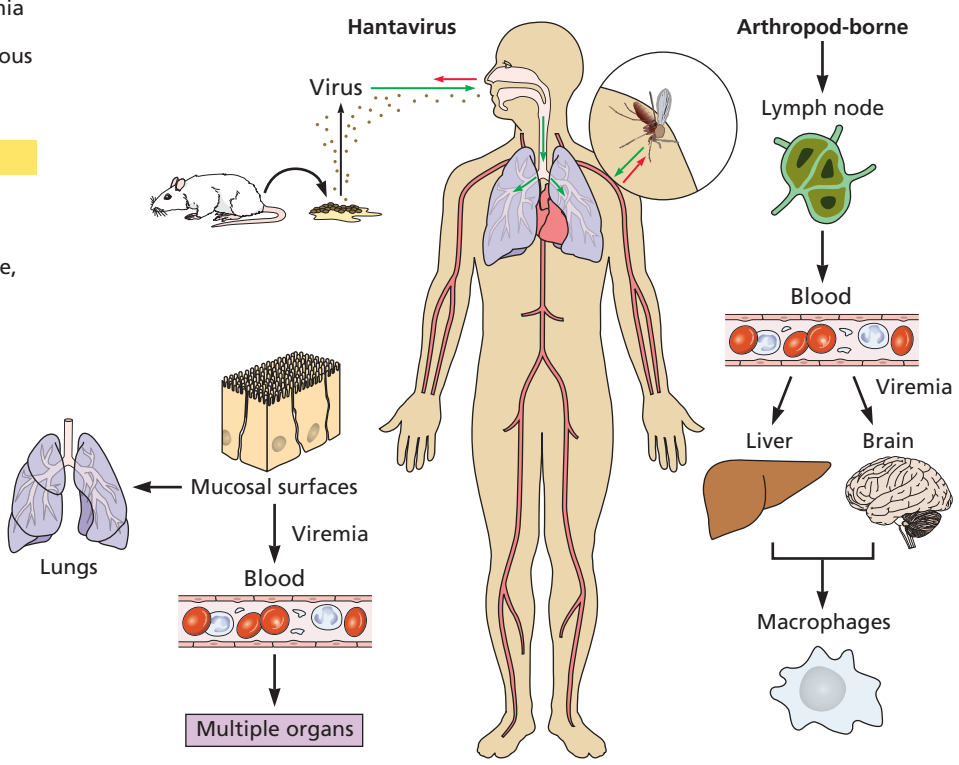


Figure 3

Caliciviruses

Virus	Disease
Logovirus *Norovirus* • Norwalk virus	Gastroenteritis
Sapovirus • Sapporo virus	Gastroenteritis

Epidemiology

Transmission
- Fecal-oral route from contaminated water and food
- Virus particles are resistant to detergents, drying, and acid

Distribution
- Worldwide
- No seasonal incidence

At risk or risk factors
- Children in day care centers and schools
- Resorts, hospitals, nursing homes, restaurants, cruise ships

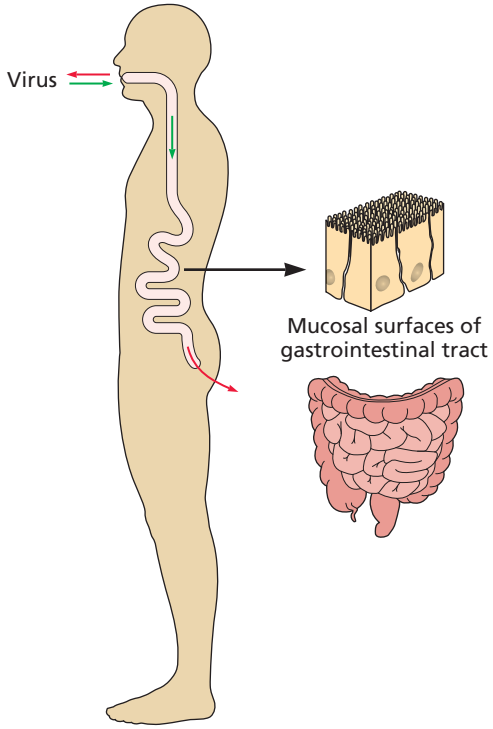
Vaccines or antiviral drugs
- None

Pathogenesis

Infection of intestinal brush border, prevents proper absorption of water and nutrients

Cause diarrhea, vomiting, abdominal cramps, nausea, headache, malaise, and fever

May cause persistent infection, but usually resolved



Human Infections

Noroviruses: Of 348 outbreaks during Jan. 1996–Nov. 2000, 39% occurred in restaurants; 29% occurred in nursing homes and hospitals; 12% in schools and day care centers; 10% in vacation settings, including cruise ships; and 9% in other settings

Figure 4

Filoviruses

Virus	Disease
Marburgvirus • Lake Victoria	Hemorrhagic fever
Ebolavirus • Reston • Sudan • Zaire • Bundibugyo • Tai Forest	Hemorrhagic fever

Epidemiology

Transmission
- Fruit bats are reservoirs
- Contact with infected fruit bats, monkeys, or their tissues, secretions, or body fluids
- Contact with infected humans or body fluids
- Accidental injection, contaminated syringes

Distribution
- Africa, Philippines
- No seasonal incidence

At risk or risk factors
- Bushmeat hunting and preparation
- Family members of the sick
- Burial preparation
- Health care workers attending sick persons

Vaccines or antiviral drugs
- In clinical trials

Pathogenesis

Virus reproduction causes necrosis in liver, spleen, lymph nodes, and lungs

Hemorrhage causes edema and shock

Death from dehydration

Human Infections

Case fatality: 40–90%

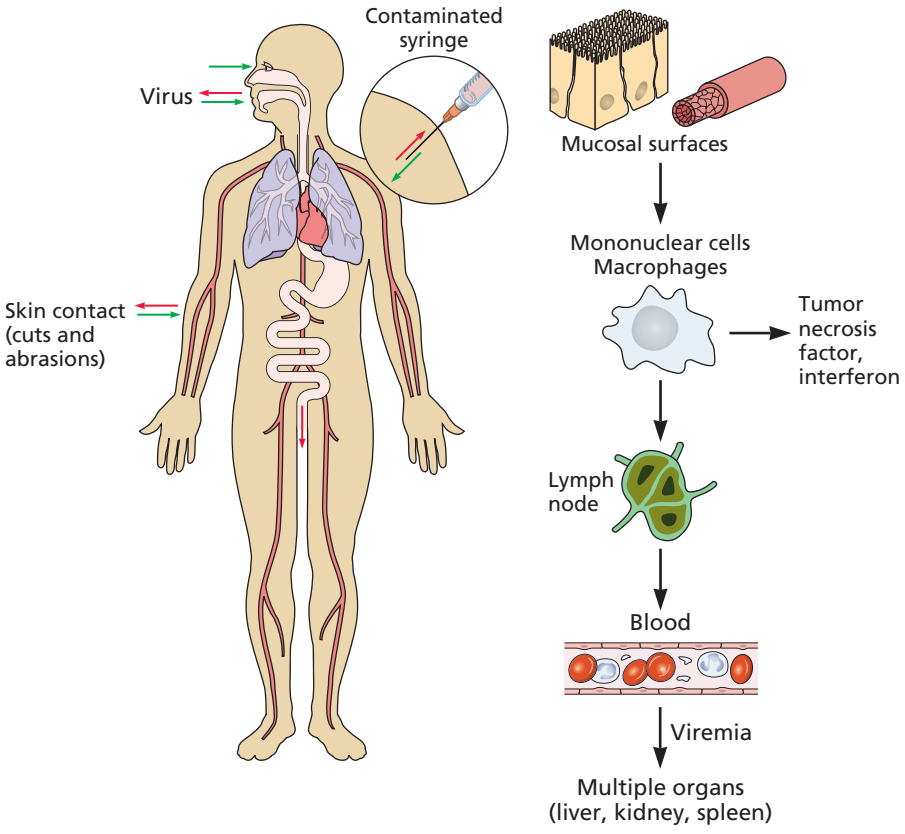


Figure 5

Flaviviruses

Virus	Vector	Disease
Flavivirus		
• Yellow fever virus	*Aedes* mosquitoes	Hepatitis, hemorrhagic fever
• Powassan virus	*Ixodes* ticks	Encephalitis
• Dengue virus	*Aedes* mosquitoes	Breakbone fever, dengue hemorrhagic fever, dengue shock syndrome
• Japanese encephalitis virus	*Culex* mosquitoes	Encephalitis
• St. Louis encephalitis virus	*Culex* mosquitoes	Encephalitis
• West Nile virus	*Culex* mosquitoes	Fever, encephalitis, hepatitis

Epidemiology

Transmission
- Mosquito or tick vectors

Distribution
- Determined by habitat of vector
  Aedes mosquito (urban areas)
  Culex mosquito (forest, urban areas)
- More common in summer

At risk or risk factors
- Proximity to vector

Vaccines or antiviral drugs
- Attenuated vaccines for yellow fever and Japanese encephalitis
- No antiviral drugs

Pathogenesis

Viruses cause viremia and systemic infection

Nonneutralizing antibodies can facilitate infection of monocytes/macrophages via Fc receptors

Human Infections

Dengue cases worldwide/year:
50 million–100 million (estimated);
419 dengue cases in U.S. since its identification

Yellow fever cases worldwide:
200,000 (90% in Africa)

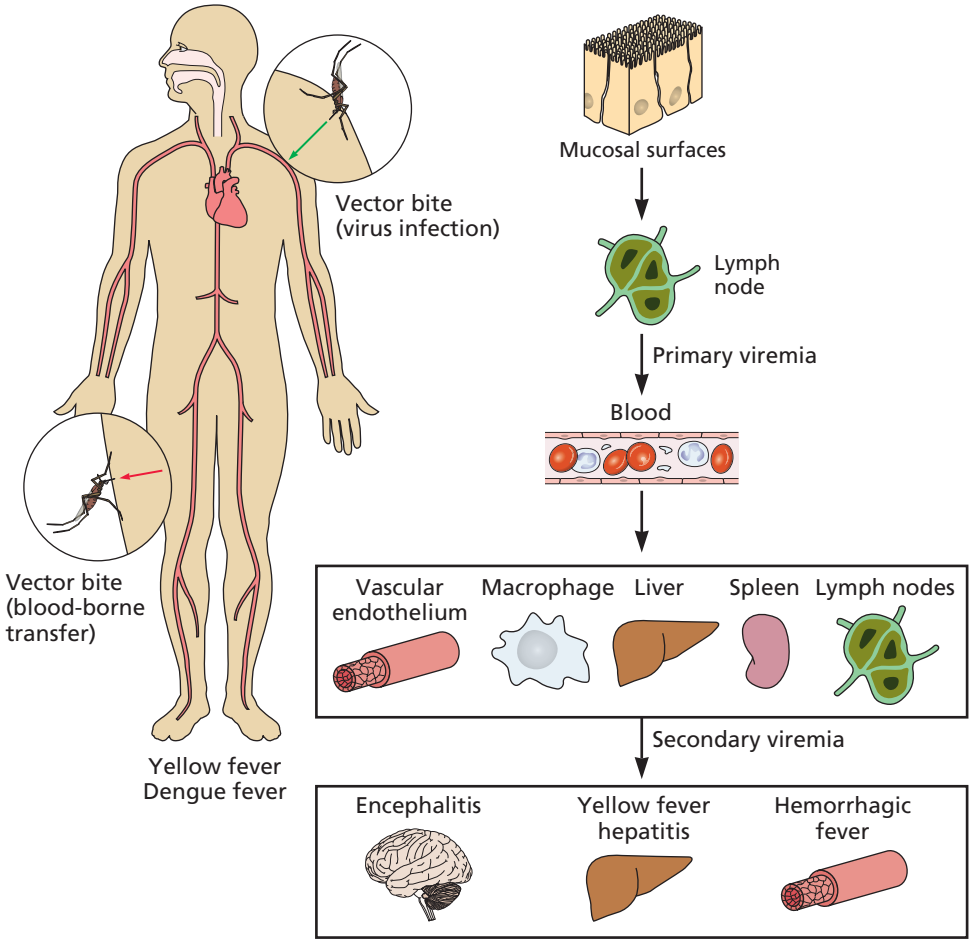


Figure 6

Flaviviruses (Hepatitis C)

Virus	Vector	Disease
Hepacivirus • Hepatitis C virus	None	Hepatitis

Epidemiology

Transmission
- Blood
- Sex

Distribution
- Worldwide
- No seasonal incidence

At risk or risk factors
- IV drug users
- Health care workers

Vaccines or antiviral drugs
- Currently 7 FDA-approved antivirals
- No vaccines

Pathogenesis

Viruses are noncytolytic and chronic

Disease caused by ongoing immune response

Liver cancer can result from chronic cirrhosis

Human Infections

Persons living with hepatitis C
Worldwide: 130 million–150 million
In United States: 3.2 million

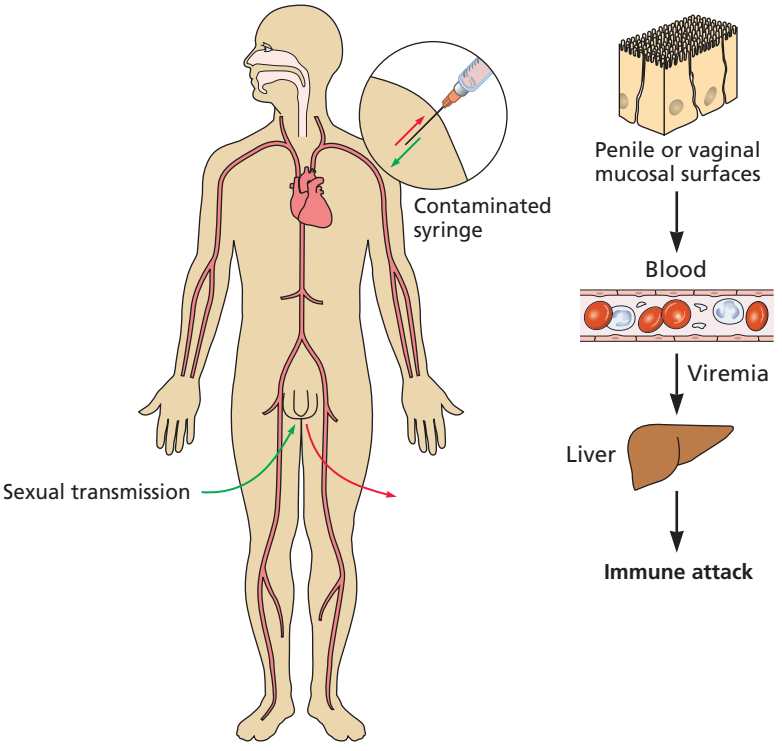


Figure 7

Hepadnaviruses

Virus	Disease
Hepatitis B virus	Hepatitis, primary hepatocellular carcinoma, cirrhosis • Children (mild, chronic infection) • Adults (insidious hepatitis) • Adults with chronic hepatitis

Epidemiology

Transmission
- Blood, semen, vaginal secretions, transfusions, sex, breast-feeding, saliva, contaminated needles

At risk or risk factors
- IV drug use
- Promiscuity

Distribution
- Worldwide
- No seasonal incidence

Vaccines or antiviral drugs
- Currently 6 FDA-approved antiviral drugs
- Subunit vaccine

Pathogenesis

Primary reproduction in hepatocytes followed by viremia

Tissue damage caused by cell-mediated immune response

Acute infections

Chronic infections that can lead to hepatocellular carcinoma

Human Infections

Worldwide incidence of hepatitis B: 240 million

Annual number of deaths: ~1 million

Hepatitis B virus vaccine efficacy: >95%

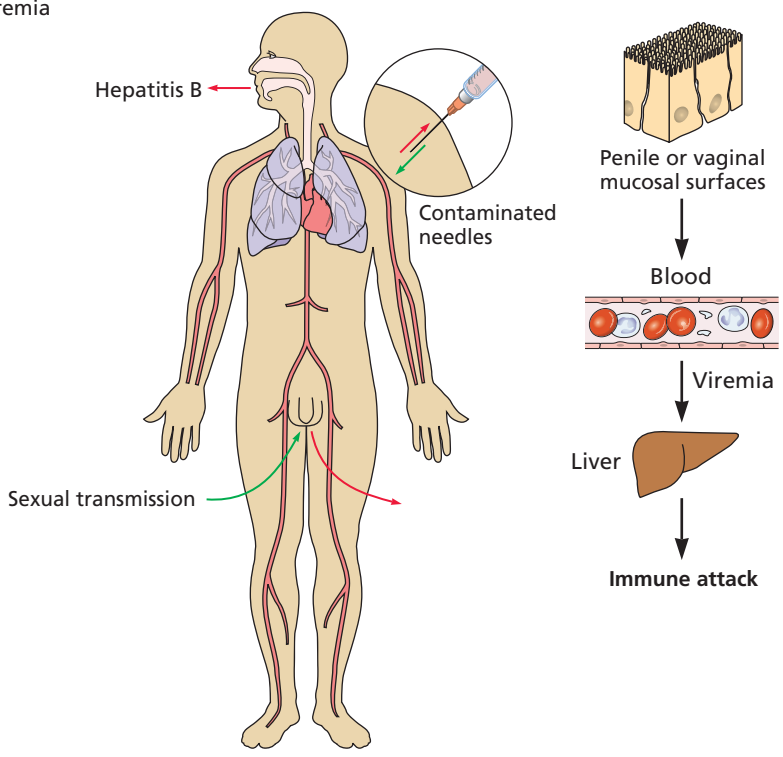


Figure 8

Herpesviruses (Herpes simplex virus)

Virus	Disease
Alphaherpesviruses • Herpes simplex virus types 1 and 2	Mucosal lesions, encephalitis • Children (HSV-1) • Sexually active people (HSV-2)

Epidemiology

Transmission
- Saliva, vaginal secretions, secretions from blisters in oral and anogenital tracts
- Eyes; skin lesions
- Herpes simplex virus type 1: mainly oral; herpes simplex virus type 2: mainly sexual

At risk or risk factors
- Promiscuity

Distribution
- Worldwide
- No seasonal incidence

Vaccines or antiviral drugs
- Antivirals: acyclovir, ganciclovir

Pathogenesis

Cell-to-cell spread, not neutralized by antibody

Establishes latency in neurons

Reactivated from latency by stress or immune suppression

Human Infections

HSV-1 seroprevalence: >65%

HSV-2 seroprevalence: 16.2%

Incidence increases with number of sex partners

Demographic	(Females)	(Males)
1 Lifetime sex partner	5.4%	1.7%
2–4 Lifetime sex partners	18.8%	7.3%
5–9 Lifetime sex partners	21.8%	10.1%
≥10 Lifetime sex partners	37.1%	19.1%

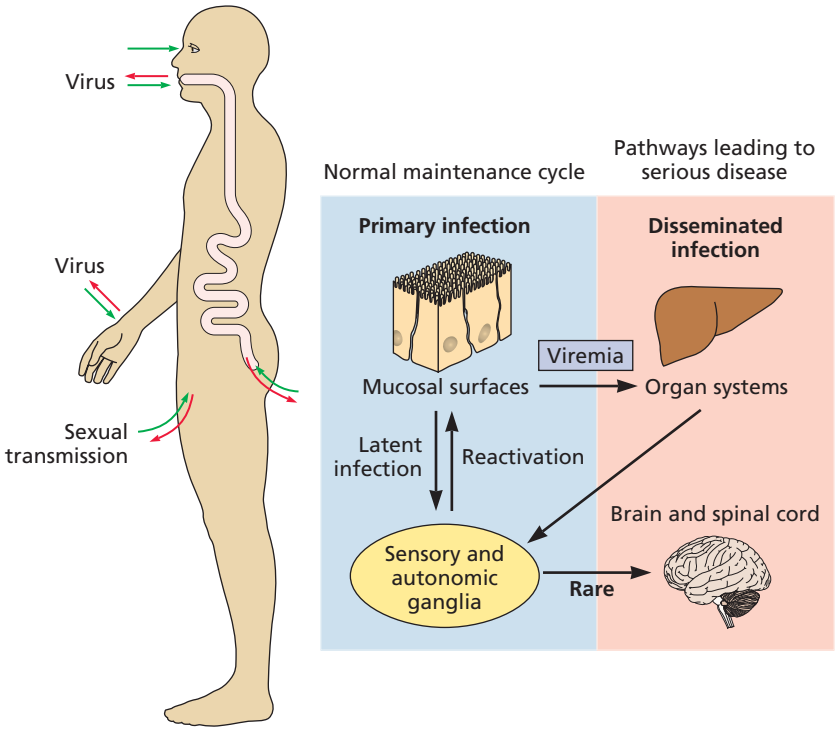


Figure 9

Herpesviruses (Varicella-zoster virus)

Virus	Disease
Alphaherpesviruses • Varicella-zoster virus	Chickenpox, shingles • Children (ages 5–9 years) (mild disease) • Teenagers and adults (more severe disease, possibly pneumonia) • Elderly, immunocompromised (recurrent zoster)

Epidemiology

Transmission
- Aerosol

Distribution
- Worldwide
- No seasonal incidence

At risk or risk factors
- Immunosuppression
- Shingles: previous exposure

Vaccines or antiviral drugs
- Attenuated vaccine
- Antiviral drugs: acyclovir, foscarnet

Pathogenesis

Infects epithelial cells and fibroblasts, spread by viremia to skin, causes lesions of chickenpox

Latent infection in neurons

Reactivation by immune suppression, stress, or other infections leads to zoster or shingles, formation of lesions over entire dermatome

Human Infections

99.5% of people are seropositive

30% chance of shingles following primary infection

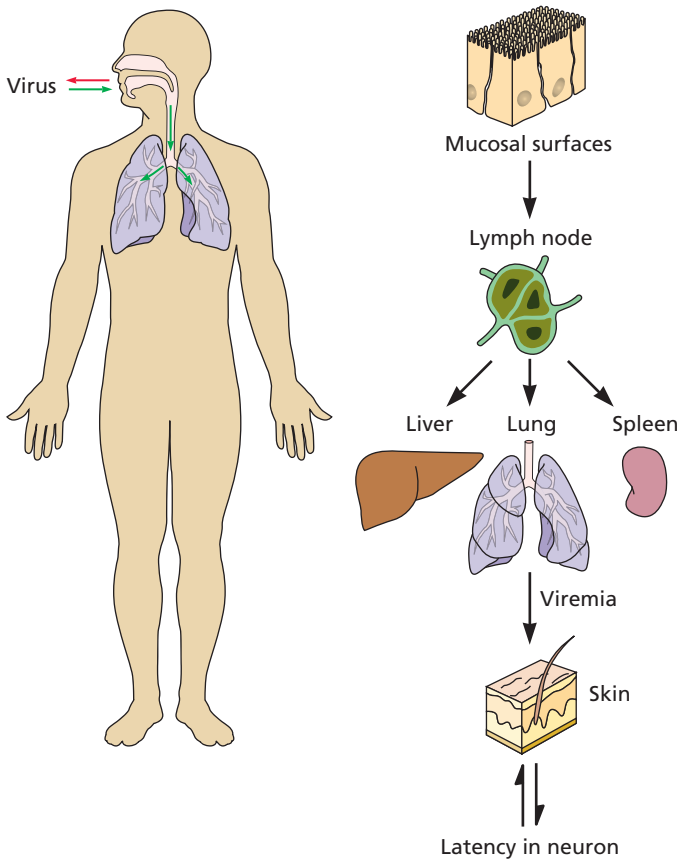


Figure 10

Herpesviruses (Cytomegalovirus)

Virus	Disease
Betaherpesviruses • Cytomegalovirus	Congenital defects (cerebral palsy) Disseminated disease in immunosuppressed patients

Epidemiology

Transmission
- Blood, tissue, and body secretions (urine, saliva, semen, cervical secretions, breast milk, tears)

Distribution
- Worldwide
- No seasonal incidence

At risk or risk factors
- Babies whose mothers become infected during pregnancy
- Sexual activity
- Transplant recipients
- Burn victims
- Immunosuppression

Vaccines or antiviral drugs
- No vaccines
- Antiviral drugs: acyclovir, ganciclovir

Pathogenesis

Infects epithelial and other cells

Mainly causes subclinical infections

Latent infection in $CD34^+$ bone marrow progenitor cells, macrophages, other cells

Immunosuppression leads to recurrence and severe disease

Human Infections

60–70% seropositive in resource-rich countries

100% seropositive in resource-poor countries

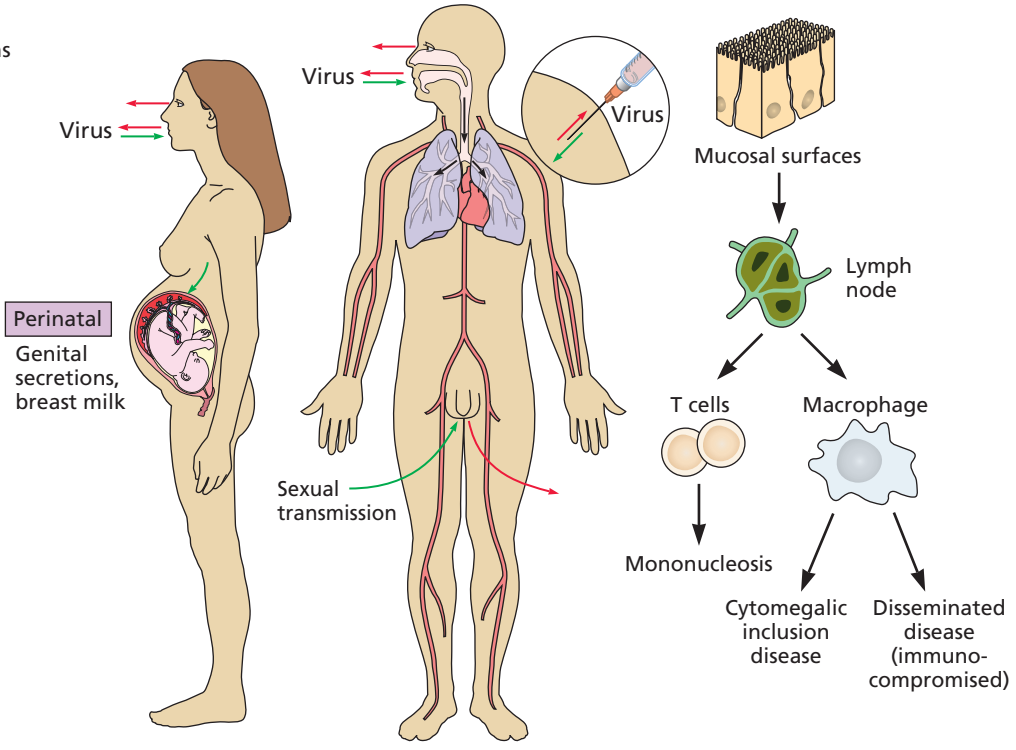


Figure 11

Herpesviruses (Gammaherpesviruses)

Virus	Disease
Gammaherpesviruses	
• Epstein-Barr virus (EBV)	Infectious mononucleosis; associated with a variety of lymphomas
	Nasopharyngeal carcinoma
• Human herpesvirus 8 (HHV-8; Kaposi's sarcoma-associated herpesvirus [KSHV])	Kaposi's sarcoma, rare B cell lymphoma

Epidemiology

Transmission
- Saliva, close oral contact, or shared items (cup or toothbrush)

Distribution
- EBV: Worldwide
- KSHV: Mediterranean basin
- Burkitt's lymphoma in malaria belt
- No seasonal incidence

At risk or risk factors
- Immunosuppression
- Malaria (Burkitt's lymphoma)
- Kissing

Vaccines or antiviral drugs
- None

Pathogenesis

EBV:
Infects oral epithelial cells, B cells

Immortalizes B cells

HHV-8:
Characteristic skin rash

Human Infections

In the U.S.: 90–95% of adults show evidence of prior EBV infection

HHV-8: Prevalence highly variable:
Japan: 0.2%
Africa: >50%

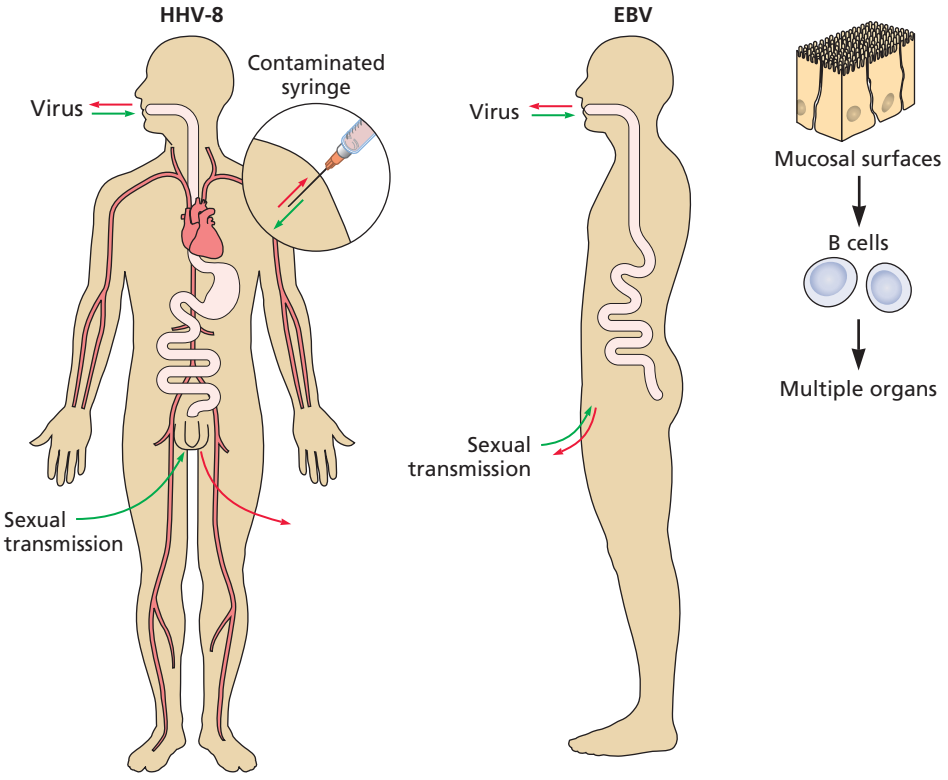


Figure 12

Orthomyxoviruses

Virus	Disease
Influenza A, B, and C viruses	Acute febrile respiratory tract infection Children may also have abdominal pain, vomiting, otitis media, muscle inflammation, croup Primary viral pneumonia Reye's syndrome

Epidemiology

Transmission
- Aerosols; fomites

At risk or risk factors
- Age (very young and elderly)
- Immunosuppression
- Pregnancy

Distribution
- Worldwide
- More common in winter

Vaccines or antiviral drugs
- Inactivated vaccine against annual strains of influenza A and B viruses; Flublok subunit vaccine
- Attenuated influenza A and B vaccine (nasal spray)
- Antiviral drugs: amantadine, oseltamivir (Tamiflu)

Pathogenesis

Infects upper and lower respiratory tract

Pronounced systemic symptoms caused by cytokine response to infection

Susceptibility to bacterial superinfection because of compromised natural epithelial barriers

Human Infections

Worldwide deaths due to flu epidemics:
1918: >50 million
2009: 151,000–575,000

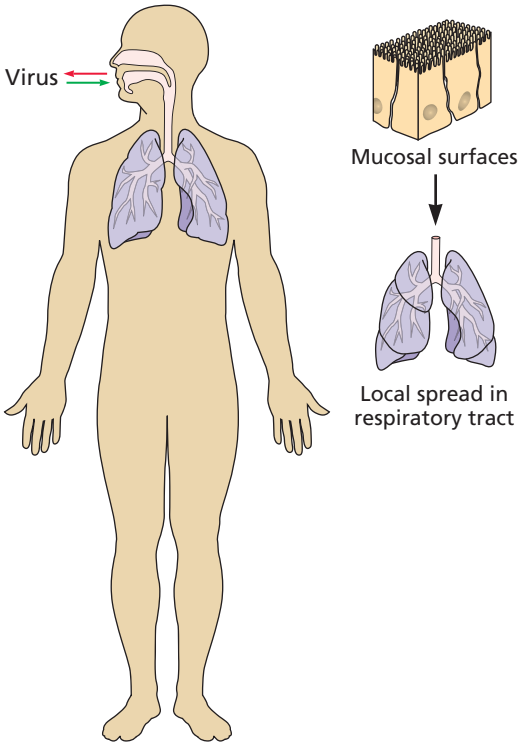


Figure 13

Papillomaviruses

Virus	Disease
Papillomavirus (120 genotypes)	Skin warts: plantar, common, and flat warts; epidermodysplasia verruciformis Head and neck tumors: laryngeal, oral, and conjunctival papillomas Anogenital warts: condyloma acuminatum, cervical intra-epithelial neoplasia, cancer

Epidemiology

Transmission
- Direct contact, sexual contact
- During birth, from infected birth canal

Distribution
- Worldwide
- More common in winter

At risk or risk factors
- Genital and oral sex

Vaccines or antiviral drugs
- Vaccine against types 6, 11, 16, and 18 (e.g., Gardasil)

Pathogenesis

Infect epithelial cells of skin, mucous membranes

Reproduction depends on stage of epithelial cell differentiation

Cause benign outgrowth of cells into warts

Some types are associated with dysplasia that may become cancerous

Human Infections

Head and neck cancers in U.S. annually:
Males: 10,000
Females: 2,500

Cervical cancers: 12,000

Penile cancers: 1,000

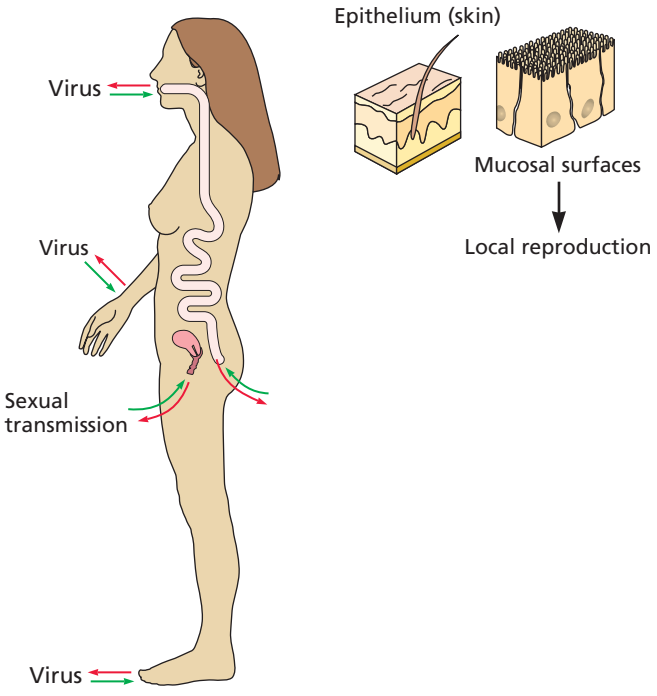


Figure 14

Paramyxoviruses (Measles virus)

Virus	Disease
Morbilliviruses • Measles virus	Ear infections, croup, bronchopneumonia, encephalitis, congestion, immunosuppression, skin rash

Epidemiology

Transmission
- Aerosols
- Highly contagious

Distribution
- Worldwide
- Endemic from autumn to spring

At risk or risk factors
- Malnutrition
- Unvaccinated
- Immunosuppression

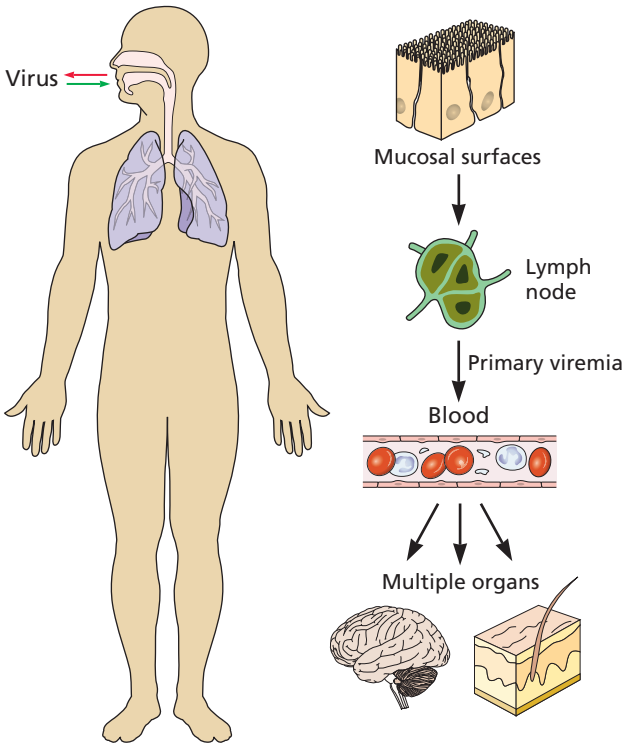
Vaccines or antiviral drugs
- Attenuated vaccine
- No antiviral drugs

Pathogenesis

Infects epithelial cells of respiratory tract, spreads in lymphocytes and by viremia

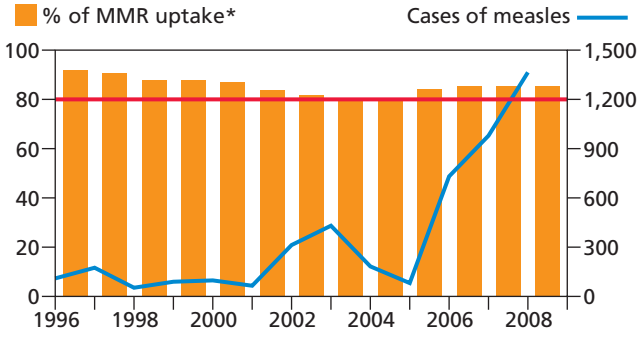
Reproduces in conjunctivae, respiratory tract, urinary tract, lymphatic system, blood vessels, and central nervous system

T cell response to virus-infected capillary endothelial cells causes rash



Human Infections

Increased incidence of measles with decreased herd immunity



*Figures relate to financial years 1996/97, 1997/98, etc.

Figure 15

Paramyxoviruses (Respiratory syncytial virus)

Virus	Disease
Pneumoviruses	
• Respiratory syncytial virus	Bronchiolitis, pneumonia, febrile rhinitis, pharyngitis, common cold

Epidemiology

Transmission
- Aerosols

Distribution
- Worldwide
- Winter and spring

At risk or risk factors
- Age (<6 months)
- Immunosuppression
- Adults with other respiratory problems (chronic obstructive pulmonary disease)

Vaccines or antiviral drugs
- Monoclonal antibody for treatment
- Antiviral drug: ribavirin for infants

Pathogenesis

Infects the respiratory tract, does not spread systemically

In newborns, the infection may be fatal because narrow airways are blocked by virus-induced pathology

Infants are not protected from infection by maternal antibody

Reinfection may occur after a natural infection

Human Infections

Most common cause of bronchiolitis and pneumonia in children <1 year of age in the U.S.

Almost all children will have had respiratory syncytial virus infection before 2 years of age, though symptoms vary from severe respiratory disease to mild

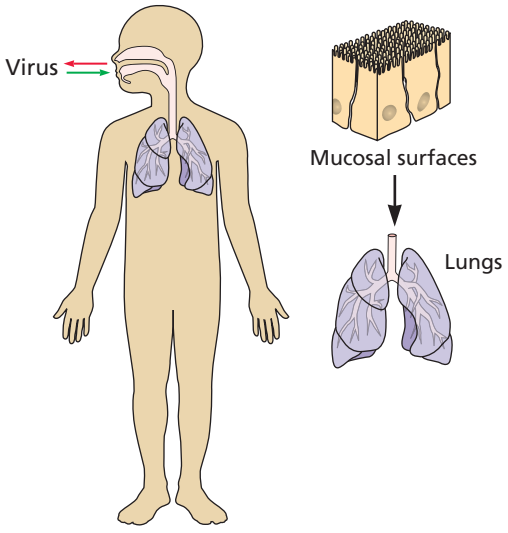


Figure 16

Picornaviruses (Poliovirus and Hepatitis A)

Virus	Disease
Polioviruses	
• Poliovirus types 1–3	Paralytic disease, encephalitis, meningitis, respiratory tract infections, undifferentiated fever, disease in immunodeficient patients
Hepatitis A virus	Hepatitis

Epidemiology

Transmission
- Fecal-oral

At risk or risk factors
- **Polio:** Poor sanitation
- **Hepatitis A:** Intravenous drugs, sex, contaminated food supply

Distribution
- Nearly eradicated (polio)
- Worldwide hepatitis A

Vaccines or antiviral drugs
- Attenuated oral or inactivated polio vaccines
- No licensed antiviral drugs
- Hepatitis A virus: inactivated vaccine

Pathogenesis

99% of infections are mild or asymptomatic

Only 1% associated with paralysis

Reversion of attenuated vaccine may lead to vaccine-associated paralysis

Human Infections

Polio continues to be endemic in Pakistan, Afghanistan, Nigeria

Only 350–400 cases in recent years

Hepatitis A: 1.4 million cases annually

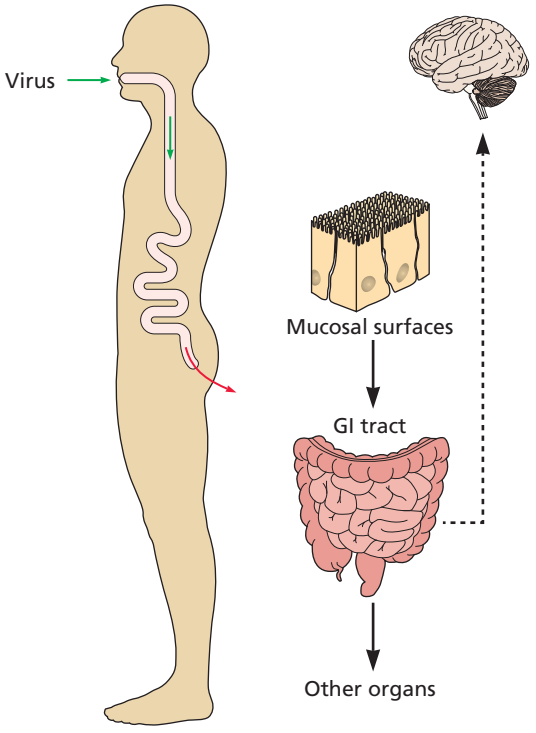


Figure 17

Picornaviruses (Rhinoviruses)

Virus	Disease
Rhinoviruses • A, B, and C (>150 genotypes)	Respiratory tract infections Major cause of common cold

Epidemiology

Transmission
- Aerosols
- Contact with contaminated hands

Distribution
- Worldwide
- Disease most common in early autumn, late spring

At risk or risk factors
- Preexisting respiratory conditions

Vaccines or antiviral drugs
- No vaccines
- No licensed antiviral drugs

Pathogenesis

Enter upper respiratory tract and may remain localized or spread to lower respiratory tract

Major factor in asthma exacerbations

Human Infections

Millions of cases per year in U.S. alone

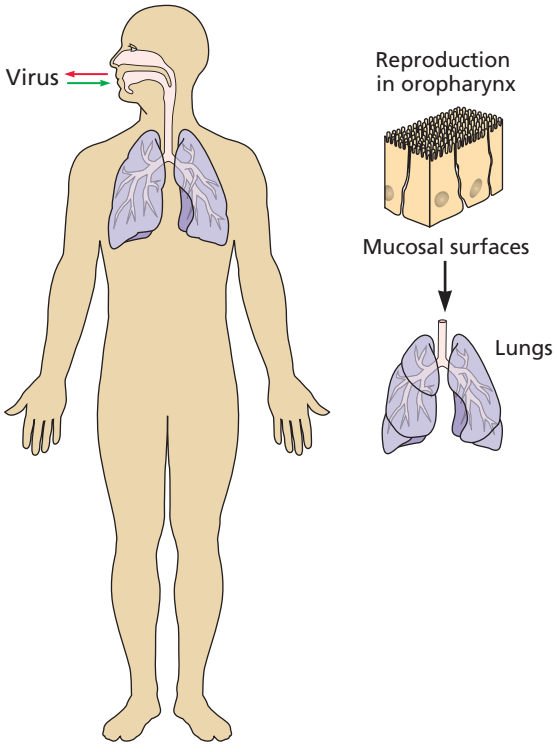


Figure 18

Picornaviruses (Enteroviruses)

Virus	Disease
Enteroviruses	
• Enterovirus 68	Severe respiratory disease
• Enterovirus 70	Paralytic disease, acute hemorrhagic conjunctivitis
• Enterovirus 71	Paralytic disease, encephalitis, meningitis, hand-foot-and-mouth disease

Epidemiology

Transmission
- Fecal-oral

Distribution
- Worldwide
- Disease most common in summer

At risk or risk factors
- Poor sanitation
- Age (newborns and neonates)

Vaccines or antiviral drugs
- No vaccines
- No licensed antiviral drugs

Pathogenesis

Enter oropharyngeal or intestinal mucosa

Serum antibody blocks spread

Virus may be shed in feces

High asymptomatic infection rate

Human Infections

10 million–15 million enterovirus infections in the U.S. each year

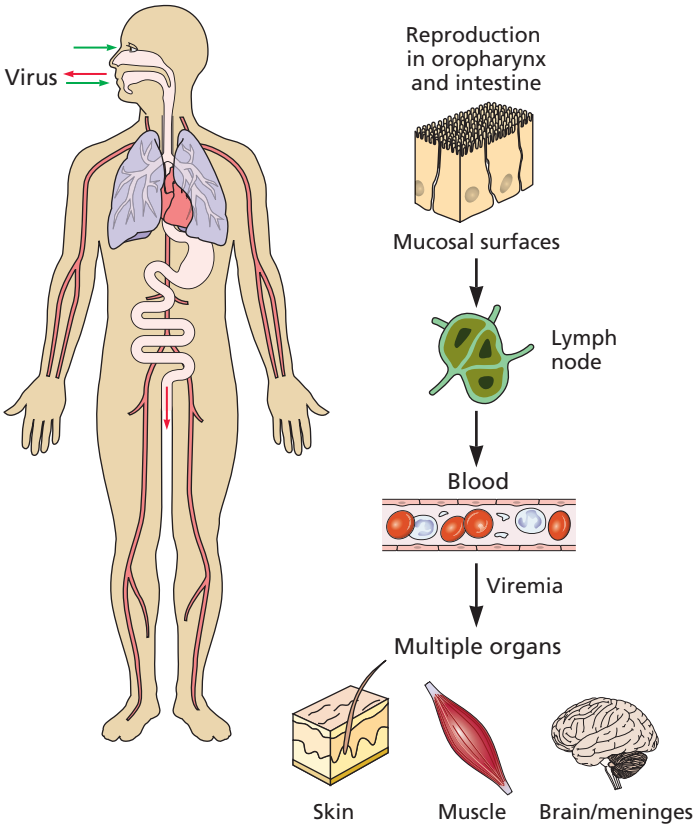


Figure 19

Polyomaviruses

Virus	Disease
Polyomavirus	
• BK virus	Renal disease in immunosuppressed patients
• JC virus	Progressive multifocal leukoencephalopathy (PML) in immunosuppressed patients

Epidemiology

Transmission
- Aerosols

Distribution
- Worldwide
- No seasonal incidence

At risk or risk factors
- Immunocompromised persons (HIV infection)

Vaccines or antiviral drugs
- None

Pathogenesis

Acquired through the respiratory route, spread by viremia to kidneys early in life

Infections are usually asymptomatic

Virus establishes persistent and latent infection in organs such as the kidneys and lungs

In immunocompromised people, JC virus is activated, spreads to the brain, and causes progressive multifocal leukoencephalopathy; oligodendrocytes are killed, causing demyelination

Human Infections

Ubiquitous in human population

PML rare, even in immunosuppressed patients

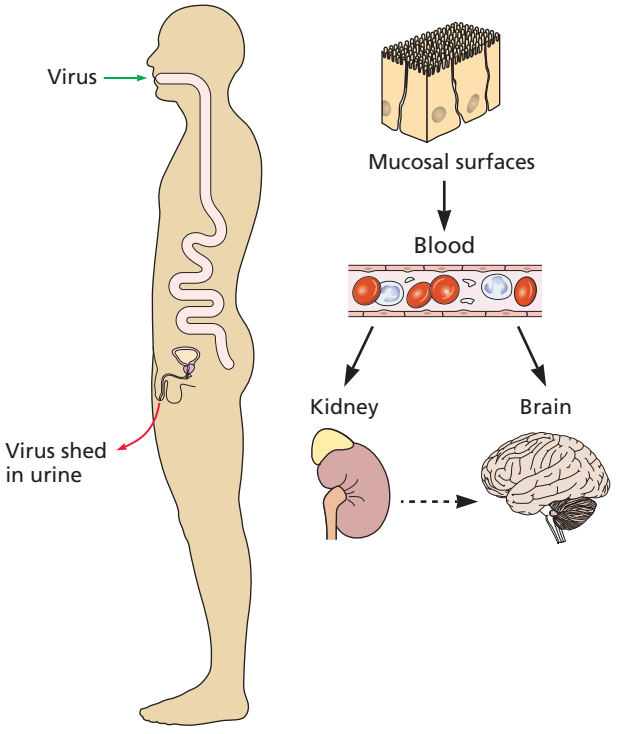


Figure 20

Poxviruses

Virus	Disease
Variola virus	Smallpox
Vaccinia virus (smallpox vaccine)	Encephalitis and vaccinia necrosum (complications of vaccination)
Cowpox virus	Localized lesion
Monkeypox virus	Generalized disease
Molluscum contagiosum virus	Disseminated skin lesions

Epidemiology

Transmission
- Smallpox: respiratory droplets, contact with virus on fomites
- Other poxviruses: direct contact or fomites

Distribution
- Worldwide
- No seasonal incidence

At risk or risk factors
- Molluscum contagiosum: sexual contact, wrestling
- Pet owners, animal handlers (contact with lesion)

Vaccines or antiviral drugs
- Attenuated vaccine against smallpox (vaccinia virus)

Pathogenesis

Infects respiratory tract, spreads through lymphatics and blood

Sequential infection of multiple organs

Skin lesions are prominent

Human Infections

Smallpox is the only human virus to be eradicated

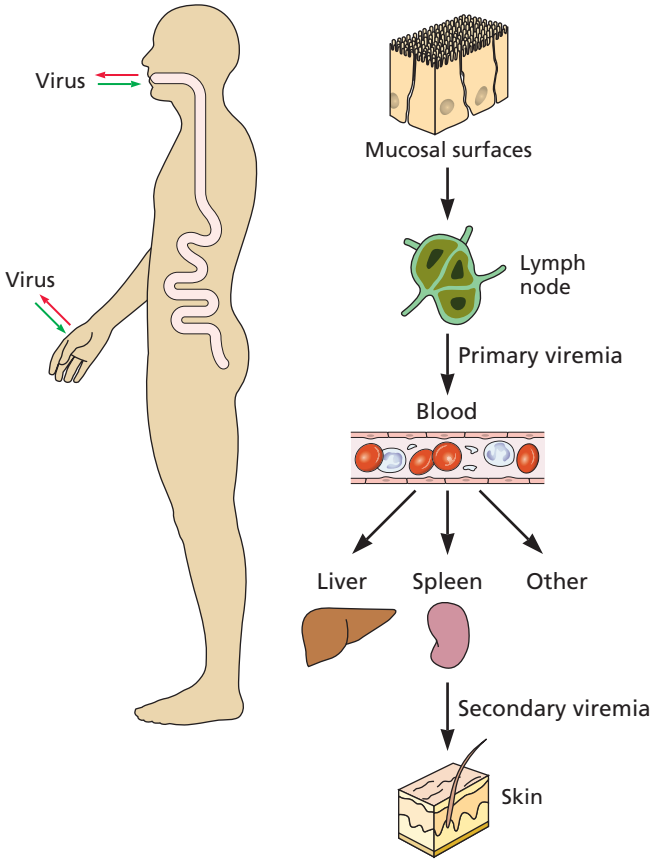


Figure 21

Reoviruses

Virus	Disease
Orthoreovirus	Mild upper respiratory tract disease, gastroenteritis, biliary atresia
Coltivirus	Colorado tick fever: febrile disease, headache, myalgia
Rotavirus	Gastroenteritis

Epidemiology

Transmission
- Fecal-oral route
- Arthropod

Distribution
- Worldwide; type B prevalent in China
- Less common in summer

At risk or risk factors
- Malnourishment
- Age (<2 years)
- Proximity to vectors

Vaccines or antiviral drugs
- Attenuated, oral vaccines available

Pathogenesis

Rotavirus: Large quantities of virus particles released in diarrhea

Colorado tick: persists in red blood cells up to 120 days

Human infections

Rotavirus:
Estimated worldwide cases annually: 111 million
Estimated deaths annually: 440,000
82% of deaths occur in the poorest countries from dehydration

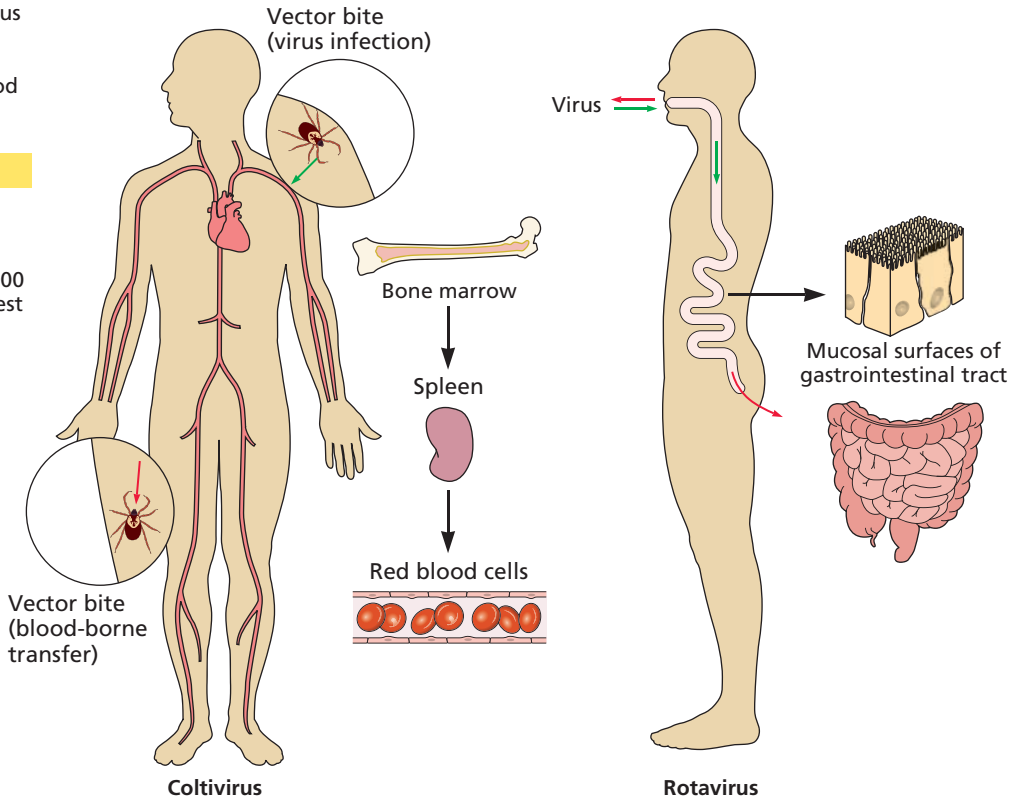


Figure 22

Retroviruses (Human T-lymphotropic virus type 1)

Virus	Disease
Deltaretrovirus	
• Human T-lymphotropic virus type 1	Adult T-cell leukemia, tropical spastic paraparesis
• Human T-lymphotropic virus type 2	Hairy-cell leukemia
• Human T-lymphotropic virus type 5	Malignant cutaneous lymphoma

Epidemiology

Transmission
- Transfusions, needle sharing among drug users
- Virus in semen
  Anal and vaginal intercourse
- Perinatal transmission

At risk or risk factors
- Intravenous drug users
- Homosexuals and heterosexuals with many partners
- Newborns of virus-positive mothers

Distribution
- Worldwide
- No seasonal incidence

Vaccines or antiviral drugs
- No vaccines
- Some antivirals may be useful

Pathogenesis

Infects T lymphocytes

Remains latent or reproduces slowly, induces clonal outgrowth of T cell clones

Long latency period (30 years) before onset of leukemia

Infection leads to immunosuppression

Human Infections

Highest incidence in Japan and islands in the Caribbean

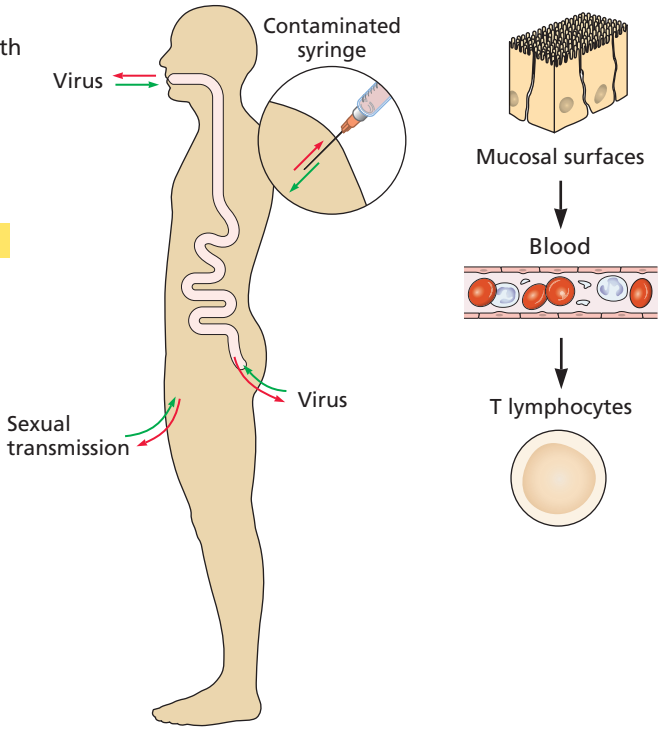


Figure 23

Retroviruses (Human immunodeficiency virus types 1 and 2)

Virus	Disease
Lentivirus	
• Human immunodeficiency virus types 1 and 2	Acquired immune deficiency syndrome (AIDS)

Epidemiology

Transmission
- Transfusions, needle sharing among drug users
- Virus in semen
  Anal and vaginal intercourse
- Perinatal transmission

Distribution
- Worldwide
- No seasonal incidence

At risk or risk factors
- Intravenous drug users
- Homosexuals and heterosexuals with many partners
- Prostitutes
- Newborns of virus-positive mothers

Vaccines or antiviral drugs
- No vaccines
- Antiviral drugs
  Nucleoside analog reverse transcriptase inhibitors (e.g., azidothymidine, dideoxycytidine)
  Nonnucleoside reverse transcriptase inhibitors (e.g., nevirapine, delavirdine)
  Protease inhibitors (e.g., saquinavir, ritonavir)
  Integrase inhibitors (e.g., raltegravir, elvitegravir)
  Fusion inhibitors (e.g., enfuvirtide, maraviroc)

Pathogenesis

Infects mainly $CD4^+$ T cells and macrophages

Lyses $CD4^+$ T cells, persistently infects macrophages

Infection alters T cell and macrophage function; immunosuppression leads to secondary infection and death

Infects long-lived cells, establishing reservoir for persistent infection

Infected monocytes spread to brain, causing dementia

Human Infections

Percentage of adults (ages 15-29 years) infected worldwide (2011)

Afghanistan	<0.1
Argentina	0.5
Belgium	0.3
Botswana	23.4
Canada	0.3
Ethiopia	1.4
India	0.3
Philippines	>0.1
Russia	1.1
South Africa	17.3
Swaziland	26.5
United States	0.6

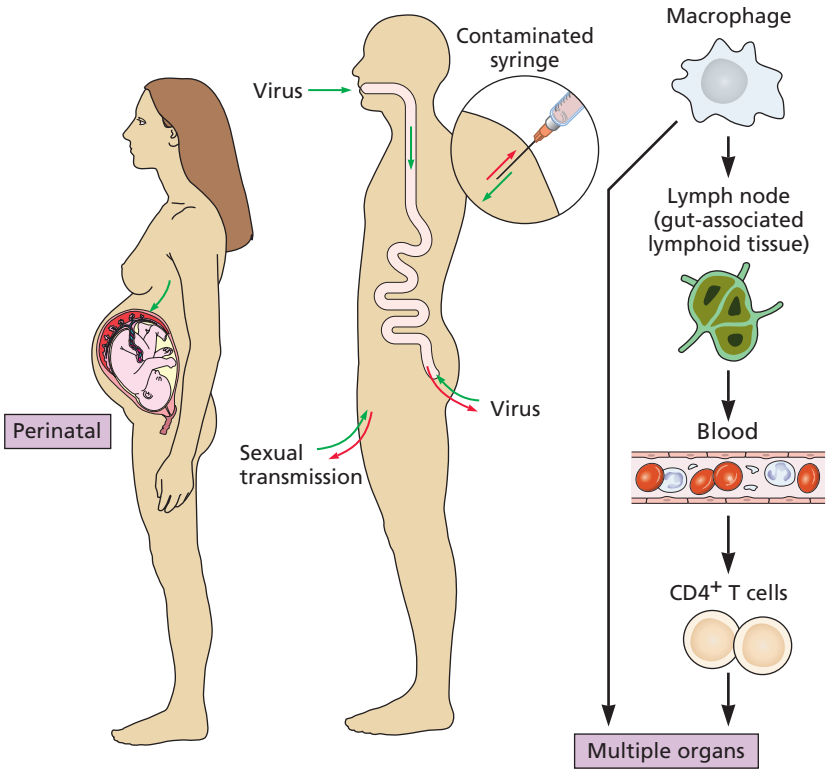


Figure 24

Rhabdoviruses (Rabies virus)

Virus	Disease
Lyssavirus	
• Rabies virus	Rabies
Vesiculovirus	
• Vesicular stomatitis virus	Flu-like illness

Epidemiology

Transmission
- Bites of wild animals and unvaccinated dogs and cats

At risk or risk factors
- Animal handlers, veterinarians
- Those in countries with no pet vaccinations or quarantine

Distribution
- Worldwide, except certain islands and U.K.
- No seasonal incidence

Vaccines or antiviral drugs
- Vaccines for pets and wild animals
- Inactivated virus vaccine for at-risk personnel, postexposure prophylaxis
- No antiviral drugs

Pathogenesis

Reproduces in muscle at bite site

Incubation period of weeks to months, depending on inoculum and distance of bite from central nervous system

Infects peripheral nerves and travels to brain

Reproduction in brain causes hydrophobia, seizures, hallucinations, paralysis, coma, and death

Spreads to salivary glands of nonhuman animals, from which it is transmitted

Postexposure immunization can prevent disease due to long incubation period

Human Infections

Rabies deaths estimated 20,000 per year worldwide

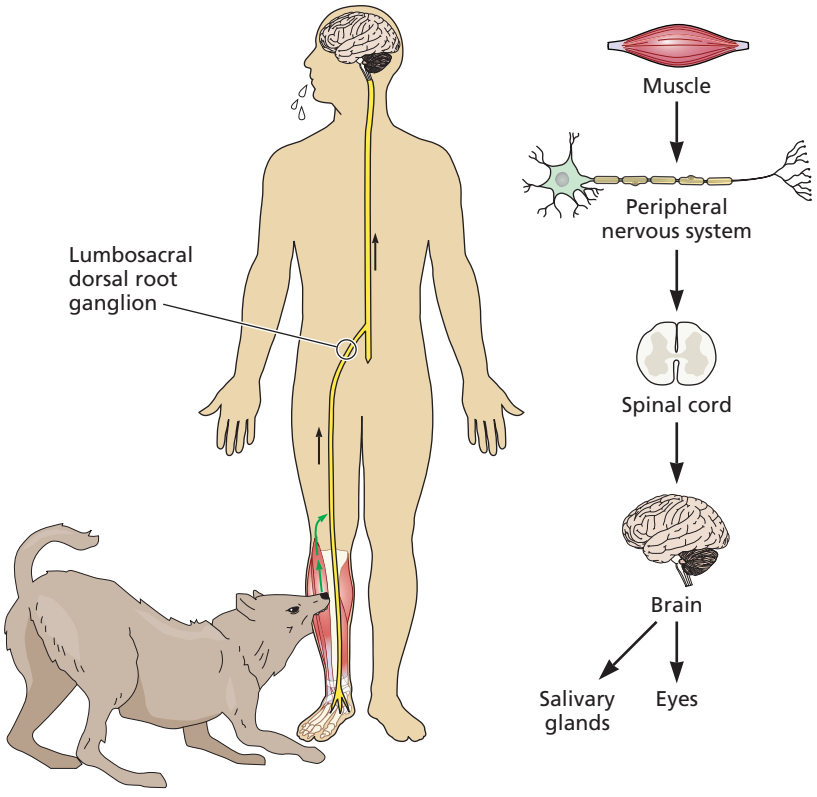


Figure 25

Togaviruses (Alphaviruses)

Virus	Vector	Disease
Alphaviruses		
• Venezuelan equine encephalitis virus, Eastern equine encephalitis virus, Western equine encephalitis virus	*Aedes, Culex, Culiseta* mosquitoes	Mild systemic; severe encephalitis
• Chikungunya virus	*Aedes* mosquitoes	Fever, arthralgia, arthritis

Epidemiology

Transmission
- Mosquito vectors

Distribution
- Range determined by habitat of vector
- Most common in summer

At risk or risk factors
- Proximity to vector

Vaccines or antiviral drugs
- None

Pathogenesis

Antibodies limit virus spread by viremia (e.g., to fetus in pregnant host)

Cell-mediated immunity important to resolve infection

Human Infections

Chikungunya endemic in >40 countries and associated with sporadic epidemics

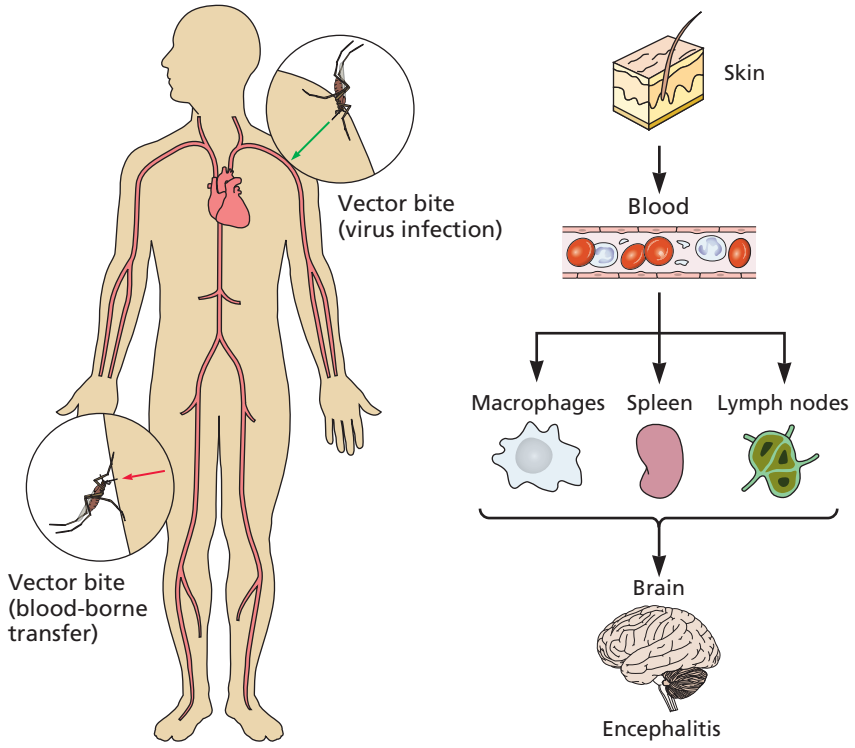


Figure 26

Togaviruses (Rubella virus)

Virus	Disease
Rubella virus	Rubella Cerebral palsy

Epidemiology

Transmission
- Aerosols

Distribution
- Worldwide
- No seasonal incidence

At risk or risk factors
- Age (<20 weeks)
- Lack of vaccination

Vaccines or antiviral drugs
- Attenuated vaccine

Pathogenesis

Antibodies limit virus spread by viremia (e.g., to fetus in pregnant host)

Cell-mediated immunity important to resolve infection

Human Infections

U.S. rubella epidemic 1964–65 (pre-vaccine):
12.5 million cases
2,000 cases of encephalitis
2,100 deaths
11,200 abortions

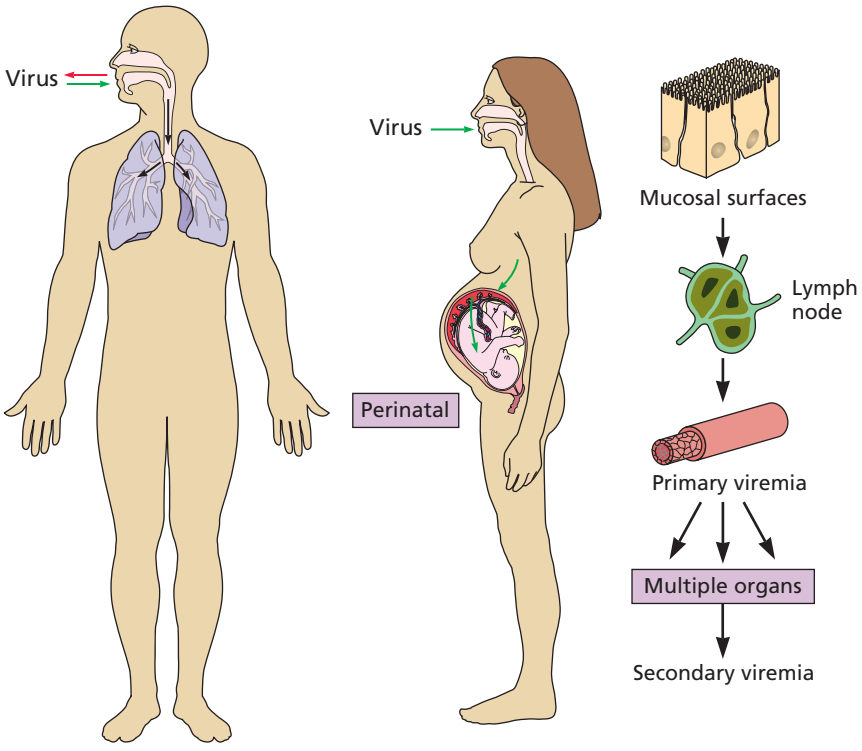


Figure 27

Glossary

Abortive infection An incomplete infectious cycle; virions infect a susceptible cell or host but do not complete reproduction, usually because an essential viral or cellular gene is not expressed. *(Chapter 5)*

Accessibility An attribute that describes the physical availability of cells to virus particles at the site of infection. *(Chapter 2)*

Active immunization The process of inducing an immune response by exposure to a vaccine; contrasts with passive immunization. *(Chapter 8)*

Acute infection A common pattern of infection in which virus particles are produced rapidly, and the infection is resolved quickly by the immune system; survivors are usually immune to subsequent infection. *(Chapter 5)*

Adaptive response The immune response consisting of antibody (humoral) and T lymphocyte-mediated responses; unlike the innate response, the adaptive response is tailored to the particular foreign invader; the adaptive response has memory: subsequent infections by the same agent are met with a robust and highly specific response. Also known as the acquired immune response. *(Chapters 2 and 4)*

Adjuvant A compound or mixture that stimulates immune responses to an antigen *(Chapter 8)*

Adoptive transfer The transfer of cells, usually lymphocytes, from an immunized donor to a nonimmune recipient. *(Chapters 4 and 8)*

Alternative pathway One of three pathways in the complement system; activates the C3 and C5 convertases without going through the C1-C2-C4 complex. *(Chapter 3)*

Anchorage independence The ability of some cells to grow in the absence of a surface on which to adhere; often detected by the ability to form colonies in semisolid media. *(Chapter 6)*

Antibody-dependent cell-mediated cytotoxicity The process in which binding of an anti-viral IgG antibody to Fc receptors on macrophages and some NK cells targets these cells to kill infected cells that carry on their surfaces the antigen recognized by the antibody; also known as ADCC. *(Chapter 4)*

Antigen Protein, DNA, lipid or polysaccharide that induces an immune response. *(Chapter 4)*

Antigenic drift The appearance of virus particles with a slightly altered surface protein (antigen) structure as a result of the accumulation of point mutations following passage and immune selection in the natural host. *(Chapter 5)*

Antigenic shift A major change in one or more surface proteins of a virus particle when genes encoding markedly different surface proteins are acquired during infection; this process occurs when viruses with segmented genomes exchange segments, or when nonsegmented viral genomes recombine after coinfection. *(Chapter 5)*

Antigenic variation The display by virus particles or infected cells of new protein sequences that are not recognized by antibodies or T cells that responded to previous infections. *(Chapter 5)*

Antiviral state A condition in which cells cannot support reproduction of viruses as a result of binding and responding to interferon. *(Chapter 3)*

Apoptosis Cell death following a sequence of tightly regulated reactions induced by external or internal stimuli that signal DNA damage or other forms of stress; characterized by chromosome degradation, nuclear degeneration and cell lysis; a natural process in development and the immune system, but also an intrinsic defense of cells to viral infection. Also called programmed cell death. *(Chapters 2 and 3)*

Attenuated Having mild or inconsequential instead of normally severe symptoms or pathology as an outcome of infection; having a state of reduced virulence. *(Chapter 5)*

Autocrine growth stimulation Stimulation of cell growth by proteins produced and sensed by the same cell. *(Chapter 6)*

Autophagy A process in which cells are induced to degrade the bulk of their cellular contents for recycling within specialized membrane-bounded compartments called autophagolysosomes. *(Chapter 3)*

Blind screening Screening for antiviral compounds without regard to a specific mechanism. *(Chapter 9)*

Case fatality ratio The number of deaths divided by the number of clinically confirmed infections. *(Chapter 1)*

Caspases Crirical proteases in apoptosis; members of a family of cysteine proteases that specifically cleave after aspartate residues. *(Chapter 3)*

CD markers *See* Cluster-of-differentiation markers.

CD4$^+$ T cells T lymphocytes that carry the coreceptor protein CD4 on their surfaces. *(Chapter 4)*

CD8$^+$ T cells T lymphocytes that carry the coreceptor CD8 on their surfaces. *(Chapter 4)*

Cell cycle The orderly and reproducible sequence in which cells increase in size, duplicate the genome, segregate duplicated chromosomes, and divide. *(Chapter 6)*

Cell-mediated response The arm of the adaptive immune response consisting of helper and effector T lymphocytes. *(Chapter 4)*

Central memory T cells Self-renewing memory T cells that are abundant in lymph nodes and other lymphoid tissues. *(Chapter 4)*

Chemokines Small proteins that attract and stimulate cells of the immune defense system; produced by many cells in response to infection. *(Chapter 3)*

Circadian rhythm The cycle (roughly 24 hours in humans) that regulates many physiological processes, such as sleep-wake cycles. *(Chapter 1)*

Clades Subtypes of human immunodeficiency virus that are prevalent in different geographic areas. *(Chapter 7)*

Classical pathway One of three complement pathways that lead to activation of C3-C5 convertases; activation occurs by direct interaction of C1q or C3b proteins with a viral protein/antibody complex on the surface of an infected cell or a virus particle. *(Chapter 3)*

Clinical latency A state of persistent viral infection in which no clinical symptoms are manifested. *(Chapter 7)*

Cluster-of-differentiation markers Distinct surface proteins that are recognized by specific monoclonal antibodies; these antibodies bind to various cluster-of-differentiation markers and are used to distinguish different cell types (e.g., CD4 on helper T cells). Also called CD markers. *(Chapter 4)*

Cold chain A supply chain in which the low temperatures required to preserve vaccines (and other biological agents) are maintained continuously. *(Chapter 8)*

Complement A general term referring to all the components of the complement system. *(Chapter 3)*

Complement system A set of blood plasma proteins that act in a concerted fashion to destroy extracellular pathogens and infected cells; originally defined as a heat-labile activity that lysed bacteria in the presence of antibody (it "complemented" antibody action); the activated complement pathway also stimulates phagocytosis, chemotaxis, and inflammation. *(Chapter 4)*

c-Oncogene A cancer-causing gene encoded in cellular genomes: may be formed via mutagenesis of a gene that does not cause cancer, known therefore as a proto-oncogene. *(Chapter 7)*

Contact inhibition Cessation of cell division when cells make physical contact, as occurs at high density in a culture dish. *(Chapter 6)*

Control group A group of experimental subjects, such as patients in a clinical trail, who receive no treatment or a placebo. *(Chapter 9)*

Cutaneous immune system The lymphocytes and scavenger antigen-presenting cells (Langerhans cells) that comprise the skin-associated lymphoid tissue. *(Chapter 4)*

Cytokines Soluble proteins produced by cells in response to various stimuli, including virus infection; they affect the behavior of other cells both locally and at a distance, by binding to specific cytokine receptors. *(Chapters 3 and 4)*

Cytokine storm See systemic inflammatory response syndrome *(Chapters 1 and 5)*

Cytopathic effect Deleterious morphological changes induced in cells by viral infection. *(Chapter 3)*

Cytopathic virus A virus that causes characteristic visible cell damage and death upon infection of cells in culture. *(Chapter 5)*

Damage-associated molecular patterns Host components that are released upon cellular damage. *(Chapter 3)*

Defensins Small (29- to 51- residue), cysteine-rich, cationic proteins produced by lymphocytes and epithelial cells that are active against bacteria, fungi, and enveloped viruses; usually found in the gut. *(Chapter 2)*

Delayed-type hypersensitivity A reaction caused by $CD4^+$ T cells that recognizes antigens in the skin; the reaction typically occurs hours to days after antigen is injected, hence its name; it is partially responsible for characteristic local responses to virus infections, such as rashes. *(Chapter 4)*

Diapedesis The process by which viruses cross the vascular endothelium, while being carried within monocytes or lymphocytes. *(Chapter 2)*

Direct-acting antivirals Drugs that inhibit viral enzymes. *(Chapter 9)*

Disseminated infection An infection that spreads beyond the primary site; often includes viremia and infection of major organs such as the liver, lungs, and kidneys. *(Chapter 2)*

DNA synthesis phase *See* S phase.

DNA vaccine A preparation of DNA containing the genes for one or more antigenic proteins; when the pure DNA preparation is injected into a test subject and enters cells, the proteins are synthesized, and an immune response to those proteins is elicited. *(Chapter 8)*

Double blind A trial in which neither the investigators nor the patients know which patients belong to treated and control groups. *(Chapter 1)*

Effector memory T cells Memory T cells that produce cytokines rapidly upon re-encountering a viral antigen, and are generally present in the circulatory system. *(Chapter 4)*

Emerging virus A viral population responsible for a marked increase in disease incidence, usually as result of changed societal, environmental, or population factors. *(Chapter 11)*

Endemic A disease or condition typical of a particular population or geographic area; persisting in a population for a long period without reintroduction of the causative agent from outside sources. *(Chapter 1)*

Endogenous antigen presentation The cellular process by which viral proteins are degraded inside the infected cell, and the resulting peptides are loaded onto major histocompatibility complex class I molecules that move to the cell surface. *(Chapter 4)*

Enhancing antibodies Antibodies that can facilitate viral infection by allowing virus particles to which they bind to enter susceptible cells. *(Chapter 7)*

Epidemic A pattern of disease characterized by rapid and sudden appearance of cases spreading over a wide area. *(Chapter 1)*

Epidemiology The study of the incidence, distribution, and spread of infectious disease in populations with particular regard to identification and subsequent control. *(Chapter 1)*

Epitope The parts of an antigen that are bound by an antibody or that are recognized by a T cell receptor in the context of major histocompatibility proteins. *(Chapter 4)*

Error threshold A mathematical parameter that measures the complexity of the information that must be maintained to ensure survival of a population. *(Chapter 10)*

Etiology The cause or causes of a disease. *(Chapter 1)*

Exogenous antigen presentation The cellular process in which viral proteins are engulfed from the outside of the cell, degraded, and the resulting peptides loaded onto major histocompatibility complex class II molecules that then move to the cell surface for presentation to T cells. *(Chapter 4)*

Extrinsic pathway Pathway by which apoptosis is induced when a proapoptotic ligand binds to its cell surface receptor. *(Chapter 3)*

Fitness The degree to which an organism is able to reproduce its environment. *(Chapter 10)*

Foci Clusters of cells that are derived from a single progenitor and share properties, such as unregulated growth, that cause them to pile up on one another. A single such cluster is called a **focus** *(Chapter 6)*

Fomites Inanimate objects that may be contaminated with microorganisms and become vehicles for transmission. *(Chapter 1)*

Gap phases (G_1 and G_2) Phases in the cell cycle between the mitosis (M) and DNA synthesis (S) phases. *(Chapter 6)*

Genetic bottleneck A descriptive term evoking the extreme selective pressure on small populations that results in loss of diversity, accumulation of selected mutations, or both. *(Chapter 10)*

Genetic drift Diversity in viral genomes that arises as a result of errors during genome replication and immune selection. *(Chapter 10)*

Genetic shift Diversity in viral genomes that arises as a result of re-assortment of genome segments or recombination between genomes. *(Chapter 10)*

Granzymes Members of a family of serine proteases that are released from activated cytotoxic T cells and induce apoptosis of target cells. *(Chapter 4)*

G_0 *See* Resting state.

Helper virus A virus that provides viral proteins needed for the reproduction of a coinfecting defective virus or subviral agents. *(Chapter 12)*.

Hematogenous spread Spread of virus particles through the bloodstream. *(Chapter 2)*

Hepatitis Inflammation of the liver. *(Chapters 2 and 5)*

Herd immunity The immune status of a population, rather than an individual. *(Chapter 8)*

Heterologous T cell immunity A secondary T cell response to antigen that is related but not identical to the immunodominant antigens that elicited the primary T cell response. *(Chapter 5)*

Humoral response The arm of the adaptive immune response that produces antibodies. *(Chapter 4)*

Immortality The capacity of cells to grow and divide indefinitely. *(Chapter 6)*

Immune memory A property provided by specialized B and T lymphocytes (memory B and T cells) that respond rapidly upon reexposure to an antigen. *(Chapter 8)*

Immunodominant Having the property of being recognized most efficiently by cytotoxic T lymphocytes and antibodies; said of peptides and epitopes. *(Chapter 5)*

Immunological synapse A specialized, organized structure formed upon aggregation of the T cell receptors of a cytotoxic T cell bound to peptide presented by MHC on the target cells; this structure allow prolonged signaling from the engaged T cell receptors and associated co-receptors, and facilitates polarization of the T cell secretion machinery. *(Chapter 4)*

Immunopathology Pathological changes caused partly or entirely by the immune response. *(Chapters 1 and 5)*

Immunotherapy A treatment that provides an infected host with exogenous antiviral cytokines, other immunoregulatory agents, antibodies, or lymphocytes in order to reduce viral pathogenesis. *(Chapter 8)*

Inactivated vaccine A vaccine made by taking a disease-causing virus and treating it (e.g., with chemicals) to reduce infectivity to undetectable levels. *(Chapter 8)*

Incidence The frequency with which a disease appears in a particular population or area (e.g., the number of newly diagnosed cases during a specific period); distinct from the prevalence (i.e., the number of cases in a population on a certain date). *(Chapter 1)*

Incubation period The period before symptoms of disease appear after an infection. *(Chapter 5)*

Index case The human or other animal originally infected in an epidemic. *(Chapter 1)*

Infectious mononucleosis An infectious disease caused by Epstein-Barr virus; characterized by an increase in the number of lymphocytes with a single nucleus. *(Chapter 5)*

Inflammation A general term for the elaborate response that leads to local accumulation of white blood cells and fluid; initiated by local infection or tissue damage; many different forms of this response, characterized by the degrees of tissue damage, capillary leakage, and immune cell infiltration, occur after infection with pathogens. *(Chapter 3)*

Innate response The first line of immune defense; able to function continually in the host without prior exposure to the invading pathogen. This elaborate system includes cytokines, sentinel cells, complement, and natural killer cells. *(Chapters 2 and 3)*

Insertional activation The mechanism of oncogenesis by nontransducing retroviruses; integration of a proviral promoter or enhancer in the vicinity of a proto-oncogene results in inappropriate transcription of that gene, making it a cellular oncogene (c-oncogene) *(Chapter 6)*

Interfering antibodies Antibodies that can bind to virus particles or infected cells and block interaction with neutralizing antibodies. *(Chapter 7)*

Interferons Cytokines that activate antiviral programs. *(Chapter 3)*

Interleukins Secreted cytokines that allow communication among leukocytes. *(Chapter 4)*

Intrinsic cellular defenses The conserved cellular programs that respond to various stresses, such as starvation, irradiation, and infection; intrinsic defenses include apoptosis, autophagy, and RNA interference. *(Chapters 2 and 3)*

Intrinsic pathway Pathway of apoptosis in which cell death is induced in response to indicators of *internal* stress, such as DNA damage. *(Chapter 3)*

Koch's postulates Criteria developed by the German physician Robert Koch in the late 1800s to determine whether a given agent is the cause of a specific disease. *(Chapter 1)*

Koplik's spots Small spots inside the mouth that are hallmarks of measles virus infection. *(Chapter 1)*

Kupffer cells Macrophages of the liver that are part of the reticuloendothelial system. *(Chapter 2)*

Latency-associated transcript RNA produced specifically during a latent infection by herpes simplex virus. *(Chapter 5)*

Latent infection A class of persistent infection that lasts the life of the host; few or no virus particles can be detected, despite continuous presence of the viral genome. *(Chapter 5)*

Lectin pathway One of three complement pathways that lead to activation of C3-C5 convertases; mannose-binding, lectin-associated proteases cleave the C2 and C4 proteins. *(Chapter 3)*

Lethal mutagenesis The elevation of mutation rates by exposure to a mutagen or an error-prone polymerase to the point at which the resulting population of genomes has lost fitness and is incapable of propagating. *(Chapter 10)*

Long-latency retrovirus A retrovirus that causes cancer in a host many years after infection; the viral genome does not encode cellular oncogenes, nor does it cause cancer by perturbing the expression of cellular oncogenes. *(Chapter 6)*

Maximum tolerated dose The highest dose of a drug or other treatment that does not cause unacceptable side effects. *(Chapter 9)*

M cell Microfold or membranous epithelial cell; cells of mucosal surfaces specialized for delivery of antigens to underlying lymphoid tissues. *(Chapters 2 and 4)*

Memory cells A subset of B and T lymphocytes maintained after each encounter with a foreign antigen; these cells survive for years and are ready to respond and proliferate upon subsequent encounter with the same antigen. *(Chapter 4)*

Metagenomic analysis Nucleic acid sequencing of samples recovered directly from the environment, and comprising multiple genomes. *(Chapter 10)*

Metastases Secondary tumors, often at distant sites, that arise from the cells of a malignant tumor. *(Chapter 6)*

MHC restriction The recognition of an antigen by T cells only when it is presented by MHC of the haplotype identical to that of the T cells. *(Chapter 4)*

Microbicides Creams or ointments that inactivate or block virus particles before they can attach and penetrate tissues. *(Chapter 9)*

Mitogens Extracellular signaling molecules that induce cell proliferation. *(Chapter 6)*

Mitosis The phase of the cell cycle in which newly duplicated chromosomes are distributed to two new daughter cells as a result of cell division. Also called M phase. *(Chapter 6)*

Molecular mimicry Sequence similarities between viral peptides and self-peptides that result in the cross-activation of autoreactive T or B cells by virus-derived peptides. *(Chapters 5 and 8)*

Monoclonal antibody-resistant mutants Viral mutants selected to propagate in the presence of neutralizing monoclonal antibodies; often carry mutations in viral genes encoding structural proteins. *(Chapter 4)*

Morbidity The percentage of individuals in a specified population who show symptoms of infection in a given period. *(Chapter 1)*

Mortality The percentage of deaths in a specified population of infected individuals. *(Chapter 1)*

M phase *See* Mitosis.

Muller's ratchet A model positing how small, asexual populations decline in fitness over time if the mutation rate is high. *(Chapter 10)*

Natural killer cells An abundant lymphocyte population that comprises large, granular cells; distinguished from other lymphocytes by the absence of B and T cell antigen receptors; these cells are part of the innate defense system. Also called NK cells. *(Chapter 3)*

Negative selection (re: T cells) Elimination of T cells that recognize target cells that display "self" peptides on their surfaces. *(Chapter 4)*

Neuroinvasive virus A virus that can enter the central nervous system (spinal cord and brain) after infection of a peripheral site. *(Chapter 2)*

Neurotropic virus A virus that can infect neurons. *(Chapter 2)*

Neurovirulent virus A virus that can cause disease in nervous tissue, manifested by neurological symptoms and often death. *(Chapter 2)*

Neutralizing antibodies Antibodies that block the infectivity of virus particles. *(Chapter 7)*

NK cells *See* Natural killer cells.

Noncytopathic virus A virus that produces no visible signs of infection in cells. *(Chapter 5)*

Nontransducing oncogenic retroviruses Retroviruses that do not encode cell-derived oncogene sequences but can cause cancer (at low efficiency) when their DNA becomes integrated in the vicinity of a cellular oncogene, thereby perturbing its expression. *(Chapter 6)*

Oncogene A gene encoding a protein that causes cellular transformation or tumorigenesis. *(Chapter 6)*

Oncogenesis The processes leading to cancer. *(Chapter 6)*

Original antigenic sin A secondary immune response to an antigen that is related, but not identical to, the antigen that elicited the primary response. *(Chapter 5)*

Pandemic A worldwide epidemic. *(Chapter 1)*

Pantropic virus A virus that replicates in many tissues and cell types. *(Chapter 2)*

Passive immunization Direct administration of the products of the immune response (e.g., antibodies or stimulated immune cells) obtained from an appropriate donor(s) to a patient; contrasts with active immunization. *(Chapter 8)*

Pathogen A disease-causing virus or other microorganism. *(Chapters 1 and 4)*

Pathogen-associated molecular patterns Molecules or molecular features unique to pathogens. *(Chapter 3)*

Pattern recognition receptors Protein receptors of the innate immune system that bind definitive molecular features of pathogens; present in sentinel cells, such as immature dendritic cells and macrophages. *(Chapter 3)*

Permissive Able to support virus reproduction when the viral genome is introduced; refers to cells. *(Chapter 2)*

Permissivity A cellular environment that provides all cellular components required for viral reproduction. *(Chapter 2)*

Persistent infection A viral infection that is not cleared by the combined actions of the innate and adaptive immune response. *(Chapter 5)*

Phagocytosis Engulfment of dying cellular debris and virus particles by myeloid cells, including dendritic cells. *(Chapter 4)*

Plasma cells Mature B cells that synthesize secreted antibodies. *(Chapter 4)*

Polymorphic gene A gene that has many allelic forms in outbred populations. *(Chapter 4)*

Positive selection (re: T cells) The process in which only T cells with T cell receptors that can bind to MHC proteins are retained during T cell differentiation. *(Chapter 4)*

Power The probability that a meaningful difference or effect can be detected, if one were to occur. *(Chapter 1)*

Prevalence The proportion of individuals in a population having a disease; the number of cases of a disease present in a particular population at a given time. *(Chapter 1)*

Primary antibody response The response of B cells following first exposed to a pathogen. *(Chapter 4)*

Primary viremia Progeny virus particles released into the blood after initial virus propagation at the site of entry. *(Chapter 2)*

Prodrug An inactive precursor to an active antiviral compound. *(Chapter 9)*

Prions Infectious agents comprising an abnormal isoform of a normal cellular protein but no nucleic acid; implicated as the causative agents of transmissible spongiform encephalopathies. *(Chapter 12)*

Professional antigen-presenting cells Dendritic cells, macrophages, and B cells; defined by their ability to take up antigens and present them to naïve T lymphocytes in the groove of an major histocompatibility complex class II molecule. *(Chapter 4)*

Prospective studies Studies in which cohorts of subjects with and without the condition(s) or treatment(s) of interest are examined for a specified period. *(Chapter 1)*

Proteome The total protein repertoire of a sample, such as a preparation of virus particles or a type of host cell. *(Chapter 9)*

Proto-oncogene A normal cellular gene that, when altered by mutation or misregulation, can contribute to cancer; thereafter called a cellular oncogene (c-oncogene). *(Chapter 6)*

Quasispecies Virus populations that exist as dynamic distributions of nonidentical but related replicons. *(Chapter 10)*

Recombinant vaccine A vaccine produced by recombinant DNA technology. *(Chapter 8)*

Replication-competent, attenuated vaccine A vaccine made from viral mutants that have reduced virulence but can reproduce; they often also have reduced capacity for transmission. *(Chapter 8)*

Reservoir The host population in which a viral population is maintained. *(Chapters 1 and 11)*

Resting state A state in which the cell has ceased to grow and divide and has withdrawn from the cell cycle. Also called G_0. *(Chapter 6)*

Reticuloendothelial system Macrophages that line sinusoids present in organs such as liver, spleen, bone marrow, and adrenal glands. *(Chapter 2)*

Retrospective study A study that looks backwards in time and examines exposures to suspected risk or protection factors in relation to a particular outcome. *(Chapter 1)*

Satellites Small, single-stranded RNA molecules that lack genes required for their replication, but are replicated in the presence of another virus that can supply the required proteins (the **helper virus**). *(Chapter 12)*

Satellite RNA An RNA that does not encode capsid proteins, and is packaged by a protein(s) encoded in a helper virus genome. *(Chapter 12)*

Satellite virus A satellite with a genome that encodes one or two proteins. *(Chapter 12)*

Secondary antibody response The antibody response produced after a subsequent infection or challenge with the same antigen or virus. *(Chapter 4)*

Secondary viremia Delayed appearance of a high concentration of infectious virus in the blood as a consequence of disseminated infections. *(Chapter 2)*

Sentinel cells Dendritic cells and macrophages; migratory cells that are found in the periphery of the body and can take up proteins and cell debris for presentation of peptides derived from them on major histocompatibility complex molecules. These cells respond to recognition of a pathogen by synthesizing cytokines such as interferons. *(Chapters 2, 3, and 4)*

Sepsis Uncontrolled, systemic inflammation induced by infection with a pathogen. *(Chapter 5)*

Shedding The release of virus particles from an infected host. *(Chapter 2)*

Signal transduction cascade A chain of sequential physical interactions among, and biochemical modifications of, membrane-bound and cytoplasmic proteins. *(Chapter 6)*

Sinusoids Small blood vessels characterized by a discontinuous basal lamina, with no significant barrier between the blood plasma and the membranes of surrounding cells. *(Chapter 2)*

Slow infection An extreme variant of the persistent pattern of infection; has a long incubation period (years) from the time of initial infection until the appearance of recognizable symptoms. *(Chapter 5)*

Slow viruses Viruses characterized by long incubation periods, typical for the genus lentivirus in the family *Retroviridae. (Chapter 7)*

S phase The phase of the cell cycle in which the DNA genome is replicated. *(Chapter 6)*

Structural plasticity The ability of virus particles to tolerate large numbers of amino acid substitutions in surface proteins without losing infectivity. *(Chapter 5)*

Subunit vaccine A vaccine formulated with purified components of virus particles, rather than intact virus particles. *(Chapter 8)*

Superantigen Extremely powerful membrane bound T cell proteins that nonspecifically activate many subsets of T cells. *(Chapter 5)*

Susceptibility The property of a cell that enables it to be infected by a particular virus (e.g., the presence of a viral receptor[s] on the cell surface). *(Chapter 2)*

Susceptible Producing the receptor(s) required for virus entry; refers to cells. *(Chapter 2)*

Systemic infection An infection that results in spread to many organs of the body. *(Chapter 2)*

Systemic inflammatory response syndrome A disproportionate host response that leads to large-scale release of inflammatory cytokines and stress mediators, resulting in severe pathogenesis or death. Also known as a cytokine storm. *(Chapter 5)*

Systems biology An approach in which experimental methods to identify all the components of a biological system and their interactions are linked to properties and functions of the system using computational models. *(Chapter 1)*

Transcytosis A mechanism of transport in which material in the intestinal lumen is endocytosed by M cells, transported to the basolateral surface, and released to the underlying tissues. *(Chapters 2 and 4)*

Transducing oncogenic retroviruses Retroviruses that include oncogenic, cell-derived sequences in their genomes and carry these sequences to each newly infected cell; such viruses are highly oncogenic. *(Chapter 6)*

Transformed Having changed growth properties and morphology as a consequence of infection with certain oncogenic viruses, introduction of oncogenes, or exposure to chemical carcinogens. *(Chapter 6)*

Transforming infection A class of persistent infection in which cells infected by certain DNA viruses or retroviruses may exhibit altered growth properties and proliferate faster than uninfected cells. *(Chapter 5)*

Tropism The predilection of a virus to invade, and reproduce, in a particular cell type. *(Chapter 2)*

Tumor A mass of cells originating from abnormal growth. *(Chapter 6)*

Tumor suppressor gene A cellular gene encoding a protein that negatively regulates cell proliferation; mutational inactivation of both copies of the genes is associated with tumor development. *(Chapter 6)*

Vaccination Inoculation of individuals with attenuated or related microorganisms, or their antigenic products, in order to elicit an immune response that will protect against later infection by the corresponding pathogen. *(Chapter 8)*

Variolation Inoculation of healthy individuals with material from a smallpox pustule, or in modern times from a related or attenuated cowpox (vaccinia) virus preparation, through a scratch on the skin (called scarification). *(Chapter 8)*

Vector A carrier, often an arthropod, that transmits a virus or other infectious agent from one host to another. *(Chapter 1)*

Viral pathogenesis The processes by which viral infections cause disease. *(Chapters 1, 2, and 5)*

Viremia The presence of infectious virus particles in the blood. *(Chapter 2)*

Viroceptor A viral protein that modulates cytokine signaling or cytokine production by mimicking host cytokine receptors. *(Chapters 3 and 5)*

Viroids Unencapsidated, small, circular, single-stranded RNA molecules that replicate autonomously when introduced mechanically into host plants. *(Chapter 12)*

Virokine A secreted viral protein that mimics cytokines, growth factors, or similar extracellular immune regulators. *(Chapters 3 and 5)*

Viroporin Hydrophobic viral protein that forms pores in cellular membranes; many facilitate release of progeny virus particles. *(Chapter 5)*

Virtual screening Computational methods for iterative docking of chemical compounds into a chosen site in a protein target to identify drug leads. *(Chapter 9)*

Virulence The relative capacity of a viral infection to cause disease. *(Chapter 5)*

Viruria The presence of viruses in the urine. *(Chapter 2)*

Virus evolution The constant change of a viral population in the face of selection pressures. *(Chapter 10)*

v-Oncogene An oncogene that is encoded in a viral genome. *(Chapter 6)*

Xenophagy The capture of virus particles for degradation in lysosomes. *(Chapter 3)*

Zoonoses (zoonotic infections) Diseases that are transferred from other animals to humans. *(Chapters 1 and 11)*

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