STUDIES IN VIRAL ECOLOGY

STUDIES IN VIRAL ECOLOGY
Microbial and Botanical Host Systems

Volume 1

Edited By

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DEDICATION

I dedicate these two volumes to the memory of my brother in spirit, Henry Hanssen. To me, he seemed a hero and I remember him most for his unfailing ability to present a sense of humanity in times of tragedy. We first met while studying together for our doctorates in Houston, Texas.

Henry was born in Colombia near Medellín and tragically orphaned as a young child after which he was lovingly raised by an aunt in Bogotá. Henry may have gained his tremendous sense of humanity from that experience. He had no biological children of his own but helped to raise two daughters. The first of those came into his life by a twist of luck while one day Henry was walking along a street in Colombia and heard what he thought might be a cat trapped inside of a garbage bin. Henry went over to free the cat and discovered instead a crying infant child in a plastic bag, presumably discarded there by a distraught mother. Henry took the baby to the police, and when no one stepped forward as a parent Henry adopted the child and eventually even helped to pay for her college tuition. The second daughter came through Henry's marriage to the love of his life.

When there arose need for representing humanity, Henry was undaunted by circumstance. His accomplishments included establishing an infant vaccination program against poliomyelitis in Angola at the personal request of Jonas Salk. Angola was in a state of civil war at that time and no one else was willing to undertake the necessary but frightening task. Henry showed equal humanitarianism to civilians and military on both sides of that conflict. Subsequently, Henry initiated

a similar poliomyelitis vaccination program during a period of civil war in Central America and for his efforts was awarded honorary citizenship by one of the countries there. He then initiated a poliomyelitis vaccination program in his native Colombia, while that country's continuing civil war was in full strength.

I was proud to address Henry by the name of "brother" and always will think of him in that way. He addressed me by that same term of affection and he is lovingly remembered by everyone whom his life touched.

HENRY HANSSEN VILLAMIZAR (1945–2007)

PREFACE

Virology is a field of study which has grown and expanded greatly since the viruses as a group first received their name in 1898. Many of the people who presently are learning virology have come to perceive these acellular biological entities as being merely trinkets of nucleic acid to be cloned, probed, and spliced. However, the viruses are much more than merely trinkets to be played with in molecular biology laboratories. The viruses are indeed highly evolved biological entities with an organismal biology that is complex and interwoven with the biology of their hosting species. Ecology is defined as the branch of science which addresses the relationships between an organism of interest and the other organisms with which it interacts, the interactions between the organism of interest and its environment, and the geographical distribution of the organism of interest.

The purpose of this book is to help define and explain the ecology of viruses, i.e., to examine what life might seem like from a "virocentric" point of view, as opposed to our normal "anthropocentric" perspective. As we begin our examination of the virocentric life, it is important to realize that in nature both the viruses of macroorganisms and the viruses of microorganisms exist in cycles with their respective hosts. Under normal conditions, the impact of viruses upon their natural host populations may be barely apparent due to factors such as evolutionary coadaptation between the virus and those natural hosts. However, when viruses find access to new types of hosts and alternate transmission cycles, or when they encounter a concentrated population of susceptible genetically similar hosts such as occurs in densely populated human communities, communities of cultivated plants or animals, or algal blooms, then the impact of the virus upon its host population can appear catastrophic. The key to understanding these types of cycles lies in understanding the viruses and how their ecology relates to the ecology of their hosts, their alternate hosts, and any vectors which

they utilize, as well as their relationship to the availability of suitable vehicles that can transport the different viral groups.

I hope that you will enjoy the information presented in this book set as much as I and the other authors have enjoyed presenting it to you. The written word is a marvelous thing, able to convey understanding and enthusiasm across unimaginable distances and through time.

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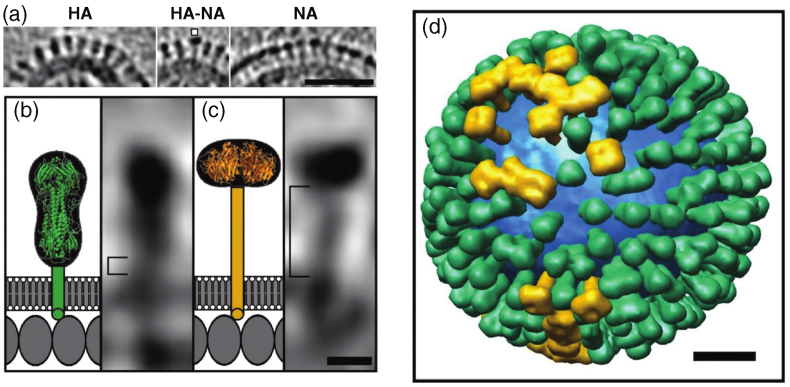


FIGURE 3.3 Model virus with HA and NA spikes by cryo-ET analysis. (*See text for full caption.*)

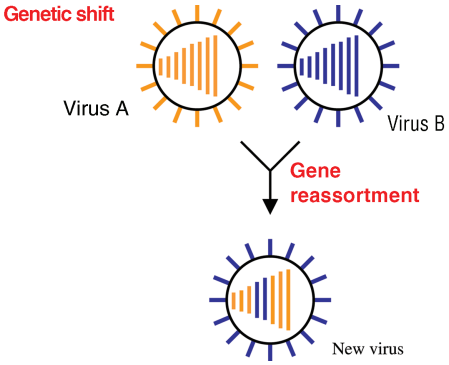


FIGURE 3.10 Reassortment of influenza virus RNA segments. (*See text for full caption.*)

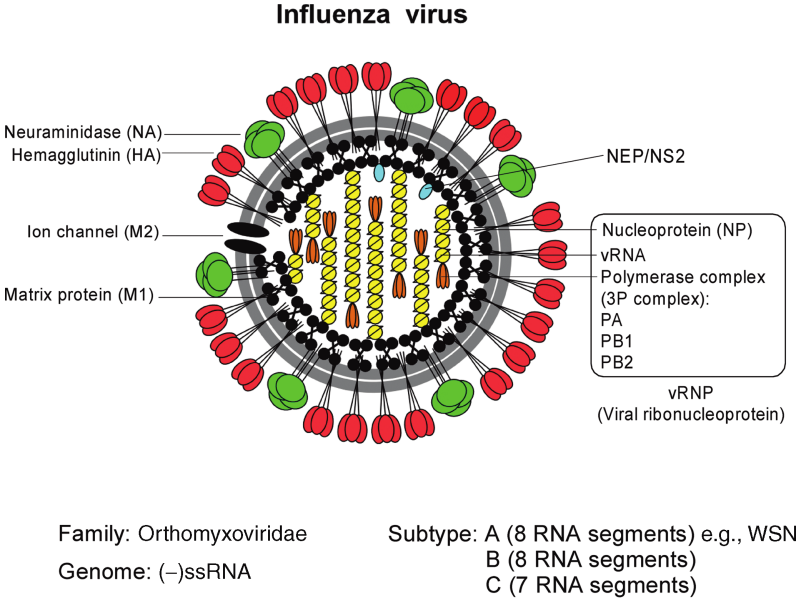
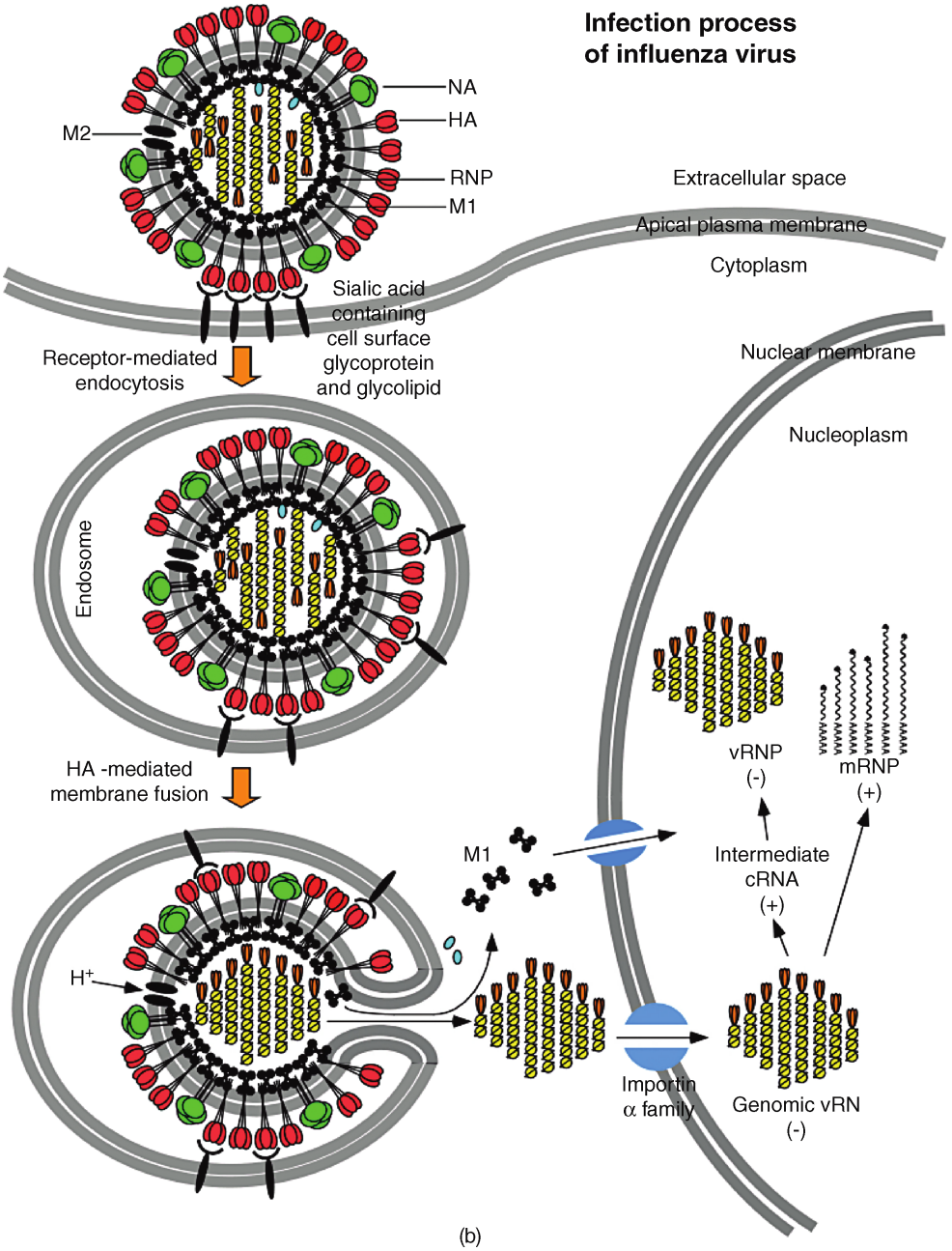
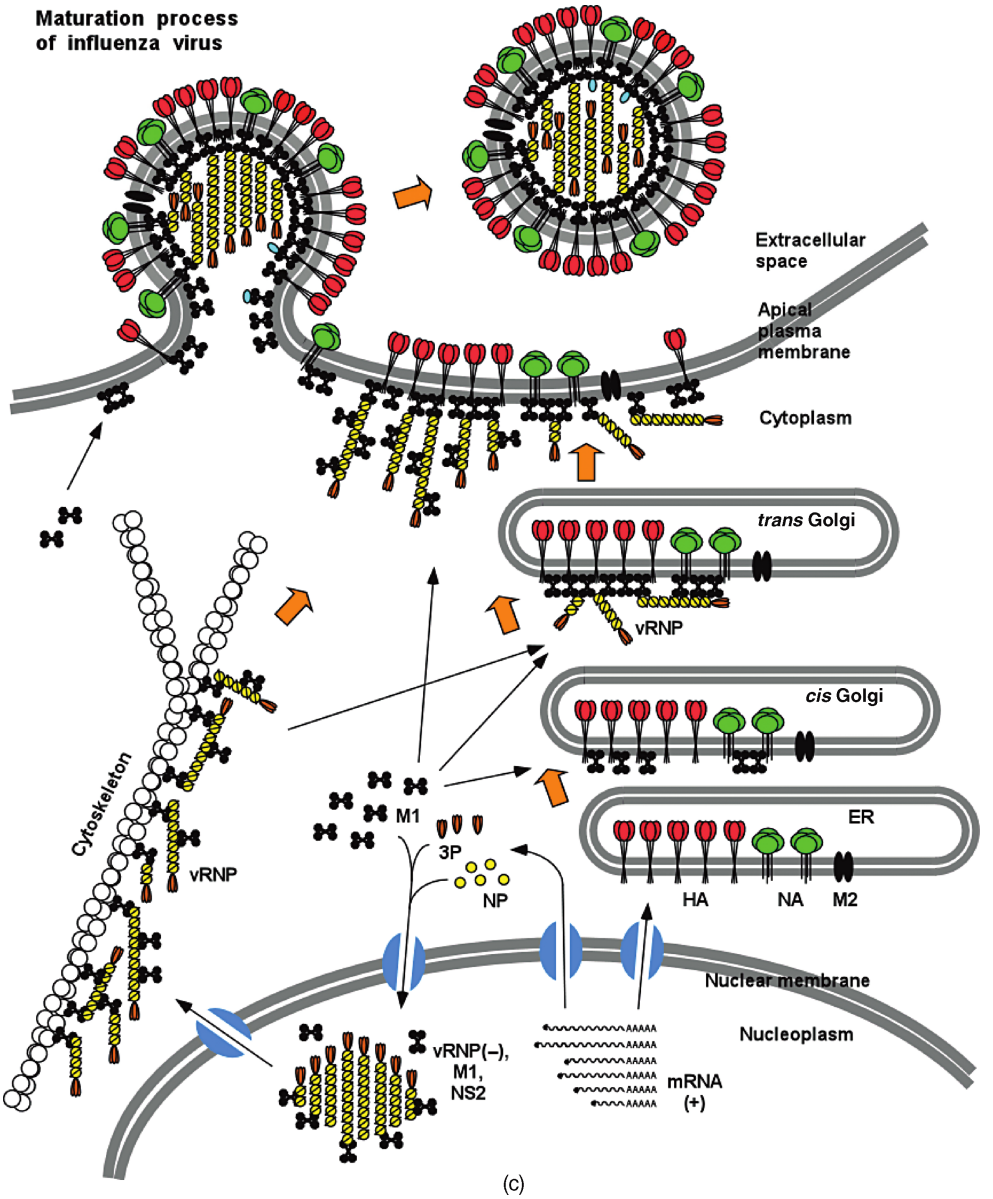


FIGURE 3.11 Schematic presentation of the infectious cycle of an influenza virus. (a) Schematic presentation of influenza virus structure.



(b)

FIGURE 3.11 (*Continued*) (b) Schematic presentation of influenza virus infection showing attachment, entry, and uncoating of a virus particle. The steps in the replication cycle are attachment mediated through HA and sialic acid receptor, entry into the cell via endosome, HA-mediated fusion of virus membrane with endosomal membrane at low pH, release of vRNP, transport of vRNP into the nucleus, and transcription (mRNA synthesis) and replication (cRNA and vRNA synthesis) of vRNP in the nucleus.



(c)

FIGURE 3.11 (*Continued*) (c) Schematic presentation of influenza virus infectious cycle showing export, assembly, and budding of a virus particle. The steps include export of vRNP from nucleus into cytoplasm, export of virus proteins, vRNP to the budding site, bud formation, and bud release by fusion and fission viral and cellular membranes.

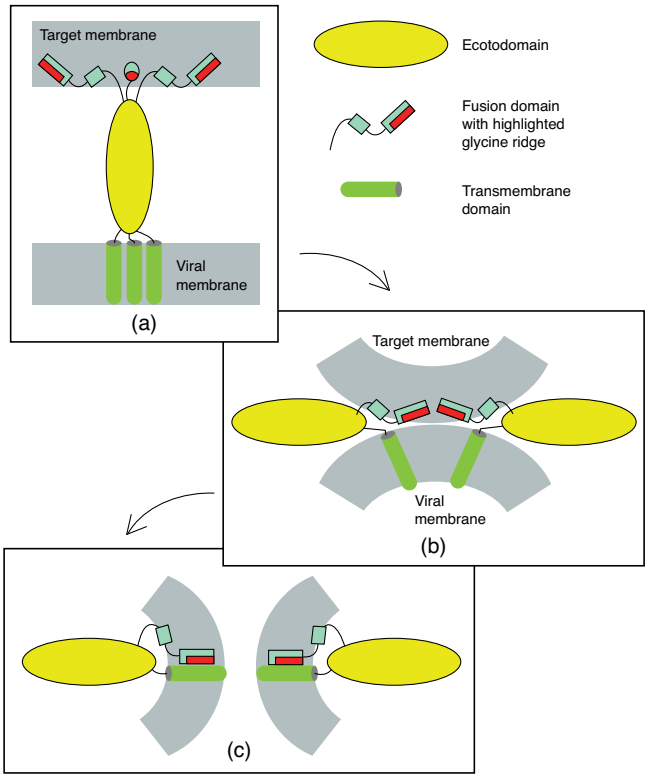


FIGURE 3.14 Boomerang model of influenza virus HA-mediated membrane fusion. (*See text for full caption.*)

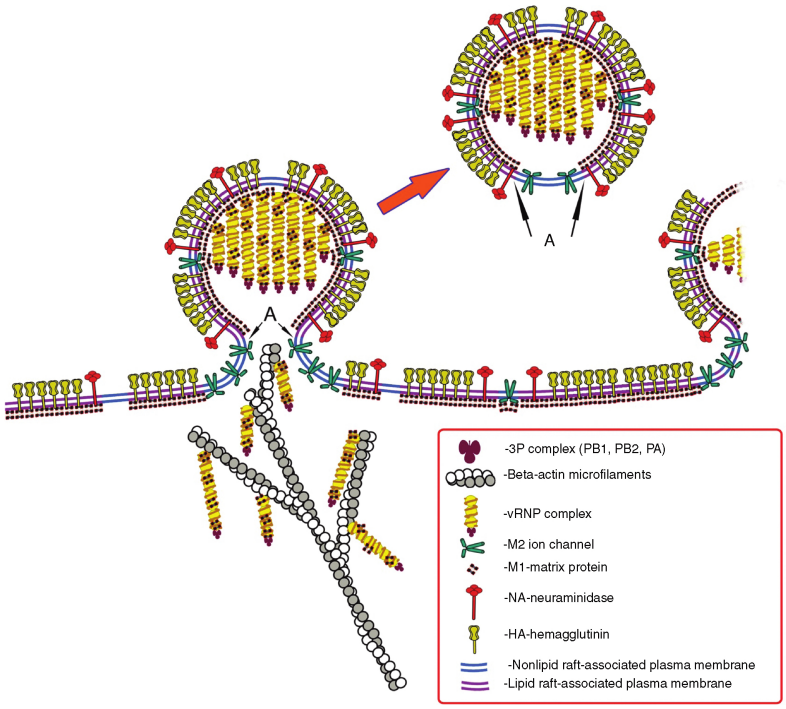


FIGURE 3.22 Schematic illustration of the pinching-off process of influenza virus bud. (*See text for full caption.*)

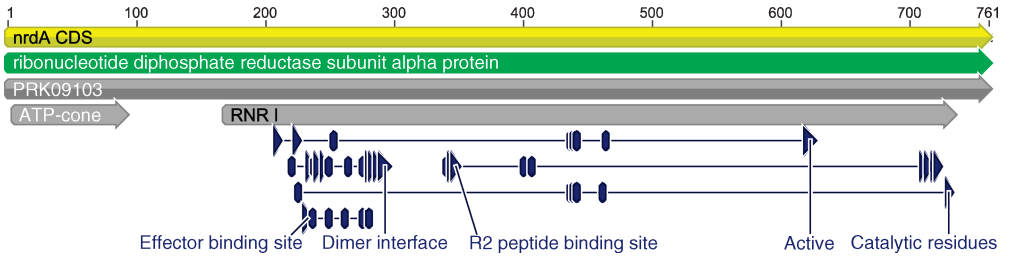


FIGURE 5.2 A gene map of NrdA from *E. coli* (Acc. No. YP_002927204). The putative catalytic residue is Cys-439 and was conserved among all reference sequences and contigs. The extracted region used to create the NrdA phylogenetic tree ranged from Arg-389 to Ile-644 in this sequence.

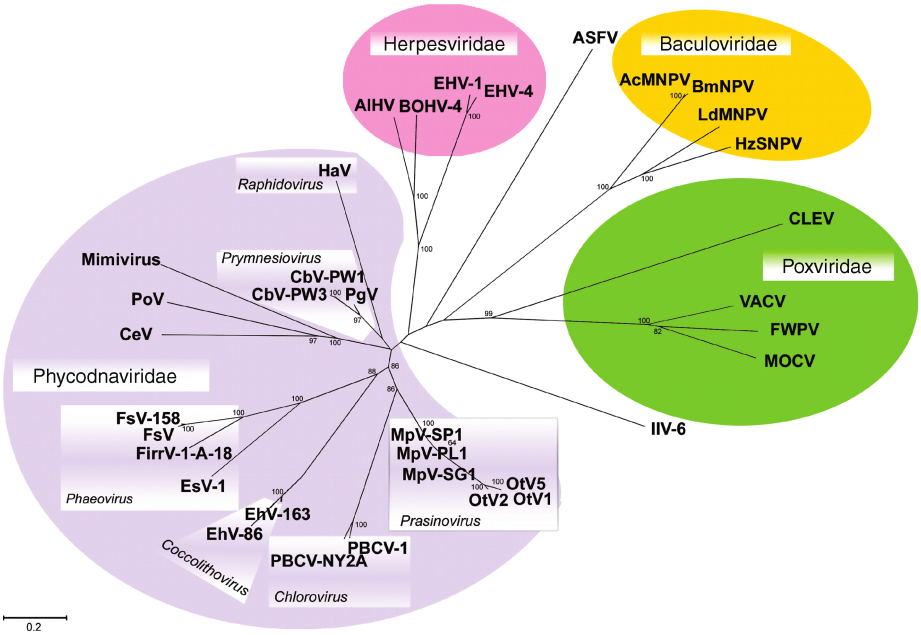


FIGURE 7.2 Phycodnaviridae taxonomy. Phylogenetic analysis of members of algal viruses based on a distance matrix algorithm between the DNA *pol* gene fragments of the family Phycodnaviridae and the other large dsDNA viruses (Neighbor in PHYLIP, version 3.61). (*See text for full caption.*)

FIGURE 7.5 True color satellite image of a milky *E. huxleyi* bloom in the English Channel, south of Plymouth, UK, on July 30, 1999 (*Source*: Remote Sensing Group, Plymouth Marine Laboratory http://rsg.pml.ac.uk/). This bloom was effectively "dead" and up to 1 million *E. huxleyi*-specific coccolithoviruses per teaspoon of water were found in the middle of the high reflectance water.

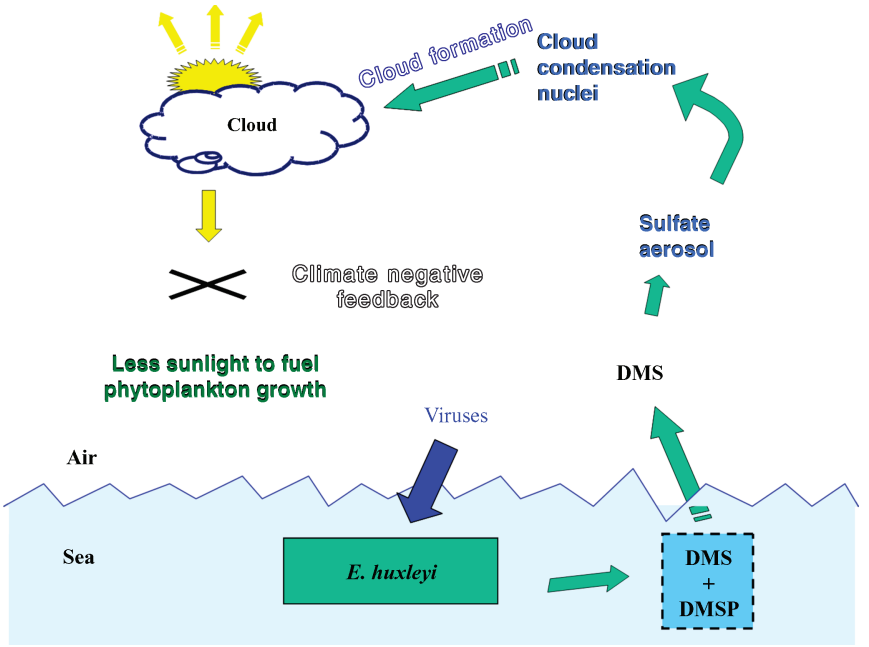


FIGURE 7.6 The Gaia hypothesis states that the Earth is a self-regulating organism. This may seem plausible when the activity of coccolithoviruses is taken into consideration. (*See text for full caption.*)

FIGURE 9.1 Vegetative (or heterokaryon) incompatibility in filamentous fungi results in programmed cell death in the chestnut blight fungus, *Cryphonectria parasitica*, when cells (hyphae) of incompatible individuals fuse, and restricts the horizontal transmission of CHV-1 (family Hypoviridae and genus *Hypovirus*). (a) Macroscopic view of vegetative (mycelial) incompatibility when pairs of isolates are grown on solid medium.

FIGURE 9.5 Superficial canker on a European chestnut tree (*Castanea sativa*) caused by CHV-1-infected individuals of *Cryphonectria parasitica*. Photo by Paolo Cortesi, University of Milan.

FIGURE 9.6 Healthy stand of European chestnut trees (*Castanea sativa*) in Portofino Park, near Genoa, Italy in 2003. Most trees had one or more superficial cankers caused by CHV-1-infected individuals of *Cryphonectria parasitica*. Photo by Paolo Cortesi, University of Milan.

FIGURE 9.7 A stand of American chestnut tree (*Castanea dentata*) in northern Michigan in 2008 where naturally occurring hypovirulence allowed its survival from chestnut blight. Photo by Alice C. L. Churchill, Cornell University.

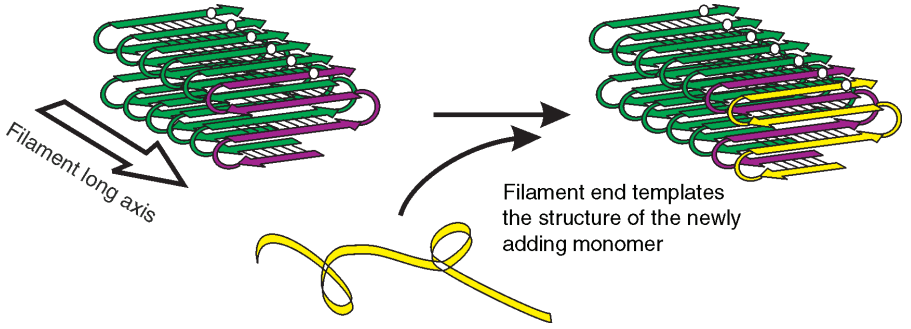


FIGURE 10.1 Prion domain structures explain propagation of structures (prion variants). (*See text for full caption.*)

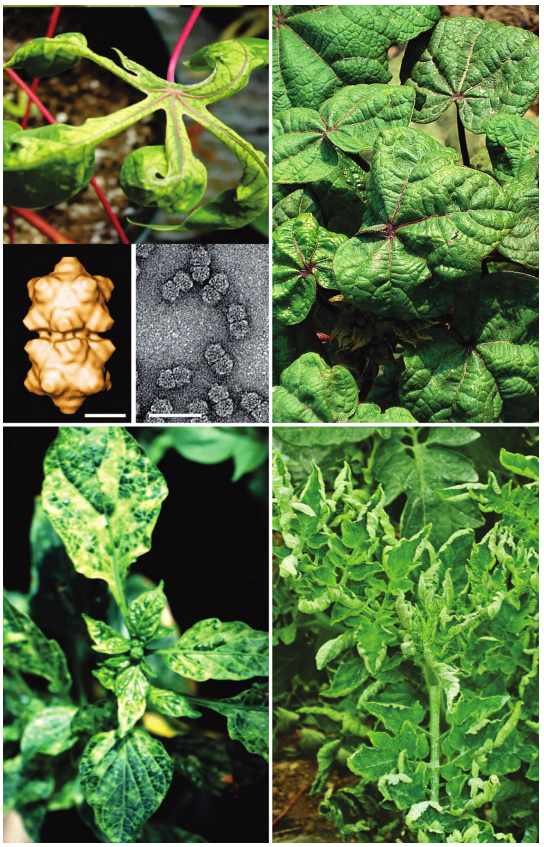


FIGURE 11.1 Symptoms caused by several geminiviruses: cassava mosaic disease (top left), cotton leaf curl disease in Pakistan (top right), pepper golden mosaic disease in Mexico (bottom left), and tomato yellow leaf curl disease from Jordan (bottom right). The geminivirus particles are shown in the top left panel with a computer rendering of a structure of maize streak virus, obtained via cryomicroscopy (left: the bar represents 5 nm) and a transmission electron microscope picture (right: the bar represents 40 nm).

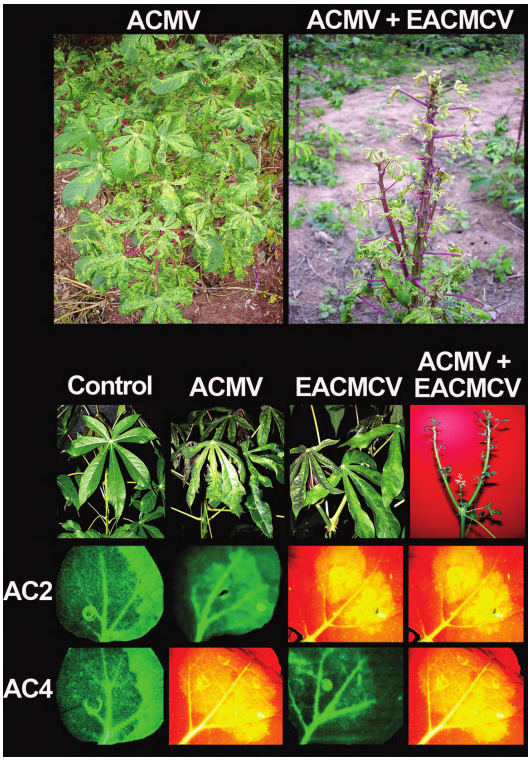


FIGURE 11.4 Example of synergism between two geminiviruses, ACMV and East African cassava mosaic Cameroon virus (EACMCV). Top panel shows a single infection of cassava with ACMV (left) and a dual infection of cassava with ACMV and EACMCV (right) in Ghana. Bottom panel: reproduction of synergistic interaction between ACMV and EACMCV in the lab; from left to right: control cassava plant, ACMV, EACMCV, and dual infection of cassava. Lower panel: picture of the effect of gene silencing suppression of the geminivirus AC2 and AC4 proteins of both viruses on tobacco. Green color indicates no PTGS suppression, while yellow color indicates PTGS suppression. Dual PTGS suppression by AC2 and AC4 from two geminiviruses corresponds to the collapse of cassava (top right).

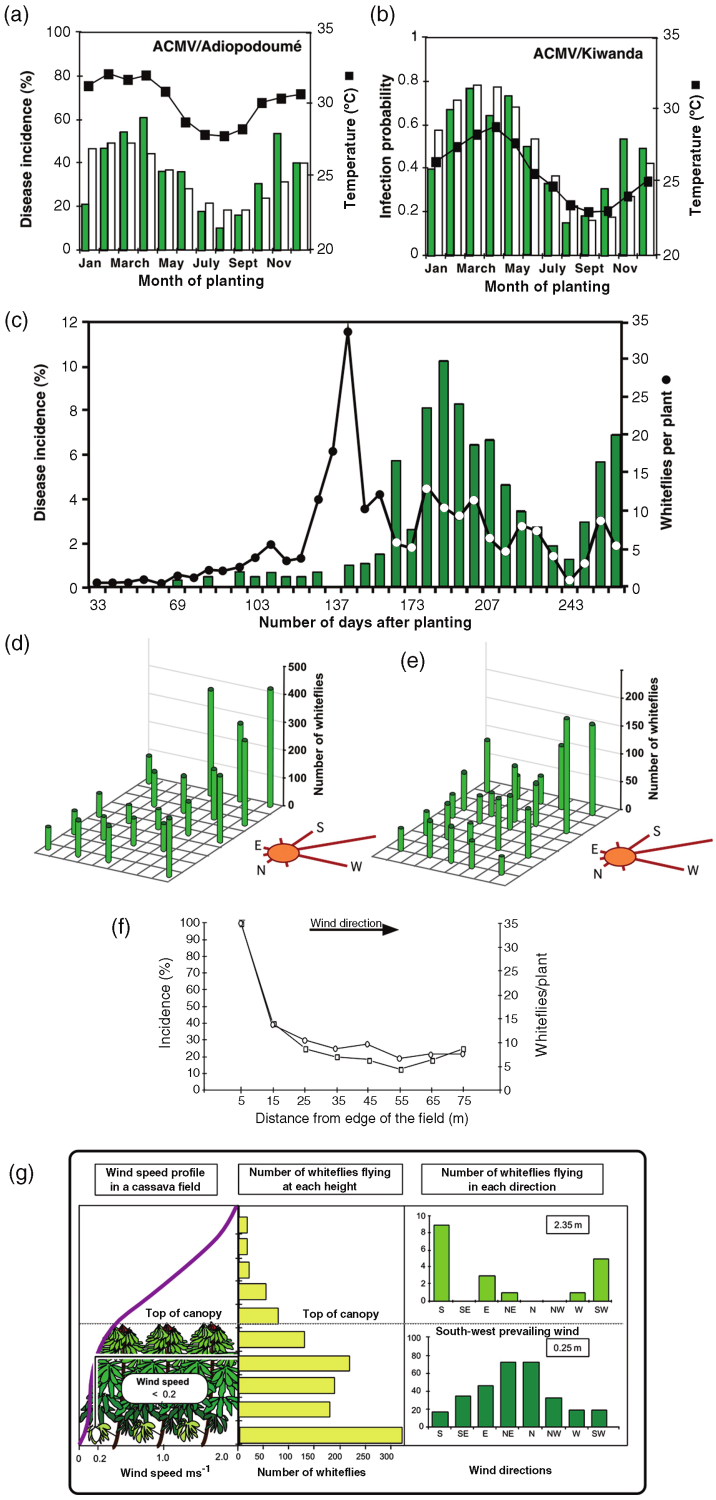


FIGURE 11.5 Correlation between ACMV incidence and monthly mean temperatures in Ivory Coast (a) and Tanzania (*See text for full caption.*)

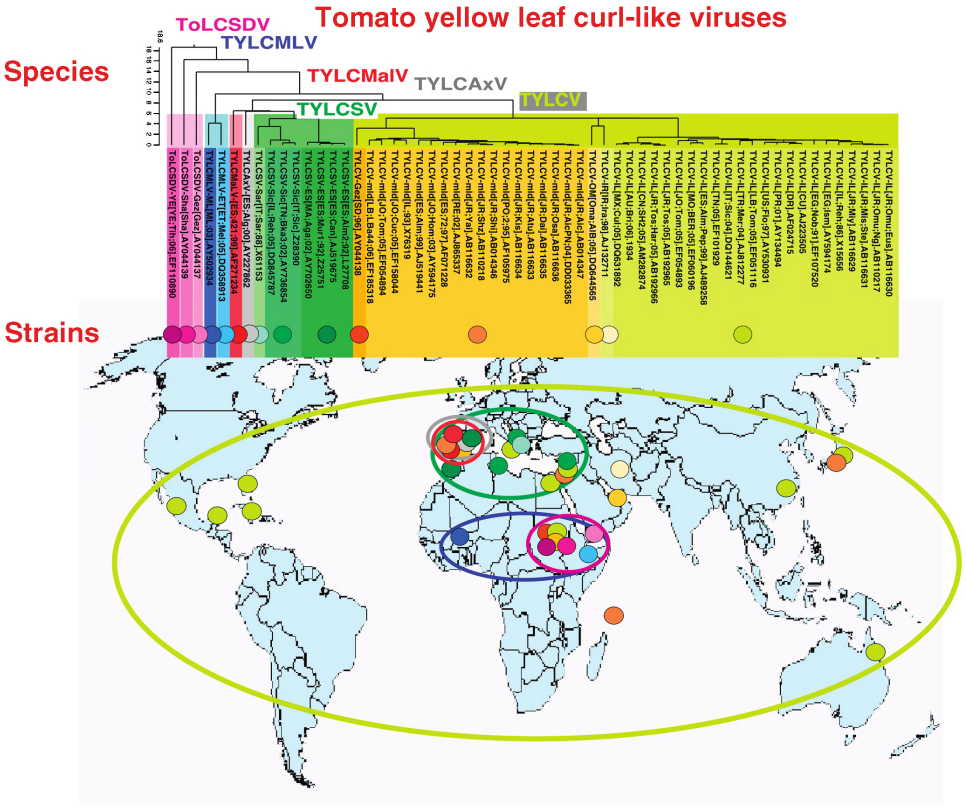


FIGURE 11.6 World map on which each of the 59 members represents 6 begomovirus species of the TYLCV cluster. The upper part of the diagram shows a phylogenetic tree of these 59 viruses using their complete A component sequence. The Clustal V algorithm of the program MegAlign from DNAStar has been used and distances in percentage difference are indicated on the left. The tree shows a partition in six major clusters, one for each of the six designated species, TYLCV, TYLCSV, TYLCAxV, TYLCMalV, TYLCMLV, and ToLCSDV, respectively, in yellow, green, gray, red, blue, and purple colors. These six species constitute the so-called TYLCV cluster of the OW begomoviruses. The individual viruses composing these clusters are positioned on the world map, as dots of various colors representing their pertaining to one of the 15 specific strains of the 6 species, as indicated in the colored boxes at the bottom of the tree. On the world map, the individuals pertaining to the same species are circled with the same color as indicated by the name of the species of the boxes on the phylogenetic tree. Adapted from Abhary et al. (2007).

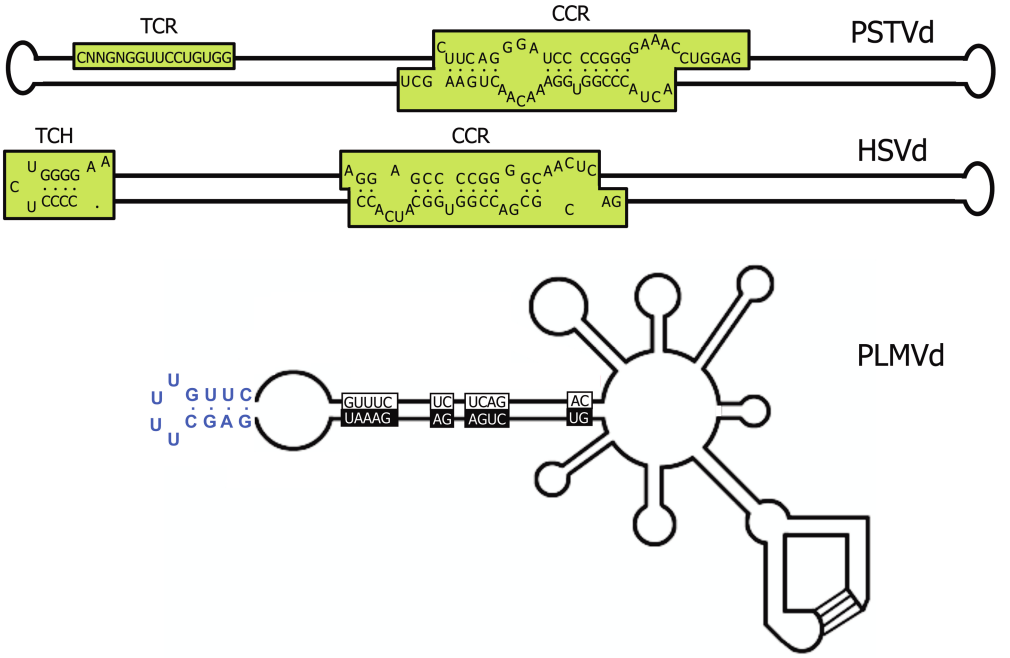


FIGURE 12.1 Structure of viroids. *Upper and middle panels*: schemes of the characteristic rod-like secondary structures of the genomic RNAs of *Potato spindle tuber viroid* (PSTVd) and *Hop stunt viroid* (HSVd), respectively (family Pospiviroidae), with the central conserved region (CCR), the terminal conserved region (TCR), and the terminal conserved hairpin (TCH). (*See text for full caption.*)

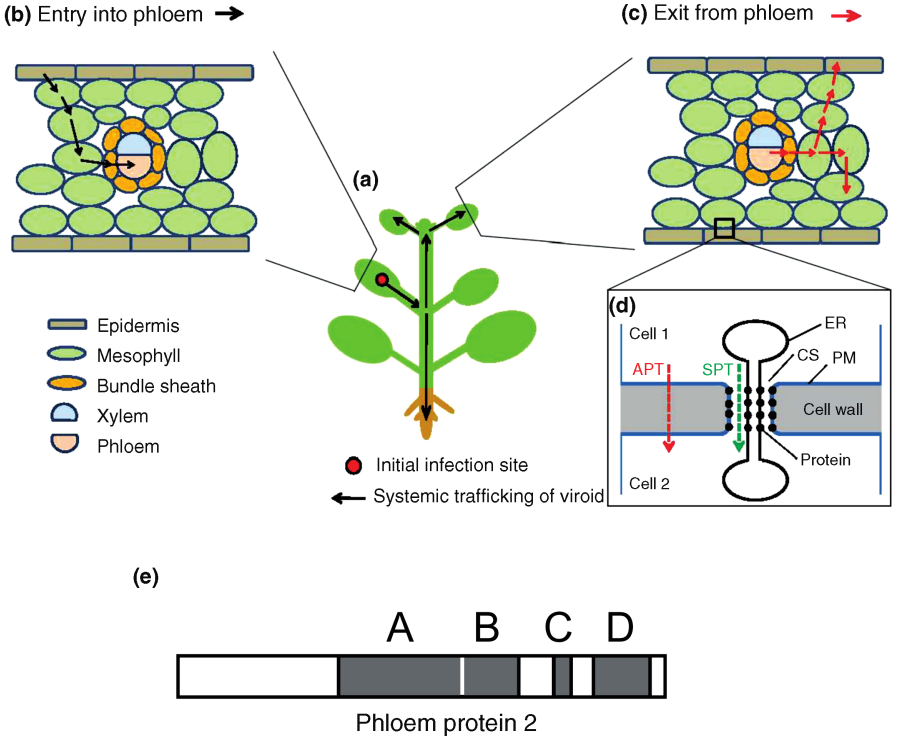


FIGURE 12.3 Viroid movement pathways. (*See text for full caption.*)

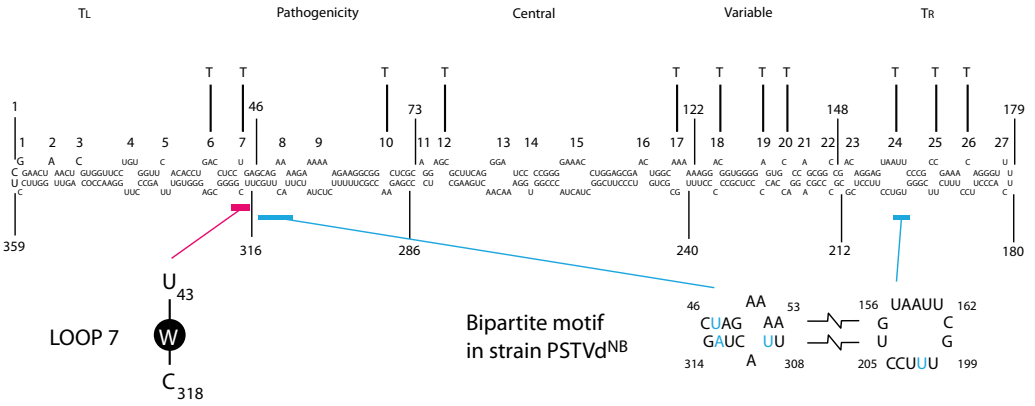


FIGURE 12.4 Secondary structure of PSTVd showing the relative locations of cell-to-cell and long-distance trafficking motifs identified by site-directed mutagenesis (Zhong et al., 2007), as well as the five structural domains proposed by Keese and Symons (1985). (*See text for full caption.*)

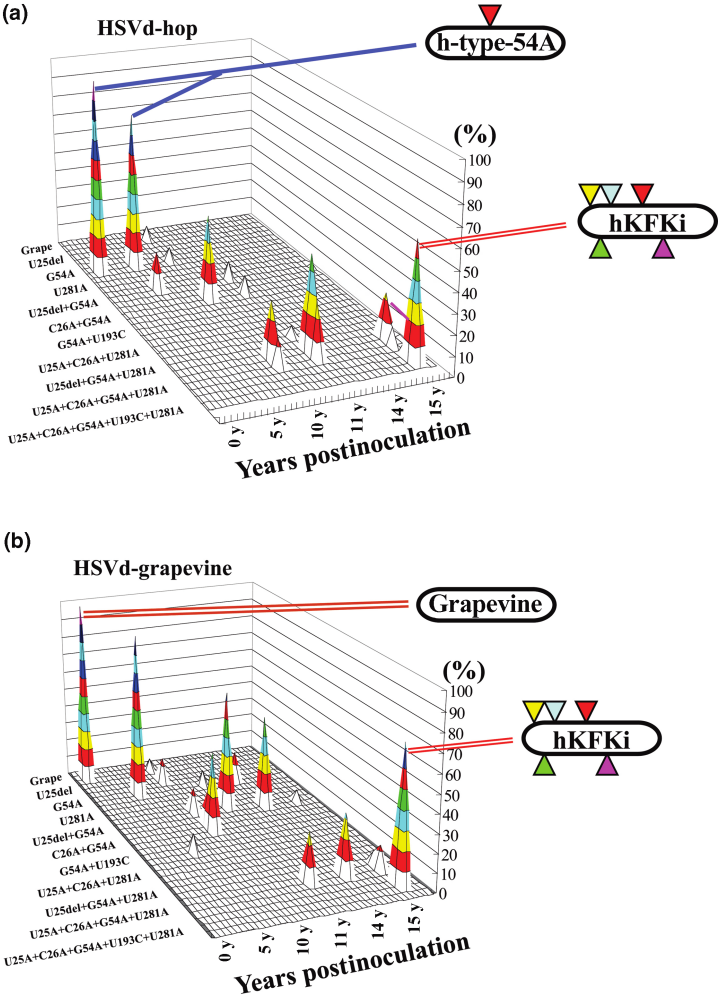


FIGURE 12.5 Convergent evolution of two natural isolates of HSVd during prolonged passage in hop. The upper (a) and lower (b) panels summarize data for HSVd-hop and HSVd-grapevine, respectively. (*See text for full caption.*)

SECTION I

AN INTRODUCTION TO THE STRUCTURE AND BEHAVIOR OF VIRUSES

CHAPTER 1

DEFINING THE ECOLOGY OF VIRUSES*

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CONTENTS

1.1 INTRODUCTION

The goal of virology is to understand the viruses and their behavior. Virology is an interesting subject and even has contributed to the concepts of what we consider to represent dieties and art. Sekhmet, an ancient Egyptian goddess, was for a time considered to be the source of both causation and cure for many of the diseases that we now know to be caused by viruses (Figure 1.1). Influenza, a viral-induced disease of vertebrates, was once assumed to be caused by the influence of the stars, and that is represented by the origin of it's name which is derived from Italian. The following was a

*This chapter represents a revision of "Defining the ecology of viruses", which appeared as chapter 1 of the book *Viral Ecology*, edited by Christon J. Hurst, published in 2000 by Academic Press. All of the artwork contained in this chapter appears courtesy of Christon J. Hurst.

Studies in Viral Ecology: Microbial and Botanical Host Systems, Volume 1, First Edition. Edited by Christon J. Hurst.

FIGURE 1.1 Image of Sekhmet, "Bust Fragment from a colossal statue of Sekhmet", Cincinnati Art Museum, John J. Emery Fund, Accession #1945.65 Cincinnati, Ohio. Originally the warrior goddess of Upper Egypt, Sekhmet was for a time believed to be the bringer of disease. She would inflict pestilence if not properly appeased, and if appeased could cure such illness.

rhyme which children in the United Sates sang while skipping rope during the influenza pandemic of 1918–1919:

I had a little bird

It's name was Enza

I opened a window

And in-flew-Enza.

(Source: The flu of 1918, by Eileen A Lynch, The Pennsylvania Gazette November/December 1998 (http://www.upenn.edu/gazette/1198/lynch.html).

And a bit more recently an interesting poem was written about viruses (Source: Michael Newman, 1984):

"The Virus"

Observe this virus: think how small
Its arsenal, and yet how loud its call;
It took my cell, now takes your cell,
And when it leaves will take our genes as well.
Genes that are master keys to growth
That turn it on, or turn it off, or both;
  Should it return to me or you
  It will own the skeleton keys to do
  A number on our tumblers; stage a coup.

But would you kill the us in it,
The sequence that it carries, bit by bit?
The virus was the first to live,
Or lean in that direction; now we give
Attention to its way with locks,
And how its tickings influence our clocks;
  Its gears fit in our clockworking,
  Its habits of expression have a ring
  That makes our carburetors start to ping.

This happens when cells start to choke
As red cells must in monoxic smoke,
When membranes get the guest list wrong
And single-file becomes a teeming throng,
And growth exists for its own sake;
Then soon enough the healthy genes must break;
  If we permit this with our cells,
  With molecules abet the clanging bells;
  Lend our particular tone to our death knells.

The purpose of this book is to define the ecology of viruses and, in so doing, try to approach the question of what life is like from a "virocentric" (as opposed to our normal anthropocentric) point of view. Ecology is defined as the branch of science which addresses the relationships between an organism of interest and the other organisms with which it interacts, the interactions between the organism of interest and its environment, and the geographic distribution of the organism of interest. The objective of this chapter is to introduce the main concepts of viral ecology. The remaining chapters of this book set, Studies in Viral Ecology volumes 1 and 2, will then address those concepts in greater detail and illustrate the way in which those concepts apply to various host systems.

1.1.1 What is a Virus?

Viruses are biological entities which possess a genome composed of either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA). Viruses are infectious agents which do not possess a cellular structure of their own, and hence are "acellular infectious agents". Furthermore, the viruses are obligate intracellular parasites, meaning that they live (if that can be said of viruses) and replicate within living host cells at the expense of those host cells. Viruses accomplish their replication by usurping control of the host cell's biomolecular machinery. Those which are termed "classical viruses" will form a physical structure termed a "virion" that consists of their RNA or DNA genome surrounded by a layer of proteins (termed "capsid proteins") which form a shell or "capsid" that protects the genomic material. Together, this capsid structure and its enclosed genomic material are often referred to as being a "nucleocapsid". The genetic coding for the capsid proteins generally is carried by the viral genome. Most of the presently known virus types code for their own capsid proteins. However, there are some viruses which are termed as being "satellite viruses". The satellite viruses encapsidate with proteins that are coded for by the genome of another virus which coinfects (simultaneously infects) that same host cell. That virus which loans its help by

giving its capsid proteins to the satellite virus is termed as being a "helper virus". The capsid or nucleocapsid is, in the case of some groups of viruses, surrounded in turn by one or more concentric lipid bilayer membranes which are obtained from the host cell. There exist many other types of acellular infectious agents which have commonalities with the classical viruses in terms of their ecology. Two of these other types of acellular infectious agents, the viroids and prions, are included in this book set and are addressed within their own respective chapters (Volume 1, chapters 10 and 12). Viroids are biological entities akin to the classical viruses and likewise can replicate only within host cells. The viroids possess RNA genomes but lack capsid proteins. The agents which we refer to as prions were once considered to be nonclassical viruses. However, we now know that the prions appear to be aberrant cellular protein products which, at least in the case of those afflicting mammals, have acquired the potential to be environmentally transmitted. The natural environmental acquisition of a prion infection occurs when a susceptible host mammal ingests the bodily material of an infected host mammal. The reproduction of prions is not a replication, but rather seems to result from a conversion of a normal host protein into an abnormal form (Volume 1, chapter 10). The Acidianus two-tailed virus, currently the sole member of the viral family Bicaudaviridae, undergoes a morphological maturation following its release from host cells and this is unique among all of the biological entities now considered to be viruses suggesting that this species may represent the initial discovery of an entirely new category of biological entities.

1.1.2 What is Viral Ecology?

Ecology is the study of the relationships between organisms and their surroundings. Viral ecology is, therefore, the relationship between viruses, other organisms, and the environments which a virus must face as it attempts to comply with the basic biological imperatives of genetic survival and replication. As shown in Figure 1.2, interactions between species and their constituent individual organisms (biological entities) occur in the areas where there exist overlaps in the temporal, physical, and biomolecular (or biochemical) aspects of the ecological zones of those different species. Many types of interactions can develop between species as they share an environment. One of the possible types of interactions is predation. When a microorganism is the predator, that predator is referred to as being a pathogen and the prey is referred to as being a host.

When we study viral ecology we can view the two genetic imperatives that every biological entity must face, namely, that it survive and that it reproduce, in the perspective of a biological life cycle. A generalized biological life cycle is presented in Figure 1.3. This type of cycle exists, in its most basic form, at the level of the individual virus or individual cellular being. However, it must be understood that in the case of a multicellular being this biological life cycle exists not only at the level of each individual cell, but also at the tissue or tissue system level, and at the organ level. This biological life cycle likewise exists on even larger scales, where it operates at levels which describe the existence of each species as a whole, at the biological genus level, and also seems to operate further upward to at least the biological family level. Ecologically, the life cycles of those different individuals and respective species which affect one another will become interconnected both temporaly, geographically, and biologically. Thus, there will occur an evolution of the entire biological assemblage and, in turn, this process of biotic evolution will be obliged to adapt to any abiotic changes that occur in the environment which those organisms share. While a species physiologic capacities establish the potential limits of the niche which it could occupy within this shared environment, the actual operational boundaries of it's niche are more restricted and defined by it's interspecies connections and biological competitions.

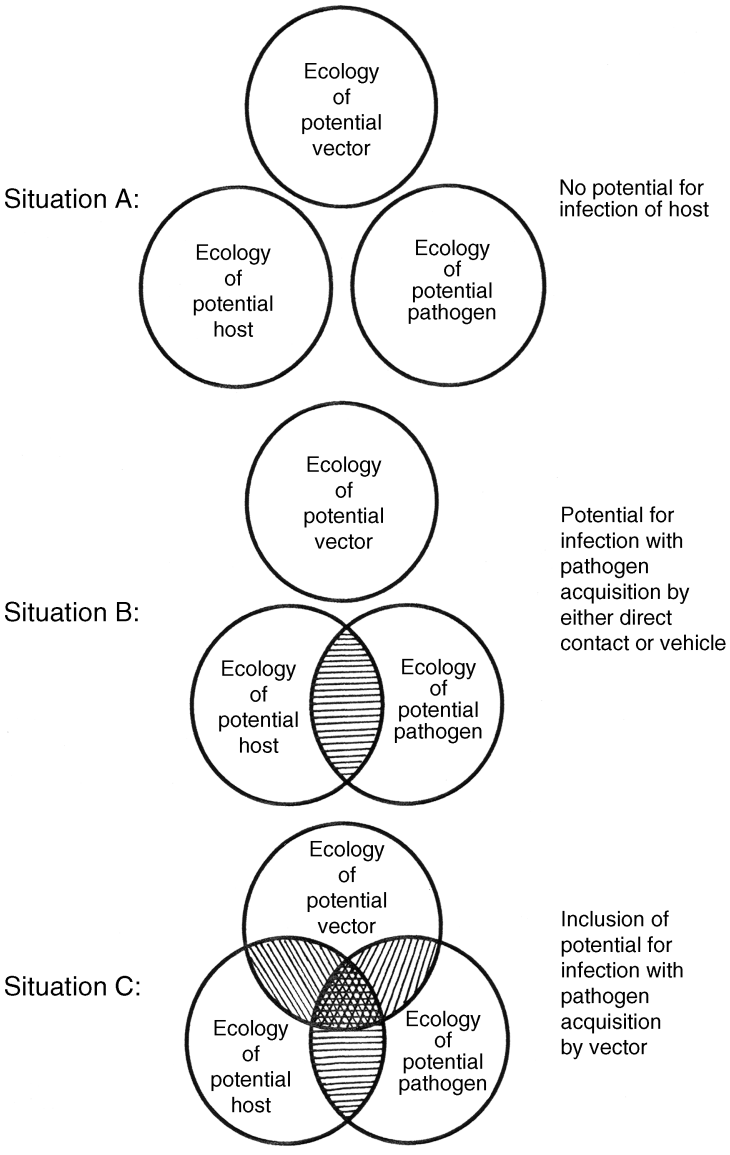

FIGURE 1.2 Interactions between organisms (biological entities) occur in the areas where the physical and chemical ecologies of the involved organisms overlap. Infectious disease is a type of interaction in which a microorganism acts as a parasitic predator. The microorganism is referred to as a pathogen in these instances.

1.1.3 Why Study Viral Ecology?

The interplay which occurs between a virus and the living organisms which surround it, while all simultaneously pursue their own biological drive to achieve genetic survival and replication, creates an interest for studying the ecology of viruses (Doyle, 1985; Fuller, 1974; Kuiken et al., 2006; Larson, 1998; Morell, 1997; Zinkernagel, 1996). While examining this topic, we improve our understanding of the behavioral nature of viruses as predatory biological entities. It is important to realize that in nature both the viruses of macroorganisms and the viruses of microorganisms normally

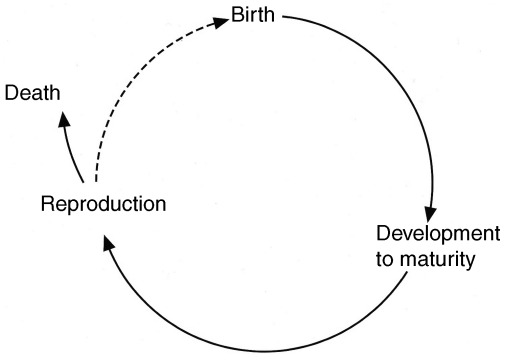

FIGURE 1.3 Generalized biological life cycle. Ecologically, the life cycles of different organisms which affect one another are temporally interconnected.

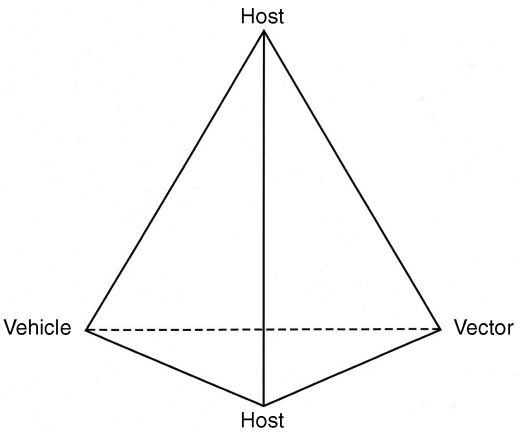

FIGURE 1.4 The lines connecting the four vertices of this tetrahedron represent the possible routes by which a virus can move from one host organism to another host organism.

exist in a cycle with their respective hosts. Under normal conditions, the impact of viruses upon their natural hosts may be barely apparent due to factors such as evolutionary coadaptation between the virus and its host (evolutionary coadaptation is the process by which species try to achieve a mutually acceptable coexistence by evolving in ways which enable them to adapt to one another). However, when viruses find access to new types of hosts and alternate transmission cycles, or when they encounter a concentrated population of susceptible genetically similar hosts such as occurs in densely populated human communities, communities of cultivated plants or animals, or algal blooms, then the impact of the virus upon its host population can appear catastrophic (Nathanson, 1997; Subbarao et al., 1998).

As we study viral ecology we come to understand not only those interconnections which exist between the entities of virus and host, but also the interconnections between these two entities and any vectors or vehicles which the virus may utilize. As shown in Figure 1.4, this interplay can be represented by the four vertices of a tetrahedron. The possible routes by which a virus may move from one host organism to another host organism can be illustrated as the interconnecting lines between those vertices which represent two hosts (present and proximate) plus one vertice apiece representing the concepts of vector and vehicle. Figure 1.5, which represents a flattened form of the tetrahedron shown in the previous figure (Figure 1.4) can be considered our point of reference as we move forward in examining viral ecology. The virus must survive when in association with the present host and then successfully move from that (infected) host organism (center of Figure 1.5) to another host organism. This movement, or transmission, may occur via direct contact between the two host organisms or via routes which involve vectors and vehicles (Hurst and Murphy, 1996). Vectors are, by definition, animate (living) objects. Vehicles are, by definition, inanimate (non-living) objects. Any virus which utilizes either vectors or vehicles must possess the means to survive when in association with those vectors and vehicles in order to sustain its cycle of transmission within a population of host organisms. If a virus replicates enough to increase its population while in association with a vector, then that vector is termed to be "biological" in nature. If the virus population does not increase while in association with a vector, then that vector is termed to be "mechanical" in nature. Because viruses are obligate intracellular parasites, and vehicles are by definition non-living, then we must assume that the virus cannot increase its population while in association with a vehicle.

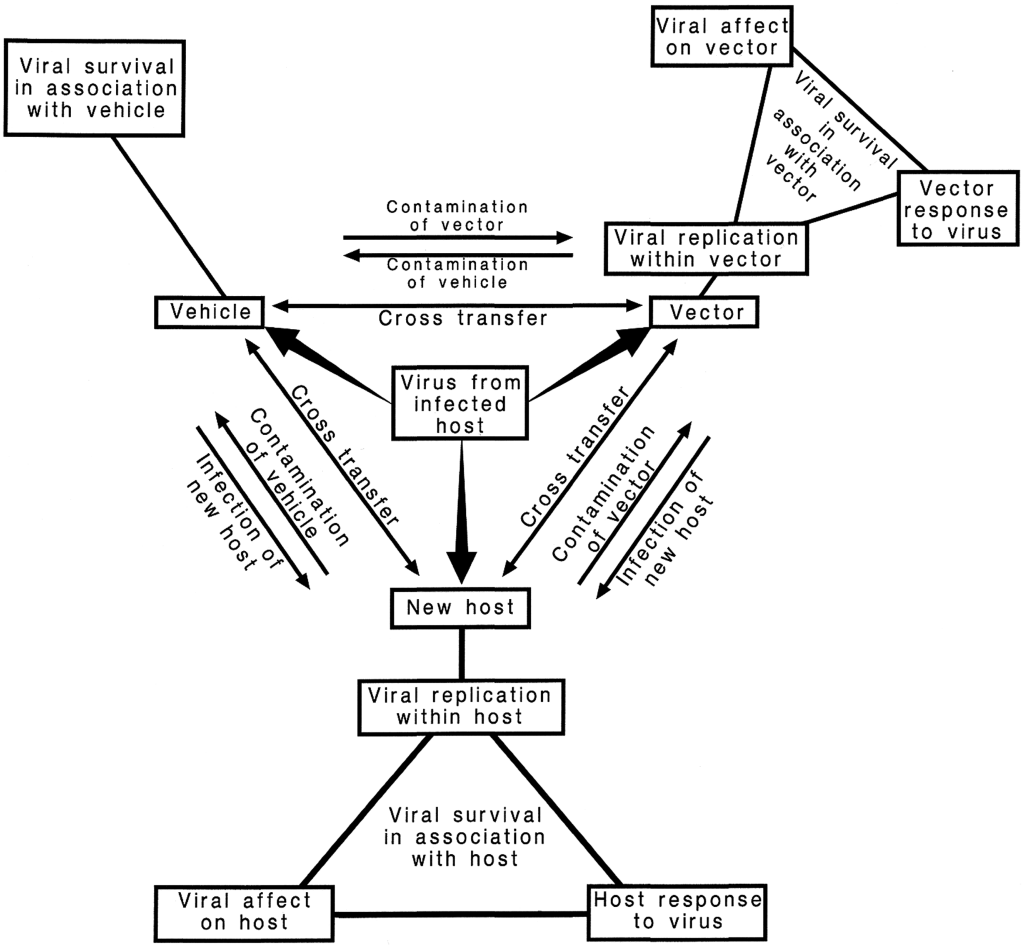

FIGURE 1.5 Viral ecology can be represented by this diagram, which represents a flattened form of the tetrahedron shown in the previous figure (Figure 1.4). The virus must successfully move from an infected host organism (center of figure) to another host organism. This movement, or transmission, may occur via direct transfer or via routes which involve vehicles and vectors. In order to sustain this cycle of transmission within a population of host organisms, the virus must survive when in association with the subsequently encountered hosts, vehicles and vectors.

Environmentally, there are several organizational levels at which a virus must function. The first and most basic of those levels is the individual host cell. That one cell may comprise the entire host organism. Elsewise, that host cell may be part of a tissue. If within a tissue, then the tissue will be contained within a larger structure termed either a tissue system (plant terminology) or an organ (plant and animal terminology). That tissue system or organ will be contained within an organism. The host organism is exposed to the open (ambient) environment, where it is but one part of a population of other organisms belonging to its same species. The members of that host species will be surrounded by populations of other types of organisms. Those populations of other types of organisms will be serving as hosts and vectors for either the same or other viruses. Each one of these organizational levels represents a different environment which the virus must successfully confront. A virus' affects upon it's hosts and vectors will draw responses against which the virus must defend

itself if the virus is to survive. Also, the virus must always be ready to do battle with it's potential biological competitors. Contrariwise, the virus must be open to considering newly encountered (or reencountered) species as possible hosts or vectors. Because of their acellular nature, when viruses are viewed in the ambiental environments (air, soil and water) they appear to exist in a form that essentially is biologically inert. However, they have a very actively involved behavior when viewed in these many other organismal environments.

Considering the fact that viruses are obligate intracellular parasites, their ecology must be presented in terms which also include aspects of the ecology of their hosts and any vectors which they may utilize. Those factors or aspects of viral ecology which we study, and thus which will be considered in this book set, include the following:

Host Related Issues

1. what are the principal and alternate hosts for the viruses;
2. what types of replication strategies do the viruses employ on a host cellular level, host tissue or tissue system level, host organ level, the level of the host as a whole being, and the host population level;
3. what types of survival strategies have the viruses evolved that protect them as they confront and biologically interact with the environments internal to their host (many of those internal environments are actively hostile, as the hosts have developed many powerful defensive mechanisms);
4. what direct effects does a virus in question have upon its hosts, i.e. do the hosts get sick and, if the hosts get sick, then how severe is the disease and does that disease directly threaten the life of the host;
5. what indirect effects does the virus have upon its hosts, i.e., if the virus does not directly cause the death of the hosts or if viral-induced death occurs in a temporaly delayed manner as is the case with slow or inapparent viral infections, then how might that virus affect the fitness of the host to compete for food resources or to avoid the host's predators;

General Transmission-Related Issue

6. what types of transmission strategies do the viruses employ as they move between hosts, including their principal and alternate transmission routes which may include vehicles and vectors; and

Vector-Related Issues

7. in reference to biological vectors (during association with a biological vector the virus will replicate and usually is carried within the body of the vector), what types of replication strategies do the viruses employ on a vector cellular level, vector tissue or tissue system level, vector organ level, the level of the vector as a whole being, and also on a vector population level;
8. in reference to biological vectors, what types of survival strategies have the viruses evolved that protect them as they confront and biologically interact with the environments internal to their vectors (those internal environments may be actively hostile, as vectors have developed many powerful defensive mechanisms);
9. in reference to biological vectors, what direct effects does a virus in question have upon its vectors, i.e. do the vectors get sick and, if the vectors get sick, then how severe is the disease and does that disease directly threaten the lives of the vectors;
10. in reference to biological vectors, what indirect effects does the virus have upon its vectors, i.e., if the virus does not directly cause the death of the vectors or if viral-induced death occurs in a temporaly delayed manner as is the case

with slow or inapparent viral infections, then how might that virus affect the fitness of the vectors to compete for food resources or to avoid the vector's predators;

11. in reference to mechanical vectors, what types of survival strategies have been evolved by those viruses which are transmitted by (and during that event usually carried on the external surfaces of) mechanical vectors, since while in association with a mechanical vector the virus must successfully confront any compounds naturally present on the body surface of the vector plus confront the passively hostile ambiental environments of either air, water or soil through which the vector will be moving; and

Vehicle-Related Issue

12. what types of survival strategies have been evolved by those viruses which are transmitted by way of vehicles and which thereby must successfully confront the passively hostile ambiental environments of either air, water or soil as the virus itself is transferred through those environments.

If biological curiosity alone were not a sufficient reason for studying viral ecology, then perhaps we would study the viruses out of a desire to both understand them as predators and to contemplate the ways in which we might enlist their aid as ecological tools.

1.2 SURVIVING THE GAME: THE VIRUS AND IT'S HOST

Remember that: *so long as the virus finds a new host, whether or not the current host survives is unimportant*. Although it may be beneficial to not kill a current host until that host has reproduced to help provide a new generation of potential host organisms, if the host to virus ratio is large enough, then even this latter point may be unimportant.

This section presents in general terms the relationship between a virus and host. The generalities of relationships between viruses, vectors, and vehicles will be discussed in section 1.3 of this chapter. The specific subject of the practical limits to viral virulence in association with hosts and vectors will be addressed in section 1.4 of this chapter.

While in association with a host, the virus has only one principle goal. This goal is for the virus to replicate itself to a sufficient level that it can achieve transmission to another host. This goal can be attained by one of two basic strategies. The first of these strategies would be a productive infection, for which five basic patterns can be defined. The second strategy would be a non-productive infection. The goal of a productive infection is for the virus to produce infectious viral particles (those capable of infecting cells) which are termed "virions", during the virus' association with the current host. Subsequent spread of the infection to the next host occurs by transfer of these produced virions. Contrastingly, some of those agents which exhibit a non-productive pattern may either seldom or never produce actual virions. Thus, the usual goal of a non-productive strategy of infection is to pass the infection to the next host by directly transferring only the viral genomic sequences (van der Kuyl et al., 1995). The patterns of productive infection are:

"Short term - initial" in which viral production has only a short term initial course, after which the viral infection ends and there no longer is a presence of that virus within the body of the host individual although subsequent reinfection can occur, the outcome from this pattern of infection depends upon the virus type and historical exposure to that type within the host population, the situation being that in otherwise healthy members of a multicellular host population with which the virus has coevolved, these infections are usually mild and by

themselves normally associated with a fairly low incidence of mortality;

"Recurrent" in which repeated episodes of viral production occur, this pattern often has a very pronounced initial period of viral production, after which the virus persists in a latent state within the body of the host with periodic reinitiations of viral production that usually are not life threatening;

"Increasing to end-stage" in which viral infection is normally associated with a slow, almost inocuous start followed by a gradual progression associated with an increasing level of viral production and eventual death of the host, in these instances death of the host may relate to destruction of the host's immunological defense systems which then results in death by secondary infections;

"Persistent-episodic" is a pattern that represents a prolonged nonfatal infection which may persist for the remainder of the hosts natural lifetime associated with a continuous production of virions within the host, but interestingly the infection only episodically results in symptoms, the viral genome does not become quiescent, the host remains infectious throughout the course of this associative interaction, and very notably some members of the family Picobirnaviridae often produce this pattern of productive infection;

"Persistent but inapparent" is a pattern that represents a prolonged nonfatal infection which seemingly never results in overt symptoms of illness attributable to that particular virus, the viral genome never becomes quiescent and viral infections that follow this pattern are persistently productive with the host often remaining infectious for the remainder of their natural lifetime, with notable examples of viruses which produce this pattern being members of the family Anelloviridae, and it also occurs in certain rare instances of infection by Human immunodeficiency virus 2 which is a member of the genus *Lentivirus* of the family Retroviridae.

There are two options to the "short term - initial" pattern. The first option is a very rapid, highly virulent approach which is termed "fulminate" (seemingly explosive) and usually results in the rapid death of the host organism. This first option usually represents the product of an encounter between a virus and a host with which the virus has not coevolved. The second option is for the virus to be less virulent, causing an infection which often progresses more slowly, and appears more benign to the host. The "recurrent" and "increasing to end-stage" patterns incorporate latency into their scheme. Latency is the establishment of a condition in which the virus remains forever associated with that individual host organism and generally shows a slow and possibly only sporadic replication rate that, for some combinations of virus and host, may never be life threatening to the host. The strategy of achieving a non-productive, or virtually non-productive, pattern of infection involves achieving an endogenous state (Terzian et al., 2001). Endogeny implies that the genome of the virus is passed through the host's germ cells to all offspring of the infected host (van der Kuyl et al., 1995; Villareal, 1997).

The product of interspecies encounters between a virus and it's natural host will usually lead to a relatively benign (mild, or not directly fatal), statistically predictable, outcome that results from adaptive coevolution between the two species. Still, these normal relationships do not represent a static coexistance between the virus and the natural host, but rather a tenuous equilibrium. Both the virus species and it's evolved host species will be struggling to get the upper hand during each of their encounters (Moineau et al., 1994. The result will normally be some morbidity and even some mortality among the host population as a result of infection by that virus. Yet, because the virus as a species may not be able to survive without this natural host species

(Alexander, 1981), excessive viral-related mortality in the host population is not in the long term best interest of the virus. Some endogenous viruses have evolved to offer a survival-related benefit to their natural host, and this can give an added measure of stability to their mutual relationship. Two examples of this type of relationship are the hypovirulence element associated with some strains of the Chestnut blight fungus, and the endogenous retroviruses of placental mammals. The hypovirulence (reduced virulence) which the virus-derived genetic elements afford to the fungi that cause Chestnut blight disease reduce the virulence of those fungi (Volume 1, chapter 9). This reduced virulence allows the host tree, and in turn the fungus, to survive. Placental mammals, including humans, permanently have incorporated species of endogenous retroviruses into the chromosomes of their genomes. It has been hypothesized that the incorporation of these viruses has allowed the evolution of the placental mammals by suppressing maternal immunity during pregnancy (Villareal, 1997).

However, the impact of a virus upon what either is, or could become, a natural host population can sometimes appear catastrophic. The most disastrous, from the host's perspective, are the biological invasions which occur when that host population encounters a virus which appears new to the host (Kuiken et al., 2006). Three categories of events can lead to biological invasions of a virus into a host population. These categories are: first, that this virus species and host species (or sub-population of the host species) may never have previously encountered one another (examples of this occurring in human populations would be the introduction of measles into the Pacific islands and the current introduction of HIV); second, if there have been previous encounters, the virus may have since changed to the point that antigenically it appears new to the host population (an example of this occurring in humans would be the influenza pandemic of 1918–1919); and third, that even if the two species may have had previous encounters, this subpopulation of the host species subsequently may have been geographically isolated for such a length of time that most of the current host population represents a completely new generation of susceptible individuals (examples in humans are outbreaks of viral gastroenteritis found in remotely isolated comunities on small islands as related to the occasional arrival of ill passengers by aircraft or watercraft). Sadly, the biological invasion of the HIV viruses into human populations seems to be successful (Caldwell and Caldwell, 1996), and the extreme host death rate associated with this invasion can be assumed to indicate that the two species have not had time to coevolve with one another. The sporadic, but limited, outbreaks in human populations of viruses such as those which cause the hemmorrhagic fevers known as Ebola and Lassa represent examples of unsuccessful biological invasions. The limited chain of transmission for these latter two illnesses (for Lassa, see: Fuller, 1974), with their serial transfers often being limited to only two or three hosts in succession, represents what will occur when a virus species appears genetically unable to establish a stabile relationship with a host species. The observation of extremely virulent and fulminate symptomatology, as associated with infections by Lassa and Ebola in humans, can generally be assumed to indicate either that the host in which these drastic symptoms are observed is not the natural host for those viruses or, at the very least, that these two species have not had time to coevolve. In fact, the extreme symptomatology and mortality which result in humans from Ebola and Lassa fevers seems to represent an overblown immune response on the part of the host (Spear, 1998). While having the death of a host individual occur as the product of an encounter with a pathogen may seem like a dire outcome, this outcome represents a mechanism of defense operating at the level of the host population. If a particular infectious agent is something against which members of the host population could not easily defend themselves, then it may be better to have that particular host individual die (and die very quickly!) to reduce the possible spread

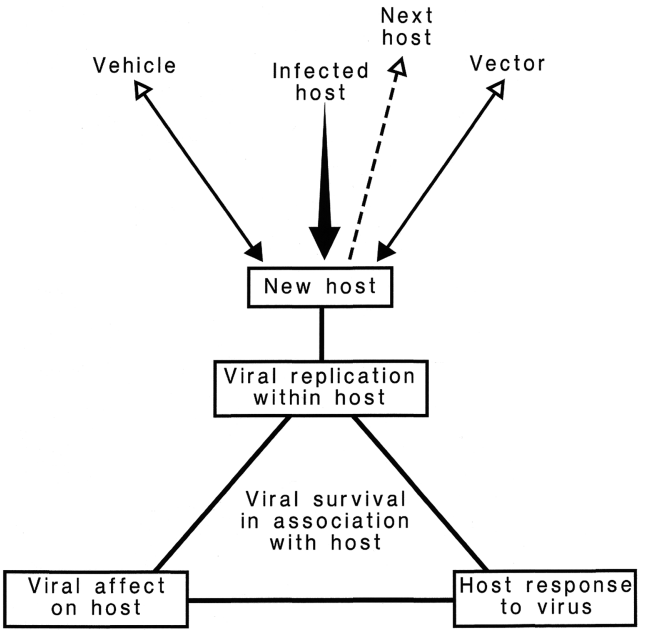

FIGURE 1.6 Viruses can arrive at their new host (filled arrows) either directly from the previously infected host, via an intermediate vehicle, or via an intermediate vector. Viral survival in association with that new host depends upon: viral replication within that new host, the effects which the virus has upon that host, and the response of that host to the virus. Successful viral survival in association with this new host will allow a possible subsequent transfer of the virus (open arrows) to its next host either directly, via a vehicle, or via a vector. This represents a segment from Figure 1.5.

of the contagion to the other members of the host population.

1.2.1 Cell Sweet Cell, and Struggles at Home

As diagramed in Figure 1.6, viruses can arrive at their new host (solid arrows) either directly from the previously infected host, via an intermediate vehicle, or via an intermediate vector. Viral survival in association with the new host will first depend upon the virus finding it's appropriate receptor molecules on the host cell's surface (Spear, 1998). After this initial location, the virus must be capable of entering and modifying the host cell so that the virus can reproduce within that cell. If the host is multicellular, then the virus may first have to successfully navigate within the body of the host until it finds the particular host tissue which contains it's correct host cells.

Within a multicellular host, the virus may face anatomically associated barriers including membranous tissues in animals. The virus also may face non-specific, non-immune biological defenses (Moffat, 1994), including such chemical factors as the enzymes found in both tears and saliva, and the acid found in gastic secretions. The types of anatomical and non-specific, non-immune defenses encountered can vary depending upon the viral transmission route and the portal by which the virus gains entry into the host's body. After a virus finds it's initial host cell and succeeds in beginning it's replication, the effects which the virus has upon the host can then draw a defensive biological response. The category of non-specific non-immune responses which a virus may encounter at this stage include even such things as changes in host body temperature for mammals. As if in a game of spy versus spy, the virus most importantly must survive the host's specific immune defenses

(Beck and Habicht, 1996; Gauntt, 1997; Levin et al., 1999; Litman, 1996; Ploegh, 1998; Zinkernagel, 1996).

The listing and adequate explanation of antiviral defense techniques would by itself be enough to nearly fill a library. But, I will attempt to summarize some of them here and help the reader to track those through this book set.

Molecular antiviral defenses begin at the most basic level which would be non-specific mechanisms. These conceptually include DNA restriction and modification systems (volume 1, chapter 5), progressing upward with greater complexity to the use of post transcriptional processing (Russev, 2007). Countering these defenses is done by such techniques as using virally-encoded restriction-like systems to chop-up the DNA genome of their host cells to provide a ready source of nucleic acids for the production of progeny viral genomes. There also are viruses which try to shut down the the post-transcriptional defenses, most clearly noticed among some viruses infective of plants. Plants in fact heavily rely upon molecular defenses such as post-transcriptional control, (volume 1, chapter 11) and beyond that technique the plants try to wall off an infection, essentially trying to live their lives despite presence of the infectious agent and hoping not to pass the infection along to their offspring through viral contamination of their germ cells.

Antimicrobial peptides are a defensive mechanism found in all classes of life, and represent a main part of the insect defensive system (volume 2, chapter 10). Higher on the scale of defensive responses are things which we term to be immunological in nature (Danilova, 2006). Some of these we term to be innate, others we call adaptive. A good starting point for this discussion of immunological responses is the capacity for distinguishing self versus non-self, accompanied by the capability for biochemically destroying cells that are determined to be non-self. This approach exists from at least the level of fungi (volume 1, chapter 9) upwards for the non-animals, and among the animals this approach begins with at least the corals (volume 2, chapter 5). Determining and acting upon the distinction of self versus non-self likely may have developed as a system that helps to support successful competition for growth in a crowded habitat, but it serves well against pathogenic organisms. As a health issue, this process sadly plays a role in autoimmune diseases and we try to suppress it when hoping to use organ and tissue transplantation to save human lives.

Apoptosis, the targeting of individual cells within the body of the host for selective destruction by the host, commonly exists across the animal kingdom. This mechanism is used by many invertebrates (volume 2, chapters 6 and 7) as wells as vertebrates to destroy any virally infected cells which may be present within their bodies. However, apoptosis is a weapon that can be used by both of the combatants. Using apoptosis to destroy virally-infected cells before the virus contained within those cells can assemble progeny virions is an effective approach when used carefully by the host. As might be expected, some viruses therefore defensively try either to shut-down the process of apoptosis, or at least to shut-down that process until the virus is ready to use apoptosis as a mechanism for assisting in the liberation of assembled virions from the infected host cell.

Vertebrates, and some of the invertebrates, have more complex body plans and can use them with good effectiveness in combating infections. With the evolutionary development of more complex body plans, comes the possibility of dedicating cells and even organs to the task of fighting pathogenic invaders. Those invertebrates with more complex body plans are represented in the anti-viral fight by their use of lymphoid organs to actively collect and either sequester or actively assault and destroy the microbial offenders. Some of the aquatic crustaceans (volume 2, chapter 7) tend to rely upon sequestering an infection and must hope to breed a new generation of their own progeny before they, themselves, are killed by the infection which they have sequestered within their

body. At the same time, the infected parents must hope not to pass along the sequestered infection to their offspring through contamination of their eggs and sperm. Such collection and sequestration techniques are found upward through the evolutionary line and likewise used by the vertebrates. Many viruses have found ways around these issues, as is the case with endogenous viruses and retrotransposons that insert and maintain themselves in the genome of their host, passing directly through the germ cell line. Some viruses infect and replicated within the immune cells! Some viruses are shed along with the eggs of inertebrates and thus are ready to await the hatching of those offpsring. Still other viruses, as in the case of viviparous mammals, simply cross the placenta to infect the fetus.

Interferons and their homologues are protein systems which vertebrates have developed and use effectively against some viruses, and correspondingly many viral groups contain mechanisms for suppressing interferon production (Muñoz-Jordán and Fredericksen, 2010). Although the "walling-off" of a pathogen still occurs in vertebrates, with an example being the development of tubercules in some mycobacterial infections, active mechanisms for hunting down and destroying pathogens and pathogen-infected cells within their bodies is highly developed. With vertebrates, the end goal can be percieved as ridding the body of the pathogen even if that end goal is not always achieved. The jawed vertebrates possess immune systems which are termed adaptive, and these produce protein antibodies that can be highly specific (volume 2, chapters 8, 9, 11–14).

Options for surviving the immune defenses of the host can include such techniques as:

- **"You don't know me"** (a virus infecting an accidental host, in which case a very rapid proliferation may occur, an example being Lassa fever in humans);
- **"Being very, very quiet"** (forming a pattern of latency in association with the virus' persistence within that host, an example being herpesviruses);
- **"Virus of a thousand faces"** (antigen shifting, an example being the lentiviruses);
- **"Keep to his left, that's his blind spot"** (maintaining low antigenicity, an approach used by viroids and prions);
- **"Committing the perfect crime"** (infecting the immune system, an approach taken by many retroviruses and herpesviruses); and
- **"Finding a permanent home"** (taking up permanent genetic residency within the host and therefore automatically being transmitted to the host's progeny, an approach taken by viroids, endogenous retroviruses, and LTR retrotransposons).

Each virus must successfully confront it's host's responses while the virus tries to replicate to sufficient numbers that it has a realistic chance of being transmitted to another candidate host. Failure to successfully confront the host's responses will result in genetic termination of the virus and, on a broader scale, such failure may eventually result in extinction for that viral species.

1.2.2 I Want a Niche, Just Like the Niche, That Nurtured Dear Old Mom and Dad

The initial tissue type in which a virus replicates may be linked inextricably with the initial transmission mode and portal (or site) of entry into the body of the host. For example, those viruses of mammals which are acquired by fecal - oral transmission tend to initiate their replication either in the nasopharyngial tissues or else in the gastrointestinal tissues. There then are subsequent host tissue and organ types affected, some of which may be related to the virus' efforts at trying to reach it's proper portal of exit. Others of the host tissues affected by the virus may be unrelated to interhost viral transmission, although the affect upon those other tissues may play a strong role in the severity of illness which is associated with that viral infection. An example of the latter would be the

encephalitic infection of brain neurons in association with echoviral conjunctivitis, an infection which initially would be acquired from fomites as part of a fecal-oral transmission pattern. In this case, the encephalitis causes nearly all of the associated morbidity but does not seem to benefit transmission of the virus (personal observation by author C. J. Hurst).

1.2.3 Being Societal

Successful viral survival in association with this new host will allow a possible subsequent transfer of the virus (Figure 1.6, open arrows) to its next host either directly, via a vehicle, or via a vector. The movement of a viral infection through a population of host organisms can be examined and mathematically modeled. An epidemic transmission pattern, characterized by a short term, higher than normal rate of infection within a host population is represented by the compartmental model shown in Figure 1.7 (Hurst and Murphy, 1996). An endemic transmission pattern, characterized by a long term, relatively constant incidence rate of infection within a host population is represented by the compartmental model shown in Figure 1.8 (Hurst and Murphy, 1996).

1.3 STEPPIN' OUT AND TAKING THE A TRAIN: REACHING OUT AND TOUCHING SOMEONE BY VECTOR OR VEHICLE

Remember that: *host-vector choices, cycles and vehicle utilizations as they exist today may (and probably do!) reflect evolutionary progression from prior species interactions and ecological relationships.*

After a virus has successfully replicated within the body of it's current (present) host, it must seek successful transmission to it's next (proximate) host. The resulting chain of transmission usually is the end-all of viral reproduction. These are three basic approaches by which this can be attained: transmission by direct contact between the present and proximate hosts, transmission mediated by a vector (Brogdon and McAllister, 1998; Hurst and Murphy, 1996; Mills and Childs, 1998), and transmission mediated by a vehicle (Hurst and Murphy, 1996). While considering these approaches, it is important to keep in mind that the chains of transmission originate by random chance followed by evolution.

1.3.1 "Down and Dirty" (Just Between Us Hosts)

This heading is one which can be used to describe host to host transmission (transmission by host to host contact). While this is one of the most notorious, it is not the most common route of viral transmission between animals. This route only serves to a limited extent in microbes. Even worse, this route essentially does not seem to function in vascular plants due to the relative immobility of those hosts.

1.3.2 "The Hitchhiker" (Finding a Vector)

Transmission by vectors may be the most prevalent route by which the viruses of plants are spread among their hosts. This route clearly also exists for some viruses of animals. However, this route has not yet been defined in terms of viruses which infect microbes. Vectors are, by definition, animate objects, and more specifically they are live organisms. Being a vector implies, although by definition does not require, that the entity serving as vector has self-mobility. Thus, plants could serve by definition as vectors, although when we consider the topic of viral vectors we usually tend to think in terms of the vectors as being invertebrate animals. Vertebrate animals can also serve as vectors, as likewise can some cellular microbes.

There are two categories of vectors: biological and mechanical. As was stated earlier, if the virus increases it's numbers while in association with a vector, then that vector is termed

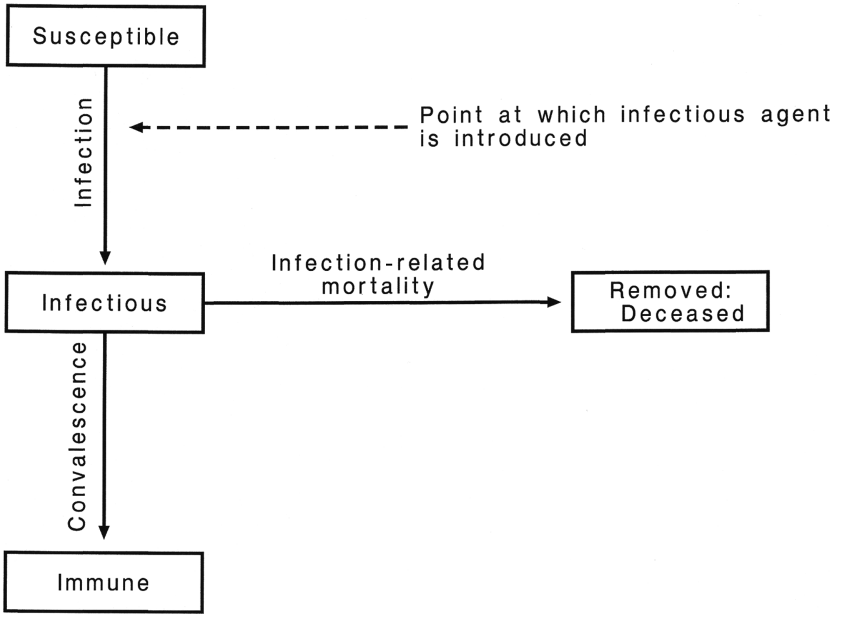

FIGURE 1.7 Epidemic transmission of a virus within a host population is represented by this type of compartment model (Hurst and Murphy, 1996). Each of the boxes, referred to as compartments, represents a decimal fraction of the host population with the sum of those decimal fractions equaling 1.0. The compartments which represent actively included members of the host population are those labeled susceptible, infectious, and immune. This model incorporates only a single category of removed individuals, representing those whose demise was due to infection related mortality. The solid arrows represent the rates at which individual members of the host species move between the different compartments during the course of an epidemic. Those rates of movement are often expressed in terms of individuals per day as described by Hurst and Murphy (1996). Used with permission of the author and Cambridge University Press.

as being biological. Conversely, the vector is termed to be mechanical if the virus does not increase it's numbers while in association with that vector. Beyond this there lie some deeper differences between mechanical and biological vectors. These differences include the fact that the acquisition of a virus by a biological vector usually involves a feeding process. Phagic habits of the biological vector result in the virus being acquired from an infected host when the vector ingests virally contaminated host body materials acquired through a bite or sting. Subsequent transfer of the infection from the contaminated biological vector to the virus' next host occurs when the biological vector wounds and feeds upon the next host. Actual transference of the virus to that next host occurs incidentally when the vector contaminates the wound by discharging viruses contained either in the vector's saliva, regurgitated stomach or intestinal contents, or else discharged feces and urine. Essentially any animal is capable of serving as a potential biological vector provided that the wound which it inflicts while feeding upon a host plant or animal will not result in the death of that new host until the virus would have had the chance to replicate within and subsequently be transmitted onward from that new host. There are many issues surrounding the question of what makes a good biological vector. These issues include: physical contact between the virus' host and the potential vector during a feeding event, viral reproduction within that potential vector, and that the infected vector be able to survive long enough to transmit the virus to a new host. It also helps if there is some factor driving the vector to pass along the infection, such as the

Waning immunity
Host progeny
Removed: Live, susceptible
Removed: Deceased
Removed: Live, immune
Susceptible individuals moving out
Susceptible individuals moving in
Vaccine-related mortality
Natural mortality
Infection-related mortality
Natural mortality
Natural mortality
Immune individuals moving out
Immune individuals moving in
Susceptible
Infection
Infectious
Convalescence
Immune
Host progeny
Host progeny
Host progeny
Infectious individuals moving out
Infectious individuals moving in
Host progeny
Removed: Live, infectious
Effective vaccination

virus finding it's way into the vector's saliva, or the virus increasing the physical aggressiveness of the vector.

The fact that biological vectors usually acquire the viral contaminant while wounding and ingesting tissues from an infected host brings us to another distinguishing difference between biological and mechanical vectors: viral contamination of a biological vector usually is associated with the virus being carried internal to the body of the vector. Replication of the virus then occurs within the body of the biological vector. Contrastingly, viral contamination of a mechanical vector usually occurs on the external surface of the vector and the virus subsequently tends to remain on the external surface of the mechanical vector. One possible example of mechanical vectoring would be the acquisition of plant viruses by pollinating animals such as bees and bats during their feeding process. These pollinators can serve as mechanical vectors if subsequently they are able to passively transfer the virus from their body surface to the next plant from which they will feed. In the case of these pollinators, the acquired virus presumably is carried external to the pollinator's body. Conversely, it is possible that a plant being visited by a pollinator might become contaminated by viruses afflicting that pollinator, and the plant could then passsively serve as a mechanical vector if subsequent pollinators should become infected when they visit that plant. Biting flies can serve as biological vectors if, during feeding, they ingest a pathogen which can replicate in association with that fly and then be passed onward when the fly bites it's next victim (Hurst and Murphy, 1996). Non-biting flies can passively serve as mechanical vectors if they feed upon contaminated material and then subsequently transmit those microbial contaminants to the food of a new host without that pathogen having been able to replicate while in association with the non-biting fly (Hurst and Murphy, 1996). Arthropods such as wasps, which repeatedly can sting multiple animals, could serve as mechanical vectors by transporting viruses on the surfaces of their stingers. Also, passive surface contamination of pets that occurs unrelated to a feeding event can result in the pets serving as mechanical vectors (Hurst and Murphy, 1996).

When a virus is transported inside the body of the vector, then that transportation is referred to as being an "internal carriage". Contrastingly, transportation of a virus on the external body surfaces of a vector is referred to as being an "external carriage". As will be described in volume 1, chapter 11, there are some plant viruses which are transported through internal carriage by invertebrates that represent mechanical vectors (because the virus does not increase its population level when in association with those invertebrates). Thus, although the biological vectoring of a virus usually involves internal carriage, the fact of internal carriage does not alone always indicate that

FIGURE 1.8 Endemic transmission of a virus within a host population is represented by this type of compartment model (Hurst and Murphy, 1996). This model is essentially an extension of the model presented in Figure 1.7. This model contains the same three compartments (susceptible, infectious, and immune) representing actively included individuals and the category of individuals removed by infection related mortality as were described for Figure 1.7. This model differs in that it must also consider the various possible categories of live removed individuals which can move into and out from the compartments of actively included individuals. Their removal represents the fact that they do not interact with the actively included individuals in such a way that the virus can reach them, often due to spatial isolation. This model also includes the fact that the immune status of individuals can naturally wane or diminish with time such that immune individuals return to the compartment labeled susceptible; production of host progeny, representing reproductive success of the members of the host species; natural mortality, as a means of removing members of the population; and the possible use of vaccination to circumvent the infectious process plus the associated vaccine - related mortality. Please notice that the progeny of infectious individuals may be susceptible, infectious, or immune at the time of their birth depending upon the type of virus which is involved and whether or not that viral infection is passed to the progeny. Used with permission of the author and Cambridge University Press.

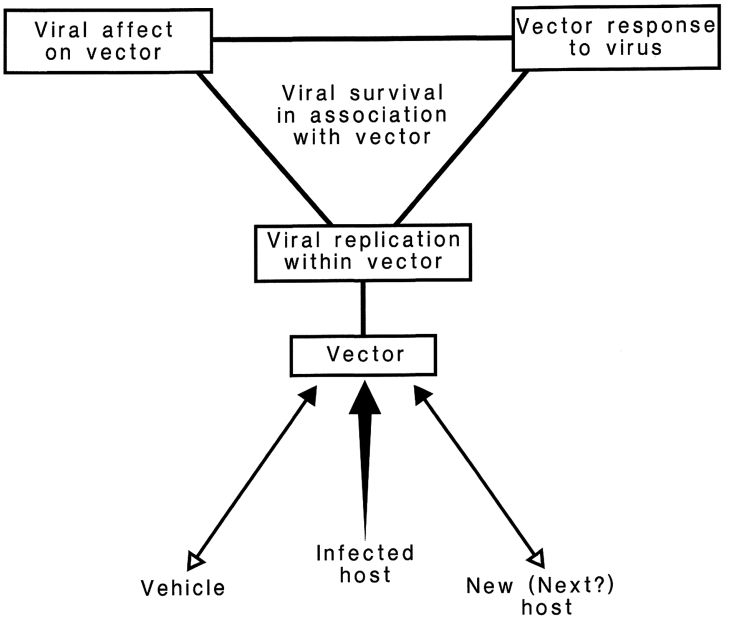

FIGURE 1.9 This figure addresses viral association with a biological vector and represents a segment from Figure 1.5. Vectors are, by definition, animate objects and are categorized either as 'biological', meaning that the virus increases in number during association with that vector, or 'mechanical', meaning that the virus does not increase in number during association with that vector. Biological vectors seem to have far greater importance than do mechanical vectors in terms of the spread of viral infections. Viruses can arrive at the biological vector (filled arrows) either directly from an infected host or via an intermediate vehicle. Transmission of the virus, via this vector, to a new host (or perhaps more accurately the 'next' host since, in the case of viruses, biological vectors may be considered as alternate hosts) requires that the virus both survive and replicate while in association with that biological vector. Thus, examining viral survival in association with a biological vector also involves considering the effects which viral replication has upon that vector and the response of that vector to the virus. Successful viral survival in association with the vector will allow a possible subsequent transfer of the virus to its next host either directly or via a vehicle (open arrows).

the vectoring is biological. Humans, interestingly, can serve as mechanical vectors via internal carriage for plant viruses that would be consumed with food and later excreted in feces (Zhang et al., 2006).

Because a virus must (by definition!) replicate in association with the biological vector; we can view the viral - vector association (Figure 1.9) in the same manner as was done for that of a virus and it's host (Figure 1.6). Indeed, it often is difficult to know which species is actually the viral host and which is actually the viral vector; to distinguish which is the victim and which serves as the messenger. Traditionally, we have often taken the view that humans are a high form of life and that there is a decreasing heirarchy down to the microbes. From this traditional, and sadly very anthropocentric, viewpoint we might assume that any living thing that transmits a virus between humans must be the vector as humans surely must be in the respectible position of serving as the host. Another version of this philosophy would consider a vertebrate to be the host and any invertebrate to be the vector. Still a third version has been based upon relative size, with the largest creature considered as the host and the smaller considered as the vector. Since we stated earlier that this chapter is intended to consider life from a virocentric perspective, we could easily accept the virocentric view which finds that there may be no clear distinction

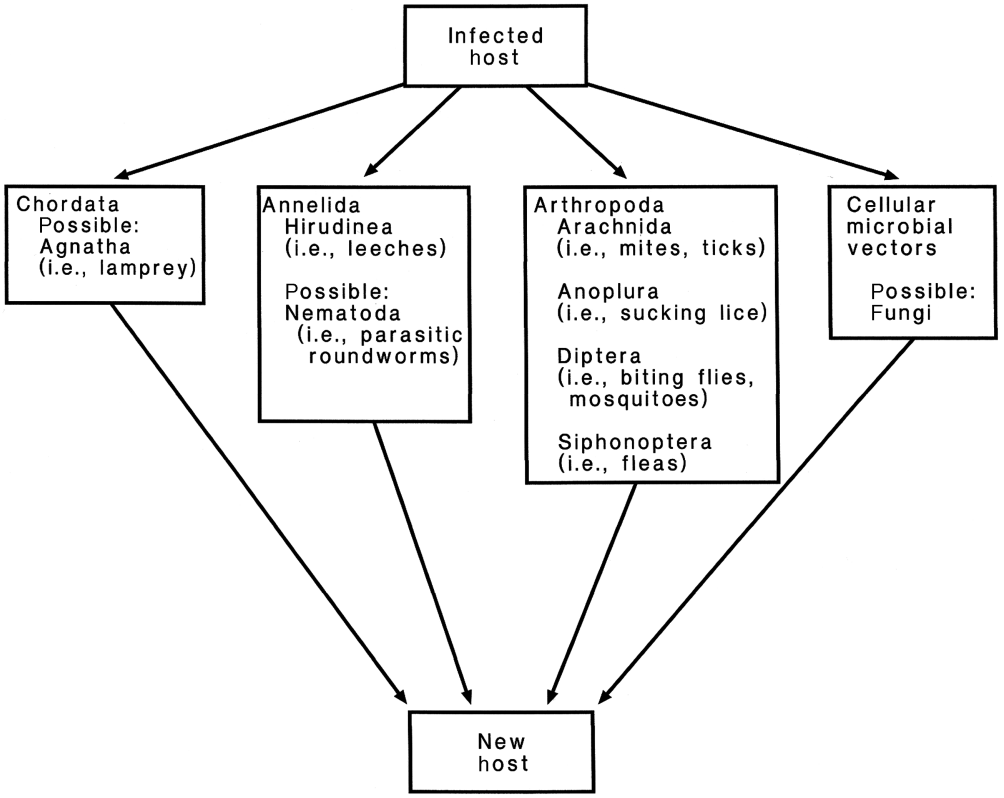

FIGURE 1.10 The transmission of a virus via a biological vector can be represented by this diagram. The virus is acquired as the biological vector feeds upon natural bodily fluids or else enzymatically liquified bodily components of the infected host. Subsequent transmission of the virus to a new host results when the vector releases contaminated excretions or secretions while feeding upon that new host.

between host and vector. Rather, any biological vector can likewise be viewed as a host. The argument as to which one, the traditional host or traditional vector, really serves as the host would then become moot.

Because many types of viruses are capable of infecting more than a single species of host, we are also left to ponder about determining which is the principle host versus those which serve as alternate hosts. Settlement of the distinction asked by this latter question is usually done by examining the comparative virulence of the virus in the different types of hosting species. That species for which the virus seems less virulent is assumed to be the more natural, most coevolved, host. It then is assumed that the species for which the virus seems to have greater virulence are alternate hosts. While trying to appreciate this conundrum, it must be understood that from a virocentric perspective both the principle and alternate hosts, as well as any biological vectors utilized by a virus, will all represent hosting species, and thus we may never be able to sort out the answers. Any further discussion of this particular issue is best left to only the most insistent of philosophers! Perhaps the only things left to be said of this issue are that examples of the transmission of a virus by a biological vector are represented in Figure 1.10, and that ecological interactions between a virus and it's principle hosts, alternate hosts, and biological vectors can be represented by the example shown in Figure 1.11.

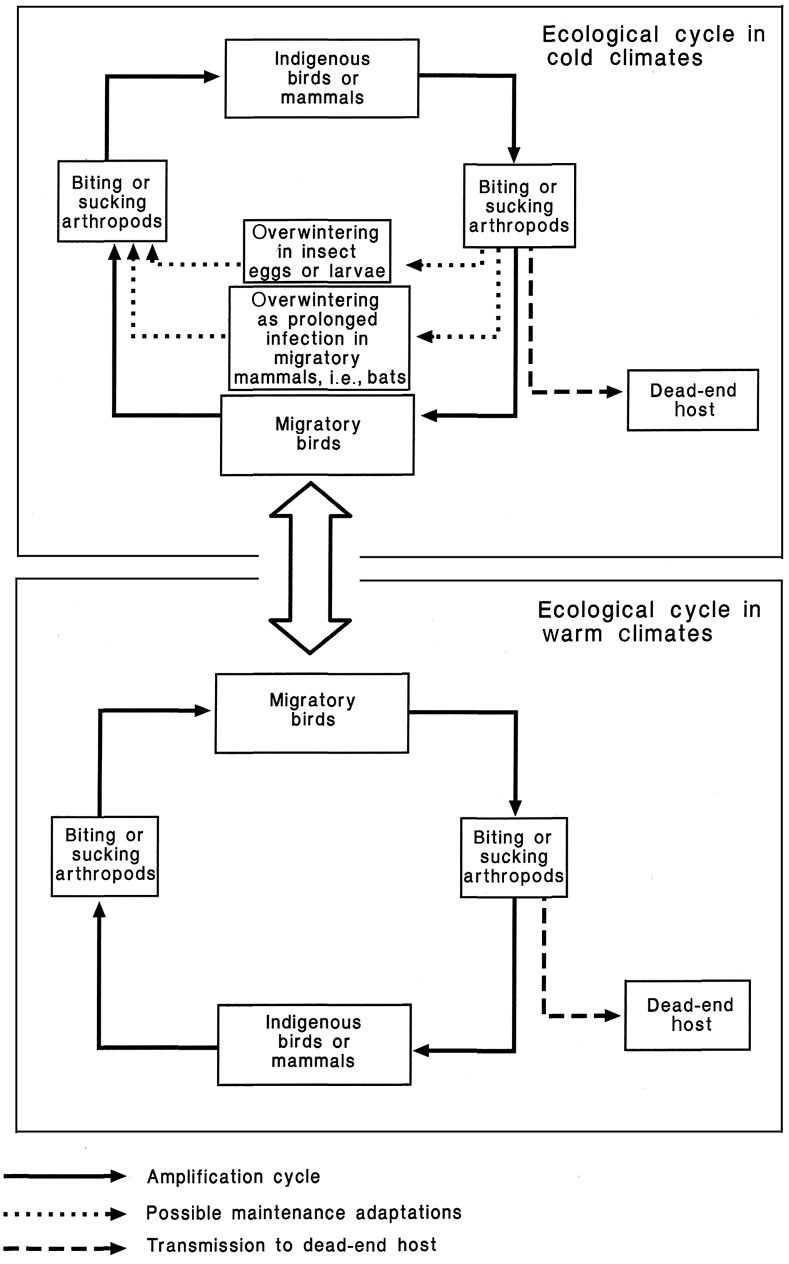

FIGURE 1.11 This figure represents a generalization of the ecological interactions which lead to insect-transmitted viral encephalitids. These infections generally are either enzootic or epizootic, meaning that their natural hosts are animals. Humans normally represent dead - end hosts for these viruses, meaning that the virus is not efficiently transmitted from infected humans to other hosts. The example shown in this figure is of a virus which has evolved ecological cycles both in warm, tropical climates and in cold, temperate climates. The cycle that has evolved in the warm climates can utilize arthropod vectors which do not have to go through the process of overwintering, thus allowing for an active year-round transmission cycle. Migratory birds, which may travel thousands of miles during their seasonal migrations, can shuttle the virus infection to the temperate zones. In the temperate zones, the virus' ecological cycle may need to include strategies for overwintering in insect eggs or larva and the possibility of survival as a prolonged infection in animals which may migrate lesser distances, such as bats.

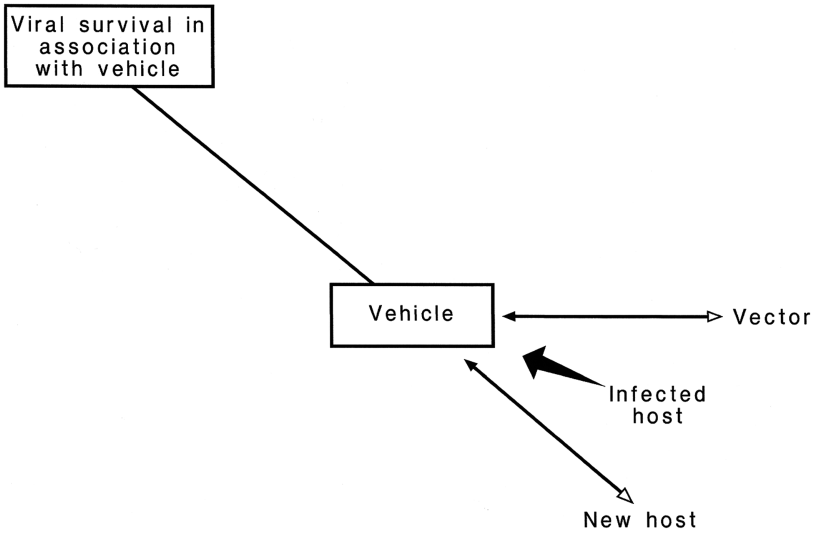

FIGURE 1.12 This figure addresses viral association with a vehicle and represents a segment from Figure 1.5. Viral transmission between hosts can occur by means of a vehicle. Vehicles are by definition inanimate objects. Viral contaminants can reach the vehicle (filled arrows) either directly from an infected host or via an intermediate vector. Transmission of the virus, via this vehicle, to a new host requires that the virus survive in association with the vehicle. Transference of the virus to its next host can occur either directly or via a vector (open arrows).

1.3.3 "In a Dirty Glass" (Going There by Vehicle)

Viruses also can be transmitted by vehicles. Vehicles are, by definition, inanimate objects. More specifically, the term vehicle applies to all objects other than living organisms. There are four general categories of vehicles and these are: foods, water, fomites (pronounced fo mi tez, defined as contaminated environmental surfaces which can serve in the transmission of pathogens), and aerosols. Figure 1.12 represents viral association with a vehicle. Transmission of the virus, via a vehicle, to a new host first requires contamination of that vehicle (shown by the filled arrows in Figure 1.12). The virus must then survive while in association with the vehicle. Because viruses are by definition obligate intracellular parasites, and by definition vehicles are non-living, then a virus neither can replicate on nor within a vehicle. Likewise, because vehicles are by definition non-living, we do not expect that any specific antiviral response will be produced by the vehicle. Transference of the virus to its next host can occur either directly or via a vector (shown as the open arrows in Figure 1.12). One possible indication as to the difference between a vector and a vehicle is that, while a live mosquito can serve as a biological vector, after it's death that same mosquito instead represents a vehicle. The transmission of a virus via a vehicle can be represented by the diagram shown in Figure 1.13. Acquisition of the virus by the next host or vector from that contaminated vehicle results from either ingestion of the vehicle (associated with foods and water), surface contact with either contaminated water or a contaminated solid object (a fomite), or inhalation (aerosols). Although, from a human perspective, we might tend to associate waterborne transmission with animals and in particular human diseases (volume 2 chapter 13); the waterborne approach will play a major role in viral transmission for viruses that infect cyanobacteria (volume 1 chapter 6), algae (volume 1 chapter 7) and seaweeds (volume 1

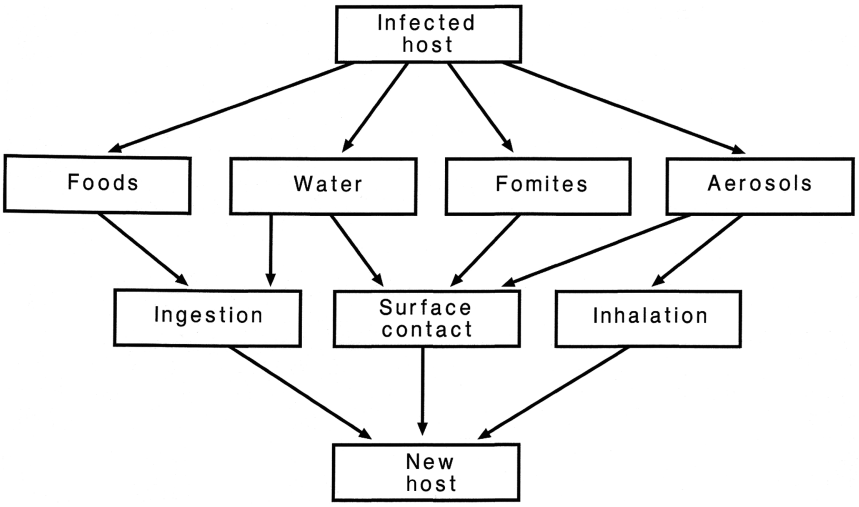

FIGURE 1.13 The transmission of a virus via a vehicle can be represented by this diagram. Food items can be contaminated by the action of an infected host. Alternatively, the food in question may actually be the body of an infected host that subsequently is consumed by a susceptible, predatory new host. Viral contaminants present in water can be acquired by a new host either directly, as the result of external or internal exposure to the contaminated water including ingestion of the water; or indirectly, following contact between the new host and an environmental surface (serving as a secondary, intermediate vehicle) that has been contaminated by that water. Fomites are solid environmental (non-food) objects whose surfaces may be involved in the transfer of infectious agents. Viral aerosols may result in the infection of a new host either directly through inhalation of the aerosol, or indirectly following contact between the new host and some other vehicle (either food, water, or a fomite) contaminated by that aerosol.

chapter 8). The are even viruses of terrestrial plants, including some carmoviruses of the viral family Tombusviridae, which seem as though they might be transmitted by water. The list of vehicles associated with viral transmission even includes agricultural tools and other work implements. The topic of vehicle-associated transmission of pathogens is discussed at length in the reference by Hurst and Murphy (1996).

1.3.4 Bringing Concepts Together

Biological entities exist over a spectrum of complexities, ranging from the viruses, viroids and prions (yes, even the prions are biological entities!) to multicellular organisms. The process of maintaining the viability of even the largest of organisms is, and perhaps must, be organized at small levels. Biologically, this has been achieved by a highly evolved process of internal compartmentalization of functions with a systemic coordination. If we consider for a moment one of the most enormous of the currently living multicelled organisms, the blue whale (*Balaenoptera musculus*), we notice that this kind of compartmentalization and coordination begins all of the way down at the level of the subcellular structures and organelles within each individual cell. The compartmentalization and coordination then continue upward through a number of levels including the various individual types of cells, the tissues into which those cells are organized, the organs which the tissues comprise, and finally the total internal coordination of all of these through nerve signaling and hormonal regulation. At every one of these biological levels there is a "taking from" and a "leaving behind" exchange of material with respect to the immediate surrounding environment. This results in the existence of dramatic environmental differences at all levels, even down to the many microenvironments which exist within the organizational regions of a single cell.

Every virus must try to comply with the basic biological imperatives of genetic survival and replication. While complying with these imperatives the viruses must, as obligate intracellular parasites, not only face but also survive within and successfully be transported through the various environments which are internal to the host. Those viruses which are transmitted by biological vectors must also have evolved the capability to survive and be transported through internal environments faced within the vector. Viruses which are transmitted by mechanical vectors generally must possess an additional evolved ability to survive on the surface of that vector. Likewise, both those viruses transmitted by mechanical vectors and viruses transmitted by vehicles must possess the ability to survive exposure to natural ambiental environments encountered either in the atmosphere, hydrosphere or lithosphere. These numerous environments are summarized in Figure 1.14. Conditions confronted at the interface zones, as indicated by the dashed lines in Figure 1.14, represent areas of still additional environmental complexity. While viruses appear biologically inert when viewed in the ambiental environments, they display their biology and interact with their surroundings when they reach the environments internal to their hosts and biological vectors.

The adaptability of a species in terms of its biological cycle and biological needs will determine that species' potential distribution range. This potential distribution range is limited in actuality to a smaller range based upon interspecies relationships and competitions. Ourselves being large multicellular creatures, we humans normally think of a distribution range as being geographical in nature. As microbiologists, many of us have come to understand the concept of distribution range in finer detail; an example being the depth within a body of water where a particular species of microorganism normally will be found. At the level of viral ecology, the concept of species distribution range encompasses everything from tissue and organ tropisms (those tissues and organs which a virus seems to attack preferentially) upwards to the geographical availability of host species, vector species, and the prevailing directional flow of appropriate vehicles such as air and water. The larger, geographical end of this scale is represented in Figure 1.15.

While considering the factors addressed in Figure 1.15, it is important to keep in mind that albeit the virus' election of hosts, vectors, and routes of transmission would all originate by random chance, the attainment of reliable continued viral success would require that such random selection events be followed and strengthened by evolution. This explains the reason why viruses do not appear suddenly to develop the ability to use a different vehicle. Indeed, it is perhaps likely that in order to use a vehicle such as air or water, the virus must have preadapted itself to the conditions which it will encounter in association with that vehicle. Nearly each individual species of virus which achieves transmission by vehicles, seems invariably to use only one type of vehicle. This trait likewise seems to hold true for all species belonging to any given viral genus. Furthermore, this identification seems to nearly always hold true at the level of viral family. In fact, this is one of the defining characteristics of the ecology of a viral group. The only virus which seems to have evolved the ability to utilize more than a single vehicle is the Hepatitis A virus (Hurst and Murphy, 1996), which has evolved a most remarkable ability to be effectively transmitted both by water and on fomites. Perhaps accordingly, the Hepatitis A virus currently exists in a genus (*Hepatovirus*) of its own. We should not be surprised if we eventually would discover other members of that viral genus, and subsequently discern those other members to likewise use these same two vehicles. It is for these reasons, that fears expressed in the public press that viruses such as Ebola will suddenly take flight and be transmitted over large distances via aerosol transmission amount to nothing more than frightening speculation. Why is it just speculation? Because that route of transmission is not a part of the

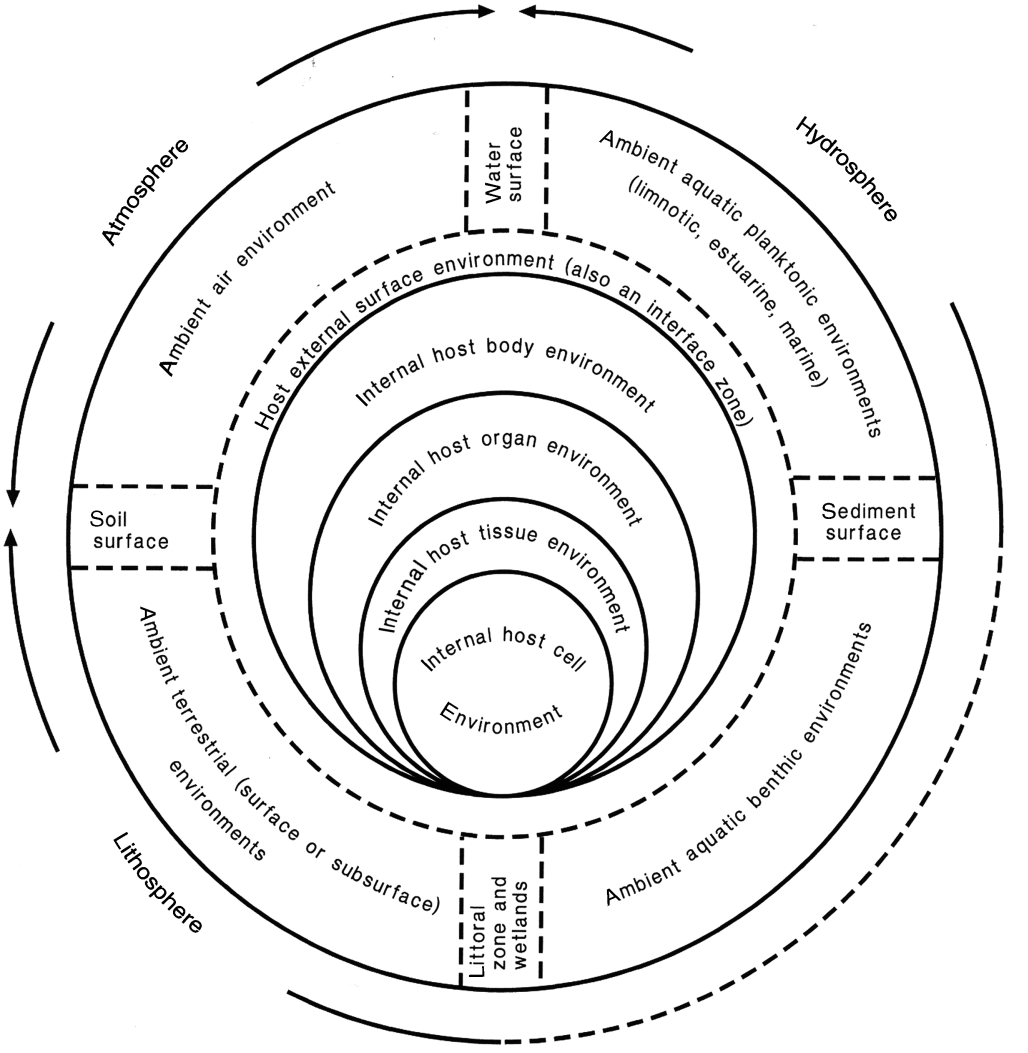

FIGURE 1.14 This figure integrates the concepts of host, vehicle and biological vector by representing the environments potentially faced by a virus. As obligate intracellular parasites, the viruses must face, survive within, and successfully be transported through environments which are internal to the host. Those viruses which are transmitted by biological vectors must also have evolved the capability to survive and be transported through internal environments faced within the vector. Viruses which are transmitted by vehicles and mechanical vectors must additionally possess an evolved ability to survive in natural ambiental environments (atmosphere, hydrosphere and lithosphere). Conditions confronted at the interface zones, as indicated by dashed lines, represent areas of additional environmental complexity.

virus' ecology. Invasive medical devices such as syringes, endoscopes and other surgical implements, plus transplanted animal tissues including transfused blood and blood products, and grafted plant material, represent exceptions to this rule. These devices and transplanted tissues represent unnatural vehicles which, by their nature, allow the virus an abnormal access to the interior of a new host (Hurst and Murphy, 1996). Any virus which would naturally be transmissible by direct contact with either an infected host or any type of

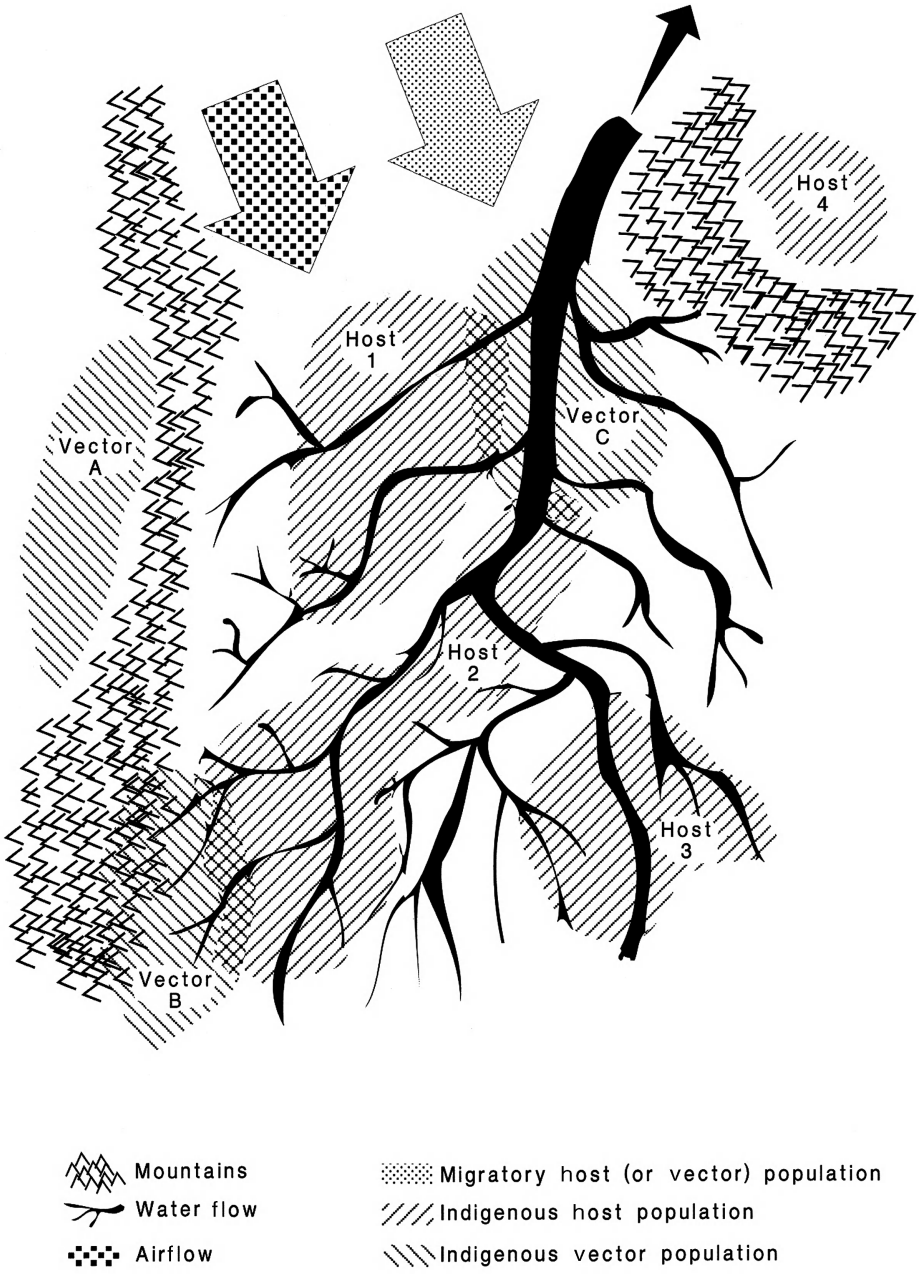

FIGURE 1.15 This figure presents a hypothetical example of the way in which the ecology of a virus is delineated by the spatial relationships between its potential hosts, vectors, and vehicles. The figure represents a viral infection existing in a watershed basin whose area covers tens of millions of hectares. An assumption is made that the four potential indigenous host populations and three potential indigenous vector populations are terrestrial organisms whose ecological areas are delineated and that these organisms do not migrate outside of their own respective ecological areas. Indigenous host populations 1, 2, and 3 reside in riverine ecological areas within the basin. Indigenous vector population B has a highland ecology, while vector population C has a lowland ecology, and both of these vector populations reside within the basin. Indigenous vector population A and indigenous host population 4 are excluded from participation in the viral infection cycle due to their geographical isolation and, because of their

vector can also be transmitted by one of these unnatural vehicular routes.

Viruses occasionally will appear in association with "apparently new" (unexpected) hosts and biological vectors. These latter occurrences with unexpected hosts or vectors represent the identification of sporadic events which occur when geographical boundaries are breached by the movement of those potential hosts and vectors for which the virus in question already has a preevolved disposition. These preevolved dispositions may represent, at some basic level, the renewal of old acquaintances between a virus, vector, and host. Alternatively, if these particular viral, host, and vector species truely never have met before, then an important aspect which can factor into these encounters is the biological relatedness between these "apparently new" hosts or vectors and those other hosts or vectors which the virus more normally would use.

1.3.5 Is There no Hope?

Many host-related factors do play a role in the transmission of viral-induced illnesses. These include:

"Finding the wrong host"– the "oops" or accidental occurrence factor wherein viruses occasionally will encounter and successfully infect living beings other than their natural hosting species, an event which represents a mistake not only for the host (which often will be fated to die for want of having inherited an evolved capability to mount an effective defense against that virus) but also is a mistake for the virus (which often will not be able to subsequently find one of its natural hosts and hence also loses it's existence);

"Only the good die young"– culling the herd for communal protection can have some advantage for the host population as a whole if those individuals that demonstrate a lesser ability to resist the virus are weakened enough by the infection that they then are more easily killed by predators (this is an act that both reduces the likelihood that other members of the host population will become infected by that virus strain and also may improve the gene pool of the host species by selectively eliminating it's most susceptible members);

"Being your own worst enemy"– behavioral opportunities for disease transmission do exist, and ethnic or social customs often play a role in disease transmission (including the probable reality that a lack of male circumcision has spelled disaster for the human population of Africa by facilitating the heterosexual transmission of HIV) (Caldwell and Caldwell, 1996), and in fact most of those vector borne diseases that aflict humans can be avoided by changes in host behavior.

If we view this situation from the human perspective, there does exist a basis for hope in terms of the health of hosts. Our most important

geographical exclusion from the basin, we do not need to be concerned with the nature of their ecological zones. Vector population B is capable of interacting in a cycle of transmission involving host population 2. Vector population C is capable of interacting in a cycle of transmission invloving host populations 1 and 2. None of the indigenous vector populations is capable of interacting in a cycle of transmission involving host population 3. A virus capable of being transmitted by surface waters could move from host population 3 to host population 2, since host population 2 is located downstream of host population 3. That same surface waterborne route could not spread the virus to host population 1, because host population 1 is not situated downstream of either host populations 2 or 3. Likewise, neither could the surface waterborne route spread the virus in a upstream direction from host population 1 to host population 2, nor from host population 2 to host population 3. Alternatively, a migratory host or vector population could carry the virus from host population 1 to host populations 2 and 3, as likewise could air flow if the virus is capable of being transmitted as an aerosol.

TABLE 1.1 Categories of Physical Barriers

Thermal
Acoustic (usually ultrasonic)
Pressure
barometric
hydrostatic
osmotic
Radiation
electronic
neutronic
photonic
protonic
Impaction (includes gravitational)
Adhesion (adsorption)
electrostatic
van der Waals
Filtration (size exclusion)
Geographic features
Atmospheric factors (includes such meterological aspects as humidity, precipitation, and prevailing winds)

advantage lies in the use of barriers, which represent a very effective means by which we can reduce the transmission of all types of infectious agents. Barriers can be classified by their nature as physical (Table 1.1), chemical (Table 1.2), and biological (Table 1.3). In many cases, these barriers already exist in nature. Natural examples of barriers include both high and low temperatures (thermal, a physical barrier), sunlight (radiation, a physical barrier), the natural salinity of water (both osmotic, a physical barrier and also dessicant, a chemical barrier), and ecological competition (competitive, a biological barrier). The intentional use of barriers can involve both individual and combined applications. One example of a combined barrier application is the retorting of canned products, a process which employs a combination of elevated temperature and hydrostatic pressure to achieve either disinfection or sterilization (this process is similar to autoclaving). Many of these barrier concepts, such as filtration acting as a physical barrier, can be applied at different levels. For example: some particle exclusion filtration devices have pore sizes small enough that they can act as a filtration barrier against virus particles themselves; natural latex condoms and disposable gloves act as filtration barriers against a liquid vehicle (they contain pores which are larger than the virus particles yet smaller than the droplets of liquid in which the virus is contained); window screens and mosquito netting act as filtration barriers against flying vectors; and walls, fences, doors and gates can act as filtration barriers against infected hosts. The ingestion of food and water is associated with digestive treatments such as pH changes and secreted enzymes, both of which represent chemical barriers. When viewed from the virocentric perspective, the use of barrier techniques for preventing viral transmission would represent cause for despair instead of hope. There is, however, a notable exception represented by the idea of some viruses such as the polyhedrin- forming members of the viral families Baculoviridae and Reoviridae seem to require digestive treatment as an aid to their infectivity for their insect hosts.

TABLE 1.2 Categories of Chemical Barriers

Ionic (includes pH and salinity)
Surfactant
Oxidant
Alkylant
Desiccant
Denaturant

TABLE 1.3 Categories of Biological Barriers

Immunological (includes specific as well as nonspecific)
naturally induced (intrinsic response)
naturally transferred (lacteal, transovarian, transplacental, etc.)
artificially transferred (includes injection with antiserum and tissue transfers such as transfusion and grafting)
Biomolecular resistance (not immune-related)
lack of receptor molecules
molecular attack mechanisms (includes nucleotide-based restrictions)
antibiotic compounds (metabolic inhibitors, either intrinsic or artificially supplied)
Competitive (other species in ecological competition with either the virus, its vectors, or its hosts)

1.4 WHY THINGS ARE THE WAY THEY ARE

The ability of a virus to pass on it's genetic content is the key consideration of the virus. We now understand how this gets done on a molecular level. What still remains to be understood are how this thing gets done and has come about at the species level.

1.4.1 To Kill or Not to Kill - A Question of Virulence

One of the nagging questions which a virus must face is what should be the extent of it's virulence, i.e., whether or not it should kill it's hosts and biological vectors as a consequence of their encounters (Ewald, 1993; Lederberg, 1997). When considered in purely evolutionary terms, virulence is the ability of the disease agent to reduce reproductive fitness of that host. The relative virulence of a virus with respect to one of it's hosts or biological vectors is generally presumed to be a marker of co-evolution. More specifically stated, it seems that the less virulent is the virus for one of it's hosts or vectors, the more greatly coevolved is the relationship. Why should this be so? It should clearly be the case that, were a virus to infect an individual member of a host or biological vector population prior to that individual having reached reproductive age, it would be in the virus' best interest to not kill that host or vector. Contrariwise, in a very strict sense, death of that host or biological vector should not matter to the virus if that individual host or biological vector has passed the end of the normal reproductive lifespan. The reason for this latter philosophy is that, even if this particular host were to survive, it would not produce more susceptible offspring. Additionally, within each species of potential host or biological vector, there would be a strong genetic drive to enable their infants to mount sufficient immunological defense so as to reach the age of reproductive maturity. That same genetic drive does not, by definition, act upon the preservation of individuals who have passed their reproductive years. One example of the result from interaction of these forces is the fact that infections caused by the Hepatitis A virus can go nearly unnoticed in human infants, yet Hepatitis A virus infections can be disastrous in human adults.

Figure 1.16 represents the question of how the success of a virus relates to its' virulence. The virus will not be successful if the result of viral infection is too deleterious in terms of affecting the ability of the present host or biological vector to survive before that virus has been able to achieve transmission to it's next host or biological vector.

1.4.2 Genetic Equilibrium (versus Disequilibrium)

One of the hallmarks of relationships between virus species and their host species is their apparent goal of reaching a mutually acceptable genetically-based equilibrium (Dennehy et al., 2006; Lederberg, 1997; Zinkernagel, 1996). Some viruses also seem to have interchanged genetic material with their hosts while striving to evolutionarily reach a level of mutual coexistence.

There are many considerations associated with an apparent genetic equilibrium. In most instances of endemic viral infection in populations of a coevolved host or biological vector, the infections appear relatively unnoticed or relatively innocuous. This may change when the virus encounters a concentrated population of genetically similar susceptible hosts or biological vectors concentrated within a small radius, perhaps resulting in an epidemic. It also may change when the virus invades a population of novel hosts or vectors (hosts or vectors to which that virus appears to be new); this is termed a "biological invasion". Excessive virulence may represent reduced genetic fitness with respect to the virus, host, or biological vector. Limited virulence on the part of the virus seems to represent a state of coevolution but with some remaining flux in the virus-host

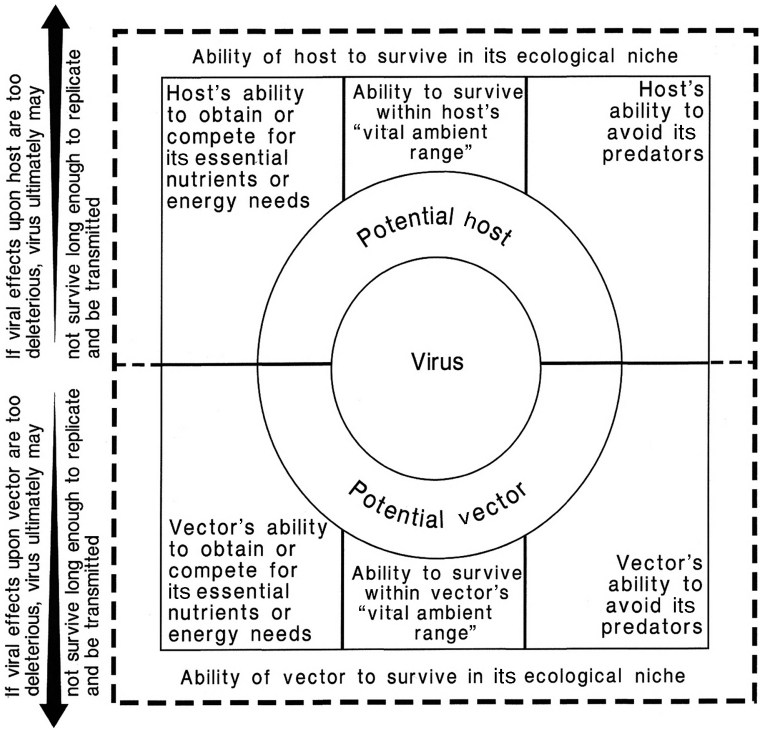

FIGURE 1.16 This figure represents the question of how the success of a virus relates to its' virulence. Success requires that the virus replicate within the bodies of its hosts and any biological vectors to concentrations which are high enough that the virus has a reasonable chance of being passed onward to infect either its next host or its next biological vector. The virus will not be successful if, within this period of replication, the result of viral infection is too deleterious in terms of affecting the ability of the present host or biological vector to survive within its own respective ecological niche. The survival requirements of those potential hosts and biological vectors include: the respective ability of those hosts and biological vectors to compete for their essential needs; their ability to survive within their own vital ambiental range as defined by factors such as temperature, plus either humidity and altitude (if terrestrial) or depth and salinity (if aquatic); and their ability to avoid being consumed by predatory individuals.

interaction. This state may have a beneficial effect by acting as a genetic screening upon both the host species and the viral species. In contrast, avirulence may represent a far more evolved steady state, although evolutionarily it may not be the final state, between the viral and host populations. Avirulence is normally acquired by repeated successive passage of the virus through members of a host or biological vector population.

What are the considerations associated with an apparent genetic disequilibrium? If the virus seems to make all of the members of a species extremely sick, then presumably it normally may not be hosted or vectored by that species. If a virus causes a reduction in the genetic fitness of the host (ability of the host to pass on it's genetic heritage) then the virus is viewed as being in disequilibrium with the host. Incompatible genetic differences may both fuel the fires of virulence and allow a constant state of genetic disequilibrium to exist. Genetic equilibria need time to establish. Constant disequilibrium may be viewed as a competitive strategy effected via "Evolutionary Cheating" (included in loving memory of Dr. Alex Frasier who taught me to understand evolution). Evolutionary cheating involves finding ways to change the rules of fair competition and thereby tilt the playing field in favor of your species. One good

example of evolutionary cheating would be to eat your competing species. Viruses tend to steal genes from their hosts (Balter, 1998), and this would represent another example of evolutionary cheating.

1.4.3 Uniqueness versus Commonality (There Are Hussies and Floozies in the Virus World)

1.4.3.1 Numbers of Major Viral Groups (Viral Families and Floating Genera) Affecting Different Host Categories:

From examining the list of approved viral taxonomic groups published by the ICTV (International Committee on Taxonomy of Viruses, Master Species list for November 2009, which is available as 2009_5F00_v3 on their website http://www.ictvdb.org/) is was possible to determine the host ranges of the 100 major viral groups (88 families plus 12 unassigned or 'floating' genera). These groups are listed alphabetically in Table 1 of Chapter 2 (if you are curious, searching each of those 2,289 viral species on the internet took 8 days of diligence). From that knowledge, the relative specificity of those major viral groups can be ascertained with regard to the host categories for which they are infective. Each of the major viral groups was associated only with either prokaryotic hosts or eukaryotic hosts. As such, none of the major viral groups crossed the imposing biochemical divide between prokaryotes and eukaryotes.

1.4.3.1.1 Prokaryotic Host Categories

There are 18 known major viral groups that are associated with prokaryotic hosts, and summarizing these by category of host the results are:

Archaea - a total of 10 major viral groups contain member species which infect archaea, with 8 of those viral groups being unique to only this host category, and the other 2 viral groups being common which means that they include viral species infective of additional host categories;

Bacteria - a total of 10 major viral groups contain member species which infect bacteria, with 7 of those viral groups being unique to only this host category, and the other 3 viral groups being common which means that they include viral species infective of additional host categories;

Cyanobacteria - a total of 2 major viral groups contain member species which infect cyanobacteria with none of those viral groups being unique to only this host category.

Among those major viral groups associated with prokaryotes, we can assess which groups have commonality as expressed in terms of their possesing a general capacity for association with more than one host category (the hussies!), and those are:

1 viral group is common to Archaea + Bacteria

1 viral group is common to Archaea + Bacteria + Cyanobacteria

1 viral group is common to Bacteria + Cyanobacteria

1.4.3.1.2 Eukaryotic Host Categories

There are 82 known major viral groups that are associated with eukaryotic hosts, and summarizing these by category of host the results are:

Algae – a total of 4 major viral groups contain member species which infect algae, with 1 of those viral groups being unique to only this host category, and the other 3 viral groups being common which means that they include viral species infective of additional host categories;

Fungi – a total of 14 major viral groups contain member species which infect fungi, with 6 of those viral groups being unique to only this host category, and the other 8 viral groups being common which means that they include viral

species infective of additional host categories;

Invertebrates – a total of 22 major viral groups contain member species which infect invertebrates, with 9 of those viral groups being unique to only this host category, and the other 13 viral groups being common which means that they include viral species infective of additional host categories;

Plants – a total of 33 major viral groups contain member species which infect plants, with 25 of those viral groups being unique to only this host category, and the other 8 viral groups being common which means that they include viral species infective of additional host categories;

Protozoa – a total of 3 major viral groups contain member species which infect protozoans, with 1 of those viral groups being unique to only this host category, and the other 2 viral groups being common which means that they include viral species infective of additional host categories; and

Vertebrates – a total of 33 major viral groups contain member species which infect vertebrates, with 22 of those viral groups being unique to only this host category, and the other 11 viral groups being common which means that they include viral species infective of additional host categories.

Among those major viral groups associated with eukaryotes, we can assess which groups have commonality as expressed in terms of the general capacity for association with more than one host category (the hussies!), and those are:

Viruses Infecting only Microbial or Botanical Hosts

1 viral group is common to Algae + Protozoa

1 viral group is common to Fungi + Protozoa

3 viral groups are common to Plants + Fungi

Viruses Infecting Invertebrate Animal Hosts

1 viral group is common to Invertebrates + Fungi + Plants

1 viral group is common to Invertebrates + Fungi + Plants + Algae

Viruses Infecting Vertebrate Animal Hosts

7 viral groups are common to Invertebrates + Vertebrates

1 viral group is common to Invertebrates + Vertebrates + Fungi

2 viral groups are common to Invertebrates + Vertebrates + Plants

1 viral group is common to Invertebrates + Vertebrates + Fungi + Plants + Algae

The absolute floozies were the Reoviridae, a viral family that produces infectious virions and presently is known to have representation in five host categories of eukaryotes excepting only the protozoa; and the Metaviridae and Pseudoviridae which are the two viral families that represent LTR (long terminal repeat) retrotransposons and are known to each be associated with four host categories of eukaryotes.

Table 1.4 gives an asessment of relative specificity in terms of the percentage of major viral groups that were determined associated with (unique to) only a single host category, plus those major viral groups that were associated with only one additional host category. The absolute numbers of viral groups associated with each host category differed, with the greatest numbers of viral groups being known for vertebrates and plants. This relative wealth of information may be an absolute indication that in fact some host categories are more fertile ground for the evolution of new viral groups, but there also is an important associated truth which is that this difference in numbers of identified viral groups likely reflects the far greater amount of time and money that have been spent on researching viruses of vertebrates and plants. Among the eukaryotic host categories, those major viral groups infective for plants and vertebrates tended to be more

TABLE 1.4 Relative Specificity of the Viral Taxonomic Groups as Compared by Category of Host

Host Category	Viral groups unique to that host category	Viral groups common to one additional host category	Summary of viral groups either unique to that host category or common to just one additional host category
Eukaryotes			
Algae	25% (1 of 4)	25% (1 of 4)	50% (2 of 4)
Fungi	43% (6 of 14)	29% (4 of 14)	71% (10 of 14)
Invertebrates	41% (9 of 22)	32% (7 of 22)	73% (16 of 22)
Plants	76% (25 of 33)	9% (3 of 33)	85% (28 of 33)
Protozoa	33% (1 of 3)	67% (2 of 3)	100% (3 of 3)
Vertebrates	67% (22 of 33)	21% (7 of 33)	88% (29 of 33)
Prokarytoes			
Archaea	80% (8 of 10)	10% (1 of 10)	90% (9 of 10)
Bacteria	70% (7 of 10)	20% (2 of 10)	90% (9 of 10)
Cyanobacteria	0% (0 of 2)	50% (1 of 2)	50% (1 of 2)

The viral taxonomic groups represented in this table are the 88 families and 12 floating genera currently listed by the ICTV (International Committee on Taxonomy of Viruses, Master species list of November 2009 (2009_5F00_v3) which is available on the website http://www.ictvdb.org/) and those groups are listed along with their host ranges in Table 1 of Chapter 2).

unique, ranging from 67–76%, with the extent of uniqueness being either 43% or less for viral groups associated with the other categories of eukaryotes. Among the prokaryotic host categories, those major viral groups infective for archaea and bacteria tended to be more unique, ranging from 70–80%, while the extent of uniqueness was zero for viral groups associated with the only other category of prokaryotes, which was the cyanobacteria. The vast majority of the major viral groups either were unique to a single host category or common to only one additional host category (71–100%) except for the viruses of algae and cyanobacteria (50%).

1.4.3.2 What Might be Reflected When We Look at the Concept of Uniqueness versus Commonality for the Major Viral Groups?

Figure 1.17 gives a visual representation for this concept of assessing uniqueness versus commonality. The most obvious separation was observed to be an apparently absolute distinction between those major viral groups associated with eukaryotic host categories (Figure 1.17a) versus prokaryotic host categories (Figure 1.17b). The second most obvious separation is not quite as absolute, but nevertheless represents a clear distinction between viral groups associated with animals versus non-animals. Among those major viral groups associated with animals, the majority of commonalities were limited to the host categories of vertebrates and invertebrates, with only a relatively small percentage of those viral groups extending between the animals and non-animals. Among those major viral groups associated with non-animals, the majority of commonalities were between the host categories of fungi and plants. Half of those viral groups which were common to fungi and plants were able to cross the divide into invertebrates.

Invertebrates often serve as biological vectors for viruses, and this accounts for many, but not all, of the viral group associations which exist between the host categories of invertebrates and either vertebrates, fungi, or plants. It also is very possible that the apparent separations or 'divides' visualized as we examine Figures 1.17a. and 1.17b. can give us clues as to when the presently known major viral groups evolved, i.e., that all presently known viral groups may have arisen since the separation of prokaryotes and eukaryotes, with there being a second major point representing the development of animals.

1.4.4 Evolution

As we look at the relationships between viruses and their hosts and vectors, we might ask ourselves that age-old question of "Which came first, the virus or the cell?" (Koonin et al., 2006). It is perhaps more likely that the viruses and cells arose simultaneously. Presumably they have been struggling to come to terms for a long time, (Claverie, 2006; Forterre and Prangishvili, 2009). We do not

Figure 1.17a

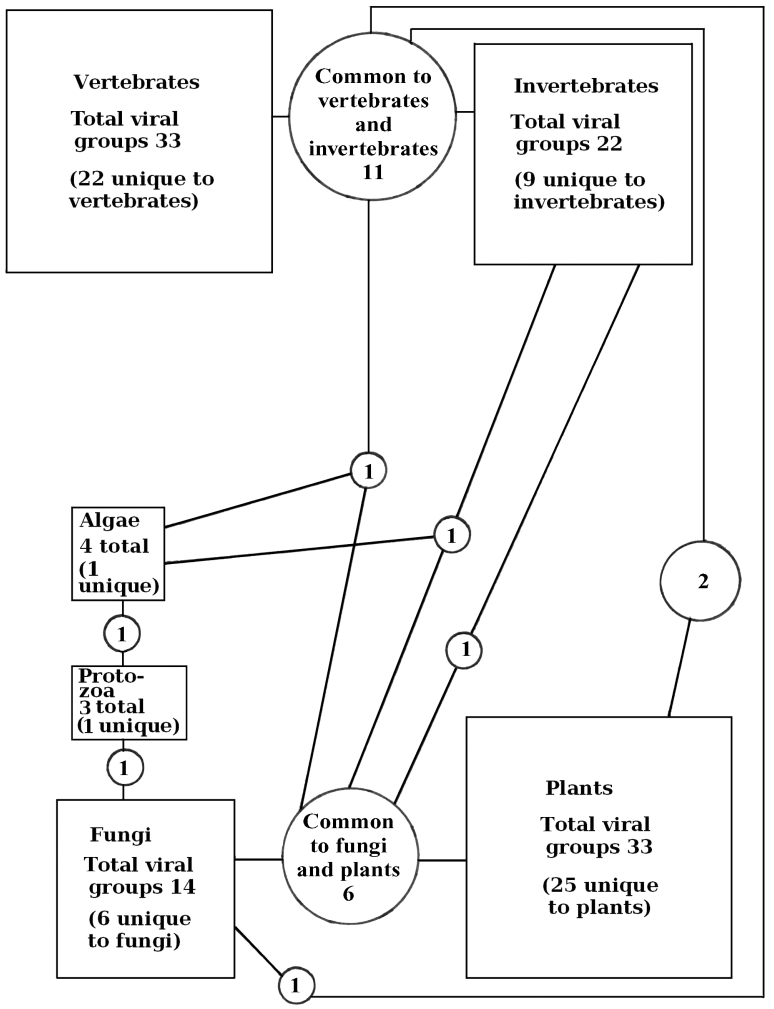

FIGURE 1.17 This figure represents the number of major viral groups, those having the taxonomic classification level of either family or unassigned "floating" genus, know to be associated with eukaryotic host categories (Figure 1.17a) and prokaryotic host categories (Figure 1.17b). The boxes represent host categories. The circles represent interconnections, which are zones that illustrate the fact that many of the major virus groups overlap and are common to more than a single host category. The areas within the boxes and circles are in relative proportion to the numbers of viral families and floating genera being represented, thus giving a visual presentation of viral diversity. The connecting lines represent possibilities for viral-mediated gene flow between host categories. To date, there are no viral families or floating genera known capable of crossing the boundary between eukaryotic and prokaryotic host categories. The names of the virus families and floating genera are listed in Table 1 of chapter 2.

Figure 1.17b

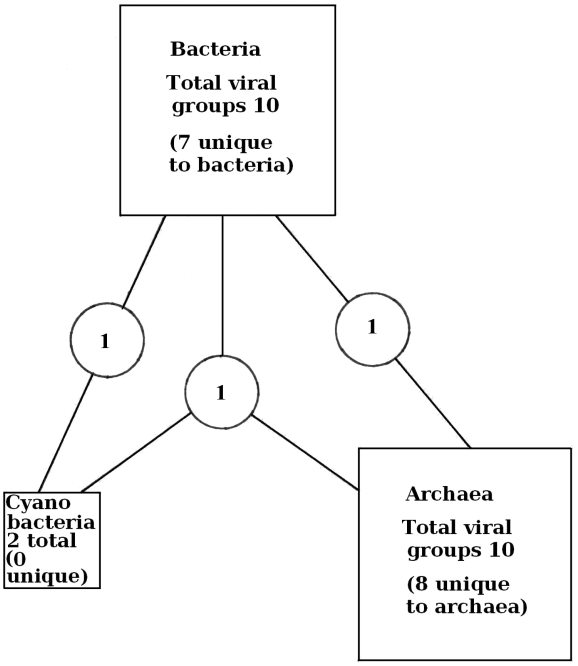

FIGURE 1.17 *(Continued).*

know either to what, or to where, the viruses are leading. Although in a true biological sense it is not necessary for the viruses to "lead" anywhere. From a virocentric view, a perfectly organized virus reproducing from host to host (perhaps with a few vectors included for spice) and transmitting its genetic information over time is a sufficient trend. In considering the evolution of viruses, we must remember the wisdom of Niles Eldredge (1991), that no existing biological entity can be said to represent an end product of evolution. Rather, it is only the extinct biological life forms that clearly can be said to have represented end products of evolution. Likewise, we do not and perhaps never may know if viruses arose only once or else have arisen at many times, with their evolutionary arisal bounded only by the practical limits of some definable adaptive zone. Understanding this comes from the realization that thus far, sabre-toothed cats have evolved at three different times during history and that they evolved from different lineages (Eldredge, 1991). Their evolution at each time would have corresponded to the opening of the appropriate niche, and each of their extinctions would have corresponded to the closing of that niche. For just as it is true that the availability of a niche can drive evolution, so too can the closure of a niche drive extinction.

Although the lack of viral fossils restricts our efforts at following the evolution of viruses, we can draw hypotheses by looking at parallels between a few of the virus groups and their hosts. To begin this process, we have seen that some of the presently existing viral families (we know nothing about those viral families that may be extinct) seem restricted to different host groups. It is likely that as time has gone by, these viruses and their hosts have coevolved and perhaps even undergone phylogenation (the evolution of phylogenetic groupings) in parallel. For example, those viruses which we know as the Myoviridae seem restricted to

infecting prokaryotic cells. This could suggest either that the ancestors of the Myoviridae are relatively new or else relatively ancient. Members of the Siphoviridae, which also infect prokaryotes, have developed a relatively stabile mechanism of endogeny (in their case referred to as lysogeny), which may be suggestive of these viruses having had a long period of coevolutionary adaptation with their host cells. We can see that the viroids of plants, which genetically bear a link to the viruses (chapter 2 addresses viral taxonomy, and prions are specifically addressed in chapter 12 of volume 1) seemingly have developed such a highly evolved endogenous state that they never produce anything resembling a virion and indeed may not use or even need a natural route of transmission because they remain internal to their host. Additional examination of the existing viral groups, and the establishment of parallels between these and the known evolution of animal phyla, reveals that virus groups such as the Iridoviridae, which do produce virions, seem restricted to invertebrates and poikilothermic vertebrates. This latter examination could lead to the suggestion that ancestors of the iridoviruses followed the animal phylogenation pathway upward to a point just short of the evolution of euthermia. The retroviruses have gone onward to infect euthermic animals, and it has been hypothesized that at least some retroviruses have coevolved with their hosts to the extent that they allowed development of the placental mammals (Villareal, 1997).

Why are the viruses still around? The viruses might serve as an evolutionary benefit to the cellular organisms by gradually transferring genetic information between different sources and serving as a source for genomic development (de Lima Fávaro et al., 2005; Piskurek and Okada, 2007; Todorovska, 2007; Williams, 1996). Perhaps this is the reason why their hosting species continue allowing the viruses to exist. Perhaps the pure beauty of a virus, when viewed as an evolutionary element, is that it can break free from one host to enter another host. Gradually, that virus could coevolve until at last it might settle upon a permanent home as some endogenous genetic element within a single hosting species. Alternatively, the virus may play the role of eternally being a rebel in search of a cause. Oh, to be so free as a species!

What will the viruses become with time? As stated above, in a strictly evolutionary sense it is not necessary for the viruses to be leading to anywhere. However, if we can draw parallels and make the assumption that the relationship between virus and host moves with time towards avirulence and an eventual genetic equilibrium, then we can make hypotheses. Perhaps some of the viruses will indeed continue the way of being predatory outsiders. Others, however, seem destined for symbiosis and thus to become a part of us. We see at least two clues pointing to the latter type of destiny. One of these lies in Villareal's hypothesis (Villareal, 1997) that by evolving to have the same biological agenda as their placental mammalian hosts, the endogenous retroviruses have symbiotically joined with their hosts to create a single species. The hypovirulence elements of the fungi which cause Chestnut blight disease are another clue (Volume 1, Chapter 9), these elements apparently evolved from a virus and seemingly have achieved symbiosis. The hypovirulence elements sustain their existence by reducing the virulence of their host fungi, so that in turn the host fungus does not kill the tree upon which the fungus feeds, enabling all to survive.

Alas, it might also be true that the evolution of viruses represents a question which we cannot yet even try to answer.

1.5 SUMMARY (CAN THERE BE CONCLUSIONS?)

The ecology of a virus primarily consists of it's interactions with the organisms that serve as it's hosting species (principle hosts, alternate hosts, and vectors. The routes by which viruses achieve transmission between these other organisms represent a second aspect of the ecology of viruses. Furthermore, an

examination of the interactions between a virus and it's hosts and biological vectors brings up many questions. Principle among these questions is the reason why the outcome of viral infections sometimes appears to be so disastrous, and yet at other times appears unnoticeable.

One of the founding principles in biology is that natural selection serves as the basis for the population dynamics which produce the many different outcomes that we observe as scientists. When we use this principle as the lense through which to examine interactions between viruses and their host and vector species, we notice that many possible strategies exist, more than can be explained. The strategies which we do find in evidence began at random and exist because selection has not done away with them. While we do not know how the viruses have arisen, or what will be their destiny, we can assume that there may be viruses for as long as there are cells.

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CHAPTER 2

AN INTRODUCTION TO VIRAL TAXONOMY WITH EMPHASIS ON MICROBIAL AND BOTANICAL HOSTS AND THE PROPOSAL OF AKAMARA, A POTENTIAL DOMAIN FOR THE GENOMIC ACELLULAR AGENTS*

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2.1 INTRODUCTION

Taxonomy is literally the naming of taxons (in plural also termed taxa), which by definition are groupings of items based upon identifiable similarities. The viruses are a group of biological entities which have in common the fact that they possess a nucleic acid genome that is composed either of DNA or RNA. That nucleic acid genome is surrounded and protected by a shell of proteins which is termed either to be a nucleocapsid or, more simply, a capsid. The nucleocapsids of some viruses are, in turn, surrounded by a lipid membrane. The viruses have been grouped by many different methods. Those viral taxonomic groupings which presently are recognized by the ICTV (International Committee on Taxonomy of Viruses) divide these biological entities into families, genera and species. There also currently exist a few recognized viral order groups.

This chapter also introduces the idea that the taxonomy of the viruses and their biological relatives could be extended to the domain level. There currently exist three biological domains, Archaea, Bacteria and

*This chapter represents a revision of "An Introduction to Viral Taxonomy and the proposal of Akamara, a potential domain for the genomic acellular agents", which appeared as chapter 2 of the book *Viral Ecology*, edited by Christon J. Hurst, published in 2000 by Academic Press. All of the artwork contained in this chapter appears courtesy of Christon J. Hurst.

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Eukarya, which consist only of cellular organisms. The establishment of these three existing domains and the taxonomic placement of biological entities within them largely is based upon the ribosomal RNA nucleotide sequence of those constituent organisms. This article proposes the creation of an additional biological domain that would represent the acellular infectious agents which possess nucleic acid genomes (termed 'genomic acellular infectious agents' for the purpose of this proposal). The proposed constituents of this domain are the agents commonly termed to be either viruses, satellite viruses, virusoids or viroids. The proposed domain title is Akamara (ακαμαρα), whose derivation from Greek would translate as meaning 'without chamber' or 'without vault', and is suggested as describing the fact that these agents lack a cellular structure of their own. A possible organizational structure within this proposed new domain is also suggested, with its occupants shown as being divided into two kingdoms, plus phyla and classes premised upon basic characteristics of the organisms' genomic biochemistry. The kingdom Euviria (true viruses) is suggested as containing the 'conventional' viruses plus those viral-like agents which likewise possess genomes that code for their own structural 'shell' or 'capsid' proteins. The kingdom Viroidia would contain the genus Deltavirus plus the viroids and virusoids, whose members are RNA agents that have in common the trait that their genomic structure has endowed them with the capacity for evolutionary survival even though their genomes do not code for such structural proteins. The members of the kingdom Euviria are suggested as being subdivided into two phyla based upon whether their genome is of RNA (phylum Ribovira) or DNA (phylum Deoxyribovira), and these are further subdivided into classes based upon whether their genomes are 'negative sense' single stranded RNA versus 'plus sense' single stranded RNA, double stranded RNA, single stranded DNA or double stranded DNA. The kingdom Viroidia is suggested to contain one phylum, Viroida, encompassing the viroids, virusoids, and genus Deltavirus, all of which possess RNA genomes.

2.2 THE EXISTING VIRAL FAMILIES

The viruses recognized by the ICTV have been assigned into genera, and nearly all of these genera have been grouped into families. Those genera which have not been incorporated into families are considered to be "floating genera". This concept is very fluid (pun intended! never accept taxonomy as though it were written into stone tablets either by some God or biological committee). The viral family groupings and floating genera which existed at the time when the ICTV published its Master Species List of November 2009 (2009_5F00_v3), which is available on the website of the International Committee on Taxonomy of Viruses http://www.ictvdb.org/, are listed in Table 2.1. Some of these viral families have been placed into higher taxonomic levels up to that of order (Table 2.2). Those viral families and floating genera which affect either microbial or botanical hosts are depicted, along with their basic morphological characteristics, in Figures 2.1–2.5. An examination of the drawings of the virus groups presented in those first five figures reveals that most of the known viral capsid structures can be categorized as being either helical or icosahedral in form. The basic form of a helical capsid structure is represented in Figure 2.6. A sculpture which interestingly and unintentionally resembles the membrane envelope with its associated proteins that encloses the helical capsid of a filovirus is presented in Figure 2.7. The icosahedral capsid structure is represented in Figures 2.8 and 2.9. There are four recognized viral families, although no floating genera, that are associated with either microbial or botanical hosts but which are not presented in Figures 2.1–2.5 because they are not known

TABLE 2.1 Listing of Viral Taxonomic Groups: Families and Floating Genera

Viral Group (Name)	Taxonomic Level (Family vs. Unassociated or "Floating" Genus)	Nature of Genome	Host Range as Presently Known	Refer to Figure Number
Adenoviridae	Family	DNA, double-stranded	Vertebrates	Vol. 2[c]
Alloherpesviridae	Family	DNA, double-stranded	Vertebrates	Vol. 2
Alphaflexiviridae	Family	RNA, single-stranded (+ sense)[a]	Fungi, plants	2.5
Ampullaviridae	Family	DNA, double-stranded	Archaea	2.1
Anelloviridae	Family	DNA, single-stranded	Vertebrates	Vol. 2
Arenaviridae	Family	RNA, single-stranded (± sense)[b]	Vertebrates	Vol. 2
Arteriviridae	Family	RNA, single-stranded (+ sense)	Vertebrates	Vol. 2
Ascoviridae	Family	DNA, double-stranded	Invertebrates	Vol. 2
Asfarviridae	Family	DNA, double-stranded	Vertebrates	Vol. 2
Astroviridae	Family	RNA, single-stranded (+ sense)	Vertebrates	Vol. 2
Avsunviroidae	Family	RNA, single-stranded (viroid)	Plants	None[d]
Baculoviridae	Family	DNA, double-stranded	Invertebrates	Vol. 2
Barnaviridae	Family	RNA, single-stranded (+ sense)	Fungi	2.5
Benyvirus	Floating genus	RNA, single-stranded (+ sense)	Plants	2.5
Betaflexiviridae	Family	RNA, single-stranded (+ sense)	Plants	2.5
Bicaudaviridae	Family	DNA, double-stranded	Archaea	2.1
Birnaviridae	Family	RNA, double-stranded	Invertebrates, vertebrates	Vol. 2
Bornaviridae	Family	RNA, single-stranded (– sense)[e]	Vertebrates	Vol. 2
Bromoviridae	Family	RNA, single-stranded (+ sense)	Plants	2.5
Bunyaviridae	Family	RNA, single-stranded (± sense)	Invertebrates, plants, vertebrates	2.4
Caliciviridae	Family	RNA, single-stranded (+ sense)	Vertebrates	Vol. 2
Caulimoviridae	Family	DNA, double-stranded	Plants	2.1
Chrysoviridae	Family	RNA, double-stranded	Fungi	2.3
Cilevirus	Floating genus	RNA, single-stranded (+ sense)	Plants	2.5
Circoviridae	Family	DNA, single-stranded	Vertebrates	Vol. 2
Closteroviridae	Family	RNA, single-stranded (+ sense)	Plants	2.5
Coronaviridae	Family	RNA, single-stranded (+ sense)	Vertebrates	Vol. 2
Corticoviridae	Family	DNA, double-stranded	Bacteria	2.1
Cystoviridae	Family	RNA, double-stranded	Bacteria	2.3

(*continued*)

TABLE 2.1 (*Continued*)

Viral Group (Name)	Taxonomic Level (Family vs. Unassociated or "Floating" Genus)	Nature of Genome	Host Range as Presently Known	Refer to Figure Number
Deltavirus	Floating genus	RNA, single-stranded (– sense)	Vertebrates	Vol. 2
Dicistroviridae	Family	RNA, single-stranded (+ sense)	Invertebrates	Vol. 2
Emaravirus	Floating genus	RNA, single-stranded (± sense)	Plants	2.4
Endornaviridae	Family	RNA, double-stranded	Fungi, plants	None
Filoviridae	Family	RNA, single-stranded (– sense)	Vertebrates	Vol. 2
Flaviviridae	Family	RNA, single-stranded (+ sense)	Invertebrates, vertebrates	Vol. 2
Fuselloviridae	Family	DNA, double-stranded	Archaea	2.1
Gammaflexiviridae	Family	RNA, single-stranded (+ sense)	Fungi	2.5
Geminiviridae	Family	DNA, single-stranded	Plants	2.2
Globuloviridae	Family	DNA, double-stranded	Archaea	2.1
Guttaviridae	Family	DNA, double-stranded	Archaea	2.1
Hepadnaviridae	Family	DNA, partially double-stranded	Vertebrates	Vol. 2
Hepeviridae	Family	RNA, single-stranded (+ sense)	Vertebrates	Vol. 2
Herpesviridae	Family	DNA, double-stranded	Vertebrates	Vol. 2
Hypoviridae	Family	RNA, double-stranded	Fungi	2.3
Idaeovirus	Floating genus	RNA, single-stranded (+ sense)	Plants	2.5
Iflaviridae	Family	RNA, single-stranded (+ sense)	Invertebrates	Vol. 2
Inoviridae	Family	DNA, single-stranded	Bacteria	2.2
Iridoviridae	Family	DNA, double-stranded	Invertebrates, vertebrates	Vol. 2
Leviviridae	Family	RNA, single-stranded (+ sense)	Bacteria	2.5
Lipothrixviridae	Family	DNA, double-stranded	Archaea	2.1
Luteoviridae	Family	RNA, single-stranded (+ sense)	Plants	2.5
Malacoherpesviridae	Family	DNA, double-stranded	Invertebrates	Vol. 2
Marnaviridae	Family	RNA, single-stranded (+ sense)	Algae	2.5
Metaviridae	Family	RNA, single-stranded (+ sense?)	Fungi, invertebrates, plants, vertebrates	2.5
Microviridae	Family	DNA, single-stranded	Bacteria	2.2
Mimiviridae	Family	DNA, double-stranded	Protozoa	2.1
Myoviridae	Family	DNA, double-stranded	Archaea, bacteria, cyanobacteria	2.1
Nanoviridae	Family	DNA, single-stranded	Plants	2.2

TABLE 2.1 (*Continued*)

Viral Group (Name)	Taxonomic Level (Family vs. Unassociated or "Floating" Genus)	Nature of Genome	Host Range as Presently Known	Refer to Figure Number
Narnaviridae	Family	RNA, single-stranded (+ sense)	Fungi	None
Nimaviridae	Family	DNA, double-stranded	Invertebrates	Vol. 2
Nodaviridae	Family	RNA, single-stranded (+ sense)	Fungi (experimentally), invertebrates, vertebrates	2.5
Ophioviridae	Family	RNA, single-stranded (– sense)	Plants	2.4
Orthomyxoviridae	Family	RNA, single-stranded (– sense)	Invertebrates, vertebrates	Vol. 2
Ourmiavirus	Floating genus	RNA, single-stranded (+ sense)	Plants	2.5
Papillomaviridae	Family	DNA, partially double-stranded	Vertebrates	Vol. 2
Paramyxoviridae	Family	RNA, single-stranded (– sense)	Vertebrates	Vol. 2
Partitiviridae	Family	RNA, double-stranded	Fungi, plants	2.3
Parvoviridae	Family	DNA, single-stranded	Invertebrates, vertebrates	Vol. 2
Phycodnaviridae	Family	DNA, double-stranded	Algae, protozoa	2.1
Picobirnaviridae	Family	RNA, double-stranded	Vertebrates	Vol. 2
Picornaviridae	Family	RNA, single-stranded (+ sense)	Vertebrates	Vol. 2
Plasmaviridae	Family	DNA, double-stranded	Bacteria	2.1
Podoviridae	Family	DNA, double-stranded	Bacteria, cyanobacteria	2.1
Polemovirus	Floating genus	RNA, single-stranded (+ sense)	Plants	2.5
Polydnaviridae	Family	DNA, double-stranded	Invertebrates	Vol. 2
Polyomaviridae	Family	DNA, partially double-stranded	Vertebrates	Vol. 2
Pospiviroidae	Family	RNA, single-stranded (viroid)	Plants	None
Potyviridae	Family	RNA, single-stranded (+ sense)	Plants	2.5
Poxviridae	Family	DNA, double-stranded	Invertebrates, vertebrates	Vol. 2
Pseudoviridae	Family	RNA, single-stranded (+ sense)	Algae, fungi, invertebrates, plants	2.5
Reoviridae	Family	RNA, double stranded	Algae, fungi, invertebrates, plants, vertebrates	2.3

(*continued*)

TABLE 2.1 (*Continued*)

Viral Group (Name)	Taxonomic Level (Family vs. Unassociated or "Floating" Genus)	Nature of Genome	Host Range as Presently Known	Refer to Figure Number
Retroviridae	Family	RNA, single-stranded (+ sense)	Vertebrates	Vol. 2
Rhabdoviridae	Family	RNA, single-stranded (– sense)	Invertebrates, plants, vertebrates	2.4
Rhizidiovirus	Floating genus	DNA, double-stranded	Fungi	2.1
Roniviridae	Family	RNA, single-stranded (+ sense)	Invertebrates	Vol. 2
Rudiviridae	Family	DNA, double-stranded	Archaea	2.1
Salterprovirus	Floating genus	DNA, double-stranded	Archaea	2.1
Secoviridae	Family	RNA, single-stranded (+ sense)	Plants	2.5
Siphoviridae	Family	DNA, double-stranded	Archaea, bacteria	2.1
Sobemovirus	Floating genus	RNA, single-stranded (+ sense)	Plants	2.5
Tectiviridae	Family	DNA, double-stranded	Bacteria	2.1
Tenuivirus	Floating genus	RNA, single-stranded (± sense)	Plants	2.4
Tetraviridae	Family	RNA, single-stranded (+ sense)	Invertebrates	Vol. 2
Togaviridae	Family	RNA, single-stranded (+ sense)	Invertebrates, vertebrates	Vol. 2
Tombusviridae	Family	RNA, single-stranded (+ sense)	Plants	2.5
Totiviridae	Family	RNA, double-stranded	Fungi, protozoa	2.3
Tymoviridae	Family	RNA, single-stranded (+ sense)	Plants	2.5
Umbravirus	Floating genus	RNA, single-stranded (+ sense)	Plants	2.5
Varicosavirus	Floating genus	RNA, single-stranded (– sense)	Plants	2.4
Virgaviridae	Family	RNA, single-stranded (+ sense)	Plants	2.5

This information has been summarized from the ICTV Master Species List of November 2009 (ICTV Master Species List 2009_5F00_v3 available on the website of the International Committee on Taxonomy of Viruses, http://www.ictvdb.org/).

[a]The RNA genome has the same sense as messenger RNA and can be translated directly.

[b]The RNA genome is considered to be ambisense, meaning that it has some sections which are of the same sense as messenger RNA, but other sections of the genome must be copied to produce an opposite strand which in turn can be translated.

[c]The members of this group are presented in Volume 2.

[d]No traditional viral structure is produced. These are the viroids and other genetic elements which are considered to be "virus-like" and they are included here because their ecologies are similar to those of the "true" viruses. They are not presented in Figures 2.1–2.5 because they do not produce viral particles, for this reason they are instead represented in the text of this chapter.

[e]The RNA genome must be copied to produce an opposite strand which in turn can be translated.

TABLE 2.2 Viruses Which have been Assigned to Taxonomic Orders

Order	Family	Subfamily	Genus
Caudovirales	Myoviridae	Undesignated	*"T4-like viruses"*
	Podoviridae	Autographivirinae	*"PhiKMV-like viruses"*
			"SP6-like viruses"
			"T7-like viruses"
		Picovirinae	*"AHJD-like viruses"*
			"Phi29-like viruses"
		Undesignated	*"BPP-1-like viruses"*
			"Epsilon15-like viruses"
			"LUZ24-like viruses"
			"N4-like viruses"
			"P22-like viruses"
			"Phieco32-like viruses"
	Siphoviridae	Undesignated	*"c2-like viruses"*
			"L5-like viruses"
			"Lambda-like viruses"
			"N15-like viruses"
			"PhiC31-like viruses"
			"PsiM1-like viruses"
			"SPbeta-like viruses"
			"T1-like viruses"
			"T5-like viruses"
Herpesvirales	Alloherpesviridae	Undesignated	*Batrachovirus*
			Cyprinivirus
			Ictalurivirus
			Salmonivirus
	Herpesviridae	Alphaherpesvirinae	*Iltovirus*
			Mardivirus
			Simplexvirus
			Varicellovirus
		Betaherpesvirinae	*Cytomegalovirus*
			Muromegalovirus
			Proboscivirus
			Roseolovirus
		Gammaherpesvirinae	*Lymphocryptovirus*
			Macavirus
			Percavirus
			Rhadinovirus
	Malacoherpesviridae	Undesignated	*Ostreavirus*
Mononegavirales	Bornaviridae	Undesignated	*Bornavirus*
	Filoviridae	Undesignated	*Ebolavirus*
	Paramyxoviridae	Paramyxovirinae	*Avulavirus*
			Henipavirus
			Morbillivirus
			Respirovirus
			Rubulavirus
		Pneumovirinae	*Metapneumovirus*
			Pneumovirus

(*continued*)

TABLE 2.2 (*Continued*)

Order	Family	Subfamily	Genus
	Rhabdoviridae	Undesignated	*Cytorhabdovirus*
			Ephemerovirus
			Lyssavirus
			Novirhabdovirus
			Nucleorhabdovirus
			Vesiculovirus
Nidovirales	Arteriviridae	Undesignated	*Arterivirus*
	Coronaviridae	Coronavirinae	*Alphacoronavirus*
			Betacoronavirus
			Gammacoronavirus
		Torovirinae	*Bafinivirus*
			Torovirus
	Roniviridae	Undesignated	*Okavirus*
Picornavirales	Dicistroviridae	Undesignated	*Cripavirus*
	Iflaviridae	Undesignated	*Iflavirus*
	Marnaviridae	Undesignated	*Marnavirus*
	Picornaviridae	Undesignated	*Aphthovirus*
			Avihepatovirus
			Cardiovirus
			Enterovirus
			Erbovirus
			Hepatovirus
			Kobuvirus
			Parechovirus
			Sapelovirus
			Senecavirus
			Teschovirus
			Tremovirus
	Secoviridae	Comovirinae	*Comovirus*
			Fabavirus
			Nepovirus
		Undesignated	*Cheravirus*
			Sadwavirus
			Sequivirus
			Torradovirus
			Waikavirus
Tymovirales	Alphaflexiviridae	Undesignated	*Allexivirus*
			Botrexvirus
			Lolavirus
			Mandarivirus
			Potexvirus
			Sclerodarnavirus
	Betaflexiviridae	Undesignated	*Capillovirus*
			Carlavirus
			Citrivirus
			Foveavirus
			Trichovirus
			Vitivirus

TABLE 2.2 (*Continued*)

Order	Family	Subfamily	Genus
	Gammaflexiviridae	Undesignated	*Mycoflexivirus*
	Tymoviridae	Undesignated	*Maculavirus*
			Marafivirus
			Tymovirus

This information has been summarized from the ICTV Master Species List of November 2009 (ICTV Master Species List 2009_5F00_v3 available on the website of the International Committee on Taxonomy of Viruses http://www.ictvdb.org/).

to produce true viral particles. The summary information for these four groups is:

Family: Avsunviroidae
Nucleic acid: RNA
Genome: Single-stranded, sense unspecified (non-coding), 1 circular segment (246–375 bp)
Morphology: None
Virion: None
Nucleocapsid: None

Family: Endornaviridae
Nucleic acid: RNA
Genome: Double-stranded, 1 linear segment (14–18 Kbp)
Morphology: None
Virion: None
Nucleocapsid: None

Family: Narnaviridae
Nucleic acid: RNA
Genome: Single-stranded, Positive sense, 1 linear segment (2.3–3.6 Kb)
Morphology: None
Virion: None
Nucleocapsid: None

Family: Pospiviroidae
Nucleic acid: RNA
Genome: Single-stranded, sense unspecified (non-coding), 1 circular segment (approx. 359 nt)
Morphology: None
Virion: None (Mw of genome approx. 1×10^6)
Nucleocapsid: None.

2.3 THE PROPOSED DOMAIN AKAMARA

It has been suggested (Morell, 1996) that with the sequencing of an archaeon microbe (Bult et al., 1996), the last of life's three domains has been elucidated, and that these domains are the Archaea, Bacteria and Eukarya. That assessment leaves out something very important, namely, the viruses and the other acellular infectious agents. Indeed, it must be remembered that the first life forms whose genomes were sequenced in entirety were not cellular in nature, but rather the viruses MS2 (having an RNA genome) and SV40 (having a DNA genome), and we have had knowledge of their full genomes for more than 30 years (Fiers et al. 1976, 1978).

Perhaps the time has come to suggest a fourth biological domain to give a higher taxonomic home to the viruses and their genomic relatives. One logical suggestion would be to include as a group the 'conventional' viruses plus those satellite viruses whose genomes likewise code for their own structural 'shell' or 'capsid' proteins, and which are also commonly defined as 'viruses'. A second group within this domain might consist of the viroids, virusoids, and the viral genus Deltavirus, which share strong commonalities with respect to their RNA genomic structure and the fact that they do not code for such structural proteins. These infectious agents are excluded from the three existing domains for two reasons: first, their genomes do not code for ribosomal RNA which is the defining characteristic for membership in the three domains and second, they lack a cellular structure of their own, a fact which also kept them officially excluded from the older kingdom classifications.

The conventional viruses are very heterogenous with respect to their genomic structure and vary widely in the extent of genetic coding

Plate 2.1.1 Family: Ampullaviridae. Nucleic acid: DNA. Genome: Double-stranded, linear segment (23.9–40.5 Kbp). Morphology: Enveloped. Virion: Bottle shaped, (Mw not specified). Nucleocapsid: Helical (presumably). Distinguishing feature: The virions have a bottle-like appearance.

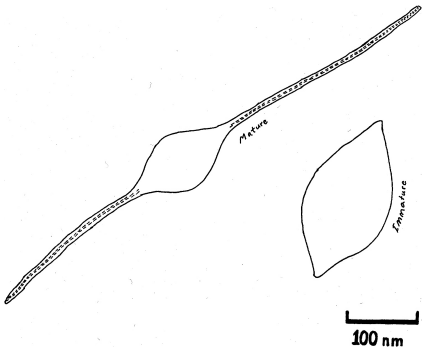

Plate 2.1.2 Family: Bicaudaviridae. Nucleic acid: DNA. Genome: Double-stranded 1 circular segment (62.7 Kbp). Morphology: Presumably non-enveloped. Virion: Lemon-shaped with two opposing tubular tails (Mw not specified). Nucleocapsid: Complex. Distinguishing feature: Lemon-shaped particles which may have either one, or more often two, opposing tapered tail-like structures extending from the pointed ends of the particles. Volume of the central body seems to be decreased if tail structures are present.

Plate 2.1.3 Family: Caulimoviridae. (was: genus *Caulimovirus*). Nucleic acid: DNA. Genome: Double-stranded, 1 circular segment (6.8–8.2 Kbp). Morphology: Non-enveloped. *Category One*: (was: genus Caulimovirus). Virion: Icosahedral (Mw = 2.0×10^7). Nucleocapsid: Icosahedral. *Category Two*: (was: genus Badnavirus). Virion: Bacilliform (Mw not specified). Nucleocapsid: Tubular, comprised of repeating hexamer subunits. Distinguishing feature: Polyhedral, nucleocapsid structure based on an icosahedra cut across its 3-fold axis.

Plate 2.1.4 Family: Corticoviridae. Nucleic acid: DNA. Genome: Double-stranded, 1 circular segment (9.5–12 Kbp). Morphology: Non-enveloped. Virion: Icosahedral (Mw = 5.8×10^6). Nucleocapsid: Icosahedral. Distinguishing feature: Nucleocapsid consists of two concentric protein shells enclosing an internal lipid bilayer.

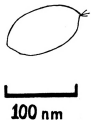

Plate 2.1.5 Family: Fuselloviridae. Nucleic acid: DNA. Genome: Double-stranded, 1 circular segment (14.8–17.3 Kbp). Morphology: Enveloped. Virion: Lemon-shaped with short tail fibers attached to one of the polar ends (Mw not specified). Nucleocapsid: Unspecified (possibly helical).

Plate 2.1.6 Family: Globuloviridae. Nucleic acid: DNA. Genome: Double-stranded, 1 linear segment (28–30 Kbp). Morphology: Enveloped. Virion: Spherical, (Mw not specified). Nucleocapsid: Unspecified (nucleoprotein core). Distinguishing feature: Spherical protrusions are present on the virion surface.

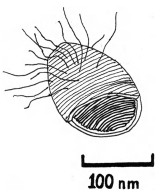

Plate 2.1.7 Family: Guttaviridae. Nucleic acid: DNA. Genome: Double-stranded, 1 closed circular segment (20 Kbp). Morphology: Enveloped. Virion: Ovoidal or 'droplet-shaped' (Mw not specified). Nucleocapsid: Helical (possibly). Distinguishing feature: Virions consist of a densely-wound oblong nucleoprotein core internal to a covering coat that results in a ribbed 'Beehive-like' appearance. The virions have a 'beard-like' covering of filaments at their smaller end.

FIGURE 2.1 Relative sizes and basic information for those viruses which possess double stranded DNA genomes.

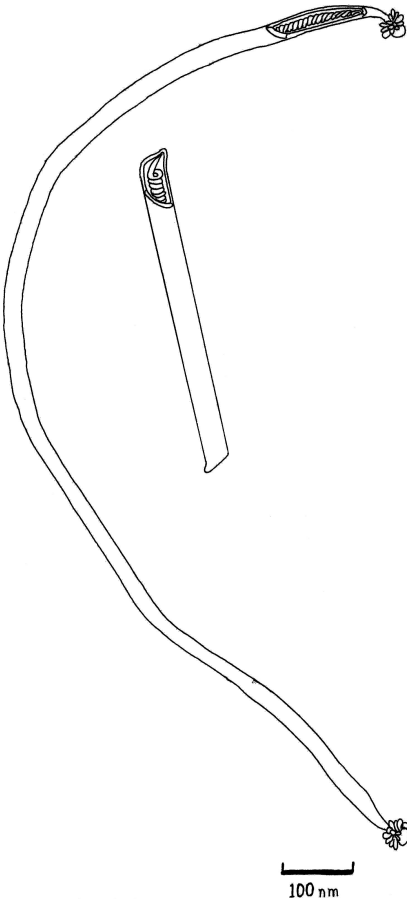

Plate 2.1.8 Family: Lipothrixviridae. Nucleic acid: DNA. Genome: Double-stranded, 1 linear segment (16–56 Kbp). Morphology: Enveloped. Virion: Rod-like (Rigid or slightly flexous) (approx. Mw = 3.3×10^8). Nucleocapsid: Helical. Distinguishing feature: The virions of some species are described as having a bottle brush appearance, being extremely long and thin with distinct and very different terminal structures which contain short filaments.

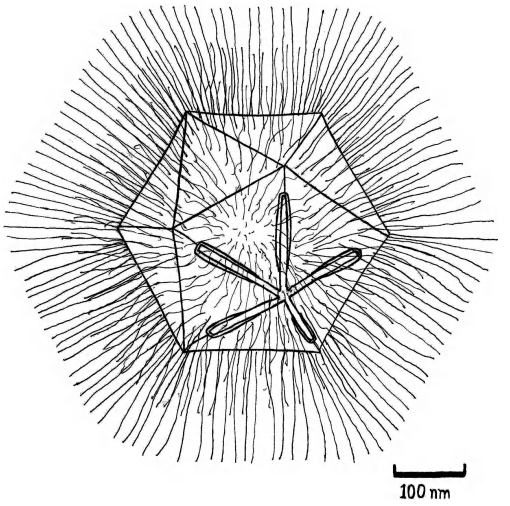

Plate 2.1.9 Family: Mimiviridae. Nucleic acid: DNA. Genome: Double-stranded, 1 linear segment (1,181 Kbp). Morphology: Non-enveloped. Virion: Icosahedral, (Mw not specified). Nucleocapsid: Icosahedral. Distinguishing feature: Long protein filaments extend from the capsid. One vertex per capsid has an unusual starfish-shaped structure which may be the exit point for the nucleoprotein.

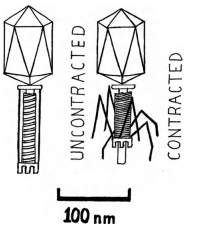

Plate 2.1.10 Family: Myoviridae. Nucleic acid: DNA. Genome: Double-stranded, 1 circular segment (approx. 90–170 Kbp). Morphology: Non-enveloped. Virion: Tailed (Mw = 2.1×10^8). Nucleocapsid: Elongated head with contractile tail and long tail fibers. Distinguishing feature: Contractile tail.

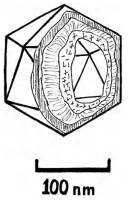

Plate 2.1.11 Family: Phycodnaviridae. Nucleic acid: DNA. Genome: Double-stranded, 1 linear segment (160–380 Kb). Morphology: Non-enveloped. Virion: Icosahedral (Mw = 1.0×10^9). Nucleocapsid: Icosahedral (multilaminate) with internal lipid membrane.

FIGURE 2.1 (*Continued*)

Plate 2.1.12 Family: Plasmaviridae. Nucleic acid: DNA. Genome: Double-stranded, 1 circular segment (12 Kbp). Morphology: Enveloped. Virion: Spherical (Pleomorphic) (Mw not specified). Nucleocapsid: None (contains an asymmetric nucleoprotein condensate).

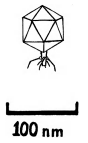

Plate 2.1.13 Family: Podoviridae. Nucleic acid: DNA. Genome: Double-stranded, 1 linear segment (approx. 40 Kbp). Morphology: Non-enveloped. Virion: Tailed ($Mw = 4.8 \times 10^7$). Nucleocapsid: Isometric head with short rigid tail and short tail fibers.

Plate 2.1.14 Floating genus: *Rhizidiovirus*. Nucleic acid: DNA. Genome: Double-stranded, 1 linear segment (16.8–25.5 Kbp). Morphology: Non-enveloped. Virion: Icosahedral (Mw not specified). Nucleocapsid: Icosahedral.

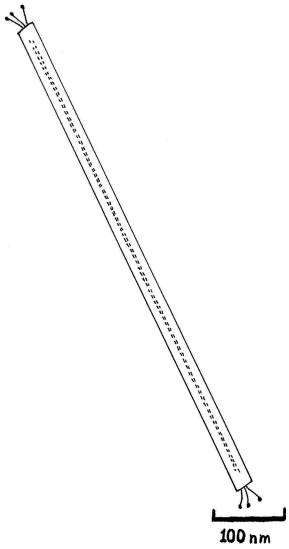

Plate 2.1.15 Family: Rudiviridae. Nucleic acid: DNA. Genome: Double-stranded, 1 linear segment (24–35 Kbp). Morphology: Non-enveloped. Virion: Rod-like (rigid), (Mw not specified). Nucleocapsid: Helical (presumably helical, although also reported as being polyhedral). Distinguishing feature: Three tail fibers at each end.

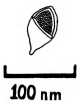

Plate 2.1.16 Floating genus: *Salterprovirus*. Nucleic acid: DNA. Genome: Double-stranded, 1 linear segment (14.5 Kbp). Morphology: Enveloped. Virion: Lemon-shaped (flexible), (Mw not specified). Nucleocapsid: Unspecified (possibly helical). Distinguishing feature: One pole of the virion contains short tail-like fibers that extend from the interior through the membrane.

Plate 2.1.17 Family: Siphoviridae. Nucleic acid: DNA. Genome: Double-stranded, 1 linear segment plus 1–2 very short single stranded multimeric segments (approx. 48.5 Kbp total). Morphology: Non-enveloped. Virion: Tailed ($Mw = 6.0 \times 10^7$). Nucleocapsid: Isometric head with long, non-contractile flexible tail and short tail fibers.

Plate 2.1.18 Family: Tectiviridae. Nucleic acid: DNA. Genome: Double-stranded, 1 linear segment (150 Kbp). Morphology: Non-enveloped. Virion: Icosahedral ($Mw = 7.0 \times 10^7$). Nucleocapsid: Icosahedral. Distinguishing feature: An internal lipid envelope surrounds the nucleoprotein and this is contained within an icosahedral capsid that has external spikes.

FIGURE 2.1 (*Continued*)

Plate 2.2.1 Family: Geminiviridae. Nucleic acid: DNA. Genome: Single-stranded, Ambisense, 1–2 circular segments (per virion) (2.5–3 Kb). Morphology: Non-enveloped. Virion: Geminate (Mw not specified). Nucleocapsid: Geminate. Distinguishing feature: Virion capsid consists of multiple (usually two) adjoined incomplete icosahedra. Members of the genus Begomovirus have independently encapsidated bipartite genomes.

Plate 2.2.3 Family: Microviridae. Nucleic acid: DNA. Genome: Single stranded, Positive sense, 1 circular segment (4.5–6 Kb). Morphology: Non-enveloped. Virion: Icosahedral (Mw = 6.0–7.0 × 10^6). Nucleocapsid: Icosahedral.

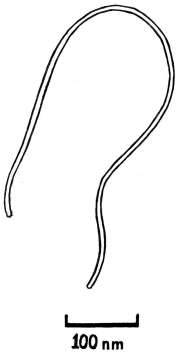

Plate 2.2.2 Family: Inoviridae. Nucleic acid: DNA. Genome: Single-stranded, Positive sense, 1 circular segment (4.4–8.5 Kb). Morphology: Non-enveloped. Virion: Rod-like (flexible) (Mw = 1.2–2.3 × 10^7). Nucleocapsid: Helical.

Plate 2.2.4 Family: Nanoviridae. Nucleic acid: DNA. Genome: Single-stranded, 6–11 circular segments (approx. 1 Kb per segment). Morphology: Non-enveloped. Virion: Icosahedral (Mw not provided). Nucleocapsid: Icosahedral.

FIGURE 2.2 Relative sizes and basic information for those viruses which possess single stranded DNA genomes.

that they carry. Some of them such as T4, a member of the family Myoviridae, carry a major amount of the genetic coding which is necessary to replicate themselves. Other viruses, such as the human polioviruses which belong to the family Picornaviridae, carry just barely more than the limited amount of genome needed to code for their structural proteins. In comparison, the RNA genomes of the viroids and virusoids as a group are more homogenous and uniquely seem to evidence an evolutionary stability as infectious agents despite the fact that their genomes do not code for any such structural proteins. The variety of agents known as virusoids "borrow" encapsulating proteins from a helper virus. The viroids either have done away with the need for encapsulating proteins or perhaps never possessed them. The genome of the Hepatitis D virus, which is the constituent species of the floating genus Deltavirus, represents what seems to be an interesting evolutionary anomaly. This agent of humans is essentially identical to that of the viroids which are plant pathogens, with exception of the fact that the Hepatitis D virus' genome carrys the genetic coding for a protein that it apparently has picked-up from a cellular host (Brazas and Ganem, 1996; Robertson, 1996). Despite its very limited coding capacity, as with the virusoids, the hepatitis D virus needs to "borrow" enveloping structural proteins which are coded for by a helper virus.

The assignment of taxonomic levels for cellular organisms was initially based upon their similarities at the level of physical traits

Plate 2.3.1 Family: Chrysoviridae. Nucleic acid: RNA. Genome: Double-stranded, 3–4 linear segments (12.6 Kbp total). Morphology: Non-enveloped. Virion: Icosahedral (Mw not specified). Nucleocapsid: Icosahedral.

Plate 2.3.2 Family: Cystoviridae. Nucleic acid: RNA. Genome: Double-stranded, 3 linear segments (Approx. 14 Kbp total). Morphology: Enveloped. Virion: Spherical ($Mw = 9.9 \times 10^7$). Nucleocapsid: Icosahedral.

Plate 2.3.3 Family: Hypoviridae. Nucleic acid: RNA. Genome: Double stranded, 1 linear segment (9–13 Kbp). Morphology: Encapsulating lipid vesicle. Virion: No true virion (Mw not specified). Nucleocapsid: None.

Plate 2.3.4 Family: Partitiviridae. Nucleic acid: RNA. Genome: Double-stranded, 2 linear segments (3–10 Kbp total). Morphology: Non-enveloped. Virion: Icosahedral ($Mw = 6.0–9.0 \times 10^6$). Nucleocapsid: Icosahedral.

Plate 2.3.5 Family: Reoviridae. Nucleic acid: RNA. Genome: Double stranded, 10-12 linear segments (1–3.9 Kbp per segment). Morphology: Non-enveloped. Virion: Icosahedral ($Mw = 1.2 \times 10^8$). Nucleocapsid: Icosahedral. Distinguishing feature: Nucleocapsid contains several concentric protein layers. Members of the genus *Cypovirus*, which infect insects, have capsids with only a single layer and form protein polyhedra.

Plate 2.3.6 Family: Totiviridae. Nucleic acid: RNA. Genome: Double stranded, usually 1 linear segment but may be segmented (4.7–6.7 Kbp total). Morphology: Non-enveloped. Virion: Icosahedral ($Mw = 1.2 \times 10^7$). Nucleocapsid: Icosahedral.

FIGURE 2.3 Relative sizes and basic information for those viruses which possess double stranded RNA genomes.

and aided by a trail of fossilized remains. This approach has since been superseded by the suggestion that such assignments could be based upon molecular chemistry, specifically the nucleotide sequence of the organism's ribosomal RNA. These assignments based upon RNA sequence are assumed to represent the phylogenetic origin and evolutionary history of the organisms and they have largely confirmed the preexisting eukaryote classifications that had been based upon physical traits. Similarly, defining the genetic relatedness of the viruses on the taxonomic levels of order, family, genus and species, as elaborated by the ICTV was initially based upon morphologic and antigenic characteristics of the viruses. These older viral classifications have subsequently been refined and largely confirmed based upon the nucleotide sequence and organizational structure of the viral genomes. The proposed taxonomic structure for the genomic acellular infectious agents (Figure 2.10) suggests a logical placement of the existing ICTV taxonomic classifications into a higher-level schematic by progressing upwards using successively more basic attributes of the viral genomes. The suggested domain name is Akamara (ακαμαρα), whose derivation from Greek [α (without) + καμαρα (vault, chamber)] could represent the fact that these life

Plate 2.4.1 Family: Bunyaviridae. Nucleic acid: RNA. Genome: Single-stranded, 3 linear or pseudo-circular segments which differ by genus with regard to their sense, often at least one is Ambisense and the others variously are either Negative sense or Ambisense, (10.5–22.8 Kb). Morphology: Enveloped. Virion: Spherical (pleomorphic), (Mw = 3.0–4.0 × 10^8) Nucleocapsid: Helical.

Plate 2.4.2 Floating genus: *Emaravirus.* Nucleic acid: RNA. Genome: Single-stranded, Negative sense and possibly Ambisense, 4 linear segments (approx. 12.3 kb total) with reports of double stranded RNA. Morphology: Enveloped. Virion: Spherical (Mw not specified). Nucleocapsid: Helical (presumably).

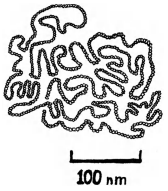

Plate 2.4.3 Family: Ophioviridae. Nucleic acid: RNA. Genome: Single-stranded, Negative sense, 3–4 linear segments (12.4 Kb total). Morphology: Non-enveloped. Virion: Filamental (highly flexible) (Mw not specified). Nucleocapsid: Undefined (possibly either helical or a 'stacked disc' structure; the latter would physically resemble a string of beads, with the 'beads' being globular capsid proteins that are strung together by a genomic nucleic acid strand which passes through a central hole in the three dimensional structure of those proteins). Distinguishing feature: The genomic segments are encapsidated separately resulting in two generally distinct size classes of viral particles. Positive sense RNA segments also can be encapsidated, but the Negative sense strands are numerically predominate by an overwhelming factor which can exceed 50 fold.

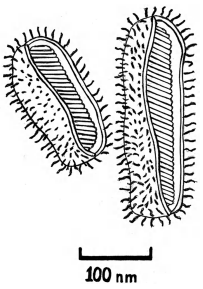

Plate 2.4.4 Family: Rhabdoviridae. Nucleic acid: RNA. Genome: Single-stranded, Negative sense, 1 linear segment (11–15 Kb). Morphology: Enveloped. Virion: Bullet as well as bacilliform (Mw = 0.3–1.0 × 10^9). Nucleocapsid: Helical.

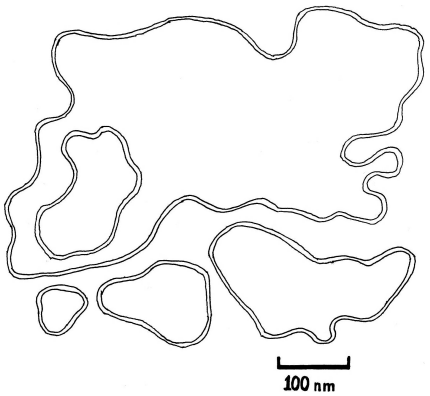

Plate 2.4.5 Floating genus: *Tenuivirus.* Nucleic acid: RNA. Genome: Single-stranded, both Ambisense and Negative sense, 4 to 5 linear segments (Approx. 16 Kb total). Morphology: Non-enveloped. Virion: Filamental (Mw not specified). Nucleocapsid: Helical. Distinguishing feature: The genomic segments are encapsidated independently resulting in different size categories of viral particles.

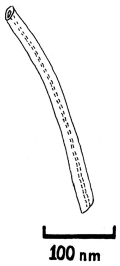

Plate 2.4.6 Floating genus: *Varicosavirus.* Nucleic acid: RNA. Genome: Single-stranded, Negative sense, 2 linear segments (12.9 Kb total). Morphology: Non-enveloped. Virion: Rod-like (slightly flexous), (Mw not specified). Nucleocapsid: Helical. Distinguishing feature: The genomic segments are encapsidated independently resulting in two generally distinct size classes of viral particles. Positive sense RNA segments also can be encapsidated, but the Negative sense strands are numerically predominate.

FIGURE 2.4 Relative sizes and basic information for those viruses which possess single stranded RNA genomes having either negative sense or ambisense coding.

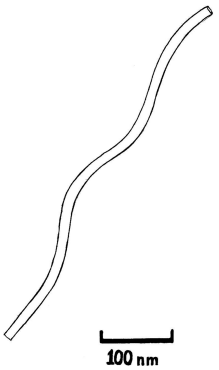

Plate 2.5.1 Family: Alphaflexiviridae (was: genus *Potexvirus*). Nucleic acid: RNA. Genome: Single stranded, Positive sense, 1 linear segment (5.0–9.4 Kb). Morphology: Non-enveloped. Virion: Rod-like (flexous) ($Mw = 3.5 \times 10^6$). Nucleocapsid: Helical.

Plate 2.5.2 Family: Barnaviridae. Nucleic acid: RNA. Genome: Single stranded, Positive sense, 1 linear segment (4 Kb). Morphology: Non-enveloped. Virion: Bacilliform ($Mw = 7.1 \times 10^6$). Nucleocapsid: Polyhedral.

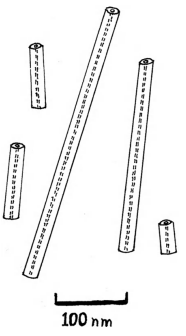

Plate 2.5.3 Floating genus: *Benyvirus.* Nucleic acid: RNA. Genome: Single stranded, Positive sense, 4–5 linear segments (Approx. 16 Kb total). Morphology: Non-enveloped. Virion: Rod-like (rigid), (Mw not specified). Nucleocapsid: Helical. Distinguishing feature: The genomic segments are encapsidated independently resulting in different size classes of viral particles.

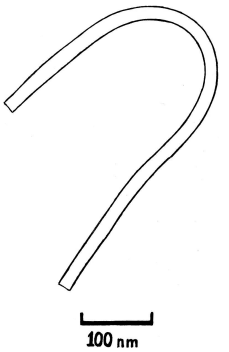

Plate 2.5.4 Family: Betaflexiviridae (was "Floating genera": Capillovirus, Carlavirus, Trichovirus). Nucleic acid: RNA. Genome: Single stranded, Positive sense, 1 linear segment (6.5–9.0 Kb). Morphology: Non-enveloped. Virion: Filamental (slightly flexous), [$Mw = 4.2 \times 10^6$–6.0×10^7]. Nucleocapsid: Helical.

Plate 2.5.5 Family: Bromoviridae. Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 3–4 linear segments (8.6–9.5 Kb total). Morphology: Non-enveloped. Virion: Icosahedral (the genomic RNA segments are encapsidated individually) ($Mw = 3.5$–6.9×10^6). Nucleocapsid: Icosahedral.

Plate 2.5.6 Floating genus: *Cilevirus.* Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 2 linear segments (approx. 13.7 Kb). Morphology: Enveloped. Virion: Bacilliform (Mw not specified). Nucleocapsid: Helical.

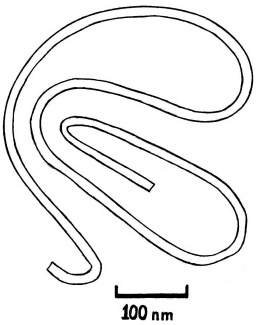

Plate 2.5.7 Family: Closteroviridae (was: genus *Closterovirus*). Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 1–2 linear segments (7.5–19.5 Kb total). Morphology: Non-enveloped. Virion: Filamental (flexous) [Mw = (estimate) 8.0–9.0×10^6]. Nucleocapsid: Helical. Distinguishing feature: For those viruses whose genomes have two segments, the segments are of different length and encapsidated independently resulting in different size classes of viral particles.

FIGURE 2.5 Relative sizes and basic information for those viruses which possess single stranded RNA genomes having positive sense coding.

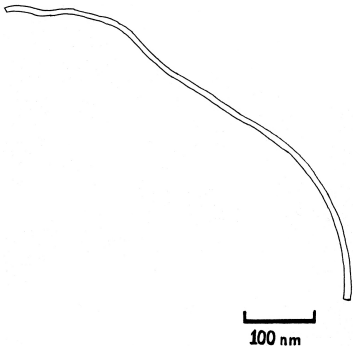

Plate 2.5.8 Family: Gammaflexiviridae. Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 1 linear segment (6.5–7.0 Kb). Morphology: Non-enveloped. Virion: Rod-like (flexous) (Mw not specified). Nucleocapsid: Helical.

Plate 2.5.9 Floating genus: *Idaeovirus.* Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 3 linear segments (8.6 Kb total). Morphology: Non-enveloped. Virion: Icosahedral ($Mw = 7.5 \times 10^6$). Nucleocapsid: Icosahedral. Distinguishing feature: The genomic segments are encapsidated separately resulting in viral particles that may be distinguished by density.

Plate 2.5.10 Family: Leviviridae. Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 1 linear segment (3.6–4.2 Kb). Morphology: Non-enveloped. Virion: Icosahedral ($Mw = 3.6–4.2 \times 10^6$). Nucleocapsid: Icosahedral.

Plate 2.5.11 Family: Luteoviridae (was: genera *Enamovirus* and *Luteovirus*). Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 1 linear segment (5.6–6.9 Kb). Morphology: Non-enveloped. Virion: Icosahedral ($Mw = 5.0–6.5 \times 10^6$). Nucleocapsid: Icosahedral.

Plate 2.5.12 Family: Marnaviridae. Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 1 linear segment (8.6–9 Kb). Morphology: Non-enveloped. Virion: Icosahedral (Mw not specified). Nucleocapsid: Icosahedral.

Plate 2.5.13 Family: Metaviridae. Nucleic acid: RNA. Genome: Single-stranded, Positive sense 1 linear segment (4–11 Kb). Morphology: Enveloped. Virion: None. Nucleocapsid: These are retrotransposons for which no capsid has been identified although they do form intracellular aggregations of irregularly ovoid enveloped particles presumably possessing nucleoprotein cores. These are termed "viral-like particles" because they are not considered infectious, and thus by definition are not virions.

Plate 2.5.14 Family: Nodaviridae. Nucleic acid: RNA. Genome: Single stranded, Positive sense, 2 linear segments (4.5 Kb total). Morphology: Non-enveloped. Virion: Icosahedral ($Mw = 8.0 \times 10^6$). Nucleocapsid: Icosahedral.

Plate 2.5.15 Floating genus: *Ourmiavirus.* Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 3–4 linear segments (4.7 Kb total). Morphology: Non-enveloped. Virion: Bacilliform (Mw not specified). Nucleocapsid: Polyhedral. Distinguishing feature: The genome segments presumably are encapsidated separately resulting in the appearance of viral particles of different lengths, with their respective capsids constructed by varying the number of capsid discs per virion.

Plate 2.5.16 Floating genus: *Polemovirus.* Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 1 linear segment (approx. 4.6 Kb). Morphology: Non-enveloped. Virion: Icosahedral (Mw not specified). Nucleocapsid: Icosahedral.

FIGURE 2.5 (*Continued*)

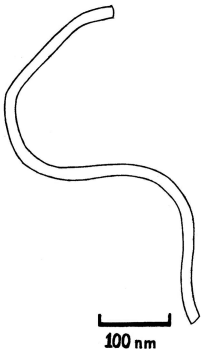

Plate 2.5.17 Family: Potyviridae. Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 1 linear segment (9–12 Kb). Morphology: Non-enveloped. Virion: Filamental flexous, [Mw = (estimate) 1.0×10^7]. Nucleocapsid: Helical.

Plate 2.5.18 Family: Pseudoviridae. Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 1 linear segment (4.2–12 Kb). Morphology: Non-enveloped. Virion: None. Nucleocapsid: Icosahedral. Distinguishing feature: These are retrotransposons and form intracellular aggregations of particles with icosahedral capsids termed "viral-like particles" but those are not considered infectious and therefore by definition are not virions.

Plate 2.5.19 Family: Secoviridae (was: Families Comoviridae and Sequiviridae). Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 1-2 linear segments (10–12 Kb total). Morphology: Non-enveloped. Virion: Icosahedral (Mw = 3.2–3.8 $\times 10^6$). Nucleocapsid: Icosahedral. Distinguishing feature: Those members with multipartite genomes encapsidate their genomic segments separately.

Plate 2.5.20 Floating genus: *Sobemovirus.* Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 1 linear segment (4.1–5.7 Kb). Morphology: Non-enveloped. Virion: Icosahedral (Mw = 6.6×10^6). Nucleocapsid: Icosahedral. Distinguishing feature:

Plate 2.5.21 Family: Tombusviridae (was: genera *Dianthovirus*, *Machlomovirus*, and *Necrovirus*). Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 1-2 linear segments (3.8–5.3 Kb total). Morphology: Non-enveloped. Virion: Icosahedral (Mw = 6.1–8.9 $\times 10^6$). Nucleocapsid: Icosahedral.

Plate 2.5.22 Family: Tymoviridae (was: genera *Marafivirus* and *Tymovirus*). Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 1 linear segment (6–7.5 Kb). Morphology: Non-enveloped. Virion: Icosahedral (Mw = (estimate) 3.6–9.6 $\times 10^6$). Nucleocapsid: Icosahedral.

Plate 2.5.23 Floating genus: *Umbravirus.* Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 1 segment (4.2–6.9 Kb). Morphology: Non-enveloped. Virion: Icosahedral (Mw not specified). Nucleocapsid: Icosahedral. Important note: Umbraviruses require a coinfecting helper virus to provide them with capsid proteins, a role that can be fulfilled by some members of the Luteoviridae which otherwise are capable of replicating independently.

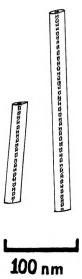

Plate 2.5.24 Family: Virgaviridae (was: genera *Furovirus*, *Hordeivirus*, *Tobamovirus*, and *Tobravirus*). Nucleic acid: RNA. Genome: Single-stranded, Positive sense, 1-4 linear segments (encapsidated separately) (6.4–19.9 Kb total). Morphology: Non-enveloped. Virion: Rod-like (Rigid), (Mw = 1.1–5.0 $\times 10^7$). Nucleocapsid: Helical. Distinguishing feature: The genome segments are encapsidated separately resulting in different size classes of viral particles.

FIGURE 2.5 (*Continued*)

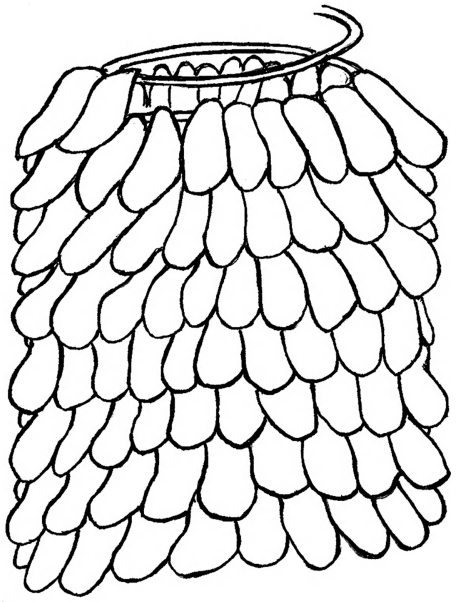

FIGURE 2.6 Drawing of a helical capsid structure showing how the capsid proteins attach to the helical coil of the viral nucleic acid genome. Presumably all of the capsid proteins are identical to one another in a helical structure.

forms do not possess a cellular structure of their own.

All of the groups of infectious agents shown in Figure 2.10 are depicted as belonging to a common domain as it would seem perhaps improbable to premise an accurate grouping of these agents based upon which of the three commonly suggested evolutionary sources represented their respective origins. In examining this point we should remember the three possible theories about how viruses began. These are: that the viruses may be remnants of the primordial soup, might represent degenerated cellular organisms, or be regulatory cellular elements that have gone awry.

The suggested subdominal classifications as shown in Figure 2.10 would group together the numerous agents whose genomes code for their own structural 'shell' or 'capsid' proteins as one kingdom (Euviria, signifying 'true' viruses). The viroids, a group of agents which share a unique and very homogenous single-stranded RNA genomic organization which somehow has enabled them to evolutionarily persist despite the fact that they do not code for proteins, are suggested as constituting a second kingdom (Viroidia) along with other groups of related agents whose genomes likewise do not code for their structural 'shell' or 'capsid' proteins. This may be perceived as a key biological difference, since all of the cellular organisms as well as the Euviria completely code for their own structural proteins. The kingdom Euviria is suggested as being divided into two phyla, which separate the Euviria with respect to whether their genomes are composed of RNA (Ribovira) or DNA (Deoxyribovira). It is suggested in turn, that these two phyla of the Euviria could logically be subdivided into classes based upon whether their genomes are double stranded or single stranded. Those Euviria which possess single stranded RNA genomes could logically be divided as to whether their genomes are "plus" sense, meaning that they can be directly translated by ribosomes, or are "negative" sense. This assignment of phyla and classes is premised upon principal biochemical differences in the agents' genomes and also follows basic commonalities in terms of the molecular strategies of these infectious agents. As noted above, the viroids, virusoids, and floating genus Deltavirus are shown as being assigned a separate kingdom level, named Viroidia, and the suggested schematic carries their placement intact to the phylum level as all of the agents grouped into that category possess RNA genomes. The current ICTV nomenclature structure groups these genomic acellular infectious agents at the levels of order through species and could be adopted directly into this proposed new domain. An example of that existing ICTV structure is shown within the box that is inside of Figure 2.10. That ICTV nomenclature structure is now considered to be based upon viral nucleotide sequence commonalities at the lowest levels, progressing through commonalities in genome organization according to a 'bottom up' philosophy. The levels of taxonomy proposed here can be seen as logically continuing that trend by progressing upward to the trait of strandedness at the class level, where a

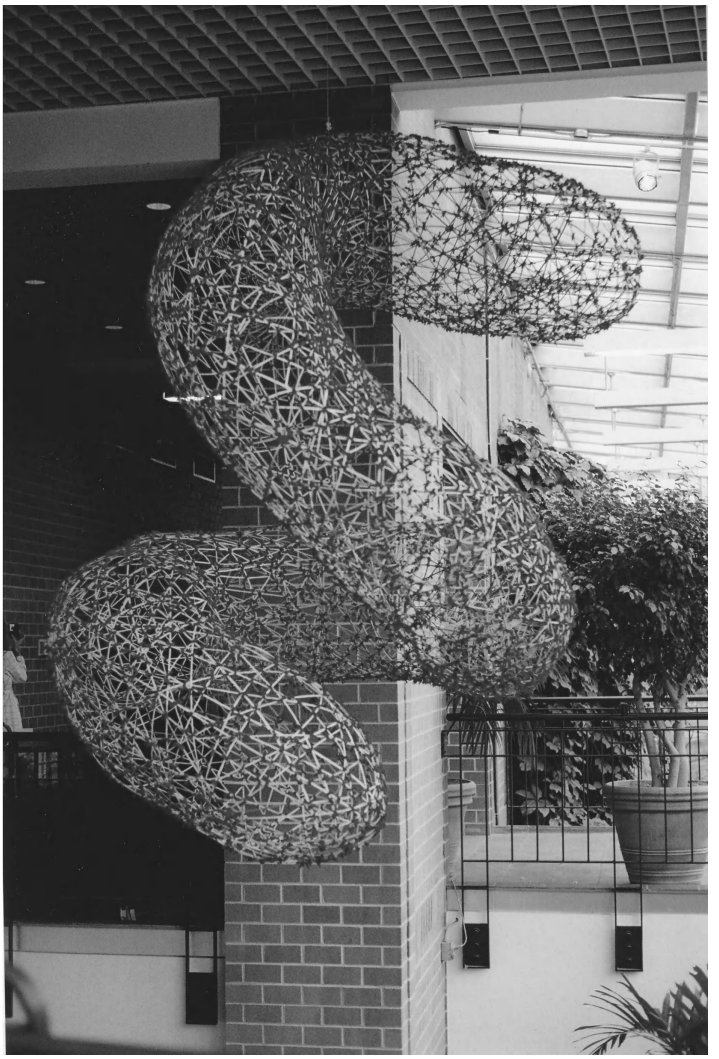

FIGURE 2.7 "Vivaldi" by Shigeo Kawashima, 2008; Collection of the Franklin Park Conservatory, Columbus, Ohio, USA. A sculpture made of bamboo, plastic zip ties and yarn, that has an interesting similarity of appearance to the filoviruses.

distinction of single versus double stranded genome is used and aided by the designation of plus versus negative sense in the case of those viruses whose strategies are based upon single stranded RNA genomes, and to the still more basic distinction of RNA (Ribovira) versus DNA (Deoxyribovira) genome at the phylum level. Simultaneously, this proposed structure could also be perceived as progressing from the top downward to meet the current ICTV structure. Perhaps someday, the ICTV will allow me to present this concept in the open journal literature!

Perhaps it is necessary to ask if these genomic acellular infectious agents do indeed deserve to be considered among the living and therefore assigned into a taxonomic structure. In examining this point, we should remember the three possible theories about how viruses began, id est, that these agents as we know them represent the evolutional product from either primordial remnants, degenerated cells, or

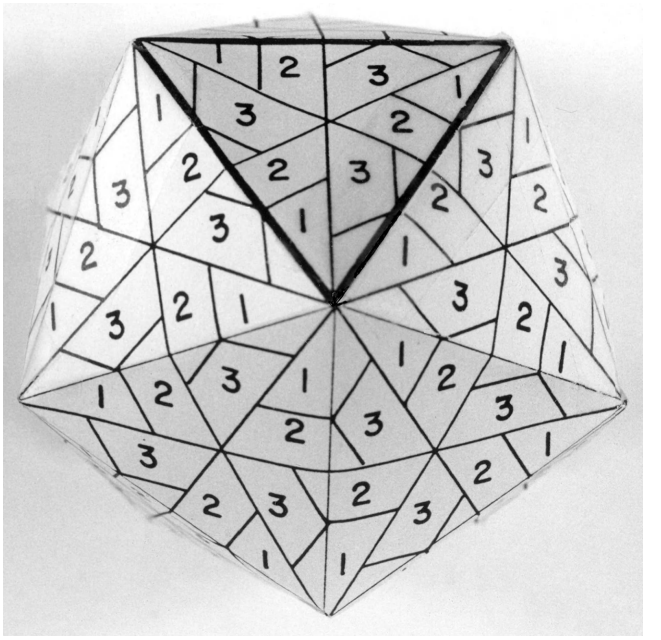

FIGURE 2.8 Photograph of the assembled model published by Hurst et al. (1987) showing the protein arrangement in an icosahedral capsid structure. This particular structure is a representation of the viral family Picornaviridae. The members of this viral family produce capsids that contain multiple copies of three major (larger sized, numbered 1, 2, and 3) capsid proteins and one minor (smaller sized) capsid protein. The relative positions of the three major capsid proteins are shown as the trapezoids numbered 1, 2, and 3. The trapezoidal shape is used for illustrative purposes, as the true shapes of these proteins is more complex and not truely trapezoidal. The darkly outlined triangle represents one of the twenty sides of the viral capsid. Although these sides are often referred to as "faces", the word icosahedron literally interprets from the greek as meaning that this structure has twenty surfaces upon which it could sit.

rogue cellular elements. It is indeed possible and perhaps likely that the origin and evolutionary course which the various groups of genomic acellular infectious agents have followed to attain their present forms does in fact reflect a combination of contributions from all three sources. Many of the large viruses certainly have replicative traits that seem to mimic

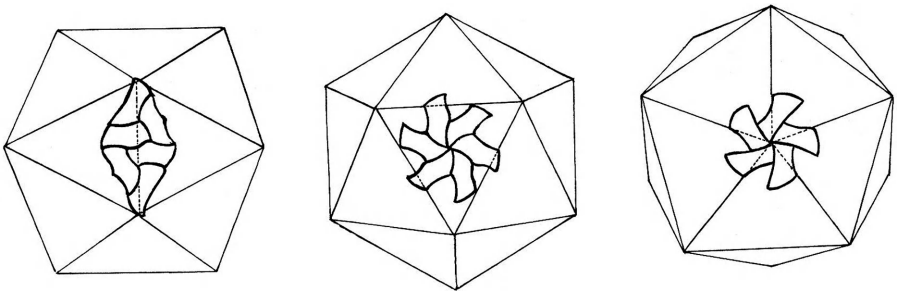

FIGURE 2.9 Drawing of an icosahedral capsid structure showing what would be a mirror image of the shape of the capsid proteins for the viral family Bromoviridae. Unlike the picornaviral model, the bromoviral capsid seems to contain multiple copies of only one type of capsid protein. Presumably, those copies of the same protein would be rotated into different relative positions such that they can arrange into an icosahedron. This drawing shows how those capsid proteins combine to produce the two-fold (left image), three-fold (center image) and five-fold (right image) axes of symmetry which define an icosahedral structure.

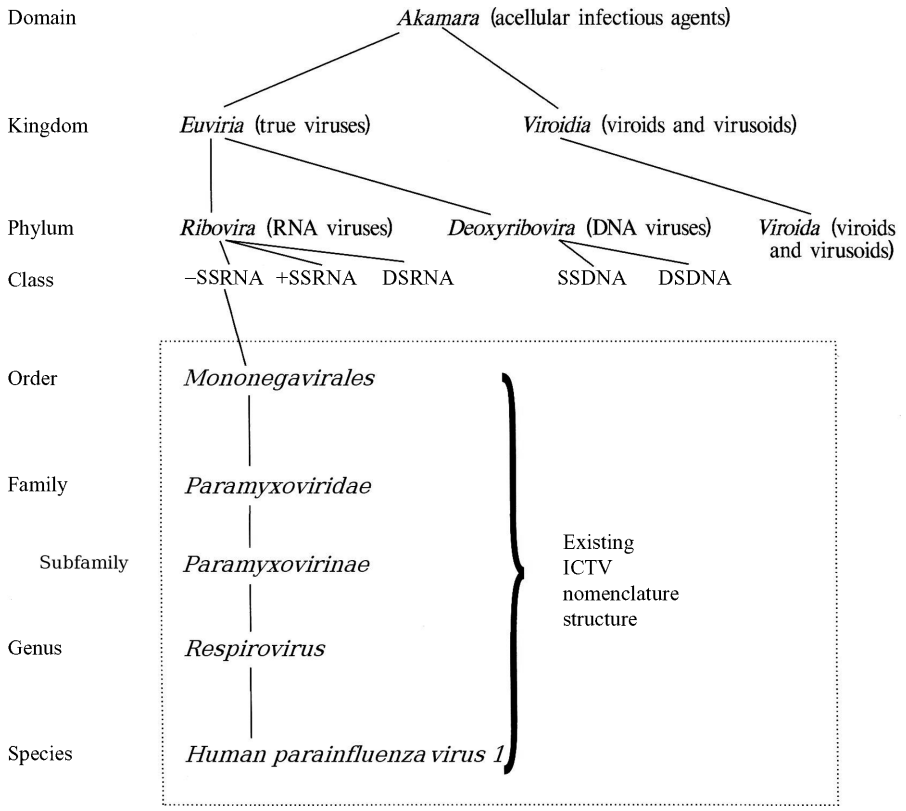

FIGURE 2.10 Proposed new domain, Akamara, plus proposed taxonomic structure at the levels of kingdom, phylum, and class. The abbreviated designations at the class level represent: –SSRNA, 'negative' sense single stranded RNA genome; +SSRNA, 'positive' sense single stranded RNA genome; DSRNA, double stranded RNA genome; SSDNA, single stranded DNA genome; and DSDNA, double stranded DNA genome. The existing ICTV nomenclature structure for viruses, an example of which is shown within the inset box, covers only taxonomic levels from order through species and could be adopted directly into this domain.

some of the molecular complexity which we associate with cellularity. In contrast, the idea that these groups of acellular infectious agents began as sub-cellular elements that gained an independence is certainly suggested by some of the smaller viruses, which scarcely carry any more genetic coding than that which is required for their few capsid proteins, and by the viroids and virusoids which do not code for capsid proteins. However, since these groups of acellular infectious agents seem to have evolutionarily taken on an identity of their own, then perhaps we should recognize that identity as a life form. When considering the possibility that these groups of acellular infectious agents might in fact have begun as cellular organisms that have since lost biochemical complexity, if we should consider them now to be non-living based upon the application of complex definitions of living creatures, then we would be faced with deciding at which exact point in the process of evolutionary simplification the term 'life' would cease to be applicable. If, alternatively, we could more simply define life by indicating that living things are naturally existing organic entities which are capable of catalyzing their biochemical self replication, then yes, these genomic acellular infectious agents are a form of life.

It is not necessarily implied that the suggested taxonomic separation of these genomic acellular infectious agents into kingdoms,

phyla and classes represents a strict phylogeny. This is a departure from the current philosophy that the existing ICTV taxonomic nomenclature, which goes no higher than the level of order, does carry strong evidence for common phylogeny. Taxonomic systems change with the evolution of our scientific philosophy, and the proposed categories presented in this chapter are only a suggestion. However, it might be necessary to admit that we may never be able to use strict phylogeny to base an exact classification of these agents at the very highest taxonomic levels. This is due to the idea that after presumably billions of years of coevolution and biochemical interactions within their host cells, the physical appearance of these modern descendants may be very different from that of their evolutionary ancestors and they have left no identifiable fossilized remains to guide us. Instead, basic biochemistry has been used as the basis for this proposed division of the genomic acellular infectious agents into kingdoms, phyla and classes. If there is a fault to be found with this proposal, it is perhaps that this organizational structure could be seen as relating to a cellular origin for the genomic acellular infectious agents. However, this proposal is not intended to imply that cellularity was an initiating condition for the evolution of these acellular agents. Rather, as indicated earlier in this article, we do not and perhaps cannot know the conditions under which these life forms initially began, and the same present-day endpoint could have resulted from gradual evolution regardless of whether either the viruses or other genomic acellular infectious agents began as primordial components, cellular organisms, or rogue cellular elements. Likewise, it would not be possible to place these organisms into the three domains described by Woese et al. (1990), since those domains are defined by the nucleotide sequences of their constituent organism's genes that code for ribosomal RNA whereas the genomes of the viruses, satellite viruses, virusoids and viroids do not code for ribosomal RNA. There is a suggestion that single point evolutionary connections exist at the branch junctures where the three domains that are used to describe the cellular organisms separate from one another. However, assigning a single point of evolutionary connection between the life forms contained in this newly proposed domain Akamara and the domains of Woese et al. (1990) may not be possible as that might necessitate stating that these acellular organisms either evolved into or from the cellular organisms which are represented by the three existing domains.

2.4 CONCLUSIONS

The purpose of this chapter is to help us take stock of what we as virologists now have available in terms of taxonomy for the viruses and their relatives. As we now have passed the century mark for use of the name virus (Beijerinck, 1898) and official biological recognition of the viruses by a scientific commission (Loeffler and Frosch, 1898), it would seem time that the scientific community consider extending the existing viral taxonomy by recognizing the viruses on a domain level. The taxonomic schematic proposed in this chapter is, of course, only one possible suggestion and no taxonomic scheme can be considered infallable. However, this proposal is logically based and represents an assessment which relies upon the successive generations of sound biochemical research that has been conducted and published by tens of thousands of virologists world wide. What does this proposal leave out? Classification of those RNA viruses which utilize reverse transcription to produce a DNA equivalent of their genome during the course of their replication could be perceived as requiring a separate class or even phylum within the proposed kingdom Euviria. Those viruses which possess single stranded RNA genomes that are positive sense imitate messenger RNA molecules. Those viruses that possess single stranded RNA genomes which are ambisense, meaning that their genomes are partially negative sense and partially positive sense, may be seen as representing a significant departure from those viruses which possess single stranded RNA genomes that strictly are

positive sense. The reason why this represents a molecular departure is because the negative sense regions of those viruses' RNA genomes must be copied to form a matching positive sense strand before they can be translated into protein. Perhaps the ambisense single stranded RNA viruses could be grouped with the negative sense single stranded RNA viruses because the members of this latter viral group also have incorporated the same molecular departure into their biochemistry. This commonality is the reason why the ambisense and negative sense single stranded RNA viruses were combined together into Figure 2.4.

What else is left in the field of biological entities? There are reasons for biologists to potentially consider at least some plasmids to represent a type of infectious agent, and perhaps therefore to be a form of life and worthy of an eventual home within some taxonomic scheme. A noteworthy example of this might be the conjugational plasmids which carry coding for specific protein structures that are then expressed by their cellular host organisms and are important in facilitating the transmission of that plasmid to a new cellular host. We certainly allow the LTR (long terminal repeat) retrotransposons to be considered viruses and presume that these may have had an initial viral origin, the ICTV has grouped those into the viral families Metaviridae and Pseudoviridae. The viral species called Acidianus two-tailed virus, which currently is the sole member of the viral family Bicaudaviridae, has the unique trait of undergoing a dramatic morphological rearrangement which is considered to be a maturation following its release from the host cell (Prangishvili et al, 2006) and this is a strong departure from any of the other viruses. This difference seems sufficient to potentially suggest that at some future time the Acidianus two-tailed virus will become the first identified member of a new non-viral category of biological entities. The prions of mammals are considered infectious agents and in that sense might eventually be thought to also represent a form of life despite the fact that they apparently do not carry any genomic coding of their own (and thus would be defined as agenomic or non-genomic) as they move from one host to another. Indeed, we must consider that perhaps an infectious agent would not need to carry any genomic material with it if its new host cells already possessed all of the coding necessary for replicating that agent. In fact, while two of the traits which prions possess, a measure of resistance to acidic conditions and to proteolytic enzymes (Volume 1, Chapter 10), would seem to contribute to the pathogenic process associated with the prions, these same two traits could also be seen as representing an evolutionary adaptation to the acidic conditions and enzymatic milieu encountered in the digestive tract of their host animals during the course of these prions' natural transmission as an enterically acquired infection. These remaining questions represent fodder for future thought.

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CHAPTER 3

VIRUS MORPHOLOGY, REPLICATION, AND ASSEMBLY*

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CONTENTS

3.1 INTRODUCTION

Viruses are unique life forms different from all other living organisms, either eukaryotes or prokaryotes, for three fundamental reasons: (1) the nature of environment in which they grow and multiply, (2) the nature of their genome, and (3) the mode of their multiplication. First, they are obligate intracellular parasites, that is, can function and multiply only inside another living organism that may be a prokaryotic or eukaryotic cell depending on the virus. Viruses are acellular and metabolically inert outside the host cell. Although there are other examples of obligatory parasites among the eukaryotes and prokaryotes, the nature of the intimate relationship between viruses and

* This is a revised version of the chapter that appeared in *Viral Ecology*, edited by Christon J. Hurst, published in 2000 by Academic Press.

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their host (i.e., environment) is much different. For example, some viruses extend their parasitic behavior to another level of mutual coexistence with their host; that is, they not only exist intracellularly but can also, and do in some cases, integrate their genome into the genome of their host and thus tie their fate to the fate of the host. In fact, under these conditions, the integrated viral genome behaves as a host gene(s), undergoing similar regulatory control in transcription and replication and similar evolutionary changes as do the host gene(s). Second, whereas all other living forms can use only DNA (and not RNA) as their genetic material (genome) for information transmission from parent to progeny, viruses can use either DNA or RNA as their genome; that is, some viruses can use only RNA (and not DNA) as their genetic material. Therefore, these classes of RNA viruses have developed new sets of enzymes for replicating and transcribing RNA from an RNA template, as such enzymes (RNA-dependent RNA polymerase (RDRP)) are not normally found in eukaryotic or prokaryotic cells. Finally, all eukaryotic and prokaryotic cells divide and multiply as a whole unit, that is, $1 \rightarrow 2 \rightarrow 4 \rightarrow 8$ and so on. However, viruses do not multiply as a unit. In fact, they have developed a much more efficient way to multiply just as complex machines are made in a modern factory. Different viral components are made separately from independent templates, and then these components are assembled into the whole and infectious units, also called virus particles (virions), just as the complex machines are efficiently assembled from individual components. Similarly, disassembly of the virus components occurs during the infection process, leading to genome replication, transcription of messenger RNAs (mRNAs), translation of viral proteins, and assembly of the virus particles and their release from the infected cell into the environment for continuing the next infectious cycle. In this chapter, aspects of viral morphology, mode of viral replication, and viral morphogenesis including budding and release are discussed.

Although all viruses exhibit this common mode of replication or infectious cycle, viruses are a heterogeneous group of microorganisms that vary with respect to size, morphology, and chemical composition. The size of virions ranges from 20 nm (parvovirus, family Parvoviridae) to ~300 nm (poxvirus, family Poxviridae) in diameter, compared to the size of *Escherichia coli*, which is about 1000 nm in length. However, some filamentous viruses such as filoviruses (family Filoviridae) may be 800 nm or even longer. In addition to size, the shape of viruses also varies. Some viruses are round (spherical, spheroidal), others filamentous, and still others pleomorphic. Usually, naked (nonenveloped) viruses have specific shapes and sizes (Figure 3.1), whereas some enveloped viruses (particularly those possessing helical nucleocapsids) are highly pleomorphic (e.g., orthomyxoviruses), with shapes varying from spherical to filamentous (Table 3.1, Figures 3.1–3.5). Viruses are different from viroids and prions. Viroids are small, circular, single-stranded infectious RNA molecules without a protein coat or capsid and cause a number of plant diseases including potato spindle tuber disease, cucumber pale fruit disease, citrus exocortis disease, and cadang-cadang (coconuts) disease, and so on. On the other hand, prions are infectious protein molecules without any DNA or RNA and thought to cause transmissible and/or inherited neurodegenerative diseases known as transmissible spongiform encephalopathies. These include Creutzfeldt–Jakob disease, kuru, and Gerstmann–Straussler syndrome in humans, as well as scrapie in sheep and goats and mad cow disease in cattle. The infectious prion proteins are modified forms of normal proteins encoded by a host gene. The normal prion protein that has alpha helices in its secondary structure is converted into beta sheets for the secondary structure in diseased animals.

3.2 CHEMICAL COMPOSITION

The chemical composition of a virus depends on the nature of that virus, that is, the nature of

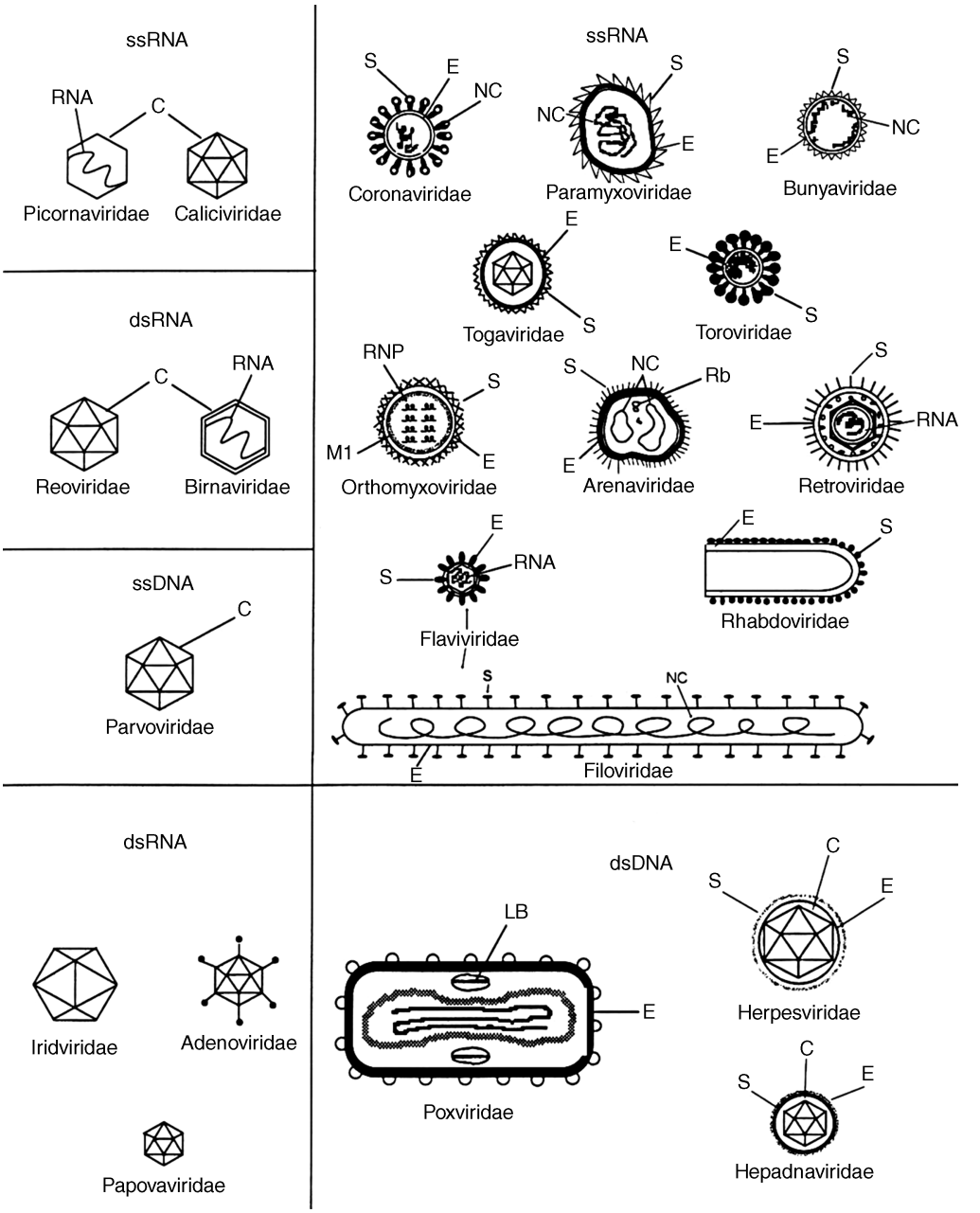


FIGURE 3.1 Schematic presentation of different forms of viral structures. C, capsid; S, spike on viral envelope; E, viral lipid envelope; NC, nucleocapsid (i.e., capsid proteins in association with RNA or DNA); M1, matrix protein of influenza virus; LB, lateral bodies present in poxviruses; ss, single-stranded, ds, double-stranded RNA or DNA.

the viral genome (RNA or DNA), the composition of the protein shell called the viral "nucleocapsid" surrounding the genome, and the presence or absence of viral membrane depending on whether the virus is enveloped or naked. All viruses have nucleocapsids and therefore contain nucleic acids and proteins. The nucleic acid is the genome that contains the

TABLE 3.1 Properties of the Virions of the Major Genera of DNA and RNA Animal Viruses

Viruses	Genome Nature of Nucleocapsid	Envelope	Shape	Genome Polarity	Size (nm)	Transcriptase in Virion	Symmetry
RNA viruses							
Enterovirus	S,[a]1[b]	−	Icosahedral	+	~20–30	−	Icosahedral
Rhinovirus	S, 1	−	Icosahedral	+	20–30	−	Icosahedral
Calicivirus	S, 1	−	Icosahedral	+	20–30	−	Icosahedral
Alphavinis	S, 1	+	Spheroidal	+	50–60	−	Icosahedral
Flavivirus	S, 1	+	Spheroidal	+	40–50	−	Icosahedral
Orthomyxovirus	S, 8	+	Spheroidal[c]	−	80–120	+	Helical
Paramyxovirus	S, 1	+	Spheroidal	−	100–150	+	Helical
Coronavirus	S, 1	+	Spheroidal	+	80–220	−	Helical
Arenavirus	S, 2	+	Spheroidal	±[d]	85–120	+	Helical[e]
Bunyavirus	S, 3	+	Spheroidal	±[d]	90–100	+	Helical[e]
Retrovirus	S, 1[f]	+	Spheroidal	+	100–120	+[g]	Icosahedral[h]
Rhabdovirus	S, 1	+	Bullet shaped	−	175 × 70	+	Helical
Reovirus	D, 10	−	Icosahedral	±	70–80	+	Icosahedral
Orbivirus	D, 10	−	Icosahedral	±	50–60	+	Icosahedral
Filovirus	S, 1	+	Filamentous	−	≥80 × 800	+	Helical
DNA viruses							
Papillomavirus	D, circular	−	Icosahedral	±	55	−	Icosahedral
Polyomavirus	D, circular	−	Icosahedral	±	45	−	Icosahedral
Adenovirus	D, linear	−	icosahedral	±	70–80	−	Icosahedral
Hepadnavirus	D, circular partiae	+	Spheroidal	±	40–50	+[l]	Icosahedral
Herpesvirus	D, linear	+	Spheroidal	±	150	−	Icosahedral
Iridovirus[i]	D, linear	+[i]	Spheroidal	+	125 × 300	+	Icosahedral
Poxvirus	D, linear	+	Brick shaped	+	300 × 240 × 140[j]	+	Complex
Parvovirus	S, linear	−	Icosahedral	+,−[k]	20	−	Icosahedral

[a] D = double-stranded; S = single-stranded.
[b] Genome, the number indicates the segments of RNA present in the virus particle. All RNA genome is haploid except retrovirus (diploid).
[c] Pleomorphic including filamentous forms.
[d] Ambisense (contains coding for protein on both genomic and complementary RNA strands).
[e] Circular helical nucleocapsid.
[f] Diploid, two molecules of the same RNA (+ strand) segment are present in one virus particle.
[g] Reverse transcriptase (RT).
[h] The capsid structure of mature retroviruses is not fully known, although it appears icosahedral.
[i] Insect iridoviruses have no envelope; vertebrate members are enveloped.
[j] Length × width × thickness.
[k] Some virus particles contain plus-strand and others contain minus-strand DNAs.
[l] P protein functions as reverse transcriptase.

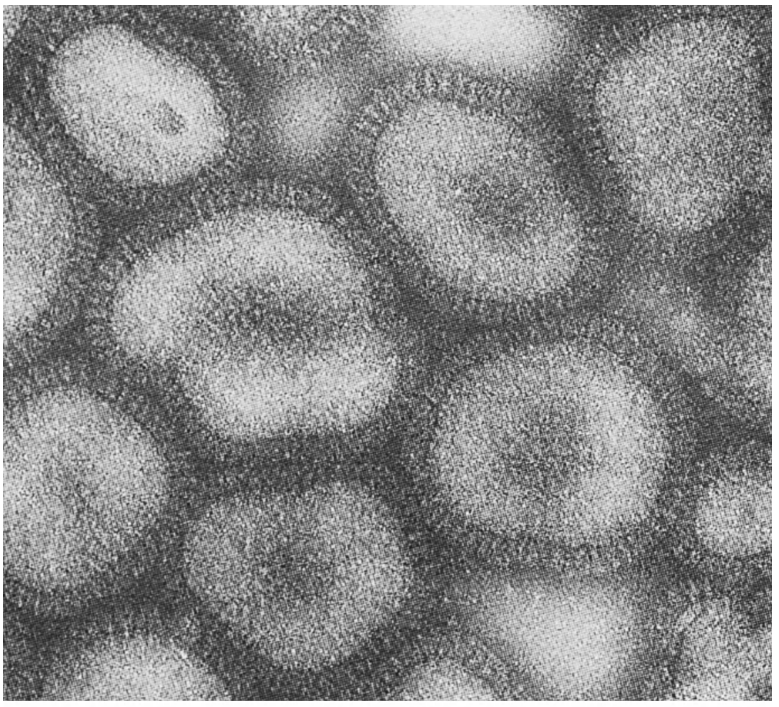


FIGURE 3.2 Influenza virus morphology. Transmission electron micrographs of *Influenzavirus A*. Courtesy of K. G. Murti of St. Jude Children's Research Hospital of Memphis, Tennessee.

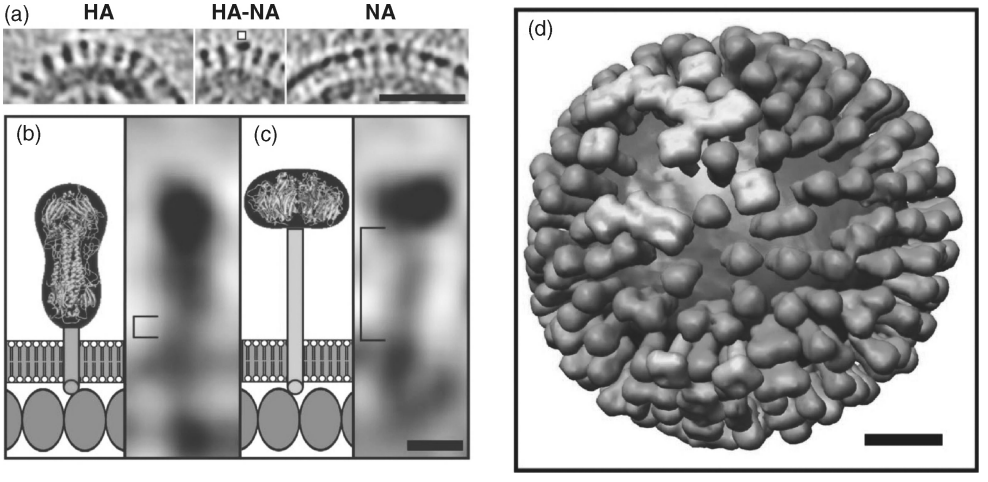


FIGURE 3.3 Model virus with HA and NA spikes by cryo-ET analysis. (a) HA cluster (left), (b) single NA (marked) in a cluster of HA (middle), and (c) cluster of mainly NA spikes (right). (b and c) The stem length of HA and NA (square brackets in (b) and (c), respectively). The structures of the stem, transmembrane domain, and ectodomain are shown schematically. Molecules in the matrix layer are inferred to be packed in a monolayer (scale bar 5 nm). (d) Model of distribution of HA (green), NA (gold), and lipid bilayers (blue) in a single virion (scale 20 nm). Reproduced from Harris et al. (2006) with permission. (*See the color version of this figure in Color Plate section.*)

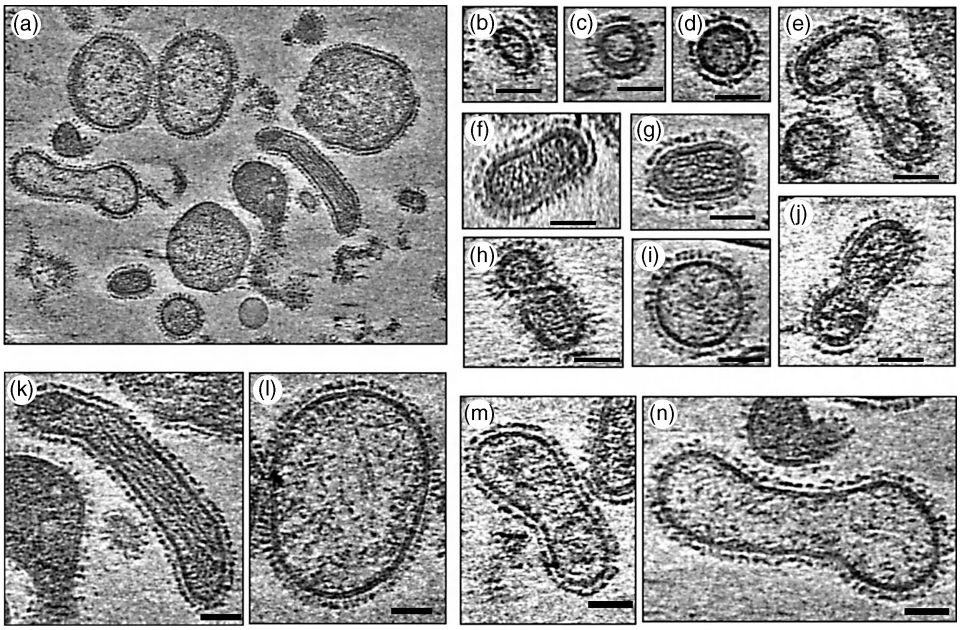


FIGURE 3.4 Cryoelectron tomography of A/PR8 (an antigenic variant of influenza A virus, a member of the genus *Influenzavirus A*) showing highly pleomorphic virion architecture. (a) A density slice from a 3D cryoelectron tomography reconstruction of influenza A virus strain PR8. PR8 virus was grown in MDCK cells at 0.001 MOI. The tilt series spanning –70° to 70° sample tilt were recorded in a TF20 cryoelectron microscope using the Batch Tomography program (FEI Company) and then reconstructed using the Inspect3D (FEI Company) and refined by Protomo program (Winkler and Taylor, 2006). (b–n) Comparison of central slices of viral particles extracted from different cryoelectron tomograms. Different virus particles were picked at random. No attempt was made to determine the percentage of each virus form in the population. Each virus particle contained electron dense spots (RNP) inside and spikes outside. Both HA and NA spikes, as identified based on morphology described in Harris et al. (2006), were visible on the outer membrane (scale bar 50 nm). Reproduced from Nayak et al. (2009).

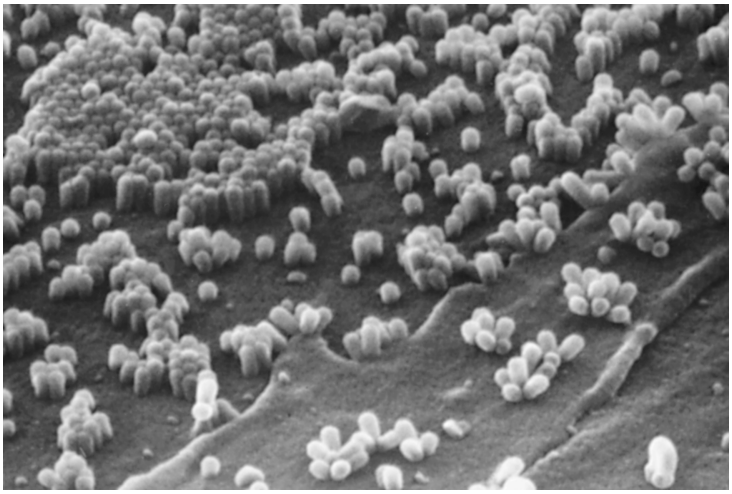


FIGURE 3.5 Scanning electron micrographs of influenza viruses budding from infected cells. Spherical virus particles nearly complete are seen budding from infected cells (×40,000). These micrographs were provided by David Hockley of the National Institute for Biological Standards and Control at Hertfordshire, UK and reproduced from Nayak et al. (2009).

information necessary for viral function and multiplication, and this information is passed from the parent to progeny viruses. Some viruses contain extragenomic nucleic acid, for example, tRNA in retroviruses (family Retroviridae) and ribosomal RNA in arenaviruses (family Arenaviridae). Viral proteins have three primary functions: (1) they provide the shell to protect the nucleic acid from degradation by environmental nucleases, (2) facilitate transfer of the genome from virus to host and from one host to another, and (3) provide many of the enzymatic and regulatory functions needed for transcription and replication so that viruses can survive, multiply, and perpetuate. In addition to the capsid shell, many viruses also possess an envelope (or viral membrane) around the nucleocapsid. The envelope in these viruses is critical for their transmission from one host to another. The naked nucleocapsids of enveloped viruses are noninfectious or poorly infectious because they lack the viral receptor binding protein for attachment to the host receptor. The viral envelope contains lipids and carbohydrates in addition to "envelope- or membrane-associated" viral proteins. The viral genome codes for most, if not all, of the proteins associated with the viral envelope. Lipids of the viral membrane, on the other hand, are synthesized by the host cell and derived from it. Therefore, viral lipid composition varies depending on the host cell in which the virus grows and also on the type of the cellular membrane (e.g., ER, Golgi, plasma, or nuclear membrane) from which the particular type of virus buds. However, lipid composition of viral membrane does not completely mimic that of the cellular membrane but rather is selectively enriched in specific host lipid components. Enveloped viruses in most cases are assembled and bud from specialized membrane microdomains called lipid rafts that are enriched in long saturated fatty acids, cholesterol, and sphingolipids (Nayak and Hui, 2004; Nayak et al., 2009). The carbohydrate content of the viral envelope is usually determined by the nature of glycosylation (*N*-glycosylation, *O*-glycosylation, complex versus simple sugar addition) of the viral envelope proteins, which may in turn undergo other modifications, such as myristoylation, palmitoylation, sulfation, and phosphorylation.

3.2.1 Viral Nucleic Acid (Genome)

Genomes of different viruses are widely diverse in size and complexity. Some are composed of DNA, while others of RNA. As mentioned earlier, only in viruses is RNA known to function as a genome. Viral DNA genomes vary in complexity ranging from 5 kb containing 5–6 genes (parvoviruses, members of the viral family Parvoviridae; SV40 (Simian virus 40), family Polyomaviridae, genus *Polyomavirus*) to over 300 kb (avipoxviruses, family Poxviridae, genus *Avipoxvirus*) containing more than 200 genes and having complex organization. Some DNA genomes are double-stranded (SV40), some are partially double-stranded (hepatitis B virus (HBV), family Hepadnaviridae, genus *Orthohepadnavirus*), and still others are single-stranded (parvoviruses) (Tables 3.1 and 3.2). The single-stranded viral DNAs can be of plus or minus polarity. Some DNA genomes are circular (and supercoiled), while others are linear. Some linear DNA genomes become circular intermediates during replication. Many viral DNA genomes are terminally redundant in their nucleotide sequences.

RNA genomes of viruses also vary in length and complexity but not as widely as do DNA genomes. For the RNA viruses known to date, the range of variation is from ~7 kb for rhinoviruses, which are divided into the species of human rhinovirus A, B, and C, all belonging to genus *Enterovirus* of the family Picornaviridae, to ~30 kb for coronaviruses (family Coronaviridae). Coronavirus RNA represents the largest stable single-stranded RNA found in nature. Viral RNA can be single- or double-stranded (Tables 3.1 and 3.3).

The viral RNA genome may be nonsegmented, consisting of a single RNA molecule, or segmented, consisting of multiple segments. Usually, viral genomes are haploid, but some are diploid (e.g., retroviruses; Figure 3.6). Some viral RNA genomes may be linear, whereas others have partial terminal complementarity

TABLE 3.2 Replication of DNA Viruses

Virus	Form of DNA	Polymerase	Activity	Presence in Virion	Replication Site in Cell
Papovaviruses	ds[a]	Host	DNA *pol*	–	Nucleus
Adenoviruses	Ds	Viral	*DNA pol*	–	Nucleus
Herpesviruses	Ds	Viral	DNA *pol*	–	Nucleus
Poxviruses	Ds	Viral	DNA *pol*	–[b]	Cytoplasm
Parvoviruses	Ss	Host	DNA *pol*	–	Nucleus
Hepadnaviruses	Partially Ds	Viral	Reverse transcriptase	+	Nucleus/cytoplasm

[a] ds, double-stranded; ss, single-stranded.
[b] Virions contain DNA-dependent RNA transcriptase and many other enzymes, but not DNA-dependent DNA polymerase.

assuming panhandle structures (e.g., orthomyxoviruses, family Orthomyxoviridae). Some of the single-stranded RNA genomes are of plus or "positive" polarity, meaning that they can be translated directly into proteins, and others are of minus or "negative" polarity, meaning that they cannot function as mRNAs and as such cannot be directly translated into proteins. Therefore, these negative-strand viral RNAs should be used as a template to synthesize a translatable complementary strand (mRNA), and still other viral RNAs are ambisense (Table 3.1). The plus-polarity naked viral genomes (except for retroviruses), completely free from all viral proteins, are infectious when introduced into a permissive cell, whereas minus-polarity naked genomes are noninfectious. Viruses possessing the minus-polarity genome therefore must carry an enzyme, RDRP, inside the virus particle to initiate the infectious cycle.

TABLE 3.3 Replication of RNA Viruses

	Virus	Form of RNA	Source of Nucleic Polymerase	Nature of Polymerase Activity	Presence of Polymerase in Virion	Viral Replication Site Within Host Cell
A	Paramyxovirus, rhabdovirus	ss[a] (–), unsegmented	Viral	RDRP	+	Cytoplasm
B	Bunyavirus, arenavirus	ss[b] (±), segmented	Viral	RDRP	+	Cytoplasm
C	Orthomyxovirus (influenza virus)	ss (–), segmented	Viral	RDRP	+	Nucleus
D	Rotavirus, reovirus, and orbivirus	ds[c] (±), segmented	Viral	RDRP	+	Cytoplasm
E	Picornavirus (poliovirus, hepatitis A), togavirus (Sindbis virus) Coronavirus	ss (+), unsegmented	Viral	RDRP	–	Cytoplasm
F	Retrovirus (HIV)	ss (+), unsegmented, diploid	Viral	Reverse transcriptase	+	Nucleus

[a] ss = single-stranded.
[b] ± = ambisense genome.
[c] ds = double-stranded; (+) or (–) indicate positive or negative polarity, respectively.

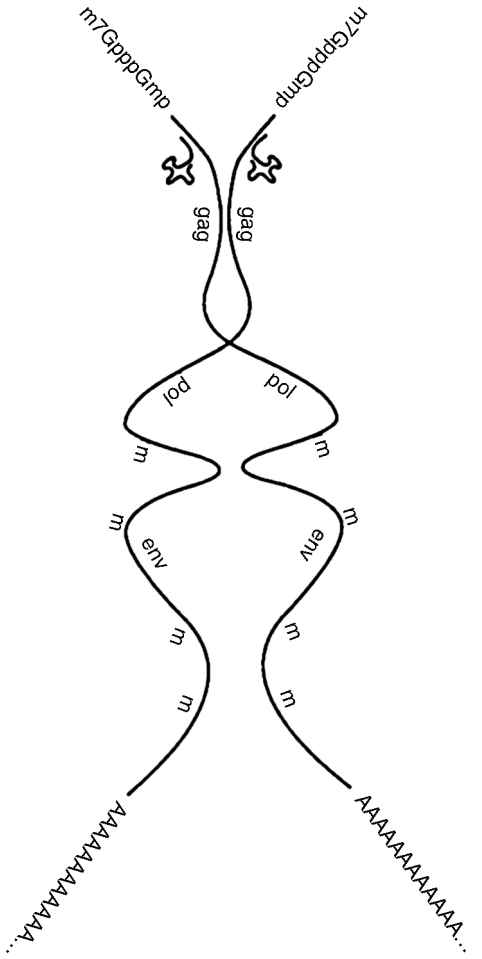


FIGURE 3.6 Features of the retrovirus (family Retroviridae) genome. The diploid RNA genome includes the following from 5′ to 3′: the m^7Gppp capping group, the primer tRNA, the coding regions, the M_6A residues (m), and the 3′ poly(AAAAA) sequence. Reprinted with permission from Fields and Knipe (1990).

Similarly, retroviruses must possess reverse transcriptase (RT, RNA-dependent DNA polymerase) in virus particles to initiate the infectious cycle inside host cells. However, using reverse genetics (RNA → DNA → RNA), many of the RNA genomes of both plus and minus polarity can be converted into infectious double-stranded DNA, thus permitting artificially induced mutational changes and genetic analysis of the viral genome, as well as use in DNA vaccination and as vectors in gene therapy. Some of the DNA viral genomes (adenoviruses, family Adenoviridae; hepadnaviruses, family Hepadnaviridae) and RNA (polioviruses, antigenic variants of human enterovirus C, family Picornaviridae, genus *Enterovirus*) viral genomes possess a covalently linked terminal protein at the 5′-end of a genomic nucleic acid strand, which provides critical functions for initiating DNA or RNA replication. Some positive-strand RNA viral genomes are also capped at the 5′-end and polyadenylated at the 3′-end (togaviruses, family Togaviridae), while others are not capped at the 5′-end (polioviruses) but possess polyadenylation (poly(A)) at the 3′-end. The minus-strand RNA genomes do not possess the cap at the 5′-end or the poly(A) at the 3′-end. Usually, the 5′ and 3′ ends of the minus-strand RNA genome are partially complementary, often forming panhandles by intrastrand hybridization and functioning as their own promoters for transcription and replication.

Organization of genes in the RNA genome varies between different groups of viruses. For positive-strand naked RNA viruses (e.g., polioviruses), which are translated into a single large polyprotein, the 5′-end of the genome is not capped but is rather covalently linked to a small protein VPg (Figure 3.7). The 5′-end of these viral genomes contains an untranslated region possessing a highly ordered secondary structure for internal ribosome entry, followed next in sequence by the genes of capsid proteins (VP4, VP2, VP3, VP1). The genes for nonstructural proteins including proteases and viral replicase (an RNA-dependent RNA polymerase) are located in the 3′-half of the genome. However, for the plus-strand enveloped RNA viruses (e.g., Sindbis virus, Family Togaviridae, genus *Alphavirus*), the genes for the nonstructural proteins are present at the 5′-end and structural proteins including capsid and envelope proteins are present in the 3′-half of the genome. Structural genes of this latter type of viruses are translated from a separate subgenomic mRNA, whereas their nonstructural proteins are translated from the genomic RNA. The large plus-strand coronavirus RNA

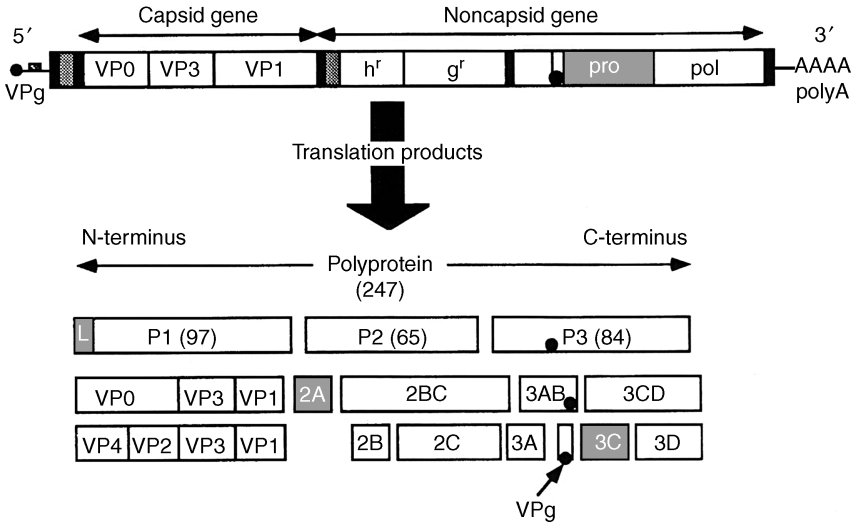


FIGURE 3.7 Organization of picornaviral (family Picornaviridae) genome (plus-strand RNA) and its translation products. The virus RNA has VPg protein attached to its 5′-end and poly(A) at its 3′-end. The order and the position of virally encoded proteins are shown. P1, P2, and P3 indicate three intermediate precursor proteins cleaved from the polyprotein. These precursor proteins are further cleaved by virus-encoded proteases into mature functional proteins. Numbers in parentheses indicate molecular weights in thousands. h^r and g^r indicate host range and guanidine resistance determinants, respectively. 2A and 3C are proteinases involved in cleavage of the polyprotein and precursor proteins into mature viral proteins. VPg, VPO, and so on indicate specific viral proteins.

genome possesses the nonstructural genes in the 5′-half and structural genes in the 3′-half of the genome. The gene for the highly abundant nucleoprotein (N protein) of coronaviruses is present at the 3′-end of the genome.

For unsegmented minus-strand RNA genomes, the order of genes for both vesiculoviruses (family Rhabdoviridae) and paramyxoviruses (family Paramyxoviridae) are similar. Structural genes for capsid (N and P proteins) and envelope proteins are at the 3′-half, and the large polymerase (L) gene occupies the entire 5′-half of the minus-strand RNA genome (Figure 3.8). The 3′-end of the template (minus-strand) RNA is transcribed into a leader (ℓ) sequence not present in the mRNA, and the region between two genes is separated by an element called the EIS. It consists of an "E" (end) sequence for transcription termination and polyadenylation of a gene, an "I" (intergenic) sequence that allows the viral transcriptase to escape (therefore the "I" sequence is not represented in the mRNA), and "S" (the start) sequences that denote the start of the next gene. EIS sequences in the genome vary for different viruses in these groups.

3.2.2 Viral Proteins

Proteins are major constituents of the viral structure, and their main functions, as indicated previously, are to protect the nucleic acid from nucleases and provide receptor binding site(s) for virus attachment, which is required for efficient transmission of virus from one host to another. Viral proteins can be classified as nonstructural or structural. Nonstructural proteins are those proteins that are encoded by the virion genome and expressed inside the virus-infected host cells, but not found in the virion particles. These nonstructural proteins usually have regulatory or catalytic functions that are involved in viral replication or transcription processes, as well as in modifying host functions. Structural proteins are broadly defined as proteins found in virus particles. The majority of these structural proteins constitutes the viral

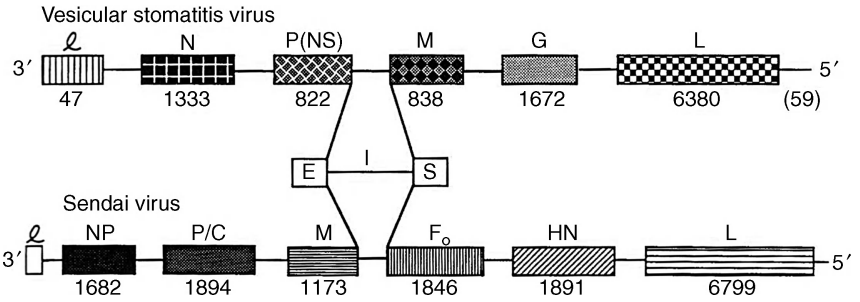


FIGURE 3.8 Genome of unsegmented negative-strand RNA viruses (vesicular stomatitis virus (VSV, refers to three species belonging to the family Rhabdoviridae and genus *Vesiculovirus*)) and Sendai virus, a species of the family Paramyxoviridae, genus *Respirovirus*. Numbers underneath rectangles represent the number of nucleotides in each gene (shown above the line), ℓ, leader sequence; E, end (or transcription termination) sequence; I, intergenic sequence (not transcribed); S, start sequence of mRNA of the next gene; N, NP = nucleoproteins; P/C, P (NS) = phosphoprotein; M = matrix protein; G, F, HN = glycoproteins; L = polymerase protein.

capsid or core and are intimately associated with the viral genome to form the nucleocapsid. The cores of some viruses also contain regulatory or catalytic proteins as minor structural proteins (e.g., proteins with enzymatic functions, such as transcriptase (RDRP) or reverse transcriptase) (Tables 3.2 and 3.3). In addition, some viruses include host proteins such as histones associated with the viral genome in virus particles (e.g., SV40 minichromosome; Simian virus 40, family Polyomaviridae, genus *Polyomavirus*) or ribosomes, as is the case with arenaviruses. Although these minor virus- and host-coded proteins are critically involved in virus replication and infectivity, they are not essential for formation of viral capsids.

In addition to having viral capsids, the enveloped viruses possess membranes (or envelopes) surrounding the viral capsids. These viral membranes, as noted earlier, contain lipids derived from the host membrane and proteins specified by the viral genome. Two types of proteins are found in the viral membrane: transmembrane (TM) proteins and matrix proteins.

3.2.2.1 Transmembrane Proteins

Transmembrane proteins can be type I (such as influenza virus hemagglutinin (HA), family Orthomyxoviridae; VSV G protein, family Rhabdoviridae, genus *Vesiculovirus*), type II (such as influenza virus neuraminidase (NA)), and type III (such as influenza virus M2), depending on their molecular orientation, or complex proteins, containing multiple transmembrane domains (TMDs) (such as E1 glycoprotein of coronaviruses). Enveloped viruses may contain only one (as in the G protein of VSV (vesicular stomatitis virus, refers to three species belonging to the family Rhabdoviridae, genus *Vesiculovirus*)), two (as in the HN and F proteins in paramyxoviruses), or multiple transmembrane proteins (as in the influenza viruses, herpesviruses, family Herpesviridae; poxviruses, family Poxviridae, etc.) on their envelope. Again, viruses containing multiple transmembrane proteins may have proteins of different orientations such as type I, type II, and type III (e.g., influenza viruses, family Orthomyxoviridae). These transmembrane proteins are often glycosylated via *N*- or *O*-glycosidic bonds and their carbohydrate moieties can be composed of simple sugars, usually consisting of mannose molecules, complex sugars, including galactose, glucosamine, galactosamine, fucose, and mannose, and sialic acid residues. Proper glycosylation of viral proteins is often important to provide the necessary molecular stability, solubility, oligomer formation, and intracellular transport of viral proteins, as well as for modulating the host immune response, including epitope masking and unmasking. These glycans may also play an important role in apical sorting of proteins within the

polarized epithelial cells. It often is the case that one or more of these transmembrane proteins are involved in providing important functions in the processes of virus life cycle such as receptor binding (e.g., HA in influenza viruses, HN in paramyxoviruses, G protein in VSV). The same protein (influenza viruses, VSV) or a different protein (e.g., F protein in paramyxoviruses) can be involved in fusion of the viral envelope with cellular membranes, uncoating, and entry of the viral genome inside the cell. In addition, some other viral membrane protein can aid in releasing mature viruses from the infected cells and spreading of viruses from cell to cell (e.g., function of the neuraminidase protein in releasing influenza viruses after budding). These envelope proteins are important not only in virus infectious cycle but also for host defense, where they elicit both neutralizing antibodies and CTL (cytotoxic T lymphocyte) responses against the virus infection in infected hosts and therefore play a critical role in vaccination and protection against viral infections.

3.2.2.2 Matrix Proteins In addition to the transmembrane proteins, the majority of these enveloped viruses also contain another type of membrane protein called a matrix protein (e.g., M1 protein of influenza viruses) that forms a shell underneath the membrane enclosing the capsid (Figure 3.3). The matrix proteins are therefore likely to interact with the lipid bilayer and transmembrane proteins of the viral envelope on the outer side and with the nucleocapsid on the inner side. Matrix proteins are also usually the most abundant proteins in enveloped virus particles and are critical for the budding of enveloped viruses. Some enveloped viruses containing icosahedral capsids do not possess typical matrix proteins around the nucleocapsids underneath the membrane (e.g., togaviruses).

Viruses vary greatly in size and shape. They can be spherical, cylindrical (rod shaped), or even pleomorphic (Figures 3.1 and 3.4). Primarily, the virus structure is determined by the nature of the capsid and whether the capsid is naked or surrounded by an envelope. The structure of the capsid is in part determined by the protein and nucleic acid (nucleocapsid) interactions, but principally by the protein–protein interactions of the capsid protein(s). In most cases, the nucleic acid is incorporated after the majority of the protein shell of the capsid has been formed, or capsids can remain empty, resulting in noninfectious virus particles. The capsids are composed of repeating protein subunits called capsomeres. Capsomeres are composed of multimeric units of a single protein, or often heteromeric units of more than one protein.

3.2.3 Lipids

In addition to nucleic acids (DNA or RNA) and proteins, enveloped viruses contain lipids in their membrane. These lipids constitute integral components of viruses and are critically involved in many aspects of virus life cycle including entry, fusion, uncoating, and delivery of viral genome into the host cell for initiating the infectious cycle; transport and assembly of viral components; and budding and release of virus particles. Although viruses bud from host membranes and all viral lipids are acquired from the host membranes, some of the viral lipids do not match quantitatively with that of the host membranes. A number of factors are involved in the selection of viral lipids, including the budding site of enveloped viruses in the infected cells and the type of cells in which viruses are grown, as well as the type of organelles such as plasma membrane, Golgi complex, nucleus, and so on from which the virus buds. Since different cell types and subcellular organelles possess varying lipid composition, viruses budding from membranes of different organelles will have different lipid composition. For example, herpesvirus, a complex DNA virus, buds from the inner nuclear membrane. However, fully mature infectious herpesvirus exits from the basal layer of infected epithelial cells. Hepadna, rota, and spuma viruses bud from the endoplasmic reticulum (ER). Coronaviruses (family Coronaviridae) and vaccinia virus (family Poxviridae, genus

Orthopoxvirus) acquire their envelope from the intermediate pre-Golgi compartment (IC). Vaccinia virus is further surrounded by membrane envelope, which is a part of complex maturation process, before being released from the plasma membrane. Bunyaviruses (family Bunyaviridae) and togaviruses (family Togaviridae, genera *Alphavirus* and *Rubivirus*) acquire their envelope on the Golgi complex. However, whereas Sindbis virus (SIN, family Togaviridae, genus *Alphavirus*) exits from the apical membrane, Semliki Forest virus (SFV, also family Togaviridae, genus *Alphavirus*) buds from the basolateral membrane. The assembly and budding of some viruses such as orthomyxo-, paramyxo-, filo-, retro-, and rhabdoviruses occur only at the plasma membrane, although orthomyxoviruses and paramyxoviruses bud from the apical plasma membrane whereas the filo-, retro-, and rhabdoviruses bud from the basolateral plasma membrane. Viruses budding from the different domains of the same membrane will have different lipid composition. Furthermore, both cellular and viral membranes are mosaic in nature and contain different lipid microdomains. Among these, lipid rafts are known to play many important functions in both cellular and viral biology and often function as the budding site for many viruses.

3.2.3.1 Lipid Rafts Lipid rafts are operationally defined as cholesterol-dependent microdomains resistant to solubilization by nonionic detergents such as TX-100 at low temperature. Lipid rafts consist of sphingolipid–cholesterol clusters, usually varying in size and are present in the plasma membrane, apical transport vesicles, and Golgi and *trans*-Golgi membranes. Lipid rafts vary in size, ~50 nm in diameter (Pralle et al., 2000) and smaller than the caveolae that also exhibits TX-100 insolubility similar to the lipid rafts. Lipid raft microdomains are formed by lateral organization and phase separation of lipids between l_o phase and l_d or l_a phases (Nayak and Hui, 2004). l_o and l_d or l_a phases refer to ordered and disordered phases of lipid in the membrane, respectively. l_o phase separation also leads to asymmetric distribution of different lipids in the exoplasmic versus cytoplasmic lipid leaflets. These lipid microdomains containing l_o phase have been variously called by different names, such as detergent-insoluble GSL (glycosphingolipid)-enriched domains (DIGs), GSL-enriched membranes (GEMs) or microdomains, detergent-resistant membranes (DRMs), Triton-insoluble membranes (TIMs), GSL/sphingolipid–cholesterol rafts, lipid rafts, or simply rafts.

Lipid raft microdomains contain glycerophospholipids, (glyco)-sphingolipids, GPI lipids bearing predominantly saturated fatty acids, cholesterols, and gangliosides such as GMI and GM2. These lipids form tight packing and cholesterol contributes to tight packing by filling the interstitial space between the long saturated acyl chains and sphingolipids resulting in the formation of l_o state of lipids in lipid raft microdomains. The tight lateral packing of sphingolipids and cholesterol leads to TX-100 insolubility at low temperature. Lipid rafts exclude most of the membrane proteins including the TM proteins except for proteins with GPI anchor, and palmitoylation, prenylation, acylation, myristoylation are partitioned in these microdomains. However, some TM proteins such as influenza virus HA and NA without acyl modification are included in the lipid raft microdomain of influenza virus envelope. Protein–lipid and protein–protein interactions may contribute to coalescence, growth, and stability of lipid rafts.

3.3 MORPHOLOGY

Viruses vary greatly in size and shape. They can be spherical, cylindrical (Figure 3.1), or even pleomorphic (Figure 3.4). Primarily, the virus morphology is determined by the nature of the capsid structure and whether the capsid is naked or surrounded by envelope. The structure of the capsid is in part determined by the protein and nucleic acid (nucleocapsid) interactions but principally by the protein–protein interactions of capsid proteins. In most cases,

nucleic acid is incorporated after the majority of the protein shell of the capsid has been formed, or capsids can remain empty, resulting in production of noninfectious virus particles. The capsids are composed of repeating protein subunits called capsomeres. Capsomeres are composed of multimeric units of single or often heteromeric units composed of more than one protein. Formation of the viral capsid and its shape is primarily determined by three-dimensional (3D) structure of the capsid proteins, which in turn is determined by the specific amino acid sequence encoded by the viral nucleic acid. The amino acid sequence is considered the primary structure of the protein, whose three-dimensional structure is composed of secondary structures such as α helices, β sheets, and random coils. These secondary structures interact with each other, forming the tertiary and quaternary structures, which are usually stabilized by noncovalent interactions (sometimes by covalent disulfide linkages) and represent folding of the proteins into relatively stable structures of microdomains (e.g., globular heads). In addition, extended and flexible regions of proteins, called hinges, are also present, and these hinges become important for interaction with other members of the protein subunits that form the capsomeres. In most viruses, contacts between capsomeres are repeated, exhibiting symmetry. This is a process of self-assembly driven by the stability of interaction among the protein subunits forming the capsomeres and the capsomeres forming the capsid. Viral capsids have a helical (springlike) or icosahedral-based (cuboidal or spherical) symmetry.

Until recently, the morphology of viruses was based on transmission electron microscopy (TEM) images of negatively stained viral particles or thin sections of virus-infected cells. However, staining and sectioning procedures often introduce artifacts in the shape, size, and morphology of virus particles during sample processing owing to the use of heavy metal stain at nonphysiological pH and sample drying. Viruses such as influenza viruses are particularly sensitive to these procedures owing to the flexible, pH-sensitive viral envelope. Recently, electron tomography (ET) has been used to reconstruct the 3D structure of viral particles in thin sections by combining different tilt views of the same sample. In addition, the size and shape of virus particles vary with different virus isolates and laboratory strains. Recently, cryoelectron microscopy (cryo-EM) and cryoelectron tomography (cryo-ET) have been used to examine the structure of these viruses in their natural state without fixing and staining (Calder et al., 2010). Furthermore, cryo-ET can be used to determine the 3D structure of each viral particle by combining different tilt views of the same viral particles (Baumeister, 2002). The 3D structures can then be computationally sliced to reveal the structural arrangement of proteins, nucleic acid, and lipids and their possible interactions in their native state within the virus particles. Some examples of morphology of different viruses are shown in Figures 3.3–3.5 and 3.9.

3.3.1 Helical Capsids

Helical capsids are usually flexible and rod like. The length of the helical capsid is usually determined by the length of the nucleic acids; that is, some defective interfering (DI) viruses having shorter nucleic acids will have a shorter helical nucleocapsid (e.g., DI RNA of VSV). Helical capsids can be naked, that is, without an envelope (e.g., tobacco mosaic virus, family Virgaviridae, genus *Tobamovirus*). However, there is no known example of an animal virus with a naked helical nucleocapsid. All animal viruses with helical capsids found to date are enveloped. However, such helical capsids when enclosed in an envelope can exhibit various morphologies, including filamentous (filoviruses), rod shaped (e.g., rhabdoviruses) or spherical, spheroidal or elongated (e.g., orthomyxo- or paramyxoviruses), and even pleomorphic (Figure 3.4), indicating that the helical capsid in these viruses is flexible. Some helical capsids can be further folded, forming supercoiled nucleocapsids (e.g., orthomyxoviruses). Helical capsids can package only

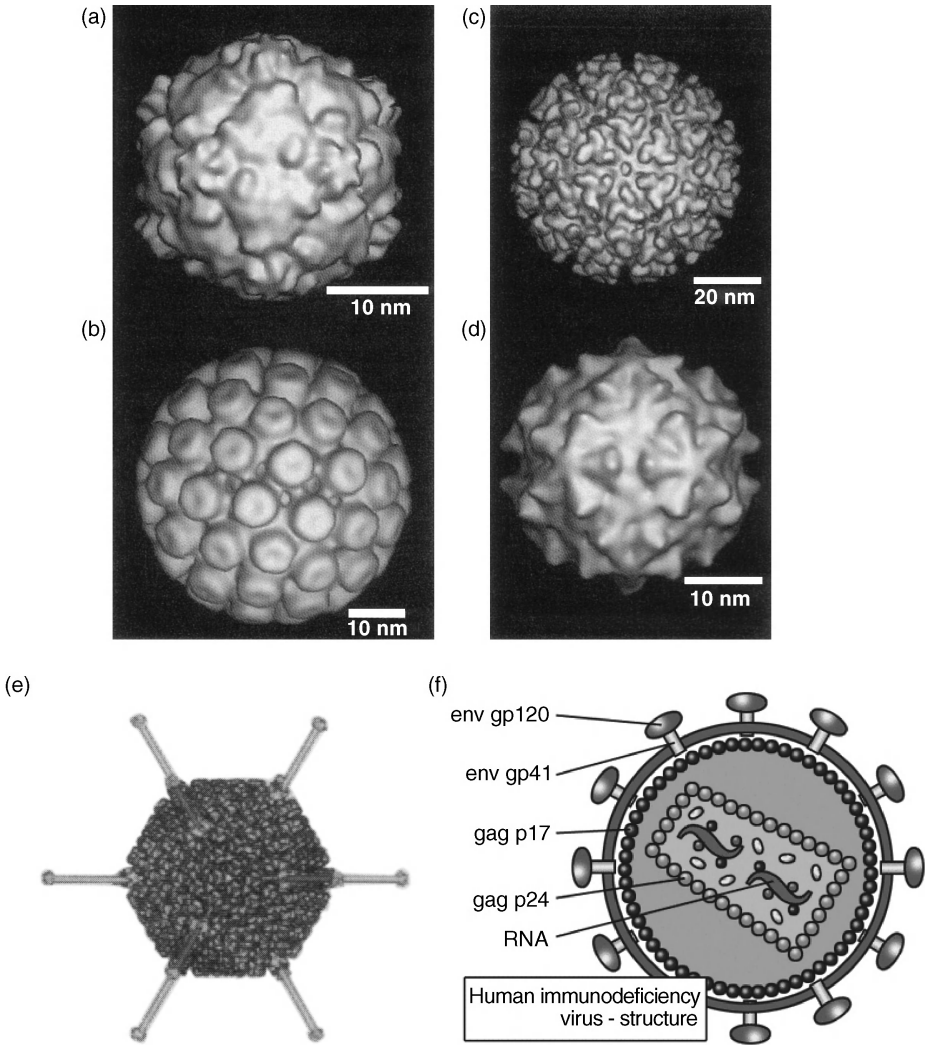


FIGURE 3.9 Structure of representative RNA and DNA viruses as determined by cryoelectron microscopy. (a) human rhinovirus 14, a ssRNA virus that is an antigenic variant of human rhinovirus B, family Picornaviridae, genus *Enterovirus*; (b) SV40, Simian virus 40, a dsDNA virus of the family Polyomaviridae, genus *Polyomavirus*; (c) Sindbis virus capsid, an ssRNA virus of the family Togaviridae, genus *Alphavirus*; (d) flock house virus, a positive-strand bipartite ssRNA insect virus of the family Nodaviridae, genus *Alphanodavirus*; (e) adenovirus, a dsDNA virus of the family Adenoviridae. The micrographs of human rhinovirus 14, SV40, Sindbis virus, and flock house virus were provided by and are reprinted with permission from Norm Olson and Jim Baker of Purdue University. The adenovirus micrograph was provided by and is reprinted with permission from Phoebe Stewart of UCLA. (f) Schematic presentation of HIV reproduced from Avert with permission.

single-stranded RNA, but not double-stranded DNA or RNA, possibly because of the rigidity of the double-stranded nucleic acids. However, some viruses with helical capsids may possess only one capsid containing one virion RNA (unsegmented) molecule (rhabdoviruses, paramyxoviruses) or multiple capsids containing multiple RNA segments (orthomyxoviruses) (Calder et al., 2010). Viruses containing multiple RNA segments can undergo reassortment

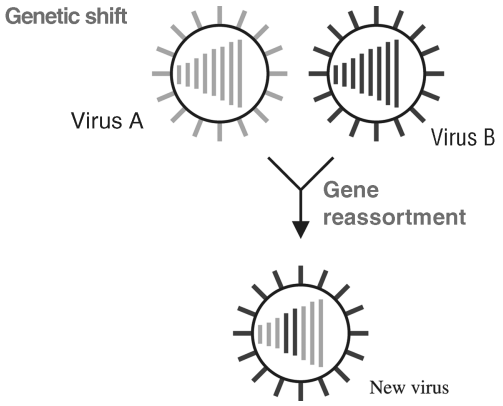


FIGURE 3.10 Reassortment of influenza virus RNA segments. When two influenza viruses (virus A orange and virus B blue color RNA segments) infect a single cell, progeny viruses from the infected cells will possess different combinations of vRNA segments. One progeny virus particle shown here contains six RNA segments (PB1, PB2, PA, NP, M, and NS RNA) from virus A and two RNA segments (HA and NA) from virus B. The new progeny virus will be antigenically different from virus A and will emerge as a potentially pandemic virus. In addition to gene reassortment, mutations in vRNA segments will facilitate adaptation, growth, and spread in the human population. (*See the color version of this figure in Color Plate section.*)

with other related viruses (Figure 3.10), thus exchanging different RNA segments and giving rise to new viruses with different antigenic and virulence determinants (e.g., the antigenic shift that occurs in influenza viruses). The genomic RNA is protected by the helical capsid in some viruses (e.g., paramyxo- and rhabdoviruses) but remains exposed in others (e.g., orthomyxoviruses). A single viral protein (e.g., NP protein of orthomyxoviruses) is usually involved in helical capsid formation.

3.3.2 Icosahedral Capsids

Viruses with icosahedral capsids possess a closed shell enclosing the nucleic acid inside (Figure 3.1). An icosahedron has 20 triangular faces, 30 edges, and 12 vertices and is characterized by a 5:3:2-fold rotational symmetry. Unlike helical nucleocapsids that package only single-stranded nucleic acid, icosahedral capsids can be used to package single- or double-stranded RNA and DNA molecules. However, although plus- or minus-strand DNA segments are found in the icosahedral capsids of parvoviruses, there are as yet no examples of an icosahedral virus with minus-strand RNA. An icosahedral virus can be naked or enveloped, but, unlike the helical enveloped viruses, the enveloped icosahedral viruses are less pleomorphic in their shape because the icosahedron capsid structure is rather rigid and, in addition, with icosahedral capsids, the overall size is fixed for a particular virus. The virus particle's formation, stability, and size do not depend on the amount of nucleic acid in the capsid. Although the packaging of the nucleic acid inside the icosahedral capsid is relatively fixed and does not vary greatly, noninfectious viruses containing empty capsids (i.e., without nucleic acid) can often be seen in virus populations. In recent years, the complete three-dimensional structures of several icosahedral viruses have been determined at the atomic level using the powerful tools of cryoelectron microscopy and X-ray diffraction analysis. Such analyses have led to the rational design of a number of antiviral drugs. Some examples of such three-dimensional viral structures are presented in Figures 3.3 and 3.9.

3.4 VIRAL REPLICATION CYCLE

To survive, viruses must multiply. Since viruses cannot multiply outside the host cell, they must infect host cells and use cellular machinery and energy supplies to replicate and produce the progeny viruses that must in turn infect other hosts and the cycle continues. Host–virus interaction at the cellular level is therefore obligatory for virus replication. Specific host cells can be susceptible (i.e., permissive) or nonsusceptible (i.e., resistant or nonpermissive) to a particular virus. Nonsusceptibility of cells can be at the attachment (e.g., lack of a suitable receptor for a virus at the cell surface) and entry/uncoating phases, at the intracellular

phase (i.e., a block in synthesis of viral macromolecules), or at the assembly and exit phases. Furthermore, following infection, viruses can cause abortive (nonproductive) or productive infection. Only productive infection yields infectious progeny virus particles. Following abortive or productive infection, the host cell may survive or die, i.e., the cytopathic effect (CPE). CPE caused by a virus does not necessarily indicate the permissiveness of a cell to a virus leading to productive infection. The viral genome in abortive infection may be degraded, may become integrated into the host DNA, or exist as extrachromosomal (episomal) DNA in the surviving cell. The growth properties of such cells may be altered, including the possibility that they may become transformed and cancerous. Alternatively, cells containing the integrated viral DNA may behave normally, exhibiting little change in their normal properties. Malignant transformation of infected cells often depends on the site of viral genomic integration, leading to activation of cellular oncogenes, disruption or inhibition of tumor suppressor genes, or synthesis of viral oncogene products that are encoded by the virus in its genome. In the infected cells, the viral genome may remain dormant, resulting in a latent infection, and it can be activated later, producing infectious viruses, as occurs with herpesviruses. Alternatively, infected cells may yield virus at a low level without affecting cell survival, resulting in persistent infection, as occurs with LCMV (lymphocytic choriomeningitis virus, family Arenaviridae, genus *Arenavirus*). The effect of virus infection has been studied at both the cellular and organismic levels. At the organismic level, it is called "viral pathogenesis," while at the cellular level, it is called the CPE. Under these conditions, cells may undergo morphological changes, including rounding, detachment, cell death and cell lysis (either apoptotic or necrotic), and syncytium (giant multinucleated cell) formation, as well as inclusion body formation. Many of these changes are caused by the toxic effects of viral proteins affecting host macromolecular synthesis, including DNA replication, DNA fragmentation, mRNA transcription, translation, protein modification, and degradation, as well as other cellular synthetic and catalytic processes. Furthermore, since the same cellular machineries are directed toward viral macromolecular synthesis, the host is deprived of their functions. In addition to direct cell killing, virus infection can indirectly cause injury to tissues in a complex organism, as a result of both complex host–viral immune interactions (i.e., immunopathology) and by cytokine production causing inflammatory reactions. Usually, lytic viruses cause cell death and when a sufficient number of cells in a given tissue (e.g., lungs, liver, etc.) die, it leads to the loss of function of the tissue and the production of specific disease syndrome (pneumonia, hepatitis, etc.). Since infection of the host usually begins at a very low MOI (multiplicity of infection, expressed as virus:cell or virus:host ratio), the virus must be able to replicate efficiently and produce a large number of progeny viruses in a short period to infect and kill a sufficient number of cells to cause the disease syndrome. It is evident from the foregoing discussion that for successful replication, a virus must find susceptible host cells and it must be able to attach itself to and penetrate into the host cell and be uncoated, rendering the viral genome available for interaction of the viral and cellular machineries for transcription, translation, and replication of the viral genome. Finally, the newly synthesized viral components must be assembled into progeny viruses and released into the medium (i.e., outside environment) to infect other hosts. Whether with cultured cells in laboratory or the complex organisms in nature, the virus–host interaction always occurs at the level of single cells. Thus, the viral infectious cycle (also known as the viral growth cycle, replication, or multiplication cycle) can be divided into different phases, namely, (1) adsorption (attachment), penetration, and uncoating; (2) transcription, translation, and replication; and (3) assembly and release. The replication cycle of influenza (orthomyxo) viruses is diagrammatically shown in Figure 3.11.

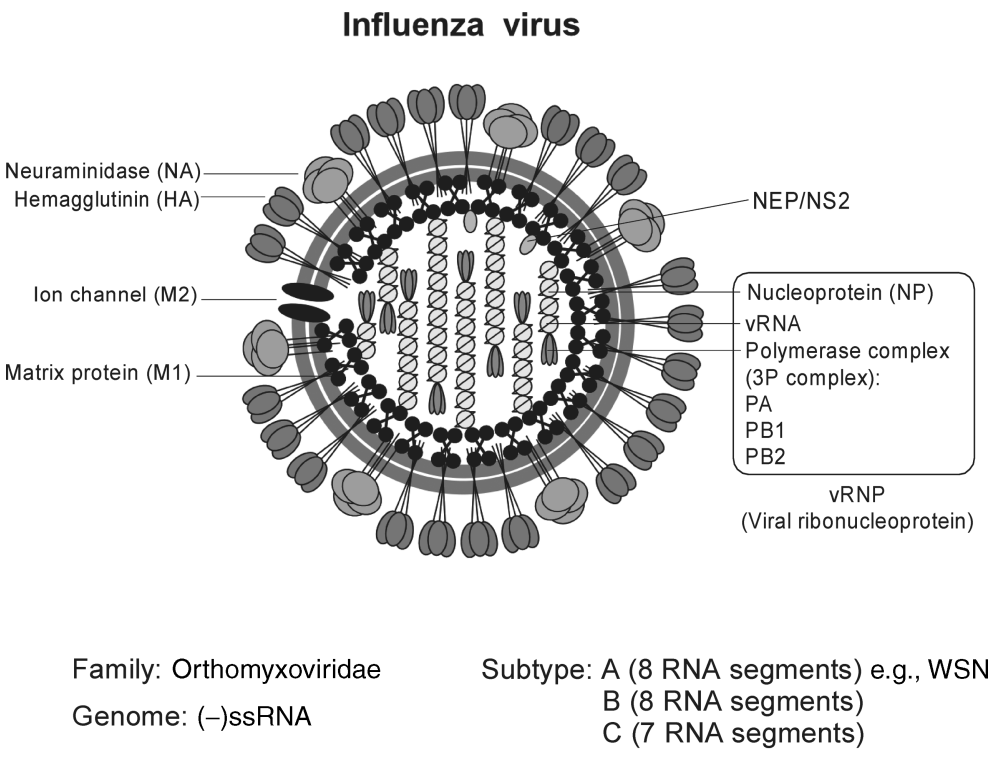


FIGURE 3.11 Schematic presentation of the infectious cycle of an influenza virus. (a) Schematic presentation of influenza virus structure. (b) Schematic presentation of influenza virus infection showing attachment, entry, and uncoating of a virus particle. The steps in the replication cycle are attachment mediated through HA and sialic acid receptor, entry into the cell via endosome, HA-mediated fusion of virus membrane with endosomal membrane at low pH, release of vRNP, transport of vRNP into the nucleus, and transcription (mRNA synthesis) and replication (cRNA and vRNA synthesis) of vRNP in the nucleus. (c) Schematic presentation of influenza virus infectious cycle showing export, assembly, and budding of a virus particle. The steps include export of vRNP from nucleus into cytoplasm, export of virus proteins, vRNP to the budding site, bud formation, and bud release by fusion and fission viral and cellular membranes. (*See the color version of this figure in Color Plate section.*)

3.4.1 Adsorption

Viral adsorption is defined as the specific binding of a virus to a cellular (host) receptor. It is the first step for the virus to enter into the cell. Viruses cannot cause disease if this first step is blocked. Vaccines and the resulting antibodies are designed primarily to block this step in the virus replication cycle. It is a receptor–ligand interaction in which viruses function as specific ligands and bind to the receptors present on the cell surface. Ligand functions of the virus are provided by the specific viral proteins present at the surface of the virus. For naked (i.e., nonenveloped) viruses, this function is performed by one of the capsid proteins and for enveloped viruses, one of the membrane proteins functions as the ligand (also variously known as the receptor binding protein, viral attachment protein, or antireceptor) for the host receptor. Usually, only one viral protein provides the receptor binding function, although one or more cellular proteins can function as receptor and coreceptor. For enveloped viruses, a classic example of a viral ligand (i.e., receptor

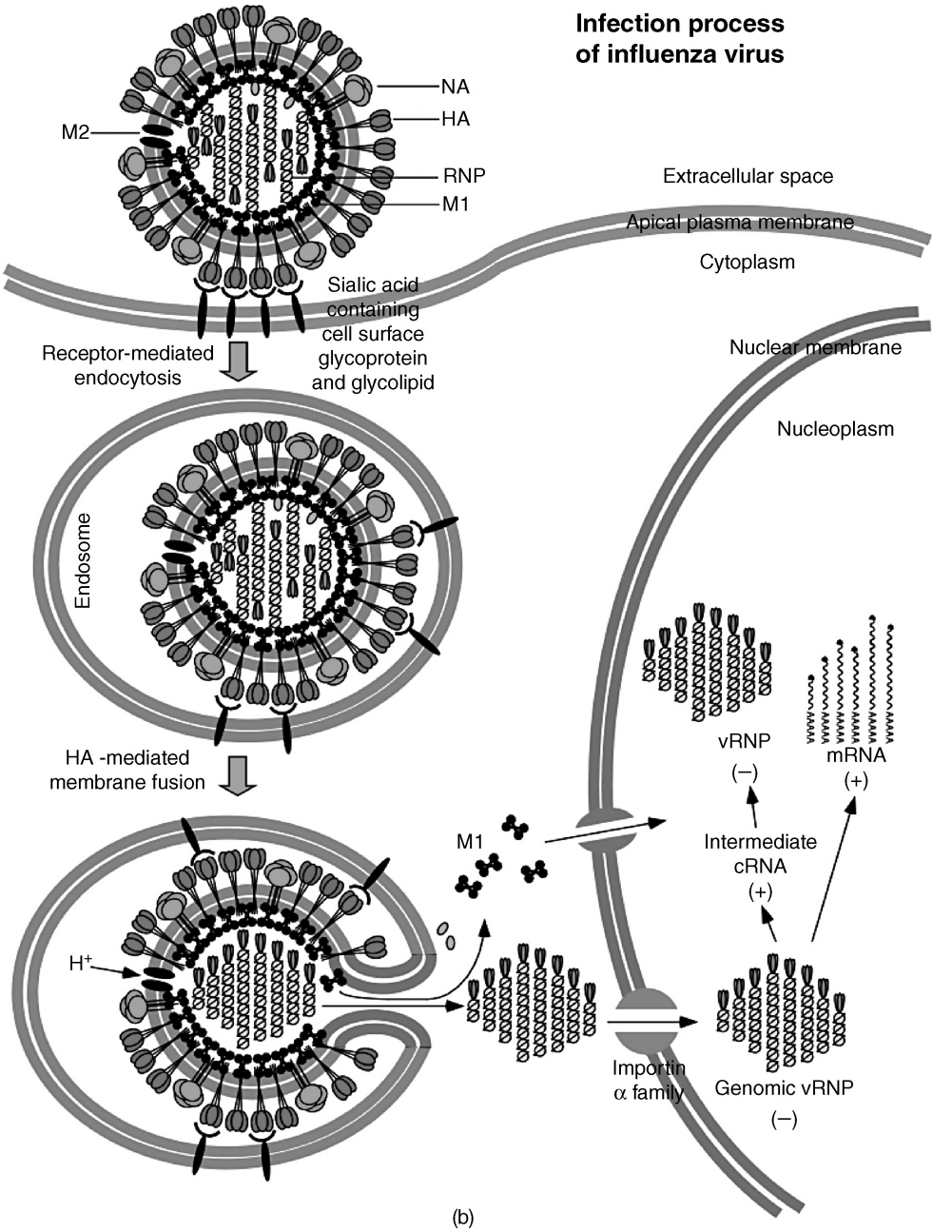


FIGURE 3.11 (*Continued*)

binding protein) is the influenza virus hemagglutinin and its receptor binding site is present on the globular head of the HA spike. Variation in the amino acid sequence of the receptor binding site of HA is a critical factor in species-specific susceptibility of influenza viruses (e.g., chicken versus human). For non-enveloped viruses, a classic example of a viral

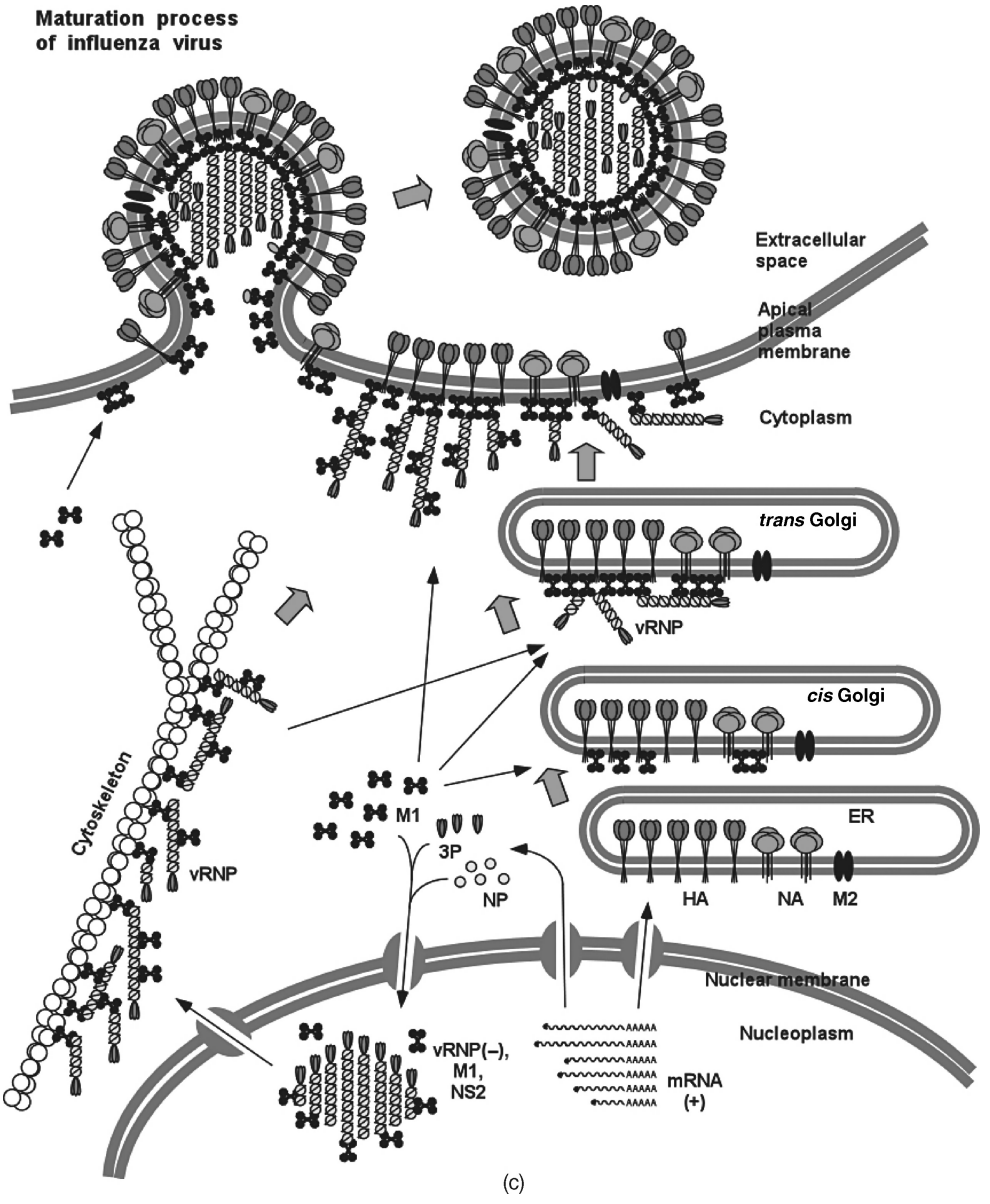


FIGURE 3.11 (*Continued*)

ligand is the VP1 of rhinoviruses. When five VP1 proteins are packed together within the viral capsid structure, the confluence of these grooves forms a depression called a canyon. The canyon is shown to be the site for interaction between human rhinovirus 14 (HRV14; an antigenic variant of human rhinovirus B, family Picornaviridae, genus *Enterovirus*) and the cellular molecule ICAM-1 (receptor for rhinovirus). The amino acids lining the floor of these canyons are highly conserved, but residues on the surface of the canyon are variable (Figure 3.12). Antibodies can bind to the surface epitopes in and around the proximity of

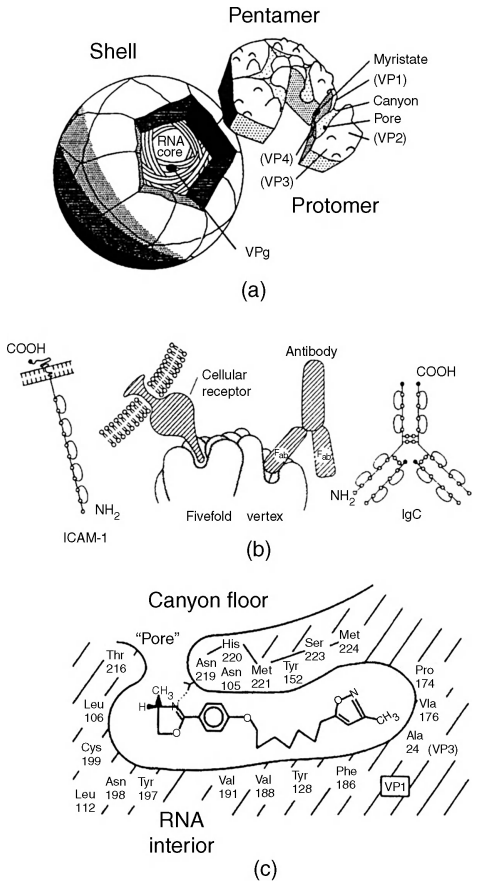


FIGURE 3.12 Key features in the function of cellular receptor interactions with an invading virus, similar to a typical picornavirus. (a) Exploded diagram showing internal location at the canyon-like center of the pentamer fivefold vertex with myristate residues on the NH_2 terminus of VP4. (b) Binding of cellular receptor (ICAM-1 molecule) to the floor of the canyon. Note that the binding site of the ICAM-1 molecule, identified as a major rhinovirus (species human rhinovirus A, B, and C) receptor, has a diameter roughly half that of an IgG antibody molecule. (c) Location of a drug binding site in VP1 of HRV14 (human rhinovirus 14) and identity of amino acid residues lining the wall. The drug shown here, WIN 52084, prevents attachment of HRV14 by deforming part of the canyon floor. The pentamer vertex lies to the right. Reprinted with permission from Fields and Knipe (1990).

the receptor binding site and thus interfere with virus attachment by steric hindrance. Viruses can accept mutations in these surface epitopes and thereby escape (and are thus known as escape mutants) neutralization by specific antibodies, but the receptor binding site usually does not undergo mutational changes because of its location inside the canyon and therefore remains conserved. This also appears to be the case with influenza virus hemagglutinin and other viral receptor binding sites that remain conserved despite the variation in the neutralizing epitopes of the same viral protein. Thus, the receptor binding site in the viral protein is usually a depression or canyon and is therefore protected from the mutational pressure of antibodies because antibodies do not have direct access to this region.

The cellular receptors of many viruses have recently been identified. Cellular receptors should be present at the cell surface and are carbohydrates, lipids, or proteins. Sialooligosaccharides present in glycoproteins or glycolipids function as receptors for orthomyxoviruses, paramyxoviruses, or polyomaviruses, as well as immunoglobulin superfamily molecules (CD4 for HIV, human immunodeficiency virus 1 and 2, of the family Retroviridae, genus *Lentivirus*, and many members of the family Picornaviridae, including ICAM-1 for both rhinoviruses (genus *Enterovirus*) and encephalomyocarditis virus (genus *Cardiovirus*), as well as Pvr for polioviruses (genus *Enterovirus*)). Hormone or neurotransmitter receptors function as receptors for a number of other viruses (e.g., epidermal growth factor for vaccinia virus, β-adrenergic receptor for reovirus (family Reoviridae), and acetylcholine receptor for rabies virus (family Rhabdoviridae, genus *Lyssavirus*)) and heparan sulfate for herpesviruses (family Herpesviridae). Some viruses have more than one receptor, one being the primary receptor and the other a coreceptor. A classic example of this is the case of CD4 and chemokine receptors (CXCR4, CCR5, etc.) functioning as the receptor and coreceptor for HIV, respectively. Both the receptor and the coreceptor are needed for productive HIV infection, although only one viral protein (gp120) provides the receptor binding sites for both receptor and coreceptors. Receptor–virus interaction is the major reason for the host and tissue tropism of viruses. It has been shown that lack

of a specific coreceptor on surface of the cell provides resistance to HIV infection in some persons. Receptor–virus interactions are specific, and this is a noncovalent binding independent of energy or temperature. Thus, the kinetics of viral binding to cells can be determined at 4°C, which serves as a research aid since their interaction at this temperature prevents viral penetration and uncoating. Therefore, binding virus to cells at 4°C and subsequently raising the temperature to 37°C can be used to infect cells synchronously and to study the subsequent events such as uncoating and penetration of virus into host cells. The time course of viral adsorption follows first-order kinetics and is dependent on virus to cell concentration. Usually, susceptible cells contain a large number of virus receptors, in the range of 10^4–10^5 per cell.

3.4.2 Penetration and Uncoating

Following specific ligand to receptor interaction, the next steps in virus replication include entry/penetration of virus into the host cell and uncoating of the viral genome, which are energy-dependent processes and can be prevented experimentally in the laboratory by subjecting the virus–cell complex to low temperatures (4°C). Viruses attached to the cell surface can be detached by specific enzymatic treatment (e.g., neuraminidase treatment in the case of influenza virus). However, once the virus enters into the cell, it can be neither separated from the cell nor neutralized by antibodies. Penetration refers to the entry of the surface-bound virus particles inside the cell, where they exist free in the cytoplasm or inside the host cell vesicles (usually within endosomes). Quantitatively, penetration of virus particles is measured by the loss of the ability of antiviral antibodies to neutralize the cell-bound virus particles after adsorption, an effect that occurs because after the viral particles have entered the cell, they are protected and no longer accessible to antibodies outside the cell. Uncoating, on the contrary, refers to disruption of virus particles, causing partial or complete separation of nucleic acid from the capsid, and is needed for initiation of transcription and translation of the viral genome. Uncoating can be assessed by, among other things, changes in viral morphology or viral density, release of nucleocapsid and membrane proteins from enveloped virus particles, and the accessibility of viral genome to nucleases. For viruses such as orthomyxovirus and poliovirus, these processes are separated temporally (i.e., penetration is followed by uncoating in the cytoplasm), but for some viruses, both penetration and uncoating occur simultaneously at the cell surface (e.g., paramyxoviruses, HIV). Uncoating refers to the step in which the viral genome becomes functional transcriptionally or translationally. However, complete separation of nucleic acid from all capsid proteins is not required for most viruses. For naked viruses, uncoating is a post-penetration process that occurs in the endosome or nucleus. Viruses that undergo uncoating in the cytoplasm following endocytosis require low pH (∼5) in the endosome for uncoating, whereas viruses that undergo fusion at the cell surface can undergo uncoating in a pH-independent manner.

Naked viruses such as the RNA-based picornaviruses enter into the cytoplasm of the infected cells via receptor-mediated endocytosis (Figure 3.13) or by phagocytosis (also called viropexis). In the endosome, the virus particle undergoes alteration in structural and antigenic properties and becomes acid labile and noninfectious. During uncoating, VP4 (a capsid protein) is released and the viral RNA is extruded from the capsid structure through the hole in the capsid caused by VP4 release into the cytoplasm. How the viral RNA gets through the endosomal membrane is not clear, but it is speculated that pore formation may occur by the interaction of the myristoylated NH_2 terminus of VP4 with the endosomal membrane (Flint et al., 1999). The viral RNA now becomes available for translation and replication (Figure 3.13, step 4a). However, only a small fraction of the viruses in the endosomes undergo successful uncoating. The majority of the virus particles in the endosomes, however,

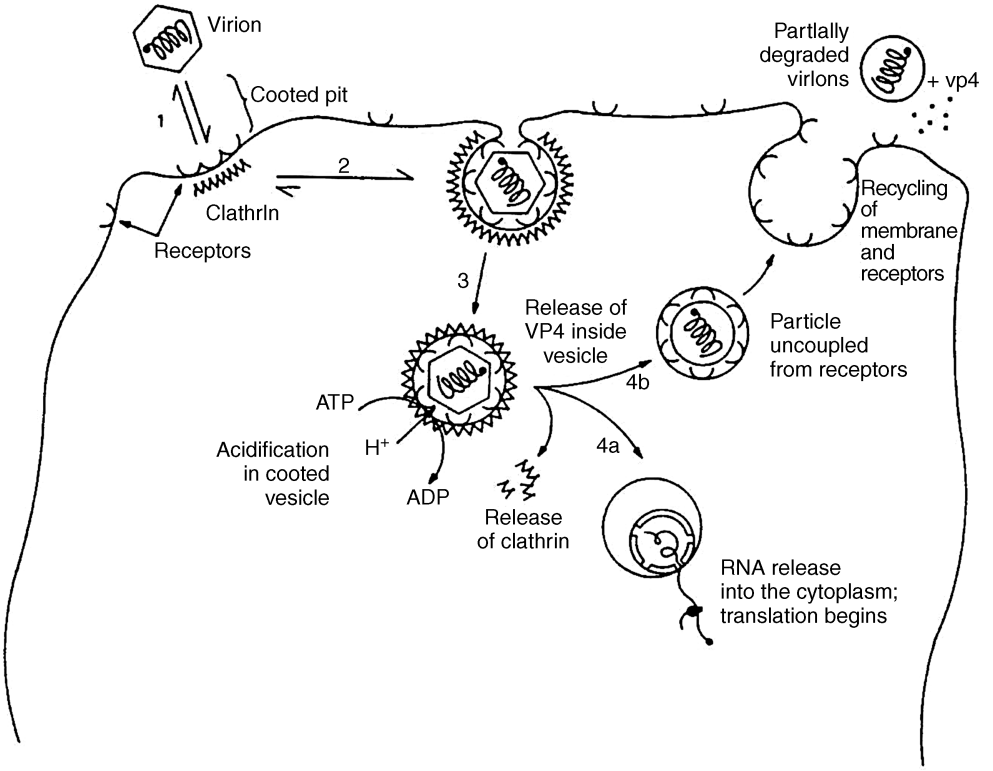


FIGURE 3.13 Receptor-mediated endocytosis of viruses such as polioviruses (steps 1 through 4a,b). The virus binds to cell surface receptors, usually glycoproteins, that undergo clustering at clathrin-coated pits (step 1) and is followed by invagination (step 2) and internalization (endocytosis) to form clathrin-coated vesicles (step 3). Acidification inside the coated vesicles, brought about by an energy-requiring ATPase-coupled proton pump, triggers the release of VP4 and unfolding of hydrophobic polypeptide patches previously buried inside the viral capsid. Fusion of the lipid bilayer with hydrophobic patches in the acid-unfolded capsid protein presumably triggers release and transfer of RNA from virion into the cytosol (step 4a), where ribosomes can begin translating the plus-strand viral genome. Fusion of uncoated vesicles with other kinds of intracellular lysosome-like vesicles may also be involved in the uncoating process. Some virus particles are not fully uncoated after acid-induced changes in the endosomes and are released into the extracellular medium via an abortive pathway (step 4b). These partially degraded extracellular virus particles are noninfectious. Reprinted with permission from Fields and Knipe (1990).

become noninfectious due to acid-induced structural alteration and are released outside the cell by the abortive pathway (Figure 3.13, step 4b). SV40 virus, a naked DNA virus, also enters into the cytoplasm via receptor-mediated endocytosis. Some alteration in the SV40 virion structure occurs in the endosome as VP3, a viral capsid protein, becomes exposed. However, in the case of SV40, the virus is extruded essentially intact from the endosome into the cytoplasm and targeted to the nucleus. Therefore, the uncoating of the SV40 genome occurs in the nucleus and not in the cytoplasm. In the nucleus, the viral minichromosome (viral DNA containing associated histone proteins) is released from the capsid and becomes available for transcription and replication. Therefore, although entry of SV40 virus into the cell likewise occurs via an endosome, its uncoating takes place within the nucleus in a pH-independent manner. However, how the SV40 virus is released from the endosome into the cytoplasm prior to nuclear entry remains unclear. Reovirus, a double-stranded naked RNA virus,

uses the host proteolytic enzymes present in the lysosome to partially remove the outer capsid proteins and activate the core RNA transcriptase for initiation of viral mRNA synthesis.

For enveloped viruses, uncoating occurs through fusion of the viral membrane with the cellular membrane using pH-independent or pH-dependent pathways. As mentioned previously, in the pH-independent pathway, virus penetration and uncoating occur simultaneously at the cell surface after virus–host interaction. This is best illustrated by the entry process of paramyxoviruses and retroviruses (e.g., HIV). In both cases, viruses bind to the cell surface receptors (i.e., sialic acid present on the cell surface glycolipids or glycoproteins for paramyxoviruses and the receptor protein CD4 and coreceptors for HIV). Either one (gp160 for HIV) or two (F and HN for paramyxovirus) separate viral glycoproteins are involved in this binding and fusion process. Fusion-inducing proteins in the infecting virus must be cleaved for causing fusion to occur (e.g., gp160 → gp120 and gp41 for HIV and F → F1 and F2 for Sendai virus, the latter belonging to the family Paramyxoviridae, genus *Respirovirus*). For HIV, the gp120/gp41 complex undergoes conformational changes after binding to the cellular receptor and coreceptor, releasing the hydrophobic domain of gp41 that then functions as a fusion peptide and mediates fusion of the viral membrane with the plasma membrane, thereby releasing the nucleocapsid containing the viral RNA and reverse transcriptase into the cytoplasm. Subsequently, cyclophilin A, present in HIV particles, aids in the uncoating process by destabilizing the capsid and initiating reverse transcription of the viral RNA. For paramyxoviruses, HN protein binds to the sialic acid on the cell surface receptor and induces, in some way, conformational changes in the other viral envelope protein, known as the F1/F2 complex, thereby facilitating the fusion domain of F1 to cause fusion between the viral membrane and the plasma membrane and release of the viral nucleocapsid containing the RNA-dependant RNA transcriptase (RDRP) into the cytoplasm. For paramyxovirus, the entire viral replication process takes place in the cytoplasm, whereas for retroviruses the proviral DNA is formed in the cytoplasm after reverse transcription of the viral RNA and then transported to the nucleus for integration and transcription. How the receptor–protein interaction facilitates conformational changes leading to fusion of the viral and cellular membranes in a pH-independent manner is not fully understood. Furthermore, fusion for these viruses occurs not only between viruses and host cells but also between virus-infected cells expressing the cleaved viral membrane proteins on the cell surface and uninfected cells containing the receptors (and coreceptors) present on the cell surface. These cell to cell interactions lead to the formation of syncytium or multinucleated giant cells. Such multinucleated giant cells are important diagnostic markers for a number of viral infections (e.g., human respiratory syncytial virus (RSV), family Paramyxoviridae, genus *Pneumovirus*; mumps virus, family Paramyxoviridae, genus *Rubulavirus*; and measles viruses, family Paramyxoviridae, genus *Morbillivirus*). The process of fusion of HIV-infected cells with uninfected CD4+ T cells is implicated in the pathogenesis of AIDS, which causes depletion of CD4+ T cells in HIV-infected people.

For other enveloped viruses such as VSV and influenza viruses, penetration and uncoating are two separate events. Following receptor binding, these viruses enter the cytoplasm by receptor-mediated endocytosis, and fusion and uncoating occur within the endosome in a pH-dependent (low pH of ~5) manner. The fusion and uncoating of these viruses can be blocked by agents such as monensin, which increases endosomal pH. For VSV, the G protein binds to the receptor and becomes activated for fusion at low pH, even though it remains uncleaved. Although the VSV G protein contains a hydrophobic fusion region, the mechanism of its fusion process within the endosome is not well understood. The fusion and uncoating processes are best understood at the molecular level for influenza viruses. Again, for influenza viruses, although fusion and uncoating occur

simultaneously, they are considered two separate events. Following binding to sialic acid on the cell surface receptor, influenza virus undergoes receptor-mediated endocytosis and the cleaved HA trimer (i.e., HA1/HA2 heterotrimer complex) present on the viral membrane undergoes conformational changes at the low pH (~5) of endosomes. Acidic pH specifically alters the structure of HA2, which attains the fusiogenic state (Figure 3.14). In conjunction with this process, HA1 is dissociated from the stem of the HA spike and the fusion peptide present at the NH_2 terminus of HA2, which normally remains buried in the protein interior of the HA trimer, is released and the polypeptide structural loop is transformed into a helix to form an extended coiled-coil structure that relocates and thrusts the boomerang-shaped hydrophobic fusion peptide toward and into the target (endosomal) membrane (Figure 3.14). This process first leads to hemifusion by mixing of the outer lipids of the bilayers of both viral and endosomal membranes and then to complete fusion of both lipid bilayers of the membranes, leading to the formation of a pore between the two compartments. Subsequently, the pore dilates leading to mixing of the cytosol and virion contents and delivery of the viral nucleocapsid into the cytoplasm (Figure 3.14). In addition to causing fusion, low pH also aids in the uncoating of the influenza virus nucleocapsid. Uncoating (Figure 3.11b) in this case is defined as the separation of a nucleocapsid from the virus matrix protein (M1). Therefore, with this type of virus, low pH (–5) is not only crucial to the outside of the virus particle (virion) for inducing conformational changes of HA1 and HA2 but is also needed inside the virus particle for separation of M1 from the nucleocapsid. Acidification of the virion interior is carried out by a viral protein called M2. A small number of M2 tetramers (16–20 per virus particle) are formed by the type III transmembrane M2 protein present on the viral membrane. These M2 tetramers constitute ion channels that remain closed at neutral pH and open at low pH (~5) to allow protons (H^+) to enter from the endosomes into the core of the virus particle. The resulting acidic pH inside virus particles causes dissociation of M1 from the viral RNP (also known as the vRNP or nucleocapsid) containing vRNA (minus strand), and so the M1-free viral RNP is released into the cytoplasm (Figure 3.11b). Both the opening of the M2 ion channel and the uncoating of some members of the species influenza A virus can be blocked by amantadine (or rimantadine), a drug currently used to treat influenza infection. The dissociation of M1 from the vRNP is important since the released vRNP can now be translocated into the host nucleus, where the transcription and replication of vRNA can occur. M1, on the other hand, interferes with the transport of vRNP into the nucleus and also inhibits the vRNP transcription. However, mutation(s) in the M2 channel can make the mutant virus resistant to amantadine (or rimantadine). Many of the epidemic/pandemic viruses have become resistant to amantadine (or rimantadine).

3.4.3 Targeting Viral Nucleocapsids to the Replication Site

Viral replication occurs either in the nucleus or in the cytoplasm of infected cells. For those viruses that replicate in the cytoplasm, which customarily are those with RNA genomes, except for the DNA-containing poxviruses, the uncoating process releases the viral nucleocapsid directly into the cytoplasm, which is the site of transcription and replication. For viruses that replicate within the nucleus, which tend to be the ones having DNA genomes with notable exceptions such as the RNA-containing influenza viruses and retroviruses, the nucleocapsids of these viruses, released in the cytoplasm after uncoating, must be targeted into the nucleus. Nuclear targeting requires that these viral nucleocapsids contain proteins possessing nuclear targeting signals (NTSs) or nuclear localizing signals (NLSs) that are recognized by the cellular nuclear targeting machinery and translocated into the cell nucleus via nuclear pores. However, the stage of uncoating at which nuclear targeting takes place varies with

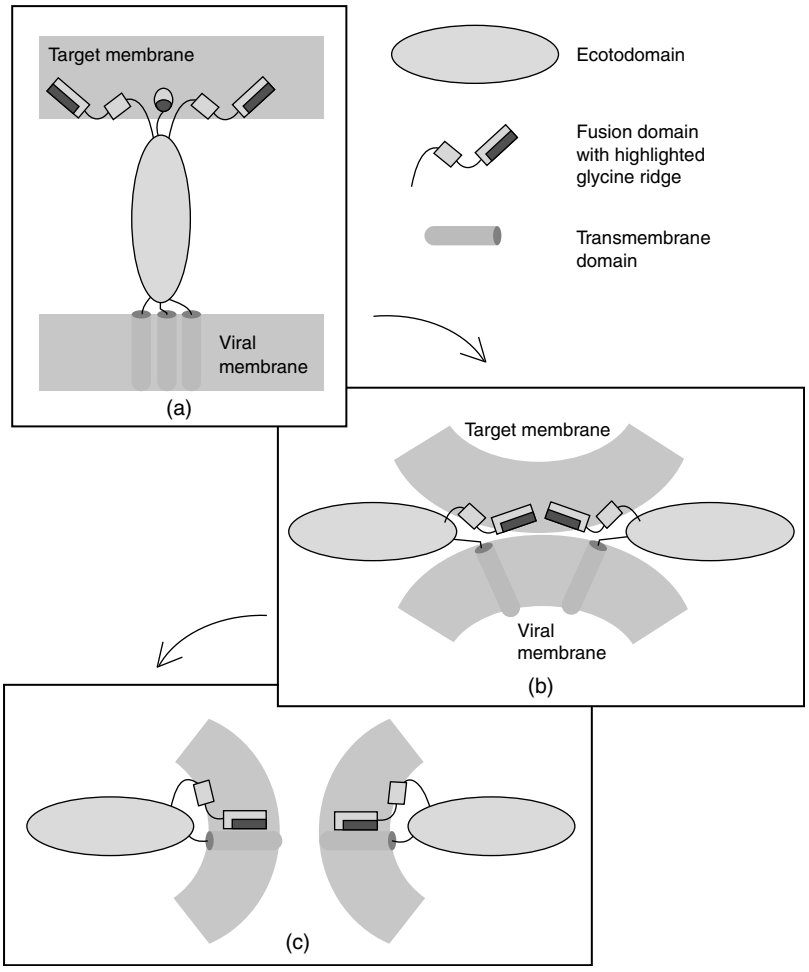


FIGURE 3.14 Boomerang model of influenza virus HA-mediated membrane fusion. (a) Cleaved influenza virus HA (HA0 into HA1 and HA2) undergoes pH-induced conformational change in the endosome and thrusts the boomerang-shaped fusion peptide toward the target cellular membrane where it inserts. (b) The ectodomain tilts to the plane of membranes. The boomerangs retrieve the target membrane and bring it to the close juxtaposition with the viral membrane such that lipid exchange forming hemifusion can occur. In this state, lipids of the outer leaflets, but not the inner leaflets, mix. At the point of hemifusion, the aqueous contents of the two vesicles still remain separated. (c) Eventually the fusion peptides and the transmembrane domains interact by virtue of the glycine edge fusion peptide, causing complete fusion of both lipid bilayers and leading to formation of the initial fusion pore opening. Multiple HA trimers are required for causing and opening the fusion pore. After opening of the initial narrow fusion pore, the pore dilates (not shown) releasing the viral nucleocapsid into the cytoplasm of the infected cell. Reprinted with permission from Tamm (2003). (*See the color version of this figure in Color Plate section.*)

viruses. For SV40, essentially the entire virus particle taken into the cytoplasm is transported into the nucleus, and it is only in the nucleus that uncoating of the capsid occurs concomitant with release of the viral minichromosome. For adenoviruses, uncoating occurs at the nuclear pore where the viral nucleocapsid docks and the viral DNA is delivered into the nucleus through the nuclear pore. For influenza viruses, uncoating occurs by dissociation of M1 from the vRNP during introduction of the nucleocapsid into the cytoplasm. This M1-free

vRNP is then transported into the nucleus. For retroviruses, not only uncoating but also additional biosynthetic processes—including reverse transcription of the RNA genome and synthesis of the double-stranded proviral DNA—occur in the cytoplasm. Then the retroviral DNA along with integrase is translocated into the nucleus for integration of the proviral DNA into the host genome. Transcription of the retroviral genomic and subgenomic mRNAs occurs only from the integrated proviral DNA in the nucleus. For hepatitis B virus, the partially double-stranded DNA, the viral genome following uncoating in the cytoplasm, becomes fully double-stranded and circularized in the cytoplasm and then it is translocated into the nucleus for subsequent transcription of genomic and subgenomic mRNAs.

3.4.4 Postuncoating Events

The "immediate events" in the viral replication cycle, those that occur following uncoating, vary with the nature of the viral genome. For plus-strand RNA viruses except retroviruses, translation of the viral RNA follows immediately after uncoating. The viral RNA extruded from the capsid is then used by the host translation machinery for directing protein synthesis (Figure 3.13). For all other viruses, whether of DNA or RNA genome, the step immediately following uncoating is either transcription of the genome yielding functional mRNAs or reverse transcription of vRNA yielding proviral DNA (retroviruses).

3.4.5 Transcription of Viral Genes

From the transcription viewpoint, viruses can be classified into two major categories, that is, whether they possess a DNA genome or an RNA genome. Of the first group, the DNA genome of different viruses varies greatly in complexity between virus families, encoding from only 4–5 genes (polyomaviruses, family Polyomaviridae) to more than 200 genes (poxviruses) or open reading frames (ORFs). DNA viruses use DNA-dependent RNA polymerase that can be either virus specified (e.g., poxviral RNA polymerase) or host specified (e.g., RNA *pol* II) to generate their mRNAs. RNA viruses, however, must use RDRP, which is always virus specified and is therefore different and specific for each virus group.

3.4.5.1 Transcription of DNA Viruses

All DNA viruses except the poxviruses transcribe and replicate their genomic material in the host cell nucleus. Poxviruses transcribe and replicate in the cytoplasm. In addition, all DNA viruses except poxviruses use host *pol* II for transcription of their DNA into mRNAs. Poxviruses use virus-specific RNA polymerase for transcription of their genome. Viral DNA genomes as host DNA often possess the *cis*-acting elements, which are essential for successful transcription of their DNA. These DNA elements are called the viral promoter and enhancer. The promoter is the RNA polymerase binding site on viral DNA (e.g., TATA box, CAT box, GC box) localized in the vicinity (usually upstream) of the transcription initiation point. The enhancer element, which enhances transcription of the viral mRNA over the basal level, is found in the proximal or distal region of the promoter and may be located upstream or downstream of the promoter element. Transcription of the viral DNA genome can broadly be divided into the early and late phases. Early genes are usually catalytic and regulatory in nature, involved in regulating transcription of mRNAs and replication of viral DNA. Late genes usually produce mRNAs for structural viral proteins, which are the major components of the viral capsid or envelope. Early genes are usually transcribed prior to the initiation of viral DNA synthesis, and late genes are transcribed only after viral DNA synthesis is initiated. Thus, synthesis of the progeny viral DNA demarcates the dividing line between early and late gene transcription. However, for complex viruses such as HSV (human herpesvirus 1 and 2 of the family Herpesviridae, genus *Simplexvirus*), the different classes of regulatory genes, for example, immediate early (α), delayed early (β), and late

(γ1, γ2) are transcribed at different phases of the viral replication cycle, each having different regulatory functions for turning on or shutting off other viral genes. Viral genes can be transcribed from either of the two DNA strands, with the coding sequences thus running in a direction opposite to a duplex DNA. These viral genes usually possess the structural features of eukaryotic cellular genes, and the viral mRNAs similarly undergo posttranscriptional processing similar to cellular genes. The majority of viral mRNAs, like host mRNAs, are usually capped at the 5′-end, polyadenylated at the 3′-end, and may undergo posttranscriptional splicing prior to their exit from the host cell nucleus. However, poxviral mRNAs, which are also capped and polyadenylated, do not undergo splicing since they are made in the cytoplasm.

An example of transcription of a small double-stranded viral DNA genome (SV40) is shown in Figure 3.15. Transcription of the SV40 genome is carried out by the host cell's RNA polymerase II. Early mRNAs (large T and small T) are transcribed from the early promoter of the early DNA strand, whereas late mRNAs (i.e., the mRNAs for VP1, VP2, VP3, and agno proteins) are transcribed from the late promoter and the opposite DNA strand. Both early and late transcription in SV40 is initiated from the common control region in opposite directions at different phases of the replication cycle. This control region also regulates SV40 viral DNA replication. This region consists of a series of repeat elements with different functions: three 21-base repeats that together contain six copies of GC-containing hexamers serve as the promoter for early transcription. Downstream of these repeats is a TATA box and upstream are two 72-base repeats constituting the enhancer element (Figure 3.15, bottom). These three regulatory elements bind specific cellular factors and are important in regulating early transcription. Of these, the 21-bp repeats and the enhancer elements are also important in regulating late transcription. The switch from early to late transcription is brought about by binding of large T antigen to specific sites in the control region and a change in the replicative state of the viral DNA. Large T (LT) and small T (ST) antigens are two early proteins translated from two different mRNAs produced by differential splicing. The two late mRNAs have a common untranslated region and a common poly(A) addition site but are generated by differential splicing. Each of these late SV40 mRNAs is bicistronic, with alternative initiation codons. One of these late mRNAs is translated into VP2 and VP3, and the other into VP1 and the agno protein.

On entry into the cytoplasm of the infected cell, HBV, a partially double-stranded DNA virus, uses virus-specific reverse transcriptase (P) to synthesize the complete circular DNA that is then transported into the nucleus. Host cell *pol* II subsequently transcribes its genomic and subgenomic mRNAs from different initiation points (Figure 3.16). They are all capped at the 5′-end, unspliced, and have a common termination and poly(A) addition site at the 3′-end. Different classes of genomic-length (3.5 kb) RNAs, possessing different 5′ but common 3′ termini, function as a template for making cDNA or are translated into both the Pre-C and C proteins and the P protein. Subgenomic mRNAs are translated into the Pre-S1, Pre-S2, and S proteins as well as the X protein (Figure 3.16).

3.4.5.2 Transcription of RNA Viruses

Among the different families of RNA viruses, the RNA viral genome appears to be much less complex compared to the genomes of the highly complex DNA viruses. However, these RNA viruses use multiple strategies to encode different mRNAs and proteins. Unlike DNA viruses, the majority of the RNA viruses (except for retro-, orthomyxo-, and related viruses) replicate in the cytoplasm, so that their mRNAs cannot undergo RNA splicing. RNA viruses also possess genes both for regulatory and catalytic proteins and for structural proteins. However, transcription of mRNAs encoding these proteins is not as strictly demarcated with respect to the timing of their genomic nucleic acid replication as is found for DNA viruses. On the other hand, with RNA viruses, there is a

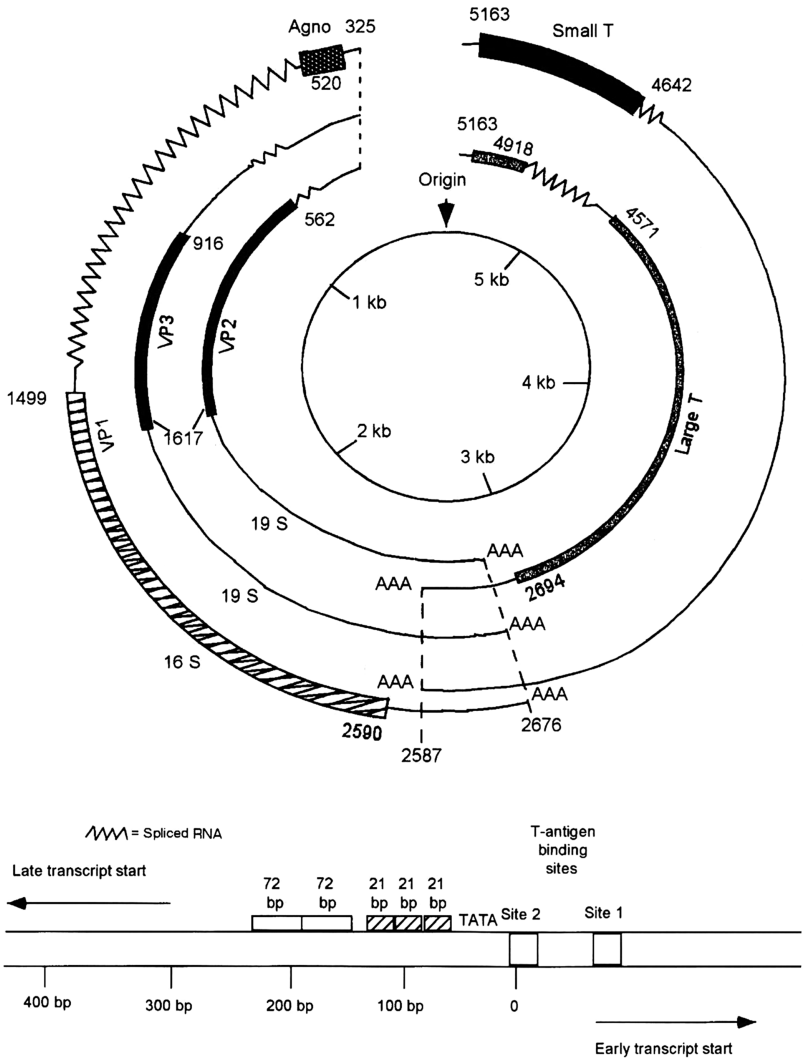


FIGURE 3.15 Genome and transcription map of SV40 (top). The origin of replication is shown at the top of the inner circle. The numbers indicate the nucleotide position in the SV40 DNA, while zigzag markings indicate spliced introns. Different shaded regions indicate different protein-coding sequences. The bottom drawing shows the details of the transcription regulatory elements in the proximity of the "origin" region and the direction of the early and late transcription.

great deal of variation at the level of transcription of different viral mRNAs. The mRNAs of the major structural proteins—such as the nucleoprotein (NP), matrix (M), and glycoproteins—are usually made in larger amounts compared to the lower amount of mRNAs synthesized for catalytic (e.g., polymerases) proteins. For nonsegmented negative-strand RNA viruses, the level of mRNA transcription is regulated by the promoter-proximal position of a gene (e.g., for VSV or paramyxoviruses, see Figure 3.8) or by an internal promoter that produces subgenomic mRNAs (e.g., togaviruses). The strategy used by different RNA viruses for mRNA transcription depends on the nature of the RNA genome (+ or − strand, segmented or nonsegmented) and whether the nucleocapsid is icosahedral or helical.

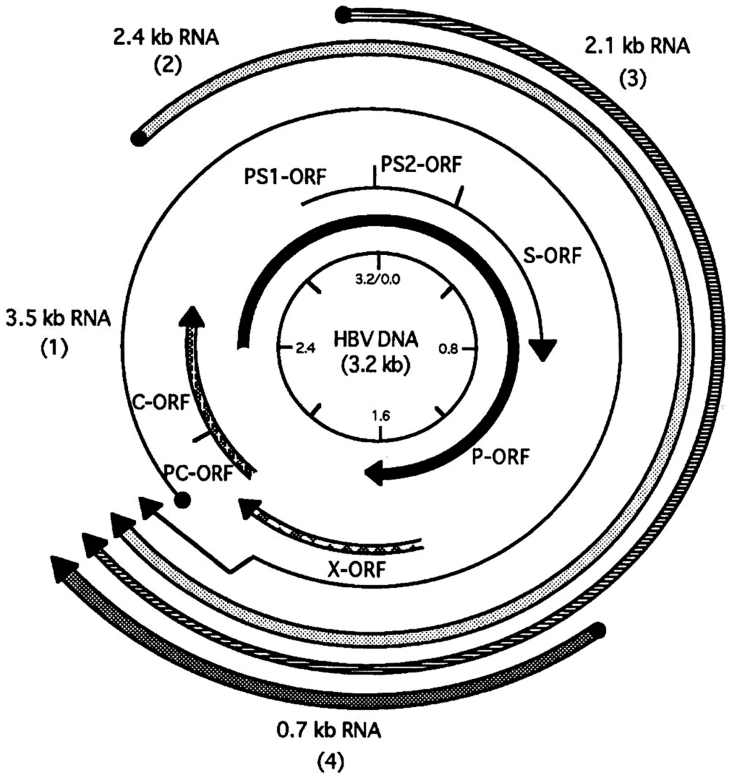


FIGURE 3.16 Replication, transcription, and translation of hepatitis B virus (family Hepadnaviridae, genus *Orthohepadnavirus*) DNA. Four RNA classes: 3.5 kb (1), 2.4 kb (2), 2.1 kb (3), and 0.7 kb (4) are transcribed. The 3.5 kb product (#1) is used for full-length DNA (minus-strand) synthesis. Different classes of 3.5 kb product also function as mRNAs whose translation products are HBcAg, the polypeptide consisting of the PC-ORF (precore), C-ORF (core) and P-ORF (P protein, also called polymerase or reverse transcriptase). The 2.4 kb mRNA (#2) makes a large protein consisting of the polypeptides PS1-ORF, PS2-ORF (presurface), and S-ORF (surface protein). The 2.1 kb mRNA (#3) makes the S-ORF (surface) protein, and the 0.7 kb mRNA (#4) encodes the X-ORF protein.

For plus-strand naked icosahedral RNA viruses (e.g., poliovirus), the entire viral genomic RNA functions as the only mRNA and is translated from one ORF into a large polyprotein that is then cleaved by specific proteases into different functional proteins representing the RNA polymerase and the capsid proteins (VP1, VP2, VP3, VP4) and so on (Figure 3.7).

For some enveloped plus-strand RNA viruses (e.g., togaviruses), the 5′-half of the viral genomic RNA encodes and is translated into nonstructural (catalytic) proteins involved in RNA transcription and replication, whereas a separate subgenomic 26S mRNA (+), made from an internal promoter on the minus-strand RNA template, encodes the structural proteins (i.e., capsid and envelope proteins). This 26S mRNA is synthesized in larger quantities than is the genomic-length RNA. However, another group (flaviviruses, family Flaviviridae) of enveloped plus-strand RNA viruses possesses one large ORF in its genomic RNA encoding a single large polyprotein that, as is the case with picornaviruses, is cleaved into specific proteins by a virus-encoded proteinase.

For coronaviruses, which contain a large plus-strand RNA genome of ~30 kb, multiple subgenomic mRNAs are found. However, each of these mRNAs possesses the same 5′-leader

(i.e., leader-primed transcription) and the common 3′-end containing poly(A) sequences. These mRNAs therefore contain the nucleotide sequence of more than one ORF. Usually, however, only the first ORF at the 5′-end of mRNA is translated into protein.

Minus-strand RNA (–) viruses replicating in the cytoplasm may possess either one large genomic RNA molecule (nonsegmented) or two or more different subgenomic RNAs (segmented). For those viruses that possess one nonsegmented genomic RNA molecule (e.g., VSV), the viral genes are arranged sequentially in the genomic RNA (–) with stop, intergenic, and start (EIS) sequences (Figure 3.8). The viral RNA polymerase (RDRP) synthesizes the virus mRNAs by initiating transcription at the 3′-end (one entry) and then terminates at the stop sequence (E) of that gene, skips the intergenic sequence (I), and initiates at the start (S) sequence of the next gene, and so on. Therefore, the viral RNA polymerase sequentially transcribes the downstream genes and there is no independent internal entry of the RNA polymerase on the viral genome. Since RNA polymerase randomly falls off during transcription and cannot initiate *de novo* internally, the mRNA level (and, consequently, the protein level) is determined by the location of a particular gene in the viral genome. For example, mRNA of the capsid protein (N or NP) is present at the extreme 3′-end of the minus strand (i.e., proximal to the promoter) just after the leader (ℓ) sequence, and it is therefore made in the most abundant amount because it is the first gene to be transcribed by RDRP into mRNA. On the other hand, the L (polymerase) gene, encompassing nearly half of the genome, is located at the 5′-end of the viral RNA (distal to the promoter), so the L mRNAs and L proteins are made in the least amount (Figure 3.8). Each mRNA is capped at the 5′-end and polyadenylated at the 3′-end by the virally encoded RDRP.

Orthomyxoviruses, which are segmented minus-strand RNA viruses, possess 8 (genera *Influenzavirus* A and B) or 7 (genus *Influenzavirus* C) RNA segments that in total encode 10 mRNAs and 11 proteins for type A and B viruses. Orthomyxoviruses are transcribed and replicated in the nucleus. Orthomyxoviruses use a unique strategy to initiate transcription. They cannot initiate *de novo* mRNA transcription without a primer and must use the host's capped RNA as the primer at the 5′-end for mRNA transcription. One of the three proteins (PB2) of the viral polymerase complex (PB1/PB2/PA) recognizes the newly synthesized capped host RNA and PA possessing the endo nuclease activity cleaves it around 12–15 nucleotides from its 5′-end. Then another protein (PB1) of the polymerase complex uses the capped primer for viral mRNA initiation and chain elongation. Therefore, each influenza viral mRNA possesses at its 5′-end a capped nonviral RNA sequence acquired from the host nuclear RNA (Figure 3.19). In addition, two viral RNA segments (segments #7 and #8) generate both unspliced and spliced mRNAs, causing translational shift to a different reading frame. Furthermore, another small (87 amino acids) protein PB1-F2 is translated from an alternative reading frame of PB1 mRNA. In this process, eight influenza viral RNA segments of type A and B viruses give rise to 10 mRNAs and 11 proteins.

Segmented ambisense RNA viruses (e.g., arenaviruses) on infection produce a subgenomic mRNA using the 3′-end of the genomic RNA as the template, and later on in the infectious cycle use the antigenomic RNA as the template to generate the mRNA with the same polarity as the 5′-end of genomic RNA.

Viruses that possess double-stranded (ds) RNA viral genomes, such as reoviruses, are segmented and replicate in the cytoplasm. Their viral transcriptase, which is also present within the virus particles, synthesizes single monocistronic mRNAs from each dsRNA segment.

Retroviruses, although possessing a plus-strand RNA genome, contain reverse transcriptase in the virion. Transcription of retroviral mRNAs occurs in the nucleus from the integrated proviral DNA template by the host RNA *pol* II. Usually, both the unspliced genomic-length mRNA and the subgenomic mRNA, the

latter being produced by splicing in the nucleus, function in protein translation.

3.4.6 Translation

Virions have evolved to become very efficient organisms that package a relatively small amount of genomic information as DNA or RNA in their capsids but use this information efficiently to generate the maximum number of functional proteins required to produce infectious progeny virions and cause the disease syndrome. For some viruses like VSV, all the viral proteins encoded by the genome and produced in the infected cells including the transcriptase are incorporated into the virion and become structural components of virus particles. For these viruses, there are by definition no nonstructural proteins; that is, there are no proteins that are encoded in the virion genome and produced in the infected cells but not incorporated into the virion. However, for the majority of viruses, one or more nonstructural proteins, either catalytic (enzymatic) or regulatory, are synthesized in virus-infected cells. These nonstructural proteins are required for the infectious cycle but are not incorporated into virion particles. Both structural and nonstructural proteins are translated from viral mRNAs, and the majority of viral mRNAs (except in the case of picornaviruses) possess structural features similar to that of the host mRNA (i.e., they possess a cap at the 5′-end, a translation initiation triplet (AUG) in the context of Kozak's rule, and translation termination triplets and poly(A) sequences at the 3′-end). These viral mRNAs undergo cap-dependent ribosome binding and ribosome scanning to locate the proper initiation triplet, a process that does not provide any advantage over the host mRNAs during translation. Therefore, after infection, the virus must overcome two major problems to achieve successful replication: (1) viruses must somehow overcome competition from host mRNAs for using translation machineries, and (2) viruses that possess only a limited amount of coding information must still be able to generate the considerable number of functional proteins needed for replication.

Viruses have developed a number of strategies to compete with host mRNAs for efficiently using the host translation machinery. These include the following: (a) Viral transcription machinery (especially in RNA viruses) are more efficient in generating high levels of mRNAs, so that they can outcompete host mRNAs in translation. (b) Some viral proteins target and interfere with the host transcription machinery, so that the host transcription level goes down or shuts off. Influenza viruses, however, use a novel system to their advantage. As mentioned previously, one of the influenza polymerase proteins, PB2, recognizes, binds to, and cleaves the newly synthesized capped host hnRNAs (heterogeneous nuclear RNAs) around 13–15 nucleotides, and the capped oligonucleotide is used as the primer for mRNA synthesis. The cleavage of host hnRNAs, in turn, prevents host mRNA synthesis and processing. In addition, this virus interferes with nuclear export of the host mRNAs. (c) Some viruses modify the host translation machinery to use that machinery for their advantage, while simultaneously shutting off host mRNA translation. This latter mechanistic approach is particularly evident for picornaviruses, which inactivate the cap binding protein and modify the host translational factors (e.g., eIF2, eIF3/4B) and thus shut off cap-dependent host mRNA translation. However, picornaviral mRNA can still be translated efficiently because it does not have a cap at the 5′-end but rather possesses a unique RNA secondary structure known as an internal ribosome entry site (IRES) and is independent of Kozak's rule (Kozak, 1986). Kozak's rule states that most eukaryotic mRNAs contain a short recognition sequence (ACCATGG) that facilitates binding of mRNA to the small subunit of the ribosome for initiation of protein translation (Kozak, 1986). The picornaviral mRNAs possessing an IRES can be translated efficiently in a cap-independent manner, while capped host mRNAs cannot be translated because of viral-mediated inactivation of some of the host translational factors.

Viruses have developed different strategies to produce a relatively large number of functional proteins from a small amount of genetic information using both transcriptional (or posttranscriptional) and translational (or posttranslational) processing.

3.4.6.1 Transcriptional (or Posttranscriptional) Generation of Different mRNAs Double-stranded DNA viruses can use both of their DNA strands to transcribe mRNAs, thereby increasing potential transfer of information into proteins. Some viruses that make mRNAs in the nucleus (RNA or DNA viruses) can generate different mRNAs from the same genomic strand by using unspliced mRNA or electing alternative splicing sites, thus even causing frameshifts in the subsequent translation. Influenza viral proteins M1, M2, NS1, NS2, and SV40 (such as VP1, VP2, and large T and small T antigens) are classic examples of generating different mRNAs and proteins through splicing. Some viruses use RNA editing (i.e., nontemplated nucleotide addition in the mRNA) to shift the translation frame. This latter technique is frequently used by paramyxoviruses to generate their V and C proteins. Hepatitis delta virus (family unassigned, genus *Deltavirus*) uses adenosine deaminase for RNA editing as part of the transcription process to generate its δ Ag-L antigen. Other viruses selectively use different promoters to generate genomic and subgenomic mRNAs (e.g., HBV, togaviruses). Also, as mentioned earlier, influenza virus PB1 mRNA often uses an alternative reading frame to produce PB1-F2 protein.

3.4.6.2 Translational (and Posttranslational) Generation of Different Viral Proteins The most common way to generate a number of functional proteins after translation is by proteolytic cleavage. These endoproteases, usually encoded by the virus, are sequence specific and can generate a number of functional proteins from one large viral polypeptide. Classic examples of this type of cleavage activity are found with poliovirus (picornavirus) and flavivirus proteins. Poliovirus RNA is translated into a large polypeptide that sequentially undergoes endoproteolytic cleavage by different poliovirus proteases at specific amino acid sites, generating 11 viral proteins (VP4, VP2, VP3, VP1, 2A, 2B, 2C, 3A, VPg, 3C, 3D) and other intermediate proteins (Figure 3.7). The importance of virus-specific proteases has been demonstrated in HIV infection, during which HIV protease inhibitors alone or in combination with RT inhibitors can be used in the treatment of AIDS to reduce virus load of the patient. Cleavage by host proteases is also sometimes critical to render viral proteins functional and viral particles infectious (e.g., conversion of influenza viral HA to HA1 and HA2, HIV gp160 to gp120 and gp41).

Different initiation codons are also used in bicistronic mRNAs to translate different proteins. Depending on the initiation codon used, either one or the other protein can be translated (e.g., NB protein and NA protein from the same mRNA in *Influenzavirus B* and PB1 and PB1-F2 in *Influenzavirus A*). Usually, one of the initiation codons is favored, thus regulating the levels of the two proteins produced from one bicistronic messenger RNA. Another strategy, often used by retroviruses, is translational frameshift or translational suppression of termination codons. Translational frameshift owing to ribosomal slippage causes generation of the *gag*-pro-*pol* fusion protein in avian leukosis virus (family Retroviridae, genus *Alpharetrovirus*). This protein is then cleaved by a virus-specific protease (usually aspartic proteases) to generate individual functional proteins. Similarly, some retroviruses use translational termination suppression to continue translation in the same reading frame. In the *gag*-UAG-*pol* sequence, translation is normally terminated after the *gag* protein at the UAG codon. Occasionally, termination at UAG can be suppressed by a minor host tRNA capable of inserting glutamine and thereby generating a *gag–pol* fusion protein that subsequently is cleaved by a viral protease to generate *gag* and *pol* proteins. Again, both frameshift and in-frame

suppression produce only a minority of fusion proteins with *pol*, thus regulating the amount of *pol* protein needed in small amounts in virus-infected cells.

3.4.7 Replication of Viral Genome

The replication pathway of different viral genomes varies depending on the nature of the viral genome. The overall strategy of viral genome replication can be grouped into seven pathways depending on the nature of the genome (Figure 3.17). While all DNA viruses of eukaryotes except poxviruses replicate in the nucleus of their host cells, some use cellular DNA polymerase and others use DNA polymerase encoded by the virus genome (Table 3.2). Poxviruses replicate their genome in the

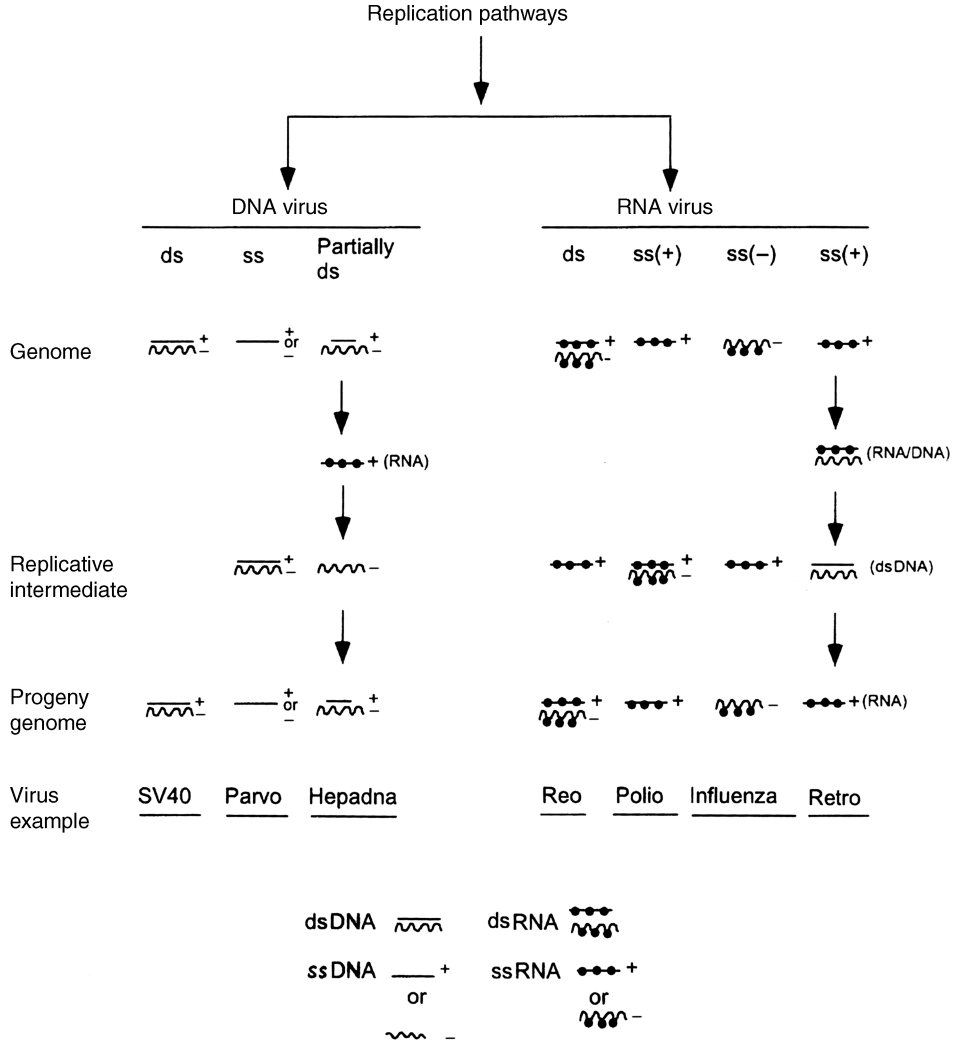


FIGURE 3.17 Seven replication pathways of the DNA and RNA genomes of viruses. Examples of different viruses with DNA or RNA genomes are indicated as ds, double-stranded; ss, single-stranded; and (+) and (–), positive and negative polarity, respectively. *Note*: the name "Polio" is now considered to represent antigenic variants of the species human enterovirus C belonging to the genus *Enterovirus*; the name "Parvo" refers to members of the family Parvoviridae and the name "Papova" refers to members of the families Papillomaviridae and Polyomaviridae.

cytoplasm and use polymerase encoded by the viral genome. All RNA viruses except retroviruses use an RDRP encoded by their own genome (Table 3.3). Some of these (minus-stranded RNA viruses) carry RDRP in the virus particle to initiate transcription/replication of viral RNA following their entry and uncoating inside the cell. Retroviruses require reverse transcriptase, an RNA-dependent DNA polymerase, in the virion particle to initiate replication. The majority of RNA viruses of eukaryotes replicate in the cytoplasm, except the orthomyxo- and related viruses and the retroviruses. Orthomyxoviruses require cellular capped 5′-RNAs as primers for mRNA transcription, and retroviruses require production of proviral DNA and its integration into the host DNA as a prelude to both transcription and replication of the viral genome.

3.4.7.1 Replication of DNA Genome

Smaller DNA viruses including the papillomaviruses, members of the family Papillomaviridae; polyomaviruses, members of the family Polyomaviridae; and parvoviruses, members of the family Parvoviridae, rely on the host cell DNA polymerase, whereas more complex DNA viruses use their own virus-encoded DNA polymerase (Table 3.2). The step for switching from transcription to replication of DNA viral genomes is primarily determined by the level of early viral proteins, which often are both regulatory and catalytic in nature. For SV40, when a sufficient amount of large T antigen is synthesized, binding of the LT antigen initiation to the transcription start site of early mRNAs (Figure 3.15) causes suppression of early mRNA transcription. The helicase activity of the virus LT antigen then unwinds the DNA molecule, creating a replication bubble, whereupon the host DNA primase–polymerase complex initiates DNA synthesis using an RNA primer, creating a replication fork. Synthesis of the SV40 DNA continues bidirectionally, creating circular intermediates (Figure 3.18c). Adenoviruses use asymmetric DNA replication, which initiates DNA synthesis at the 3′-end of one strand (template strand). At the 5′-end of that strand, a 55 kDa protein, covalently linked to the DNA, is needed for initiation of DNA replication. The new growing opposite DNA strand then displaces the preexisting opposite strand. The displaced strand forms a panhandle structure by pairing the inverted terminal repeats before its own replication begins (Figure 3.18a). In poxvirus DNA, two complementary forms are joined at the terminal repeat sections forming palindromes. During replication, concatamers of two genomic-length strands are formed. Unit length genomic molecules are then formed by separating the staggered ends and ligation (Figure 3.18e). Linear herpesvirus DNA becomes circularized inside the host cell nucleus and then replicates as a rolling circle, forming tandem concatamers. Finally, the unit length genomic DNA molecules are excised from concatamers (Figure 3.18b). Single-stranded parvoviral linear DNA has terminal palindromes that form hairpin structures. These hairpins then serve to covalently link the plus and minus DNA strands and self-prime the replication. The progeny viral DNA genomes are then made by strand displacement (Figure 3.18d).

Hepatitis B virus DNA uses reverse transcription for replication (Figure 3.18f). The partially dsDNA in the virion contains a complete minus and a partial plus strand. After infection of the cell, the virion-associated reverse transcriptase renders the partially double-stranded viral DNA into a circular duplex DNA in the cytoplasm that is then translocated into the nucleus and transcribed into a full-length plus-strand RNA by the host RNA *pol* II already present in the nucleus (Figure 3.16). This full-length plus-strand viral RNA is encapsidated, transported into cytoplasm, and reverse transcribed into a full-length minus-strand and a partial plus-strand DNA before being released as infectious virion.

3.4.7.2 Replication of RNA Genome

Viral RNA genomes can be single-stranded and composed of a plus or minus strand, or it can be double-stranded. Furthermore, while the genomes of some RNA viruses are segmented

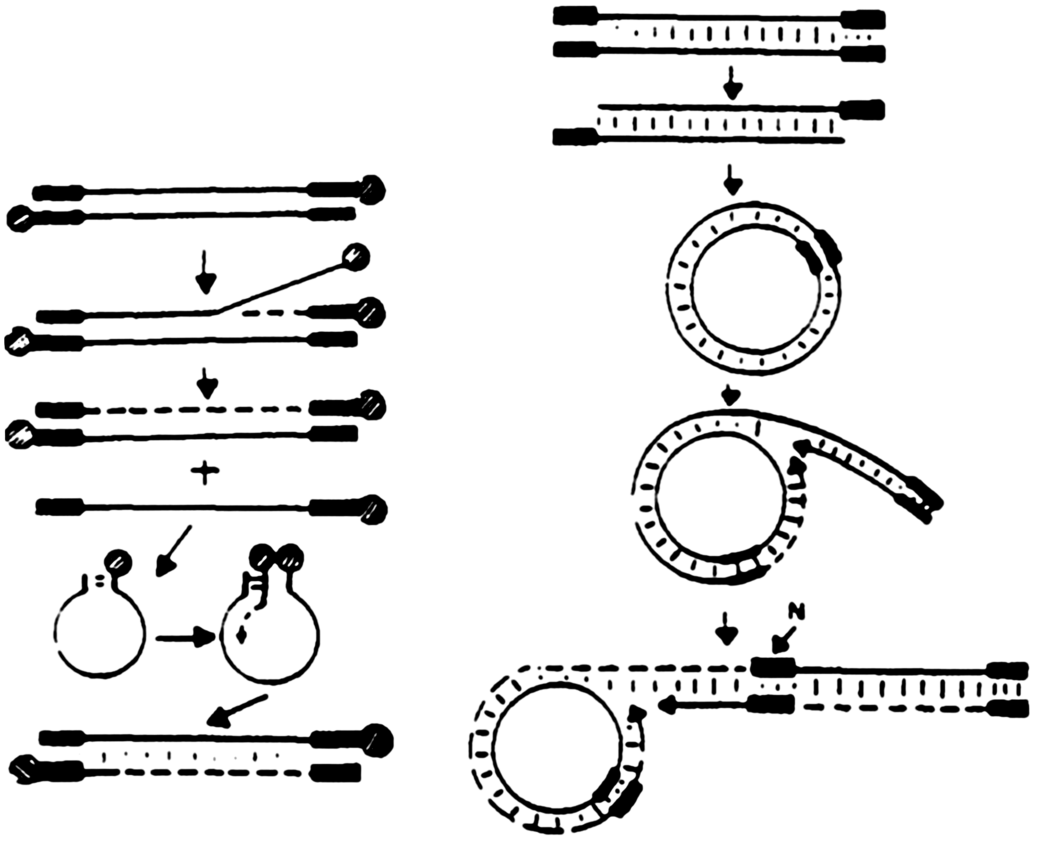


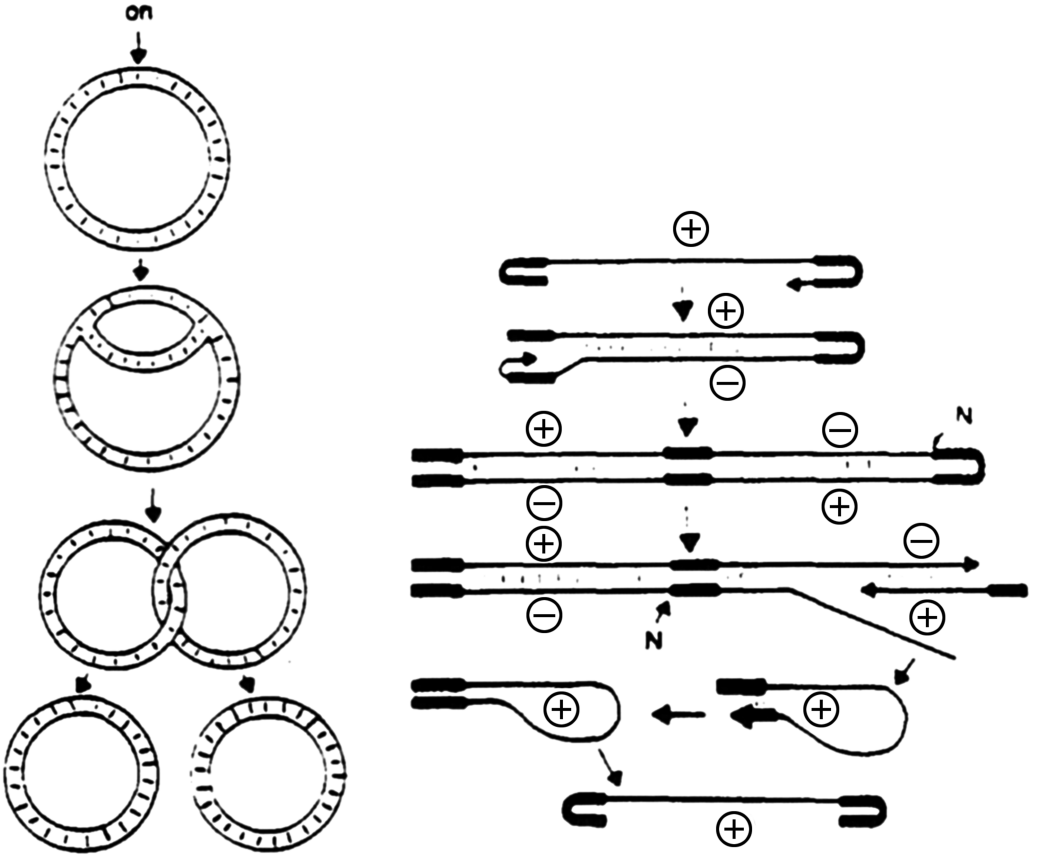
FIGURE 3.18 Replication pathways for viral DNA genomes. Dashed circles in (a) are terminal viral proteins attached to the 5′-end of the DNA strands. N in (b and d) represents endonuclease cleavage site. The heavy lines shown in (d) are palindromes and self-priming steps, with (+) and (–) representing strand polarity. The wavy lines in (f) represent RNA and the dashed lines represent DNA coding for direct repeats DR1 and DR2. Reprinted with permission from Davis et al. (1990).

(multiple RNA molecules), others are nonsegmented (i.e., one RNA molecule) (Tables 3.1 and 3.3). Switching from transcription to replication in the viral infectious cycle usually occurs after sufficient amounts of the capsid protein (e.g., nucleoprotein) are synthesized. The nucleoprotein functions as a regulator for switching from transcription to replication of the viral RNA genome.

Inhibition of nucleoprotein or protein synthesis will also inhibit vRNA (genomic RNA present in the virion) replication without necessarily interfering with mRNA synthesis. The same core enzyme (i.e., RDRP) is used for both transcription and replication, but the enzyme (and possibly the template RNA) becomes modified by viral nucleoprotein and cellular factors to effect the switch from transcription mode to replication mode. There are five different classes of RNA genomes, based on the different strategies that these viruses use for genome replication (Table 3.3).

Replication of Single-Stranded Viral RNA

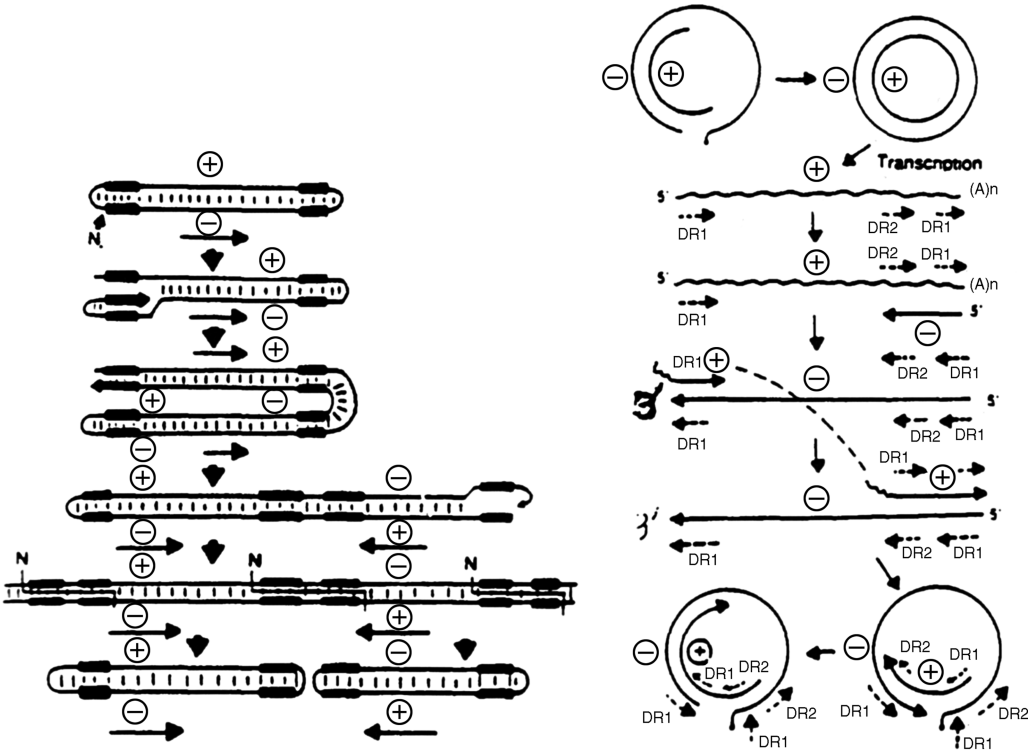
Plus-strand (+) RNA viruses are copied into a complete minus-strand RNA that then serves as a template for synthesis of more plus strands via replicative RNA intermediates



(c) Papovavirus (d) Parvovirus

FIGURE 3.18 (*Continued*)

(Figure 3.17). Minus-strand nonsegmented RNA genomes are transcribed into two types of plus-strand RNAs: subgenomic mRNAs (plus sense), which represent specific portions of the genome and are translated into proteins, and full-length cRNA (plus sense), which represents a complete copy of the entire minus-strand genome and serves as the template for genomic RNA (minus sense) synthesis (Figure 3.19). The synthesis of cRNA is regulated by a switch from transcription to replication mode that occurs after sufficient amounts of capsid proteins (e.g., NPs in influenza viruses) are synthesized. The capsid proteins provide the antitermination factor required for full-length cRNA synthesis. The cRNA is then copied back into full-length minus-strand viral RNA, which is incorporated into virions. Orthomyxoviruses, which possess a segmented minus-strand RNA genome, likewise synthesize two classes of plus-strand RNA from the same minus-strand RNA template. However, the mRNAs and cRNAs of these viruses are different at their both 5′ and 3′ ends (Figure 3.19). As indicated earlier, the orthomyxoviral mRNAs possess nonviral sequences from capped host mRNAs at their 5′ ends and terminate 18–22 nucleotides prior to the 3′-end with the addition of poly(A) sequences. However, the orthomyxoviral template cRNAs are complete copies of vRNA from end to end without any nonviral sequence



(e) Poxvirus (f) Hepatitis B virus

FIGURE 3.18 (*Continued*)

at the 5′-end or poly(A) sequences at the 3′-end. Therefore, for orthomyxoviral cRNA synthesis to occur, the viral polymerase must be able to initiate RNA synthesis without any host capped primer at the 5′-end and the RNA synthesis must not terminate until the complete 3′-end is reached, thus fully copying the entire template vRNA from the 3′-end to the 5′-end and without any polyadenylation. Such complete cRNAs then function as templates for vRNA (minus-strand) synthesis.

Replication of Double-Stranded Viral RNA

Each segment of double-stranded viral RNA genome is replicated independently. First, the genome is transcribed to generate plus-strand mRNAs within the incoming virion core by the virion-associated RDRP. Next, the mRNA is used as a template by RDRP to synthesize the minus RNA strand, and thereby mRNAs are converted into double-stranded RNA (Fig. 3.17) that is then packaged into progeny virion capsids.

Replication of RNA via a DNA Intermediate

Retroviruses contain a diploid genome consisting of two identical RNA molecules, a tRNA primer (Figure 3.6) and a reverse transcriptase, an RNA-dependent DNA polymerase that also possesses both RNase H and integrase activities. Conversion of the plus-strand viral RNA into double-stranded DNA is initiated by viral reverse transcriptase using the tRNA as a primer. The RNA-dependent DNA replication process is complex and requires strand switching twice (Figure 3.20). Eventually, a double-stranded proviral DNA is made in the cytoplasm that is translocated into the nucleus and integrated into the host genome. The integrated proviral DNA is then

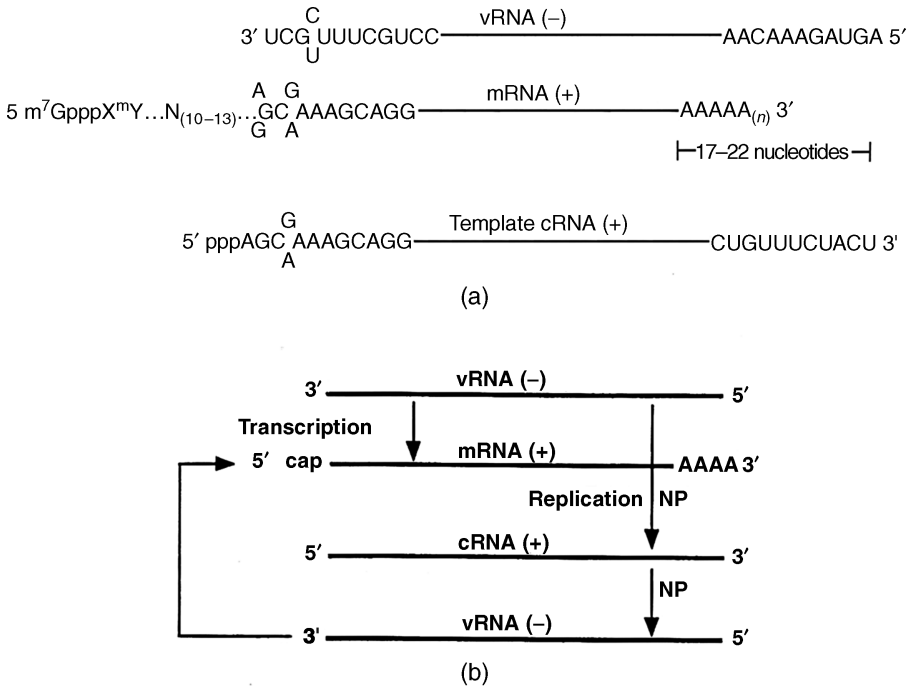


FIGURE 3.19 Transcription and replication of the influenza virus RNA (vRNA). (a) The three classes of influenza virus-specific RNAs found in the virus-infected cells, vRNA of minus (–) polarity; cRNA and mRNA, both of the latter two possessing (+) polarity. Note that the viral mRNA (+) possesses nonviral (host) capped sequences at the 5′-end and lacks sequences of 17–22 nucleotides from the 3′-end but contains poly(A) sequences. The template cRNA (+ strand), on the other hand, is an exact copy from end to end of the vRNA (– strand) and does not possess either cap at the 5′-end or poly(A) at the 3′-end. (b) The transcriptive and replicative processes of influenza viral RNA.

transcribed by the host RNA *pol* II into full-length plus-strand RNA and the full-length RNA is then transported into the cytoplasm and encapsidated into progeny virions.

3.5 ASSEMBLY AND MORPHOGENESIS OF VIRUS PARTICLES

As indicated earlier, compared to eukaryotes or prokaryotes, viruses use a unique multiplication strategy to produce their progeny. All cells, prokaryotic or eukaryotic, multiply as a whole unit from parent to progeny and in a geometric order, that is, 1, 2, 4, 8, and so duplicatively on. Viruses, however, do not multiply as units. Rather, they are assembled from component parts. Each component part of progeny virus particles is made separately, and they are often made in different amounts and at different locations and compartments within the host cell. These viral components are then put together to form the whole (infectious) virus particles (virions). In this assembly line type of process, all individual viral components need not be assembled at the same time, and in fact some components may be put together separately to form higher ordered structures, that is, subviral particles (e.g., capsid, nucleocapsid, RNPs), before they are assembled into a whole progeny virus particle. The number of steps involved and the complexity of the assembly process may vary greatly from one virus type to another. Some viruses, such as the polioviruses, have only a few components to assemble, and yet others, such as

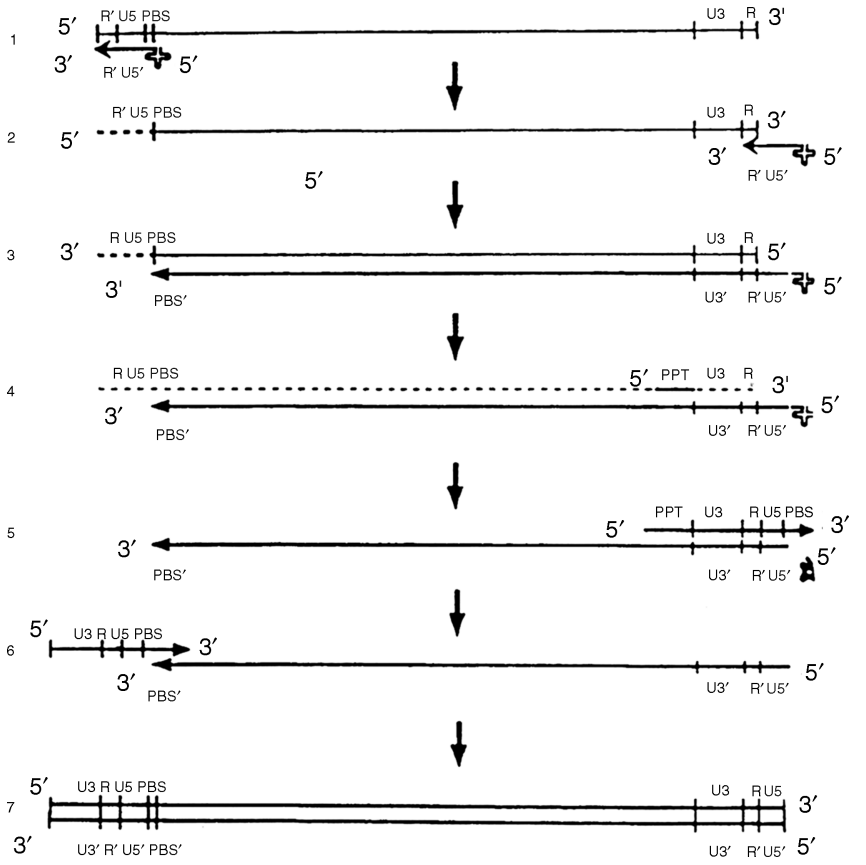


FIGURE 3.20 Reverse transcription of retroviral genomic RNA into double-stranded proviral DNA. Step 1: annealing of primer tRNA (shown as a cross-shaped symbol) to the primer binding site (PBS) and synthesis of minus-strand strong-stop DNA. The R and U5 RNA is degraded by the RNase H activity of the reverse transcriptase, and the strong-stop DNA is released. Step 2: The first strand switch (or transfer). The minus-strand strong-stop DNA is annealed to the 3′-terminus of the genomic RNA via R–R′ hybridization (first strand jump). Steps 3 and 4: Further synthesis of minus-strand DNA, during which the genomic RNA is further degraded by RNase H. However, a small piece of RNA (the polypurine tract (PPT)) remains undergraded and serves as a primer for synthesis of plus-strand strong-stop DNA (step 5). Step 5: Termination of plus-strand DNA synthesis at 18 nucleotides into the primer tRNA, thus generating the new primer binding site (PBS) sequence; the plus-strand DNA is then released from the minus-strand DNA. Step 6: The second jump (or the second transfer). The plus-strand strong-stop DNA is annealed to the 3′-terminus of the minus-strand DNA via PBS–PBS hybridization, completing the second jump. Step 7: Completion of the synthesis of the double-stranded proviral DNA. R, terminally redundant identical sequences at the 5′ and 3′ ends of viral RNA; U5, unique nucleotide sequences near the 5′-end of the viral genome between the R and PBS (primer binding site); U3, the region near the 3′-end of the viral RNA between the initiation site of the plus-strand DNA synthesis and R sequences; PPT, polypurine tract that escapes RNase H digestion and serves as a primer for the second strand DNA synthesis. Strong-stop DNA is the DNA copy of the region between the primer binding site (PBS) and the 5′-end of the viral RNA genome. Reprinted with permission from Mak and Kleiman (1997).

the poxviruses or herpesviruses, have many components to assemble and their assembly compared to polioviruses is a far more complex process involving multiple steps.

With respect to the assembly processes, viruses can be classified into two major subclasses: naked viruses and enveloped viruses. Naked viruses consist of only a nucleocapsid,

that is, the capsid containing the genome (DNA or RNA) and no envelope. The assembly of the protein capsid and incorporation of genomic nucleic acid into the capsid to create this nucleocapsid will render the virus particle infectious. For these viruses, the virus receptor binding proteins are part of the capsid proteins. Enveloped viruses, however, are those in which the nucleocapsid is surrounded by a lipid membrane containing the transmembrane viral proteins. In enveloped viruses, one of the transmembrane viral proteins (and not the capsid protein) contains the receptor binding protein.

3.5.1 Assembly and Morphogenesis of Naked Viruses

The assembly of naked viruses occurs in the cytoplasm (most RNA viruses) or nucleus (DNA viruses). Nearly all cytoplasmic viruses with the exception of poxviruses are RNA viruses (e.g., the plus-sense RNA picornaviruses). The entire genomic RNA of these viruses is translated into a single giant polyprotein (Figure 3.7) that is cleaved by a virus-specific protease into P1 (a coat precursor protein); P1 is then further cleaved by the protease 3C into VP0, VP3, and VP1 (5S promoter). Five subunits (5S promoter)—each containing one molecule of VP0, VP3, and VP1—then assemble into pentamers (14S). Twelve pentamers form the 60-subunit protein shell (capsid) for the picornaviruses. The viral RNA genome is then incorporated into the capsid, forming what is called the "provirion." Subsequently, VP0 molecules in the provirion are cleaved into VP4 and VP2 (Figure 3.7), converting the provirion nucleocapsid into an infectious virion. This process of picornaviral capsid assembly is basically a self-assembly process whose rate depends on viral protein concentration.

For assembly of a naked virion to occur inside the nucleus, one of at least two distinct strategies can be used. The first of these would require that all capsid proteins, after their translation in the cytoplasm, must be transported into the nucleus either independently or cooperatively by forming a complex with other capsid proteins and that nucleocapsid assembly occurs around the viral genome in the host nucleus. This option is used by polyomaviruses whose DNA genomes, or minichromosomes, contain a single closed circular duplex DNA molecule complexed with cellular histone, which is organized into a nucleosome within the host nucleus. Polyomaviral capsid assembly then proceeds in a stepwise fashion around the viral minichromosome. The capsid of SV40, which is a member of this virus group, contains 360 copies of its major viral protein (VP1) assembled into 72 pentamers plus 30–60 copies of internal proteins VP2 and VP3. VP2 contains the full VP3 sequence plus 100 extra amino acids at the NH_2 terminus, which are critical for interacting with the SV40 minichromosome. The polyomaviral capsid proteins and minichromosomes first assemble into 200S structures called provirions that then mature into infectious virions. During this maturation, H1 histone protein is removed from the viral minichromosome and degraded.

Adenoviruses use a second type of strategy in which the capsid shell is first formed by the assembly of viral capsid proteins. Viral DNA, including core proteins, is then inserted into the empty capsid shells to form infectious virions. Both these nuclear DNA viruses and the cytoplasmic naked RNA viruses are primarily released to the extracellular environment by cell lysis.

3.5.2 Assembly, Morphogenesis, and Budding of Enveloped Viruses

The assembly and budding of enveloped viruses is much more complex than that of naked viruses. It involves not only assembly and formation of nucleocapsids but also envelopment of the nucleocapsid and budding of enveloped nucleocapsids from different cellular organelles and membranes specific for each group of viruses. Subsequently, buds are pinched off and the virus particles are

released into the extracellular environment. The assembly and the budding site on the cellular membrane vary with different groups of viruses. Some viruses, such as poxviruses and rotaviruses, bud from the ER, while others, such as bunyaviruses, bud from the Golgi complex, and still others bud from the nuclear membrane, such as herpesviruses. Still other viruses (e.g., orthomyxo-, paramyxo-, rhabdo-, and retroviruses) use the plasma membrane (apical or basolateral) as the budding site.

Assembly, morphogenesis, and budding of enveloped viruses require multiple steps: (1) transport and assembly of viral components to the budding site and (2) the budding process including bud initiation, bud growth, and pinching off from the plasma membrane. Budding is a complex process and involves physical and structural as well as functional requirements of multiple biological components of both virus and host cell and the processes involved in budding are not fully understood in viral biology. Since the assembly and budding processes of orthomyxoviruses have been well studied, the steps involved in morphogenesis and budding of these viruses will be discussed in some detail. For comparison, rhabdovirus and retrovirus assembly will also be mentioned as needed. As noted earlier, orthomyxo- and paramyxoviruses are enveloped RNA viruses containing single-stranded RNA genomes of negative (minus) polarity, and they are assembled into nucleocapsids having helical symmetry (Table 3.1). Electron microscopic studies have demonstrated that these viruses bud from the plasma membrane into the outside environment and that complete virions are usually not found inside the cell during the productive infectious cycle.

For budding to occur, all viral components must be brought to budding site. With the majority of viruses enveloped or nonenveloped, assembly implies the formation of complete capsid, either helical or icosahedral including incorporation of the genome into the capsid. Furthermore, with the majority of enveloped viruses, capsid formation is a requirement for bud formation and bud release as is shown for retroviruses such as human immunodeficiency viruses (Ganser-Pornillos et al., 2008) and alphaviruses such as SFV (Garoff et al., 1994). Even for some enveloped viruses possessing helical nucleocapsids such as VSV, formation of nucleocapsid is critical for bud formation and bud release. In fact, the size of the nucleocapsid assessed by the size of vRNA determines the size and shape of the released VSV particles. For example, smaller DI virus particles contain shorter vRNA/RNP (nucleocapsid) compared to the bullet-shaped elongated virus particles of wild-type viruses containing the complete and longer vRNA/RNP (Pattnaik and Wertz, 1991). Therefore, with these viruses, capsid assembly is a critical requirement for virus budding. However, requirements of capsid assembly and budding are much more complex for viruses with segmented genome (e.g., influenza viruses) for a number of reasons. First, budding may occur in the absence of vRNPs (capsids) and/or with incomplete vRNPs. Furthermore, the viral genome consists of multiple segments of vRNAs/vRNPs. Therefore, budding of infectious virus particles requires that each segment of multiple vRNPs must be successfully incorporated into the bud. Second, all the components of the virus, that is, envelope containing the transmembrane proteins (HA, NA, and M2) and M1 and vRNPs, must be brought individually or as a complex to the budding site for bud initiation, bud growth, and finally release of infectious virus buds.

For elucidating the budding process of influenza viruses, the viral structure can be separated into three major subviral components, each of which must be brought to the assembly site for morphogenesis. These subviral components are (a) the viral nucleocapsid (or viral ribonucleoprotein (vRNP)) containing the vRNA, NP, and transcriptase/polymerase complex that together form the inner core of virus particle; (b) the matrix protein (M1), which forms an outer protein shell around the nucleocapsid and constitutes the bridge between the envelope and nucleocapsid; and (c) the envelope (or membrane), which forms the outer-

most barrier of these enveloped virus particles. The viral envelope contains virally coded transmembrane proteins and host cell lipids. Each of these subviral components must be brought to the budding site for interactions among themselves and for budding to occur. Depending on the virus groups, the budding site on the cell membrane varies. For example, viruses belonging to orthomyxo-, paramyxo-, rhabdo-, and retroviruses bud from the plasma membrane of infected cells. However, while the orthomyxo- and paramyxoviruses bud from the apical domain of plasma membrane in polarized epithelial cells, both *in vivo* (e.g., in bronchial epithelium) and in cultured polarized epithelial cells (e.g., Madin Darby canine kidney (MDCK) cells), the rhabdoviruses and retroviruses bud from the basolateral surface of polarized epithelial cells.

3.5.3 Assembly and Transport of Viral Components to the Budding Site

As mentioned above, for budding and release of influenza viruses to occur, all viral components must be brought to the budding site. Therefore, two questions arise: (1) How these subviral complexes are brought to the budding site? (2) What factors determine the selection of budding site? As mentioned above, there are three major subviral components within the influenza virus particle, namely, virus envelope, M1 (matrix protein), and the viral core (vRNP/nucleocapsid). The influenza virus envelope consists of a mosaic lipid bilayer and viral transmembrane proteins (HA, NA, and M2). Transport of the transmembrane envelope proteins (HA, NA, and M2) has been studied extensively (Nayak et al., 2004). As mentioned earlier, the viral membrane is a mosaic containing both raft- and nonraft-associated lipids. Both HA and NA proteins are inserted in the raft domains, whereas M2 is present in the nonraft lipid domains. These transmembrane proteins use cellular exocytic transport pathway for apical transport and possess the determinants for both lipid raft association and apical transport in their TMD. Lipid raft association of TMD is responsible for apical transport of both HA and NA (Lin et al., 1998; Barman and Nayak, 2000). However, as discussed later, transport of the envelope proteins is not the only or major determinant for the selection of the budding site of influenza viruses.

Next question is how the M1 protein, the most abundant viral protein, present underneath the lipid bilayer and forms the bridge between the envelope and viral core (vRNP) is brought to the budding site. M1 is not known to possess any apical determinant but possesses determinants for lipid binding, for RNA, RNP, or NP binding (Baudin et al., 2001; Noton et al., 2007; Watanabe et al., 1996; Ye et al., 1999), and for associating with HA and NA tails (Ali et al., 2000) and M2 tails (Chen et al., 2008). Therefore, it is likely that some M1 can be transported to the budding site of apical plasma membrane on the piggyback of HA and NA and also as a complex with vRNP.

Finally, how is the virus core (viral nucleocapsid) that consists of vRNP (minus-strand vRNA associated with NP), minor amounts of NEP (nuclear export protein), and 3P protein complex (three polymerase proteins PA, PB1, and PB2 forming a heterotrimeric complex) brought to the apical budding site. Helical nucleocapsid assembly occurs during the synthesis of minus-strand viral RNA for both segmented (influenza) and nonsegmented (VSV, Sendai) RNA viruses. In the absence of the capsid protein (N or NP), minus-strand vRNA synthesis does not occur. Each influenza viral nucleocapsid (vRNP) is a supercoiled helix with ribbon structure and a terminal loop, where the vRNA is coiled around NP monomer to form a hairpin structure and vRNA is exposed on the outer surface of NP (Elton et al., 2006). Therefore, RNP assembly involves the formation of these subviral complexes and their transport to the budding site, that is, the apical domain of the plasma membrane in polarized epithelial cells whether in cultured cells in laboratory or respiratory epithelium of infected animals. Furthermore, since influenza RNP is synthesized in the nucleus, it must be exported from the nucleus into cytoplasm be-

fore being transported to the apical plasma membrane. M1, a small protein possessing nuclear localization signal (NLS), can enter the nucleus, interact with both vRNP and NEP forming the daisy-chain complex of (Crm1 and RanGTP)–NEP–M1–RNP, and mediate nuclear export of v-RNP (Akarsu et al., 2003; Whittaker and Digard, 2006). M1–RNP complex has been demonstrated both in infected cells and in virions (Zhirnov, 1992; Ye et al., 1999). Interaction of M1 with RNP preventing transcription is critically required for the exit of vRNPs into cytoplasm and incorporation into virions (Nayak et al., 2004) since it is only transcriptionally inactive vRNPs with the polymerase complex present at the end of vRNP that are found in virus particles (Murti et al., 1988). Furthermore, lack of chain elongation of preexisting RNA molecules and requirements of capped RNA primers for *de novo in vitro* RNA transcription of vRNP molecules also support the presence of transcriptionally active vRNP within influenza virus particles.

Recent studies suggest that the viral NP or ribonucleoprotein (RNP) complex may possess an as yet undefined determinant for apical transport (Carrasco et al., 2004; Nayak et al., 2009). It was recently shown that influenza NP/RNP exits the nucleus from its apical side of nucleus and is transported to the apical plasma membrane of polarized MDCK cells. NP/RNP was also shown to interact with actin microfilaments (Avalos et al., 1997) and associate with lipid rafts (Carrasco et al., 2004). Therefore, it is likely that RNP along with the associated M1 can be directed to the apical budding site via its association with cortical actin microfilaments and lipid rafts. However, neither the apical determinant(s) of NP/RNP nor the cellular machinery involved in its apical transport has been identified.

Finally, since the genome of influenza virus is segmented, multiple vRNA/vRNP segments (eight separate segments for members of the genera *Influenzavirus A* and *B* versus seven segments for members of the genus *Influenzavirus C*) must be correctly assembled and incorporated into each infectious virus particle (see later). Each of these vRNA segments replicates independently in infected cells and can undergo reassortment during assembly and budding (Figure 3.10). When two or more viruses infect a single cell, released virus particles will have a set of different RNA segments arising from one or more viruses infecting the same cell (Figure 3.10). This is the major cause of antigenic shift and responsible for the emergence of pandemic influenza viruses. Depending on the set and combination of vRNAs, the virus particles will possess different antigenic epitopes and will have selective advantage of growth, virulence, and spreading.

However, although packaging of different RNP segments in the virus bud is critically important for infectivity of virus particle, assembly or incorporation of genomic segments does not appear to play a critical role in the budding of virions. However, M1 has been shown to play an important role in the assembly of virion components as it interacts with multiple components, such as viral RNA or viral RNP, and envelope proteins (HA, NA, and M2) and brings viral components together. M2 interacts with M1 via cytoplasmic tail and thereby plays an important role in virus assembly, genome packaging, and budding (Iwatsuki-Horimoto et al., 2006; McCown and Pekosz, 2005, 2006; Chen et al., 2008).

Although both orthomyxo- and paramyxoviruses bud from the apical domain of plasma membrane, there are two major differences: (1) as mentioned previously, since the viral genome of orthomyxoviruses is segmented, multiple RNA segments (eight separate RNA segments for members of the genera *Influenzavirus A* and *B* versus seven RNA segments for members of the genus *Influenzavirus C*) must be incorporated into infectious virions, whereas only one large RNA molecule is packaged in infectious paramyxovirus particles. (2) Since the transcription and replication of orthomyxovirus RNA and assembly of these viral nucleocapsids (vRNP) occur in the host nucleus, the viral nucleocapsids must be exported out of the nucleus into the cytoplasm for the final stages

of viral assembly and for budding. In contrast, paramyxoviruses possess one single nonsegmented minus-strand vRNA, and all these steps, including assembly of viral nucleocapsids, take place in the cytoplasm. The processes involved in the assembly and transport of other nonsegmented minus-strand RNA viruses such as rhabdoviruses (VSV) are essentially similar to that of paramyxoviruses. However, VSV buds from the basolateral membrane, whereas paramyxoviruses bud from the apical domain of the plasma membrane.

3.5.4 Selection of Budding Site

As mentioned previously, different enveloped viruses bud from different membrane compartments of infected cells, and budding site plays an important role in the pathogenesis of specific viruses (Nayak,). Therefore, it becomes important to ask, how is the budding site of enveloped viruses (e.g., apical domain of plasma membrane in polarized epithelial cells for influenza viruses) selected? For the majority of the viruses, viral glycoproteins are thought to be important in the selection of the budding site since virus glycoproteins, even when expressed alone in the absence of other viral components, predominantly accumulate at the site of virus budding. For example, viruses such as hepatitis B virus, bunyaviruses, coronaviruses, and others that bud from the internal subcellular organelles possess intrinsic determinants for the same subcellular localization as the site of virus budding (Hobman, 1993). On the other hand, for viruses budding from the plasma membrane, the viral glycoproteins possess apical or basolateral sorting signals and are directed to the specific site where virus assembly and budding occur in polarized epithelial cells. The surface glycoproteins of viruses such as influenza virus (family Orthomyxoviridae) and human respiratory syncytial virus (family Paramyxovidae) budding from the apical plasma membrane possess apical sorting signal(s) and predominantly accumulate at the apical plasma membrane in polarized epithelial cells. Conversely, for viruses released from the basolateral membrane, their surface glycoproteins, possessing basolateral sorting signal, are transported basolaterally in polarized epithelial cells even when these proteins are expressed alone. VSV, SFV, vaccinia virus, and certain retroviruses including human immunodeficiency virus type 1 (HIV-1) exhibit basolateral budding. Furthermore, in different cells and tissues where some viruses bud from the opposite domains of the plasma membrane, their glycoproteins are distributed accordingly. For example, SFV buds apically from FRT (a Fisher rat thyroid-derived cell line) cells but basolaterally from CaCo-2 (human epithelial colorectal carcinoma) cells. Similarly, in the absence of any other viral protein, p62/E2, the envelope glycoproteins of SFV, is targeted apically in FRT cells but basolaterally in CaCo-2 cells (Zurzolo et al., 1992). However, there are examples of polarized virus budding occurring independently of the polarized envelope viral glycoprotein sorting. For example, although measles virus glycoproteins H and F are transported in a random fashion or to basolateral membrane, respectively, virus budding was observed to have occurred predominantly from the apical surface of polarized MDCK cells (Maisner et al., 1998). Similarly, the spike protein of coronavirus is not involved in the polarized budding of this virus (Rossen et al., 1998). Moreover, Lake Victoria marburgvirus (family Filoviridae, genus *Marburgvirus*) buds predominantly from the basolateral surface, while its glycoprotein is transported to the apical surface (Sanger et al., 2001).

However, accumulation of viral glycoproteins may not be the only or the major determinant in selecting the budding site. For example, using a mutant transfectant influenza virus (HAtyr) containing basolaterally targeted HA (Cys543 → Tyr543), it was shown that the basolateral targeting of HA did not significantly alter the apical budding of influenza virus (Barman et al., 2003; Mora et al., 2002). Over 99% of the virus particles containing the HAtyr were released from the apical side even though the majority of HAtyr viruses were directed to the basolateral side. However, when virus bud-

ding was examined by thin section transmission electron microscopy, empty virus-like structures (Barman et al., 2003) with the same size diameter as the virus particles at apical surface were often observed only in HAtyr-infected cells (Barman et al., 2003). Likely, these particles represent abortive virus buds containing HA and M1 but not vRNP, suggesting that vRNP may play a role in polarized budding of influenza virus. Furthermore, apical targeting of NP was also shown to be independent of M1 and NEP that did not accumulate at the apical membrane (Carrasco et al., 2004; Nayak et al., 2009). We also observed that NP/vRNP in VLP-infected polarized MDCK cells lacking the expression of viral envelope proteins accumulated at the apical plasma membrane similar to that observed in wt virus-infected cells (Nayak et al., 2009) and NP exited through apical side of the nucleus in both wild-type (wt) virus-infected and VLP-infected polarized MDCK cells. These results demonstrate that NP/vRNP can be transported independently to the apical plasma membrane of polarized epithelial cells in the absence of transmembrane viral proteins. Therefore, transmembrane proteins alone do not determine the site of virus budding and NP also plays an important role in apical budding of influenza A viruses. Both cortical actin microfilaments and lipid rafts may aid in apical transport since NP binds to both these host components.

Two steps are obligatory for virus assembly and morphogenesis to occur. First, as mentioned above, all viral components (or subviral particles) must be directed and brought to the assembly site, that is, the apical plasma membrane in polarized epithelial cells for assembly and budding of orthomyxoviruses and paramyxoviruses. Obviously, this step is the first obligatory requirement in virus assembly and morphogenesis, since if different viral components are misdirected to different locations or parts of the cell, virus assembly and morphogenesis cannot take place. Second, the viral components must interact with each other to form the proper virus structure during morphogenesis. It is possible that viral components may be directed to the assembly site but that defective interaction among these components will not yield infectious particles. However, although these two steps are obligatory, they alone may not be sufficient to form and release infectious virus particles. Therefore, virus components may be directed correctly to the assembly site and then they interact with each other to form virus particles, yet infectious viruses may not be released into the medium. For example, abortive virus morphogenesis in HeLa (human cervical carcinoma) cells infected with influenza viruses has been observed where virus particles are formed at the plasma membrane but not released (Gujuluva et al., 1994). Therefore, there are other factors regulating the pinching-off process causing bud release.

In addition, with influenza viruses, correct assembly of multiple vRNA/vRNP segments (eight separate segments for *Influenzavirus A and B* and seven segments for *Influenzavirus C*) will be required for incorporation into each infectious virus particle. Although packaging of different RNP segments in the virus bud is absolutely essential for infectivity of virus particle, assembly or incorporation of all eight RNA genomic segments is not critical for budding and bud release of virus particles. However, since infectivity of a virus particle depends on the correct incorporation of each vRNA segment, it becomes important to determine how all vRNA segments are selectively incorporated into infectious virions. Two models have been proposed for the incorporation of eight vRNA/vRNP segments into virions: "random packaging" and "specific packaging." The "random packaging" model predicts the presence of common structural elements in all vRNPs, causing them to be incorporated randomly into virions, and therefore incorporation of vRNPs will be concentration dependent. Support for this model comes from the observation that influenza A virions can possess more than eight vRNPs (9–11 vRNAs per virion) (Bancroft and Parslow, 2002; Enami et al., 1991), and at most 1 in 10 or more virus particles are infectious. On the other hand, the "specific packaging" model assumes that spe-

cific structural features are present in each vRNA/vRNP segment, enabling them to be selectively incorporated into virions. Evidence for this model is deduced mainly from the finding that the various vRNAs are equimolar within viral particles even though their concentrations in infected cells may vary (Smith and Hay, 1982). The selective packaging model has been favored by the earlier studies demonstrating that the small DI (also called von Magnus particles) vRNAs can competitively inhibit the packaging of their normal counterparts but not that of other vRNAs (Duhaut and McCauley, 1996; Nakajima et al., 1979; Nayak et al., 1985, 1989; Odagiri and Tobita, 1990). DI RNAs are smaller internally deleted viral RNA segments. They possess all the structural features of the wild-type viral RNA segments for replication and incorporation into virus particles. They selectively replace their progenitor viral RNA in virions. Recent studies have demonstrated the presence of segment-specific packaging signal (s) in 3′- and 5′-UTR as well as adjacent coding regions (varying with both specific RNA segment and 3′ or 5′ ends). Specific packaging signals are found for all eight RNA segments (Watanabe et al., 2003; Fujii et al., 2003, 2005; Liang et al., 2005; Muramoto et al., 2006; Ozawa et al., 2007) and incorporation of some specific RNA segments is critical for the incorporation of other RNA segments (Muramoto et al., 2006; Marsh et al., 2008).

ET studies of serially sectioned *Influenzavirus A* particles have shown that the RNPs of influenza A virus are organized in a distinct pattern (seven segments of different lengths surrounding a central segment). This finding argues against random incorporation of RNPs into virions and supports the "specific packaging" model (Noda et al., 2006). Such a model would require that specific vRNA–vRNA interaction among the specific eight vRNP segments should form multisegmental vRNP macromolecules prior to or during incorporation into virus particles and that these large vRNP complexes containing eight unique vRNPs should be stable. However, such intracytoplasmic multi-RNA/RNP complexes have not yet been demonstrated. More important, bud closure and virus release should not occur until such vRNP complexes containing eight specific vRNP segments are formed and incorporated in the bud. In support of this model, Fujii et al. (2003) demonstrated that the efficiency of infectious virion production correlated with the number of different vRNA segments. They observed that the higher the number of different vRNA segments, the higher was the efficiency of virion production. Recently, specific nucleotide residues in 3′ and 5′ ends (coding and noncoding) of PB1, PB2, and PA (Liang et al., 2008; Marsh et al., 2008), as well as in HA (Marsh et al., 2007), have been further shown to play a critical role for packaging of specific vRNA segment into progeny virions. The major weakness of this model is that bud closure and virus release do not appear to depend on the incorporation of eight specific RNA segments and particles with fewer RNP segments are found. Therefore, it is possible that segment-specific complex formation and incorporation of viral RNA may occur but may not affect bud closing and bud release.

3.5.5 Budding Process

For enveloped viruses, budding of infectious virus particles requires that all structural viral components be brought to the budding site for assembly and incorporation into the virus particles. For influenza viruses that selectively bud from the apical domain of polarized epithelial cells, all viral components must be brought to the budding site at the apical plasma membrane, and highly specific interactions are required both prior and subsequent to their arrival in order to achieve successful assembly. As mentioned earlier, transporting viral components to the budding site requires involvement of the exocytic pathway and its components. Similarly, during the assembly process, multiple cellular components including actin microfilaments and lipid rafts also play critical roles in concentrating the viral components and providing a favorable environment for their interaction and the progressive formation of higher order

subviral complexes. The final budding process itself requires three major steps: bud initiation, bud growth, and bud completion, including releasing of the virus from the host cell membrane. Each of these steps involves interaction of multiple host and viral components.

3.5.5.1 Bud Initiation Bud initiation requires outward bending of the plasma membrane and involves transition of a more planar membrane structure to a curved structure at the budding site. Although the structural nature and biochemical properties as well as the physical forces at these sites responsible for membrane bending and bud initiation are unknown, it is likely that both lipid rafts and raft-associated proteins present at the budding site play an important role in causing membrane curvature and bud initiation. Lipid rafts producing asymmetry in lipid bilayers can cause intrinsic curvature of one lipid monolayer relative to the other monolayer leading to membrane bending (Nayak and Hui, 2004). Membrane deformation can be caused by selective transfer of lipids between the lipid bilayers, interaction of cholesterol with the budding leaflet, and hydrolytic cleavage of phosphocholine head groups of sphingomyelin by sphingomyelinase generating smaller head groups (Holopainen et al., 2000). In addition, BAR (Bin/amphiphysin/Rsv) domain is shown to cause membrane curvature (Peter et al., 2004) and is known to be present in a number of proteins involved in vesicle formation and recycling. (BAR domains are helical domains found in proteins involved in vesiculation processes including endocytosis, intracellular trafficking, budding, and so on that require membrane bending. BAR domains interact with endocytic and cytoskeletal machinery including GTPase, dynamin and possess dimerization motifs sensing and inducing membrane curvature. BAR domain containing proteins include endophilins, GTPase activating proteins, amphiphysin, arfaptin, and others.) However, the specific role of any of these host proteins in influenza virus budding largely remains unknown. In addition to lipid raft microdomains, accumulation of viral proteins including HA, NA, and M2 on the outer side of membrane and M1 proteins on the inner leaflet of the membrane plays a critical role in further facilitating the membrane bending at the budding site (Nayak and Hui, 2004). Among these, M1 interacting with the inner leaflet of lipid bilayers is likely to play a major role in bud initiation. Currently, we do know that clustering of M1 owing to M1/M1 interaction underneath the lipid bilayers can cause outward membrane bending and bud initiation.

3.5.5.2 Bud Growth Bud growth leading to bud maturation is the intermediate stage between the bud initiation and the bud release. Bud growth determines the size and the morphology of released virus particles. However, what factors or forces determine and regulate bud growth remains unclear. For most viruses, regardless of whether they contain icosahedral (e.g., SFV) or helical (e.g., VSV) nucleocapsids, the size of the nucleocapsids determines the size of the virions. However, there is room for variability in virion size. For example, influenza viruses are highly pleomorphic and the size of the released particles can vary from spheroidal to elongated and even filamentous (Figures 3.4 and 3.5), and the content of the nucleocapsids is not the major factor for bud growth. Influenza virus bud growth rather appears to depend on two forces, pulling and pushing. The pulling force is primarily provided by the transmembrane proteins along with M1 that pull nucleocapsids into the bud. On the other hand, the host cortical actin microfilaments that bind to viral RNPs provide the pushing force for incorporating the nucleocapsids and M1 into the bud. Electron tomography analysis of virus buds attached to the cell surface shows that helical nucleocapsids are oriented perpendicularly to the cell membrane while being incorporated into the buds and that buds essentially complete and still remaining attached to the cell membrane are of similar size (Figures 3.5 and 3.21).

As mentioned earlier, influenza virus particles are highly pleomorphic in shape and size

FIGURE 3.21 Virus buds at the cell surface by cryo-EM. At 12 hpi, WSN-infected MDCK cells were processed for thin section and examined by cryotomography. This picture represents one slice through inner core of the virus buds. One can see the parallel arrangement of the vRNPs inside the bud perpendicular to cell surface. The bud neck (⇒) shows gaps indicating possible absence of M1. HA and NA spikes are seen on the bud envelope. Reproduced from Nayak et al. (2009).

(Calder et al., 2010). Basically, there are two types of pleomorphism observed among influenza viruses: (1) strain specific, that is, strain to strain variation that may also vary depending on the host cell and (2) variation within the population of plaque purified virus in the same cell. Clearly, the genome of the virus strain is an important factor in determining the particle size and shape of a specific virus strain (e.g., Udorn versus WSN, both of which are antigenic variants of influenza A virus, belonging to the genus *Influenzavirus A*). Specific viral genes involved in determining filamentous versus spheroidal forms have been identified. Similarly, the roles of polarized epithelial cells and intact actin microfilaments were found to be critical in maintaining the filamentous form of Udorn virus. However, the cause of pleomorphism in plaque purified influenza viruses is not well understood. Whatever may be the viral and cellular factors involved in viral pleomorphism, these are likely to affect bud growth and closing and will eventually affect the shape and size of the virus particles. This is not to state that factors affecting bud closing will always affect bud size and bud shape; however, factors affecting bud shape and bud size will always affect bud closing. The viral and host factors affecting the size of virus particles will hinder or facilitate bud closing.

3.5.5.3 Bud Closing Bud closing is the final step for the scission of the bud and release of the virus particle into the outer environment. Bud closure would involve fusion of two ends of the apposing viral membranes as well as that of the apposing cell membranes leading to fission of the virus bud from the infected cell membrane (Figures 3.11c and 3.22). This would require bringing and holding the apposing membrane ends next to each other in close proximity, so that each end can find its counterpart causing fusion of corresponding lipid bilayers. Virus buds would then become separated from the membrane of the parent-infected cell. This model holds that two lipid bilayers are to be held in very close proximity for fusion to occur. Host and viral factors could have both positive and negative impact on bud release.

Some factors could interfere in bringing the apposing ends close to each other and therefore should be removed. Host factors such as actins and lipid rafts may interfere with bud release. Cellular actin microfilaments pushing the viral RNP into the bud may interfere with the final step of bud closing, and actin depolymerization is known to facilitate bud release. Other host factors could help in bringing and holding the membrane ends close to each other for fusion to occur and therefore should be brought to the pinching-off site. On the other hand, a number

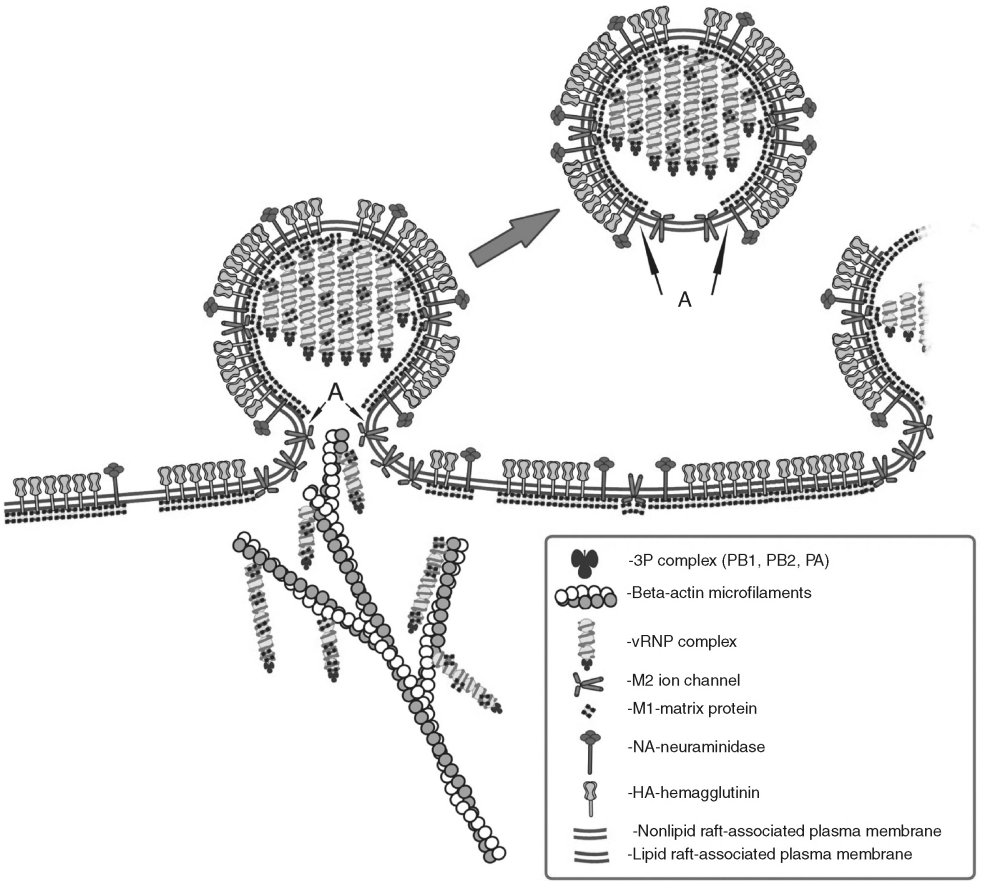


FIGURE 3.22 Schematic illustration of the pinching-off process of influenza virus bud. The pinching-off region (neck) is shown to be viral membrane devoid of lipid rafts (Barman and Nayak, 2007), devoid of HA and NA spikes outside and M1 inside the lipid bilayers (Harris et al., 2006), and may contain M2 (Schroeder et al., 2005). Reproduced from Nayak et al. (2009). (*See the color version of this figure in Color Plate section.*)

of host components have been shown to activate bud release. For example, VPS (vesicular protein sorting) components are shown to facilitate bud release of HIV and other enveloped viruses. Bud scission of these viruses depends on the interaction of their L domain(s) with the components(s) of VPS pathways involved in giving rise to multivesicular bodies (MVBs). Tsg101 and AIP1/Alix, the components of ESCRT (endosomal sorting complex required for transport), interact with L domains and require the function of AAA-ATPase of Vps4 for bud release of HIV (Fujii et al., 2007; Demirov and Freed, 2004). However, some other type of action must occur in the case of orthomyxoviruses, since influenza virus proteins do not contain any identifiable L domain (s) and influenza virus budding is not affected by dominant Vps4 (Chen et al., 2007) or proteasome inhibitors (Hui and Nayak, 2001; Khor et al., 2003).

Among the orthomyxoviral components, three viral proteins, namely, NA, M2, and M1, have been shown to play critical roles in both virus morphogenesis and bud release of influenza viruses. Specific NA mutants in TMD and CT (cytoplasmic tail) affected virus morphology, generating elongated virus buds (Jin

et al., 1997; Barman et al., 2004). Similarly, some M1 mutants produced elongated virus particles indicating the involvement of M1 in the last step of bud release (Burleigh et al., 2005; Nayak et al., 2004). Complete or partial deletion of the WSN M2 tail was also shown to cause attenuation of virus growth and to produce elongated or even filamentous particles in some mutants, indicating an important role of the M2 tail in viral assembly and morphogenesis (Iwatsuki-Horimoto et al., 2006) and also suggesting that the M2 tail affected particle release in VLP (virus-like particle) assay and affected viral morphology (Chen et al., 2008). VLPs are virus-like particles possessing virus-like morphology, but are noninfectious because they do not contain any viral genetic material (DNA or RNA). VLPs are produced by expression and self-assembly of viral structural proteins in a variety of cell culture systems, including mammalian cell lines, insect cell lines, yeast, and plant cells.

As mentioned previously, lipid rafts and cortical actin microfilaments, though critical in many aspects of the budding process, are inhibitory in the final step of bud closing and therefore should be removed from the pinching-off site. Clearly, the role of specific host factors varies in the bud release of different enveloped viruses. Although some host factors critical for bud release of HIV and other enveloped viruses are identified, other host factors required for bringing and holding the apposing viral and cellular membranes next to each other for facilitating fusion and fission are yet to be identified. Among the better understood viral components, M2 may play a critical role in the pinching-off process of influenza viruses. M2 when present in the neck of the bud may aid in bud release (Schroeder et al., 2005) by bringing together lipid microdomains that are devoid of lipid rafts in this region (Figure 3.22). Absence of M1 protein underneath the lipid bilayers and absence of spikes on the outer surface may indicate the absence of lipid rafts (Figures 3.3 and 3.4). From CT analysis, such lipid microdomains are proposed to be the preferred sites for the bud pinching off (Harris et al., 2006).

As mentioned previously, bud closing for influenza viruses is very inefficient and only a small fraction of virus buds are released, while the majority of virus buds remain attached to the cell membrane even though they appear mature (Figures 3.5 and 3.21). Both host and virus factors may be contributing toward the rate-limiting step of the pinching-off process. Influenza virus budding also appears to be an active, energy-dependent process and metabolic inhibitors, such as antimycin A (AmA), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP), and oligomycin that prevent the synthesis of ATP, and ATP analogues, such as ATPyS (adenosine 5′-*O*-(3-thiotriphosphate) and AMP-PNP (5′-adenylylimidodiphosphate), are shown to inhibit influenza virus budding (Hui and Nayak, 2001). Therefore, limited energy at the end of infectious cycle may be a factor for the inefficient release of virus particles. Among other host factors, actin microfilaments may interfere with bud closing, and, conversely, disassembly of cortical actin microfilaments may facilitate it. This notion is supported by several observations, including the release of virus particles in abortively infected HeLa cells (Gujuluva et al., 1994), conversion of filamentous Udorn (H3N2) virus to spherical virus, and enhanced release of WSN and PR8 (antigenic variants of influenza A virus) spherical particles in polarized MDCK cells (Roberts and Compans, 1998; Simpson-Holley et al., 2002) by microfilament-disrupting agents.

Scission of influenza virus buds from infected cells is the last step in the completion of virus life cycle. This step appears to be rate limiting and morphological analysis by thin section transmission microscopy (Barman and Nayak, unpublished), scanning electron microscopy (Figure 3.5), and cryotomography (CT) (Figure 3.21) shows that a large number of mature virus particles remain attached to the cell membrane and only a relatively small fraction of virus buds (∼10%) are released. The kinetics of virus release relative to the presence of virus buds awaits further investi-

gation in order to understand the cause(s) and mechanism(s) for such inefficient bud completion.

3.5.6 Role of Viral Budding in Viral Pathogenesis

Since the host is usually infected at very low MOI and the severity of the disease syndrome caused by lytic viruses largely depends on the number of the cells of the affected organs (or tissues) killed by the infecting virus, factors contributing to productive replication, release of infectious progeny virus, virus yield, and budding will have a major role in the development and severity of disease production. In addition, the site of budding can be an important contributory factor in viral pathogenesis, particularly for such respiratory viruses as influenza and Sendai viruses. The influenza and Sendai viruses bud from the apical surface of polarized epithelial cells (e.g., bronchial epithelial cells) into the lumen of the lungs and are therefore usually pneumotropic, that is, restricted to the lungs, and do not cause viremia or invade other internal organs. However, occasionally some influenza viruses such as the fowl plague viruses (H5 or H7, both being antigenic variants of influenza A virus, with the designation of H5 or H7 indicating the hemagglutinin subtype specificity) and WSN (H1N1) virus are not restricted to the lungs and produce viremia infecting other internal organs (pantropism) and cause a high degree of mortality in infected animals. The viruses restricted to lungs are called "pneumotropic," whereas the viruses that cause viremia and spread to other internal organs are called "pantropic." In humans, most of the influenza viruses are pneumotropic and do not spread to other internal organs. However, it is not clear whether the Spanish influenza of 1918, the most devastating influenza pandemic in recorded human history that killed 20–40 million people worldwide, particularly affecting young healthy adults, was only pneumotropic, that is, restricted in lungs or also pantropic and invaded other internal organs. During 1918, some people died due to influenza pandemic, in addition to pneumonia, showing evidence of massive pulmonary hemorrhage and edema (Taubenberger, 1998). The 1918 flu virus, like fowl plague virus, may have caused viremia and infected other organs. Therefore, it is possible that 1918 highly virulent viruses were not restricted to lungs in chicken or humans. In recent years H5N1, the Hong Kong chicken influenza virus, which is extremely virulent and pantropic for chicken, causing viremia and spreading to other internal organs, also caused high morbidity and mortality in infected humans. The majority of H5N1-infected people exhibited clinical pneumonia (or acute respiratory distress syndrome), gastrointestinal symptoms, and impaired hepatic and renal function and therefore exhibited pantropic characteristics in humans. However, since this virus did not spread from human to human, it did not emerge as a major pandemic. On the other hand, unlike the avian H5N1 virus, the recent H1N1 swine influenza virus spread efficiently among humans and developed as a pandemic. Luckily, H1N1 swine flu virus was mostly pneumotropic and caused only a moderate pandemic. Why some influenza viruses are pneumotropic and others are pantropic is an important question for predicting the outcome of a major influenza epidemic or pandemic.

The severity of viral pathogenesis depends on both viral and host factors, including host immunity and cytokine production. The virulence determinants of influenza viruses are complex and multigenic. However, one factor that is thought to be critical in viral growth and virulence is the cleavability of HA into HA1 and HA2. Influenza virus is normally restricted to the lungs because its HA can be cleaved by tryptase Clara, a serine protease restricted to the lungs. However, some HA variants containing multiple basic amino acids at the HA1–HA2 junction, found only in H5 and H7 avian subtypes, can be cleaved by furin and subtilisin-type enzymes that are present ubiquitously throughout the body, enabling such viruses to grow in other organs and possibly contributing to pantropism. In

addition, the NA of some influenza viruses (e.g., WSN virus) binds to plasminogen and activates its conversion into plasmin in the vicinity of HA, and the activated plasmin cleaves HA into HA1 and HA2, rendering the virus infectious. This, therefore, enables viruses such as WSN, which lack multiple basic residues in its HA, to grow and multiply in tissues other than the lungs.

However, although the cleavage of HA into HA1 and HA2 is a major virulence factor, it is not the only factor contributing to the pantropism of a normally pneumotropic flu virus. For example, although WSN virus is pantropic and neurovirulent in the mouse, gene reassortment experiments demonstrated that the WSN NA gene responsible for the cleavage of HA was not sufficient for neurovirulence in chickens or mice. Other WSN genes, such as the M and NS genes, in addition to the NA gene, were required for neurovirulence and, therefore, likely affected pantropism. The function of M and NS genes in neurovirulence is not known. The M gene in Sendai virus has been shown to affect apical versus basolateral budding and contribute to the pantropism of F1-R Sendai virus mutant (Tashiro and Seto, 1997). Therefore, it is possible that, in addition to increased cleavability of HA, the pantropic virus causes alteration in apical budding, releasing more virus basolaterally. Since blood vessels are proximal to the basolateral surface of cells, basolateral budding would facilitate more viruses entering into the blood, causing viremia, and invading other internal organs. Therefore, pantropic influenza viruses such as WSN/33 virus or highly virulent Hong Kong H5N1 and H7N1 fowl plague viruses may also cause altered budding from apical and basolateral surfaces. Thus, altered budding may be considered an important trait for the virulence of a specific strain of influenza virus. However, the role of the altered budding in influenza virus pathogenesis remains to be determined.

Sendai virus, like influenza virus, is a pneumotropic mouse virus that buds apically. However, a Sendai virus mutant F1-R that exhibited pantropism possessed two potentially important characteristics (Tashiro and Seto, 1997): (1) ubiquitous cleavage mutation of F into F1 and F2 due to the presence of multiple basic residues and (2) altered budding from both apical and basolateral surfaces. Contrastingly, the Sendai virus mutants that exhibited only one of these two traits, either cleavage of F into F1 and F2 or altered budding, did not cause viremia or pantropism in the mouse. This would also support the argument that altered budding may be a factor that facilitates viremia and pantropism. Therefore, altered apical versus basolateral budding could be an important factor in the release of virus into the blood, invasion of internal organs, pantropism, and higher virulence of a specific virus strain.

3.6 CONCLUSIONS

The replication and morphogenesis processes of viruses are different from those of prokaryotic or eukaryotic organisms. In this chapter, some of the general steps involved in the viral infectious cycle, including entry, uncoating, transcription, translation, replication, and assembly processes and the possible role of budding in viral pathogenesis have been presented. Of these, viral morphogenesis is the most obscure phase in the virus life cycle. Yet knowledge of how the particles are formed during this morphogenetic stage is fundamental to understanding virus growth and multiplication and, therefore, is crucial in defining viral infectivity, transmission, virulence, tissue tropism, host specificity, and pathogenesis and contributes to an overall understanding of the disease process and progression of disease, including host morbidity and mortality. In addition, the site of budding can affect virus virulence and pathogenesis. Elucidation of the viral replication and assembly processes is critical in terms of enabling us to find ways to block these steps and thereby intervene in the viral life cycle and disease process. Much remains to be done to achieve these necessary research goals, particularly in terms of elucidating those stages of the viral assembly process that relate to how viral

components are brought to the assembly site, how those components interact with each other at the assembly site, and how viral budding actually occurs. A better understanding of viral replication and morphogenesis may lead us to develop novel therapeutic agents capable of interfering with these critical steps in viral multiplication, pathogenesis, and virulence.

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ABBREVIATIONS AND DEFINITIONS

Ambisense RNA	These RNAs are of partly positive-sense and partly negative-sense polarity.
ATPyS	(adenosine 5′-*O*-(3-thiotriphosphate) and AMP-PNP (5′-adenylylimidodiphosphate) are ATP analogues.
BAR (Bin–amphiphysin–Rvs) domains	BAR domains are helical domains found in proteins involved in vesiculation processes including endocytosis, intracellular trafficking, budding, and so on that require membrane bending. BAR domains interact with endocytic and cytoskeletal machinery including GTPase dynamin and possess dimerization motifs sensing and inducing membrane curvature. BAR domain proteins include endophilins, GTPase activating proteins, amphiphysin, Arfaptin, and so on.
Capsid coat or shell	The protein shell in contact with or directly surrounding the viral nucleic acid (genome).
CAP	The 5′-cap is found on the 5′-end of a eukaryotic mRNA molecule with the exception of some viral RNAs and consists of an altered guanine nucleotide connected to the mRNA via an unusual 5′- to 5′-triphosphate linkage. This guanosine is methylated on the 7 position and is referred to as a 7-methylguanosine cap, abbreviated m^7G. The 5′-cap of mRNA facilitates nuclear export, prevents mRNA degradation by exonucleases, and promotes ribosome binding and protein translation.
Capsomeres	These are morphological units that form capsids. Capsomeres consist of oligomers of one or more viral proteins.
CPE	Cytopathic effect; could be due to apoptosis, necrosis, or syncytium formation.
cRNA	Full-length plus-strand template RNA complementary to the minus-strand genomic RNA.
DI	Defective interfering viruses. DI virus particles contain a smaller viral genome, are noninfectious, and need the help of infec-

tious (wild-type) virus for replication but, in turn, interfere with replication of homologous infectious (standard) viruses.

Dominant negative — A mutation whose gene product interacts with the intracellular components as the wild-type gene product and thereby adversely affects the function of normal, wild-type gene product within the same cell.

Ectodomain — The portion of the transmembrane protein that remains exposed outside the cell or virus particle.

EIS — These are the *cis* elements of the unsegmented minus-strand RNA genome (e.g., VSV). "E" denotes the end of transcription termination and polyadenylation sequence; "I" stands for intragenic sequence not transcribed in messenger RNA (mRNA); "S" indicates the start sequence for the next mRNA.

Endocytic pathways (endocytosis) — The process of internalization of external macromolecules or viruses, which involves specific binding to cell surface receptors. Viruses use this mechanism to enter into host cells. In this process, clathrin-coated vesicles and subcellular organelles such as endosomes and lysosomes are involved.

Envelope — The viral membrane containing the lipid bilayer and associated proteins that surround the nucleocapsid of enveloped viruses and form the outermost barrier of the enveloped virus particle.

Enveloped viruses — Viruses that possess an envelope or membrane surrounding the nucleocapsid. For enveloped viruses, the naked nucleocapsid is not infectious.

Episomal, extrachromosomal — The state of existence of nucleic acid molecules that do not became integrated into host cell chromosomes. They exist and multiply independently within the cell nucleus or cell cytoplasm.

Escape mutants — Virus mutants that are not neutralized by antibodies. These viruses possess amino acid change in the epitope and therefore no longer bind to the neutralizing antibody.

Exocytic pathways (exocytosis) — The mechanism for transporting intracellular transmembrane or secretory proteins from intracellular compartments to the cell surface or extracellular environment. In this process, various subcellular compartments, such as endoplasmic reticulum and Golgi complexes, are involved in protein transport.

Genome — The complete genetic information (DNA or RNA) of an organism.

Glycosylation — In this process, one or several carbohydrate groups

are attached to proteins during their transport through the exocytic pathways. Sugar residues are attached at specific sites to amino acids such as serine or threonine (for O-linked) or asparagine (for N-linked) carbohydrate moieties. These carbohydrate moieties are also called glycans.

HBV Hepatitis B virus (family Hepadnaviridae, genus *Orthohepadnavirus*).

Helical capsids These structures are spiral, spring-like, and flexible rods. The RNA genome in a helical capsid is exposed (influenza viruses) or enclosed (paramyxoviruses, rhabdoviruses) by the nucleoprotein molecules constituting the nucleocapsid.

H1N1 Antigenic variant of influenza A virus (family Orthomyxoviridae, genus *Influenzavirus A*). H denotes hemagglutinin (H1–H15) and N stands for neuraminidase (N1–N9) subtypes.

HIV Human immunodeficiency virus 1 and 2 (family Retroviridae, genus *Lentivirus*).

hnRNA Heterogeneous nuclear RNA (hnRNA), also called pre-mRNA or immature mRNA, is an incompletely processed single strand of ribonucleic acid (RNA) present in the nucleus. It contains introns and exons and is processed by splicing to eliminate introns and become mature mRNA that can be translated into protein.

HSV Human herpesvirus 1 and 2 (family Herpesviridae, genus *Simplexvirus*).

HRV14 Human rhinovirus strain 14 (antigenic variant of human rhinovirus B, family Picornaviridae, genus *Enterovirus*)

ICAM-1 Intracellular adhesion molecule-1, the receptor for rhinoviruses.

Icosahedron, icosadeltahedron, icosahedral symmetry, icosahedral capsids Icosahedron is a structure with a two-, three-, and fivefold rotational symmetry. It is a polyhedron with 20 faces, 12 vertices, and 30 edges. Most icosahedral viruses have 60 (multiple of 60) subunits (e.g., polioviruses, togaviruses).

Inclusion bodies Microscopic structures, produced in some virus-infected cells consisting of viral proteins, nucleic acids, and cellular elements (particularly cytoskeletal elements). Inclusion bodies can be intranuclear (herpesviruses) and intracytoplasmic (paramyxoviruses).

Kozak's rule Most eukaryotic mRNAs contain a short recognition sequence (ACCATGG) that facilitates binding of mRNA to the small subunit of the ribosome and initiation of protein translation (Kozak, 1986).

LB	Lateral bodies found in poxviruses.
LCMV	Lymphocytic choriomeningitis virus (family Arenaviridae, genus *Arenavirus*).
Lipid rafts	These are lipid microdomains containing increased levels of glycosphingolipids, including sphingomyelins, cholesterol, and long saturated fatty acids but decreased phosphatidylcholines. These specialized membrane microdomains are relatively resistant to nonionic detergents, such as Triton X-100 or Brij-98, at low temperatures (e.g., 4°C). These specialized membrane microdomains play important roles in the assembly of signaling molecules, influence membrane fluidity and membrane protein trafficking, and regulate neurotransmission and receptor trafficking, virus assembly, and virus budding. Lipid rafts are more ordered and tightly packed compared to the surrounding nonraft lipid bilayer.
LT	Large T antigen of SV40.
MOI	Multiplicity of infection, that is, infectious units adsorbed per cell.
Naked or nonenveloped viruses	These viruses do not have any membrane and the nucleocapsids represent the infectious virus.
Nucleocapsid	The complete nucleic acid–protein complex of a virus particle. Sometimes the term viral ribonucleoprotein (vRNP) is used to indicate nucleocapsid (e.g., vRNP of influenza viruses).
Panhandle	A circular nucleic acid structure of single-stranded (ss) DNA or RNA with a double-stranded stem at the end produced by intrastrand hybridization due to partial complementarity of the nucleic acid sequences at both the 5′ and 3′ termini of ssRNA or DNA. The panhandle structures function as the promoter and are important for transcription and replication.
pfu	Plaque-forming unit.
Phagocytosis, viropexis	Uptake of particles by cells not totally dependent on receptor-mediated endocytosis. The particle on the surface is engulfed by the cell membrane into a phagocytic vesicle. These phagocytic vesicles then undergo similar changes as the endosome. Poxviruses enter cells by phagocytosis.
Poly(A)	Polyadenylation at the 3′-end of an RNA molecule.
Prions	These are infectious protein molecules without any DNA or RNA and thought to cause transmissible and/or inherited neurodegenerative diseases known as transmissible spongiform encephalopathies. These include Creutzfeldt–Jakob disease, kuru, and Gerstmann–Straussler syndrome in humans, as well as scrapie in sheep

and goats and mad cow disease in cattle. The infectious prion proteins are modified forms of normal proteins encoded by a host gene. The normal prion protein having alpha helices in its secondary structure is converted into beta sheets for the secondary structure in diseased animals.

Protomer — The term often used to indicate a structural unit containing one or more nonidentical protein subunits. Promoters are used as a building block for virus capsid assembly.

RDRP — RNA-dependent RNA polymerase, also called RNA transcriptase and RNA replicase.

RNA of positive and negative polarity — The RNA strand of the same polarity as the mRNA-encoding proteins and is called positive-, plus-, or (+) strand RNA. When the RNA is of polarity opposite to the mRNA (i.e., cannot code for a protein), it is called negative-, minus-, or (–) strand RNA.

RNP — Ribonucleoprotein. Viral nucleoprotein binding to vRNA is called vRNP.

RT — Reverse transcriptase, RNA-dependent DNA polymerase.

ST — Small T antigen of SV40.

Structural and nonstructural proteins — Structural proteins are those proteins that are found in virions as components of capsid or envelope. Nonstructural proteins are those virally encoded proteins that are produced in the infected cells but not found in virions. Nonstructural proteins are usually catalytic and regulatory in nature and are also involved in modifying host functions.

Synchronous infection — When all cells in the culture are infected simultaneously. Cells are infected at a high MOI (>5) and at low temperatures (4°C). Then the temperature is raised to 37°C to permit entry and uncoating of all cell-bound viruses at the same time.

Syncytium (multinucleated giant cells) — Cells possessing multiple nuclei are formed due to fusion among a number of cells. Usually, viruses that can undergo fusion at a neutral pH (paramyxoviruses, retroviruses) produce syncytium.

Temperature-sensitive (ts) mutant — A mutant virus that will replicate at a permissive (low) temperature but not at the nonpermissive or restrictive (high) temperature. This phenotype is usually caused by missense mutations of one or more nucleotides, causing alteration of amino acid(s) of a protein that cannot assume the functional configuration at the

	nonpermissive (restrictive) temperature.
TGN	Trans-Golgi network.
Transmembrane proteins	These are membrane proteins that are anchored to the membrane by spanning the lipid bilayer of the membrane via transmembrane domains. These proteins can be classified as type I (e.g., influenza virus HA), type II (e.g., influenza virus NA), type III (e.g., influenza virus M2), or complex (e.g., coronaviral E1) depending on the orientation of the NH_2 and COOH termini (type I, II, or III), cleavage of signal peptide (type I), and multiple transmembrane spanning domains (complex).
Virion	The entire virus particle. It usually refers to infectious or complete virus particle as opposed to noninfectious or defective virus particles.
Viroids	These are small, circular, single-stranded infectious RNA molecules without a protein coat or capsid and cause a number of plant diseases, including potato spindle tuber disease, cucumber pale fruit disease, citrus exocortis disease, cadang-cadang (coconuts) disease, and so on.
VLPs or virus-like particles	These possess virus-like morphology but are noninfectious because they do not contain any viral genetic material (DNA or RNA). VLPs are produced by expression of viral structural proteins, such as envelope or capsid proteins, in a variety of cell culture systems, including mammalian cell lines, insect cell lines, yeast, and plant cells. VLPs are produced from a wide variety of virus families, including Orthomyxoviridae (influenza virus), Parvoviridae (e.g., adeno-associated virus), Retroviridae (e.g., HIV), Flaviviridae (e.g., hepatitis C virus), and so on. VLPs can be used as vaccine (e.g., hepatitis B virus, human papilloma virus) and as a delivery system for genes and therapeutics.
WSN/33 (H1N1)	A neurotropic variant of WS/33 (H1N1), a human influenza virus isolated in 1933 (Francis and Moore, 1940).

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CHAPTER 4

THE (CO)EVOLUTIONARY ECOLOGY OF VIRUSES

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CONTENTS

4.1 VIR-OLUTION: SETTING THE SCENE

There is much debate on the precise status of viruses: Can they be considered alive? Do they have a place on the tree of life? How long have they existed? Do they predate the first living cells? Should the different types of viruses really be considered under the same "virus" banner? Regardless of the answer to these questions, it is undeniable that, whatever their status, viruses have had and continue to have a profound influence on the composition and function of the planet's living biota (Villarreal and Witzany, 2010). By their very definition, viruses, as obligate intracellular parasites, manipulate and selfishly hijack their host organisms purely for their own survival. This in itself leads to an interesting paradox: any virus that is too successful will ultimately go extinct since it will have no host left to infect. This paradox has effectively led to the field of viral ecology whereby viruses and their hosts are in a continuous, yet hugely dynamic and intricate relationship. These complex relationships between hosts and their viruses are at least as old as life on Earth itself. Clearly, the roots run deep in viral family trees and their

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interaction with their host(s) will run just as deep. The diverse nature of viral genomic material betrays their multiple and ancient ancestral origins (i.e., single- versus double-stranded, RNA versus DNA genomes). This polyphyletic group consists of many distinct lineages with independent origins that are all grouped under the "virus" banner by virtue of their lifestyle. Thus, a comprehensive tome on the topic of virus–host coevolution would need to encompass such a wide range of systems of such varying nature that it would justify at least a book all to itself, not merely a chapter! For this reason, I will attempt to provide an overview of the issues and processes associated with virus–host coevolution, using specific examples wherever necessary to illustrate points, but retaining a more generalist approach to the topic. Working with viruses has taught us many things in the life sciences, chiefly expect the unexpected and that there are exceptions to every rule. With this in mind, I invite the reader to read on with an open mind, never take anything at face value, question all ideas and hypotheses herein, but most importantly retain your wonder and amazement at the sheer audacity and beauty of this truly wonderful group of selfish and uncompromising biological replicators!

4.2 THE OBSESSION WITH DEATH: MORTALITY FROM A VIRAL PERSPECTIVE

From our human-centric perspective, viruses are associated with illness, disease, and often death. Yet, from a viral perspective every infection ultimately ends in death: either of the host cell or of the virus itself. A key difference between multicellular (the so-called "complex") organisms and their unicellular counterparts is that a successful infection in a multicellular organism does not usually lead to the death of the entire organism. This applies to hosts at all levels of complexity from fungi to mammals and trees. A successful infection in a single-cell organism will always lead to an untimely death of that cell. A successful infection in a multicellular organism will lead to the death of some cells, but usually leave the remainder of the host intact. Indeed, despite some incredibly virulent viruses ultimately causing the total death of their multicellular hosts (and not just the subpopulation of cells they actually infect), rarely does the physical loss of the infected cells cause death: the mortality is usually a product of "particularly" nasty viral dispersal mechanisms such as hemorrhage and diarrhea that are induced to aid the transfer of the virus to new hosts. A multicellular host offers a unique environment to a virus: a homogeneous population of cells within a contained system. Although a classic viral infection (e.g., by the influenza viruses, members of the family Orthomyxoviridae) is usually regarded by the patient as a single infection, the symptoms observed are actually a product of thousands of cells being infected. If a virus can successfully infect one type of cell within an organism, there is usually no reason for viral progeny to subsequently infect every other identical cell type within the organism. Clearly, this would have disastrous consequences, and this is why multicellular organisms have evolved defensive strategies (such as immune systems) against such an occurrence. These systems can actively seek out and destroy both virus and infected cells to stop the infection from spreading out of control. When viruses attack the cells involved in these processes, such as in the case of HIV infection (human immunodeficiency virus 1 and 2, both of the family Retroviridae, genus *Lentivirus*), the results are catastrophic to the host concerned. Crucially though, it is not HIV infection *per se* that causes mortality in such cases, but the compromised immune system function (and development of AIDS) that leads to susceptibility to opportunistic infections (sometimes other viruses, but usually from the other domains of life such as bacteria, fungi, or protozoan) and tumor growth. Thus, death of a multicellular organism through viral infection should be regarded as an exception to the

rule. It is an unfortunate by-product of the viral infection of a subpopulation of cells within an organism. With the loss of their particular and specific function, goes the loss of whole organism integrity, leading to an untimely death.

It is important to realize that viral infection can be regarded on a cell-by-cell basis, regardless of whether that infection occurs to a single-cell organism or to a single cell *within* an organism. Viral infection is a fact of life. Despite our obsession with biology that is visible to the naked eye, it is a microbial/cellular world in which we live. In our oceans, virus-induced mortality is estimated to account for about 40% of the loss of microbial cells on a daily basis (Suttle, 2005). Microbial populations can withstand this sort of loss due to their rapid growth rates, a luxury not available to most complex multicellular organisms composed of a majority of cells that undergo irreversible differentiation and slower regeneration rates. Viruses that infect multicellular organisms are subjected to additional selection pressures that single-cell host viruses simply do not have to contend with.

4.3 A MARRIAGE MADE IN HELL

In dealing with the topic of viral ecology and evolution, we must always remember the polyphyletic nature displayed by viruses. The sheer wealth of diversity displayed by these "biological entities" makes any comprehensive study of the subject an almost impossible task. Yet, all viruses share one overarching property that defines them: they are entirely dependent upon the intracellular infection of their hosts for survival. This concept, despite the weird, wonderful and incredibly diverse strategies in which they act (and which you will read about within the pages of this book), binds all viruses together. The interaction between any virus and its host will be ingrained in the history of both lineages and, crucially, will have left and continues to leave, its mark on both host and virus. Put simply, the history of a host will help shape the future of both itself and any virus that infects it. Equally, this applies to a virus as well. Yet, even if a virus could have a memory of its illustrious past, it would have no care for this history. A virus, if it does "live" in the philosophical sense, lives only in the moment. At the population level, hosts and viruses are entwined in the closest marriage imaginable. It is far from a happy marriage though constant arguing ensues since only one partner (the virus) wants the marriage. Like all marriages, there are only two options available as a get-out clause: death (of either or both virus and host populations) or divorce. Ironically, divorce in this sense is always instigated by the virus (which wanted the marriage in the first place!), never the host and, crucially, the viral divorcee requires an immediate remarriage to whichever suitor (host) has turned its eye. Thus, from an evolutionary perspective, jumps by viruses across apparent species barriers (i.e., promiscuous extramarital activity) are mere examples of viruses taking advantage of an opportunity that has presented itself to them. If this new partnership is successful, it can be considered a divorce and immediate remarriage as the new selective pressures of interacting with a new system become applied. Crucially, the previous host will most likely still remain married to the original virus. The viruses really do have all the fun at their hosts' expense.

4.4 THE NUMBERS GAME

It is important to deal with the issue of species jumps early in this chapter since it is often the issue that most people mistakenly consider as the most important when thinking about viral ecology. For the vast majority of infections, a virus will infect a host that is similar to the host that it infected last. Despite the ease with which we mistakenly assign a conscious thought to the process, it is merely biochemical

interaction and compatibility between host and virus that will determine if an infection occurs or not. The last host a virus successfully infected is most likely the most compatible future host. An increase in the abundance of a viral population will lead to an increase in the occurrence of physical interactions with potential hosts. An increase in viral diversity will lead to an increase in potential biochemical compatibility following a successful physical interaction. It is purely about numbers in a relentless game of chance. Despite viruses being highly specific for their hosts, a virus has only to infect a single cell of another host successfully to begin the natural selection process for the new host. Such opportunistic infections are spontaneous and the selection pressures against viruses are so strong they often fail to become established in their new host. These are incredibly rare events in relation to "normal" viral infections, but surprisingly common due to the sheer number of infections that take place. A useful analogy can be taken from the aviation industry: aeroplane crashes are very rare because of stringent safety regulations; however, due to the high number of flights made on a daily basis, aeroplane crashes do occur frequently. Usually, cross species barrier infections are associated with increased virulence (as would be expected for a host exposed to a new virus) and typically generate a disproportionate amount of attention from virologists and the media. The expanding human population coupled with globalization (which itself increases the chance of viral infection from interactions between human viruses and humans) has led to intensive farming methods to meet the increasing food demand. Intensive farming is exactly this: large populations of quite often genetically homogeneous animals (and plants) in relatively small areas, increasing not only the potential for viral infection within the population but also the chances of transmission of "animal" viruses to humans. Transmission of plant viruses to humans is possible but more unlikely: a human is more similar to a pig or chicken than wheat. Thus, according to the numbers game that viruses play so well, it is inevitable that species jumps will occur. While they are usually unwelcome, they are also inevitable and a product of simple viral ecology.

4.5 FIGHT TO DEATH: GENES ARE THE WEAPONS

We have already begun to touch on many of the issues associated with viral ecology. However, we should not be fooled into thinking that any host actively welcomes viral infection; a virus cares little for its host and serves only its own selfish requirements. It is not a one-way battle, and no cell takes a viral infection lying down. Furthermore, despite the subject matter of this volume, the viruses and hosts themselves should not be considered the lowest common dominator in the study of viral ecology. It is the genes that reside within them that are the driving force behind organic life. Hosts and viruses are examples of groups of genes clubbing together (into genetic lineages) for mutual success. Over time, these genetic groupings become so established that genes work together to produce (as a by-product) the weird and wonderful forms of life we see today. The longer the genes coevolve in these groups, the stronger the dependency that develops within the groups. Over time, complex regulation systems and multilayered interaction networks evolve as systems become increasingly more complex. Out of chaos, comes order. Viruses (as simple but ordered systems) ruthlessly exploit their hosts' ordered systems for their own benefit.

The host will, of course, develop countermeasures to ensure that this fails to occur. It has nothing to lose (except cellular integrity that will be lost to the virus anyway) by throwing every biochemical trick it has up its sleeve at the virus. Thus, an infection can be regarded as a winner-takes-it-all conflict between a virus and a host. This battle takes place both internally and externally to the cellular environment

and provides scope and opportunity for natural selection and evolution to occur at a multitude of places.

This volume discusses such evolutionary selection. As stated previously, selection occurs at many different levels, each with widely varying degrees of pressure. Ultimately, these evolutionary processes are reflected in the genetic structure of both viruses and their hosts. Selection related to external environmental factors results in the *evolution* of systems in the classical sense, a process that all biological entities are subjected to (a host cell is an external factor to a virus and *vice versa* when no physical, biological, or chemical interaction is taking place between them). However, selection occurring within the biochemical components of the cellular environment when both host and virus genomes and their products are interacting directly can be considered a *coevolution* (Woolhouse et al., 2002), a process that symbiotic and parasitic organisms are subjected to and viruses take to the extreme by their very nature of being obligate intracellular parasites. Over the billions of years that organic life has been evolving on Earth, this has led the development of increasingly complex interactions between viruses and hosts. Let us not get distracted from the raison d'être of a virus' existence, which is to replicate at the expense of its host. Examples of beneficial effects to the host will be discussed within this chapter, but these examples are few and far between and could be considered accidents and quirks of biology. Quite simply, while a virus needs a host to replicate, no host ever relies on the virus that infects it. Hosts can survive without viruses, but the opposite is never true. Dinosaur viruses (and their genes) unable to infect other living organisms at the time of the alleged meteor strike went extinct alongside their dinosaur hosts.

Although viruses instigate the premature death of cells, this is not to say that viruses are not necessary to *support* life: without the constant virus-induced cellular mortality in our oceans in particular, entire ecosystems would undoubtedly collapse. A staggering 10^{23} viral infections are predicted to occur every second in our oceans, causing the constant cycling of nutrients through all trophic levels (Suttle, 2007). Quite simply, relentless and uncompromising viral infection should be considered the *status quo* in any system. Despite their small size, through their sheer abundance and activity viruses are the essential and unappreciated giants of the nutrient cycling realm. However, in addition, through their role as merciless predators, viruses play a crucial role in the natural selection and evolution of their hosts profoundly altering their appearance at the genomic, proteomic, and metabolic levels.

4.5.1 The Arms Race: Winner Takes it All in the Battle, But Not the War

Viruses are in a constant and ongoing battle with their hosts. Evolution by natural selection continually acts to tip the balance in favor of either host or virus. The direction and extent of this change is somewhat transient though, and depends on a plethora of variables that can change almost at will. Thus, a virus or host selected at one moment in time is by no means guaranteed to thrive under the next cycle of selection pressure. Yet, no host will ever take viral infection lying down, resistance mechanisms exist and are implemented ruthlessly. Indeed, there will usually be a natural population with an increased resilience and resistance that will be selected, thus tipping the balance, albeit momentarily, in the host's favor. It is crucial to remember though that population breeds disease: any resilient population that then flourishes has a greater chance of being destroyed by a future infection because it contains less diversity (having recently come through a selective bottleneck) compared to its abundance (Domingo et al., 1996).

Virulence leading to mass host death is no problem for a virus provided the host population is suitably abundant and future-proof. Virulence causing rapid decline in host population levels

in turn leads to decreased infection rates (there are fewer hosts to infect), thus allowing host population recovery. Crucially, this selection can occur only after the host population has been decimated. It is about survival into the future, yet this is selected only after a virus' short-term fate has been sealed.

The ecological dynamics within a host–virus system can have vastly different outcomes depending on whether the host is single or multicellular. The dynamics also differ dramatically depending on whether a lytic or lysogenic lifestyle is adopted (or something in between). Further complications arise when factors such as virus dispersal have to be considered and selected for, for example, in microbial systems (perhaps exemplified by the marine environment); this mechanism is fairly equal for all viruses, in terrestrial plant or animal systems the pressures are somewhat higher.

Thus, there are conflicting, yet complementary, aspects of virus ecology. This ongoing arms race of adaptation and counteradaptation has been described in the Red Queen hypothesis. Taken from Lewis Carroll's *Through the Looking-Glass, and What Alice Found There* (1871, by Charles Lutwidge Dodgson, Macmillan Publishers Ltd., London), "Now, here, you see, it takes all the running you can do to keep in the same place." Applied to host–parasite systems, it can be translated as "For an evolutionary system, continuing development is needed just in order to maintain its fitness relative to the systems it is co-evolving with" (van Valen, 1973).

4.5.2 The War of the (Viral) World: the Battlegrounds

Before discussing individual examples of interacting and coevolving systems, we must consider the entire landscape of host–virus systems and identify the main sites where the battles are fought. To this end, the virus life cycle can be broken into two main parts: time spent within a cell and time spent outside the cell; this roughly correlates with being metabolically active and metabolically inactive (gray areas exist of course: for example, often whole and intact viruses need to be internalized and targeted to specific intracellular locations prior to release of the genomic contents). Where selection occurs on the host or virus when the virus is not metabolically active and has yet to instigate any biochemical influence over its host cell, this leads to evolutionary change for both host and virus that can be considered almost independent of each other. However, once the virus is metabolically active or is at least interacting with its host (through binding to the surface), any selection that occurs (for host or virus) can be considered a truly coevolutionary process since the two systems are inherently, intrinsically, and undeniably biochemically linked as just one system. At this stage, there can be only one winner: host or virus.

4.5.3 Without a Cell: the Vulnerability of Being in Limbo

When not battling within a cell for survival, metabolically inactive viruses are left exposed to the environment. The environment in this context varies hugely depending on the nature of the virus and the type of host it infects. The infection of multicellular organisms can also create a suite of additional environmental conditions that must be overcome by viruses. For example, a virus such as influenza virus has to cope with both biotic and abiotic environments in between infection cycles. Successful infection and release of influenza viruses within the body places the virus directly in contact with the human internal environment (with its specific temperature, biochemical composition, and immune system). One good sneeze can then catapult the virus from this relatively homogeneous and safe environment, yet biologically harsh due to the actions of the immune system, into the outer world where factors such as temperature, pressure, and UV exposure vary dramatically and where they represent a nutritious food packet for all

manner of life forms. Crucially, when in this "limbo" state, viruses are completely at the mercy of whatever is thrown at them. Viral losses are thus nearly catastrophic at this stage and account for the large burst sizes observed when viruses infect cells (when one cell produces at least an order of magnitude more viruses, usually two to three orders more). However, the high wastage of viruses does ensure that only the most fit and robust viruses survive and ensures that evolutionary rates within viral populations are incredibly fast, far faster than in their hosts.

If we consider viral entry as the starting point in the infection process, there exists a range of strategies employed by viruses for ensuring their genomic material is safely delivered to the appropriate location. Binding to receptor sites (proteins, carbohydrates, lipids, and glycolipids) followed by injection of genomic material, absorption, and merging with the membrane, phagocytic engulfment, and many other mechanisms are all employed to pierce the hosts' outer armor. Even in the simple task of obtaining entry to the cell, there is enormous scope for evolutionary battle: modification of the receptors to avoid viral binding can ensure host resistance; conversely, modification of the viral binding receptor can ensure increased virus binding. It is these types of adaptation and subsequent counteradaptation that give rise to the previously mentioned Red Queen dynamics. Although cellular binding and internalization are essential for a successful infection under natural conditions, it can be bypassed in the laboratory with genetically compatible viruses that are "physically" incompatible; that is, the barrier represented by the cell surface only creates a physical spatial separation between where a virus is inactive and where it can safely and efficiently replicate. The artificial introduction of viruses to the intracellular regions of cells (effectively bypassing the membrane barrier) that under natural conditions are completely off limits by virtue of being "receptorless" often results in successful viral replication; for example, human poliovirus (human enterovirus C, family Picornaviridae, genus *Enterovirus*) can replicate happily when introduced artificially to the inside of mouse cells which, not having the CD155 receptor found in primates, would otherwise be resistant to infection (McLaren et al., 1959; Holland et al., 1959).

4.5.4 Within a Cell: Out of the Pan, Into the Fire

Before viral takeover, the host cell can represent the most hostile environment that a virus will encounter in its life cycle. Viruses suddenly become a huge threat to the long-term survival of a cell once they have breached the outer surface and find themselves inside the cell. Outside the cell, threats to a virus are almost all random and nonspecific. A slight exception to this are multicellular organisms with innate and adaptive immune responses, even though the immune system should be considered random: low immunogenic viruses will eventually be targeted by previously unexposed immune systems, but initially survival rates will be fairly high. As virus numbers increase, it will increase the opportunity for the "right" immune cells to interact with virus particles, which in turn triggers the specific immunogenic cascade.

However, within the cell, the intracellular host response to foreign DNA is harsh and uncompromising. Defense mechanisms involving RNA interference, RNases, and endonucleases are used to combat the invading viral genome. RNA interference works by using small RNA molecules to inhibit gene transcription (a defense that can be applied to all types of virus infection regardless of the nature of their genomic material) and can cause the direct degradation of dsRNA viral genomes (Marques and Carthew, 2007). Restriction endonucleases cleave DNA at specific recognition sequence sites (often found in viral genomes, but not present or protected in host genomes) and provide general protection from DNA viruses. Other cellular defenses include the apoptosis (programmed

cell death) pathway in eukaryotes that can be induced in order to prevent the infection from spreading, a case of sacrificing a cell to save the larger population. With obvious advantages to multicellular organisms, this process has also been suggested to occur within single-cell systems whereby the sacrifice of single cells acting individually may be undertaken to prevent viral infection spreading to the neighboring natural population.

Following infection of a cell, in order to survive and to infect another cell, a virus must successfully replicate its genome and create functional virions. This intracellular infectious time should be regarded as the primary battleground where the majority of directly coevolving host–virus systems can be observed. Following successful virion production, the virus must then be able to exit the cell. This is done in a variety of ways from budding to total cellular rupture. The nature of the host cell will then determine what the virus is exposed to. For free-living single-cell hosts, the released viruses will be exposed directly to aquatic, aerosol, or solid surfaces. For hosts involved in symbiotic or parasitic relationships, their viruses may be exposed to contained biotic environments (e.g., the gut). Depending on the stage of infection and the scale of the host response, viruses infecting multicellular organisms will either continue to infect cells within the same organism or transfer to and attempt to infect a new host.

4.6 THE SILENCE OF THE VIRUSES

Viruses often lie dormant inside their hosts, in what are known as latent, lysogenic, or endogenous lifestyles. Provided their incorporation into the host genome induces no direct negative effects (e.g., the disruption or deregulation of useful gene function), this is usually a safe strategy for the virus with negligible impact on host fitness. When viruses undertake this strategy, it is a reflection of their close relationship with their host: they can afford to sit out, let the host take the strain until such time in the future when either conditions are favorable for mass viral production or, alternatively, the host is approaching cell death and has outlived its usefulness to the virus as a low copy number safe haven. It could be argued that when in a prolonged latent phase, since viruses are essentially part of their host genome, they cease to be viral in nature. Indeed, any host death that is not associated with the virus in question would result in the premature end of the "silent" virus, an outcome which is not uncommon and displays the deep-rooted "trust" shown by viruses in their hosts for ensuring their long-term survival. Furthermore, an inactive virus does not produce progeny and thus can be considered to be at an evolutionary standstill. This does not necessarily apply to the host organism over the same time period: multicellular organisms in particular will continue to evolve provided they remain reproductively active. However, if during a period of inactivity the virus is replicated as part of the host genomic cellular division (because either the virus has infected a stem cell or a single-cell organism), it will continue to diverge at a similar rate to the host. Only when the virus becomes active will selection occur and evolutionary rates accelerate. Viruses that become integrated into their host genomes can be considered as the crudest form of coevolving systems, where, by definition, every piece of their genetic makeup is coevolving alongside their hosts. The advent of the genomic era has heralded unique insights into this phenomenon. The human genome, for example, is thought to comprise up to a staggering 8% of its material from viral origin (Lander et al., 2001).

4.7 GIVING UP THE VIRAL GHOST

The success viruses gain from incorporating into their hosts' genome is perhaps exemplified by the endogenous retroviruses. These viruses lie dormant almost indefinitely within genomes after infecting the germ cells of many vertebrate genomes. While other viruses strive to replicate at their hosts' expense, these viruses

have effectively stopped fighting the war and have become permanently incorporated into their host's genome: if you can't beat them, join them. This strategy has led to the permanent integration of huge amounts of previously viral genetic material into genomes. Most is deemed to be inactive or the so-called junk DNA. However, this may not be necessarily the case, diverging genes under no strong selection pressure over time can quite often assume new functions providing an advantage for either the host or for other active viruses (see Section 4.10.1).

4.8 THE MAKINGS OF VIRUS–HOST COMPATIBILITY

The total dependency and reliance that viruses have on their hosts is reflected in their genomic composition and metabolic potential. In order for viral genes (and proteins) to function correctly inside their hosts, they must be suitably adapted to and compatible with their host genetic background (e.g., composition, size, regulation, codon usage, folding, and post-translational modification). This intricate host–virus genetic compatibility creates the opportunity for genes to move between lineages in the process known as horizontal gene transfer. This can occur in either direction and provides an interesting aspect to viral ecology and evolution. Since viruses act as vectors for moving and shuttling genes between different lineages, no gene can or should ever be considered as being restricted indefinitely to a particular lineage. If a virus picks up a gene from its host, the gene becomes, by definition of its current location, a viral gene (despite its evolutionary history). The same applies vice versa or when transfer occurs between any genetic lineages. Where a gene hangs its hat is its home. In this context, viruses represent a mammoth hat stand, containing the largest reservoir of genes on the planet. It also adds a layer of complexity to the study of viruses and their hosts from an evolutionary perspective! Much has been written on the evolution of viruses and much more on the evolution of the organisms they infect. Yet, the subject of coevolution of viruses and hosts when considered as two intermingled parts of a whole is often neglected and forms the basis for the rest of this chapter. Quite often, the distinction between evolution and coevolution can become somewhat blurred, as is the distinction between what can be deemed viral or host with regard to genomic material with a shared history. Yet, it is at the interface that this shared and/or closely integrated biochemical machinery occupies where the fundamental selection occurs that is paramount to host–virus coevolution. I will attempt to dissect these issues and provide insights into and examples of the ever-changing landscape that is the ecology and (co)evolution of viruses.

4.9 THROWING LIGHT ON VIRUS–HOST EVOLUTION

Without doubt, the genomic era has instigated a change in attitude toward viruses. No longer thought of as merely bags of virus genes performing purely viral functions, it has become increasingly apparent that in reality many viruses harbor within their genomes homologous genes to their hosts. Debate still rages as to the nature of these genes, such as whether they originated as viral genes or host genes, the likelihood of further and ongoing recombination and transfer between host and viruses, their function in the viral system in relation to the "normal" host function. However, despite these issues, many of which are beginning to be resolved and many of which will never be resolved to satisfaction, there can be no doubt that these genes offer a unique insight into the process of virus–host coevolution. We are now blessed with an abundance of examples that can be utilized to illustrate our point from which I will take a select few to illustrate the types of evolutionary interaction that can occur between viruses and their hosts.

To begin with, we shall take our first example from the cyanobacteria *Synecoccus* and

Prochlorococcus. Viruses of the families Podoviridae and Myoviridae infecting these photosynthetically active organisms have been found to contain an assortment of photosynthesis-related gene products such as the photosystem II core reaction center proteins D1 and D2, a high light-inducible protein, plastocyanin and ferredoxin (Lindell et al., 2004; Mann et al., 2005). During infection, as the homologous host transcripts are in decline, viral transcripts become expressed and help to maintain the functioning of the photosynthetic system that in turn allows optimal viral production (Lindell et al., 2005, 2007). It remains to be determined whether the virus acquisition of the host photosynthetic genes came as a direct response to the host actively shutting photosynthesis down in response to infection or whether it is a mechanism used merely to increase the efficiency of the infection process, that is, providing more bang for the virus' buck. In addition to the photosynthesis-related genes, these interesting viruses possess homologues for stress response genes found in their hosts. Intriguingly, during infection, while the vast majority of host genes become downregulated as infection progresses, a few dozen are actually upregulated. These genes belong to two broad groups: stress response and nucleotide metabolism. It is likely that the stress response genes encoded by the viruses are involved in some aspect of this transcriptional regulation. Again, it is unclear whether this is a last ditch attempt by the host to slow or stop the infection or if they are actually induced by the virus and used against the host. This example shows the boundary between host defense and viral offense where true coevolutionary processes take place. This metabolic battleground consisting of both host and virus systems utilizing, exploiting, or manipulating the same processes, often through shared genes is a recurring theme mirrored in other host–virus systems. The cyanophage system does offer further insights that may shed light on other systems: there is a strong connection between the upregulated host genes, their position on the host genome (in hypervariable islands thought to be mobilized by phage), and the presence of viral homologues (Lindell et al., 2007). Thus, the cyanophage–cyanobacteria system provides clear directions to the site where biochemical confrontation and an intricate metabolic battle take place. Of course, different hosts and different viruses will all battle it out in different manners. Over time, the direct interaction and manipulation of metabolic pathways and processes lead to very interesting, intricate, and subtle host–virus coevolution dynamics. Indeed, the concept that viruses use the host systems against them (and vice versa) is becoming increasingly clear as full genomic sequencing lifts the lid on the Pandora's box of molecular evolution.

4.10 SOMETIMES IT TAKES MORE THAN THE ODD GENE

The previous example of a few genes involved in photosynthesis being acquired by a virus to aid infection provides an excellent illustration on how a few genes can be used by a virus to manipulate the host system. Yet, some viruses have taken the need to manipulate metabolic functions during infection to the extreme. The coccolithoviruses (family Phycodnaviridae, genus *Coccolithovirus*) are one such group of viruses. Amazingly, these viruses have acquired an almost complete metabolic pathway for the synthesis of sphingolipids from their algal host, the coccolithophore *Emiliania huxleyi* (see Chapter 7 for further information on this remarkable virus family) (Wilson et al., 2005). The reasons for the acquisition of this pathway are unclear at present, but clearly an important component of the battle between this host and virus is played out in the sphingolipid arena (Han et al., 2006; Monier et al., 2009). At this stage, the reasons behind these unique horizontal gene transfer events (genomic positioning suggests separate events were necessary) or why one gene is "missing" is not clear. However, crucially the sphingolipid

pathway provides useful guidance on how the virus and host genome products interact during infection (Pagarete et al., 2009). For example, sphingolipids are well known for their role as signaling molecules in apoptosis (programmed cell death). As mentioned previously, apoptosis is a well-known antiviral defense mechanism, albeit more commonly used in multicellular organisms. Caspases (a type of proteinase) are usually the vehicles used to induce the process. Accordingly, caspase induction has been found upon viral infection of this system (Bidle et al., 2007). However, the story does not stop there. Caspase induction may be actually necessary for successful infection. Furthermore, many of the viral gene products are predicted to have caspase cleavage sites that presumably require cleavage (by the host system) before they become active. This is an excellent example of complex metabolic pathways becoming a site for host–virus coevolution and involves both gene products with a shared origin and gene products and metabolites that regulate and/or manipulate associated pathways. The cellular environment is composed of an intricate network of biochemical pathways, and during the early stages of infection, viral activity will target and impact the function of particular pathways. Importantly, the ripple effect will be felt in the closest interacting pathways first. The coccolithophore–coccolithovirus system provides an excellent example of this process (Allen et al., 2006). However, it is important to note that manipulation of these particular pathways is not limited to the coccolithoviruses. This battleground is common to many host–virus systems and is reflected in the examples of apoptosis inhibitors found in a diverse range of viral genomes such as those belonging to the baculoviruses (family Baculoviridae), adenoviruses (family Adenoviridae), human cytomegalovirus (family Herpesviridae, genus *Cytomegalovirus*), herpesviruses (family Herpesviridae), African swine fever virus (family Asfarviridae), poxviruses (family Poxviridae), human papillomaviruses (family Papillomaviridae), and myxoma virus (family Poxviridae, genus *Leporipoxvirus*) (Alcami and Koszinowski, 2000; Hanada, 2005). Although every host and virus interaction will be highly specific and niche adapted, there are strong themes running through the infection and coevolution process. Particular pathways and networks are continually targeted by widely diverse viruses. Ultimately, the entire biochemical network will break down and cellular integrity will be lost; but in the early stages of infection, the outcome of infection often depends upon the control and manipulation of just a few precise metabolic pathways, crucially either utilizing the same molecular machinery or manipulating existing function.

4.10.1 Immunity, Protection, and Infection

The previous examples show how viruses can manipulate their hosts, often utilizing and turning the host molecular machinery and biochemical pathways against itself. However, we should not think of this as a one-way battle. Hosts also pick up viral machinery and use it in the battle against viruses. A striking example is that of endogenous retroviruses. Consisting of just three gene products, group-specific antigen, polymerase, and envelope protein (known as gag, pol, and env, respectively) (Villesen et al., 2004), the endogenous retroviruses (commonly referred to as ERVs, of the family Retroviridae) differ from their "exogenous" retrovirus counterparts because they integrate into the genomes of the germ cells of their hosts, thus becoming transmitted to future generations (Arnaud et al., 2007). Comprising a staggering 8% of the genome, there are an estimated 450,000 copies of ERVs within the human genome (Lander et al., 2001). This genomic colonization is mirrored in all vertebrate genomes. Presumably, there is a strong selection against their integration into essential genomic loci or to positions with deleterious effects: their integration into primary germ cells allows for an easy selection process. Deleterious

insertions will be immediately selected and only germ cells with stable inserts will be able to grow and develop normally following fertilization. Thus, despite our obsession with viruses being bad for our heath, it is undeniable that human (and animal) evolution is closely mired with that of our viruses.

Although the vast majority of ERVs are inactive after accumulating genetic defects, their sheer abundance ensures that some will be transcriptionally active and will be capable of producing functional gene products (Stoye, 2009). Indeed, their prevalence in genomes is thought to provide an advantage to their hosts. In a case of poacher-turned gamekeeper, endogenous (and therefore stably integrated) retroviruses are thought to provide protection from exogenous retrovirus infection. For example, expression of the ERV envelope glycoproteins provides protection from infection by exogenous retroviruses by blocking the entry through receptor competition (Malik and Henikoff, 2005). ERV Gag expression has been shown to protect mice against some murine leukemia virus strains (Villarreal, 1997).

But the story does not end here with viruses solely offering protection to their hosts from other viruses. We mentioned previously that apoptosis and cellular signaling is a pathway specifically targeted by a variety of viruses for manipulation during infection. Unchecked, apoptosis has the capacity to severely inhibit successful infection. Organisms with immune systems also provide a target that must be neutralized as efficiently as possible by the viruses for successful infection. Therefore, many viruses, including the retroviruses, harbor genes whose products can inhibit host immune responses. In similar fashion to the strategies employed to manipulate apoptosis, various viruses manipulate the immune response by either using virally encoded homologues of the host immune genes or by using genes whose products can interact with the molecular functioning of the host immune system (Alcami and Koszinowski, 2000). Targets include the humoral (antibody) response (poxviruses, coronavirus (family Coronaviridae), cytomegalovirus, herpesviruses, and HIV); interferon response (adenoviruses, poxviruses, reoviruses (family Reoviridae), baculoviruses, HIV, polioviruses, influenza viruses, rotaviruses (family Reoviridae, genus *Rotavirus*), and Sendai virus (family Paramyxoviridae, genus *Respirovirus*)); cytokine and chemokine response (African swine fever virus, adenoviruses, poxviruses, and Epstein–Barr virus (human herpesvirus 4, family Herpesviridae, genus *Lymphocryptovirus*)); and major histocompatibility complex (cytomegalovirus, HIV, herpesviruses, and adenoviruses).

Thus, as you can see, the incorporation of large amounts of viral material into a genome could potentially have severely deleterious effects and create a ticking time bomb especially with regard to crucial functions such as immunity. However, if harnessed correctly it does provide the host genome with the opportunity to have localized areas with inhibited immunological performance. While under most circumstances there would be little or no call for this situation, the evolution of the mammalian placenta has created the opportunity for the evolved products of stably integrated retrovirus genomes (which could/should actually be deemed host material because of their long-term integration) to perform such a function. The foreign fetus is thought to be protected from the maternal immune system through the actions of an immunosuppressive domain located on the envelope protein of an ERV (Villarreal, 1997). Furthermore, some ERV envelope glycoproteins, such as those of HERV-W group (family Retroviridae), have fusogenic effects and play a crucial role during the formation of the placental syncytium (Blaise et al., 2003). Despite being labeled as "syncytin" genes of the host, they are clearly coopted retroviral genes. A variety of syncytin genes have been identified suggesting that capture and utilization of retroviral genes is a recurrent theme in

mammalian placental evolution (Heidmann et al., 2009). However, given the content of this chapter, it is not surprising that this event has occurred on so many occasions: it is almost inevitable given what we know about the effects of viruses on cells and the pathways they target to ensure that their infection is successful.

4.10.2 The End of the Concept of the Host Gene?

Our knowledge of host–virus coevolution derived from some of the examples described in this chapter has been entirely dependent on genomic sequencing. In particular, our knowledge is heavily biased toward human and animal viruses because these are the most economically relevant to justify the research on them. I have tried to avoid overly referring to these viruses since life is far more diverse than our human-centric obsession would have us believe. Nevertheless, I hope you have now obtained a taste of the issues and themes involved in the study of host and virus coevolution in any system. This is a field very much in its infancy, but is growing rapidly as we realize that viruses have and continue to shape the evolution of all living organisms in ways we are only just beginning to grasp. As more types of viruses become sequenced from diverse hosts, it will be unavoidable for us to realize that many of the genes that we have previously considered as being bacterial or eukaryotic in origin will actually reveal themselves to be viral in nature. The study of viral ecology will have to deal with the realization that all too often what is thought of as host function is actually virus in origin, what is virus function is actually host in origin, and there exists a large gray area in between where the quirkiness of nature expresses itself with beautiful intricacy. Evolutionarily, viruses and their hosts should no longer be considered separate entities, the boundaries between them have been exposed for what they are: an approximate line drawn in the sand, accurate at any given moment but never set in stone.

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SECTION II

VIRUSES OF OTHER MICROORGANISMS

CHAPTER 5

BACTERIOPHAGE AND VIRAL ECOLOGY AS SEEN THROUGH THE LENS OF NUCLEIC ACID SEQUENCE DATA

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CONTENTS

5.1 THE UBIQUITY OF VIRUSES IN THE BIOSPHERE

The first hint at the abundance of viruses in the environment came in 1979 when Torrella and Morita used transmission electron microscopy (TEM) to estimate the concentration of viral particles in Yaquina Bay to be $>10^4$ viruses mL^{-1} (Torrella and Morita, 1979). Despite this report and several others that followed, the impact of viruses on ecological processes remained largely ignored (Weinbauer, 2004). However, the past two decades have witnessed a dramatic resurgence of interest in viruses, their role in microbial communities, and their impact on ecosystems. The seed of this flourishing research enterprise came through the discovery that viruses are not only extraordinarily abundant within aquatic environments but also typically outnumber coexisting microbial host cells by 10-fold or more (Wommack and Colwell, 2000). More recent work in porous media environments such as soils (Ashelford et al., 2003; Williamson et al., 2003) and aquatic sediments (Maranger and Bird, 1996; Drake et al., 1998; Danovaro et al., 2001; Hewson et al., 2001; Helton et al., 2006) has further substantiated the ubiquity and predominance of viruses within natural environments and has shown that the abundance of free viral particles can exceed that of microbial cells by over 1000-fold. Assuming the average length of a bacteriophage is 1×10^{-7} m and that the global abundance of phage within the biosphere is on the order of 10^{31} individuals (Whitman et al., 1998; Hendrix et al., 1999; Hendrix, 2002), lined end-to-end phages span a distance of 10^{24} m or

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10 million light years, a distance equal to that of the nearest 60 galaxies (Suttle, 2003, personal communication). It is the extraordinary abundance and ubiquity of viruses that substantiates hypotheses about the influence of viruses on global biogeochemical cycling of nutrients and the generation of unique genetic diversity through rare recombination events.

Exploration into the far reaches of the biosphere has shown viruses to be responsible for a large proportion of carbon turnover in the deep sea (Danovaro et al., 2008) and potential gene transfer in deep-sea hydrothermal vents (Williamson et al., 2008a). Observations of viruses infecting the resident populations of archaea of extreme geothermal environments have led to the discovery of strange and novel viral families with equally novel and unknown morphologies and gene content (Prangishvili et al., 2006; Lawrence et al., 2009). From these baseline observations, we can confidently predict that viruses will exist in nearly every natural microbial environment and influence the ecology of their co-occuring microbial hosts. Direct enumeration of viruses over time scales ranging from hours to months has shown that viral assemblages within aquatic (Winget and Wommack, 2009), soil (Srinivasiah et al., 2008), and benthic (Hewson et al., 2001) environments are active and responsive to shifting environmental conditions. Process-level investigations of viral activity found that aquatic viral assemblages also exhibit an extraordinary capacity for growth, with turnover times as fast as half a day in productive coastal environments (Winget and Wommack, 2009); a few days for pelagic surface waters (Parada et al., 2007; De Corte et al., 2010); around two days for cold deep-sea sediments (Danovaro et al., 2008). The fact that all viruses are obligate parasites means that these observed responses are intimately connected to changes in the growth, activity, and composition of microbial host communities.

Connecting estimates of viral production to rates of viral-mediated bacterial and phytoplankton mortality has been challenging mainly due to uncertainties surrounding the magnitude of viral burst sizes among autochthonous viral–host systems (i.e., number of virus particles produced upon cell lysis) (Wommack and Colwell, 2000; Weinbauer et al., 2002). However, even more conservative estimates find that around 20% of bacterioplankton cells are lost to viral lysis each day (Suttle, 1994), a mortality rate similar to that induced by grazing zooplankton in coastal ocean waters (Suttle, 2005). Among the immediate impacts of viral-induced mortality on ecosystems is the increase in available nutrients through the conversion of particulate (cellular)-to-dissolved organic matter. Thus, through their direct control on the productivity of microbial host populations, viruses indirectly influence the flow of carbon, nutrient elements, and energy through ecosystems.

Pressing global environmental concerns over climate change and the productivity of agricultural systems including fisheries have driven efforts to understand the inner workings of the biogeochemical processes that govern carbon and nutrient element cycles. Certainly, the discovery that viral infection plays a significant role in microbial cell mortality reminds us that microbiological systems may provide many more surprises that are important to our understanding of how ecosystems work. Efforts to restrict the impact of viral activity on specific biogeochemical cycles continue to drive process-level investigations of viral ecology. However, a deeper understanding of the intricacies of autochthonous viral–host interactions and the diverse biochemical pathways responsible for the chemical transformations within carbon and nutrient cycles has been an important rationale behind the application of genomic and metagenomic approaches within viral ecology. To date, the compendium of research in viral ecology has included the examination of metabolic constraints regarding the broad impact of viral processes on the flow of C and nutrients through aquatic ecosystems (Suttle, 2005) and to a much lesser extent porous media environments.

5.2 POSSIBLE ECOLOGICAL INFLUENCES OF THE PHAGE LIFE CYCLE

Yet, it is important to remember that the impact of viruses on ecosystem services rest upon a plethora of individual virus–host interactions and environmental conditions. The ebb and flow of viral lysis on specific host populations can shape the composition and diversity of microalgal (Martinez et al., 2007) and bacterial communities (Sandaa et al., 2009). These predator–prey dynamics are influenced by host abundance and growth rate, as maximum phage production corresponds to optimal host growth conditions (Lenski, 1988; Weinbauer and Rassoulzadegan, 2004). Increased viral lysis under more productive environments where prokaryotic host abundance is large enough to sustain lytic infection (Weinbauer and Rassoulzadegan, 2004) reduces the dominance of abundant prokaryotes. Through this "kill-the-winner" approach, phage-induced mortality promotes sufficient biodiversity within the community to efficiently utilize available resources (Rodriguez-Valera et al., 2009; Winter et al., 2010).

This influence spreads beyond lysis as both temperate and virulent phages can alter the phenotypic characteristics of their host cells during infection or within the prophage state. One notable example is the presence of restriction-modification (R-M) systems in bacteria. R-M systems consist of a restriction endonuclease and methylase and play a role in antiviral defense by recognizing and cleaving unmodified sites of foreign DNA (Kobayashi, 2001; Danilova, 2006). Remarkably, these enzymes are often encoded by prophages rather than by the bacterial genome (Danilova, 2006). The best known example, however, is the transfer of virulence genes among pathogenic bacteria through temperate phages (Brüssow et al., 2004). This phenomenon is so prevalent among pathogenic strains that chromosomally encoded virulence is believed to be the exception (Canchaya et al., 2003). The extraordinary frequency of prophage-encoded bacterial virulence has led some to hypothesize that this is an ancient strategy exploited by temperate phages to aid their bacterial host cell in avoiding predation by bacterivorous protists (Brüssow, 2007). The present-day fact that pathogenic bacteria are capable of evading "bacterivorous" elements of animal immune systems is an outcome of a billion years of phage–host interactions in the face of bacterial predation by microeukaryotes. Indeed, a hallmark of many pathogenic bacteria is the ability to survive within the phagosomes of neutrophil white blood cells, a trait sometimes linked to prophage-encoded virulence determinants within their genomes (Brüssow, 2007). The ecological outcome of prophage-encoded virulence is the increased survival and fitness of lysogenic cells despite the fact that the ultimate fate of some lysogenic cells will be death through the induction of prophage.

In ecological terms, we can assume that lysogenic viral–host relationships ultimately provide a net improvement in host cell fitness. The challenge is to determine the mechanistic basis of these "fitness improvements." Beyond the well-documented occurrence of virulence genes within temperate phage genomes and prophage elements, very few lysogenic phages have been examined in sufficient detail to uncover other unique host phenotypes that are encoded within their genomes. Recent work examining possible lysogenic conversion phenotypes in *Vibrio harveyi* cells harboring VHML prophage found substantial alterations in substrate utilization profiles over nonlysogenized cells. Paradoxically, lysogens showed reduced substrate utilization capacity (Vidgen et al., 2006). A similar outcome was observed when comparing the substrate utilization profiles of *Listonella pelagia* cells carrying phi HSIC (an unclassified member of the family Siphoviridae), the main pseudolysogenic phage with wild-type (wt) cells (Williamson et al., 2001; Williamson and Paul, 2006). Out of 42 substrates and a no-substrate control, phi HSIC-carrying cells showed reduced growth in

29 cases (Paul, 2008). Although phi HSIC is not an integrative temperate phage, many aspects of its pseudolysogenic life cycle are similar to those of true lysogenic phage. The most detailed confirmation of the negative impact of a temperate phage on the growth of its lysogenic host came through studies comparing the expression profiles of phage Lambda (family Siphoviridae, genus *Lambda-like viruses*) lysogens to wt *Escherichia coli* cultures. Surprisingly, phage Lambda C1 repression was linked to the downregulation of several host genes, most notably *pck*A encoding phosphoenolpyruvate carboxykinase. Loss of this central enzyme in gluconeogenesis substantially lowered the growth rate of Lambda lysogens in glucose-free environments leading the authors to hypothesize that increased fitness may come through better survival under carbon-limited environments (Chen et al., 2005).

Examination of nearly two decades of work investigating the incidence of lysogeny in marine environments tends to support this hypothesis. Although field investigations examining the incidence of lysogeny within natural bacterial communities have often yielded highly variable results, the most consistent conclusion of these studies has been that environmental conditions unfavorable to rapid host growth tend to support larger populations of lysogenic cells. Among the notable examples of this trend was a seasonal study that documented a higher proportion of lysogenic marine *Synechococcus* in the winter samples from Tampa Bay, a season that offers unfavorable conditions for growth of these sun- and warmth-loving photoautotrophs (Chen et al., 2005). Examination of lysogen frequency within prokaryotic communities spanning the 2500 m water column overlying Pacific deep-sea hydrothermal vents found that lysogens were most frequent within the sulfide and metal-laden warm water diffusing from the vents (Williamson et al., 2008a). Williamson et al. (2008a) concluded that the increased incidence of lysogens within diffuse-flow waters was an emergent response of prokaryotes to the challenging growth conditions at the vents. Perhaps not surprisingly, more stochastic soil environments have consistently shown high lysogen frequencies (Williamson et al., 2007, 2008b), again suggesting that assemblages of temperate phage may provide a collection of genes and regulatory networks to promote host survival in adverse environments.

Thus, initial indications are that the increased fitness of lysogens can take at least two mechanistic forms – resistance to predation and survival through lowered metabolic rate – however, it is likely that there are substantially more genetic and regulatory strategies within the vast populations of temperate phage. Moreover, it is likely that these strategies have been tuned, through steady selective pressure, to fit the specific challenges prokaryotes face within a given environmental niche. At present, the best data set to explore the genetic inner workings of temperate phage influence on host cell phenotypes exists within the collection of bacterial whole genome sequence (WGS) data. This data set is substantially biased toward pathogenic bacteria and thus the specific and somewhat unique ecology of pathogens. However, this situation is rapidly changing with the move to expand the representation of WGS data across the entire tree of life (Wu et al., 2009) and targeted programs such as the Marine Microbiology Initiative funded by the Gordon and Betty Moore Foundation (Seshadri et al., 2007; Moore Foundation, 2010). A careful survey found that 43% of the 113 bacteria WGS within the Marine Microbiology Initiative collection contained one or more prophage-like regions (Paul, 2008). Interestingly, a third of these regions were small ($<$15 kb), indicating that they were possible gene transfer agents (GTAs) – defective phage-like particles capable of packaging host chromosomal DNA. Although long known from studies of *Rhodobacter capsulata* (Marrs, 1974; Lang and Beatty, 2000), the presence of GTAs within a larger collection of marine bacterial genomes and the experimentally validated GTA expression (Lang and Beatty, 2002) suggest that this mechanism of

gene transfer may be more common under marine environments than previously believed (Biers et al., 2008; Zhao et al., 2009).

5.3 GENETIC AND METAGENOMIC APPROACHES TO VIRAL DIVERSITY

In many ways, the small and coding-dense nature of their genomes makes viruses ideal candidates for the application of metagenomic approaches. Indeed, the first genome to be sequenced in its entirety belonged to the (+) ssRNA bacteriophage MS2 (family Leviviridae, genus *Levivirus*), a groundbreaking achievement by Fiers et al. (1976). A year later, Sanger et al. (1977) successfully sequenced the genome of bacteriophage phi X174 (family Microviridae, genus *Microvirus*), thus marking the first DNA genome to be sequenced. Subsequently, numerous additional viral genomes have been sequenced, providing valuable information about the genetic composition and diversity of viruses. However, the sequenced genomes belonged to cultivable viruses, while it is estimated that more than 99% of environmental viruses are not readily cultivated (Kennedy et al., 2010). Knowledge of these viruses and their diversity, biological roles, and gene content remained scant due to the limitations of cultivation-based approaches (Schoenfeld et al., 2009).

In light of the difficulty in viral cultivation from environmental samples, a complementary approach to the problem of exploring viral diversity was required. Metagenomic analysis has enabled the examination of viral assemblages at the genetic level to characterize natural assemblages of this most abundant and ubiquitous class of microorganisms. Sequence-based metagenomic analysis has provided insight into the startling scope of viral diversity in the biosphere. Unlike prokaryotic metagenomic libraries, where approximately 90% of putative genes show similarity to database sequences, viral libraries are dominated by unidentifiable sequences (60–80%) (Edwards and Rohwer, 2005; Bench et al., 2007; Wommack et al., 2008). Although this may be exaggerated by the short read length of the next-generation sequencing technology (Wommack et al., 2008), the abundance of novel genes within viral metagenomic libraries indicates viral diversity has yet to be adequately sampled. While environmental viral assemblages are dominated by bacteriophage, the gene composition of these assemblages is drastically different from that of known, cultured bacteriophages, and suggests the dominant phages in environmental samples are dissimilar to known cultivated phages (Kristensen et al., 2010).

Viral genotypes within metagenomic libraries display a high degree of richness. The most dominant genotypes typically comprise less than 5% of the assemblage, while the majority of genotypes are individually less than 0.01% of the whole (Angly et al., 2005, 2006). Combined with known viral abundances, this has led to the estimation that several thousand genotypes are present in 200 L of seawater, and potentially 1 million genotypes in 1 kg of sediment (Angly et al., 2005, 2006). While some viral genotypes appear widespread, having been sampled in locations as distant as the Gulf of Mexico and the arctic (Filée et al., 2005), most genotypes have been sampled in only a single library. As such, estimates place the number of unique viral genotypes in the biosphere at more than 10^{30}, making viruses the largest source of genetic diversity on Earth (Kristensen et al., 2010).

Annotated genes display a high degree of functional richness across libraries. Of particular interest was the discovery that phage contain genes previously believed to be restricted to the metabolic activities of cellular organisms, including those involved in photosynthesis and carbon metabolism (Breitbart et al., 2007; Dinsdale et al., 2008). Although these genes were most likely passed from host to virus through horizontal gene transfer (HGT), the resulting genes are now clearly viral in nature (Sullivan et al., 2006; Bench et al., 2007). It is now apparent that the restriction on functional types of genes carried by phage is minimal; however, the prevalence of

genes of known function in a viral assemblage appears to be related to the importance of that function in that environment. Thus, the high functional richness of annotated phage genes is contrasted by the low evenness of functional genes in viral assemblages (Dinsdale et al., 2008).

The common occurrence of certain genes within viral genomes (e.g., polymerases, terminase, and major capsid protein) has led to the search for a gene marker of viral diversity equivalent to the prokaryotic 16s rDNA gene. Such a gene would provide a means to more readily analyze the phylogenetic diversity within viral metagenomic libraries. Unlike their cellular hosts, however, viruses lack a single, universally conserved gene or genetic element such as 16S rDNA that can be used for a universal phylogenetic classification. Nevertheless, a handful of relatively conserved genes that commonly occur within aquatic viral assemblages have been used to explore viral diversity among restricted viral families. Two genes well known in bacteriophage T4 (family Myoviridae, genus *T4-like viruses*), gp 20 (Hambly et al., 2001), and gp 23 (Filée et al., 2005), encoding the terminase/vertex portal protein and major capsid protein, respectively, have been used for molecular phylogenetic investigations of marine phage diversity (Comeau and Krisch, 2008) and as a proxy measure of shifting patterns in the composition of virioplankton assemblages (Wang and Chen, 2004; Short and Suttle, 2005). Methodological approaches similar to those developed for diversity assays of prokaryotic assemblages based on 16S rDNA sequence have been applied to gp 20, including surveys of clone libraries (Zhong et al., 2002; Sullivan et al., 2008) and fingerprinting analysis of PCR amplicons through terminal restriction fragment length polymorphism (tRFLP) (Wang and Chen, 2004). The use of T4 major capsid protein, gp 23, as a tool for exploring the evolution and population dynamics of virioplankton was given a tremendous boost after the publication of the first Global Ocean Survey (GOS) data set (Rusch et al., 2007). Comparison of the previously existing collection of publicly available protein sequences in GenBank to sequence data collected during the first GOS survey indicated that 6 of the top 10 more highly represented pfams within the GOS data were viral proteins (Yooseph et al., 2007) and the most highly represented was gp 23. In this specific case, gp 23 was 230-fold more abundant in the GOS metagenome data than would have been predicted by its occurrence in public databases. Comeau and Krisch (2008) substantially expanded on earlier work examining the phylogeny of gp 23 (Filée et al., 2005) by including GOS gp 23 sequences and found that most of the GOS sequences fell into an expansive and highly divergent clade likely representing phages distantly related to T4 and infecting cyanobacterial hosts. Thus, it is possible that genes encoding T4-like major capsid proteins approach something of a universal gene among cyanophages within the Myoviridae morphological family (i.e., phages having a contractile tail) (Buechen-Osmond and Dallwitz, 1996; Valdivia-Granda and Larson, 2009).

Nevertheless, the question remains whether population surveys based on a single viral gene can be used as a proxy estimation of viral diversity (richness and evenness) or compositional changes within larger virioplankton assemblages. Early indications are that the enormous diversity of gp 23 seen under aquatic and marine environments is not encountered under soil environments with the exception of flooded rice paddy soils (Jia et al., 2007). Homologues to gp 23 were nonexistent within a collection of 13,485 viral metagenome sequences (8.45 Mb of DNA) in the Viral Informatics Resource for Metagenome Exploration database (VIROME) from viral assemblages under five different soil environments (Bhavsar et al., 2010, Beta release). In contrast, 191 gp 23 homologues were found within 257,348 metagenome sequences (195.6 Mb of DNA) from aquatic viral assemblages in VIROME. Results were similar for the vertex portal protein of T4 (gp 20) with the soil libraries containing no homologues and the aquatic libraries having

154. Although not a rigorous test, it appears that these two T4 genes, which have been used in several diversity studies of viral assemblages, occur at a rate of around one copy per Mb of metagenome sequence data from virioplankton assemblages. In contrast, gp 20 and gp 23 gene homologues appear to be substantially less common in viral assemblages from non-flooded soils. However, more definitive support will come only with the greater availability of metagenome data from soil viral assemblages.

Looking beyond genes with a specific structural function in bacteriophage, Breitbart et al. (2004) found the T7-like (family Podoviridae, genus *T7-like viruses*) DNA polymerase gene (T7 DNA *pol*) to be nearly omnipresent and highly conserved within natural assemblages of bacteriophage from aquatic environments, clustering within a single unique clade with high conservation (>99%) in multiple environments (Breitbart et al., 2004). Interestingly, a similar study examining podovirus DNA polymerase diversity demonstrated greater diversity among Podoviridae than previously appreciated (Labonté et al., 2009). Restriction fragment length polymorphism (RFLP) analysis of clone libraries from the Straight of Georgia and Gulf of Mexico revealed three novel groups of *pol* sequences containing no cultured representatives, while none of the sequences fell into the novel clade described by Breitbart et al. (2004). Although some sites were dominated by a single genotype, others contained sequences among different evolutionary groups (Labonté et al., 2009). However, the prevalence and diversity of T7 DNA *pol* appears limited to aquatic environments. BLAST searches against viral metagenome sequence libraries showed only three relatively distant homologous sequences within soil (*e* scores 10^{-9}–10^{-5}), in contrast to 984 homologues (~5 homologues per Mb) within the marine libraries, while Labonté et al. (2009) found little overlap in operational taxonomic units (OTUs) between sediment and water samples. This view of T7 DNA *pol* occurrence agrees with quantitative PCR assays that indicated a 10- to 100-fold lower density of T7 DNAp genes within samples of virus assemblages from terrestrial environments (Breitbart et al., 2004). Recent work in the Chesapeake Bay estuary has further refined our view of T7-like DNA polymerase diversity to focus on the use of this gene for specific environmental surveys of podoviruses infecting *Synechococcus* spp. (Chen et al., 2009). Phylogenetic analysis of PCR amplicon sequences from degenerate PCR primers for T7-like DNA *pol* and viral metagenome sequences (Bench et al., 2007; Rusch et al., 2007) showed that the Chesapeake tends to contain unique assemblages of cyanopodoviruses that form a large clade distinct from phages infecting bacteria within the marine cluster A *Synechococcus* or the Proteobacteria. A similar trend toward endemism was observed for Chesapeake virioplankton metagenome sequences homologous to the photosystem II reaction center protein *psbA* (Bench et al., 2007). The somewhat surprising outcome of these initial studies is that the influence of selective environmental forces can be observed at the level of primary sequence data from viral assemblages. In the case of the Chesapeake, this view was made possible only by access to shotgun metagenome sequence data where gene sequences can be obtained without the bias inherent to approaches relying solely on the use of degenerate PCR primers to explore the phylogenetic diversity of a given gene.

Similar patterns have been observed with other genetic markers and viral families. Examining cyanophage diversity from marine and freshwater samples through the *psbA* gene, Chénard and Suttle (2008) observed sequences from the same sample location within different clades. Phylogenetic analysis also presented several novel evolutionary groups and suggests that marine and freshwater *psbA* sequences do not share a common evolutionary history (Chénard and Suttle, 2008). An investigation of the prevalence and diversity of *psbA* and *psbD* (photosystem I and II) genes from myoviruses, podoviruses, and siphoviruses infecting *Prochlorococcus* and *Synechococcus* cyanobacteria by Sullivan et al. (2006) found that

88% of 42 cultured cyanophages encoded for the *psbA* gene, while only phages with broad host ranges tend to encode both *psbA* and *psbD* genes (Sullivan et al., 2006).

Among the viruses infecting unicellular microalgae, the gene for DNA polymerase has been found to be an informative phylogenetic marker, indicating a monophyletic origin for the Phycodnaviridae, a group within the nucleocytoplasmic large DNA viruses (NCLDVs) (Chen et al., 1996; Short and Suttle, 2002; Brussaard et al., 2004). RFLP analysis of PCR products from the Gulf of Mexico identified five different genotypes within the Phycodnaviridae family. Four of the five sequences were dispersed among a clade of viruses infecting *Micromonas pusilla*, while the fifth formed a novel clade. The number of distinct DNA *pol* sequences identified in a single water sample indicates the potential for high diversity among algal viruses (Chen et al., 1996; Short and Suttle, 2002). Subsequent studies using both DNA *pol* and major capsid protein (Larsen et al., 2008) as markers have demonstrated that some phycodnaviruses are widely distributed, while others appear to have narrow ranges (Clasen and Suttle, 2009). As with *psbA* studies, phylogenetic analysis indicates distinct groups between freshwater and marine phycodnaviral *pol* sequences.

Although they remain less studied, examination of RNA viral diversity using a gene marker appears to be more straightforward than for their dsDNA counterparts. Except for retroviruses, all RNA viruses encode the gene for RNA-dependent RNA polymerase (RdRp), which is vital to replication (Culley et al., 2003). The diversity of picorna-like viruses (single-stranded RNA viruses) was examined in the Straight of Georgia through the amplification of RdRp followed by DGGE, cloning, and sequencing. Surprisingly, none of the environmental sequences grouped with known picorna-like viral families; rather, the sequences fell into four novel groups representing at least two RNA viral families. In addition, three of the four novel groups of picorna-like viruses were identified in one sample, indicating high diversity in a single location (Culley et al., 2003, 2006).

Other potentially useful gene markers remain to be explored. One such example is the gene encoding ribonucleotide reductase (RNR). RNR is responsible for regulating the pool of dNTPs within a cell, which is accomplished through the removal of the 2′ hydroxyl of a ribonucleotide to generate a deoxyribonucleotide, and plays an important role in DNA repair and replication (Nordlund and Reichard, 2006). As such, the enzyme was crucial to the transition from an RNA to DNA world (Filée et al., 2003) and is found in all living organisms studied to date (Tauer and Benner, 1997; Nordlund and Reichard, 2006). Across the tree of life, three classes of RNR have been identified (class I, II, and III), with class I enzymes being further divided into subclasses Ia and Ib. Although their primary structures differ, all three classes share a common catalytic mechanism and structural features, implying a common evolutionary origin (Nordlund and Reichard, 2006). Class I RNRs are oxygen-dependent enzymes composed of two nonidentical dimeric subunits encoded by NrdA and Nrd B (class Ia) or NrdE and NrdF (class Ib) (Nordlund and Reichard, 2006). Class I RNRs appear to be the most widespread, as class Ia RNRs are found in all eukaryotes except for *Euglena gracilis*, many aerobic bacteria, and a few archaea (Filée et al., 2003; Nordlund and Reichard, 2006; Lembo and Brune, 2009) and class Ib RNRs are present in a wide spectrum of aerobic bacteria. Class II RNRs, which are encoded by a single NrdJ gene (or NrdZ in mycobacteria) and exist as a monomer or a dimer, are a class of oxygen-independent enzymes found in aerobic and anaerobic bacteria and archaea (Nordlund and Reichard, 2006; Lembo and Brune, 2009). This class of RNR depends upon adenosylcobalamin (coenzyme B12) to generate the thiyl radical necessary for catalysis among all RNR classes (Nordlund and Reichard, 2006). Class III RNRs are sensitive to oxygen and are restricted to strict and facultative anaerobes (Nordlund and Reichard, 2006; Lembo and

Brune, 2009). It has also been known that viral versions of the protein are carried by coliphages such as T4-like phages and other large DNA viruses (Breitbart et al., 2007; Lembo and Brune, 2009). Typically found in lytic phage, the presence of RNR can increase the rate of DNA synthesis by a factor of 10 and is advantageous for fast-replicating viruses or those with larger genomes (Santos et al., 2007). Interestingly, the RNR R1 subunit homologues of betaherpesviruses (family Herpesviridae, subfamily Betaherpesvirinae) have lost their catalytic activity, but are capable of blocking the signaling pathways of host cell innate immunity and inflammation through inactivation of receptor-interacting protein 1 (RIP1, a serine/threonine kinase involved in the stress response pathway and programmed cell death) (Lembo and Brune, 2009).

RNR appears to be ubiquitous in environmental viral metagenomic libraries. The RNR database (http://rnrdb.molbio.su.se/) reveals RNR genes from archaeal, bacterial, and eukaryotic viruses. In addition, metagenomic sequences annotated as putative RNR genes have been found in Chesapeake Bay (Bench et al., 2007), Wisconsin and Delaware soil (Bhavsar et al., 2010, Beta release), Bear Paw hot spring, and Octopus hot spring libraries among others (Schoenfeld et al., 2008, 2009). An examination of a single dsDNA viral metagenomic library from the Gulf of Maine offers a glimpse at the potential utility of RNR in viral diversity studies and indicates that viral RNR diversity remains undersampled.

Sequences from a Gulf of Maine dsDNA viral metagenomic library that were annotated as ribonucleoside diphosphate reductase (ribonucleotide reductase) were assembled into contigs. The contigs with $\geq$1000 nucleotides were translated and then BLAST searched against the GenBank nonredundant database. BLAST results grouped the contigs into two: those showing homology to NrdA (class Ia RNRs) and those showing homology to NrdJ/Z (class II RNRs). Division of contigs was confirmed by a phylogenetic tree of contig consensus sequences where contigs annotated as NrdA or NrdJ/Z clustered among themselves (data not shown). Putative NrdA and NrdJ/Z contigs were aligned with NrdA or NrdJ/Z reference sequences from archaea, bacteria, and viruses. Overlapping regions of 372 and 361 amino acids were used to create phylogenetic trees of the NrdA and NrdJ/Z sequences, respectively (Figure 5.1). Among the NrdA sequences, the extracted region used for phylogenetic analysis included residue Cys-439 from *E. coli*, the putative radical site (Figure 5.2) (Tauer and Benner, 1997), which was found to be conserved among all contigs and reference sequences.

Among the putative NrdA contigs, one was most closely related to *Neisseria meningitidis* (Peng et al., 2008), while two others were part of a clade containing *Aeromonas phage 31* (Petrov et al., 2006) and *Blattabacterium* sp. (Lopez-Sanchez et al., 2009), endosymbiotic bacteria of cockroaches. Four other contigs were unrelated to any of the reference sequences and formed their own clades (Figure 5.1a). Putative NrdJ/Z contigs were more closely related to each other than to the majority of reference sequences, with the closest relative being *Roseiflexus castenholzii*, a thermophilic photosynthetic bacterium (Figure 5.1b) (Hanada et al., 2002). The lack of similarity between contigs and reference sequences for both NrdA and NrdJ/Z demonstrates greater RNR diversity existing than was previously known and hints at the potential for novel families of viral RNRs. Therefore, the importance of RNR to DNA viruses, its diversity within a single environmental sample, and its widespread distribution across habitats and viral families make RNR an interesting candidate for future phylogenetic analyses.

However, all the gene markers discussed are limited by the fact that they exclude certain viral families or even entire viral nucleic acid types. The Phage Proteomic Tree (Rohwer and Edwards, 2002) utilizes a different approach to analyze phylogenetic diversity of viral assemblages. Rather than focusing on a single gene locus, the Phage Proteomic Tree considers every gene in a phage genome to determine the distance between sampled genomes and

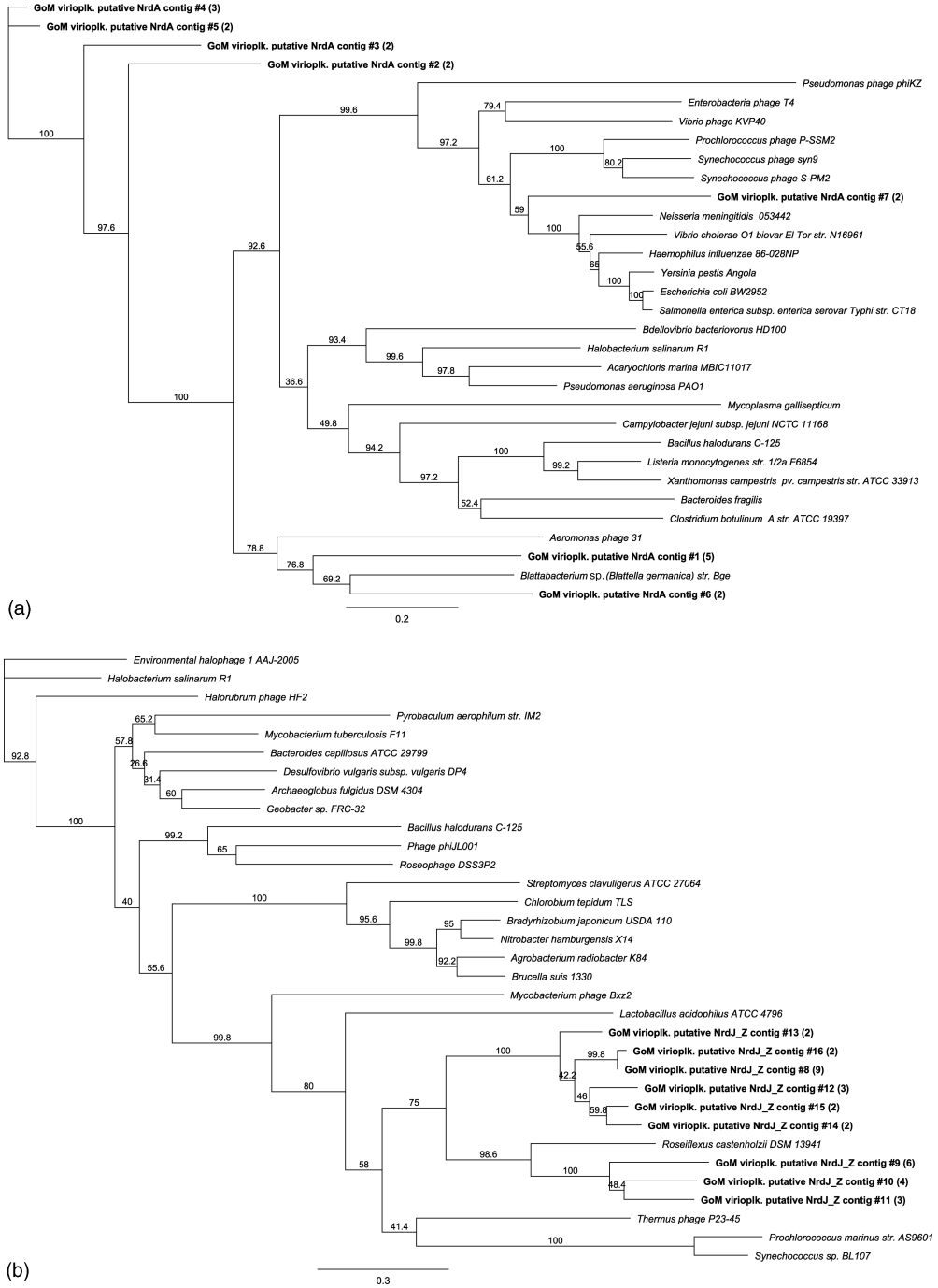

FIGURE 5.1 Phylogenetic trees of RNR consensus sequences out of a dsDNA viral metagenomic library from the Gulf of Maine with reference RNR sequences from archaea, bacteria, and viruses. Neighbor-joining trees were created using the Geneious (Drummond et al., 2010) program with no outgroup. Bootstrapping was performed with 500 replicates and a support threshold of 0%. The scale bar represents the number of amino acid substitutions per site. (a) Phylogenetic tree of putative NrdA contigs from the Gulf of Maine with reference sequences. (b) Phylogenetic tree of putative NrdJ/Z contigs from the Gulf of Maine with reference sequences.

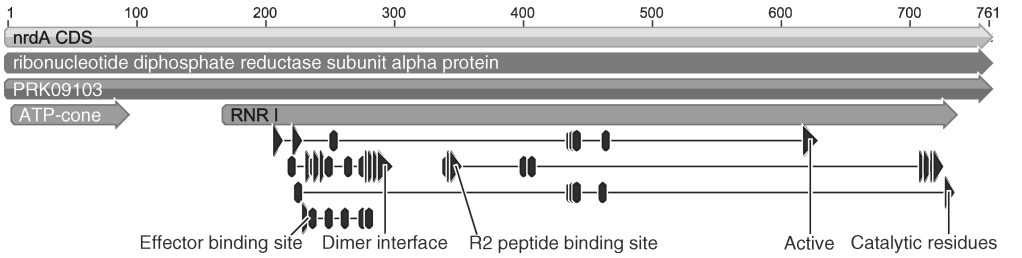

FIGURE 5.2 A gene map of NrdA from *E. coli* (Acc. No. YP_002927204). The putative catalytic residue is Cys-439 and was conserved among all reference sequences and contigs. The extracted region used to create the NrdA phylogenetic tree ranged from Arg-389 to Ile-644 in this sequence. (*See the color version of this figure in Color Plate section.*)

those of reference phage. In this way, sampled genomes are clustered into distinct clades. Incorporation of additional genomes continues to strengthen the clustering within clades; yet, approximately 20% of additional genomes fall outside of existing clades and suggest that many viral families remain unknown (Edwards and Rohwer, 2005). This approach shows promise for the taxonomic analysis of metagenomic sequences since even partial sequence fragments have been experimentally shown to predict phage identity (Edwards and Rohwer, 2005). This is particularly important since next-generation, high-throughput sequencing instruments yield sequences of short read lengths that often prevent the assembly of entire genomes. One example of the utility of this approach comes from an examination of the distribution of marine viruses from four oceanographic regions (Angly et al., 2006). Mapping sample sequences from each of the four regions on the tree revealed that most viral species show geographical specificity (84 species specific to 1 region and 102 found in 2–3 regions), while a minority were ubiquitously present throughout sampling sites (45 species) (Angly et al., 2006).

Therefore, metagenomics has provided a greater appreciation for the high diversity of environmental viruses. Although diversity among phages from environmental samples has been documented by the wide range of morphotypes observed in TEM studies (Williamson et al., 2005; Prangishvili et al., 2006), the diversity of individual genes within a single viral sample is astounding (Breitbart et al., 2007; Polson et al., 2010) and can be appreciated only at the genetic level. Differences in the composition of viral assemblages among locations, the continued emergence of novel viral groups, and the high percentage of novel sequences from viral metagenomic libraries suggest that there is still much to learn.

5.4 METHODOLOGY MATTERS: TECHNICAL CONCERNS FOR VIRAL METAGENOMICS

The two leading methodological concerns in obtaining a shotgun viral metagenome sequence library are (1) obtaining sufficient amounts of viral genomic nucleic acids for subsequent high-throughput sequence analysis and (2) elimination of contaminating cellular genomic DNA. Because of the extraordinary abundance of free virus particles, the shear copy number of a given gene within a nucleic acid sample can be very high; yet, the small size of most viral genomes means that this gene abundance will occur within nanogram to subnanogram amounts of total nucleic acid. Besides the need for certainty that viral metagenome sequences come from only viruses within an environmental sample, concerns about contamination of viral nucleic acid preparations with cellular genomic DNA arise from the fact that a typical bacterial genome contains 40- to 60-fold more DNA than a typical dsDNA bacteriophage or hundreds of

ssDNA viruses. Even small levels of cellular gDNA contamination will saturate a sequence library and eliminate any confidence that the data reflects the gene content within a viral assemblage. Some metagenome investigations have been able to utilize bioinformatic approaches such as k-mer analysis (e.g., tri- and tetranucleotides) to enhance assembly accuracy by prebinning sequences (Teeling et al., 2004; Woyke et al., 2006). These approaches examine "intrinsic" patterns within nucleotide sequences, such as oligonucleotide frequencies, which can show species-specific patterns (Teeling et al., 2004). However, there are no reliable bioinformatic means to separate viral and microbial sequences based on an intrinsic, "signature" characteristic of primary sequence data. Indeed, the few studies that have explored this issue within viral and prokaryotic whole genome sequence data have found that a given signature is often similar between viral and host genome sequences (Pride et al., 2006; Pride and Schoenfeld, 2008). Recent work indicates that dinucleotide relative abundance odds ratios can identify possible contamination of vertebrate genomic DNA within microbial and viral metagenome libraries (Willner et al., 2009); however, these signatures do not have the resolving power to identify and screen microbial gDNA sequences from a viral metagenome library.

Until more powerful bioinformatic methods are developed for segregating viral and microbial sequences, the purity of viral metagenomic libraries will continue to rely upon proper sampling procedures and precautions. A combination of filtration, gradient-based centrifugation, and nuclease treatment of samples has been adopted to remove contaminating microbial cells and DNA (Schoenfeld et al., 2009; Wommack et al., 2009a, 2009b, 2010); however, there are limitations to this method that must be taken into consideration. Samples are often passed through several filters, with the final containing a pore size of 0.2 μm. Although this is sufficient for excluding most bacteria, it is possible for the smallest cells (e.g., Mycoplasmas) (Young et al., 2010) to remain in the sample. Furthermore, phycodnaviruses can have capsid diameters of 200 nm (Brussaard, 2004), and environmental viral particles typically range in size from 25 to 300 nm in diameter (Wommack and Colwell, 2000; Breitbart et al., 2007). In addition, some filamentous phage can exceed 2 μm in length (Thurber et al., 2009). It must be acknowledged that filtering through a 0.2 μm pore size biases the metagenomic library toward smaller viral particles, while larger and filamentous particles are excluded by the filtration process. After centrifugation, contaminating free DNA/RNA is removed via nuclease addition. PCR amplification of treated samples may still yield positive amplification of 16s rDNA even after several rounds of cleanup, though, and is often the result of nuclease-resistant dsDNA. Contamination poses a potentially greater problem for RNA viral libraries since RNA viruses contain RNA in the nucleocapsid structure, and RNase addition may lead to loss of these particles (Thurber et al., 2009).

The presence of multiple nucleic acid types in viral genomes poses a unique concern when preparing a metagenomic library. The different steps required for processing various nucleic acid types have forced researchers to choose which type of library to construct for a given sample. The majority of viral metagenomic libraries are for dsDNA viruses, as viral enumeration using epifluorescence microscopy (EFM) indicates that dsDNA viruses dominate environmental samples (Suttle and Fuhrman, 2010). RNA viruses, in contrast, are small and poorly visualized by EFM, making it difficult to gauge their prevalence in environmental samples (Kristensen et al., 2010; Suttle and Fuhrman, 2010), and they remain understudied due to the technical obstacles involved in RNA analysis. Though several RNA viral metagenomes exist (Zhang et al., 2006; Kapoor et al., 2008; Victoria et al., 2008), most are from medical samples, and only one aquatic RNA viral metagenomic library has been published (Culley et al., 2006). ssDNA viruses share the same fate of poor visualization by EFM (Suttle and Fuhrman, 2010), and knowledge of their abundance remains inadequate.

As with RNA viruses, a single aquatic ssDNA viral metagenome is available (Polson et al., 2010).

Recent methodological advances, however, have made it possible to isolate different viral nucleic acid types from the same sample, thus eliminating the need to choose between viral types (Andrews-Pfannkoch et al., 2010). The procedure relies upon hydroxyapatite (HAP) chromatography. In this method, the column is coated with HAP, $Ca_{10}(PO_4)_6(OH)_2$, a compound that forms a crystalline structure with a fixed pattern of Ca^{2+}, PO_4^{2-}, and OH functional groups on its surface (Gagnon et al., 2009). When a genomic sample is passed through the column, phosphate groups on the DNA/RNA backbone form electrostatic interactions with the HAP calcium ions (Andrews-Pfannkoch et al., 2010). The binding strength between nucleic acid and column is dictated by both the size of the molecule and the number of available phosphate groups (Gagnon et al., 2009). Thus, nucleic acids with a greater number of phosphate groups (e.g., dsDNA) bind more tightly than those with fewer available phosphates, such as ssDNA (Andrews-Pfannkoch et al., 2010). By increasing the phosphate gradient of elution buffer, Andrews-Pfannkoch et al. (2010) were able to isolate ssDNA, ssRNA and dsRNA, and dsDNA from a known mixture in three fractions with high recovery. The efficiency of nucleic acid recovery in this method is especially important considering the difficulty of obtaining viral nucleic acids from environmental samples.

The small amount of genetic material present in a single viral particle poses several problems in the process of creating a library. In addition to the saturation of viral libraries by even small amounts of contaminating cellular nucleic acid, their small genome size makes it difficult to isolate enough genomic material despite high viral concentrations. Containing an average of approximately 10^{-17} g of DNA per phage and an average concentration of 10^7 phage mL^{-1} in seawater, it would require viral extraction from 10 L at 100% efficiency to isolate 1 μg of genomic material. However, the efficiency of recovery is only 10–50%, and so 10 L of seawater typically yields only 20–200 ng of viral DNA (Thurber et al., 2009). In contrast, sequencing of a metagenomic library can require up to 5 μg of genomic nucleic acids. In order to obtain the necessary amount of genomic material, large sample volumes are often required. Concentrating the sample into a reasonable volume necessitates the use of expensive filtration methods, such as tangential flow filtration (Polson et al., 2010). However, concentrating a large sample volume is often not enough to obtain sufficient quantities of nucleic acids, and PCR amplification is required. Two methods have been adopted to amplify gDNA: linker/adapter amplification and multiple displacement amplification (MDA) using Phi29 polymerase (Blanco et al., 1989) (from Bacillus phage phi29; family Podoviridae, genus *Phi29-like viruses*)

Linker/adapter amplification is a method that allows heterogeneous genomic DNA from environmental samples to be amplified *en masse* by PCR. The process involves random shearing of the DNA followed by the ligation of oligonucleotide adapters to the sheared ends. The adapters provide known sequences at the end of each fragment that can be targeted by primers, thus allowing each fragment to be amplified. Adapter-mediated PCR amplification is necessary for linker amplification shotgun library (LASL) production. LASLs are generated by cloning amplified DNA into transcription-free pSMART vectors (Schoenfeld et al., 2008; Thurber et al., 2009) and then sequenced, often using the Sanger dideoxynucleotide method. With read lengths of 600–900 bp (Kennedy et al., 2010), this method produces large contiguous sequences capable of capturing entire genes within a single read. It is also the ideal method for functional metagenomic studies since the clone library allows sequences to be readily retrieved and screened. However, the process is expensive, time consuming, and subject to cloning bias (Thurber et al., 2009). In particular, modified bases can inhibit the cloning process, and inserts may

contain genes that are toxic to the host bacterium (Henn et al., 2010). In a study by Henn et al. (2010) comparing the coverage of four known phage genomes amplified by the LASL method, the authors noted that cloning bias resulted in regions of low or incomplete coverage.

In an effort to overcome the cloning biases introduced into metagenomic libraries by the LASL method, researchers turned to clone-independent methods of amplifying metagenomic DNA samples. One such method uses Phi29 polymerase to amplify genomic DNA prior to sequencing with next-generation sequencing technology. Phi29 polymerase is a strand-displacing polymerase with high processivity (>70,000 nucleotides), and has been successfully utilized for whole genome amplification (Blanco et al., 1989; Schoenfeld et al., 2009). The method allows for the rapid generation of usable quantities of DNA from small starting amounts, and MDA has been used to create metagenomic libraries from various samples, including sediments (Abulencia et al., 2006), oceans (Angly et al., 2006), corals (Yokouchi et al., 2006), the Soudan mine (Edwards et al., 2006), and glacier ice (Simon et al., 2009). However, numerous studies have demonstrated the limitations of this method, including formation of chimeric artifacts and amplification bias, especially of circular genomes (Schoenfeld et al., 2009; Simon and Daniel, 2009). Due to these limitations, ligation of linker adapters to fragmented DNA followed by PCR amplification has become the preferred method for obtaining sufficient quantities of genomic material from environmental metagenomic samples before sequencing with next-generation sequencing technologies (Schoenfeld et al., 2009). The process is becoming increasingly streamlined, as new technologies such as Epicentre's Nextera™ technology promise to fragment, tag, and amplify genomic DNA in a single-tube reaction (www.epibio.com) in preparation for 454 pyrosequencing.

Capable of producing over 1 million reads per 10 h run, 454 pyrosequencing technology provides large amounts of data at reduced cost and time. These advantages, coupled with the longest read lengths of next-generation sequencing platforms (Shendure and Ji, 2008), have made this high-throughput sequencing technology the preferred method of sequencing viral metagenomes (Schoenfeld et al., 2009). In addition, libraries sequenced using 454 technology are never cloned into vectors and do not suffer from the cloning bias observed with libraries created using the LASL method. Henn et al. (2010) compared the coverage of the same genomes examined in the LASL experiment with the coverage of libraries amplified by emulsion PCR and sequenced using 454 pyrosequencing technology. In contrast with the LASL method, sequencing by 454 resulted in coverage of the entire genome, and variations in coverage did not appear sequence related (Henn et al., 2010).

However, pyrosequencing is not without its limitations. Although the ability to obtain sequence data in the absence of cloning circumvents the problem of cloning bias, the lack of a clone library makes retrieving sequences of interest for functional analysis difficult (Schoenfeld et al., 2009). In addition, read length remains a concern for this platform. Metagenomes created with shorter read lengths result in lots of fragmented genes that may not assemble and can go unrecognized by BLASTX searches against the GenBank nonredundant database (Wommack et al., 2008). Thus, the number of unidentifiable sequences within 454 viral metagenomic libraries may be falsely high due to read length limitations. The technology also has a well-documented problem of accurately calling homopolymeric regions, a limitation that cannot be dodged by increasing coverage (Wommack et al., 2008).

5.5 BIOINFORMATIC ANALYSIS OF VIRAL METAGENOME SEQUENCE LIBRARIES

Analysis of sequence data from metagenomic libraries seeks to answer the questions "which individuals are present?" and "what genes are

represented?" The first question relies upon the proper taxonomic assignment of the members of the library, while the second depends on proper gene annotation based on sequence similarity. In the case of viral metagenomic libraries, neither question can be answered in a straightforward manner due to the unique challenges associated with analyzing viral assemblages.

Historically, viruses have been taxonomically classified by the International Committee on the Taxonomy of Viruses (ICTV) (Mayo et al., 2003). This system relies on determining the host range and physical characteristics of the virion including capsid shape, size, structure, genome size, and nucleic acid type (Buechen-Osmond and Dallwitz, 1996). Due to the reliance on physical characteristics for the classification of a virion, taxonomic classification by the ICTV system requires viewing the particle via electron microscopy. This poses a significant problem for metagenomic studies, as identification of phage from environmental samples by electron microscopy is costly, meticulous, and time consuming. As such, many whole genome-sequenced phages in the GenBank database have no ICTV classification. Furthermore, prophage genomes cannot be classified in this manner. Thus, the ICTV classification system is inadequate for the analysis of the taxonomic origin of viral metagenomic reads. Once again, the Phage Proteomic Tree offers a potential solution to the problem. The system relies on genomic data rather than on physical characteristics, making it ideal for metagenomic studies, and could be used to taxonomically classify those genomes in GenBank that lack taxonomic classification. The tree has also been shown to recapitulate aspects of the ICTV system (Rohwer and Edwards, 2002). Thus, the two systems can complement one another and enable researchers to determine the taxonomic origin of metagenomic sequences.

Determining which genes are present in a viral metagenomic library poses a more difficult problem. The proper annotation of reads relies upon sequence similarity to known proteins. With the majority of environmental viral reads containing no homologues, up to 90% in a library may remain unidentified (Huson et al., 2009; Simon and Daniel, 2009; Polson et al., 2010). One reason for this is the divergence between the viral and the prokaryotic versions of many genes. Functional annotation of a sequence is based upon a similarity threshold between sample and database sequences, a threshold that is often not met by viral genes. The second reason that so many reads remain unidentified is the lack of terms for viral-specific proteins from the list of Gene Ontology terms and the SEED database, which are more suited to the annotation of microbial genes (Polson et al., 2010). This is a common bias among databases, and since they form the foundation for annotation pipelines such as MG-RAST, even the annotation of genes with known function may be missed.

5.6 THE NEXT FRONTIER: FUNCTIONAL VIRAL METAGENOMICS

Function-based metagenomics holds great promise for the discovery of novel enzymes. This is particularly true for viral enzymes, which are often more efficient than their prokaryotic or eukaryotic counterparts. A quick perusal of commonly used enzymes within molecular biology reveals the plethora of bacteriophage and other viral enzymes vital to the field, particularly enzymes from the bacteriophage T4 (for a review, see Schoenfeld et al., 2009). The abundance and diversity of viruses make them a promising reservoir for the discovery of enzymes that could impact tools and applications in research and medicine; yet, the divergence of viral genes from reference genes in known databases makes difficult the discovery of novel enzymes by sequence-based analysis.

In contrast, function-based viral metagenomics offers an unambiguous analysis of viral open reading frames (ORFs). The process relies on neither sequence similarity to nor annotation of known genes, but rather on direct

phenotypic screening of expressed viral genes from insert viral metagenomic DNA clone libraries. This approach is particularly advantageous for the discovery of viral enzymes that show little homology with known genes. Shotgun clone libraries are created and then assayed for enzymatic activity. The development of high-throughput technology has made it possible to screen hundreds of sequences at a time. If an enzyme is detected, the insert can then be sequenced and annotated. In addition, the protein product can be expressed, isolated, and purified for further study (Schoenfeld et al., 2009).

As with sequence-based metagenomics, functional metagenomics faces its own set of limitations. The method relies on the production of a functional protein in a foreign host cell, a process that may be hampered by several factors. The random shearing of fragments in shotgun library creation may result in the incorporation of partial ORFs, while only complete genes will be properly expressed (Simon and Daniel, 2009). The ORF must also be capable of expressing from a heterologous promoter on the cloning vector (Schoenfeld et al., 2009). Codon bias, the need for post-translational modification, or toxicity of gene products to the host cell may also hinder expression. These issues can be addressed through the incorporation of rare tRNAs or modification machinery (e.g., chaperone proteins) on a plasmid and by cloning fragments into different host cells. Although *E. coli* has traditionally been the host cell of clonal libraries, other potential host cells are currently being explored, including *Streptomyces lividans*, *Pseudomonas putida*, and *Rhizobium leguminosarum* (Simon and Daniel, 2009).

Metagenomics has only begun to address key issues in viral ecology such as diversity, gene transfer, and population dynamics. Countless environments remain to be explored, and a multitude of novel enzymes that could impact research and medicine are waiting to be found. The ability to segregate nucleic acid types should result in a greater understanding of ssDNA and RNA viruses in the environment. Advancing technology will pave the way, as longer read lengths will enable the sequencing of more full-length ORFs, and bioinformatic tools will become more amenable to viral gene annotation. In this way, metagenomics will provide insight into the microscopic world where phages reign supreme.

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CHAPTER 6

VIRUSES OF CYANOBACTERIA

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CONTENTS

6.1 INTRODUCTION

> Twas brillig, and the slithy nuisance blooms
> did gyre and gimble in the cyanobacteria.
> —(Gillian McDaniel)

Viruses impact all domains of life and algae are no exception. The term algae has no actual taxonomic meaning being only very broad grouping of simple plant-like organisms poetically termed "the grass of many waters" (Tiffany, 1958). Although typically associated with water bodies, the algal group comprises a dizzying array of species found in just about any environment, not just aquatic ones. The simplest and most ancient form of algae is the group known as the cyanobacteria. As with any living entity, cyanobacteria are susceptible to infection by viruses. Viruses that infect higher organisms are simply called viruses. However, viruses that infect bacteria are called bacteriophages or phages (meaning bacteria eating). Because of the bacterial nature of cyanobacteria, their infecting viruses are termed cyanophage. This chapter will focus on the cyanophages. Several excellent reviews have been written both on aquatic viruses and on cyanophages, including an earlier edition of this book (Suttle, 2000b, 2005; Wommack and Colwell, 2000; Mann, 2003). Therefore, this chapter will provide a brief review of previous work with the main focus on more recent findings.

6.1.1 A Little Bit About the Hosts

Cyanobacteria are ubiquitous, primitive, photosynthetic organisms found in aquatic, terrestrial,

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and sedimentary environments throughout the Earth. Cyanobacteria are not actually true plants but are photosynthetic bacteria, or a kind of plant precursor if you like. These organisms have an ancient lineage and are believed to be the first to develop oxygenic photosynthesis. These life forms or their close relatives were responsible for oxygenating the Earth's atmosphere over 3.5 billion years ago leading to an oxidized global ocean 2.1 billion years ago (Rye and Holland, 1998). This long evolutionary history has allowed them to adapt to many types of habitat including extreme environments such as thermal springs (Whitton and Potts, 2000).

Even though the cyanobacteria are relatively simple gram-negative prokaryotes, they range in morphology and lifestyle from simple unicellular forms dividing by binary fission to complex filamentous and branching forms with specialized cell types and complex life cycles, as well as colonial aggregations (Barsanti and Gualtieri, 2006). At present, the classification of cyanobacteria is in a state of flux due to the transition from classification based on physical characteristics to classification that includes molecular markers. Different schemes of classification exist, but it is generally agreed that the oxygenic photosynthetic bacteria, or cyanobacteria, form a single phylum within the *Bacteria*. According to the *Bergey's Manual of Systematic Bacteria* (Boone and Castenholz, 2001), the group is further divided into "Subsections" analogous to order. Many different features were used in the current classification including cell morphology and size, ultrastructure, colony morphology, genetic characteristics (16S and metabolic genes), physiology/biochemistry, culture conditions, and habitat/ecology.

Subsection I (formerly order Chroococcales) consists of the simplest unicellular forms that reproduce by binary fission. This group includes some of the more commonly studied "form-Genera" including *Synechococcus*, *Prochlorococcus*, and *Microcystis*. *Synechococcus* is subdivided into "clusters," which are often cited in the literature to describe the strains being studied. Cluster 1 includes the freshwater strains. Cluster 2 contains the known thermophilic (heat loving) *Synechococcus* strains isolated from hot springs. Cluster 3, formerly referred to as marine cluster C, contains euryhaline strains that can tolerate freshwater or saltwater conditions and are often isolated from estuarine environments. Cluster 5 contains the obligately marine strains that were formerly called marine clusters A and B. Cluster 5.1 (marine A) encompasses the strains containing the antenna pigment phycoerythrin (PE), making them appear shades of pink to red in culture. Cluster 5.2 (marine B) includes the nonphycoerythrin-containing strains, which makes them appear green in unialgal cultures.

Subsection II includes genera reproduced by multiple or repeated binary fission. This group includes the genera *Myxosarcina* and *Pleurocapsa*. Subsection III was formerly called the order Oscillitoriales. At present, this is a known polyphyletic group that requires more study to be fully resolved. Therefore, classification in this group is considered provisional. This subsection includes, but is not limited to, several well-studied form-genera including *Arthrospira*, *Lyngbya*, *Microcoleus*, *Oscillatoria*, *Planktothrix*, *Trichodesmium*, and *Pseudoanabaena*.

Subsection IV.I includes the heterocyst containing genera *Anabaena*, *Nodularia*, *Nostoc*, and *Scytonema*. Subsection IV.II contains only two genera, *Calothrix* and *Rivularia*, which have interesting tapered trichomes and exhibit gliding motility. The last Subsection V (formerly Stigonematales) contains the genera with the highest degree of morphological complexity.

In freshwater systems, there is a high level of morphological diversity including both unicellular and filamentous types of cyanobacteria. In contrast, the unicellular forms of cyanobacteria numerically dominate by far in the oligotrophic areas of the ocean, yet were first observed only in 1979 (Waterbury et al., 1979). Cyanobacteria numerically dominate marine ecosystems with an estimated 10^{24} cyanobacterial cells in the ocean (Barsanti and Gualtieri, 2006). "To put that

in perspective, the number of cyanobacterial cells in the oceans is two orders of magnitude more than all the stars in the sky" (Barsanti and Gualtieri, 2006).

These simple plant-like microbes continue to be vital for the earth ecosystem today. As carbon-fixing autotrophs, they function as the basis of many food webs. In fact, it is estimated that more than one-half of global primary productivity occurs in the oceans; the majority can be attributed to unicellular forms of cyanobacteria (Whitman et al., 1998; Chisholm, 2000). *Synechococcus* types are prevalent worldwide, yet prochlorophytes dominate in the tropical and subtropical ocean where they compose between 25% and 60% of the total chlorophyll *a* biomass (Barsanti and Gualtieri, 2006).

Despite the wide morphological diversity of cyanobacteria, they have the nutritional mode of oxygenic photosynthesis similar to that of land plants (Waterbury, et al., 1986). The linear electron transfer processes (light reactions) of oxygenic photosynthesis are similar in all known plants. The action takes place on three multiprotein complexes embedded within structures called thylakoid membranes. These three protein complexes are photosystem I (PSI), photosystem II (PSII), and cytochrome b_6f ($Cytb_6f$) (Mulo et al., 2009). Working in concert, these complexes utilize solar energy to produce chemical energy (ATP) and reducing power (NADPH) that are subsequently used to synthesize carbohydrates with oxygen as a by-product. All cyanobacteria contain thylakoids, membranous structures containing the photosynthetic apparatus, and all with basic chlorophyll *a* reaction centers. Different species can contain alternative secondary structures and a wide variety of accessory pigments including phycobilins, β-carotenes, and various xanthophylls (Barsanti and Gualtieri, 2006).

Cyanobacteria are not only abundant but also highly metabolically active in the environment. A recently developed, culture-independent research approach examines the activity of differing organisms in the environment based on analysis of the gene transcripts they produce. This method is called "metatranscriptomics." Analysis of the RNA transcripts of a complete microbial community (metatranscriptome) in the North Pacific subtropical gyre supported the supposition that cyanobacteria are very important components of the marine microbial community. These organisms were metabolically very active, constituting 54% of the recognizable gene transcripts, while representing only 35% of the population by cell counts (Poretsky et al., 2009).

Research has conclusively demonstrated that cyanobacteria are prevalent and ecologically important worldwide. Needless to say, cyanophages are also important because of their profound effects on the metabolism and evolutionary trajectory of the cyanobacteria.

6.1.2 Historical Perspectives

Although cyanobacteria are vital components of most ecosystems, some cyanobacteria can grow to excessive populations and/or produce toxins that lead them to be called "nuisance blooms." Cyanobacterial blooms have commonly been problematic in contained freshwater systems such as lakes, streams, and ponds. As a consequence, the first cyanophages to be isolated and studied were those of freshwater cyanobacteria. The isolation of a cyanophage was first reported by Safferman and Morris (1963). Interestingly, this finding lagged behind the discovery of bacteriophages by 50 years (Brown, 1972). Nevertheless, subsequent to their discovery, the ubiquity and ecological importance of cyanobacteria led to much early research on viruses infecting this group.

This initial discovery of a lytic cyanophage was followed by a flurry of discovery of other cyanophages, mostly infecting freshwater filamentous cyanobacteria. This surge in research activity during the 1960s and 1970s was primarily driven by the suggestion that cyanophages could be developed as an ideal algicide for problematic cyanobacterial blooms in lakes and rivers (Brown, 1972; Suttle, 2000a). After

it became clear that cyanophages were not amenable for use as control agents, these investigations were abandoned. In addition, the need for control agents became less urgent in the 1980s with the advent of environmental regulations decreasing nutrient inputs into lakes and rivers with a concomitant decline in troublesome cyanobacterial blooms (Suttle 2000b).

Subsequently, the research focus shifted to cyanophages in the marine environment. A great deal of new research focused on marine viruses after the discovery of profuse numbers of viruses in the ocean (Bergh et al., 1989). Shortly afterward, this research revealed a high abundance of viruses infecting marine cyanobacteria (Suttle and Chan, 1993; Waterbury and Valois, 1993). Since then, a large extent of the research has focused on marine cyanophages, primarily due to the global ecological significance of their hosts. Many cultured phage–host systems were initially based on isolates of marine *Synechococcus*. The ease of cultivation of these cyanobacteria under laboratory conditions was undoubtedly a contributing factor.

At present, research on cyanophage in freshwater systems is experiencing renewed interest, yet it is unclear whether the viral dynamics are similar to those of marine systems. There is some disagreement among researchers as it has been postulated that the mechanisms controlling the two may differ, preventing extrapolation of viral parameters from one system to the other (Middelboe et al., 2008).

Methodological advances have now enabled research to shift from culture-based to environment-based studies. The main advantage of this paradigm shift is that in combining knowledge gained from cultured systems, environmental studies, and mathematical modeling, it may be possible to gain quantitative understanding of viral-mediated processes on a global scale. Concerns over degradation of the environment, as well as global climate changes, have made it more imperative to understand the baseline functioning of ecosystems in order to assess the impact of potential changes.

6.2 TAXONOMY AND CHARACTERISTICS OF CYANOPHAGES

6.2.1 Morphology and Taxonomy

Lytic viruses have now been identified in numerous strains of both prokaryotic and eukaryotic algae. Many cyanobacterial phage–host systems have been isolated and described. Interestingly, the viruses infecting eukaryotic algae are almost exclusively of the nontailed polyhedral morphology (Van Etten et al., 1991). However, viruses of the prokaryotic cyanobacteria, including *Synechococcus* and *Prochlorococcus*, are very similar to bacterial viruses and have been observed to belong to one of the three well-recognized bacteriophage families, namely, Myoviridae, Siphoviridae, and Podoviridae (Safferman et al., 1983), which comprise the order Caudovirales. It appears that cyanophages are an ancient group, most likely predating the separation of bacteria and cyanobacteria since cyanophages form a monophyletic group with other tailed bacteriophages, meaning they are taxonomically related (Suttle, 2000b). Although morphology alone is of limited phylogenetic meaning, this classification scheme for cyanophages remains in general use.

An icosahedral capsid and a contractile tail, which is separated from the capsid by a neck structure, are characteristics of Myoviridae. Siphoviridae have long noncontractile tails while Podoviridae have short noncontractile tails (Figure 6.1). Early characterization and comparisons of *Synechococcus* cyanophage isolates demonstrated a typical genome size range for myoviruses of 80–85 kb and a slightly larger size range of 90–100 kb for siphoviruses (Wilson et al., 1993). An analysis of their structural proteins revealed similarities between phages of the same morphological class and clear differences between the two general types. To date, the large majority of lytic *Synechococcus* and *Prochlorococcus* phages are the myovirus type possibly because myoviruses may be easier to isolate than podo- and

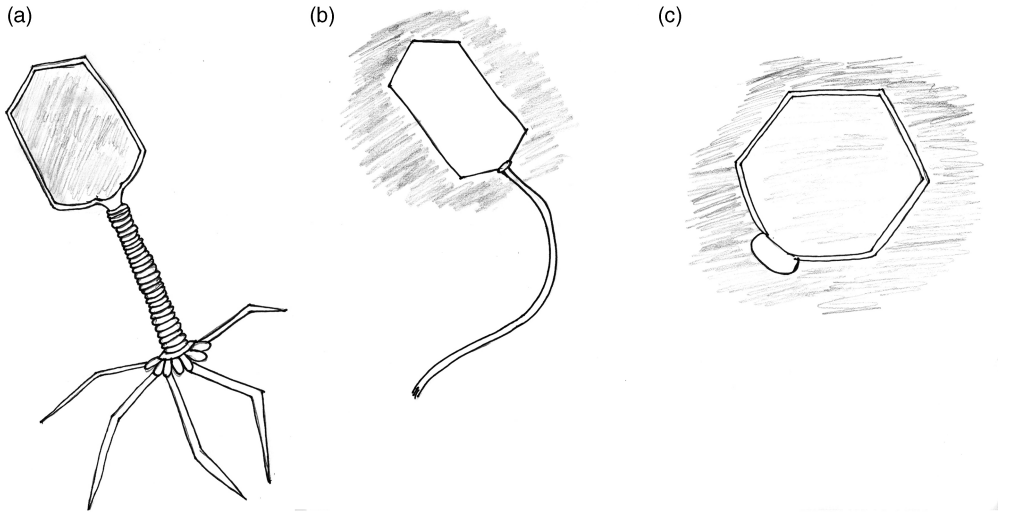

FIGURE 6.1 Three morphological types of phage: (a) myovirus, (b) siphovirus, and (c) podovirus.

siphoviruses (reviewed in Mann, 2003). As with the findings of most culture-based studies, these findings may not be representative of the pattern that exists naturally.

A general rule is used when naming a new cyanophage isolate. Generally, the phage is given a set of initials that correspond to the host organism, the area of isolation, the virus morphology, and an isolate number. For example, P-SSM2 means *Prochlorococcus* host, Sargasso sea, myovirus isolate 2 (Sullivan et al., 2003) and SPGM99-01 for *Synechococcus* phage, Gulf of Mexico 1999 isolate 1 (McDaniel et al., 2006). Although this convention is not always followed, it does help give the names meaning.

6.2.2 Characteristics

Besides morphology, there are some other measures of the viral life cycle that are important to know in order to extrapolate viral impacts to an ecosystem as a whole. These parameters include total viral abundance (biomass), viral production rates, decay rates, and burst size.

Cyanophages cannot be enumerated directly from natural samples by microscopy because they are only a subset of a complex viral community. However, they can be quantified as a subset of the total marine viral community either by plaque assay or by performing a dilution series of natural seawater, addition of a susceptible cyanobacterial host, and quantification using a most probable number (MPN) program. The MPN technique has been widely used for many algal types. However, both these techniques are limited in that detection is confined to infective viruses; furthermore, only those that are infective to the specific host are used for enumeration. Even when more than one host strain is used for detection, it cannot be determined whether the phage titers are additive or overlapping. Despite these limitations, this method has demonstrated a similar level of precision to microscopy and plaque assay techniques (Cottrell and Suttle, 1995).

Viral production rates can be calculated using one of the two methods. The first is based on increases in viral abundances in samples with many of the ambient viruses removed by a filtration method. The second approach is by determination of the proportion of infected cells in a natural population of microbes (Figure 6.2), which is called the frequency of visibly infected cells (FVICs) method (Weinbauer and Suttle, 1999; Paul, 2001; Weinbauer

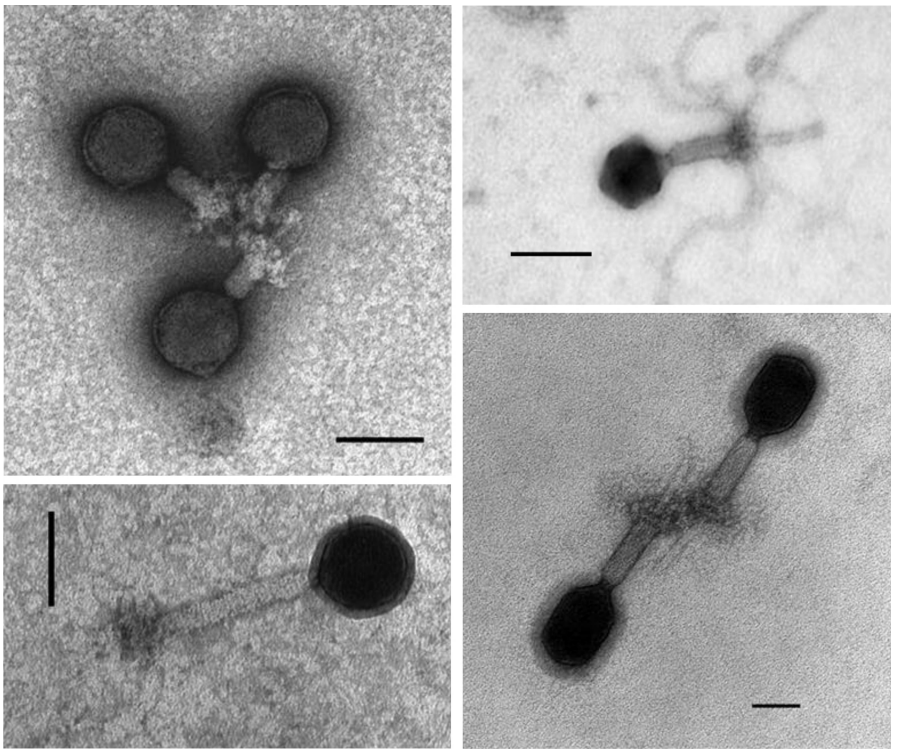

FIGURE 6.2 Several electron micrographs of typical lytic cyanomyoviruses. The scale bars equal 100 nm. Note the variability in size.

et al., 2003). Both methods have advantages and disadvantages, but the FVIC method has a commonly cited limitation in that it cannot be used for cyanophage production in a natural population since the identity of the bacterial host is not determined. Although cyanophage production rates have not been studied specifically, total viral production rates that have been measured to date are comparable in freshwater and marine environments and range from 10^8 to 10^{11} viruses liter^{-1} day^{-1} (reviewed in Wilhelm and Matteson, 2008). Because cyanobacteria are a component of the total population, it is likely that their viral production rates are constrained within this range.

Notice in Figure 6.3 that the viral particles are clearly visible within the cells. In most cases, the number of viruses may be easily counted and the average number of viruses over all the replicates for the sample gives the average burst size for that environment. It is important to note that some larger microbial cells become so completely filled with viruses that it is impossible to enumerate them accurately. This leads to potential error and often underestimation of actual viral production rates.

Similar to viruses infecting aquatic heterotrophic bacteria, ultraviolet (UV) radiation appears to be the main mechanism of deactivation of cyanophage (Suttle et al., 1993). However, adsorption to particles and colloids in seawater may play a role as well (Noble and Fuhrman, 1997). Research has demonstrated that natural *Synechococcus* cyanophage communities tend to be more UV resistant in the summer than the spring or winter, while laboratory cyanophage isolates from the same community did not exhibit this pattern (Garza and Suttle, 1998). A similar pattern was observed in freshwater cyanophages, where season and

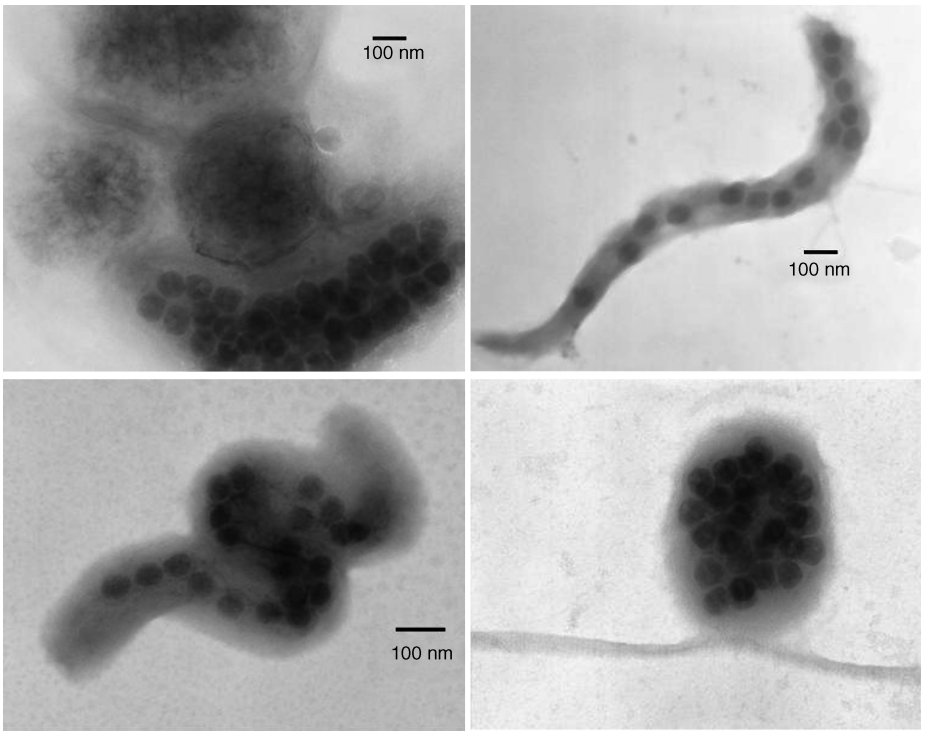

FIGURE 6.3 Examples of visibly infected bacteria from the FVIC method. Note the intact virus particles within the cells. The viruses can be enumerated and the average number for each sample gives the average lytic burst size for the area sampled.

water depth were the principal factors influencing decay (Cheng et al., 2007). In addition, cyanophage isolates tested were more UV resistant than heterotrophic bacteriophage isolates, while natural viral communities were more resistant than laboratory isolates (Weinbauer et al., 1999). In general, many researchers assume viral decay rates are comparable to production rates since total viral abundance for a given environment tends to be relatively stable over short timescales.

Burst size is the number of viral particles released per cell after the completion of a lytic cycle. Burst size is a key parameter in calculating the viral production rate using the FVIC method and varies widely by environment. Again, environmental burst size values for cyanophage have not been reported. Nevertheless, burst size estimates for studies of natural freshwater bacteria range from 28 to 40 viruses host cell^{-1} and for marine environments estimates range from 20 to 25 viruses host cell^{-1} (Wilhelm and Matteson, 2008). As with viral production, these estimates constitute average numbers from all microbial cells contained within the sample, including cyanobacteria, and a presumption is made that these ranges may be similar for cyanophage.

Viral biomass, viral production rates, burst size, and percentage of infected cells may vary somewhat between freshwater and marine systems. However, research to date indicates these parameters are more closely correlated with trophic status than with salinity (Wilhelm et al., 2006).

The host range of cyanophages is not tied to the geographical location of isolation and is highly variable between phage isolates (Mann, 2003). The cyanomyoviruses tend to have a broad host range with some strains able to infect cyanobacteria from their sister genus *Prochlorococcus* suggesting a potential

mechanism for gene transfer (Sullivan et al., 2003). Although some cyanophages have a generally broad host range, marine cyanophages may not readily infect freshwater cyanobacteria (Suttle and Chan, 1993). In contrast, the cyanopodoviruses and cyanosiphoviruses appear to be very host-specific, usually only infecting their host of isolation (Mann, 2003; Stoddard et al., 2007; Wang and Chen, 2008).

The main hypothesis to date for explaining the variability in cyanophage host range has been that there is some physiological cost to the host organism for resistance to viruses, so there will be a mixture of sensitive and resistant hosts. Most bacteriophages, including cyanophages use some sort of cell surface structure as a receptor site in order for the virus to attach and infect the host cell. Changing these receptor molecules so that the phage cannot attach is a major mechanism of resistance, but the fact that these receptor molecules serve a biological function means that their modification may affect the host ability to interact with the environment, such as the ability to absorb nutrients (Stoddard et al., 2007). It has been demonstrated experimentally that development of resistance to cyanophages leads to decreased maximal growth rates in about half the cyanobacterial strains tested (Lennon et al., 2007).

An interesting difference between cyanophages and viruses of heterotrophic bacteria is the requirement for the energy produced by photophosphorylation by the photosynthetic host for viral replication (reviewed in Suttle, 2000a; Lindell et al., 2005). One example of an experimental method that demonstrated the viral requirement for active photosynthesis was using disruption of photosynthesis by inhibitors such as DCMU, CCCP, valinomycin, nigericin, or lack of CO_2 to completely inhibit viral replication in *Synechococcus* (Sherman, 1976).

In the marine cyanobacteria that have been studied, light plays a key role during the entire phage life cycle. Phage adsorption, replication, host metabolic effects, and viral survival after lysis all have light-dependent features (reviewed in Clokie and Mann, 2006).

6.3 ECOLOGY OF CYANOPHAGES

6.3.1 Lytic Cyanophages

Lytic viruses are those that infect their host organism, immediately usurp the host metabolism, and initiate a cycle of replication leading to lysis and death of the host. Viral-induced lysis contributes significantly to nutrient and energy cycling, especially in nutrient-limited settings. Some attempts have been made to quantify the amount of viral-induced lysis occurring in the environment in order to constrain global models of nutrient and energy cycling. At present, estimates vary considerably due to lack of reliable experimental methods for most viral parameters. Nonetheless, the generally accepted range for marine environments is that viral-induced lysis removes 20–40% of the prokaryotes each day, which includes cyanobacteria (Suttle, 2005).

Lytic cyanophages are an abundant component of natural seawater and are readily isolated from most marine environments. Their concentration measured by the MPN method generally varies from $10^2\,mL^{-1}$ of seawater to around $10^5\,mL^{-1}$, generally increasing with temperature, host abundance, and salinity (Suttle and Chan, 1994; Lu et al., 2001). Of course, the titer of cyanophages depends on the host organism used for detection (Millard and Mann, 2006). The highest reported abundance of *Synechococcus* phages by this method is $10^6\,mL^{-1}$ (Mann, 2003).

Although the abundance of cyanophages does tend to remain relatively stable from one day to the next under a particular environment, it can demonstrate temporal variation over different timescales. A study of the distribution of cyanophages in the Red Sea showed that they are at the maximum abundance during the late summer and at a depth of 30 m (Millard and Mann, 2006). There can also be variability over

short timescales, at least in surface waters, with the highest abundances measured at night (Clokie et al., 2006). This is consistent with the known dependence on light for initiation of infections and typical known latent periods. Cyanophage have also been isolated from sediments where the most likely source is attachment to sinking particles (Suttle, 2000b). These particles often remain infective over a long time.

As mentioned earlier, most isolated lytic cyanophages from the marine realm are cyanomyoviruses where the dominant *Synechococcus* strains are the pink/red phycoerythrin-containing strains (marine cluster 5.1 or marine A) (McDaniel et al., 2006). Interestingly, other viral types have been readily isolated from estuarine environments where the green phycocyanin containing *Synechococcus* dominates (marine cluster 5.2 or marine B) (Wang and Chen, 2008).

This is not necessarily the case in freshwater environments, possibly due to the wider variety of host strains. A recent study of lytic cyanophages from three freshwater strains *Microcystis*, *Anabaena*, and *Planktothrix* found differing morphologies of cyanophage, including a siphovirus and the first described filamentous cyanophages (Deng and Hayes, 2008). Most of these viruses were able to infect multiple host organisms, with two of the cyanopodoviruses having the widest host ranges.

The study of algal viruses has demonstrated that these viruses can affect community composition and resistance, like viruses of heterotrophic organisms (Tarutani et al., 2000). An important component of the interactions between lytic viruses and their cyanobacterial hosts is that they coevolve. A study of long-term growth of *Plectonema boryanum*, a filamentous cyanobacterium, demonstrated that the host will develop resistance to the lytic viruses and that rapid adaptation of the virus leads to a persistent low-grade viral infection with elimination of sensitive host organisms (Colishaw and Mrsa, 1975). A similar phenomenon was also observed in the toxic bloom-forming species *Heterosigma akashiwo* (Tarutani et al., 2000). This is one of the primary reasons for the lack of success in using cyanophage to control cyanobacterial bloom; the hosts simply become resistant to the viruses in a very short time.

Nevertheless, the idea of using cyanophage as a biocontrol agent for toxic blooms of cyanobacteria is not completely defunct. A cyanophage (F1) was recently isolated that demonstrated lytic activity in the laboratory against the nuisance bloom species *Anabaena flos-aquae* (Wu et al., 2009). In addition, a PCR-based assay for detection of the toxin-producing species *Microcystis aeruginosa* and its lytic cyanophages has been developed. This assay has been tested in the environment and is intended for studying virus/host dynamics in a natural setting (Takashima et al., 2007; Yoshida et al., 2008). Although, as far as we are aware, no cyanophage has been successfully utilized in this way, some researchers still remain optimistic.

6.3.2 Temperate Cyanophages

Unlike lytic phage, some phages do not necessarily cause lysis and mortality of their host organism. If conditions are not favorable for a sustained lytic infection, they can become dormant within the host cell. Many phages do this by integrating into the host chromosome or other replicon, and in this form the virus is termed a prophage. The phage is then replicated along with the host during each cell division and can excise itself when conditions are more favorable for lytic viral production.

This process involves a high level of coevolution between the phage and its host and, of course, comes with specialized terminology to describe it. Phages with the capability to select between a lytic and a nonlytic lifestyle cycle are appropriately called temperate phages, and the entire process is called lysogeny. When a phage is quiescent, it is termed a prophage, and the process of excision with initiation of a lytic

cycle is called prophage induction. Also, since the phage and the host are so highly coevolved, temperate phages are generally more host specific. In addition, the siphovirus morphology is historically associated with temperate phage (Ackermann and DuBow, 1987).

One of the most important facets of lysogeny is that the integrated prophage can cause profound changes in the host phenotype; this process is called conversion (Paul, 2008). The phage-encoded genes sometimes confer upon the host resistance to antibiotics, allow the hosts to expand into another niche, or improve their fitness in some other way. Such genes are not essential for the phage life cycle and are often referred to as "fitness factors" (Brüssow et al., 2004), or more amusingly as "morons," an acronym for more DNA (Paul, 2008).

Lysogeny in cyanobacteria was initially demonstrated in the cultured freshwater cyanobacterial strain *Plectonema boryanum* (Cannon et al., 1971; Padan et al., 1972). The prophage of this cyanobacterium was inducible both by the common inducing agent mitomycin C, which causes direct DNA damage, and by elevated levels of heat (Rimon and Oppenheim, 1975). Similar to lytic infection by *Synechococcus* cyanophages, photosynthesis was required for induction of cyanophages in lysogenic *Plectonema* isolates (Cochito and Goldstein, 1977). Other compounds have also been implicated as triggers for induction in cyanobacteria. For instance, the freshwater strain *Anacystis nidulans* has demonstrated induction of a prophage in response to copper (Lee et al., 2006).

A more recent study screened 19 phycocyanin-rich (green) strains of freshwater *Synechococcus* for the presence of prophage induction (Dillon and Parry, 2008). This study found high levels of lysogeny with 16 of the 19 strains being inducible with much higher concentrations of the inducing agent mitomycin C than are commonly used. All the induced cyanophages were siphoviruses with low infectivity to alternative hosts, which is typical of most known temperate phages. The researchers concluded that a high level of lysogeny in freshwater cyanobacteria was likely. However, the strains used were not axenic, and their methods were based solely on electron microscopy images rendering their conclusions somewhat speculative.

Some cultured marine cyanobacteria have been documented to have inducible prophage. For example, the marine filamentous nonheterocystic cyanobacterium *Phormidium persicinum* was demonstrated to be inducible with mitomycin C (Ohki and Fujita, 1996). The filamentous cyanobacterium *Trichodesmium*, which also plays an important role in marine environments as a major nitrogen-fixing organism, was reported to have inducible prophage (Ohki, 1999).

Prophage induction in the unicellular *Synechococcus* type has also been reported. An inducible prophage was described that infected the cyanobacterial strain NKBG 042902 (Sode et al., 1994). In this case, the phage was isolated from natural marine samples and was used to lysogenize the cultured marine *Synechococcus* host. Prophage induction was subsequently observed in the laboratory-infected host in response to UV light, mitomycin C, and $CuSO_4$ (Sode et al., 1994, 1997).

Prophage induction in cyanobacteria has also been documented in nonculture-based experiments. Natural populations of marine *Synechococcus* have been found to be inducible by mitomycin C, and cyanophage induction was associated with the winter months and areas of low productivity (McDaniel et al., 2002; Ortmann et al., 2002; McDaniel and Paul, 2005; Long et al., 2008). Mitomycin C could as well stimulate viral production artificially and prophage induction was implicated as a probable mechanism of bloom collapse of the cyanobacteria *Lyngbia* (Hewson et al., 2001).

Unfortunately, unlike heterotrophic bacteria, a model system for the study of lysogeny in cyanobacteria has not been established. An ideal system would include an axenic culture of both a lysogenized and uninfected host, as well as isolated induced cyanophages, so each individual component and the interactions within the system could be studied in detail.

6.4 MOLECULAR ECOLOGY OF CYANOPHAGES

Many researchers have shifted to nonculture-based nucleic acid sequencing methods due to both the inability to culture many marine bacteria and the bias associated with culturability. For most bacteria, there are conserved metabolic genes that allow genetic comparisons between uncultured strains. Not so with viruses. Studying any type of virus in the environment is challenging due to the lack of a universal genetic marker. As a result, many environmental viruses have been sequenced in the quest for group-specific genes to use for environmental studies.

As of this writing, a search of the National Center for Biotechnology Information (NCBI, or GenBank, http://www.ncbi.nlm.nih.gov/) and the Joint Genome Institute Integrated Microbial Genomes (JGI, http://imgweb.jgi-psf.org/cgi-bin/w/main.cgi?page=home) web sites yielded 11 complete cyanophage genomes out of 2981 complete viral genomes. Clearly, cyanophages are underrepresented in the databases, yet the shift from culture-based to sequence-based methods for investigation of cyanophage communities has still thrown up a few interesting surprises.

6.4.1 Structural Genes as Potential Cyanophage Markers

An early discovery was the presence of a module of structural genes in cyanomyophages that was analogous to *Escherichia coli* phage T4 (Hambly et al., 2001). This module contains several structural genes including the capsid portal protein gene termed g20. This gene was originally touted as a universal marker for cyanophage and was utilized for several studies of environmental cyanophage diversity (Fuller et al., 1998, 1999; Zhong et al., 2002; Wilhelm et al., 2006). Questions have arisen, however, about its suitability as a cyanophage marker gene since it is not identifiable in all cyanophages, the distribution of the genes varies, and some environmental g20 sequences may not be from cyanophages at all (McDaniel et al., 2006; Wilhelm and Matteson, 2008). Freshwater cyanophages appear either to be lacking highly similar structural genes or are genetically divergent. For example, 17 cyanophage isolates infecting the filamentous strain *Nodularia spumigenea* from the brackish Baltic Sea were found to contain the g23 structural gene. However, the sequences were divergent from previously sequenced g23, forming their own closely related group (Jenkins and Hayes, 2006). Interestingly, in this group of phages the presence of the gene did not correlate at all with morphology since all three morphological types, myoviruses, siphoviruses, and podoviruses were represented among the isolates even though they carried very similar g23 sequences. A current screening of 35 freshwater cyanophages was able to identify only capsid protein genes g20 and g23 from 12 of the isolates (Deng and Hayes, 2008). In addition, the phylogeny of g20 sequences were found to be not correlated with either cyanobacterial host or habitat of isolation (Sullivan et al., 2008). Nevertheless, in cyanophages that do contain the g20 gene, expression of the sequence has shown utility in monitoring the dynamics of cyanophage infection cycles (Wharam et al., 2007). Thus, the ability of using either g20 or g23 as a marker is fraught with inconsistencies and the search continues for a useful cyanophage marker gene.

6.4.2 Cyanophage Carriage of Photosystem Genes

Analysis of sequenced cyanophages uncovered that many cyanophages carry vital photosystem II genes, including the structural proteins D1 (*psbA*) and D2 (*psbB*) (Mann et al., 2003; Bailey et al., 2004) and a high-light-inducible protein (*hli*) (Lindell et al., 2005). One sequenced cyanomyovirus also contained genes for the photosystem electron transport proteins plastocyanin (*petE*) and ferredoxin (*petF*) (Lindell et al., 2004). The cyanophage *psbA* sequences have proven to be useful marker genes with genes from different hosts as well

as freshwater and marine cyanophages having distinct evolutionary lineages (Chenard and Suttle, 2008; Wang et al., 2009).

The PSII protein D1, encoded by gene *psbA*, participates in the highly oxidative "water splitting" reaction of photosynthesis and thus is subjected to significant photodamage (Mulo et al., 2009). It has been estimated that during active photosynthesis, the D1 protein is degraded and replaced every 20 min (Mulo et al., 2009). Since this gene was historically considered a host-associated metabolic gene, it was initially very surprising to find it being carried by a cyanophage.

It was originally hypothesized that cyanophages carry photosynthesis genes in order to allow continuation of light reactions to facilitate the production of energy for the viral lytic cycle (Bailey et al., 2004). Hellweger (2009) performed *in silico* modeling experiments to investigate this question. The model demonstrated that the extra photosystem genes were not beneficial at low levels of light, but rather increased fitness of the cyanophage population at high levels of light. Furthermore, the model predicted the effect of increasing gene copy number would enable higher viral production rates in shallower depths.

A research study that examined the expression dynamics of both host and cyanophage genes during a lytic cycle gave support to this premise (Lindell et al., 2007). Several cyanophage gene transcripts related to energy generation and nucleotide metabolism, including the *psbA* and *hli* genes, were produced at high copy numbers. Interestingly, several host genes were also upregulated in comparison to the noninfected host during the cyanophage lytic cycle, a trend that is not observed in the well-studied heterotrophic system using the related *E. coli* phage T4 (Luke et al., 2002) where in most cases, transcription of host genes is completely abolished during lytic infection. These findings suggest that cyanophages use host genes for their own life cycles providing tantalizing glimpses of evolution in progress.

The function of cyanophage photosystem genes was also studied in some detail using the cultured phage/host system comprised of cyanophage S-PM2 and its *Synechococcus* host strain WH 7803 (synonyms: CCMP1334, DC2, NEPCC549) (Shan et al., 2008). The strain WH 7803 is a member of marine cluster 5.1 (formerly A), meaning it contains the phycoerythrin antenna pigment making it appear pink to red in culture. During lytic infection, the overall content of PE per cell and per phycobilisome was observed to increase (Shan et al., 2008). In addition, the total cellular content of chlorophyll *a* was increased. This phage-induced increase in light harvesting capacity could potentially meet the increased energy demands of cyanophage synthesis.

A comprehensive analysis of the distribution and evolution of cyanophage photosystem genes was performed using a collection of 33 cultured cyanophages with known morphology and host range, as well as published data on 9 additional cyanophages (Sullivan et al., 2006). The *psbA* gene was observed in 88% of the cyanophages, while 50% had both *psbA* and *psbD*. All cyanomyoviruses and *Prochlorococcus* podoviruses contained *psbA*, but it was not identified in *Synechococcus* podoviruses or any cyanosiphovirus. The authors performed phylogenetic analyses demonstrating that significant genetic exchanges have occurred from host to phage, phage to host, and between phages. These types of exchanges were suggested to be an active mechanism for photosystem evolution. In fact, this active evolution of *psbA* and *psbD* genes has been documented by analyzing genetic microdiversity between cyanophage isolates (Marston and Amrich, 2009).

However, the situation may differ in freshwater cyanophages. Two sequenced T7-like cyanopodophages, Pf-WMP3 and Pf-WMP4, isolated using the freshwater cyanobacterium *Phormidium foveolarum* were quite divergent and did not contain identifiable psb genes (Liu et al., 2007, 2008).

Although the *psbA* gene appears to be nearly universal in cyanomyoviruses, a recent analysis of three cyanopodoviruses and three cyanosiphoviruses isolated from a *Synechococcus* host

in an estuarine environment revealed that the gene was present in the podoviruses, but not in the siphoviruses (Wang and Chen, 2008). In addition, the podovirus *psbA* sequences formed a distinct cluster that included many marine environmental sequences, suggesting that *Synechococcus* cyanopodoviruses might be more prevalent in marine and estuarine habitats than originally surmised.

A metagenomic study of marine viruses from the Chesapeake Bay also recovered characteristic podovirus *psbA* and *psbB* sequences corroborating the contention that cyanopodoviruses may be more prevalent in the marine environment than originally suspected (Bench et al., 2007). Freshwater and marine cyanophages can also be separated into distinct groups based on their psb sequences (Wang et al., 2009).

The first cyanophage of the siphovirus type, isolated on the host marine *Prochlorococcus*, was recently completely sequenced (Sullivan et al., 2009). This cyanophage, designated P-SS2, was observed to have a larger than average genome of 108 kb in length. In addition, it was found to be genetically very different from other sequenced cyanophages in that it did not contain any of the commonly observed photosystem genes. Also, the structural genes that P-SS2 possessed were so divergent that most of them could not be identified on the basis of the nucleotide sequences, instead having to be documented experimentally from viral protein extracts. The researchers also surmised that it might be a temperate cyanophage since it contained many of the genes commonly associated with integration (Sullivan et al., 2009). Having this sequence information may now make it possible to determine the prevalence and ecological importance of environmental cyanosiphoviruses and the potential to investigate lysogeny in *Prochlorococcus*.

Most of the information on cyanophage photosynthesis genes was initially based on data from cultured cyanophages, which generally contain double-stranded DNA and range in size from 100 to 200 kb in size (Mann et al., 2003). Two studies of environmental virus DNA separated by size using pulsed field gel electrophoresis demonstrated the presence of cyanophage genes in uncultivated viral samples with a genome size ranging from 28 to 380 kb in length (Sandaa and Larsen, 2006; Sandaa et al., 2008). These studies were performed in a higher latitude location (60° N) than most previous studies, indicating either a differing view of diversity from that in lower latitudes or perhaps a bias from culturing.

6.4.3 Metagenomic Studies

Another recently developed tactic for studying uncultured microbes and viruses is called metagenomics. This technique essentially involves isolating purified nucleic acid, either DNA or RNA from an environmental sample of interest, and sequencing it directly without a culturing step (Committee on Metagenomics: Challenges and Functional Applications, 2007). Such samples can be selected for a desired population or type in many different ways such as size selection by filtration. Traditionally, these samples were cloned prior to sequencing, but a newer method called pyrosequencing is becoming more prevalent because the nucleic acids can be sequenced directly from the sample of interest (Margulies et al., 2006).

Metagenomic studies have confirmed and highlighted the ecological importance of cyanophages, illustrated by their identification as a dominant component of marine viral metagenomes from different laboratories and widely separated environments (Bench et al., 2007; McDaniel et al., 2008; Williamson et al., 2008). One ambitious example of the metagenomic method was the Global Ocean Sampling (GOS) expedition that gathered multiple microbial metagenomes from the surface ocean in a transect from the North Atlantic to the equatorial Pacific (Rusch et al., 2007). An analysis of the GOS data revealed that approximately 60% of the identifiable *psbA* genes in the surface ocean originated from cyanophages (Sharon et al., 2007). These researchers went a step further and collected some RNA from a

microbial sample from the Mediterranean and verified that the viral *psbA* sequence was present; they thus verified that the viral psb is actively functioning in the environment. Extrapolation of this figure to the global ocean implies that possibly 10% of total global photosynthesis could be attributed to *psbA* genes from phage (Rohwer and Thurber, 2009).

A more recent analysis of viral metagenomes indicated that besides photosystem II genes, such as the psb's, many cyanophages may also carry PSI genes (Sharon et al., 2009), and in that study the viral PSI genes were arranged in a distinct cluster suggesting a different functionality from host PSI. These researchers performed structural analysis of the deduced structure of the cyanophage-generated PSI, which indicated that the phage-produced photosystem could drive the host to perform cyclic photosynthesis causing production of excess ATP at the expense of the reductant that could be used for CO_2 fixation. This excess ATP would be an obvious benefit for a lytic phage, being required for the viral lytic cycle.

Besides photosynthesis, another way cyanophages influence host metabolism and evolution is by the carriage of phosphate metabolic genes. In many aquatic environments, phosphate is a limiting nutrient so organisms need efficient mechanisms for uptake and reuse of a variety of phosphorus-containing compounds, both organic and inorganic. A case in point is the unicellular cyanobacterium *Prochlorococcus* that exists in extremely oligotrophic areas of the ocean. It has been observed in this organism that the genomic content of phosphate utilization genes is linked to the environmental conditions under which the isolate was found, rather than its phylogenetic affiliation (Martiny et al., 2009). Many of these phosphate utilization genes were found in genomic islands, suggesting they may have been acquired from cyanophage (Coleman et al., 2006). Similar to heterotrophic bacteria, these researchers found that most of the strain-specific differences among *Prochlorococcus* strains were attributable to genomic islands.

6.4.4 Cyanophages as a Genetic Reservoir

The host metabolic genes carried by cyanophages and viruses in general are not limited to photosynthesis or phosphate utilization (Coleman et al., 2006; Lindell et al., 2007). The analysis of viral metagenomes has illustrated the capacity of viruses to function as gene reservoirs, allowing host adaptation to new niches is a prevalent and vastly underestimated phenomenon (reviewed in Rohwer and Thurber, 2009). Viruses were expected to carry genes for nucleotide synthesis and metabolism, yet there was also a high prevalence of genes involved in carbohydrate and protein metabolism. Indeed, many stress response genes were more prevalent in the viral metagenomes than their corresponding microbial metagenomes (Dinsdale et al., 2008).

The reason why cyanophages carry so many host genes is unknown. However, a recent comparative analysis of five cyanomyovirus genomes related to bacteriophage T4 may shed some light on the mechanisms of how this acquisition of host genes is accomplished. The analysis revealed a "core genome" of 64 shared genes (Millard et al., 2009), but more importantly, a common hyperplastic area was observed in the genomes containing many host-like metabolic genes. This discovery confirms previous findings of coevolution between cyanophages and their hosts and may provide the mechanism for this unique evolutionary process in cyanomyophages.

6.5 SUMMARY

Lytic cyanophages are an abundant component of aquatic ecosystems where they play important roles in nutrient cycling and as drivers of genetic diversification. The participation of cyanophage in global geochemical cycles is undisputed but has not been accurately quantified.

The idea of using cyanophage as a biocontrol agent is neither new nor unique to the

control of cyanobacteria and other nuisance blooms. The concept of phage therapy has been suggested for applications as divergent as wound care, aquaculture, and the treatment of coral reef disease (Nakai and Park, 2002; Skurnik and Strauch, 2006; Efrony et al., 2007). Nonetheless, phage therapy is predominantly successful using mixtures of more than one lytic phage under tightly constrained conditions. That being the case, this method is unlikely to be applicable to open natural systems.

Lysogeny has been documented in cyanobacteria, yet the topic remains sparsely studied. In natural systems, the presence of inducible cyanoviral prophage is associated with winter and low productivity environments. Recent sequencing of a possibly temperate cyanosiphovirus may provide some insight into this area, but a complete phage host system for the detailed study of lysogeny in cyanobacteria remains lacking.

At present, the shift in research methodology has demonstrated that the main role of viruses in cyanobacteria is not primarily as "pathogens" but as reservoirs of genes and drivers of evolution. Cyanophage have been conclusively demonstrated to be carriers of host metabolic genes including those involved in photosynthesis and phosphate uptake. Metagenomic studies suggest that cyanophages are very important in aquatic systems and the carriage of host metabolic genes is a strong driver of evolution. Devising ways to integrate these findings into quantitative models of global processes remains one of the greatest hurdles to be overcome by future research.

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CHAPTER 7

VIRUSES OF EUKARYOTIC ALGAE

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CONTENTS

7.1 INTRODUCTION

Eukaryotic algae are a group of oxygen-generating, photosynthetic organisms that include seaweeds (not covered in this chapter) and a large diverse group of microorganisms generically referred to as microalgae. In many aquatic ecosystems, particularly the ocean, algae are the starting point of food chains, where they fix carbon in the form of CO_2 and convert it into lipids, sugars, and carbohydrates. These generated fixed carbon compounds are passed through the food chain and sustain the oceanic ecosystem. A by-product of this global photosynthetic process is the production of 50% of the oxygen on the planet; looked at another way, every other breath we take comes from the algae in the ocean. Adsorption of light energy by algae in the ocean is considered one of the great engines of planetary control [*sic*] (the other is the adsorption of heat energy by the ocean). Sticking with this analogy, viruses that infect the algae would be considered the lubricants of this engine. If you do not lubricate an engine, it will seize; precisely the same would happen in the global ecosystem if algal viruses were not present. About a quarter of

Studies in Viral Ecology: Microbial and Botanical Host Systems, Volume 1, First Edition. Edited by Christon J. Hurst.

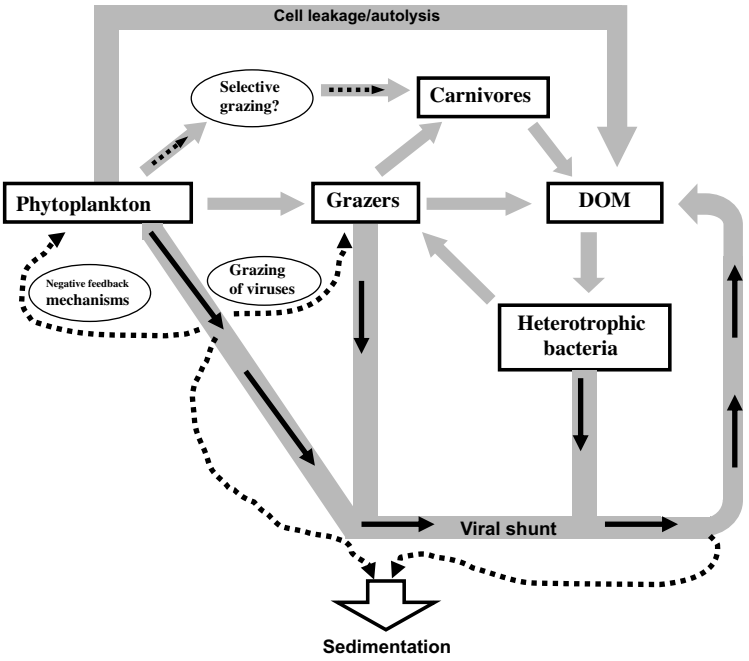

FIGURE 7.1 The virus shunt (adapted from Wilhelm, S. W. and Suttle, C. A. (1999). Viruses and nutrient cycles in the sea: viruses play critical roles in the structure and function of aquatic food webs. *Bioscience* 49, 781–788). The thick gray arrows represent the flux of photosynthetically fixed carbon (primarily by marine phytoplankton). The solid black arrows illustrate the modeled flow of dissolved organic matter (DOM) through the virus shunt, diverting fixed carbon from higher trophic levels. The broken arrows are pathways that are also thought to be significant in channeling DOM, though little data is available to confirm.

photosynthetically fixed carbon is shunted through what is termed the *virus shunt* (Figure 7.1). This is an incredibly important virus-driven process that facilitates the flow of organic nutrients from the particulate to dissolved phase where they are remineralized through the microbial loop to form inorganic nutrients, which in turn, fuels succession dynamics of algae in the ocean. For this whole process to be efficient, there needs to be an almost infinite diversity of viruses that can infect a hugely diverse global community of algae.

Eukaryotic algae range in size from the smallest known eukaryote, *Ostreococcus*, at approximately 1 μm in diameter, through numerous chain-forming and colonial species that are visible to the naked eye to the large kelp (seaweed) forests in coastal regions (seaweed viruses are covered in Chapter 8 of this book). Ubiquitously distributed photosynthetic prokaryotes such as picocyanobacteria (e.g., *Synechococcus* or *Prochlorococcus*) are not included here either; they are infected by bacteriophage-like viruses and are covered in Chapter 6 of this book. Eukaryotic algae consist of at least five distinct evolutionary lineages (plants, cercozoa, alveolates, heterokonts, and discicristates) and they are ubiquitous in marine, freshwater, and terrestrial habitats. The number of algal species (mostly microalgae) has been estimated to be as high as several million; hence their overall diversity is probably enormous. It is likely that viruses infect all these species. Most of the algal viruses described to date are assigned to the Phycodnaviridae family. They are large double-stranded DNA viruses, and this chapter will focus largely on these viruses. Other types of viruses that infect algae are being discovered and characterized all the time (e.g., ssRNA,

dsRNA, and ssDNA containing viruses), though only a few specific examples have been described. Thus, algal virology is a subject still in its infancy.

This chapter will provide a broad overview of eukaryotic algal viruses focusing primarily on the best-described family, the Phycodnaviridae, attempting to describe the novelty and incredible genetic diversity of this ancient group of viruses. The presentation of information about this virus group will start with virus propagation strategies, followed by genome structure, an analysis of known and novel genes, ecology, and finally finish with a discussion on core genes and their implication in Phycodnaviridae evolution. We will then look at future perspectives in algal virology and include an exploration of other groups of little-studied algal viruses (e.g., RNA and ssDNA), an area of research that is sure to explode in coming years.

7.2 THE PHYCODNAVIRIDAE: VIRUS LEVIATHANS OF THE AQUATIC WORLD

The Phycodnaviridae (literally translated as DNA viruses that infect algae) comprise a genetically diverse, yet morphologically and structurally similar, family of large icosahedral viruses that infect marine or freshwater eukaryotic algae with dsDNA genomes ranging from 160 to 560 kb. It is likely that viruses infect all algae although, of course, not all of these viruses will be assigned to the Phycodnaviridae family. Given the number of potential hosts, it is incredible that so few phycodnaviruses have been isolated to date. This is arguably a reflection of the low importance with which they have been regarded in the past. Yet, with 50% of the planet's oxygen produced through marine microalgae, their pivotal role in global primary productivity and the high-profile climatic changes that are being observed, it is likely that research into algae (and their viruses) will become increasingly topical and integral to global change research programs in the future.

The role of viruses in cycling nutrients is fundamental to global ecosystem function. It could be argued that if we lived in a world without the well-studied human viruses, we would all live longer; yet if we lived in a world without the poorly studied algal viruses, we would all certainly be dead. With only approximately 150 formal identifications and around 100 or so others mentioned in the literature, it is clear that most phycodnaviruses, containing an almost infinite reservoir of genetic diversity, remain to be discovered. Members of the Phycodnaviridae are grouped into six genera (named after the hosts they infect): *Chlorovirus*, *Coccolithovirus*, *Prasinovirus*, *Prymnesiovirus*, *Phaeovirus*, and *Raphidovirus.* Evolutionary analysis of sequenced representative genomes places them within the nucleocytoplasmic large DNA viruses (NCLDVs), which includes the Poxviridae, Iridoviridae, Asfarviridae, Phycodnaviridae, and Mimiviridae. The herpesviruses are also related to this family. Originally clustering as part of a major, monophyletic assemblage when restricted to a limited number of isolated viruses; as genomic information has become available for more diverse algal viruses, a multiphyletic distribution has been observed. These new members, large dsDNA viruses of algae such as *Ostreococcus* spp. *Pyramimonas orientalis* and *Chrysochromulina ericina*, are not officially assigned to the Phycodnaviridae (though this will likely change by the time this book is published) because of their high divergence from the original founder members of the family. This divergence is most likely a reflection of the ancient origins of the phycodnaviruses and the highly diverse nature of their hosts: for example, while the terrestrial plant lineage emerged "only" ~470 million years ago, the algal lineages (and likely their viruses) were already well established by this time. Indeed, phycodnaviruses have been suggested to be between 2 and 2.7 billion years old and have been witness to the eukaryotic–prokaryotic separation. It is no surprise that highly diverged phycodnavirus members lying on their own distinct branches of the NCLDV tree

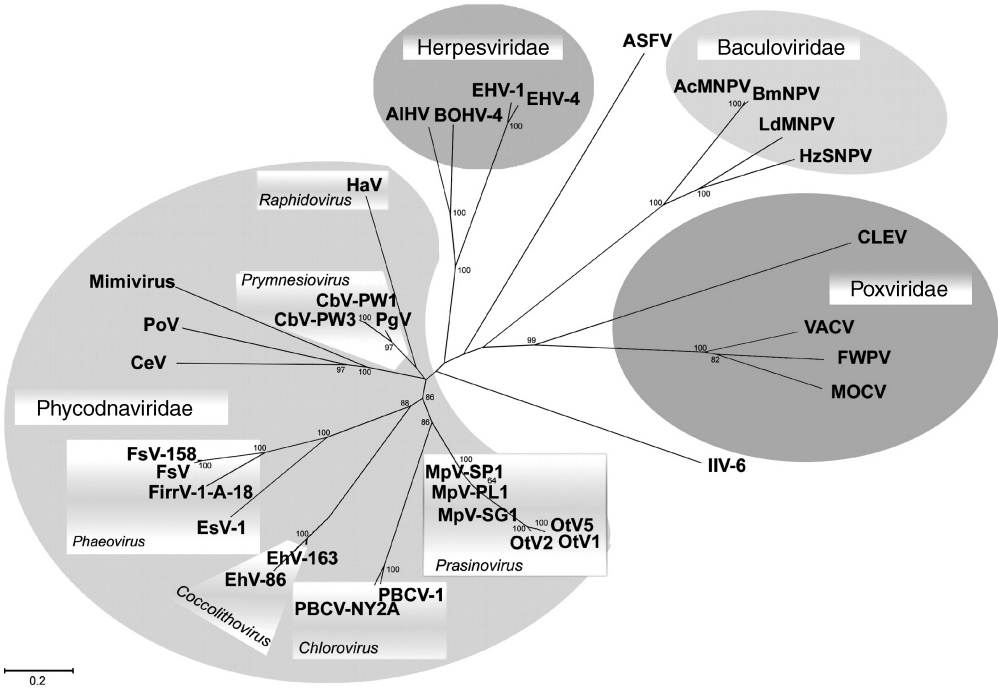

FIGURE 7.2 Phycodnaviridae taxonomy. Phylogenetic analysis of members of algal viruses based on a distance matrix algorithm between the DNA *pol* gene fragments of the family Phycodnaviridae and the other large dsDNA viruses (Neighbor in PHYLIP, version 3.61). The alignment was performed (ClustalW) on the region spanning the highly conserved regions I and IV of the DNA *pol* genes. The scale bar indicates a distance of 0.2 fixed mutations per amino acid. Courtesy of Ilana Gilg. (*See the color version of this figure in Color Plate section.*)

are beginning to appear (Figure 7.2). Indeed, PoV01 and CoV01 (infecting *P. orientalis* and *C. ericina*, respectively) actually cluster with the amoeba-infecting mimivirus, the largest virus sequenced to date. With a genome of 1.2 million base pairs, the mimiviral genome is much larger than are those belonging to some of the smallest bacteria.

7.2.1 Historical Perspectives

Reports of virus-like particles in at least 44 taxa of eukaryotic algae have appeared since the early 1970s. Initially, these reports were based upon incidental observations of VLPs (virus-like particles) in electron micrographs. For example, the first description of VLPs in *E. huxleyi* and *Chrysochromulina mantoniae* was made in 1974. Many electron microscope images of algae containing VLPs are probably labeled miscellaneous and filed into obscurity in laboratories around the world. A good example of this is an image the author uncovered from a box of old electron microscope images in 1990, which reveals an electron micrograph of a thin section of a marine *Pavlova* sp. that was full of hexagonal virus particles (Figure 7.3). It is evident that this nutritious phytoplankton, often used as a food source for feeding zooplankton and shellfish in hatcheries, was in the later stages of infection by a large virus. At the time of discovery, it was considered an exciting find, but what was more amazing was that the original samples were actually prepared back in 1978! The VLP images had been "filed" for long-term storage because there was no interest in phytoplankton viruses over 30 years ago, perhaps surprising given our current knowledge regarding the importance and ecological implications of virus infection of the

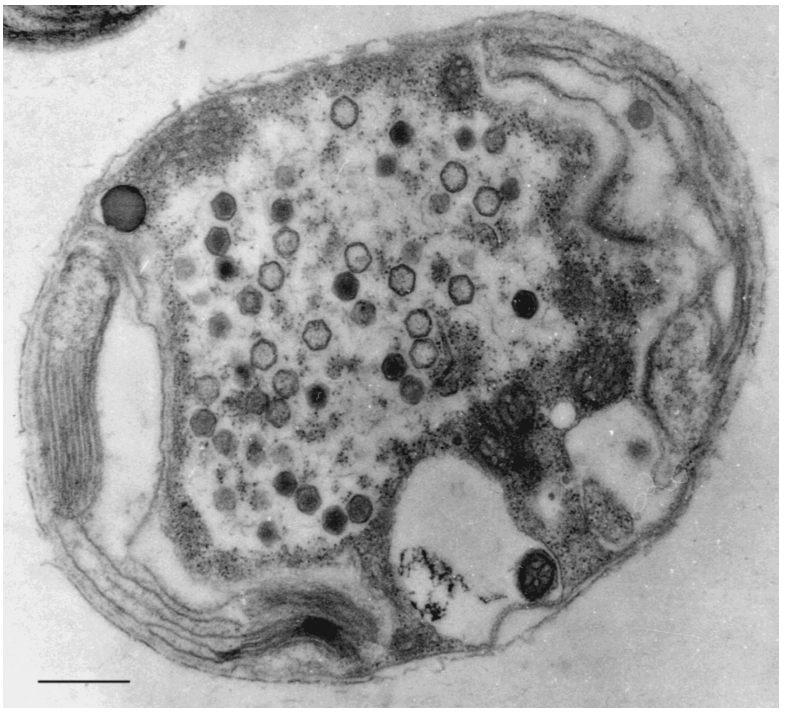

FIGURE 7.3 Final stages of infection in the marine phytoplankton *Pavlova virescens*. Note the different stages of virus assembly in the cell cytoplasm. Samples prepared by thin sectioning back in 1978. Courtesy of John Green (now retired, formerly MBA, Plymouth).

major oceanic primary producers. These old images could be a valuable resource to help identify new viruses and susceptible host strains.

It was not until 1979 that a phycodnavirus was isolated; that virus infected the marine unicellular alga *Micromonas pusilla*. However, this report was largely ignored until the early 1990s when high concentrations of viruses in aquatic environments were being described. Perhaps, most significantly, in the early 1980s a group of viruses were characterized that infect freshwater unicellular, eukaryotic, chlorella-like green algae, called chloroviruses. These reports were followed in the early 1990s by research into viruses of marine filamentous brown algae. Thereafter, the use of genetic markers such as virus-encoded DNA polymerases, which represent a core gene present in all NCLDVs, revealed that phycoviruses are a diverse and ubiquitous component of aquatic environments. The field of phycodnavirology is now firmly established and expanding rapidly.

7.3 CHLOROVIRUS

The chloroviruses are perhaps the best studied of all phycodnaviruses. Their hosts *Chlorella* are small, unicellular, nonmotile, and asexual green algae with a global distribution. While most *Chlorella* species are free living, many species have symbiotic relationships with organisms from different classes in the animal kingdom including Rhizopoda, Ciliata, Hydrozoa, and Turbellaria. To date, the only described chloroviruses infect symbiotic chlorella, often referred to as zoochlorellae, such as those associated with the protozoan *Paramecium bursaria*, the coelenterate *Hydrozoa viridis*, and the heliozoon *Ancanthocystis turfacea*.

Chlorovirus PBCV-1 (species name: *Paramecium bursaria Chlorella virus 1*), which infects *Chlorella* NC64A (a symbiont of *P. bursaria*), is the best studied phycodnavirus although complete genome sequences are available for five other chloroviruses. These six can be grouped by the host they infect: viruses PBCV-1, NY2A (*Paramecium bursaria Chlorella virus NY2A*), and AR158 (*Paramecium bursaria Chlorella virus AR158*, an unclassified chlorovirus) infect *Chlorella* NC64A; MT325 and FR483 (both of which are variants of *Paramecium bursaria Chlorella virus A1*) infect *Chlorella* Pbi; and ATCV-1 (*Acanthocystis turfacea Chlorella virus 1*, an unclassified chlorovirus) infects *Chlorella* SAG 3.83. The PBCV-1 genome is a linear 330 kb, dsDNA molecule with covalently closed hairpin termini. The termini consist of 35 nucleotide-long covalently closed hairpin loops flanked by identical 2221 bp inverted repeats. The predicted 366 PBCV-1 protein-encoding genes are evenly distributed on both strands and, with one exception, intergenic space is minimal. The exception is a 1788 nucleotide sequence near the middle of the genome that encodes 11 tRNA genes. It is not surprising that viruses infecting the same host are most similar, and overall approximately 80% of the genes are found in all six genomes. Despite the high similarity in genome content, each of the six sequenced chloroviruses contains genes that encode unique proteins and functions. For example, ATCV-1 is unique among these viruses in containing genes encoding dTDP-D-glucose 4,6 dehydratase, ribonucleotide-triphosphate reductase, and mucin-desulfatating sulfatase; MT325 encodes an aquaglyceroporin, FR483 an alkyl sulfatase, and potassium ion transporter; NY2A a ubiquitin; AR158 a calcium transporting ATPase; and a Cu/Zn superoxide dismutase is unique to PBCV-1.

Methylation status appears to be important to the chloroviruses, with all the viral genomes containing between 0.12% and 47.5% of their total cytosine in methylated form; many, but not all, chloroviruses also contain between 1.5% and 37% of the total adenine in methylated form. The methylation sites are sequence specific and the activity is presumably associated with the multiple methyltransferases found on their genomes. Many of these methyl transferases are also associated with site-specific restriction endonucleases, some of which have unique specificities such as the nickase (cleaving only one strand of dsDNA) found in strain NY2A.

The PBCV-1 virion consists of an icosahedral outer capsid covering a lipid bilayer. The 54 kDa major capsid protein (Vp54) is a glycoprotein and comprises approximately 40% of the total virus protein, the remainder composed of at least 110 different virus-encoded proteins. The glycan portion of Vp54, which is oriented to the outside of the particle, contains seven neutral sugars: glucose, fucose, rhamnose, galactose, mannose, xylose, and arabinose. Six glycans are attached to the protein (four N-linked and two O-linked); however, the four glycosylated Asn residues are not located in typical eukaryotic consensus sequences, suggesting PBCV-1 encodes most, if not all, of the machinery required to glycosylate its major capsid protein. Indeed, glycosylation of Vp54 probably occurs independent of the host endoplasmic reticulum–Golgi system.

PBCV-1 initiates infection by attaching rapidly, specifically, and irreversibly to the chlorella cell wall; attachment is immediately followed by degradation of the host wall at the point of contact by a virus-packaged enzyme (s). The chloroviruses encode several proteins involved in polysaccharide degradation that may be involved in degrading the cell wall. Following host cell wall degradation, the viral internal membrane fuses with the host membrane, resulting in entry of the viral DNA and virion-associated proteins into the cell. An empty virus capsid is left attached to the cell wall. This process triggers a rapid depolarization of the host membrane (probably triggered by a virus encoded potassium channel located in the virus internal membrane) and the rapid release of potassium ions from the cell. This depolarization is likely to serve two purposes by preventing further infection by a second

virus and by lowering the turgor pressure to aid ejection of DNA. Circumstantial evidence indicates that the viral DNA and probably DNA-associated proteins quickly move to the nucleus where early transcription is detected within 5–10 min postinfection. Shortly after infection, host chromosomal DNA begins to be degraded, presumably to aid in inhibiting host transcription and/or to provide a readily available source of nucleotides for viral DNA replication. Viral DNA replication begins 60–90 min after infection. Approximately 2–3 h postinfection, assembly of virus capsids begins in localized regions in the cytoplasm, called virus assembly centers, which become prominent at 3–4 h postinfection. By 5–6 h postinfection, the cytoplasm becomes filled with infectious progeny virus particles and by 6–8 h postinfection, localized lysis of the host cell releases progeny.

7.3.1 Chlorovirus Proteins

Many PBCV-1-encoded enzymes are either the smallest or among the smallest proteins of their family. The small sizes and the finding that many virus-encoded proteins are user-friendly have resulted in the biochemical and structural characterization of several PBCV-1 enzymes. Examples include the smallest eukaryotic ATP-dependent DNA ligase; the smallest type II DNA topoisomerase (capable of cleaving dsDNA 3–50 times faster than the human homologue); the first RNA guanylyltransferase to have its structure solved; a small prolyl-4-hydroxylase that converts Pro-containing peptides into hydroxyl-Pro-containing peptides in a sequence-specific fashion; a dCMP deaminase that is also capable of deaminating dCTP (usually enzymes from two different protein families are used for these reactions); and a small (94 amino acids, although ATCV-1 has a smaller version of only 83 amino acids) K^+ ion channel protein.

7.3.2 Chlorovirus Novel Sugar Metabolism

The chloroviruses are unusual because they encode many enzymes involved in sugar metabolism. For example, PBCV-1 encodes the enzymes glutamine:fructose-6-phosphate aminotransferase, UDP-glucose dehydrogenase, and hyaluronan synthase (HAS) that are involved in the synthesis of hyaluronan. Hyaluronan is a polymer of disaccharides, themselves composed of D-glucuronic acid and D-*N*-acetylglucosamine, linked together via alternating β-1,4 and β-1,3 glycosidic bonds. All three genes are transcribed early in PBCV-1 infection and hyaluronan accumulates on the external surface of the infected chlorella cells.

However, some chloroviruses have a chitin synthase (CAS) instead of or in addition to the hyaluronan synthase. Chitin is an insoluble linear homopolymer of D-*N*-acetylglucosamine linked by β-1,4 glycosidic bonds. Chlorella infected with these viruses produce either chitin or a chitin/hyaluronan on the surface of infected cells.

Intriguingly, a few chloroviruses appear to lack both genes and produce no extracellular polysaccharides during infection. The functional relevance of these energetically costly biosynthesis pathways remains to be elucidated; however, it is interesting to note that the chlorella viruses also encode genes for enzymes involved in sugar degradation that are essential for viral infection. PBCV-1 encodes two chitinases, a chitosanase, a glycanase, gluconase, and glucouronic lyase; one of the chitinases and the chitosanase are found packaged into the virion and are involved in cell wall digestion (as well as virus release), allowing entry of the viral DNA, following attachment of the capsid.

7.4 COCCOLITHOVIRUS

7.4.1 How Marine Viruses Influence the Weather

There are not many viruses that have a clear link to controlling the weather, yet the coccolithoviruses (cocco: derived from Greek *kokkis*, meaning berry or grain referring to

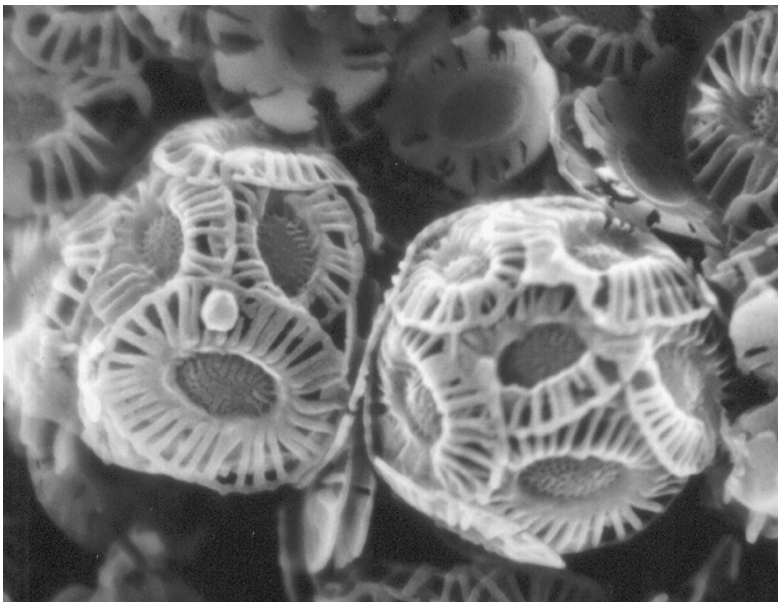

FIGURE 7.4 Virus infection of *E. huxleyi*. Virus (approx. 190 nm diameter) attached to a coccolith from an *E. huxleyi* cell.

their shape and lith from Greek *lithos*, meaning stone) are able to make this bold claim. Coccolithoviruses infect the cosmopolitan and ecologically important coccolithophore *Emiliania huxleyi*, a tiny (5 μm diameter) marine alga (Figure 7.4) that floats freely in the ocean. It is a eukaryotic cell that has an elaborate armory of calcareous (chalk) plates called coccoliths. When conditions are right, *E. huxleyi* can grow in huge numbers (up to 10,000 in a teaspoonful of seawater) to form what is known as a "bloom." Light reflected by coccoliths in these massive and impressive blooms can even be seen from space (Figure 7.5); blooms can range from the size of a small country to a whole continent. Blooms can be seen only from space when the cells are dying and the chalky shell is released into the surrounding sea. When this happens the sea looks milky white! The chalk of the White Cliffs of Dover, epitomized in Dame Vera Lynn's famous war time song, is formed from the coccoliths of algae like *E. huxleyi* killed by viruses over geological time.

Typically, these blooms collapse over a period of 2–3 days releasing a biogenic sulfur gas called dimethyl sulfide (DMS) into the atmosphere, a bit like an oceanic sneeze! DMS is the smell commonly associated with the sea but has a multitude of functions in algae. Its precursor, dimethyl sulfoniopropionate (DMSP), is thought to act as an osmolyte; but when cleaved by the membrane-bound enzyme DMSP lyase during virus infection, it forms DMS and is thought to attract certain protozoan-like grazers. It seems the virus-infected cells make a more appealing snack for grazers. High concentrations of DMS can also prevent further infection by coccolithoviruses, though the mechanisms for this antiviral property are not understood. In the upper atmosphere, oceanic DMS is oxidized into acidic particles that eventually form cloud condensation nuclei (CCN) (Figure 7.6). This process of increased cloud formation reflects heat and sunlight back out to space (termed albedo). It is a process that believers of the Gaia hypothesis (where planet Earth is a self-regulating "organism") can extol since something as small as a virus can control the weather.

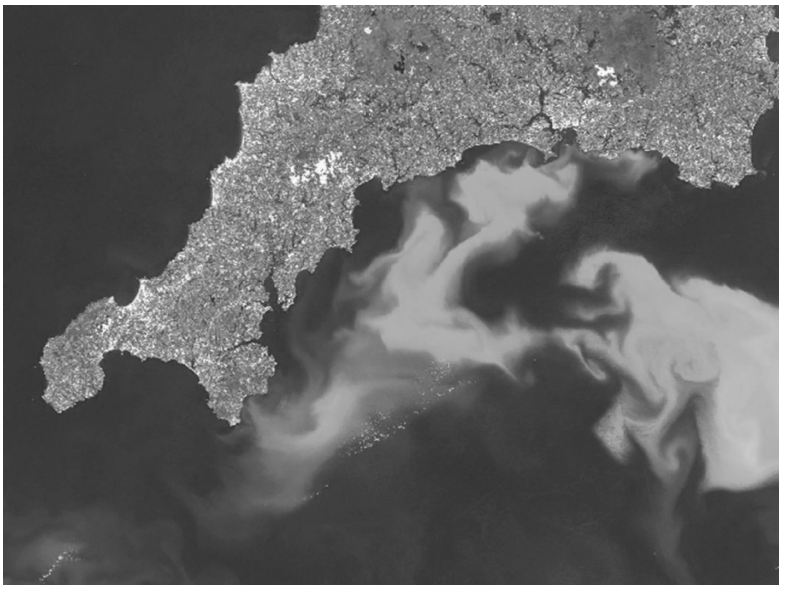

FIGURE 7.5 True color satellite image of a milky *E. huxleyi* bloom in the English Channel, south of Plymouth, UK, on July 30, 1999 (*Source*: Remote Sensing Group, Plymouth Marine Laboratory http://rsg.pml.ac.uk/). This bloom was effectively "dead" and up to 1 million *E. huxleyi*-specific coccolithoviruses per teaspoon of water were found in the middle of the high reflectance water. (*See the color version of this figure in Color Plate section.*)

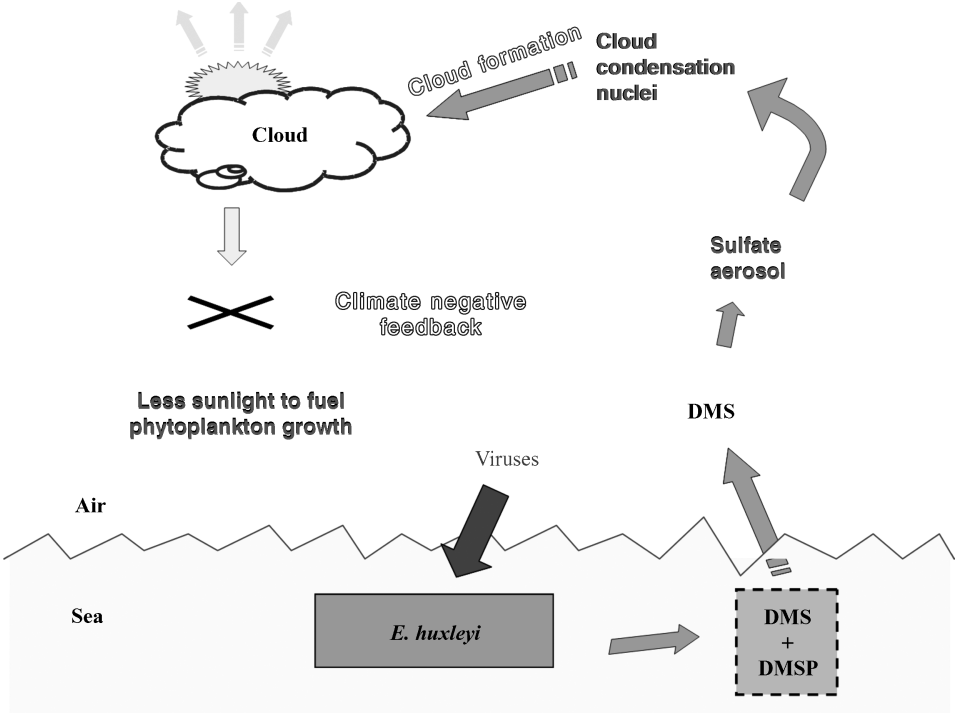

FIGURE 7.6 The Gaia hypothesis states that the Earth is a self-regulating organism. This may seem plausible when the activity of coccolithoviruses is taken into consideration. They kill continent-size blooms of their host organism *E. huxleyi* to produce a massive flux of DMS into the atmosphere that subsequently form clouds and block the vital fuel of phytoplankton growth, sunlight. (*See the color version of this figure in Color Plate section.*)

7.4.2 Cheshire Cat Dynamics: an Evolutionary Strategy to Avoid Virus Infection

The Red Queen's race in Lewis Carroll's *Alice's Adventures in Wonderland* (Charles Lutwidge Dodgson under the pseudonym Lewis Carroll, 1865, MacMillan and Company, London) is a common metaphor for an evolutionary arms race. A good example can be seen in predator– prey dynamics, particularly virus–host interactions where hosts must rapidly evolve immunity to infection for survival of the species: "Now, here, you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!" Red Queen dynamics can also help explain the bloom-bust cycles of *E. huxleyi* that seem to be controlled by the coccolithovirus. However, *E. huxleyi* has adopted a novel sex strategy to avoid coccolithovirus infection. It has been dubbed the "Cheshire Cat" escape strategy, after the disappearing antics of another famous character in Lewis Carroll's book.

The microalga responds to coccolithovirus attack by switching from its usual diploid life stage (where it is susceptible to infection) to a haploid cell, essentially changing its physical appearance, making it impenetrable to the coccolithovirus. As diploids, *E. huxleyi* are nonmotile coccolith-bearing cells, perhaps ironic given some early thinking that coccoliths actually played an antiviral role. The motile flagellated haploid cells lose their chalk armory and are instead covered by organic scales that may be the mechanism of resistance by acting as a physical barrier. It is a clever antiviral strategy and will create a reservoir of resistant haploid cells that can have sex and possibly create a huge variety of new *E. huxleyi* genotypes. Eventually, some of the fitter genotypes will succeed in generating new blooms when the environmental conditions are favorable. This constantly changing genetic landscape in response to coccolithovirus infection will be critical in buffering the effects of rapid climate change, such microorganisms will be the first to react and adapt to a changing ocean. Selection for sexual reproduction as an antiviral mechanism will also maintain a high diversity ensuring that there will always be a genetic variant that can adapt to a particular environmental condition.

With the synergistic effect of chemical warfare and selective grazing of coccolithovirus-infected cells, it seems almost implausible that coccolithoviruses would gain the upper edge with so many systematic antiviral mechanisms in place. However, with such an ancient evolutionary origin and a huge genome of novel and unknown genes, it is likely the coccolithoviruses have a barrage of equally clever counterintelligence strategies to ensure the coccolithoviruses are ultimately the victors. Clearly, it is part of a complex evolutionary arms race that Red Queen and now Cheshire Cat hypotheses are trying to embody.

7.4.3 Sphingolipid Biosynthesis: a Novel Cell Death Mechanism?

With almost 500 genes and a genome of around 410 kb, coccolithoviruses are the largest algal viruses sequenced to date. Other larger algal virus genomes are known to exist, such as a virus that infects *P. orientalis*, a marine microalga, that has a genome estimated at 560,000 bp. Coccolithovirus genomes are remarkably different from other viruses with 80% of their genes having no database matches. Such degree of novelty provides a tantalizing glimpse of the potential benefits locked within, but many of the gene secrets still remain a mystery. Of the genes that do have database matches most are similar to NCDLV core genes; for example, EhV-86 contains 21 of the core set of 40–50 conserved virus genes for NCLDVs that encode some of the principal features of virion structure, genome replication, and expression. The presence of the RNA polymerase holoenzyme, together with a family of novel promoter sequences, indicates that coccolithoviruses have their own transcription machinery. Consequently, expression of many

coccolithovirus transcripts will likely occur in the cytoplasm rather than the host nucleus.

However, there are several surprises in the coccolithovirus genome including a group of genes involved in sphingolipid biosynthesis that is thought to be responsible for the production of the sphingolipid ceramide. These genes have never been found in a virus before—they are more commonly seen in animal and plant cells. Sphingolipid production may be crucial for the production of lipid raft membrane structures that could aid viral exit from the cell. However, ceramide can also control a "death mechanism" (termed apoptosis) that prolongs the life of a cell and then kills it at will. The same mechanism is seen when a tadpole's tail disappears as it develops into a frog. Typically, apoptosis is used as a defense against virus infection and is controlled entirely by the host. By shutting down the cell, it prevents propagation of the virus and consequently acts as an antiviral mechanism preventing spread of progeny virions. Coccolithoviruses may have circumvented this defense by acquiring the ability to control the timing of the apoptotic cascade during the infection process, essentially short-circuiting host-controlled apoptosis. For an invading virus, the ability to control when your host will die and ensure your own survival is a unique propagation strategy. The virus hijacks the cell and slows down the ageing process by keeping it healthy for as long as possible. It uses the cell as a factory to replicate itself until the energy supply runs out. Host cell lysis and death finally occur when the ability to short-circuit host apoptosis subsides. Consequently, coccolithoviruses have burst sizes as large as 1000 progeny virions per algal cell.

The cell death mechanism has many implications for the development of drugs that could perhaps hold back the onslaught of life-threatening disease or the process of ageing. The discovery of coccolithovirus encoded ceramide production will therefore be of great interest to scientists and industries looking for new sources of novel compounds for use in medicines and cosmetics. As more giant viruses are discovered and their genomes sequenced, there is sure to be an explosion of exciting new genes with novel functions.

7.5 PRASINOVIRUS

7.5.1 A Giant Virus Infecting a Tiny Host

This group of viruses infects the world's smallest free-living eukaryote: the green alga *Ostreococcus*. *Ostreococcus tauri* (a marine prasinophyte) has a diameter of less than 1 μm, a naked plasma membrane, has no cell wall, and lacks scales and flagella. Typically containing just a single mitochondria, chloroplast, and Golgi body, this tiny marine algae has global distribution and is found at a wide range of depths. Distinct genotypes can be distinguished within the *Ostreococcus* genus that correlates not with geographic location but with their physiological ability to grow in a high- or low-light environment (i.e., variation appears to correlate with vertical depth and not "horizontal" geographic location). Two sequenced species of *Ostreococcus*, *O. tauri* and *O. lucimarinus*, have genomes of 12.6 and 13.2 Mbp, respectively. Despite their small size, *O. tauri* and *O. lucimarinus* each has 20 viruses isolated, so far, all of which are phycodnaviruses assigned to the family Prasinovirus.

The prasinoviruses have genomes in the approximate size range 184–191 kb and are morphologically similar to other phycodnaviruses with icosahedral capsids of around 120 nm. To date, three strains infecting *O. tauri* have been sequenced in their entirety: two high-light host-infecting strains, OtV-1 and OtV-5, and a third low-light strain OtV-2. OtV-1 has a genome size of 191,761 bp, 232 coding sequences (CDSs) and 4 tRNAs; OtV-2 has a 184,409 bp genome, 237 CDSs, and 5 tRNAs; OtV-5 has a 185,373 bp genome, 268 CDSs, and 5 tRNAs. OtV-1 and OtV-5 have similar genomes, whereas OtV-2 differs by the greatest margin. All three genomes exhibit a

high level of collinearity, despite differences in genome composition; for example, OtV-2 contains 42 unique CDSs not found in OtV-1 or OtV-5.

Among the genes of novel function found are encoded functions including *N*-myristoyltransferase, 3-dehydroquinate synthase, multiple glycosyl- and methyl-transferases, prolyl 4-hydroxylase, and 6-phosphofructokinase. Furthermore, at least 11 genes share close homology with host genes providing further evidence of horizontal gene transfer events between phycodnaviruses and their hosts. Other, standard to phycodnaviruses, functions encoded include DNA replication, recombination, and repair; nucleotide metabolism and transport; transcription; protein and lipid synthesis, modification, and degradation; signaling; sugar metabolism; and eight major capsid proteins.

The presence of so many major capsid proteins is, so far, unique to the phycodnaviruses. Aside from the OtV viruses, five of the capsid proteins have closest similarity to *P. orientalis* virus PoV-1, two have closest similarity to *Heterosigma akashiwo* virus HaV-1, and one has closest similarity to a capsid protein encoded by PBCV NY-2A. The presence and diversity of so many major capsid protein orthologues (orthologues are homologous genes whose commonality presumably arose through speciation, as contrasted with paralogues whose homology arose by gene duplication) raise interesting questions of the potential host range of these viruses, although to date host range appears restricted specifically to *Ostreococcus* species. Presumably, switching the main structural component of the virus capsid could have profound implications for future potential host interactions, although this is an avenue of research yet to be explored.

7.5.2 Common Genes Between Virus and Host

In common with the genetic promiscuity of their relatives, there is evidence of horizontal gene transfers between host and virus in all three sequenced OtV genomes. OtV-1, OtV-2, and OtV-5 contain 11, 14, and 6 genes, respectively, with close homology to *O. tauri* genes. The majority of these genes are of unknown function and the direction of transfer is yet to be determined, although to date the vast majority of horizontal gene transfers between the phycodnaviruses and their hosts has been in the host to virus direction. However, in the case of OtV-2 it appears that there is direct evidence of virus to host transfer of genes encoding DNA topoisomerase II and ribonucleotide reductase that are conserved genes among the NCLDV family.

7.5.3 A Tight Infection Process

As the smallest free-living eukaryote, *Ostreococcus* represents a significant challenge for a large virus to replicate in. With a size of less than 1 μm in diameter and a virus capsid size of around 120 nm, it is estimated that there is physically room for no more than 100 virions at any one time. When the space required for ribosomes and other essential intracellular structures is taken into consideration, it makes for a very crowded infection process. This is reflected in experimental data that suggest a typical burst size is around 6–15 viruses per cell. Following viral adsorption, genome replication occurs from 2 h postinfection, virions assemble in the cytoplasm from 6 h postinfection until 20 h postinfection, after which cellular lysis occurs. The host cell nucleus (the chromosomes contained within), mitochondria, and chloroplast remain intact through this period.

7.6 PRYMNESIOVIRUSES AND RAPHIDOVIRUSES

7.6.1 The Lesser Known Phycodnaviridae

These genera of viruses are thought to be widespread in the oceans since they infect bloom-forming species of phytoplankton.

However, very little research has been conducted on them. Of historical interest, one of the earliest reports of a phycodnavirus was that of a putative prymnesiovirus observed in thin sections of a *Chrysochromulina* sp. in 1974; this was well before the importance of algal viruses was realized.

Prymnesioviruses infect phytoplankton from the algae class Prymnesiophyceae, from the division Haptophyta (and generally referred to as Haptophytes). These algae have a global distribution and they are often associated with large-scale blooms. To date, prymnesioviruses have been isolated from members of the genera *Chrysochromulina* and *Phaeocystis*. *Chrysochromulina* is considered a cosmopolitan genus; however, information on the distribution and abundance of *Chrysochromulina* at the species level is limited. Large monospecific blooms of *Chrysochromulina* are rare; typically, they are present in low concentrations. This property has led to speculation that viruses infecting *Chrysochromulina* require only a low host density for propagation and that these viruses may even prevent bloom formation. In contrast, a lot of information is available on the biogeochemical impact the members of the genus *Phaeocystis* have on the marine ecosystem. They form dense spatially and temporally extensive monospecific blooms consisting of a mixture of colonial (within a gelatinous matrix) and unicellular cells that collapse suddenly in a virus-induced crash. This crash leads to a rapid shift in the composition of the bacterial community due to the massive flux of released organic nutrients. Intense *Phaeocystis* blooms can lead to anoxia and impressive foam formation on beaches during their decline, hence their label as harmful algal blooms (HABs). Similar to *E. huxleyi*, *Phaeocystis* spp. play important roles in CO_2 and sulfur cycling that ultimately have major implications for the global climate, and infection by viruses is known to exacerbate this process.

Raphidoviruses infect algae from the class Raphidophyceae that are often associated with toxic red tides and subsequent fish kills, particularly in the aquaculture industry. This class of algae is an important bloom-forming species found in coastal and subarctic regions of the oceans, although freshwater species also exist. To date, the only viruses reported in this genus infect the red tide-forming species *Heterosigma akashiwo*; the viruses are collectively referred to as HaV (*Heterosigma akashiwo* virus 01). *H. akashiwo* is a single species belonging to the genus *Heterosigma* and, although not usually associated with human illness, blooms of *H. akashiwo* have caused massive fish kills, typically of caged fish such as salmon and certain species of tuna. The susceptibility of *H. akashiwo* to HaV differs among clonal strains in the laboratory. Marine field surveys and cross-reactivity tests between *H. akashiwo* host strains and HaV clones suggest that this strain-dependent infection plays an important role in determining clonal composition and effectively maintaining intraspecies diversity in natural *H. akashiwo* populations. This diversity probably contributes to the success of *H. akashiwo* as a ubiquitous and problematic bloom former in coastal regions.

7.7 FUTURE PERSPECTIVES

Algal virology is still very much in its infancy and will likely go through its discovery phase for a number of years. In this chapter, we touched only upon the large dsDNA viruses, reflecting the large amount of research conducted on this group. They are lytic viruses and relatively easy to isolate and work with;

however, we do not yet have a sense of where they sit in the ecological relevance continuum when compared to other groups of algal viruses. Metagenomic analyses have revealed a wide range of unknown sequences that likely represent many different virus families. These include RNA and ssDNA viruses. Until recently, the contribution of RNA viruses to algal mortality was largely ignored (many direct counting methods favored large DNA viruses). The first algal RNA virus was reported only in 2003 and since then several RNA viruses have been isolated, for example, dinoflagellates (*Heterosigma akashiwo*), diatoms (*Chaetoceros tenuissimus*), and prasinophytes (*M. pusilla*). Their virus families have been classified as positive-sense ssRNA Marnaviridae, unclassified (though also positive-sense ssRNA), and dsRNA Reoviridae, respectively. To date, there is only a single report of an ssDNA virus, thought to be distantly related to family Circoviridae, that infects a marine diatom (*Chaetoceros salsugineum*), though metagenomic analysis of marine viriomes has revealed the presence of abundant ssDNA sequences in the Sargasso Sea.

Understanding how climate change will influence our planet is arguably one of the greatest modern-day science challenges we face. It appears inevitable that rising concentrations of atmospheric CO_2 will result in the ocean becoming both warmer and more acidic. One key aspect of the biological system that has received little attention is the response of marine algal viruses to global environmental change. Viruses are responsive to very subtle changes in host growth dynamics. This can take the form of either increasing or decreasing their effectiveness. However, host population evolution typically follows a Red Queen dynamics to eventually revert to an effective virus propagation strategy that in turn continues to catalyze biogeochemical cycling to sustain microbial loop processes. For this to be effective, there needs to be an almost infinite diversity of virus–host combinations. The research challenge is threefold: work out what is there (discovery), work out what they do (function), and then tie into ecological processes to help determine the importance of algal viruses (ecology).

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CHAPTER 8

VIRUSES OF SEAWEEDS

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CONTENTS

8.1 INTRODUSCTION

Multicellular algae, commonly known as macroalgae or seaweeds, are important inhabitants of aquatic environments around the world. They can be crudely separated into groups based on their pigmentation, that is, green (phyla Chlorophyta and Charophyceae), red (phylum Rhodophyta), and brown (phylum Phaeophyta). Our current understanding of their evolution suggests that they evolved their multicellularity independently on at least two occasions within the Plantae and Chromista kingdoms (Baldauf, 2003). Seaweeds have a long history of exploitation and cultivation. They are exploited directly as food or processed to yield fractions such as agar, carrageenans, and alginates, which are used as food additives, pharmaceuticals, and industrial chemicals (Chapman and Chapman, 1980). Moreover, seaweeds play important ecological roles in many communities. They are a food source for animals and contribute significantly to global annual primary production. They also provide shelter and a home for numerous species of fish, invertebrates, birds, and mammals. With that said, they can be significant components of fouling communities that occur on structures such as docks, buoys, and ship hulls (Baker and Evans, 1973; Henry and Meints, 1994; Van den Hoek et al., 1995; Voulvoulis et al., 1999). The importance of this group makes understanding the basics of algal evolution, biodiversity, and environmental impact of crucial importance. In addition, major concerns have been raised about the health and sustainability of ecosystems reliant on key seaweed species in response to increasing anthropogenic influences, changing environmental conditions, and pathogen-related effects.

Despite the knowledge that viruses do infect numerous taxa of eukaryotic algae, only a few seaweed viruses have been subsequently characterized (Dunigan et al., 2006). Eukaryotic algal viruses mostly fall within the Phycodnaviridae, a family of double-stranded DNA viruses (Wilson et al., 2005). Currently, this family consists of six genera: *Chlorovirus*, *Coccolithovirus*, *Prasinovirus*, *Prymnesiovirus*,

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Phaeovirus, and *Raphidovirus*. Of the six genera, only one genus (*Phaeovirus*) describes the seaweed viruses. Those unassigned seaweed viruses usually have only microscopic evidence attesting to their existence (Table 8.1). In the early 1970s, virus-like particles (VLPs) were observed in zoospores of the brown algae *Streblonema* sp. (La Claire and West, 1977) and *Sorocarpus uvaeformis* (Oliveira and Bisalputra, 1978); the vegetative cells of the brown algae species *Chorda tomentosa* (Toth and Wilce, 1972), *Sorocarpus uvaeformis* (Oliveira and Bisalputra, 1978), the red alga *Sirodotia tenuissima* (Lee, 1971); and the green algae species *Uronema gigas* (Dodds and Cole, 1980) and *Chara corallina* (Skotnicki et al., 1976). Similarly, ultrastructural investigations in the 1990s revealed VLPs in the red algae *Audouinella saviana* (Pueschel, 1995) and disease-associated symptoms such as tumors were found on *Gracilaria epihippisora* (Apt and Gibor, 1991) and dieback affected fronds in the brown kelp *Ecklonia radiata* (Easton et al., 1997). Given that there are tens of thousands of known species of seaweeds in the world, we are severely underestimating the degree and diversity of virus infection. This chapter will review what we know about these few examples and attempt to place their ecological significance in context of the major pressures influencing our world today.

8.2 DIVERSITY

Phaeoviruses appear to be by far the most encountered type of virus that infects the brown lineage (Table 8.1). They share icosahedral morphologies with internal lipid membranes and large, complex, double-stranded DNA genomes (Kapp et al., 1997). *Ectocarpus siliculosus virus* 1 (EsV-1) is the type species for this genus and its infection strategy is generally regarded as "typical" for phaeoviruses (Muller, 1996; Wilson et al., 2005). Whole genome comparisons of large dsDNA viruses have led to the general consensus that these viruses originated from a common nuclear–cytoplasmic large double-stranded DNA virus (NCLDV) ancestor (Schroeder et al., 2009). Comparative analyses performed by Iyer et al. (2001) and others demonstrate the presence of not only core conserved genes within the NCLDVs but also a high degree of diversity within this group (Allen et al., 2006; Iyer et al., 2001, 2006; Schroeder et al., 2009).

The breadth of diversity of seaweed viruses is presented in Table 8.1, indicating a range of sizes, morphological shapes, and genomic nature. The relatively large diameter range within these icosahedral virions presented is from 50–80 nm up to 390 nm. Most, if not all, are likely to be DNA viruses, although icosahedral RNA viruses are also known to have size ranges of 30–100 nm, which overlaps the range presented in Table 8.1 (Fauquet et al., 2005). What is clear, however, is that these viruses of seaweeds will eventually be separated into different families of viruses. The rod-shaped viruses of seaweeds are certainly ssRNA viruses, thought to be linked to known groups of plant viruses (van Etten et al., 1991). It is also worth noting that many of the viruses listed in Table 8.1 seem to resemble viral groups more commonly associated with plants, and these particular viruses mostly infect the red and green seaweed lineages, suggesting that these seaweed viruses could have been the progenitors of their terrestrial counterparts. This once again adds further credence to the idea that the algal viruses, in general, have ancient origins (Dunigan et al., 2006).

8.3 VIRAL INFECTION STRATEGIES

The extraordinary number of viruses in the ocean at any one time implies a constant massive production of new virus particles through infection and lysis of their respective hosts (Wilhelm and Suttle, 1999). Algal viruses replicate in their hosts through a lytic or latent infection cycle. A lytic infection occurs when the virus enters a host cell and immediately begins replication, altering "normal" host cellular processes to

TABLE 8.1 Recordings of VLPs in Seaweeds

Kingdom	Phylum	Class	Family	Species	Virus name and classification	Virion size (nm)	Virus genome size (kbp)	Genome type	Virus sequence data	References
Chromista	Heterokontophyta	Phaeophyceae	Ectocarpaceae	*Ectocarpus siliculosus*	EsV-1 *Phaeovirus*	130–150	336	dsDNA	Yes	(Delaroque *et al.*, 2001)
				Ectocarpus fasciculatus	EfasV-1 *Phaeovirus*	135–140	320	dsDNA	No	(Kapp *et al.*, 1997)
			Acinetosporaceae	*Feldmannia simplex*	FlexV-1 *Phaeovirus*	120–150	220	dsDNA	No	(Kapp *et al.*, 1997)
				Feldmannia irregularis	FirrV-1 *Phaeovirus*	140–167	180	dsDNA	Yes	(Delaroque *et al.*, 2003)
				Feldmannia sp.	FsV-158 *Phaeovirus*	150	170	dsDNA	Yes	(Schroeder *et al.*, 2009)
				Hincksia hincksia	HincV-1 *Phaeovirus*	140–170	240	dsDNA	No	(Kapp *et al.*, 1997)
				Pylaiella littoralis	PlitV-1 *Phaeovirus*	130–170	280	dsDNA	No	(Maier *et al.*, 1998)
			Chordariaceae	*Myriotrichia claviformis*	MclaV-1 *Phaeovirus*	170–180	320	dsDNA	No	(Kapp *et al.*, 1997)
				Streblonema sp.	none *Phaeovirus*-like	135150	unknown	unknown	No	(La Claire II & West, 1977)
				Sorocarpus uvaeformis	none *Phaeovirus*-like	170	unknown	unknown	No	(Oliveira & Bisalputra, 1978)

(*continued*)

TABLE 8.1 (*Continued*)

Kingdom	Phylum	Class	Family	Species	Virus name and classification	Virion size (nm)	Virus genome size (kbp)	Genome type	Virus sequence data	References
			Halosiphonaceae	*Halosiphon tomentosa*	none *Phaeovirus*-like	170	unknown	unknown	No	(Peters, 1998; Toth & Wilce, 1972)
			Lessoniaceae	*Ecklonia radiatia*	none Tobamovirus-like	25×280	unkown	ssRNA(+)	No	(Easton *et al.*, 1997)
					none Potyvirus-like	25×700–900	unknown	ssRNA(+)	No	
Plantae	Rhodophyta	Florideophyceae	Batracho-spermaceae	*Sirodotia tenuissima*	none	50-60	unknown	unknown	No	(Lee, 1971)
			Gracilariaceae	*Gracilaria epihippisora*	none	80	unknown	unknown	No	(Apt & Gibor, 1991)
			Acrochaetiaceae	*Audouinella saviana*	none	40×1000	unknown	unknown	No	(Pueschel, 1995)
	Chlorophyta	Chlorophyceae	Chaetophoraceae	*Uronema gigas*	none	390	unknown	unknown	No	(Allen Dodds & Cole, 1980)
	Charophyta	Charophyceae	Characeae	*Chara corallina/ australis*	CCV/CAV *Furovirus*-like	18×532	unknown	ssRNA(+)	No	(Skotnicki *et al.*, 1976)

optimally benefit the virus in its propagation and ultimately achieves progeny virion release when the cell bursts open and dies (lysis). The next lytic virus generation repeats the same infection cycle, often leading to a dramatic increase in virus abundance and a severe reduction in the host population. Generally, the members of the Phycodnaviridae infecting unicellular algae have a lytic infection cycle (Volume I, Chapter 7).

Latent infections differ in that the virus may lie "hidden" for long periods, during which the virus DNA becomes associated with the DNA of its host, being replicated alongside that of the host. The phaeoviruses exhibit a latent infection cycle in which the virus becomes integrated into the host DNA (Delaroque et al., 1999). Infected cells develop into mature thalli that can produce pathological symptoms in their zoidangia (overt symptoms). The best studied macroalgal virus–host dynamic is the virus–host system of *Ectocarpus* (Figure 8.1). Mature infected *E. siliculosis* plants release several millions of viral particles into the surrounding seawater through the disruption of densely packed virus zoidangia. The timing of viral release is synchronous with release of gametes and spores (Muller et al., 1998). Virus particles are formed only in prospective zoidangia cells of the host; that is, infected algae can appear normal and produce viable spores containing the viral genome (Cock et al., 2010). It is thought that one copy of the viral DNA becomes integrated into the host genome and is transmitted through mitosis to all cells of the developing alga (Bräutigam et al., 1995; Muller, 1991). The mechanism of viral integration remains unclear and, as yet, no data shows explicitly whether the genome of EsV-1 inserts only at specific locations or randomly in the host cell's genome. The virus then remains latent during the vegetative growth of the alga (Cock et al., 2010) and neither the survival nor the growth of the host is seriously impaired until the algae begin to reproduce, at which time the virus becomes very active. It is clear that phaeoviruses are unique with respect to their infection cycle and their consequential impact

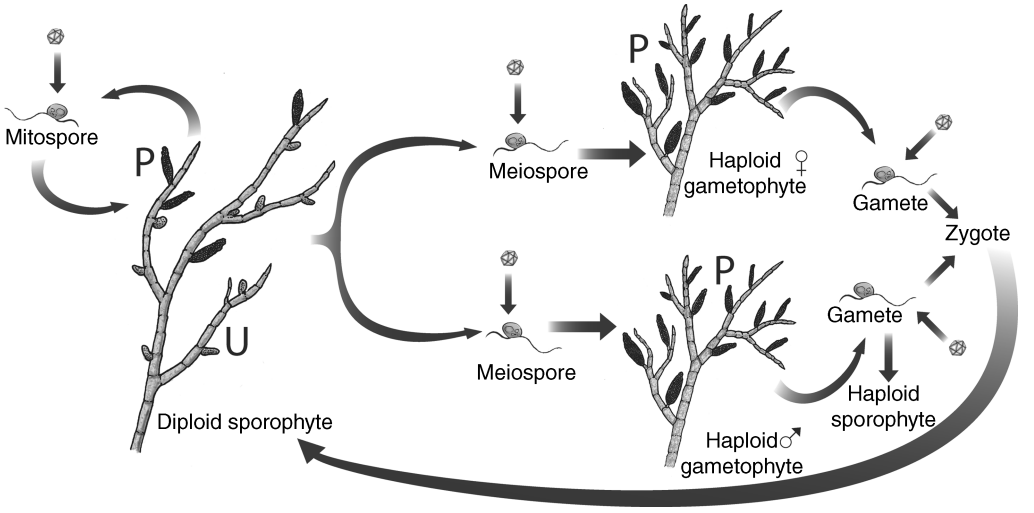

FIGURE 8.1 Life history of *Ectocarpus* – virus infection dynamic. Diploid sporophytes produce meiospores (by meiosis) in unilocular sporangia (U). Meiospores grow into male or female gametophytes. Gametophytes produce gametes in plurilocular (P) gametangia. Fusion of gametes produces a zygote that grows into a diploid sporophyte, completing the sexual cycle. Unfused gametes may grow parthenogenetically and form a parthenosporophyte (haploid sporophyte), which is indistinguishable from the diploid sporophyte. Both sporophytes and parthenosporophytes can reproduce themselves asexually by the production of mitospores in plurilocular (P) sporangia. Viruses can initiate infection of only the wall-less meiospores, mitospores, and gametes.

on their ecosystem when compared to their better characterized unicellular lytic algal viruses.

8.4 ECOLOGY

The Ectocarpales (class Phaeophyceae) have been divided and regrouped according to the method of study (Rousseau and de Reviers, 1999). This order currently includes five families: Ectocarpaceae, Scytosiphonaceae, Chordariaceae, Adenocystaceae, and Acinetosporaceae (Peters and Ramirez, 2001). Of the best studied phaeoviruses, EsV-1, FirrV-1, and FsV-1 infect different species within three different families of the order Ectocarpales (Table 8.1). Sequence comparison between these three reveal extensive genomic rearrangements (Schroeder et al., 2009). Phaeoviruses are known to have the greatest range in genome size and therefore comparative genomic analysis can provide new insights into the origin and evolution of these dsDNA viruses. The phylogenetic relationships between conserved domains among a smaller core set of NCLDV proteins added further evidence that the phaeoviruses have a close and recent evolutionary history (Schroeder et al., 2009). The inference made from the phylogenetic analysis is that the green algal viruses (e.g., *Chlorella*-infecting viruses) split from the heterokont algal viruses, that is, the viruses infecting the haptophytes and brown algae lineage, and further separating into the coccolithovirus (EhV) and phaeovirus lineages (Figure 8.2). This is congruent with our current understanding of the evolutionary history of their respective algal hosts where the Chromista separated from the Plantae around 1500 million years ago (Yoon et al., 2004). The surprising complexity of *Phaeovirus* genomes is also interesting with respect to recent arguments suggesting that a complex DNA virus could actually be the "ancestor" of the eukaryotic nucleus and, therefore, constituting the basis for "eukaryogenesis" (Bell, 2001).

The production of motile spores or gametes for dispersal is a fundamental step in the life histories of many green and brown seaweeds. During dispersal, as well as settlement, spores are exposed to heterogeneous environmental conditions, and successful settlement and survival ultimately depend on a spore's ability to sense and react to environmental cues, either to avoid unfavorable or to seek favorable conditions (Amsler et al., 1992). The sensory abilities of spores and other distributional macroalgal stages are especially important once they enter the benthic boundary layer, where they encounter new microenvironments with strong physical (e.g., surface structure, surface charge, or light), chemical (e.g., nutrient, ionic gradients, or antifouling compounds), and biological gradients (e.g., biofilms) (Amsler and Neushul, 1990; Callow et al., 1997; Harlin and Lindbergh, 1977; Kawai et al., 1990; Watanuki and Yanamoto, 1990). These factors contribute to the behavior exhibited by spores when "selecting" a favorable surface for settlement and subsequent germination and growth.

The expression of virus symptoms is often highly variable between specimens. Some plants exhibit a total takeover of all plurilocular zoidangia by the virus and thus no viable spores are produced, whereas other specimens of the same algal species indicate a reduced virulence, with normal and virus-infected sterile sporangia occurring side by side (Muller and Frenzer, 1993). Virus infection can even be in a mosaic arrangement with infected and uninfected spores occurring together in the same sporangia (Muller et al., 1990). It is also possible for an infected plant to appear phenotypically healthy, exhibiting no abnormal sporangia (Delaroque et al., 1999; Muller et al., 2000).

The frequency of overt viral infection in the field and laboratory populations of *Ectocarpus* has been shown to be negatively correlated with sea surface temperature (Dixon et al., 2000; Muller et al., 1998). In these algae, as in *Hincksia hincksiae*, overtly infected zoidangia occur less frequently at higher temperatures

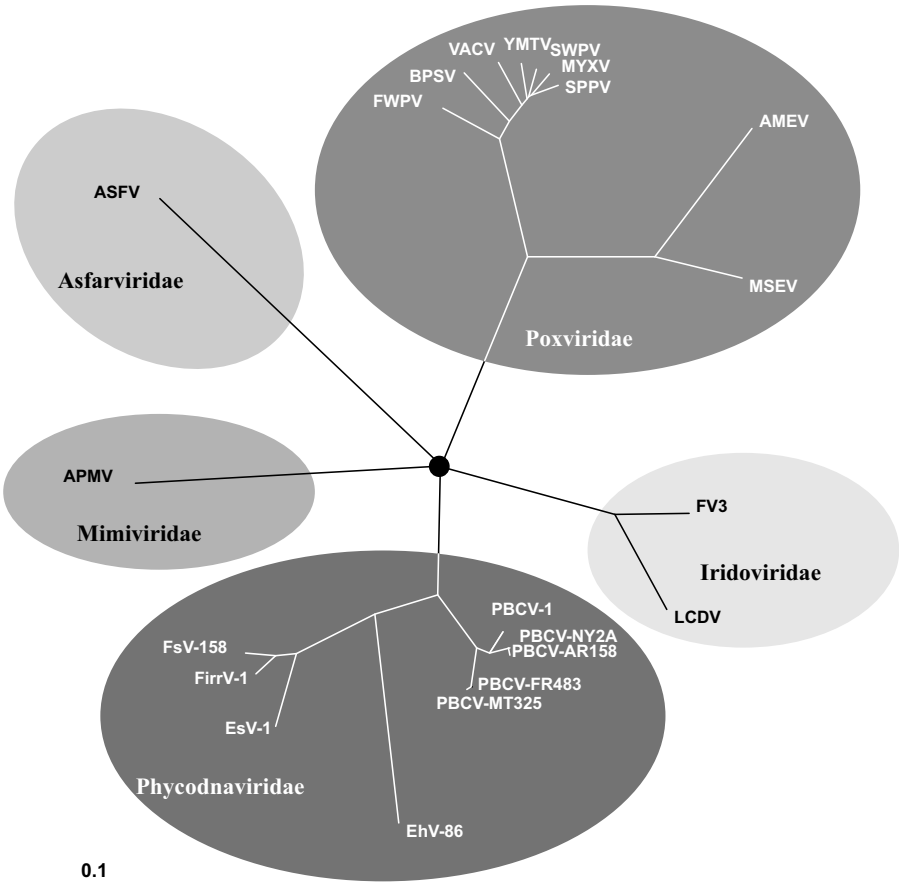

FIGURE 8.2 Unrooted phylogenetic inference tree based on a distance matrix algorithm (Neighbor, in PHYLIP version 3.6b) between the conserved concatenated domains from group I core genes (A32-like ATPase, D5-type ATPase, thiol oxidoreductase, DNA polymerase, major capsid protein, and A1L-like transcription factor) from members of the NCLDV group. Nodes with less than 900 bootstrap values from 1000 replicates for neighbor joining and in which possible parsimony analyses were collapsed. The bar length equivalent of 1 base substitution per 10 amino acids as depicted by 0.1 is shown (adapted from Schroeder et al., 2009).

(18–20°C versus 12–15°C) and are replaced with functional zoidangia (Harris, 1999). At lower temperatures, most filaments with symptoms of infection do not possess functional zoidangia (Dixon et al., 2000; Muller et al., 1998; Parodi and Muller, 1994). However, at higher temperature ranges, reversion of filaments to the formation of functional zoidangia are observed in laboratory cultures (Muller et al., 1998), thus suggesting temperature sensitivity on the part of the virus. Within the context of global climate change, temperature is an important parameter. Global sea surface temperatures have increased by 0.6 ± 0.2°C over the twentieth century, with a predicted global temperature rise of 1.4–5.8°C over the period 1990–2100 (Cox et al., 2000; Houghton et al., 2001). In view of the significance of temperature to viral infection, such a rise could dramatically affect diversity, distribution, and abundance of these marine biofoulers. Currently, polymerase chain reaction (PCR) has revealed that in many locations equal numbers of uninfected and infected plants of *Ectocarpus* co-occur (Muller et al., 1998). In addition, a proportion of below

10% of overtly infected *E. fasciculatus* filaments in the population was also observed at a site in South West England (Dixon et al., 2000). Muller and Stache (1992) reported that 2% and 15% of *E. fasciculatus* filaments collected in Ireland and California, respectively, expressed overt infections following incubation (Muller and Stache, 1992). Parodi and Muller (1994) found that up to 20% of *E. fasciculatus* and *H. hincksiae* filaments showed symptoms of infection. Overt infection has a negative feedback effect because it may cause sterility or a severe reduction in reproductive capacity of the host algae (Muller et al., 1998).

Other than manipulating the life cycle of its host, seaweed viruses have been reported to cause tumors (Apt and Gibor, 1991) and even death (Easton et al., 1997) within seaweeds. Unlike their microalgal virus counterparts (Schroeder et al., 2003), little or no evidence exist of seaweed viruses controlling macroalgal blooms. Disappearance of massive spring blooms of macroalgae have generally been attributed to differences in their competitive abilities, which are attributed to their physiological characteristics (Littler and Littler, 1980). That said, it is only due to extensive research over the past decade in the area of microalgal bloom dynamics that viruses are now accepted as key agents of their demise, with it previously being widely accepted that grazers were central in these bloom terminations. We may in future discover that viruses do indeed also affect the bloom dynamics of seaweeds.

8.5 SUMMARY

Our limited understanding of the true extent to which viruses interact with their hosts is further exemplified when the complexity of interactions surrounding seaweeds and their viruses is examined. By further characterizing this important group of viruses, we have the unique opportunity to study the evolution of viral latency in an ancient eukaryote lineage. Furthermore, the advances in "omic" technologies have the potential to build a framework and knowledge base to help us understand the nature of seaweed virus infection and ultimately the role of these viruses in the ecology of their hosts. The importance of the Ectocarpales, in particular, as biofouling organisms makes understanding the fundamentals of their evolution, biodiversity, and environmental impact to be of crucial economic importance. The field of seaweed virology will therefore contribute to our understanding of the role biotic and abiotic factors play on the success of viral interactions with their eukaryotic hosts.

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CHAPTER 9

THE ECOLOGY AND EVOLUTION OF FUNGAL VIRUSES

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CONTENTS

9.1 INTRODUCTION

Even though viruses are found in virtually every fungal taxon, very little is known about them compared to viruses in plants, animals, or many other microbes. Lack of knowledge about fungal viruses is especially acute concerning ecology and evolution. While rapid advances are being made in understanding the molecular biology of a few model systems (Hillman and Suzuki, 2004; Nuss, 2005, 2010; Schmitt and Breinig, 2006; Wickner, 2001), much less is

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known about persistence, transmission, or effects of viruses on fungal populations outside the laboratory. The fungal viruses for which most ecological information is available are those that reduce virulence of plant pathogenic fungi (Ghabrial and Suzuki, 2009; McCabe et al., 1999; Pearson et al., 2009) or have economic impacts on mushroom production (Rao et al., 2007; Romaine and Schlagnhaufer, 1989) or yeasts during fermentation (Marquina et al., 2002; Schmitt and Breinig, 2002).

In this chapter, we provide an overview of the ecology and evolution of fungal viruses. We begin by describing briefly the taxonomic diversity of fungal viruses. Much of the emphasis of this chapter, however, will relate in one way or another to virus transmission. Fungal viruses have no extracellular phase and therefore can only be transmitted by cell-to-cell contact and cytoplasmic mixing, either vertically in spores or horizontally by fusion of hyphae (anastomosis) between different individuals. The restriction of horizontal transmission by vegetative (or heterokaryon) incompatibility has led, at least in theory (explained in Section 9.4), to the evolution of viruses that have relatively little effect on their hosts' fitness. In fact, most fungal viruses have little or no detectable effect on host phenotype. Therefore, the exceptions where fungal viruses do have a significant impact on their hosts have garnered a disproportionate amount of the research with respect to ecology and evolution. These exceptions include both increases and decreases in virulence of pathogenic fungi. Decrease in virulence caused by viral infection, a phenomenon known as hypovirulence, offers the prospects of biological control of fungal diseases and has been a driving force in studies of fungal viruses. Implementing this type of control depends on understanding the ecology and evolution of fungal viruses, particularly their transmission in natural populations.

This chapter is not meant to be an exhaustive review of fungal viruses, but instead highlights the range of ecological and evolutionary interactions fungal viruses have with their hosts and provides some salient examples. In this regard, we aim to complement recent reviews on fungal viruses (Ghabrial and Suzuki, 2009; Nuss, 2010; Pearson et al., 2009; Schmitt and Breinig, 2006) by focusing more narrowly on ecology and evolution.

9.2 BIOLOGY AND DIVERSITY OF FUNGAL VIRUSES

9.2.1 Fungal Virus Diversity and Taxonomy

The diversity of fungal viruses has been described in several recent reviews (Ghabrial and Suzuki, 2009; Hillman and Suzuki, 2004; Nuss, 2010; Pearson et al., 2009). Ghabrial and Suzuki (2009) described viruses found in plant pathogenic fungi and oomycetes (often grouped with fungi because of their morphological and ecological similarities to fungi) and provided a comprehensive list of known fungal viruses, including those that have been only partially characterized and not fully recognized by the International Committee on the Taxonomy of Viruses (ICTV). Table 9.1 gives an overview of fungal virus families discussed in this chapter (modified from Kobayashi and Hillman (2005)). Detailed information about each virus group can be found in the Eighth Report of the ICTV (Fauquet et al., 2005).

Compared to plants or animals, especially vertebrates, fungi serve as hosts for a relatively narrow diversity of virus types. Almost all the "free living" (nonintegrated) fungal viruses described to date contain positive-sense single-stranded RNA genomes or double-stranded RNA genomes. No virus with a negative-sense RNA genome has yet been characterized from fungi, and only one nonintegrated virus with a DNA genome has been characterized (Yu et al., 2010). The reason for the narrow range of viruses identified in filamentous fungi to date probably reflects a combination of the actual range of viruses in fungi and a methodological bias that detects only RNA viruses. Because of the economics and the nature of the science and scientists who study viruses of fungi, fungal viruses are less well examined

TABLE 9.1 Overview of Fungal Virus Families and Groups

	Family or Genus	Example	Particle	Location	Disease?	Closest Relatives
ssRNA	Unclassified	*Diaporthe* RNA virus	None known	Cyt	Yes	Plant-infecting *Tombusviridae*
	Narnaviridae	*Ophiostoma* mitoviruses	None known	Cyt or mito	Yes	RNA phages *Leviviridae*
	Potexvirus	*Botrytis* virus X	700 × 18 nm rods	Cyt	No	Plant-infecting *Potexvirus*
	Barnaviridae	*Mushroom bacilliform virus*	20 × 50 nm bacilli	Cyt	Yes	Plant-infecting *Luteoviridae*
	Hypoviridae	*Cryphonectria* hypoviruses	None known	Cyt	Yes	Plant-infecting *Potyviridae*
	Endornaviridae	*Phytophthora endornavirus 1*	None known	Cyt	Yes	Plant-infecting *Flexiviridae*
dsRNA	*Reoviridae*	*Cryphonectria* reoviruses	70 nm icosahedra	Cyt	Yes	Animal-infecting reoviruses
	Partitiviridae	*Atkinsonella hypoxylon virus*	40 nm icosahedra	Cyt	No	Plant-infecting partitiviruses
	Totiviridae	*Saccharomyces cerevisiae virus L-A*	40 nm icosahedra	Cyt	No	Protozoan-infecting totiviruses
	Chrysoviridae	*Helminthosporium virus 145S*	40 nm icosahedra	Cyt	Yes?	Protozoan-infecting totiviruses
Reverse transcribing (RNA in particles, DNA in host genome)	*Pseudoviridae*	*Saccharomyces cerevisiae Ty1 virus* (copia-like)	30 nm icosahedra	Nuc/Cyt	No	Vertebrate-infecting *Hepadnaviridae*
	Metaviridae	*Saccharomyces cerevisiae Ty3 virus* (gypsy-like)	30 nm icosahedra	Nuc/Cyt	No	Vertebrate-infecting *Retroviridae*
DNA	*Unclassified*	*Sclerotinium sclerotiorum* DNA virus 1	20 nm icosahedra	Nuc/Cyt	Yes	Plant-infecting *Geminiviridae*

The table is modified from Kobayashi and Hillman (2005). For a more complete list of fungal viruses, refer to Ghabrial and Suzuki (2009). "Location" refers to subcellular site of virus replication and/or particle accumulation: Cyt, cytoplasm; Mito, mitochondria; Nuc, nucleus. "Disease" indicates whether or not the virus type is generally associated with a disease phenotype in the fungal host, such as reduced growth in culture or reduced virulence on a plant host. Information about each virus group can be found in Fauquet et al. (2005).

than viruses of other eukaryotes, and much less cumulative time has been spent on these viruses since they were first described in the 1960s. Broader virus screens such as virus particle purification and/or electron microscopy are expensive and time-consuming and often are inherently flawed for detecting the many fungal viruses that have no true capsids or particles. By the 1970s, the dogma developed that most fungal viruses had positive-sense ssRNA or dsRNA genomes and this led to the practice of many scientists screening for viruses in fungi by rapid dsRNA analysis, which captures both these virus types but neither DNA viruses nor, effectively, negative-sense ssRNA viruses. Thus, there is a built-in bias: we find the viruses we look for.

Assuming the search for viruses could have detected DNA viruses, their absence is not entirely unexpected. Large DNA viruses—those with genomes often in excess of 100 kb and with particle sizes in excess of 100 nm—are prevalent in aquatic lower eukaryotes such as algae and amoebae (Nagasaki, 2008; Van Etten et al., 2002), but are completely absent in higher plants and filamentous fungi. The abundance of these viruses in aquatic organisms reflects, in large part, their biology and ecology: such viruses replicate and accumulate until they lyse their hosts' cells and are released into the aquatic environment. They then infect new host cells by attachment and entry or injection of DNA, much like dsDNA bacteriophages. This lifestyle is not an option for viruses of filamentous fungi that live without free water around their cells. Even the oomycetes, or plant pathogenic "water molds," are multicellular and spend much of their lives away from water, and no large DNA viruses have been identified in this group. Until very recently, small DNA viruses—those with genomes <20 kb and particles <50 nm—were also largely or completely undiscovered in fungi. The finding of a small ssDNA-containing virus in the ascomycete *Sclerotinia sclerotiorum* that has not yet been classified by ICTV but is phylogenetically most closely related to the plant-infecting geminiviruses (Yu et al., 2010) raises a variety of questions, including whether it represents one example of many that are yet to be discovered. Discovery of viruses of this type via a screen of fungal nucleic acid extracts is only slightly more time-consuming than the targeted dsRNA analysis described above and used commonly. It requires some type of fractionation of total nucleic acid into RNA and DNA fractions, using LiCl or other salt fractionation, nuclease digestion, or a combination of these methods, combined with a sensitive method of gel analysis of various fractions. We anticipate that the description of the *Sclerotinia* DNA virus, especially as a fairly close relative of the highly economically important plant-infecting geminiviruses, will lead fungal virologists to cast wider nets in searches for extrachromosomal elements.

9.2.1.1 Cytoplasmic RNA Viruses

Most fungal viruses fall into the broad category of cytoplasmic RNA viruses. Although they differ greatly in phylogenetic and molecular characteristics (Buck, 1998; Ghabrial and Suzuki, 2009; Hillman and Suzuki, 2004), they probably are similar ecologically. Whether or not a given virus is successful in invading host populations will likely have more to do with virus virulence, transmission properties through sexual and asexual spores, and fungal population structure than virus molecular biology. So, for instance, *Cryphonectria hypovirus 2* (CHV-2, family *Hypoviridae*, genus *Hypovirus*) and *Mycoreovirus 1-Cp 9B21* (family *Reoviridae*) are unrelated viruses found in the chestnut blight fungus, *Cryphonectria parasitica*, but both cause severe host debilitation and are transmitted at less than 5% efficiency through asexual spores (conidia). Consequently, it is not surprising that both are found at low incidence (Hillman and Suzuki, 2004).

9.2.1.2 Mitochondrial Viruses

Fungi have a relatively large family of viruses, the family *Narnaviridae*, is devoid of protein coats and reside in mitochondria, in cytoplasm, or perhaps in one case both. The two

cytoplasmic viruses were identified in the yeast *Saccharomyces cerevisiae* as small RNAs that segregated in mating populations in a non-Mendelian fashion. The viruses were named *Saccharomyces 20S RNA narnavirus* (ScNV-20S) and *Saccharomyces 23S RNA narnavirus* (ScNV-23S, both family *Narnaviridae*, genus *Narnavirus*) based on the sedimentation coefficient of the RNAs originally identified (Wickner, 2001). Both have been found fairly abundantly but neither has an apparent effect on phenotype of the yeast host. A reverse genetics system to examine replication of ScNV-20S was developed (Esteban and Fujimura, 2003), but the lack of phenotype, and thus economic importance associated with the virus, resulted in relatively little use for it. ScNV-20S is more abundant than ScNV-23S in yeast strains examined to date: $\sim$20% versus $<$10% overall, and both may occur together (Lopez et al., 2002; Maqueda et al., 2010; Nakayashiki et al., 2005). Both are induced by nutritional stress (Lopez et al., 2002), but the roles and regulation of these viruses in the stress response have not been investigated.

The mitochondrial viruses in the *Narnaviridae* are in a separate genus, the genus *Mitovirus*, and are found in filamentous ascomycetes and basidiomycetes. They are of more interest than the yeast narnaviruses because many of them cause disease in fungal hosts that are plant pathogens, resulting in reduced virulence of those pathogenic fungi and possible use as biological control agents. Few of these viruses have been examined at the population level, but there are some interesting properties in different members of this genus that make them worthy of further investigation.

A single mitovirus, *Cryphonectria mitovirus 1* (CMV-1, family *Narnaviridae*, genus *Mitovirus*), was identified in *C. parasitica*. This was the first mitovirus that was characterized to the level of nucleotide sequence and was demonstrated formally to fractionate with the mitochondrial fraction in subcellular localization studies (Polashock and Hillman, 1994). CMV-1 had a measurable but not dramatic effect on virulence and phenotypic characteristics of colonies in culture. Attempts to quantify specific phenotypic effects of CMV-1 on *C. parasitica* exposed the complications of working with a nonencapsidated virus that resides in mitochondria. Completing Koch's postulates with a virus requires isolation of the virus and introduction of the purified virus to the new uninfected host. Classically, this is done by isolating virus particles to homogeneity and introducing them to an uninfected host. An even more convincing demonstration is by reverse genetics using a cDNA clone representing the complete viral genome to launch synthetic RNA transcripts that initiate infection. With no true particles, an infectious particle fraction of mitoviruses is not really an option, and attempts to date to initiate mitovirus infection from cDNA clones—which require the technically difficult step of introducing RNA into mitochondria—have been unsuccessful (B. I. Hillman, unpublished). Transmission of mitoviruses by anastomosis raises the question of what exactly is being transmitted. If whole virus-infected mitochondria are transmitted, then virus effects cannot be isolated from factors associated with the mitochondrion itself, such as plasmids or debilitating mutations arising from mitochondrial DNA recombination. Polashock et al. (1997) found that the experimental transmission of CMV-1 by anastomosis was consistently associated with mitochondrial DNA recombination.

9.2.1.3 Retrotransposons Fungal retrotransposons (Class I transposons) are classified by ICTV into two virus families: the *Metaviridae* (Ty3 or gypsy-like) and the *Pseudoviridae* (Ty1 or copia-like) (Boeke et al., 2005; Eickbush et al., 2005). Their relationships and properties (detailed below) reveal why they are included in virus classification by ICTV, whereas transposons that move around the genome via a DNA cut and paste mechanism with no RNA intermediate (Class II transposons) are not. Examples of the latter group, which are common in filamentous fungi, include the hAT-like and

Tc1/mariner-like families of Class II DNA transposons (Daboussi and Capy, 2003).

The *Metaviridae* are more closely related to metazoan retroviruses than they are to the *Pseudoviridae*, and it is likely that they are the progenitors of more familiar mammalian retroviruses such as *Human immunodeficiency virus-1* and *-2* (HIV, family *Retroviridae*, genus *Lentivirus*) or *Feline leukemia virus* (FeLV, family *Retroviridae*, genus *Gammaretrovirus*) (Boeke et al., 2005). Retrotransposons lack the gene for the envelope protein that allows retroviruses to acquire a lipid envelope, exit a cell, and enter a new host; thus retrotransposon particles, similar otherwise to retrovirus particles, are confined to their host except during fusion with a permissive host that allows their replication and integration. Retrotransposons multiply within the cell by reverse transcription via particles that encapsidate RNA, may move horizontally by anastomosis, are stress induced, and have abundant, well-documented effects on host genomes (Beauregard et al., 2008). Effects on host phenotype are not known to be associated with retrotransposon genome expression or virus particle accumulation within fungal hosts; these effects are rather associated with their integration into the host genome, for example, fungicide resistance (Kretschmer et al., 2009) and reproductive development (Nishimura et al., 2000). Thus, while they are complex and biologically interesting, retrotransposons are likely to be substantially similar to Class II transposons from the ecological perspective, moving rarely horizontally. On the other hand, approximately 70% of the genome of the barley powdery mildew fungus, *Blumeria graminis* f. sp. *hordei*, is composed of retrotransposons (Spanu et al.,), undoubtedly having a major impact on genome structure and function.

9.2.2 Relationships of Fungal Viruses to Other Eukaryotic Viruses

Eukaryotic microbes such as fungi and oomycetes often live in close and constant contact with plants and metazoans, so there is ample opportunity for cross-kingdom exchange of viruses. The phylogenies of core virus genes from these diverse hosts suggest that this has happened with regularity over time: every virus of fungi and oomycetes seems to have a close relative that infects plants and/or metazoans (Table 1). The first well-documented example of this was the sequence analysis of *Cryphonectria hypovirus 1* (CHV-1, family *Hypoviridae*, genus *Hypovirus*), in which the RNA-dependent RNA polymerase (RdRp), helicase, and papain-like proteinase genes were found to be closely related to homologs of the plant-infecting virus family *Potyviridae* (Koonin et al., 1991). Other examples are abundant and a few are provided: the closest relatives of mycoreoviruses are tick-borne, mammal-infecting reoviruses such as *Colorado tick fever virus* (family *Reoviridae*, genus *Coltivirus*) (Hillman et al., 2004); the closest relatives of the oomycete-infecting virus *Phytophthora infestans* RNA virus 1 (PiRV-1) (Cai et al., 2009) are the mammal-infecting astroviruses; *Botrytis virus F* (family *Gammaflexiviridae*, genus *Mycoflexivirus*) and *Botrytis virus X* (family *Alphaflexiviridae*, genus *Botrexvirus*) are closely related to plant-infecting potexviruses (Howitt et al., 2001). Recent examples include viruses from two plant-feeding hemipterans, a leafhopper and a treehopper, that were related to the *Totiviridae* and *Megabirnaviridae* families of fungal and lower eukaryote viruses (Spear et al., 2010), and a new virus from *S. sclerotiorum* whose closest identified relative is *Hepatitis E virus* (family *Hepeviridae*, genus *Hepevirus*) (Liu et al., 2009). Finally, the closest relatives of fungal dsRNA viruses of the family *Partitiviridae* are plant cryptic viruses of the same family (Ghabrial et al., 2005).

In some cases, the relationships between different eukaryotic hosts and related viruses are easily rationalized: in the case of the fungal reoviruses, the tick-borne, mammal-infecting coltiviruses contain 12 dsRNA segments, whereas the mycoreoviruses generally contain 11. It could be hypothesized that virus adaptation from an invertebrate to a fungal host might involve loss of a dispensable segment not

required for the fungal virus lifestyle, and, in fact, *Mycoreovirus 3* in *Rosellinia necatrix* isolate W370 (MyRV3/RnW370) may have 12 or 11 segments, with segment 8 often lost in continuous culture (Kanematsu et al., 2004). The phylogenetic relationships among many of the other dsRNA segments of the mycoreoviruses and their corresponding segments in the mammal/tick-infecting viruses are clear (Hillman et al., 2004). In another example, *Botrytis* viruses F and X are rod-shaped ssRNA viruses with particle structures, genome structures, and genome organizations similar to plant potexviruses. The fundamental difference between viruses from the different hosts is that the *Botrytis* viruses both lack homologs of the genes found in plant-infecting potexviruses that are known to be involved in cell-to-cell movement through plasmodesmata, which would be unnecessary in a fungal host. The final example of the *Partitiviridae* is of particular interest because there is nothing about the simple genome organizations of the members that infect fungi that distinguishes them from those that infect plants (Ghabrial et al., 2005). Furthermore, the plant-infecting members of the *Partitiviridae* have properties reminiscent of fungal viruses in that they cause symptomless infections in their hosts and they do not move actively through the host, but rather move passively during division of infected cells (Ghabrial et al., 2005), a combination of properties that is unique among plant viruses.

9.2.3 RIP as a Defense Against Fungal Viruses and Virus-Like Agents

Repeat-induced point (RIP) mutation is a premeiotic mechanism by which fungi detect and disable duplicated sequences greater than ~400 bases in size (Galagan and Selker, 2004). Cytosine bases in either copy of the duplicated sequence are methylated by a DNA methyltransferase called *rid* (RIP defective) (Freitag et al., 2002)—identified so far only in fungi—and subsequently deaminated to thymine. Efficiency of RIP in fungi is highly variable and it remains poorly understood in terms of mechanism and its extent through the kingdom fungi. RIP was first identified in *Neurospora crassa* and is more efficient in this fungus than in any other examined to date. A single sexual cycle of *N. crassa* can result in mutation of 30% of target cytosines in duplicated sequences, generally resulting in destruction of any duplicated ORF that was present (Cambareri et al., 1989). RIP is documented in several other fungi, generally with efficiencies of less than 10% (Galagan and Selker, 2004).

It is hypothesized that the relative dearth of DNA viruses in filamentous fungi is due to suppression of such viruses by RIP. The assumption here is that an organism having an aggressive mechanism to surveil and debilitate duplicated sequences in nuclear genomic DNA will use the same mechanism to identify and destroy repeated viral sequences within the nucleus as well. Currently, there is no experimental evidence to address this. The single fungal DNA virus, named *S. sclerotiorum* hypovirulence-associated DNA virus 1 (SsHADV-1, not yet classified by ICTV), in *S. sclerotiorum* may provide some insight, but represents a single example with no direct experimental data. SsHADV-1 is related to plant-infecting geminiviruses and, similar to geminiviruses, replicates in the *S. sclerotiorum* nucleus (Yu et al., 2010). *S. sclerotiorum* does in fact have a reasonably efficient RIP system. The genome size of SsHADV-1, 2166 nt, is in the range that should be subject to RIP, yet the virus survives. Whether RIP is acting on the SsHADV-1 genome or whether the virus escapes the RIP system is currently unknown.

9.2.4 RNA Silencing as an Active Defense Against Fungal Viruses

Possibly the most important finding in plant virology during the past 20 years is the elucidation of RNA silencing as a pathogen-specific defense system in plants and the corresponding virus counterdefense mechanism of suppressors of RNA silencing (Csorba et al., 2009; Wang and Metzlaff, 2005). RNA silencing is a component of the overall RNA interference

(RNAi) systems found in eukaryotes. It is a complex multistep process by which foreign sequences are detected in the cytoplasm at the dsRNA level, enzymatically cleaved by dsRNA-specific ribonucleases, and the resulting short RNAs are used to prime a sequence-specific surveillance system that identifies and destroys other copies of that sequence. Suppressors of silencing prevent the effective functioning of this antiviral silencing system at any of several steps, depending on the suppressor, and are identified in most all of the plant-infecting RNA viruses in which they are sought. Viruses with small genomes usually have one such silencing suppressor; viruses with larger genomes may have two or even three such suppressors (Lu et al., 2004). RNA silencing systems in filamentous fungi have many of the same properties as plant systems (Cogoni and Macino, 1999a, 1999b, 1999c). This observation led to the discovery that viral suppressors of host RNA silencing can be functional across kingdoms: plant virus suppressors function in fungi and fungal virus suppressors function in plants (Segers et al., 2006, 2007; Ghabrial and Suzuki, 2009). We still know very little about fungal virus silencing suppressors and the overall role of silencing in fungal virus ecology, but the identification of core RNA silencing machinery in genomes of fungi examined to date implies that viruses that are going to be successful in fungal hosts will need mechanisms to counter these defense systems.

9.3 TRANSMISSION OF FUNGAL VIRUSES

Fungal viruses are transmitted by direct cell-to-cell contact, with no vectors or extracellular phase. Transmission occurs vertically to offspring in sexual or asexual spores and horizontally between individuals by fusion (anastomosis) of cells. Transmission has also been documented to occur between species, both in the laboratory and in nature. All three types of transmission affect the ecology and evolution of fungal viruses.

9.3.1 Vertical Transmission

Vertical transmission is the dominant mode of transmission for most fungal viruses. In ascomycetes, the general pattern that initially emerged was that viruses are transmitted through asexual spores (conidia) but not sexual spores (ascospores) (Lecoq et al., 1979). However, transmission can be quite variable, and exceptions to this rule have emerged. In some fungi, transmission through conidia can be close to 100%, making it difficult to obtain virus-free isogenic isolates (Coenen et al., 1997; Ikeda et al., 2004; Park et al., 2006; Polashock and Hillman, 1994; van Diepeningen et al., 2006). In other systems, transmission may be intermediate and variable in frequency (Brasier, 1983; Elias and Cotty, 1996). For example, *Heterobasidion annosum virus* (HaV, family *Partitiviridae*, genus *Partitivirus*) was transmitted to 3% of conidia in one isolate of the basidiomycete tree pathogen *H. annosum* and 55% in another (Ihrmark et al., 2002). Similar variation in transmission of hypoviruses through conidia was observed among isolates of *C. parasitica* (Enebak et al., 1994; Russin and Shain, 1985), depending on specific combinations of virus and fungal isolates (Y.-C. Liu and M. G. Milgroom, unpublished). Virus-associated complexities of vertical transmission through conidia have recently been revealed in two studies of viruses in *C. parasitica*. In one study, a single CHV-1 hypovirus gene was shown to increase dramatically the vertical transmission of an unrelated reovirus through conidia (Sun et al., 2006). In the other study, rearrangement of a specific segment of that same reovirus was shown to reduce transmission of that reovirus through conidia (Eusebio-Cope et al., 2010).

Although viruses are not typically transmitted through ascospores (Brasier, 1983; Carbone et al., 2004), there are exceptions. For example, viruses were transmitted with high efficiency through ascospores of *Neosartorya hiatsukae* after self-fertilization of a virus-infected isolate (Varga et al., 1998). In *Emericella nidulans* (anamorph *Aspergillus nidulans*), 14% of

ascospores contained virus after self-fertilization of virus-infected isolates, whereas only 1 of 80 ascospores (~1%) contained virus after outcrossing between virus-infected strains (Coenen et al., 1997). In the rice blast fungus, *Magnaporthe oryzae*, viruses were transmitted to approximately 10% of ascospores (Chun and Lee, 1997). Most viruses in the Dutch elm disease fungus, *Ophiostoma novo-ulmi*, are in the genus *Mitovirus* (family *Narnaviridae*). These viruses would be expected to be maternally inherited in mitochondria but are not transmitted through ascospores (Brasier, 1983; Rogers et al., 1986).

Ascospore transmission of many of the *C. parasitica* viruses has been studied with variable results. The mitovirus CMV-1 is transmitted in mitochondria to ascospores at a frequency of ~50%, but only when the maternal parent is infected (Polashock et al., 1997). The *Cryphonectria* reoviruses MyRV-1 and MyRV-2, which are cytoplasmic and not mitochondrial, are also transmitted vertically to ~60% of the ascospores (Deng et al., 2007a). In contrast, none of the four species of *Cryphonectria* hypoviruses has been shown to be transmitted through ascospores, largely because they suppress female fertility almost completely (Anagnostakis, 1982; Carbone et al., 2004; Elliston, 1985), and thus infected individuals mate as males and rarely as females (Carbone et al. 2004). Interestingly, *Cryphonectria* hypoviruses dramatically suppress expression of two genes involved in female fertility, one a sex pheromone precursor and the other a transcriptional factor (Deng et al., 2007b). The two reoviruses do not greatly suppress expression of these genes, thus allowing female fertility in reovirus-infected strains (Deng et al., 2007a).

Among basidiomycetes, viruses are frequently transmitted through sexual spores (basidiospores), although there are exceptions. Viruses are transmitted through basidiospores produced by virus-infected parent isolates at a high frequency in some species (Castanho and Butler, 1978; Pfeiffer et al., 1996), whereas none were found among 94 basidiospore cultures of *Helicobasidium mompa* although one isolate contained a novel dsRNA segment (Ikeda et al., 2004). Transmission of *La France* isometric virus (LFIV, unassigned dsRNA virus) through basidiospores of the cultivated mushroom, *Agaricus bisporus*, can be variable, ranging from 33% to 100% among different fruiting bodies (Romaine et al., 1993).

In ascomycete yeasts, unlike filamentous ascomycetes, vertical transmission through ascospores occurs frequently. In *S. cerevisiae*, viruses appear to be preferentially included in ascospores during spore formation (Brewer and Fangman, 1980). Transmission occurs vertically during mating when haploid cells of opposite mating type fuse (Schmitt and Breinig, 2002). Although this process superficially resembles horizontal transmission (see below), cell fusion in yeasts is immediately followed by meiosis and formation of ascospores, into which viruses are transmitted. As in ascomycete yeasts, totiviruses such as *Ustilago maydis virus H1* (UmV-H1, family *Totiviridae*, genus *Totivirus*) are vertically transmitted during mating (heterokaryon formation) in the basidiomycetes corn smut fungus, *Ustilago maydis* (Day and Dodds, 1979; Martinez-Espinoza et al., 2002; Voth et al., 2006). Haploid yeast-like cells derived from basidiospores fuse during the sexual cycle and the resulting filamentous dikaryon will be virus-infected if one of the haploid strains was infected. The heterokaryon then transmits virus to a high percentage of haploid basidiospores after meiosis.

9.3.2 Horizontal Transmission

Horizontal transmission occurs when cells (hyphae) of different individuals fuse (anastomose) and viruses move from the cytoplasm of one individual to the other. Stable anastomoses are regulated in fungi by vegetative incompatibility (also called heterokaryon incompatibility). If two individuals are compatible, their hyphae can fuse and establish continuity of cytoplasm, which then allows nuclei and other intracellular elements, including viruses, to migrate freely. Vegetative incompatibility is controlled in ascomycetes by heterokaryon

incompatibility (*het*) genes at multiple loci (Glass and Kuldau, 1992; Leslie, 1993) and is independent of mating compatibility, except in *Neurospora*. These genes are referred to as vegetative incompatibility genes (*vic*) in some fungi (Cortesi and Milgroom, 1998). If two individuals share the same alleles at all *vic* (or *het*) loci, they are compatible, and if there is a different allele at one or more *vic* loci, they are incompatible and considered to be in different vegetative compatibility types or groups (vc types or VCGs). Incompatibility is evident microscopically after anastomosis in the fusion cells by vacuolation and shrinkage of the cytoplasm, followed by cell collapse and death (Biella et al., 2002; Glass and Dementhon, 2006; Glass and Kaneko, 2003). Macroscopically, vegetative incompatibility is evident in some species as zones of dead cells called a barrage (Figure 9.1a). Thus, programmed cell death breaks the continuity of cytoplasm between two anastomosing individuals and restricts horizontal virus transmission (Figure 9.1b) in a process similar to the way hypersensitive responses in plants resist infection by viruses or biotrophic cellular pathogens (Paoletti and Saupe, 2009).

The restriction of horizontal transmission by vegetative incompatibility has been shown for numerous fungi (Caten, 1972; Hoekstra, 1996). As with vertical transmission, however, horizontal transmission varies among different systems. Among basidiomycetes, somatic incompatibility (as vegetative incompatibility is called in basidiomycetes) severely restricts horizontal transmission of *Rhizoctonia virus M2* (RVM2, unclassified member of the family *Narnaviridae*) in *Rhizoctonia solani* anastomosis group 3 (AG-3), although some transmission occurs at a low frequency (Charlton and Cubeta, 2007). Transmission of *Helicobasidium mompa virus 1-17* (HmV-17, family *Totiviridae*, genus *Victorivirus*) also occurs at a low frequency among incompatible isolates in *H. mompa* (Suzaki et al., 2005). In contrast, horizontal transmission of HaV was not greatly restricted by somatic incompatibility in *Heterobasidion annosum*, even between isolates in different intersterility groups (Ihrmark et al., 2002) that by definition have diverged genetically such that they do not mate successfully. In *Agaricus bisporus*, viruses can be transmitted readily among commercially grown strains, but transmission is severely restricted to wild strains (Sonnenberg et al., 1995), presumably because of somatic incompatibility.

Among ascomycetes, horizontal transmission has been shown numerous times to be restricted by vegetative incompatibility (Brasier, 1983; Coenen et al., 1997; Park et al., 2006; van Diepeningen et al., 1997). In *O. novo-ulmi*, the incidence of viruses in nature was shown to correlate negatively with the genetic diversity of populations. Brasier (1988) showed that virus incidence was high at epidemic fronts where *O. novo-ulmi* had recently colonized, compared to low incidence in more established populations. Significant to virus transmission, populations at the epidemic front were clonal, comprising few *vic* genotypes, or vc types. Therefore, vegetative incompatibility imposed much less restriction on virus transmission in these populations compared to more diverse populations where *O. novo-ulmi* had been present longer.

Horizontal transmission of fungal viruses, in both the laboratory and in natural populations, is best characterized for CHV-1 in *C. parasitica*. Vegetative incompatibility was recognized as restricting horizontal transmission at an early stage in the deployment of CHV-1 for biological control (Grente and Berthelay-Sauret, 1978); details of this restriction were investigated later (Anagnostakis, 1983; Cortesi et al., 2001; Liu and Milgroom, 1996). Because the genetic basis of vegetative incompatibility was determined for 64 *vic* genotypes (which define vc types) of *C. parasitica* (Cortesi and Milgroom, 1998), it was possible to determine the effect of each allele at six *vic* loci on horizontal transmission (Cortesi et al., 2001). The frequency of transmission between individuals that differed at single and multiple *vic* loci was quantified *in vitro* (Figure 9.1b). The variation among alleles on transmission was striking: some *vic* alleles reduced transmission

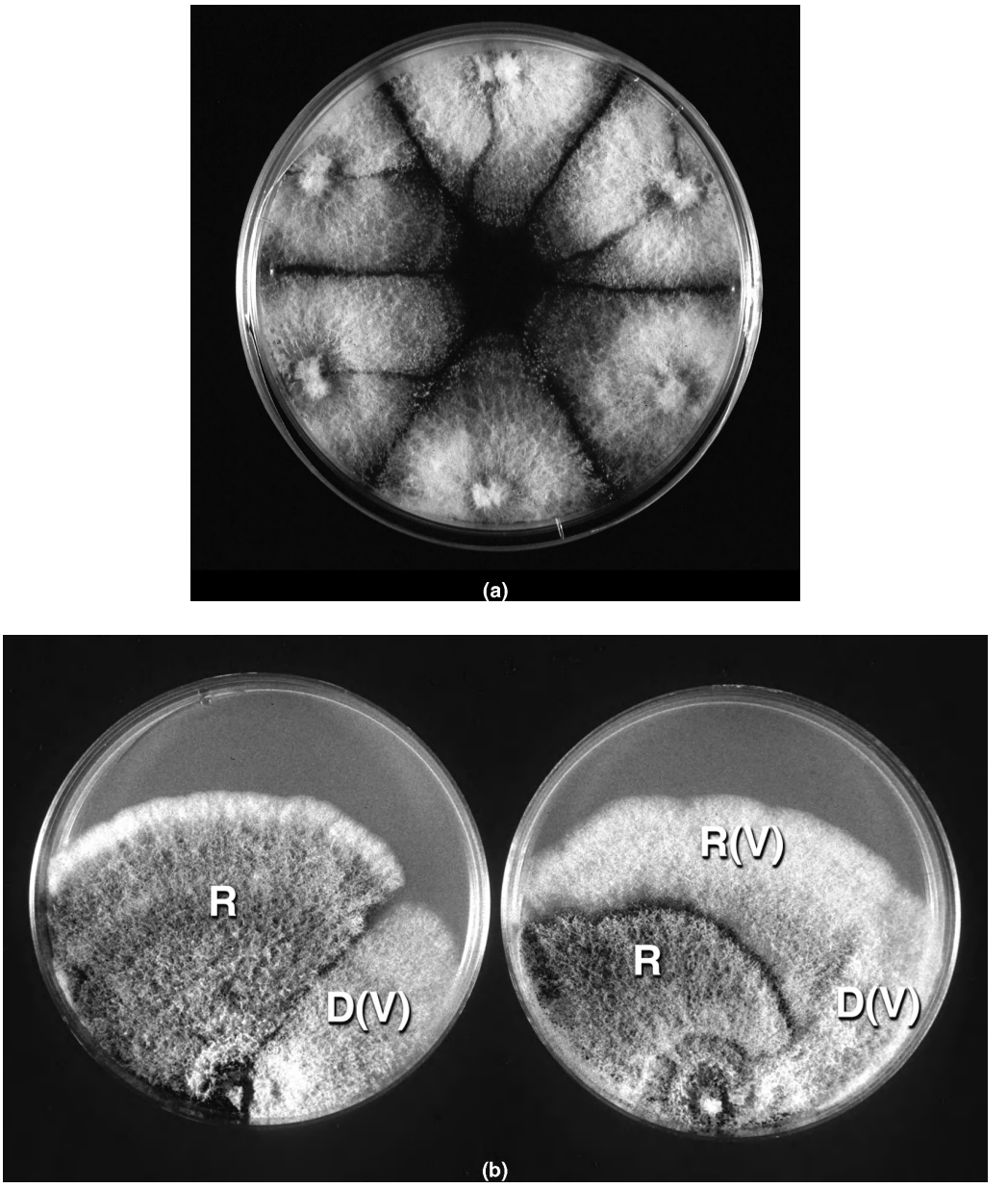

FIGURE 9.1 Vegetative (or heterokaryon) incompatibility results in programmed cell death in the chestnut blight fungus, *Cryphonectria parasitica*, when cells (hyphae) of incompatible individuals fuse, and restricts the horizontal transmission of CHV-1 (family *Hypoviridae* and genus *Hypovirus*). (a) Vegetative incompatibility between pairs of isolates grown on solid medium is evident macroscopically as zones of cell death (barrages), whereas compatible pairs (bottom and lower right pairs) have colonies that grow confluently. (b) Transmission of CHV-1 (plate on right) from virus-infected donor isolate, D(V), to the recipient isolate, R, after culturing the two isolates together on solid medium. Transmission is evident in the plate on the right because the recipient isolate, R(V), acquired the virus-infected phenotype with less pigmentation. Failure of transmission of CHV-1 (plate on left) between vegetatively incompatible isolates. Note that the recipient isolate did not change phenotype. (Reprinted from Cortesi et al. (2001) with permission of The Genetics Society of America.) Photos by Kent Loeffler, Cornell University. (*See the color version of this figure in Color Plate section.*)

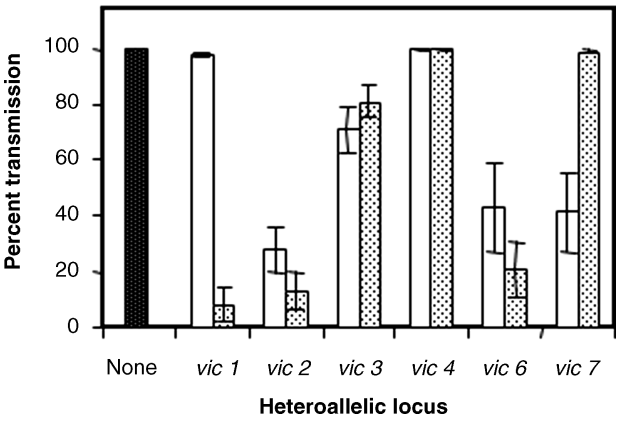

FIGURE 9.2 Effects of alleles at six vegetative incompatibility (*vic*) loci in *Cryphonectria parasitica* on transmission of CHV-1. Percent transmission represents the successful transmissions between replicated pairs of isolates with alleles that differed only at the specified *vic* loci. Open bars represent recipient isolates with allele *1* at the *vic* locus indicated, whereas stippled bars represent recipient isolates with allele *2*. Transmission was always 100% between vegetatively compatible isolates (all *vic* alleles the same, solid bar). (Reprinted from Cortesi et al. (2001) with permission of the Genetics Society of America.)

markedly, whereas others had no effect (Figure 9.2). Moreover, alleles at some loci had significant asymmetric effects such that markedly greater transmission occurred from one isolate to the other depending on which isolate was the donor and which was the recipient. The variance associated with each *vic* allele was sometimes large, suggesting that genes other than *vic* also affect virus transmission; possibly genes associated with cell death downstream of the vegetative incompatibility interaction *per se* may be involved. Furthermore, horizontal virus transmission has been reported to vary for different virus isolates (Deng et al., 2009), although other studies reported no differences in horizontal transmissibility among different viruses and plasmids (Baidyaroy et al., 2000; Liu and Milgroom, 1996).

The asymmetry in transmission of CHV-1 was correlated with asymmetry in cell death (Biella et al., 2002). For example, for fungal isolates that differed in *vic* genotype only at locus *vic1*, virus transmission occurred nearly 100% of the time from donor isolates with allele *vic1-2* to recipient isolates with allele *vic1-1*, but transmission was reduced to approximately 10% when transmission was attempted in the opposite direction (Figure 9.2) (Cortesi et al., 2001). Cell death after hyphal fusion in isolates with allele *vic1-1* was consistently delayed relative to isolates with *vic1-2*. Delayed cell death in the recipient would allow more time for viruses to move into it from the donor. Interestingly, the presence of CHV-1 in the donor reduced the rate of cell death in the recipient, possibly by suppressing host defenses (Biella et al., 2002) (M. L. Smith, personal communication).

On the basis of laboratory estimates of the effects of *vic* alleles on transmission, Cortesi et al. (2001) developed a regression model to predict the probability of transmission between any two *vic* genotypes (vc types). Not surprisingly, as populations of *C. parasitica* become more diverse for vc types, the average transmission at the population level is reduced (Milgroom and Cortesi, 2004; Papazova-Anakieva et al., 2008) (Figure 9.3). This general relationship was pointed out for *C. parasitica* in a comparison of vc type diversity between Europe and North America (Anagnostakis et al., 1986). Relatively high frequencies of CHV-1 in Europe correlate to low vc type diversity (Milgroom and Cortesi, 1999; Robin et al., 2000; Robin and Heiniger, 2001). In contrast, multiple attempts to introduce

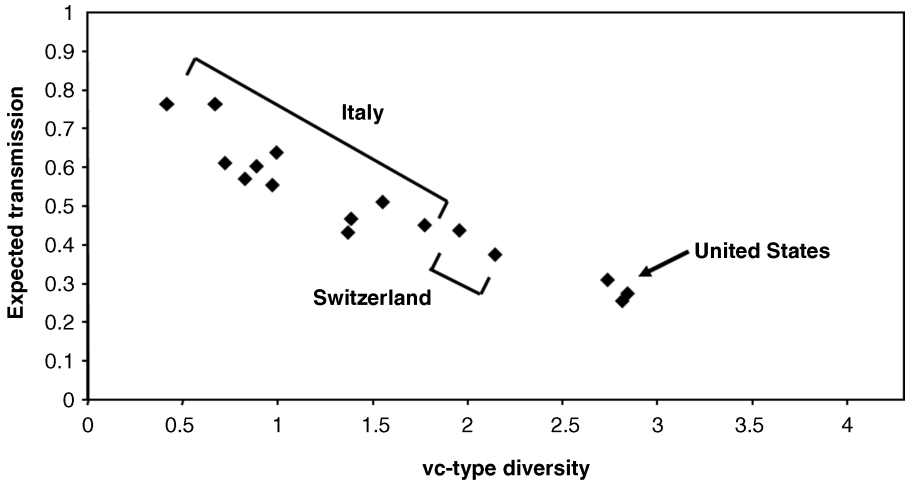

FIGURE 9.3 Expected transmission of CHV-1 in populations of *Cryphonectria parasitica* based on the diversity of vegetative compatibility types. The expected transmission was determined using a regression model (Cortesi et al., 2001) based on laboratory transmission experiments (see Figure 9.2), weighted for the distribution of vc types in each population. Diversity was estimated using the Shannon index. (Reprinted from Milgroom and Cortesi (2004) with permission of Annual Reviews.)

CHV-1 into *C. parasitica* populations in the eastern United States, which are more diverse than Europe, have failed (see below) (Milgroom and Cortesi, 2004). In China and Japan, where CHV-1 is native, vc type diversity is even greater than in North America (Liu and Milgroom, 2007; Wang et al., 1991) and virus incidence is low (Liu et al., 2003, 2007; Peever et al., 1998).

Despite the negative correlations of vc type diversity with virus incidence, the actual transmission between vc types in nature is difficult to estimate. Laboratory estimates may not be a good guide because *in vitro* experimental conditions are highly artificial. Transmission of CHV-1 in *C. parasitica* is thought to occur at higher rates than predicted from laboratory assays (Bisiach et al., 1988; Double, 1982). Transmission is likely affected by the number of contacts between individuals, the length of time individuals interact, and spatial heterogeneity of genotypes (Liu et al., 2000), all but the last of which are difficult to estimate (Dutech et al., 2008; Milgroom et al., 1991). Carbone et al. (2004) used a population genetics approach to estimate the migration of CHV-1 between dominant vc types in two populations in Italy. In contrast to predictions based on laboratory estimates, they found high rates of migration between vc types. Because the migration estimates integrate all interactions over long periods, instead of brief encounters in artificial laboratory experiments, these authors concluded the barriers that vegetative incompatibility presents with respect to horizontal virus transmission may not be as severe as previously thought for CHV-1 in *C. parasitica*. The significance of vegetative incompatibility and horizontal transmission is discussed further below in the context of biological control of fungi with viruses.

9.3.3 Interspecies Transmission

Besides horizontal transmission between individuals within species, there is mounting evidence that fungal viruses are transmitted between species. While these may be relatively rare events, they have the potential to introduce viruses into new host species. There are two types of evidence for interspecies transmission: experimental laboratory studies that demonstrate the potential for viruses to replicate and persist in other host species, and phylogenetic

or genealogical relationships among virus isolates that can be explained most easily by interspecies transmission.

Experimental studies that transmit fungal viruses to new species have been conducted in several ways. The technically simplest, and most natural, has been transmission by hyphal anastomosis. For example, viruses have been transmitted by anastomosis from *Aspergillus niger* to *A. nidulans* (Coenen et al., 1997), from *S. sclerotiorum* to *S. minor* (Melzer et al., 2002), and from *C. parasitica* to *C. nitschkei* (Liu et al., 2003). In all three examples, the viruses were stably maintained in the new species. In *S. minor*, as in *S. sclerotiorum*, virus transmission was associated with a hypovirulent phenotype.

Other experimental transmissions between species have required techniques of molecular biology. In *Aspergillus*, fungal viruses have been transmitted between species by protoplast fusion (Coenen et al., 1997; Liang and Chen, 1987; van Diepeningen et al., 1998). Interspecies transmission of CHV-1 has been achieved in two ways in addition to hyphal anastomosis: (1) by transfection of fungal spheroplasts with a synthetic viral coding-strand RNA and (2) by transformation with full-length infectious cDNA of the viral genome that integrates into the *C. parasitica* genome and gives rise to cytoplasmically replicating viral RNAs. Chen et al. (1994a, 1996) used RNA transcripts synthesized *in vitro* from a full-length cDNA clone of the CHV-1/EP713 genome, a virus isolated from *C. parasitica*, to transfect *C. cubensis*, *C. havanensis*, *C. radicalis*, and *Endothia gyrosa*, whereas van Heerden et al. (2001) transfected only *C. cubensis*. CHV-1 infection was established and resulted in reduced sporulation and alteration of pigmentation in all recipient species and in reduced virulence in *C. cubensis* and *E. gyrosa* (virulence was not tested in the other two species). Sasaki et al. (2002) extended the range of CHV-1/EP713 further by biolistic transformation of an infectious cDNA into species in two different genera in the same order as *Cryphonectria* (*Valsa* and *Phomopsis* in the Diaporthales). Double-stranded RNA from chromosomally integrated viral transgenes was detected in the cytoplasm and virulence was reduced. In all these studies with CHV-1, the authors concluded that transfection or transformation has potential for extending the range of CHV-1 into new species for biological control, although this prospect is yet to be realized.

Phylogenetic or genealogical relationships among fungal viruses from various host taxa provide compelling evidence that interspecies transmission has occurred in nature. Three criteria must be satisfied for inferring interspecies transmission from viral nucleotide sequence data: (1) the host species must be sympatric, (2) a plausible natural mode of transmission must exist, for example, by anastomosis between species, as demonstrated between some fungal taxa, and (3) genetically similar virus strains occur in different host species. High genetic similarity between virus strains in different host taxa could also result from the presence of polymorphisms in the virus in a common ancestor and recent divergence of host taxa. Therefore, to infer interspecies transmission, some virus isolates from different host species have to be more similar to one another than they are to other virus isolates from a common host species. These criteria were satisfied for CHV-1 in *C. parasitica* and *C. nitschkei*, which also can be transmitted between species by anastomosis (Liu et al., 2003), *Ophiostoma mitovirus* 5 (OMV5, family *Narnaviridae*, genus *Mitovirus*) in *O. novo-ulmi* and *O. ulmi* (Buck et al., 2003), and RVM2 among anastomosis groups of the *R. solani* species complex (Charlton et al., 2008).

The above examples demonstrate transmission of viruses between closely related species. Two additional examples suggest that transmission might also be possible between more distantly related taxa. Deng et al. (2003) found that nucleotide and amino acid sequences of the RdRp representing a mitovirus of *S. homoeocarpa* were 92% and 94% identical, respectively, to those of *Ophiostoma novo-ulmi mitovirus* 3a-Ld (OMV3a), and thus the *Sclerotinia*

mitovirus represents a strain of OMV3a. These authors concluded that viruses from the two host species are conspecific and argued that interspecies transmission must have occurred because otherwise the viral sequences would have diverged more because the two host species are distantly related. Wu et al. (2007) reported a similar finding in *Botrytis cinerea* in which the amino acid sequence of the RdRp of a virus they tentatively named *B. cinerea* debilitation-related virus (BcDRV), a presumed member of the family *Narnaviridae* and genus *Mitovirus*, is in fact closely related to another mitovirus from *O. novo-ulmi*, *Ophiostoma mitovirus* 3b (OMV3b), a tentative member of the family *Narnaviridae* and genus *Mitovirus*. When the sequence of BcDRV was completed recently, it was evident that there was a large noncoding sequence in the RNA of BcDRV that was not present in OMV3b, and thus they will not be considered strains of the same virus species (Wu et al., 2010). Interestingly, OMV3a and BcDRV reduce the fitness and virulence of *S. homoeocarpa* and *B. cinerea*, respectively, but OMV3a and OMV3b do not have any detectable effects on *O. novo-ulmi* (Cole et al., 1998). As more fungal viruses are sequenced and characterized, more such examples are sure to arise. The questions remain as to the mechanisms of virus transmission between distantly related taxa (presumably via hyphal anastomosis) and how frequently such transmission occurs.

9.4 EFFECTS OF VIRUSES ON FUNGAL FITNESS

As intracellular parasites, fungal viruses are completely dependent on their hosts for replication and transmission. However, the vast majority of fungal viruses have little or no detectable effect on host phenotype or fitness (Buck, 1986, 1998; Ghabrial, 1998; Ghabrial and Suzuki, 2009; McCabe et al., 1999; Pearson et al., 2009). The "conventional wisdom" about virulence is that associations persisting for a long time should evolve to being benign or even beneficial, and particularly so for pathogens that depend primarily on vertical transmission to host offspring (Bull, 1994; Levin, 1996). A pathogen that depends on vertical transmission and adversely affects the fitness of its host (i.e., virulence) will, on average, reduce its own fitness because vertical transmission will be reduced; in this case, therefore, virulence will be selected against. The evolution of fungal viruses can be interpreted in this context. Milgroom (1999) speculated that the barrier to horizontal transmission of fungal viruses imposed by vegetative incompatibility and their dependence on vertical transmission is a major contributing factor to their lack of virulence. Therefore, in long established fungus–virus relationships, virus infection is likely to be relatively benign, or possibly even beneficial (Ghabrial, 1998).

Although most fungal viruses appear to be benign, some have subtle deleterious effects on host fitness that are not evident without rigorous testing. These subtle effects raise the question of whether fungal viruses are always as benign as they appear superficially. Other viruses clearly have profoundly negative effects on host fitness and, therefore, may be at odds with the "conventional wisdom" paradigm described above. Marked virulence may be explained in two ways: first, when a pathogen has recently invaded a new host species, virulence may be transiently high until an evolutionarily stable level is reached. As noted above, hypovirulence in *S. homoeocarpa* and *B. cinerea* is associated with mitoviruses, whose closest related viruses are benign in *O. novo-ulmi* (Deng et al., 2003; Wu et al., 2007). The different effects in these host species are consistent with the hypothesis that these viruses have recently invaded new hosts by transmission between species. Alternatively, a virus may be transmitted to a host that is a dead end for transmission, meaning that the virus is incapable of persisting in that host population. This is often because even though the dead-end host may become diseased, it does not allow sufficient buildup of the virus for transmission. An example of this

in humans is the highly virulent *West Nile virus* (family *Flaviviridae*, genus *Flavivirus*), which is transmitted within avian populations and from birds to humans by mosquitoes but is not transmitted from human to human and thus has not spread widely in human populations (Weaver and Reisen, 2010). The second condition for which virulence may be an evolutionarily stable outcome is when horizontal transmission rates to new hosts are high (Lipsitch and Moxon, 1997). A high level of virulence may be advantageous if it enhances horizontal transmission, but selected against if virulence reduces transmission. In fungi, high rates of horizontal transmission of viruses may be possible in populations that are genetically uniform, as occurs in commercial mushroom production (Sonnenberg et al., 1995), or in clonal populations in nature, for example, populations of *O. novo-ulmi* and *C. parasitica* at epidemic fronts (Brasier, 1988; Milgroom and Cortesi, 1999; Milgroom et al., 2008).

Below are some examples where fungal viruses have their strongest effects on fungal fitness, either positive or negative. Most of these have attracted attention either because of their economic impact or for the prospects of exploiting viruses for human benefit, for example, biological control of fungi. Note that these examples are the exceptions among fungal viruses, and yet they are the best studied.

9.4.1 Mutualistic Interactions Between Viruses and Fungi

Viruses that maintain a stable or persistent relationship with their hosts through vertical transmission can evolve as mutualists (Ghabrial, 1998; Villarreal, 2007). Among fungi, beneficial symbiotic effects of viruses are evident as enhanced virulence (hypervirulence) in a few filamentous fungi or increased competitive ability of yeasts that produce toxins coded by viruses. A novel interaction recently reported involves a complex, three-way mutualism in which a virus-infected fungal endosymbiont enhances the ability of its plant host to withstand environmental stresses. These three examples of mutualisms are discussed in the following section.

9.4.1.1 Virus-Mediated Increases in Fungal Virulence (Hypervirulence)

The discovery and characterization of hypovirulence generated much interest in fungal viruses and their effects on virulence of plant pathogenic fungi (discussed below). Although many fungi have been found to contain dsRNA elements or viruses, few of them have been studied in depth because the dsRNAs or viruses have little effect on host phenotype. However, in at least two cases, discussed below, viruses have been demonstrated to increase virulence (hypervirulence) in plant pathogens. In other cases, for example with viruses of *Phytophthora infestans* (Tooley et al., 1989), there was some evidence that one or more viruses might be associated with increased virulence or enhanced growth rate in culture, but the lack of virus-free isogenic strains prevented rigorous testing of this hypothesis.

Within the *R. solani* species complex, anastomosis group 3 was known to harbor three different dsRNAs whose presence correlated to reduced fitness (Castanho et al., 1978). However, the phenotypic effects were not always consistent because different dsRNAs correlated differently with virulence (Bharathan and Tavantzis, 1990). Jian et al. (1997) subcultured sectors from a dsRNA-containing isolate in an effort to obtain isolates with specific combinations of segments. They found that a virus they designated as M1, which has a 6.4 kb dsRNA genome related to the plant-infecting family *Bromoviridae*, significantly increased virulence to potato (Jian et al., 1998), whereas a 3.6 kb dsRNA representing the genome of the mitovirus-related RVM2 counteracted this increase and reduced virulence compared to the same isolate that was cured of dsRNA. Thus, some viral dsRNAs appeared to reduce virulence, while another enhanced virulence.

In another example of hypervirulence, multiple dsRNAs were found in *Nectria radicicola*, which causes root rot in ginseng root (*Panax ginseng*) (Ahn and Lee, 2001). When

isolates of *N. radicicola* were cured of the largest (6.0 kb) dsRNA segment (L1), virulence, pigmentation, and asexual sporulation were reduced. Virulence was restored when the L1 segment was reintroduced by anastomosis (Ahn and Lee, 2001). Interestingly, the proportion of isolates containing the L1 segment was found at incidences of 9% and 4% in first cropping fields of ginseng in two provinces of Korea, whereas these proportions were 41% and 25%, respectively, in fields previously used for growing ginseng. Although samples were not collected from the same fields over time, Ahn and Lee (2001) speculated that the incidence of the L1 segment in the *N. radicicola* population increased over time because L1 was correlated with increased virulence.

In both these examples, the increase in virulence was correlated with the presence of a specific dsRNA virus. To consider this a mutualism, we have to assume that increased virulence confers increased fitness to the fungal plant pathogen and its virus. For root rot or stem pathogens such as *N. radicicola* and *R. solani*, this assumption may be reasonable because virulence is associated with greater colonization and reproduction of the fungal pathogens and consequently increased replication of mutualistic viruses.

9.4.1.2 Viruses Increase Competitive Ability of Killer Yeasts

The killer phenomenon in yeasts was first discovered in *S. cerevisiae* in 1963 because of contaminants in brewing and was later described in a number of yeast genera (Bruenn, 1980; Marquina et al., 2002; Schmitt and Breinig, 2002, 2006; Wickner, 1996). Killer yeasts are characterized as producing low molecular weight protein or glycoprotein toxins that kill closely related yeast taxa, while simultaneously being immune to the same toxin. The genetic basis of killer systems is variable and may be conferred by dsRNA viruses, linear dsDNA plasmids, or nuclear genes.

The best characterized killer viruses are those in *S. cerevisiae*. Two members of the family *Totiviridae*, genus *Totivirus*, *Saccharomyces cerevisiae L-A* (ScV-L-A) and *Saccharomyces cerevisiae L-BC* (*La*) (ScV-L-BC), which contain single dsRNA segments (L or large segment), can infect and be maintained independently, but without causing the killer phenotype. The killer phenotype is conferred only by infection with the totivirus together with one of three satellites that represent the toxin-encoding dsRNA segments (M or middle segment), namely, ScV-M1, ScV-M2, or ScV-M28. These satellite dsRNA M segments code for the toxins K1, K2, and K28, respectively; they also confer self-immunity to the same toxins (Wickner, 1996). A series of short (S) satellite dsRNA segments are present in some strains, but these short segments do not affect the killer phenotype. Thus, yeast strains bearing L and M segments can outcompete virus-free strains or those without an M segment because the toxins inhibit competitors. Other toxin-producing totiviruses, with fundamentally similar effects as the killer viruses described above, are found in the corn smut fungus, *Ustilago maydis*, which has haploid, yeast-like and dikaryotic, filamentous growth forms (Koltin, 1988).

Virus infection in yeasts is symptomless to the host cells. The icosahedral virus particles are not normally infectious. Although it is possible to transfect yeasts experimentally with virus particles, it is not known how important horizontal transmission may be in nature. Only one study, to our knowledge, has reported the natural uptake of virus particles into yeast cells from culture medium in the laboratory (El-Sherbeni and Bostian, 1987). Viruses are transmitted only in the yeast haploid stage when cells fuse during mating. Sensitive strains survive mating with killer strains and viruses are transmitted to sexual spores (ascospores). Infection with one virus strain excludes any other, so multiple infections are not found naturally.

Killer viruses are considered mutualists because their toxins kill other yeasts, increasing the competitive ability of the host strains (Starmer et al., 1987). They are found at relatively high frequencies in natural populations of yeasts (Marquina et al., 2002). The competitive advantage of killer strains,

however, depends on environmental conditions. The relationship of killer viruses to *S. cerevisiae* may vary from parasite to mutualist depending on pH (McBride et al., 2008) because the toxin only binds and is effective at inhibiting competitors in a narrow pH range. Similarly, the relationship may vary depending on host ploidy, wherein haploid cells are less likely to be killed because they are potential mates into which viruses can be transmitted (McBride et al., 2008). Regardless of these variables, killer strains are used for outcompeting unwanted contaminating strains to prevent stuck fermentations (Marquina et al., 2002; Schmitt and Breinig, 2002).

9.4.1.3 Complex Mutualisms A fascinating three-way symbiosis involving a fungal virus was reported recently. The interaction involves a virus in the fungus *Curvularia protuberata*, which is an endophyte of the tropical panic grass *Dichanthelium lanuginosum*. When *D. lanuginosum* is colonized by *C. protuberata*, it can grow in geothermal soils at high temperatures, but cannot grow at these temperatures in the absence of the endophyte (Redman et al., 2002). Marquez et al. (2007) demonstrated that thermal tolerance conferred by *C. protuberata* depends on the presence of a dsRNA fungal virus, which was named *Curvularia* thermal tolerance virus (CThTV, not yet classified by ICTV); endophytic isolates cured of CThTV no longer conferred heat tolerance. Furthermore, virus-infected fungal isolates of *C. protuberata* that colonized tomato plants also conferred heat tolerance, while those without virus did not. While there are other examples of endophytes (including plant viruses) that promote better growth in plants (Rodriguez et al., 2009), the role of fungal viruses is largely unknown, except for this one example. The detection of dsRNAs, presumably of viral origin, in 12 of 53 species in relatively small samples of fungal endophytes of grasses (Herrero et al., 2009) provides additional impetus for investigating similar effects of viruses on plant–fungal symbioses.

9.4.2 Fungal Viruses That Reduce Host Fitness

9.4.2.1 Apparently Symptomless Virus Infections Although the vast majority of fungal viruses would seem to cause symptomless infections, in which fitness is affected neither positively nor negatively, several studies question this simple interpretation. If viruses were either beneficial or truly benign, then we might expect them to occur at high frequencies in fungal populations. To some extent, this is true. For example, killer viruses in yeast are common in nature (Marquina et al., 2002) and CHV-4 is found in approximately 25% of *C. parasitica* isolates in the eastern United States (Peever et al., 1997). In contrast, many other fungal viruses are found only at low frequencies even when reported to have little or no apparent effect on fungal phenotypes. van Diepeningen et al. (2006) reported decreases in fitness associated with virus infection in isolates *A. niger*. Among 64 virus-infected isolates, they found that only one showed abnormal colony morphology. In contrast, linear growth rates, spore production, and competition *in vitro* were significantly reduced for the infected compared to isogenic virus-free isolates. These authors concluded that even though horizontal transmission is restricted in laboratory experiments (van Diepeningen et al., 1997), horizontal transmission must occur at rates high enough to counteract the observed decreases in fitness. More subtle decreases in fitness caused by viruses have also been observed. For example, small but significant reductions in fungal virulence, growth rate, or sporulation were shown in virus-infected isogenic isolates of two insect pathogens, *Metarhizium anisopliae* and *Beauveria bassiana*, but no other overt phenotypic effects were observed (Dalzoto et al., 2006; Melzer and Bidochka, 1998; Tiago et al., 2004). These studies are significant in questioning the dogma that most fungal viruses are symptomless, with no effects on fitness. Even small reductions in fitness can radically alter the dynamics of fungal viruses with their

hosts because small differences in fitness compound over time.

9.4.2.2 Diseases of Cultivated Mushrooms In contrast to the small reductions in fitness described above, fungal viruses were first described in the cultivated mushroom, *Agaricus bisporus* (Hollings, 1962), because of the drastic effects they have on host fitness. Virus-infected cultures produce slow-growing, sparse mycelium and exhibit suppressed development, dwarfing, or rapid death of fruiting bodies (Schisler et al., 1967). Diseased mushrooms produce fewer spores (basidiospores) than do healthy mushrooms and, on average, approximately 70% of the spore produced contain virus (Romaine et al., 1993). La France disease can severely reduce mushroom production, sometimes resulting in nearly complete loss of marketable crop (Fletcher et al., 1989). Symptoms of La France disease are consistently associated with the presence of LFIV in mycelium and fruiting bodies (mushrooms) (Fletcher et al., 1989; Goodin et al., 1992). Another virus, *Mushroom bacilliform virus* (MBV, family *Barnaviridae*, genus *Barnavirus*), is often associated with La France disease and is rarely found in the absence of LFIV, but MBV has not been detected in all cases of the disease, and its specific role in the disease and interactions with LFIV are unclear (Romaine and Schlagnhaufer, 1995).

Horizontal transmission of viruses occurs readily in commercial mushroom production because genetically homogeneous mycelium of *A. bisporus* is inoculated into large trays of compost. Therefore, there is little restriction due to somatic incompatibility, and small amounts of infected mycelium experimentally mixed into mushroom inoculants (spawn) results in widespread symptoms typical of La France disease in the mushroom crop (Schisler et al., 1967). In the normal disease cycle, germinating spores from infected mushrooms transmit the virus to healthy mycelia by anastomosis (Schisler et al., 1967; van Zaayen, 1979). Because large numbers of spores are sometimes produced by mushrooms under cultivation, a few infected fruiting bodies can spread viruses rapidly within and between mushroom farms (Schisler et al., 1967). The popularity of mushroom varieties that are harvested after they open, for example, "Portabello" mushrooms, increases the risks of virus spread once it is present in a crop.

Virus-infected mushrooms exhibit two phenotypes that enhance horizontal transmission. First, infected mushrooms are generally taller (longer "stems" or stipes), mature earlier, and discharge spores before healthy mushrooms (Schisler et al., 1967; van Zaayen, 1979). Therefore, infected mushrooms may go unnoticed and produce large numbers of virus-infected spores before the rest of the crop is harvested. Second, basidiospores from virus-infected mushrooms germinate more frequently and more quickly than basidiospores from healthy mushrooms. Together with high potential for transmission among cultivated strains, viruses can spread quickly and virulence is not selected against.

Another viral disease of *A. bisporus* emerged much later. Patch disease or mushroom X disease, caused by the provisionally named dsRNA virus mushroom X virus (MXV, not yet classified by ICTV), was first described in *A. bisporus* in 1996 in the United Kingdom (Rao et al., 2007). This disease results in bare patches with no mushroom primoridia (pins) next to healthy patches. Infected mushrooms also exhibit premature opening, discoloration, and distortions in shape. The etiology of this disease is under intense investigation because of the economic impact it is having across Europe, and more information on its epidemiology is likely to emerge before long.

Few viruses are described from other cultivated mushrooms; however, two viruses have been found in the oyster mushroom, *Pleurotus ostreatus*. *Oyster mushroom spherical virus 1* (OMSV-1, not yet classified by ICTV), has a positive-sense ssRNA genome (Kim et al., 2008; Yu et al., 2003), and *Pleurotus ostreatus virus 1* (PoV-1, family *Partitiviridae*, genus *Partitivirus*) has a dsRNA genome (Lim et al., 2005). Thus far, no clear association has

been found between virus infection and any adverse phenotypes in *P. ostreatus*. As oyster mushroom production increases, we predict that viral diseases are likely to emerge, either as these viruses evolve virulence because of the potential for high rates of horizontal transmission in commercial production conditions or as new viruses are discovered.

9.4.2.3 Virus-Mediated Decreases in Fungal Virulence (Hypovirulence)

The phenomenon of hypovirulence refers to fungi with reduced virulence. Most hypovirulence is caused by fungal viruses, with a few notable exceptions in which hypovirulence is conferred by defective mitochondria (Bertrand, 2000; Caten, 1972). As stated above, viruses causing hypovirulence are the exceptions, even though they have received disproportionate attention and research. We would argue that most of the search for fungal viruses has stemmed from interest in exploiting hypovirulence for biological control, particularly of plant pathogenic fungi. New examples of hypovirulence are described regularly; for recent reviews, see Ghabrial and Suzuki (2009) and Pearson et al. (2009). Much of our knowledge of the ecology and evolution of fungal viruses derives from systems that involve hypovirulence and attempts to use it for biological control, particularly for hypoviruses in the chestnut blight system. The potential for biological control with hypovirulence is explored in detail below.

9.5 POPULATION BIOLOGY OF FUNGAL VIRUSES

Because fungal viruses have no extracellular phase or vectors, they completely depend on their hosts for dispersal and migration. Voth et al. (2006) took advantage of this dependence to make inferences about the evolution of a plant pathogenic fungus by studying the population genetics of its virus. They studied the diversity and population structure of UmV-H1 and argued that its evolution was inextricably linked to that of its fungal host, *U. maydis*, because its transmission occurs only vertically during mating. The relatively high mutation rates in UmV-H1 made it possible to use nucleotide sequences to infer the divergence of viral lineages as a result of recent migration of *U. maydis* from its center of origin in Mexico to the United States with the movement of maize, the host for *U. maydis*.

The distribution of CHV-1 was also interpreted in the context of the introduction of its host. CHV-1 was first described from *C. parasitica* in Europe (Grente and Berthelay-Sauret, 1978), where it was introduced from east Asia (Anagnostakis, 1987; Milgroom et al., 1996). CHV-1 was later described in China, Japan, and Korea (Liang et al., 1992; Liu et al., 2003; Park et al., 2008; Peever et al., 1998). The diversity of CHV-1 strongly suggested that it was introduced into Europe multiple times (Allemann et al., 1999; Gobbin et al., 2003). The subtype represented by strain CHV-1/Euro7 (Chen and Nuss, 1999) is found throughout southern Europe, whereas other subtypes, including the best studied CHV-1/EP713, are rarely found and are represented by few virus isolates. Gobbin et al. (2003) hypothesized at least four separate introductions of CHV-1 because not enough time has elapsed since the introduction of *C. parasitica* into Europe in the 1930s to account for the divergence in nucleotide sequences among hypovirus subtypes. To answer this question definitively, additional studies will be needed to assess the nucleotide diversity of CHV-1 in putative source populations in Asia.

In addition to population genetic studies of migration and introductions, recombination has been found in several fungal viruses, for example, UmV-H1 (Voth et al., 2006), CHV-1 (Carbone et al., 2004), CHV-4 (Linder-Basso et al., 2005), and RVM2 in *Rhizoctonia* species (Charlton et al., 2008). Recombination complicates phylogenetic analyses such that each nonrecombining region needs to be analyzed separately (Carbone et al., 2004) or analyses that account for recombination need to be used (Charlton et al., 2008; Voth et al., 2006). As found for other viruses, recombination appears to be a significant evolutionary force for

generating diversity in fungal viruses. Intra- and intermolecular RNA recombinations are more commonly observed in positive-sense ssRNA viruses than in dsRNA viruses, but the phenomena have been documented in the latter (Suzuki et al., 1998), and the extent of their effects on viruses of fungi is unclear.

Variation in fungus–virus interactions has been documented in some populations of *C. parasitica* and CHV-1. For example, the effect that CHV-1 has on the fitness of *C. parasitica* depends on both the fungal isolate and the virus isolate (Peever et al., 2000; Sotirovski et al., 2011). Variation in these interactions, however, is small and will probably not result in virus specialization to particular host genotypes, or *C. parasitica* becoming less susceptible to some strains of CHV-1. Few other studies such as this have been done with fungal viruses because of the technical constraints of having to infect multiple host isolates with the same virus isolates.

9.6 BIOLOGICAL CONTROL OF FUNGI WITH VIRUSES

Despite numerous claims that viruses have potential for controlling fungi, the reality has not lived up to expectations in most cases. The exceptions may be the application of killer yeasts against contaminants in fermentation and some qualified successes of hypovirulence in controlling chestnut blight. Few other documented examples of biological control with fungal viruses have succeeded outside of the laboratory or in limited experimental settings.

9.6.1 Applications of Killer Viruses in Yeasts

The killer phenomenon in yeasts was originally considered problematic because killer yeasts can interfere with normal fermentation (Marquina et al., 2002; Schmitt and Breinig, 2002). This same phenomenon, however, has been exploited to reduce the risks of contaminating yeasts by engineering strains with desirable qualities for fermentation to produce killer toxins (Boone et al., 1990; Bussey et al., 1988). Although a killer virus naturally excludes the presence of another virus in the same cell, yeast strains have been genetically modified to produce multiple killer toxins and simultaneously confer immunity to these toxins. If used as starter cultures for fermentation, the engineered killer strains have the potential for broad killing activity and a competitive advantage against a variety of contaminating yeasts, especially for "triple killer" strains that produce the K1, K2, and K28 toxins simultaneously (Schmitt and Schernikau, 1997).

Many of the killer toxins exhibit killing activity against a large number of fungi, including human and plant pathogens, and have been proposed as novel sources of antifungal compounds (Schmitt and Breinig, 2002). The direct use of killer toxins has not succeeded against infections in humans, however, because they are antigenic and/or toxic. In contrast, the killer toxin KP4 produced by *Ustilago maydis virus P4* (UmV-P4, unclassified in family *Totiviridae*) has been expressed at high levels in transgenic tobacco plants (Park et al., 1996). The use of killer toxins for engineering disease-resistant plants, however, does not seem to have taken hold since these initial experiments.

9.6.2 Biological Control of Human Pathogenic Fungi

In addition to exploiting killer toxins against pathogenic fungi, an alternative mechanism is direct infection of pathogens by viruses. van de Sande et al. (2010) described the characteristics that would be desirable for developing viruses to control fungal pathogens. The ideal viruses would have extracellular transmission, replicate efficiently in fungal pathogens, cause extensive lysis, have broad host ranges, could be produced in large quantities, and could be modified in genetics and expression. Unfortunately, few of these criteria are a reality for any currently known fungal virus. Nonetheless, these authors are optimistic that genetic

engineering might someday be able to overcome these profound obstacles and argue for more research on viruses of human pathogenic fungi to achieve biological control. Experience with biological control of plant pathogens with fungal viruses, however, might suggest that these goals will not be attained easily.

9.6.3 Biological Control of Plant Pathogenic Fungi

The discovery of naturally occurring transmissible hypovirulence in *C. parasitica* in the 1950s spawned tremendous efforts to find similar phenomena in other plant pathogens with the hope that viruses could be exploited for biological control. Despite the ever-growing list of fungi in which hypovirulence is demonstrated in the laboratory (Ghabrial and Suzuki, 2009; Pearson et al., 2009), few viruses have proven to be effective for biological control. Evaluating the success of biological control with fungal viruses depends to some degree on how success is defined. Demonstrating that a virus reduces the virulence of fungus to its plant host is not sufficient. We suggest that the minimum criterion for claiming the success of biological control with fungal viruses is the transmission of viruses to new fungal individuals that then are less virulent and cause less damage to their hosts. The simplest level of success by this criterion is to treat plants therapeutically, for example, inoculating cankers on chestnut trees with hypovirulent strains of *C. parasitica* or spraying plants with mycelial fragments of virus-infected isolates of *Sclerotinia minor* to reduce lesion size and fungal reproduction (see below). The ideal success, however, is when viruses are released and spread spontaneously throughout the pathogen population. The chestnut blight hypovirulence example is frequently cited as the best success story for biological control of fungi with viruses and, therefore, will be discussed in detail below. A few other examples are first described.

Besides the chestnut blight system, biological control with hypovirulence has been demonstrated in few systems. Presence of OMV3b in *S. minor* has been shown to reduce lettuce drop disease in field and greenhouse experiments (Melzer and Boland, 1996). Mycelial suspensions of virus-infected isolates of *S. minor* sprayed onto lettuce reduced lesion size up to 50% and production of inoculum (sclerotia) up to 90%. Successful biological control was only achieved, however, if the target fungal individuals causing the lesions were vegetatively compatible with the virus-infected isolate applied. No differences were observed when lesions were treated with vegetatively incompatible isolates. Similar types of experiments were carried out with *S. homoeocarpa* infected with OMV3a (Zhou and Boland, 1998). A virus-infected isolate of *S. homoeocarpa* reduced symptoms of dollar spot, caused by *S. homoeocarpa*, on turf grass by up to 80% and some control was still evident 1 year later. When this same isolate was applied to turf grass with severe dollar spot symptoms, disease was reduced by up to 58% compared to untreated controls, which was as good as treatment with the fungicide chlorothalonil. The common use of fungicides on turf grass for control of multiple fungal diseases might make their replacement with hypovirulent fungal strains for control of a single disease problematic. We are not aware of a commercial product that has emerged from this research.

Biological control of black scurf disease caused by *R. solani* AG-3 on potato tubers has also been attempted with hypovirulence (Bandy and Tavantzis, 1990). When a virus-infected isolate was co-inoculated with a virus-free virulent isolate of *R. solani* onto surface-sterilized seed tubers in a field trial, disease severity was reduced by 56% compared to inoculation with the virulent isolate alone. In another field experiment in which seed tubers were not surface sterilized, however, hypovirulence had no effect on disease severity. Tavantzis (1994) speculated that somatic incompatibility between the inoculated virus-infected isolate and naturally occurring inoculum of *R. solani* AG-3 on the seed tubers in this latter study inhibited virus transmission.

However, successful suppression of disease on surface-sterilized tubers (Bandy and Tavantzis, 1990) may not necessarily have involved horizontal viral transmission to uninfected fungal individuals. Biological control of black scurf may also be effected by various nonpathogenic rhizoctonias that compete with pathogenic individuals of *R. solani* on the surface of tubers or induce host plant resistance (Escande and Echandi, 1991).

Viruses found in *O. novo-ulmi* have profound effects on phenotype and fitness (Figure 9.4). A total of 12 different mitovirus-related dsRNAs have been identified in the Ld isolate of *O. novo-ulmi* (Hong et al., 1999). Most of the dsRNAs appear to represent independently segregating viral genomes, but others are defective segments. Single conidial isolates containing subsets of the 12 dsRNAs show that they have variable effects on fitness (Cole et al., 1998; Sutherland and Brasier, 1997). Hypovirulence has been demonstrated experimentally because virus-infected isolates infect elm trees poorly; infection of xylem in beetle-feeding wounds is markedly inhibited and several orders of magnitude more spores are required for virus-infected isolates of *O. novo-ulmi* to infect (Sutherland and Brasier, 1997; Webber, 1987). Therefore, if viruses invaded the fungus population in the saprophytic phase in dead elm logs, where beetle vectors acquire spores before feeding on healthy elms, there would be potential biological control (Brasier, 1990). The success of hypovirulence in controlling Dutch elm disease epidemics, however, is supported by correlative evidence only. Brasier (1990) speculated that the first epidemic of Dutch elm disease in Europe declined around 1940 because of the spread of viruses in clonal populations of *O. ulmi*, in which viruses could spread rapidly because of the lack of restriction by vegetative incompatibility. In contrast, later populations were highly diverse for vc types, with a low incidence of viruses, except at epidemic fronts where populations were clonal and virus incidence high (Brasier, 1988). Unfortunately, the time scale (many years) and the need for isolated, healthy elms for observing experimental epidemics make it difficult to test the role of viruses in this system, and instead we are left with historical interpretations.

9.6.3.1 Hypovirulence in the Chestnut Blight System

By far, hypovirulence in the chestnut blight system is the best studied

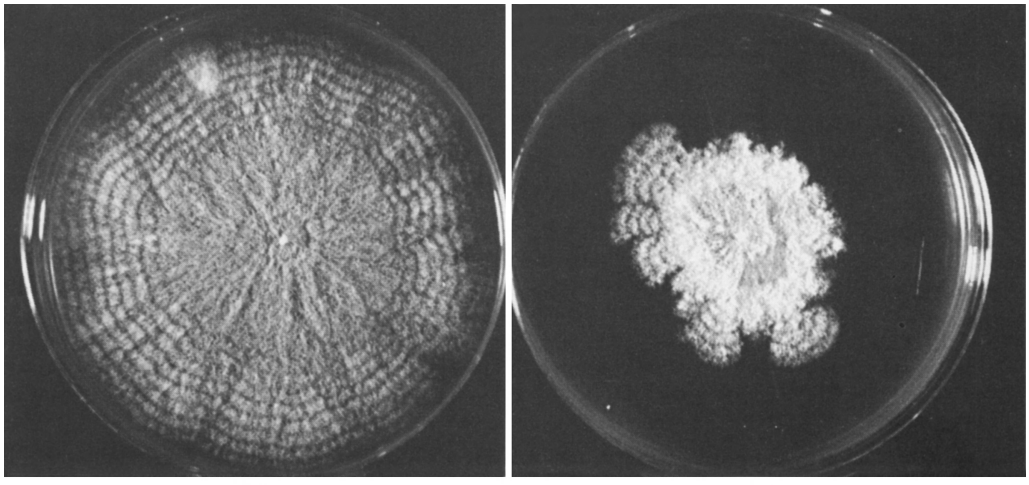

FIGURE 9.4 Cultures of isolate W2 of the Dutch elm disease fungus, *O. novo-ulmi*: (a) virus free and (b) virus infected. (Reprinted from Webber (1987) with permission of the British Society for Plant Pathology.)

example of biological control of fungi with viruses and has been extensively reviewed (Anagnostakis, 1982; Heiniger and Rigling, 1994; Hillman and Suzuki, 2004; MacDonald and Fulbright, 1991; Milgroom and Cortesi, 2004; Nuss, 1992; Van Alfen, 1982). Most hypovirulence in *C. parasitica* is caused by hypoviruses (Choi and Nuss, 1992), but numerous other viruses have been found in this fungus (Hillman and Suzuki, 2004), some of which also result in hypovirulence (Hillman et al., 2004). Many of the *C. parasitica* viruses have rarely been found or detected only in isolated populations. For example, the mitovirus CMV-1, which causes hypovirulence, has been identified in only a single isolate of *C. parasitica*, even though it seemingly has many of the properties that would allow it to invade host populations: the virus is stable in culture, it is horizontally transmitted robustly by hyphal anastomosis, and it is transmitted vertically through conidia at a very high rate, at or close to 100%, and through ascospores at a rate of ~50% when the maternal parent (mitochondrial donor) in a cross is virus-infected (Polashock and Hillman, 1994; Polashock et al., 1997). The story is similar for the two *Cryphonectria* reoviruses, each of which has been isolated only once (Hillman and Suzuki, 2004). CMV-1 and the two reoviruses, therefore, do not appear to have much potential for biological control. For the rest of this section, therefore, we will confine the discussion to hypoviruses, particularly CHV-1, which have proven more successful.

In some places in Europe, hypovirulence appears to be controlling chestnut blight extremely well (Heiniger and Rigling, 1994; Milgroom and Cortesi, 2004). When *C. parasitica* is infected with CHV-1, it produces superficial cankers that do not substantially harm the tree (Figure 9.5). Most biological control successes with hypovirulence in *C. parasitica* are anecdotal, rather than experimental, and document the natural occurrence of hypoviruses in populations of *C. parasitica*. For example, in Portofino Park, near Genoa, Italy, chestnuts (*Castanea sativa*) are the dominant tree. In 2003, nearly every mature chestnut tree had at least one superficial canker and yet only a few nonsuperficial cankers were present, not causing any obvious damage to the trees (P. Cortesi and M. Milgroom, unpublished data). This forest fits the ideal for the success of hypovirulence: CHV-1 invaded the *C. parasitica* population naturally (without being deployed) and has kept chestnut blight in check so that the forest overall looked healthy (Figure 9.6).

In contrast to this textbook case, a more critical analysis reveals some discouraging details (Milgroom and Cortesi, 2004): hypovirulence does not always spread in European forests; most successes are in managed forests or orchards; in North America, hypovirulence has failed in almost all locations where it has been deployed; forests in North America where hypovirulence does occur naturally (e.g., Michigan, USA, where *Cryphonectria hypovirus 3* (CHV-3, family *Hypoviridae*, genus *Hypovirus*) is found naturally) comprise trees that are disfigured (Figure 9.7), albeit alive, most likely because of hypovirulence. In general, treating trees with hypovirulent strains of *C. parasitica* succeeds in controlling individual cankers, but viruses may not spread to untreated trees or even on the same tree to prevent new infections. Overall, the claim of success of hypovirulence, especially in North America, is sometimes a matter of interpretation. Individual trees may survive because of hypovirulence, or disease progress may be slowed down in stands of chestnuts, but tree mortality may still be high. By some criteria, there are small signs that hypovirulence improves tree survival (Davelos and Jarosz, 2004), but it may not provide a practical level of control in North America.

One of the major constraints for the spread of hypoviruses and the success of hypovirulence in *C. parasitica* is thought to be the reduction of horizontal transmission by vegetative incompatibility (see above). In an effort to overcome this restriction, Choi and Nuss (1992) developed strains of *C. parasitica* transformed with a full-length infectious

FIGURE 9.5 Superficial canker on a European chestnut tree (*Castanea sativa*) caused by CHV-1-infected individuals of *Cryphonectria parasitica*. Photo by Paolo Cortesi, University of Milan. (*See the color version of this figure in Color Plate section.*)

cDNA of the genome of CHV-1/EP713 such that transcribed hypovirus dsRNA could be found in the cytoplasm, conferring hypovirulence (Chen et al., 1994b). In transgenic isolates, the viral transgene, integrated into a *C. parasitica* chromosome, is transmitted through 100% of the conidia and 50% of the ascospores when the transgenic isolate mates as a male. Transgenic strains were released in the field and monitored for several years (Anagnostakis et al., 1998; Root et al., 2005). Transgenes subsequently were recovered from a small percentage of isolates collected from the field, but overall failed to become established in the fungus population. Less than 3% of the isolates collected after transgenic strains were released over a 3-year period were found to contain hypovirus. No hypovirus or transgenic isolates were recovered outside the treated plot despite intensive sampling. Root et al. (2005) speculated that this failure to establish was because CHV-1/EP713 reduces the fitness of *C. parasitica* too much by markedly suppressing sporulation. Conidia transmit CHV-1 vertically

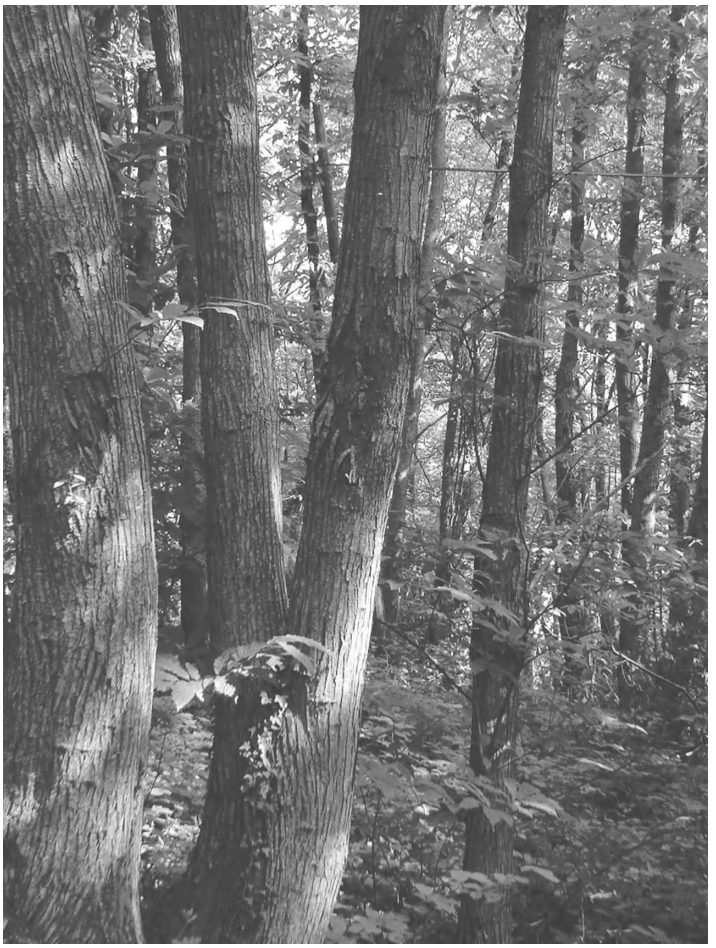

FIGURE 9.6 Healthy stand of European chestnut trees (*Castanea sativa*) in Portofino Park, near Genoa, Italy in 2003. Most trees had one or more superficial cankers caused by CHV-1-infected individuals of *Cryphonectria parasitica*. Photo by Paolo Cortesi, University of Milan. (*See the color version of this figure in Color Plate section.*)

either as asexual propagules or as male gametes in mating. As an alternative, Root et al. (2005) proposed using transgenic isolates engineered with a milder virus strain, for example, CHV-1/Euro7, which does not reduce the fitness of *C. parasitica* as much as CHV-1/EP713 (Chen and Nuss, 1999).

The counterargument to the transgenic strategy is that viral transgenes cannot invade fungal populations because they are highly deleterious and will be rapidly purged from the *C. parasitica* genome by purifying selection (Liu et al., 2000; Milgroom and Cortesi, 2004). Therefore, any beneficial effect of releasing transgenic isolates would be transient and likely be achieved by introducing hypoviruses to multiple vc types as the transgenic isolates mate and pass on transgenes to recombinant ascospores. However, hypoviruses have been released multiple times into diverse arrays of vc types in *C. parasitica* populations in North America and have failed to establish (Milgroom and Cortesi, 2004). Engineering transgenic strains with a milder virus suffers the same constraints as for CHV-1/EP713 because if the nuclear transgene reduces the

FIGURE 9.7 An American chestnut tree (*Castanea dentata*) in northern Michigan in 2008 where naturally occurring hypovirulence allowed its survival from chestnut blight. Photo by Alice C. L. Churchill, Cornell University. (*See the color version of this figure in Color Plate section.*)

fitness of *C. parasitica*, which it must for biological control to succeed, then it will be purged rapidly by selection. Furthermore, previous releases that included hypoviruses with characteristics similar to CHV-1/Euro7 using conventional methods failed, even though they were released into diverse vc types. In summary, the transgenic method of deploying hypoviruses appears at first to be a clever use of biotechnology, but it is not likely to succeed any better than previous attempts using conventional deployment methods.

The interpretation that vc type diversity is critical to the establishment of hypovirulence is based on correlations between the apparent success of hypovirulence in Europe and its failure to establish in North America (Anagnostakis et al., 1986; Milgroom and Cortesi, 1999) and from predictions extrapolated from virus transmission in the laboratory to field conditions (Figure 9.3) (Liu et al., 2000; Milgroom and Cortesi, 2004; Papazova-Anakieva et al., 2008). Estimates of migration of CHV-1 between vc types in two populations in Italy were much greater than predicted from laboratory tests (Carbone et al., 2004). Therefore, we need to question whether vegetative incompatibility is such a formidable barrier to virus transmission at the population level in *C. parasitica* that it cannot be overcome and whether indeed vc type diversity is a dominant factor in the failure of hypovirulence in North America (Milgroom and Cortesi, 2004). Clearly, there are additional factors such as excessive virulence of hypoviruses to *C. parasitica*, lack of vectors (if any are needed), host susceptibility, and environmental conditions (MacDonald and Fulbright, 1991) that yet need to be considered.

9.7 FUTURE DIRECTIONS

Study of fungal virus ecology and evolution has had a slow start relative to the rest of general viral ecology, as explored in this book and its companion volume, largely because of the difficulties outlined in this chapter: viruses have not been readily detectable in fungal hosts in natural settings, they may be lost upon initial subculture of the fungus, they are often

symptomless in their fungal hosts in culture, and finally because they are perceived (or perhaps misperceived) as having either little or no obvious economic importance. Furthermore, the number of fungal viruses characterized to a useful level is small and has begun to expand just over the last few years. What we are left with is a relatively small series of snapshots of virus–fungus interactions that are of economic importance at some level, with little understanding of their natural ecological impact. We expect this situation to change dramatically over the next few years with the advent of high-throughput sequencing associated with metagenomics projects. Rather than continuing the current paradigm of fungus isolation, followed by virus or viral nucleic acid isolation and by molecular characterization, fungal viruses are increasingly going to be discovered by sequencing of environmental samples. Good recent examples of virus discovery from hosts other than fungi include relatives of the giant *Mimivirus* of amoebae discovered in environmental sampling of the Sargasso Sea (Ghedin and Claverie, 2005) and three novel picorna-like RNA viruses, probably of insect origin, discovered during sequencing of a whole stool sample of an Afghan child (Kapoor et al., 2010). Interestingly, and pertinent to the discussion here, the presumed invertebrate host or hosts in the latter example was inferred by nucleotide composition analysis, not by actual host identification. As such methodologies mature and become less expensive, and our fungal virus database grows, relationships between viral nucleic acid sequence and fungal hosts will become easier.

Although we predict that the number of fungal viruses discovered and characterized will keep increasing, their ecology and evolution are likely to remain obscure, primarily because most fungal viruses lack obvious phenotypic effects in their hosts. The prospects of applying hypovirulence to control fungal pathogens, which have been a driving force in studies of ecology and evolution of fungal viruses, have not been realized except in a few cases. Fortunately, some researchers remain optimistic and continue to study both the molecular biology and the ecology of fungal viruses, the marriage of which is key to any success in biological control.

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CHAPTER 10

PRION ECOLOGY

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CONTENTS

10.1 INTRODUCTION

The ecology of prions is the study of the relation of prions and prion-carrying organisms to their environment. What is the distribution of prions in nature? What effects do they have on their hosts? How are they transmitted? We will begin with a definition of the term "prion" and a description of the range of known prions, and their biochemical basis and biological properties. Except for a historical introduction, we will emphasize the yeast and fungal prions.

10.2 DEFINITION OF "PRION"

In arguing that the mammalian transmissible spongiform encephalopathies (such as scrapie of sheep and human Creutzfeldt–Jakob disease) are unique among infectious entities, Prusiner coined the term "prion" for "*pro*teinaceous *in*fectious" (Prusiner, 1982; Caughey et al., 2009). He named the protein associated with purified infectious material PrP (for prion protein) (Bolton et al., 1982). But the idea of an infectious protein actually precedes the term prion by some 15 years. Alper found that the scrapie agent is far more resistant to UV irradiation than even small RNA viruses (Alper et al., 1967), suggesting that there was no nucleic acid component essential to its replication. Griffith soon proposed that, in a protein oligomer of abnormal (scrapie) form and

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normal form, the normal form could be converted by protein–protein interactions into the scrapie form—essentially the modern protein-only prion hypothesis (Griffith, 1967). What proved later to be the gene encoding PrP was first identified in 1968 as a gene determining scrapie incubation period (*Sinc*) (Dickinson et al., 1968). The structural gene for PrP was later cloned (Chesebro et al., 1985; Oesch et al., 1985), shown to be identical to *Sinc* (Carlson et al., 1986), and to be essential for propagation of the scrapie agent (Bueler et al., 1993). Showing that PrP is sufficient, however, has proven far more difficult. Caughey developed an *in vitro* system in which the protease-resistant disease-associated form of PrP (called PrP-res or PrP^{Sc}) could promote the conversion of the normal protease-sensitive form (PrP-sen or PrP^{C}) to the protease-resistant form (Kocisko et al., 1994). This system showed all the specificity of the infectious process, with, for example, a conversion barrier between PrP from different species (Kocisko et al., 1995). This system was the basis on which Soto developed a PCR-like prion amplification system (Castilla et al., 2005) that plays a large role in present work (Deleault et al., 2007).

10.3 PRION TERMINOLOGY

In yeast, prions (like viruses) appear as nonchromosomal genes, and so are given a name in brackets, for example, [URE3]. As for chromosomal genes, the dominant state is shown in upper case letters, and this generally means the presence of the prion form of the protein. The absence of the prion is recessive, and is denoted in lower case letters, for example, [ure-o]. In some cases, "+" and "–" are added to emphasize the presence and the absence, respectively, of the prions, for example, [PSI+] and [psi–]. The chromosomal gene encoding the prion protein is indicated by *URE2*, *SUP35*, and so on, with the dominant form in caps. Usually, the wild-type allele is dominant (and in capitals), but not always. Many suppressor tRNA mutations are dominant to the wild-type allele, and then the mutant is in capitals and the wild type in lower case letters. Chromosomal genes are italicized. A recessive allele (usually a mutant) is shown in lower case, for example, *sup35*. An allele number may follow after an hyphen, as in *ade2-1*, which is mutant allele 1 of the *ADE2* gene. The protein itself is shown by Ure2p, Sup35p, and so on, with only the first letter capital and no italics. The last letter "p" is for protein, so one writes "Ure2p" as an abbreviation for "the Ure2 protein." However, the convention in *Podospora anserina* is different. The heterokaryon incompatibility prion is denoted [Het-s], its absence by [Het-s^{*}], the gene encoding the small s allele by *het-s*, and the encoded protein by HET-s. The alternative allele is denoted by a large S, as in *het-S* for the gene and HET-S for the protein. This is a polymorphic locus, meaning that both *het-s* and *het-S* are found frequently in the wild, with similar frequency in this case.

10.4 HOW TO FIND A PRION

In 1994, we found that two long-known nonchromosomal genes of *Saccharomyces cerevisiae*, [PSI+] (Cox, 1965) and [URE3] (Lacroute, 1971), were actually prions of Sup35p and Ure2p, respectively (Wickner, 1994). This finding was based on the genetic properties of [URE3] and [PSI+], several of which we inferred were paradoxical if these elements were to be nonchromosomal nucleic acid replicons, but expected if they were prions (Wickner, 1994).

[URE3] is a prion of Ure2p (Wickner, 1994), a negative transcription regulator of genes encoding enzymes and transporters needed for the utilization of poor nitrogen sources (Cooper, 2002; Magasanik and Kaiser, 2002). When a good nitrogen source, such as ammonia or glutamine, is present, Ure2p binds the positive transcription factor Gln3p, keeping it in the cytoplasm and preventing expression of an array of genes. The prion form of Ure2p is inactive in its nitrogen regulation role and

many genes, including *DAL5*, encoding the allantoate/ureidosuccinate permease used to assay [URE3] presence, are inappropriately derepressed.

[PSI+] is a prion of Sup35p (Wickner, 1994), a subunit of the translation termination factor (Frolova et al., 1994; Stansfield et al., 1995). In the prion form, Sup35p is inactive in translation termination and termination codons are read through at increased frequency. This nonsense-suppression phenotype is used to assay the [PSI+] prion.

The genetic criteria that identified [URE3] and [PSI+] as prions are as follows (Wickner, 1994):

(i) [URE3] and [PSI+] could each be cured from strains carrying them by millimolar concentrations of guanidine and by high osmotic strength, respectively (Singh et al., 1979; Wickner, 1994), but from the cured strains, prion-carrying clones could again be isolated (Lund and Cox, 1981; Wickner, 1994). We called this "reversible curing." Plasmids and viruses can be cured, but do not arise again in the cured strains. Prions should arise again (rarely) because the protein capable of converting into the prion form is still present in the cell.

(ii) Overproduction of the prion protein should increase the frequency with which it converts into the self-propagating prion form. Overproduction of Ure2p increases [URE3] generation ~100-fold (Wickner, 1994) and overproduction of Sup35p similarly increases [PSI+] generation (Chernoff et al., 1993).

(iii) The phenotypes of strains carrying [URE3] and [PSI+] were similar to those of *ure2* and *sup35* mutants, and yet the *URE2* and *SUP35* genes are needed for propagation of the corresponding prion. This is the opposite of the relation between, for example, the mitochondrial genome and a chromosomal gene needed for its propagation, but is just the relation expected for a prion (Wickner, 1994).

These genetic criteria have become the gold standard for identifying a prion; but there are several ways of finding candidates. The reinvestigation of nonchromosomal genetic elements identified many years ago (Rizet, 1952; Cox, 1965; Lacroute, 1971; Kunz and Ball, 1977) has yielded several prions (Wickner, 1994; Coustou et al., 1997; Brown and Lindquist, 2009).

The prion domains of Ure2p and Sup35p are each N-terminal Q/N-rich regions, which are necessary and sufficient for prion generation and propagation, and constitute the amyloid core of the infectious filaments. Since these were the first two yeast prions identified, other protein's Q/N-rich domains became prime suspects (TerAvanesyan et al., 1994; Masison and Wickner, 1995). [PIN+] is a prion that dramatically increases the frequency of [PSI+] arising *de novo*, and was detected while studying [PSI+] generation (Derkatch et al., 1997) and later shown to be based on amyloid of the Q/N-rich Rnq1p (function unknown) (Sondheimer and Lindquist, 2000; Derkatch et al., 2001). But in the course of showing that [PIN+] is a prion of Rnq1p, it was found that overproduction of other proteins with Q/N-rich domains had [PIN+]-like activity (Derkatch et al., 2001). Two of these proteins, Swi1p (a chromatin remodeling factor) and Cyc8p (with Tup1p a transcription repressor), have proven to form prions (Du et al., 2008; Patel et al., 2009). A general search among proteins with Q/N-rich domains showed that Mot3p, another transcription factor, can also form a prion (Alberti et al., 2009).

We have used a more general approach recently, making a bank of random small yeast genome segments fused at the N-terminus of Sup35MC, the Sup35 protein lacking its own prion domain, and screening for clones in which the fusion protein acts as a prion. This led to the discovery of [MCA], a prion of

Mca1p, the yeast metacaspase homologue (Nemecek et al., 2009). Although the prion domain of Mca1p is also Q/N-rich, this method has the potential of discovering non-Q/N-rich prions (such as PrP and [Het-s]) and being used for organisms other than yeast.

It should be noted that a number of traits that prions show are too general to be considered evidence for a prion. For example, aggregation is typical of many (not all) prion proteins, but a large fraction of overproduced proteins will aggregate without being prions. Even amyloid formation is not particularly diagnostic of prions – there are over 20 human amyloid diseases, but only one known prion.

Most of the growing zoo of yeast and fungal prions (Table 10.1) consists of self-propagating amyloids of proteins with Q/N-rich prion domains, but the exceptions are exceptionally interesting. The [Het-s] prion of *P. anserina* (Coustou et al., 1997) is necessary for a normal fungal function, called heterokaryon incompatibility. When two colonies of a filamentous fungus grow toward each other, if they are identical strains, cellular processes will fuse to form a joint colony with mixture of nuclei of the parent colonies, called a heterokaryon. However, before this colony fusion is allowed to proceed, there is a trial fusion of a few cells and they somehow test the identity of alleles at about a dozen polymorphic loci (called *het* loci) scattered about the genome. If the two clones differ in any one of these loci, the trial fusion cells die, and a barrier is formed to further fusions. This reaction is called heterokaryon incompatibility. The purpose of heterokaryon formation is probably sharing of nutrients between colonies, but there is also a danger of sharing viruses and pathogenic plasmids. This danger is limited somewhat by insuring that the fusing clones are already very closely related as judged by their sharing identical alleles at the *het* loci. The *het-s* and *het-S* alleles define one such locus. Only if the HET-s protein is in the prion form does this system work correctly.

Another nonamyloid prion is [β], which is simply the active form of the vacuolar protease B (Roberts and Wickner, 2003). Yeast vacuolar protease B is made as an inactive precursor, which is normally activated by cleavage by protease A (Jones, 1991). In the absence of protease A, active protease B can itself cleave and activate its own precursor (Zubenko et al., 1982), and under conditions where precursor synthesis is derepressed, this can show all the properties of a prion (Roberts and Wickner, 2003). The [C] (for crippled growth) nonchromosomal gene of *P. anserina* may be a similar enzyme-based prion involving a self-activating MAP kinase cascade (Silar et al., 1999; Kicka et al., 2006).

Evidently, self-action is the key feature of prions, whether an amyloid templating its own synthesis or an enzyme modifying its own unmodified form. It is possible that other protein-modifying enzymes will prove, under some circumstances, to act as prions. A parallel with epigenetic chromatin states may also be drawn. Chromatin modifications can be heritable if they are self-perpetuating. DNA methylases specific for sites at which one strand is methylated and the other is not are known to be a basis for certain epigenetic states. Likewise, a histone acetylase that has high affinity for hemiacetylated chromatin will make marks that will be propagated. Once a chromatin site is acetylated, the replicated chromatin will be hemiacetylated and be a target for this enzyme. Unacetylated chromatin would only rarely be so modified.

10.5 STRUCTURAL BASIS OF YEAST AND FUNGAL PRIONS

After a great deal of indicative evidence connecting prions with amyloid formation, it was shown that amyloids formed *in vitro* from recombinant proteins could transmit the corresponding prion to cells and that this was not simply a matter of increasing the amount of the protein in the cells (see above)—it was specifically the amyloid form that was infectious (Maddelein et al., 2002; King and Diaz-Avalos, 2004; Tanaka et al., 2004;

TABLE 10.1 Known Prions

Prion	Protein/Gene	Normal Protein Function	Prion Manifestation	References
TSE	PrP/*Prnp*	Unknown	Transmissible spongiform encephalopathy (mammals)	Aguzzi et al., 2008
[URE3]	Ure2p/*URE2*	Nitrogen catabolism: In the presence of a rich N source, Ure2p binds the positive transcription factor Gln3p, keeping it in the cytoplasm	Inappropriate derepression of enzymes and transporters for the utilization of poor nitrogen sources	Lacroute, 1971; Turoscy and Cooper, 1987; Wickner, 1994
[PSI+]	Sup35p/*SUP35*	Translation termination, mRNA turnover	Increased readthrough of translation termination codons	Cox, 1965; Wickner, 1994
[PIN+]	Rnq1p/*RNQ1*	None known	Increased frequency of generation of [PSI+] and [URE3] prions	Derkatch et al., 1997; Sondheimer and Lindquist, 2000; Derkatch et al., 2001
[SWI+]	Swi1p/*SWI1*	Subunit of SWI–SNF chromatin remodeling complex	Partially defective Swi1-phenotype such as poor growth on raffinose, galactose, or glycerol	Du et al., 2008
[MCA]	Mca1p/*MCA1*	Metacaspase homologue Apoptosis?		Nemecek et al., 2009
[OCT+]	Cyc8p/*CYC8*	Transcription corepressor	Derepressed invertase, Cyc2p, other proteins	Patel et al., 2009
[MOT3]	Mot3p/*MOT3*	Transcription repressor of genes derepressed under anaerobiasis	Derepression of "anaerobic genes"	Alberti et al., 2009
[Het-s]	HET-s/*het-s*	No known nonprion function	Prion form necessary for heterokaryon incompatibility	Coustou et al., 1997
[β]	Prb1p/*PRB1*	Vacuolar protease PrB; prion form is active PrB, not amyloid	Poor sporulation, poor survival in stationary phase	Zubenko et al., 1982; Roberts and Wickner, 2003

Brachmann et al., 2005; Patel and Liebman, 2007).

Amyloid is a linear polymer of peptide or protein that has a "cross-β" structure, meaning that it is composed mainly of β-sheets with the β-strands running largely perpendicular to the long axis of the filaments (Eanes and Glenner, 1968) as reviewed in Kirschner et al. (2000). Solid-state NMR has been the most fruitful approach to examining the structures of amyloids (Tycko, 2006). Infectious amyloids of the prion domains of Sup35p, Ure2p, and Rnq1p each have been shown to have an in-register parallel β-sheet structure (Shewmaker et al., 2006; Baxa et al., 2007; Wickner et al., 2008a) (Figure 10.1), similar to the structure of amyloid of the Aβ peptide (Balbach et al., 2002; Petkova et al., 2002; Paravastu et al., 2008). In such a structure, each residue is aligned with the same residue on neighboring molecules, so that a line of identical residues is formed along the length of the filament. This structure is enforced by interactions between these aligned identical residues, such as the "$\widetilde{\beta}$ zipper" hydrogen bonds between glutamine and asparagine side chains, hydrogen bonds between serine and threonine residues, or hydrophobic interactions.

The in-register parallel structure of yeast prion amyloids can explain how a single protein sequence can stably propagate several different structures (Wickner et al., 2007, 2008b). Note that it is not remarkable that one protein can assume several structures, but that each of the several structures can be stably propagated by transmission to other protein molecules is unexplained by any other proposed model. Given the in-register parallel architecture, the small filament diameter of the prion domain of Ure2p (Taylor et al., 1999) or Sup35p (King et al., 1997) proves that each must be folded along the long axis of the filament (Figure 10.1). However, the location of the folds can vary, and once formed in the initiation of a filament, molecules that subsequently add to the end of the filament must assume the same conformation as their predecessors. This results in a heritable/infectious conformation, with several alternative structures resulting in alternative heritable/infectious phenotypes manifested as prion "variants."

Amyloid of the HET-s protein has been shown to have a β-helix structure, in which each molecule forms two turns of the helix (Ritter et al., 2005; Wasmer et al., 2008). The C-terminal prion domain of HET-s has two copies of a direct repeat sequence, and it is precisely these sequences that form the β-strands of the structure. Interestingly, the

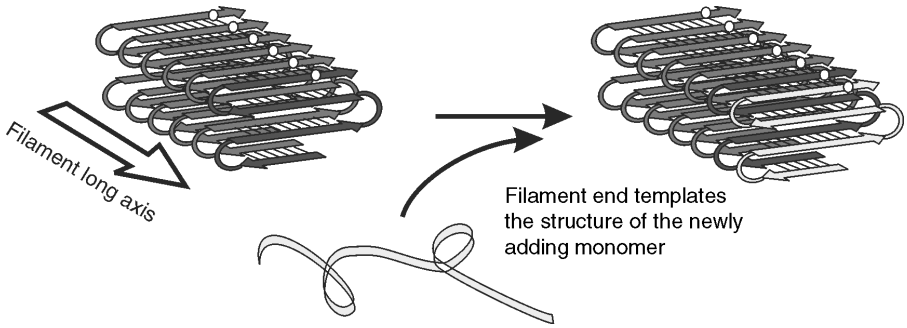

FIGURE 10.1 Prion domain structures explain propagation of structures (prion variants). The side-chain interactions among each line of identical amino acids (e.g., white circles) both make the structure be in-register and provide the templating interaction that makes the new chain assume the same structure with the turns in the same places as the molecule on the end of the filament. These side-chain interactions include the "beta zipper" interaction of glutamines and asparagines, hydrogen bonds between aligned serines or aligned threonines, and hydrophobic interactions along a line of identical hydrophobic residues. A line of charged residues would not stabilize this structure, and there are, indeed, few charged residues in the yeast prion domains. (*See the color version of this figure in Color Plate section.*)

[Het-s] prion is known to have only a single variant *in vivo*, and solid-state NMR data indicate that the HET-s prion domain forms a single structure, as judged by the unusually narrow resonance lines (Ritter et al., 2005; Wasmer et al., 2008), compared to those observed for Sup35p, Ure2p, and Rnq1p (Shewmaker et al., 2006; Baxa et al., 2007; Wickner et al., 2008a). Each of the corresponding prions, [PSI+], [URE3], and [PIN+], are known to form multiple biologically distinct prion variants (Derkatch et al., 1996; Schlumpberger et al., 2001; Bradley et al., 2002; Brachmann et al., 2005), corresponding to multiple structures (e.g., see Tanaka et al., 2004). The broad lines of the yeast prion amyloids is consistent with the mixture of structures (all in-register parallel β-sheets; see Shewmaker et al., 2009) that are reflected by their generating a mixture of prion variants on transfection into yeast (e.g., King and Diaz-Avalos, 2004; Tanaka et al., 2004; Brachmann et al., 2005). It is likely that the single [Het-s] variant and corresponding single structure of HET-s amyloid reflect the fact that HET-s has evolved to be a prion with a specific structure, while the yeast prions are molecular accidents that can occur in multiple ways (see below for details). For example, an arm bends at the elbow in one very specific way, but can break in many places.

10.6 PRION VARIANTS, PHENOTYPES, AND THE SPECIES BARRIER

As discussed above, a given prion protein sequence can form any of the several amyloid structures, each with distinguishable biological characteristics. This phenomenon is called "prion strains" in mammalian systems or "prion variants" for yeast. Different mammalian prion strains may manifest themselves as different incubation periods, different distributions of brain lesions, and different presenting signs and symptoms (reviewed in Bruce, 2003). Yeast prion variants have been identified by intensity of the prion phenotype, stability of propagation of the prion, and dependence on or sensitivity to the deficiency or overexpression of various chaperones (Derkatch et al., 1996; Kushnirov et al., 2000b; Brachmann et al., 2005).

The BSE epidemic in the United Kingdom brought to wide attention the dependence of the species barrier on prion strain. The species barrier is well known to depend on the donor and recipient prion protein sequence, but the same pair of sequences can have a high species barrier for one prion strain, but a very low barrier for another strain (reviewed by Collinge and Clarke, 2007).

A species barrier for the propagation of [PSI+] and [URE3] prions has likewise been demonstrated (Chernoff et al., 2000; Kushnirov et al., 2000a; Santoso et al., 2000; Edskes and Wickner, 2002; Baudin-Baillieu et al., 2003; Chen et al., 2007; Edskes et al., 2009). As in mammalian prions, a dependence of species barrier on prion variant has been demonstrated for the yeast prion [URE3] (Edskes et al., 2009). Originally described in *S. cerevisiae*, [URE3] can also arise in *S. uvarum* or in *S. cerevisiae* whose *URE2* gene has been replaced with that from *S. paradoxus*, *S. bayanus*, *S. cariocanus*, or *S. mikatae*. Transmission from cell to cell is efficient if the donor and recipient strains express the same Ure2p, but quite variable if the Ure2p's are from different species. For a given pair of species, the transmission rate may vary from 98% to 4%, depending on the prion variant (Edskes et al., 2009). This fact can be used as another method to classify prions. Interestingly, within limits, a prion variant maintains its species-specificity even while passing through other species (Edskes et al., 2009), a fact also previously known for mammalian prions.

Like nucleic acid genes, prion genes can "mutate" when placed under selection conditions. Kimberlin showed that the much-delayed passage of scrapie from mice to hamsters selected out a new variant of scrapie as judged by its properties when returned to mice (Kimberlin et al., 1987). Similarly, *S. cerevisiae* [PSI+] can occasionally be

transmitted to an artificial chimeric Sup35p having the prion domain of *Pichia methanolica* and the remainder from *S. cerevisiae*. At least two variants of the chimeric prion arise, showing that the transmission was not faithful, that is, mutant prions were formed (Vishveshwara and Liebman, 2009).

10.7 PRION ECOLOGY

As with any infectious agent, the incidence of yeast and fungal prions in the wild must be a complex product of frequency of generation, frequency of loss, transmission efficiency, and the biological effects of the prion on the host cell/organism. These parameters vary substantially with both host strain and prion variant; but for the commonly studied variants of [PSI+] and [URE3], prion loss is sufficiently rare so that mutants unable to maintain the prion have been isolated by several groups. The [PSI+] prion, for example, arises at around 1 in 10^5 to 1 in 10^7 cells in the presence of [PIN+], but $\sim 10^3$-fold less frequently in its absence (Derkatch et al., 1997).

10.7.1 A Beneficial Infectious Element Should be Widespread in Nature

Even detrimental bacteria, viruses, and prions are easily found in wild populations because their infectious nature can outstrip even a lethal effect on the host. The preponderance of infectious entities has resulted in an enormous impact on evolution, with a large fraction of our energy devoted to our four immune systems: cellular, humoral, innate, and RNAi-based. Nonetheless, everyone gets many viral and bacterial infections, and a very substantial fraction of our genomes are composed of parasitic infectious DNA retroelements. Prions have been known to be widespread in sheep populations for centuries (Parry, 1983), perhaps for millennia (Wickner, 2005), and chronic wasting disease of elk and deer is widespread in several areas of the United States (Sigurdson, 2008). The incidence of such elements in the wild will be a function of the balance of generation, loss, infectivity, and benefit/detriment to the host. However, it is evident that if an infectious element is beneficial to its host (e.g., mitochondrial DNA), it must be widespread in nature because infectivity and effects on the host work in the same direction.

A survey of 70 wild strains of *S. cerevisiae*, isolated from a wide variety of environments on five continents (and oceans), showed that each of the known mildly detrimental nucleic acid replicons (viruses and plasmids) of yeast could be found (Nakayashiki et al., 2005) (Table 10.2). For example, the 2 μm DNA plasmid has been estimated by two groups to impose a growth defect on the host of ~1–2% (Futcher and Cox, 1983; Mead et al., 1986; Futcher et al., 1988), and yet was found in 38 of the 70 wild strains (Nakayashiki et al., 2005). Because the 2 μm plasmid is very rarely lost, and will only arise *de novo* over geologic time, the frequency of spread (by mating) must balance the detriment to cell growth. This suggests that mating must occur at a frequency of ~1% of mitotic divisions (Futcher and Cox, 1983; Wickner, unpublished). In contrast to 2 μm DNA, the prions [URE3] and [PSI+] were not present in any of the 70 wild strains (Nakayashiki et al., 2005). Two other groups have also reported no occurrence of [PSI+] in a total of

TABLE 10.2 Incidence of Infectious Elements in Wild Yeast Strains

Infectious Element	Number of Strains with Element (70 Total Strains)
Nucleic acid replicons	
L-A dsRNA virus	15
L-BC dsRNA virus	8
20S RNA replicon	14
23S RNA replicon	1
2 μ DNA plasmid	38
Prions	
[URE3]	0
[PSI+]	0
[PIN+]	11

19 wild strains examined (Chernoff et al., 2000; Resende et al., 2003). This indicates that on the whole, [URE3] or [PSI+] are a net detriment to the host, a detriment that can be estimated to be somewhat greater than the ~1% found for 2 μm DNA because the latter is found more widely distributed in nature. The [PIN+] prion is found in occasional wild isolates at frequencies comparable to some of the mildly detrimental nucleic acid replicons (Chernoff et al., 2000; Nakayashiki et al., 2005). Sup35p is an essential protein and cells lacking Ure2p grow slowly, but there is no phenotype seen for even a complete lack of Rnq1p. This suggests that the [PIN+] prion is only mildly detrimental because one can dispense with Rnq1p altogether. However, when Rnq1p is artificially overproduced, [PIN+] is lethal (Douglas et al., 2008).

In contrast to the yeast prions, the *P. anserina* prion [Het-s] is found in 80% of wild *het-s* isolates (Dalstra et al., 2003). This result is what one would expect if the [Het-s] prion is a benefit to the fungal host. However, even in this case, another interpretation is possible. Meiotic drive is a phenomenon in which a gene promotes its own inheritance not by being a benefit to the organism but by preventing the inheritance of other alleles at the same genetic locus. Examples are known in mice (the "t locus"), *Drosophila* (segregation distorter), *Neurospora* (spore killer), and many other organisms. [Het-s] is the basis of a meiotic drive phenomenon in *Podospora*: a meiotic cross of female *het-s* [Het-s] cells with male *het-S* cells results in meiotic products in which most of the *het-S* segregants are dead (Bernet, 1965; Dalstra et al., 2003) (note that female gametes are those with substantial cytoplasm, while male gametes have little if any). This results in the gradual spread of the *het-s* allele at the expense of the *het-S* allele. Because this meiotic drive requires that the HET-s protein be in the prion form, the wide distribution of the [Het-s] prion may be simply a consequence of this meiotic drive phenomenon, rather than any benefit derived from heterokaryon incompatibility.

10.7.2 Variable Phenotypic Effects of Prions

The "phenotype" resulting from mammalian prions is inexorable progression to death, so there is little doubt that these infections are diseases. But yeast and fungal prions are compatible with survival and growth, so the issue of whether these prions are a benefit or detriment to the cells is not so easily resolved by looking at prion phenotypes. In 1997, we suggested that [Het-s] may be a beneficial prion (Wickner, 1997), but, as discussed above, it may be primarily a meiotic drive phenomenon. The notion of beneficial prion was next extended to [PSI+] by Tuite's group on the basis of data suggesting that cells with [PSI+] were more resistant to heat or high ethanol stress than were isogenic [psi–] strains (Eaglestone et al., 1999). However, in a survey of a wider range of phenotypes using a larger number of [PSI+]/[psi–] strain pairs, True and Lindquist (2000) did not find any consistent stress-resistant phenotype. In fact, except for an increased *sensitivity* to 5 mM Zn^{2+} of all [PSI+] strains, there were no common phenotypes attributable to carrying this prion (True and Lindquist, 2000). Although in three-fourths of the differences observed, [PSI+] was detrimental and [psi–] was advantageous, the authors nonetheless suggested that [PSI+] was an advantage, by somehow helping cells evolve (True and Lindquist, 2000). Whether evolvability can be selected has been questioned (Partridge and Barton, 2000).[1] A later reexamination of these phenotype differences, using the strain pairs

[1] Can "evolvability" be selected for? And what does evolvability mean? If evolvability means a gene allele that provides variability in the organism carrying it, an example might be a DNA polymerase mutant that had decreased fidelity. While such a polymerase might give rise to a favorable allele in a gene A that it replicates, unless gene A is linked to the polymerase gene, it will not produce selection for the polymerase gene except in the one clone in which the mutation in gene A was produced. On meiosis, the favorable A mutation and the mutagenic polymerase allele will segregate independently. Since most mutations in all systems are unfavorable, the mutagenic polymerase will be generally unfavorable.

obtained from True and Lindquist, found that only one-fourth of the differences previously reported could be reproduced (Namy et al., 2008). Nonetheless, there can be little doubt that a deficiency of the essential translation termination factor Sup35p, produced by the [PSI+] prion, must have a variety of phenotypic effects. That most effects of being [PSI+] were detrimental suggests [PSI+] is a disease; but if one could show that a particular condition favored by [PSI+] cells could be correlated with high-frequency isolation of [PSI+] strains from that niche in nature, a convincing case might be made (Partridge and Barton, 2000). However, as yet, no wild strains have been found to be [PSI+].

10.7.3 Prion Domains have Nonprion Functions

Is prion formation the sole function of prion domains? Mutants deleted for the Sup35p prion domain have been found to have an array of phenotypes in comparison with a normal [psi–] strain not so deleted (True and Lindquist, 2000). Although the authors did not comment on this finding, it clearly indicates that the Sup35p prion domain has function(s) independent of prion formation. A series of studies by Hoshino and coworkers has shown that the prion domain of Sup35p is necessary for the normal process of mRNA turnover in yeast (Hoshino et al., 1999; Hosoda et al., 2003). This domain interacts with the polyA binding protein (Pab1p) and with components of the polyA—degrading complexes to promote polyA shortening, a well-known step triggering mRNA degradation (Hoshino et al., 1999; Hosoda et al., 2003). This function is conserved in the corresponding human Sup35p N-terminal domain, although there is no known human Sup35p-based prion. Similarly, the Ure2p prion domain is important for the stability against degradation of the full-length protein, and this stabilization is important for the nitrogen regulation function of Ure2p (Shewmaker et al., 2007). Thus, the prion domains have normal functions that have nothing to do with prion formation, and the presence of these domains may be selected in evolution by these nonprion functions.

10.7.4 Prion Forming Ability Is not Generally Conserved

Most experiments examining prion-forming ability by Sup35p or Ure2p from species other than *S. cerevisiae* have been carried out by expression in *S. cerevisiae* of the prion domain of the foreign protein fused to nonprion parts of *S. cerevisiae*'s Sup35 (Sup35MC), not in their native context. This work has shown that the Sup35p's of *P. methanolica*, *Kluyveromyces lactis*, and *Candida albicans* have domains that can act as prion domains in *S. cerevisiae* (Chernoff et al., 2000; Kushnirov et al., 2000a; Santoso et al., 2000). Full-length Sup35p's of several *Saccharomyces* species can also be prions in *S. cerevisiae* (Chen et al., 2007). However, a survey of wild *S. cerevisiae* showed that one-fourth of the strains examined had a large deletion in their prion domains making those Sup35s incapable of becoming prions (Resende et al., 2003).

Full-length Ure2p's from a variety of *Saccharomyces* species were expressed in *S. cerevisiae*, and several were found able to form a prion, but the Ure2p of *S. castellii* could not (Edskes et al., 2009). Most striking, while the Ure2p of *S. uvarum* was able to become [URE3] in its own context, that of *S. paradoxus* could not (Talarek et al., 2005). This was surprising because the *S. paradoxus* Ure2p, when expressed in *S. cerevisiae*, can form [URE3] (Edskes and Wickner, 2002; Crapeau et al., 2009; Edskes et al., 2009). It will be of interest to learn the basis for this difference. Clearly, the ability to form [PSI+] or [URE3] is not conserved even within the *Saccharomyces*. While further studies will be needed to ascertain the distribution of prion-forming ability, what we already know suggests that it occurs sporadically.

It has been noted that the prion domains of Ure2p and Sup35p vary more significantly in evolution than do the nonprion domain parts of

each molecule, but that there are nonetheless partially conserved parts of each prion domain (Edskes and Wickner, 2002; Harrison et al., 2007). Harrison et al. (2007) interpret these results to mean that the sequence conservation in the prion domain is for the purpose of retaining prion-forming ability. However, prion-forming ability does not require sequence conservation as shuffled Ure2p or Sup35p prion domains (leaving amino acid composition unchanged) can always (five of five in each case) still be prions (Ross et al., 2004, 2005). Thus, sequence conservation of these domains cannot be explained by a need to conserve prion-forming ability. It is likely that the important nonprion functions of the prion domains explain their conservation of sequence. Moreover, whether the sequence conservation correlates with conservation of prion-forming ability is impossible to conclude until more species have been examined for ability to form prions.

10.7.5 Stress Effects on Prion Generation

It has recently been observed that the frequency of [PSI+] arising *de novo* significantly increases under certain stress conditions, but not under other stress states (Tyedmers et al., 2008). If this were a cellular adaptation to the stress condition, one would expect the prion to arise under stress conditions in which the prion favors survival and growth and not under conditions in which the prion is detrimental. In fact, under most of the stress conditions resulting in increased prion formation, the presence of [PSI+] was found to be detrimental (Tyedmers et al., 2008). For example, 10 mM H_2O_2 increases [PSI+] generation 10-fold, but [PSI+] is detrimental to cells in 10 mM H_2O_2. This suggests that increased prion formation is a consequence of antiprion systems being occupied with coping with the stress, and the prions arise because the cell's guard is down. A similar interpretation could be made of the finding that under oxidative stress, *tsa1 tsa2* mutants frequently become [PSI+] (Sideri et al., 2010). Humans dealing with the stress of cold temperature are more likely to develop a viral infection; those under nutritional stress are more likely to get tuberculosis, but none of the infections help them adapt to the respective stress.

10.7.6 Why do Prion Domains Change Rapidly in Evolution?

Mead et al. (2003) observed that heterozygosity at residue 129 of PrP makes humans relatively immune to developing any of the various prion diseases, reviewed in Collinge and Clarke (2007). They suggested that the polymorphism at this site (about half of all alleles are Val and half are Met at this residue) was selected at a time when cannibalism was more common than it is today. Indeed, among survivors of the Kuru epidemic that devastated the Fore people of New Guinea, a new PrP allele has been found with a G127V mutation, evidently selected by the epidemic. Survivors were often heterozygous for this allele, but no patients with the disease carried it (Mead et al., 2009).

Yeast prion domain sequences vary more significantly than do those of the adjoining nonprion domain sequences (Kushnirov et al., 1990; Chernoff et al., 2000; Santoso et al., 2000; Edskes and Wickner, 2002; Harrison et al., 2007), and these sequence changes have produced species barriers for both [PSI+] and for [URE3] transmission (Chen et al., 2007; Edskes et al., 2009). We have proposed that, as suggested by the Collinge group for human prion disease, sequence differences are selected in yeasts in order to protect against acquisition of a prion by infection (Edskes et al., 2009).

10.8 CONCLUSIONS

The ecology of prions is at a very early phase, particularly those of yeast and fungi. Efforts to date to isolate prion-containing strains from the wild have been unsuccessful in the cases of

[URE3] and [PSI+], but most *het-s Podospora* strains have the [Het-s] prion and it is not rare to find *S. cerevisiae* carrying [PIN+]. However, no correlation of environment with the presence of [PIN+] has yet been reported. The true extent of the prion phenomenon is only beginning to be ascertained. Few organisms have the kind of well-developed genetic system of *S. cerevisiae* or *Podospora*, so it is perhaps not surprising that these two have played a large role in the prion field. Although examining proteins for prion-forming ability in *S. cerevisiae* has been a recurring theme, the finding by Talarek et al. (2005) that *S. paradoxus* cannot become [URE3] shows that this approach may be misleading, notwithstanding Edskes et al. (2009) having shown that the *S. paradoxus* Ure2p is capable of forming [URE3] in *S. cerevisiae*. Studies in yeast have shown that even subtle changes in the environment of chaperones and other components can have dramatic effects on the ability of a prion to propagate, and one suspects that such differences between species will be substantial. With many prions already identified in *S. cerevisiae*, we expect that the true range of the prion phenomenon will prove to be wide.

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SECTION III

VIRUSES OF MACROSCOPIC PLANTS

CHAPTER 11

ECOLOGY OF PLANT VIRUSES, WITH SPECIAL REFERENCE TO GEMINIVIRUSES

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CONTENTS

11.1 INTRODUCTION

Viruses are infectious nucleoprotein complexes that must have a living cell to multiply and establish themselves in. All viruses are obligate parasites containing either an RNA or a DNA genome enveloped by a protective protein coat. Approximately, one-third of all known viruses cause diseases in plants. A plant virus may infect a variety of plant species, thus having a broad host range, or may be restricted to a single species of plant.

Plant viruses occur in different sizes and shapes but can be broadly categorized into rods (rigid or flexuous), spherical (isometric or polyhedral), or bacilliform, that is, bullet shaped (Fauquet and Stanley, 2005). Some elongated viruses are rigid rods about 15×300 nm, but most are long flexible threads 10–13 nm wide and 480–2000 nm long. Most spherical viruses have an icosahedral structure, about 13–60 nm

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in diameter. The virus particles consist of a definite number of protein subunits that package the nucleic acid genome and are spirally arranged in elongated viruses and packed along the sides in icosahedral viruses.

Each plant virus particle consists of at least one molecule of nucleic acid encapsidated in a protein shell. The nucleic acid of most plant viruses consists of RNA, but there also are some known to have DNA, which may be single (e.g., geminivirus) or double stranded (e.g., caulimovirus). The nucleic acid makes up 5–40% of the virion mass, and the protein makes up the remaining 60–95%. The genome size of plant viruses is highly diverse and can range from 2.5 kb for geminiviruses to ~12 kb for closteroviruses, in many cases with overlapping reading frames and multifunctional proteins (Elena et al., 2008). The genomes of many plant viruses are split into two or more nucleic acid components, each of which may be individually encapsidated. The coat protein of the virus not only provides protection for the nucleic acid but also plays a role in vector transmission of the virus, the kind of symptoms it causes, and its movement within the plant. Most of the proteins encoded by plant viruses, therefore, have special functions in vector transmission, movement in the plant, in cleavage of the viral polyproteins, replication of the viral genome, encapsidation, or formation of inclusion bodies.

11.1.1 Classification of Plant Viruses and Geminiviruses

Viruses are classified into different species, genera, and families using a set of established criteria based on their morphological, physical, and biochemical properties. The International Committee on Taxonomy of Viruses (ICTV) has set aside the following criteria for classification of different viruses (Fauquet and Stanley, 2005).

Genome Type: DNA versus RNA, number of strands, linear, circular, or superhelical.

Morphology: size, shape, presence or absence of an envelope, capsid size, and structure.

Physicochemical Properties: mass, buoyant densities, sedimentation velocity, and stability.

Protein: content, number, size, and function of structural and nonstructural proteins, amino acid sequences, and glycosylation.

Lipids and Carbohydrates: content and nature.

Genome Organization and Replication: gene number, characterization of transcription and translation, posttranslational control, site of viral assembly, and type of release.

Antigenic and Biological Properties: host range, transmission, distribution, and so on.

Detailed descriptions on different virus species, genera, and families can be found in Hull (2002) and Fauquet and Stanley (2005). Figure 11.2 shows the categorization of different plant geminiviruses based on the genome organization and structure of the virus particles and the viral genome, and Table 11.1 lists the most important plant virus families and the diseases they cause.

Among the different types of viruses that affect plants, those belonging to the family Geminiviridae are of economic importance. Even though one of the earliest known viruses reported in the literature was a geminivirus, which was described in the poem of Japanese Empress Koken in 752 AD (Saunders et al., 2003), it was not until the late 1970s that the etiology of the first geminivirus was revealed (Matyis et al., 1975; Galvez and Castano, 1976; Bock and Woods, 1983). Then, it took several years before the molecular details of these viruses were unraveled (Goodman, 1981; Harrison, 1985; Stanley, 1985). In recent years, geminiviruses have proved to be the single most important family of plant viruses with respect to their potential to spread rapidly throughout fields and cause tremendous losses to cereals, legumes, and several vegetable crops (Duffus, 1987; Brown, 1994; Moffat, 1999).

TABLE 11.1 Plant Viruses and Their Taxonomic Classification

Order	Family	Subfamily	Genus	Type Species	Host
The ssDNA viruses					
	Geminiviridae		*Mastrevirus*	*Maize streak virus*	P
			Curtovirus	*Beet curly top virus*	P
			Topocuvirus	*Tomato pseudocurly top virus*	P
			Begomovirus	*Bean golden yellow mosaic virus*	P
	Nanoviridae		*Nanovirus*	*Subterranean clover stunt virus*	P
			Babuvirus	*Banana bunchy top virus*	P
			Unassigned	*Coconut foliar decay virus*	P
The DNA and RNA reverse transcribing viruses					
	Caulimoviridae		*Caulimovirus*	*Cauliflower mosaic virus*	P
			Petuvirus	*Petunia vein clearing virus*	P
			Soymovirus	*Soybean chlorotic mottle virus*	P
			Cavemovirus	*Cassava vein mosaic virus*	P
			Badnavirus	*Commelina yellow mottle virus*	P
			Tungrovirus	*Rice tungro bacilliform virus*	P
	Pseudoviridae		*Pseudovirus*	*Saccharomyces cerevisiae Ty1 virus*	F, P
			Sirevirus	*Glycine max SIRE1 virus*	P
	Metaviridae		*Metavirus*	*Saccharomyces cerevisiae Ty3 virus*	F, P, I
The dsRNA viruses					
	Reoviridae	*Spinareovirinae*	*Fijivirus*	*Fiji disease virus*	P, I
			Phytoreovirus	*Wound tumor virus*	P, I
			Oryzavirus	*Rice ragged stunt virus*	P, I
	Partitiviridae		*Alphacryptovirus*	*White clover cryptic virus 1*	P
			Betacryptovirus	*White clover cryptic virus 2*	P
	Endornaviridae		*Endornavirus*	*Vicia faba endornavirus*	P

(*continued*)

TABLE 11.1 (*Continued*)

Order	Family	Subfamily	Genus	Type Species	Host
The negative stranded ssRNA viruses					
Mononegavirales	*Rhabdoviridae*		*Cytorhabdovirus*	*Lettuce necrotic yellows virus*	P, I
			Nucleorhabdovirus	*Potato yellow dwarf virus*	P, I
			Varicosavirus	*Lettuce big-vein associated virus*	P
	Ophioviridae		*Ophiovirus*	*Citrus psorosis virus*	P
	Bunyaviridae		*Tospovirus*	*Tomato spotted wilt virus*	P, I
			Tenuivirus	*Rice stripe virus*	P, I
			Emaravirus	*European mountain ash ringspot-associated virus*	P
The positive stranded ssRNA viruses					
Picornavirales	*Secoviridae*		*Comovirus*	*Cowpea mosaic virus*	P
			Fabavirus	*Broad bean wilt virus 1*	P
			Nepovirus	*Tobacco ringspot virus*	P
			Cheravirus	*Cherry rasp leaf virus*	P
			Sadwavirus	*Satsuma dwarf virus*	P
			Sequivirus	*Parsnip yellow fleck virus*	P
			Torradovirus	*Tomato torrado virus*	P
			Waikavirus	*Rice tungro spherical virus*	P
	Potyviridae		*Potyvirus*	*Potato virus Y*	P
			Ipomovirus	*Sweet potato mild mottle virus*	P
			Macluravirus	*Maclura mosaic virus*	P
			Rymovirus	*Ryegrass mosaic virus*	P
			Tritimovirus	*Wheat streak mosaic virus*	P
			Bymovirus	*Barley yellow mosaic virus*	P
			Brambyvirus	*Blackberry virus Y*	P
			Sobemovirus	*Southern bean mosaic virus*	P
	Luteoviridae		*Luteovirus*	*Barley yellow dwarf virus—PAV*	P
			Polerovirus	*Potato leafroll virus*	P
			Enamovirus	*Pea enation mosaic virus 1*	P
			Umbravirus	*Carrot mottle virus*	P

Tombusviridae	*Dianthovirus*	*Carnation ringspot virus*	P
	Tombusvirus	*Tomato bushy stunt virus*	P
	Aureusvirus	*Pothos latent virus*	P
	Avenavirus	*Oat chlorotic stunt virus*	P
	Carmovirus	*Carnation mottle virus*	P
	Necrovirus	*Tobacco necrosis virus A*	P
	Panicovirus	*Panicum mosaic virus*	P
	Machlomovirus	*Maize chlorotic mottle virus*	P
Virgaviridae	*Tobamovirus*	*Tobacco mosaic virus*	P
	Tobravirus	*Tobacco rattle virus*	P
	Hordeivirus	*Hordeivirus*	P
	Furovirus	*Soil-borne wheat mosaic virus*	P
	Pomovirus	*Potato mop-top virus*	P
	Pecluvirus	*Peanut clump virus*	P
	Benyvirus	*Beet necrotic yellow vein virus*	P
	Cilevirus	*Citrus leprosis virus C*	P
	Polemovirus	*Poinsettia latent virus*	P
Bromoviridae	*Alfamovirus*	*Alfalfa mosaic virus*	P
	Anulavirus	*Pelargonium zonate spot virus*	P
	Bromovirus	*Brome mosaic virus*	P
	Cucumovirus	*Cucumber mosaic virus*	P
	Ilarvirus	*Tobacco streak virus*	P
	Oleavirus	*Olive latent virus 2*	P
	Ourmiavirus	*Ourmia melon virus*	P
	Idaeovirus	*Rasberry bushy dwarf virus*	P
Closteroviridae	*Closterovirus*	*Beet yellows virus*	P
	Ampelovirus	*Grapevine leafroll-associated virus 3*	P
	Crinivirus	*Lettuce infectious yellows virus*	P

(*continued*)

TABLE 11.1 (*Continued*)

Order	Family	Subfamily	Genus	Type Species	Host
Tymovirales	*Alphaflexiviridae*		*Allexivirus*	*Shallot virus X*	P
			Lolavirus	*Lolium latent virus*	P
			Mandarivirus	*Indian citrus ringspot virus*	P
			Potexvirus	*Potato virus X*	P
	Betaflexiviridae		*Capillovirus*	*Apple stem grooving virus*	P
			Carlavirus	*Carnation latent virus*	P
			Citrivirus	*Citrus leaf blotch virus*	P
			Foveavirus	*Apple stem pitting virus*	P
			Trichovirus	*Apple chlorotic leaf spot virus*	P
			Vitivirus	*Grapevine virus A*	P
	Tymoviridae		*Tymovirus*	*Turnip yellow mosaic virus*	P
			Marafivirus	*Maize rayado fino virus*	P, I
			Maculavirus	*Grapevine fleck virus*	P
	Barnaviridae		*Barnavirus*	*Mushroom bacilliform virus*	F
Unassigned viruses					
Unassigned plant viruses					P
The subviral agents: viroids, satellites					
Viroids	*Pospiviroidae*		*Pospiviroid*	*Potato spindle tuber viroid*	P
			Hostuviroid	*Hop stunt viroid*	P
			Cocadviroid	*Coconut cadang-cadang viroid*	P
			Apscaviroid	*Apple scar skin viroid*	P
			Coleviroid	*Coleus blumei viroid 1*	P
	Avsunviroidae		*Avsunviroid*	*Avocado sunblotch viroid*	P
			Pelamoviroid	*Peach latent mosaic virus*	P
Satellites					P, I, F, V

Abbreviations of the virus hosts: F, fungi; I, invertebrates; P, plants; V, vertebrate.

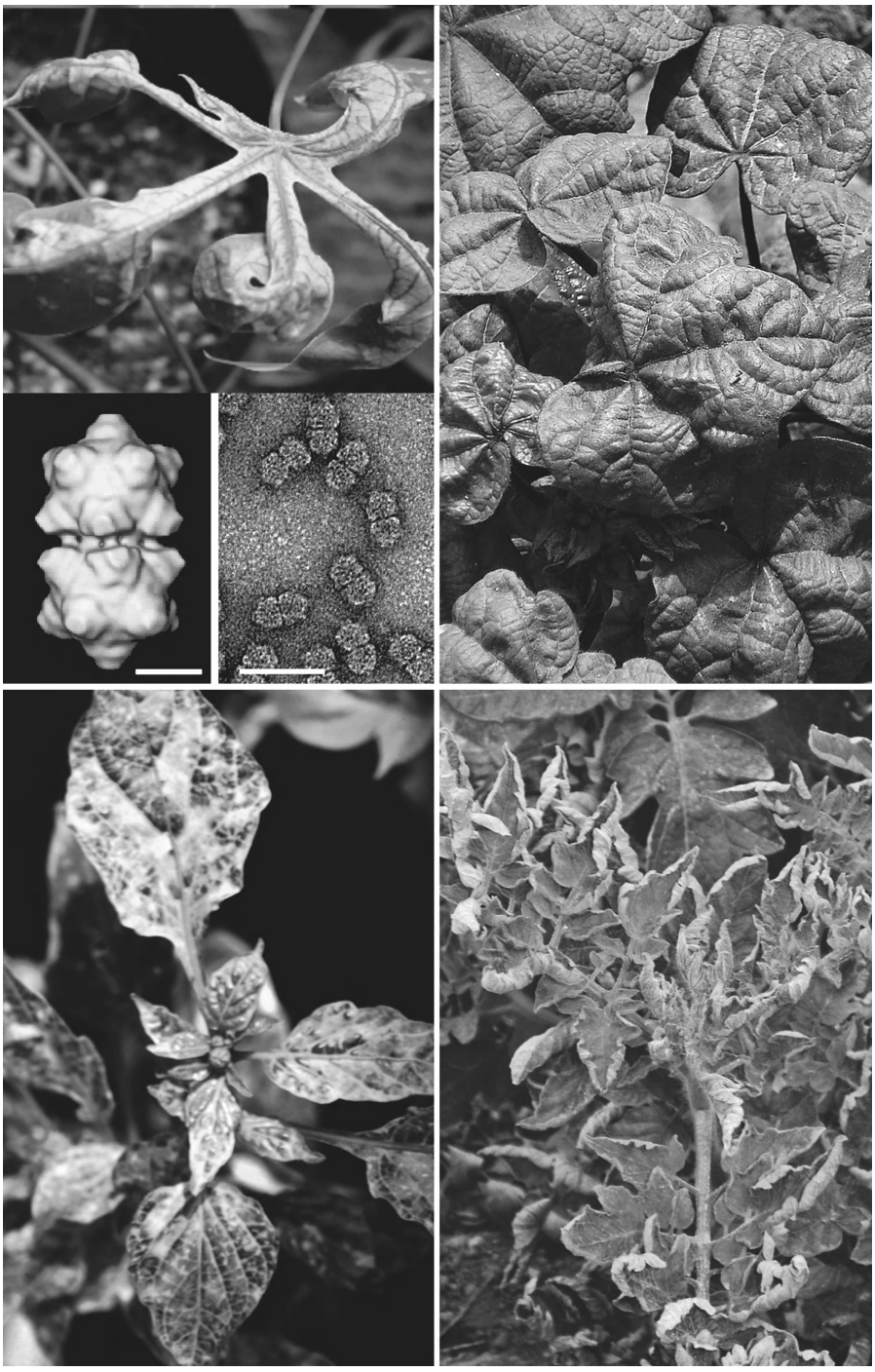


FIGURE 11.1 Symptoms caused by several geminiviruses: cassava mosaic disease (top left), cotton leaf curl disease in Pakistan (top right), pepper golden mosaic disease in Mexico (bottom left), and tomato yellow leaf curl disease from Jordan (bottom right). The geminivirus particles are shown in the top left panel with a computer rendering of a structure of maize streak virus, obtained via cryomicroscopy (left: the bar represents 5 nm) and a transmission electron microscope picture (right: the bar represents 40 nm). (*See the color version of this figure in Color Plate section.*)

Geminiviruses can infect plants, adding ornamental value (e.g., the mosaic diseases caused by Abutilon mosaic virus and Eupatorium yellow vein mosaic virus), and cause devastating plant diseases resulting in a loss of at least $1.25 billion per year on cassava crops in Africa (Thresh, 2006; Jeske, 2009). Most geminiviruses are transmitted by whiteflies, while others are transmitted either by leafhoppers or by treehoppers. All geminiviruses consist of twinned isometric (geminate) particles (Figure 11.1) containing single-stranded circular DNA genomes (Stanley et al., 1985; Lazarowitz and Shepherd, 1992; Zhang et al., 2001). They replicate via double-stranded DNA intermediates in the nuclei of infected cells and are mostly limited to phloem tissues (Hanley-Bowdoin et al., 1999). Viruses in the family *Geminiviridae* (Fauquet et al., 2008) are classified into four genera based on their genome organization, host range, and vector species (see Table 11.1): *Begomovirus*, *Mastrevirus*, *Curtovirus*, and *Topocuvirus*.

Begomoviruses (e.g., *bean golden mosaic virus*) have bipartite genomes, are transmitted by whiteflies, and infect dicotyledonous plants. Mastreviruses represented by the *maize streak virus* have monopartite genomes, are transmitted by leafhoppers, and infect mostly monocotyledonous plants. Curtoviruses, typified by *beet curly top virus,* have monopartite genomes, are transmitted by leafhoppers, and infect mostly dicotyledonous plants. The newest genus, *Topocuvirus*, has *tomato pseudocurly top virus* as its only species and is transmitted by treehoppers.

The genome organization of different members of the family Geminiviridae is illustrated in Figure 11.2. Genome size of geminiviruses ranges from 2.5 to 3.0 kb. In the case of monopartite viruses, the essential functions for virus replication and movement are located on a single genomic component (Figure 11.2). The genome of mastreviruses encodes for the movement protein (MP) and the capsid protein (CP) on the viral-sense (V-sense) strand and the RepA protein (exclusive of this genus) and the Rep protein on the complementary sense (C-sense) strand. Members of the genera *Curtovirus* (*beet curly top virus* as type species) and *Topocuvirus* (*tomato pseudocurly top virus* as type species) are transmitted by leafhoppers and treehoppers, respectively, and have a monopartite genome, although with a genetic organization different from that of mastreviruses, and infect dicotyledonous plants (Jeske, 2009). They occupy an intermediate phylogenetic position between the Mastre- and Begomoviruses. In addition to MP and CP, their genome encodes a V2 protein on the V-sense strand and has four open reading frames (ORFs) on the C-sense strand, namely, C1 (Rep: replication-associated protein), C2 (TrAP: transcriptional activator protein), C3 (REn: replication enhancer protein), and C4 (Figure 11.2).

The bipartite begomoviruses have the ORFs coding for these same proteins divided between two different DNA molecules, termed as DNA-A and DNA-B (Jeske, 2009; Patil and Fauquet, 2009). DNA-A encodes for the Rep or AC1, TrAP or AC2, REn or AC3, and AC4 on the complementary strand and the coat protein (CP or AV1) and precoat protein (AV2) on the virion strand (Figure 11.2), while DNA-B encodes for the two movement proteins responsible for nucleocytoplasmic shuttle transport (NSP or BV1) and cell-to-cell spread (MP or BC1) of the virus in the plant (Hanley-Bowdoin et al., 1999). Geminiviruses have a stretch of noncoding region (CR/IR) that has high sequence similarity in both of the DNA components of bipartite begomoviruses and a highly conserved nonanucleotide (TAATATT↓AC) sequence present where the cleavage is made during rolling circle replication (Hanley-Bowdoin et al., 1999). In addition, the intergenic region (IR) has signature sequences called iterons that are replicase binding sites and important factors for the replication compatibility of the DNA-B component in bipartite geminiviruses (Harrison, 1985; Jeske, 2009).

11.1.2 Taxonomy and Evolution of Geminiviruses

ICTV has recognized about 2000 species of plant viruses (Fauquet and Stanley, 2005),

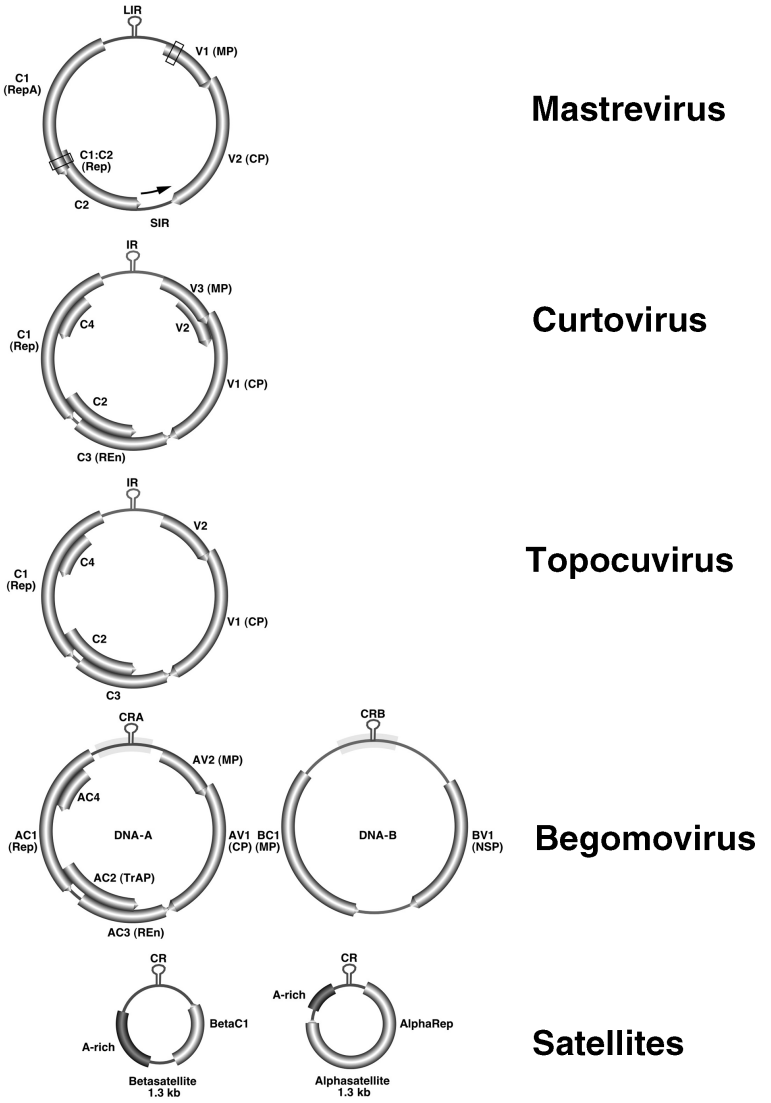


FIGURE 11.2 Schematic representation of the genome organization of members of the four genera of the family *Geminiviridae*. Abbreviations used: V, ORF encoded by the viral sense of DNA strand; C, ORF encoded by the complementary sense; RepA (C1), replication-associated protein; Rep (AC1 or C1), replication initiation protein; REn (AC3 or C3), replication enhancer protein; TrAP (AC2 or C2), transcriptional activator protein; MP (BC1), movement protein; CP (AV1 or V1), coat protein; V2 (AV2), precoat protein; and NSP (BV1), nuclear shuttle protein. The noncoding regions (or part of them) are the large intergenic region (LIR) and the small intergenic region (SIR) in mastreviruses. The small ssDNA primer at SIR (see arrow), the intergenic region (IR) in curtoviruses and topocuvirus, and the common region (CRA and CRB) for begomovirus DNA-A and DNA-B components are also shown.

which is a gross underestimation of the actual number of plant virus species that still need to be cloned and sequenced. In addition to the scant data available for virus diversity, we have a poor understanding of the level of mixed infection or temporal patterns of virus accumulation in native plants; thus, it is important to extend our knowledge beyond those combinations known to cause disease to those that might cause disease in future (Wren et al., 2006).

Phylogenetic relationships between geminiviruses are usually reflected by their geographical descent, except in cases where they have been disseminated by human intervention (Harrison and Robinson, 1999) and with the exception of some viruses originating from the Indo-China region (Nawaz-ul-Rehman and Fauquet, 2009). Phylogenetic analysis of geminivirus sequences geographically clusters them into two major categories: New World (North and South American continents) and Old World (Asian, African, and European continents). But recently New World begomoviruses were identified from Vietnam and from India (Old World), thus suggesting that the origin of New World geminiviruses might reside in the Old World, later expanding to the New World (Ha et al., 2006, 2008). Geminiviruses are named on the basis of the host plant, the most prevalent symptom, and the geographical origin of the disease. A species demarcation threshold of 89% nucleotide similarity for begomoviruses and 75% for mastreviruses has been set up by the ICTV to differentiate between the species (Fauquet et al., 2008). In the case of whitefly-transmitted begomoviruses, the helper viruses from the Old World are sometimes associated with subviral components, called satellite DNAs, which depend on the helper viruses for their proliferation, and they have been classified as betasatellites (DNA β) or alphasatellites (nanovirus-like components, previously called DNA-1) (Briddon et al., 2008; Patil and Fauquet, 2010). These satellite molecules are named on the basis of the helper virus with which they are associated, for example, *Ageratum yellow vein betasatellite* and *Ageratum yellow vein alphasatellite* are associated with ageratum yellow vein disease complex (Rigden et al., 1996; Roossinck, 1997; Saunders and Stanley, 1999; Rojas et al., 2005).

Geminiviruses are believed to have been originated from the extrachromosomal DNA replicons, present in prokaryotic or primitive eukaryotic ancestors of modern plants, followed by a number of key evolutionary steps to continue their adaptation with plants (Kikuno et al., 1984; Rojas et al., 2005; Briddon et al., 2010). Since there are no supporting fossil records, there is a lot of ambiguity surrounding the sequence of origin and evolution of geminiviruses, and it remains uncertain whether the monopartite geminiviruses evolved from the bipartites or vice versa. However, there are two different opinions based upon sequence analysis: (i) the monopartite begomoviruses captured the second component (DNA-B), which could have been a satellite molecule, or (ii) the DNA-B evolved through component duplication (Briddon et al., 2010). The New World begomoviruses are consistently associated with their cognate DNA-B component and are strictly associated with them with very few examples of pseudorecombinations among them (Jeske, 2009), whereas the Old World begomoviruses have a weak association with their cognate partner, often forming pseudorecombination complexes and are also associated with a satellite component (Duffy et al., 2008; Duffy and Holmes, 2009). The evolution rate of geminiviruses is very high with $3–5 \times 10^{-4}$ nucleotides per site per replication cycle, which is on par with the evolution rate of plant RNA viruses (Duffy et al., 2008; Duffy and Holmes, 2009). The rapid rate of mutations and their ability to undergo frequent recombinations (Gibbs et al., 2008; van der Walt et al., 2009) and to form recombinant molecules have given them extra ability to form new strains and sometimes new species with increased powers of infecting new host plants, encroaching upon new geographical areas, and leading to new viral diseases (Gibbs et al., 2008; van der Walt et al., 2009).

Although several mechanisms lead to new variants of geminiviruses, not all of the variants are capable of surviving and spreading; in general, they appear to be selected according to Darwin's theory of "survival of the fittest" (Roossinck, 1997). Their selection is controlled by several factors such as climate, insect vector, and plant host (Seal et al., 2006). The revolution in viral genomics has enabled sequencing a huge number of viral genomes, and particularly geminiviruses, which can lead to better understanding of the virus evolution,

the diversity, and the mechanisms involved in it (Fauquet et al., 2008; Roossinck et al., 2010).

11.1.3 Symptomatology of Virus-Infected Plants

Plant viruses cause a wide range of diseases, inducing a variety of symptoms. Developmental abnormalities such as stunting, uneven growth, and leaf curling are some of the striking symptoms of plant virus infections. The most easily recognizable symptoms, however, are mosaics, mottles of green and yellow, and ringspots that can appear both on the leaves and on the fruits. Some viruses cause swellings in the stem (e.g., the cocoa swollen shoot disease) or, in other cases, certain tumorous outgrowths, known as enations, may appear from the upper or lower surface of the leaf. These may be small ridges of tissues or large irregular leaf-like tissues associated with the veins. In some cases, a virus may infect a plant without causing any visible symptoms (latent infections). Besides the symptoms produced on their cultivated crop hosts, some viruses also induce a hypersensitive response in certain plant species manifested by necrotic lesions that limit the spread of the virus at the site of inoculation. Such plants are called the "local lesion hosts"and are used in quantitation of the virus concentration in infectivity assays (Sánchez et al., 1998).

Plants infected by geminiviruses often exhibit symptoms ranging from bright mottles to golden yellow mosaics and sometimes the leaves are puckered and curled (Figure 11.1). When plants are infected at an early stage, they become stunted and bushy. Plant yield is very poor when infected at older stages of plant growth, although crop losses are not so staggering in the case of later-term infections. Geminiviruses spread rapidly throughout the field because their whitefly vectors are voracious feeders and the whiteflies retain the virus for days. In recent years, both the prevalence and the distribution of whitefly-transmitted geminiviruses have increased tremendously, and, depending on the crop, stage of infection, and the whitefly population, the yield losses can range from 20% to 100% (Brown, 1994). The DNA satellites associated with the geminiviruses are also known to manifest the symptom phenotypes, either by enhancing the symptom severity or by suppressing the symptoms, more particularly the betasatellites result in characteristic vein thickening (Briddon and Stanley, 2006; Patil and Fauquet, 2010). Sometimes, generation and accumulation of defective forms of the viral genome can lead to symptom amelioration (Patil and Dasgupta, 2006; Patil et al., 2007).

Moreover, these viruses can occur single or in mixtures, the latter making their detection and accurate diagnosis very difficult. The visible symptoms induced by virus infection of plants are often a reflection of the histological changes occurring within them (Laliberte and Sanfacon, 2010). These changes could be manifested as hypoplasia (reduction in cell size), hyperplasia (enlargement of cells), or necrosis. Leaves with mosaic symptoms frequently show hyperplasia in their yellowed areas, while the leaf lamina layer is thinner in the dark green areas.

Also, in a typical mosaic pattern, the mesophyll cells are less differentiated with fewer chloroplasts and few or no intercellular spaces, while in contrast the hyperplasic cells are larger than normal, with no intercellular spaces. The vascular tissues associated with the mosaic undergo abnormal cell division, causing proliferation of the phloem cells. Recently, it has been shown that this kind of green and yellow mosaic region in the virus-infected leaf is an outcome of gene silencing (Hirai et al., 2008).

The cytological effects of virus infection have been associated with the appearance of crystalline arrays or plates in the nuclei (potyviruses), hypertrophy of the nucleolus, deposition of electron-dense particles in the nucleus, and accumulation of fibrillar rings in the nucleus (geminiviruses; Kim and Flores, 1979). The chloroplasts become aggregated, cup shaped, and accumulate starch grains, and sometimes show large vesicles or fragmentation of chloroplast structure (Laliberte and Sanfacon, 2010). Besides these characteristics,

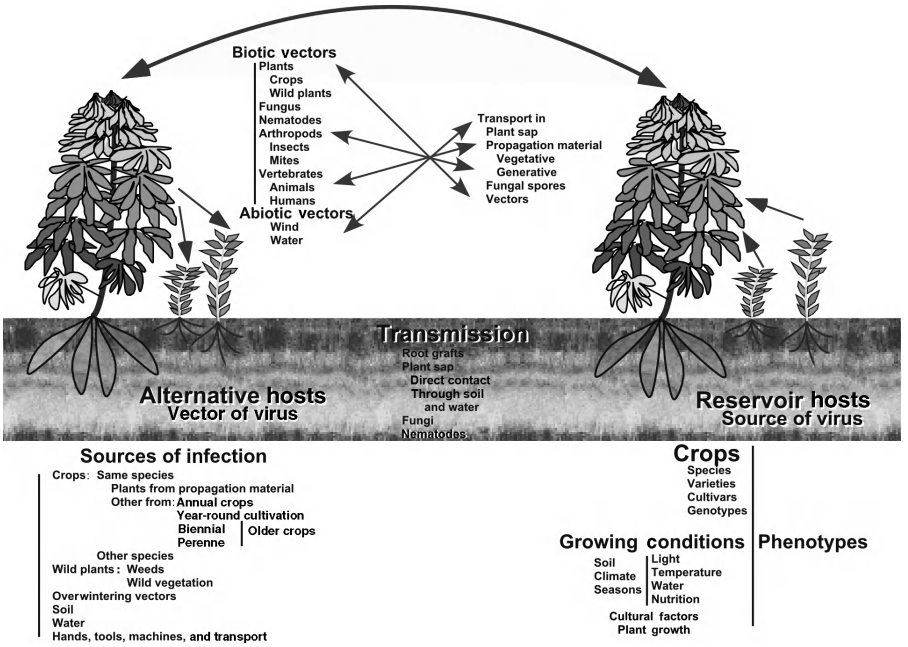


FIGURE 11.3 Interrelationships between the various environmental factors that affect virus survival and spread in the field. Adapted from Bos (1981).

the cell walls get thickened because of deposition of callose and in some cases the mitochondria may also become affected.

Most of the cytological changes induced by viruses are not due to depletion of nutrients diverted toward synthesis of the virus itself, but rather are due to disruption of normal metabolic processes in the host cells (Laliberte and Sanfacon, 2010). Virus infections generally cause a decrease in photosynthesis because of a reduction in the amount of chlorophyll and leaf area per plant. In addition, soluble nitrogen levels drop during rapid virus synthesis and in mosaic-infected tissues carbohydrate levels go down drastically.

11.1.4 Transmission of Plant Viruses

Plant viruses are transmitted to healthy plants through a variety of ways, including mechanical transmission via sap, seed, pollen, vegetative propagation; a number of insects such as aphids, leafhoppers, whiteflies, mites, and thrips; and fungi and nematodes (Harrison, 1981) (Figure 11.3). Because plant cells have a thick wall, viruses cannot easily penetrate the cells directly, and most often enter the plants through wounds created by mechanical means or by vector transmission. In other cases, the viruses are transmitted through seeds (e.g., wheat streak mosaic virus). Transmission through seeds is an effective means of introducing a virus in the field at an early stage in the life of the new host plant, often resulting in a randomized distribution of infection throughout the field. Viruses may persist in seeds for a very long time, enabling easy distribution and spread of the virus over long distances. For seed transmission to occur efficiently, the virus may reside within the tissues of the embryo; otherwise, the virus simply may be present on the surface of the seed, as is known to occur in tobacco mosaic virus (TMV). Vegetative propagation of plant material is a very effective method for perpetuating and spreading viruses.

A plant infected systemically with a virus may remain so throughout its life, and therefore all vegetative parts of the plant subsequently

used for propagation may be infected. Transmission through grafts and the parasitic plant, Dodder (*Cuscuta* spp.), are other ways by which viruses can be transmitted and spread in nature (Hosford, 1967). For the purpose of this chapter, we shall henceforth limit ourselves to the discussion of insect transmission alone. Insect vectors are the most common means of spread of virus diseases and thereby constitute an important component of viral ecology, as the viruses entirely depend on the behavior and dispersal capacity of their vectors to spread from plant to plant. Insect vectors of plant viruses belong to several orders (Hemiptera, Coleoptera, Thysanoptera, Orthoptera, Dermaptera, Lepidoptera, and Diptera), but Hemiptera is by far the most important group of vectors of plant viruses. Of the different known insect vectors, aphids constitute the largest group and transmit a great majority of all stylet-borne viruses, followed by whiteflies, leafhoppers, and thrips (Power, 2000; Fereres and Moreno, 2009). All of these homopteran vectors have piercing and sucking mouthparts and carry viruses on their stylets, consisting of four tubular structures. Homopterans, belonging to the families Aphididae (aphids) and Aleyrodidae (whiteflies), are vectors for about 55% of all known plant viruses (Nault, 1997) and are the main vectors according to the number of virus species transmitted. Aphids transmit more than 50% of the plant viruses vectored by insects (approx. 275 virus species within 19 virus genera) (Nault, 1997), while whiteflies transmit a large number of virus species belonging to five different virus genera (90% belong to the *Begomovirus* genus, 6% to the *Crinivirus* genus, and the remaining 4% are in the *Closterovirus*, *Ipomovirus*, or *Carlavirus* genera) (Jones, 2003). Furthermore, their short life cycle, high rate of population increase, and high dispersal potential have made aphids and whiteflies the two main vectors of plant viruses. Many of the associated diseases that are caused result in severe yield losses and have a great impact on agriculture across the globe (Sseruwagi et al., 2004; Fereres and Moreno, 2009).

11.1.5 Virus–Vector Relationships

The plant viruses move through the insect vector from the gut lumen into the hemolymph or other tissues and finally into the salivary glands, from which they are introduced back into the host plant. The movement and/or replication of the viruses within the insect vectors require specific interactions between virus and vector components (Hogenhout et al., 2008). Depending on how long a virus can persist in association with the vector, the viruses are categorized as nonpersistent, semipersistent, or persistent (Hogenhout et al., 2008; Fereres and Moreno, 2009). Nonpersistent viruses are acquired within seconds, retained by the vector for only a few hours, and, because they are mostly associated with the mouthparts of the vector, transmitted within minutes of being acquired (Sylvester, 1980). Transmission of nonpersistent viruses occurs mostly because the vectors tend to probe different plants in quick succession and do not settle on any single plant for prolonged, continuous feeding. Most of the aphid-borne viruses belonging to the genera *Potyvirus*, *Cucumovirus*, *Caulimovirus*, and *Carlavirus* are transmitted in a nonpersistent manner. In the case of semipersistent viruses, the virus may be acquired within several minutes or hours, and accumulate in the insect's gut before being released again through the stylets. Such viruses typically persist in the vector for 1–4 days. Typical examples of semipersistent viruses are the beet yellows virus and the citrus tristeza virus transmitted by the aphids, and the maize chlorotic dwarf virus transmitted by the leafhoppers. Persistent viruses, for example, the luteoviruses, are accumulated internally in the hemocoel, or body cavity, of the vector before they are inoculated back into the plant through the insect's mouthparts. These viruses are termed to be circulative and some of them may even multiply within their vectors such that their vector is by definition a biological vector and are then referred to as circulative–propagative viruses (Hogenhout et al., 2008). These biological vectors can retain and transmit the virus for a long period of

time. Most of the geminiviruses are believed to be circulative but nonpropagative viruses, but it has been shown that tomato yellow leaf curl virus is retained through the molt or eggs (Ghanim et al., 1998), making it a circulative–propagative virus. Nematodes and fungi transmit a very small percentage of viruses. Nematode vectors transmit the viruses by feeding on the roots of the infected plants and then moving on to healthy plants (MacFarlane and Neilson, 2009). Both juvenile and adult nematodes can acquire and transmit viruses, but the virus is not carried through the nematode's eggs.

All geminiviruses belonging to the genus *Begomovirus* are transmitted by *Bemisia tabaci* (Hemiptera: Aleyrodidae), also known as the cotton, tobacco, or sweet potato whitefly (Morales, 2007). *B. tabaci* is believed to have originated either in the Orient or in Pakistan and subsequently spread to other parts of the world (Russell, 1957; Mound, 1963; Cock, 1986; Byrne and Bellows, 1991). This whitefly species is a major pest of numerous crop species because of its polyphagous nature, and at least 506 plant species in 74 families of dicots and monocots have been reported as hosts of this species (Butler et al., 1986; Cock, 1986). This gives the viral vector ample flexibility in being able to adapt itself to feeding upon different plant hosts under unfavorable conditions (Maruthi et al., 2005). These plants then serve as carriers or reservoirs for the whitefly, allowing its survival for the next season. The viruses carried by the whiteflies are persistent and circulative, which means the whitefly can efficiently transmit them during a period of 5–20 days (Duffus, 1987). The emergence of the B-biotype of *B. tabaci*, which is extremely fecund and has a very broad host range, has led to the severe pandemics of diseases caused by geminiviruses (Colvin et al., 2004). The B-biotype, an introduction from the Middle East to the southwestern United States, has entirely displaced the A-biotype and has been colonizing a large range of crops, thereby leading to outbreaks of new begomoviral diseases (Brown, 2001). Plant viruses may modify the behavior of their vectors by inducing changes in the feeding habits of the vector, including the vector's attraction to or preference for a given species of plant host after infection (Fereres and Moreno, 2009). Fortunately, the *B. tabaci* does not tolerate temperatures below 17°C and heavy rainfall areas such as the Amazon region (Morales, 2007).

11.1.6 Replication, Transcription, and Movement of the Virus

To understand the ecology of plant viruses at the molecular level, it is important to understand the key components of the virus life cycle that significantly influence its fitness in an ecosystem and the environmental components (light, temperature, humidity, etc.) that can have an effect at any stage of virus proliferation. The key components of the geminivirus life cycle (replication, gene expression, movement, and encapsidation) are briefly described here. Geminiviruses replicate their circular ssDNA genome via double-stranded (ds)DNA intermediates either by rolling circle replication or by recombination-dependent replication mechanism in the nuclei of infected cells (Hanley-Bowdoin et al., 1999; Jeske et al., 2001). Geminivirus DNA replication cycle can be divided into two main stages: *Stage (A)* the conversion of genomic ssDNA into a double-stranded form, which serves as the template for transcription of viral genes; *Stage (B)* the production of genomic ssDNA from the double-stranded intermediate and the production and encapsidation of mature genomic circular ssDNA into viral particles. During the infection process, the insect vector injects viral particles and the viral genome is transported into the host cell nucleus by mechanisms whose molecular details are largely unknown (Lazarowitz and Shepherd, 1992; Palmer and Rybicki, 1998). Once within the nucleus, amplification of the viral genome involving an efficient DNA replication process occurs in two distinct stages as mentioned above (Gutierrez et al., 2004).

The dsDNAs formed during rolling circle replication serve as transcriptional templates and the geminivirus genomes are transcribed in a bidirectional manner resulting in mRNAs that correspond to both the virion and the complementary-sense ORFs. The bidirectional promoters are identified in the intergenic regions of both DNA-A and DNA-B (Hanley-Bowdoin et al., 1999). The begomovirus ORF AC2 is a viral transcription factor that transactivates the late viral genes AV1 and BV1 (Sunter and Bisaro, 1992; Waigmann et al., 2004).

On entering a host cell, the viruses uncoat, replicate their genomes in the nucleus, and spread to adjacent cells through plasmodesmata (for reviews, see Citovsky and Zambryski, 1993; Carrington et al., 1996; Lazarowitz and Beachy, 1999; Waigmann et al., 2004; Taliansky et al., 2008). On reaching the vascular tissues, they use the phloem to infect the entire plant (Oparka, 2004), moving rapidly toward the growing regions and other food-utilizing parts of the plant. All systemic virus infections, as in the case of geminiviruses, depend on one or more nonstructural proteins specifically required for movement within their hosts (Rojas et al., 2005). For some viruses, functional coat protein may be required for long-distance spread in the plant, while other virus types, such as the geminiviruses, encode specific movement proteins to assist cell-to-cell and long-distance movement of the virus in the plant (Lucas, 2006; Jeske, 2009). Classically, a viral movement protein is known to increase the plasmodesmal size exclusion limit and to thus facilitate viral movement from cell to cell; however, other viral proteins that do not themselves move may be essential for the movement process. Viruses that infect plants have developed a variety of strategies to move from cell to cell and are heavily dependent on endogenous host transport systems during movement, as indeed they depend upon the host in all aspects of their viral life cycles (Boevink and Oparka, 2005). Geminiviruses in particular have evolved two movement proteins, which are AV1/CP and AV2 in case of the monopartite mastreviruses, curtoviruses, and Old World monopartite begomoviruses, whereas in the bipartite begomoviruses the second genomic component (DNA-B) is specialized in movement functions (Rojas et al., 2005). The DNA-B codes for two movement proteins, respectively, the nuclear shuttle protein (NSP) and movement protein (Lazarowitz and Beachy, 1999).

11.1.7 Gene Silencing, Suppression, and Synergism

The role of gene silencing in the defense against plant viruses is well established. Gene silencing is an inducible defense pathway that targets the invading nucleic acids through production of siRNAs (Mlotshwa et al., 2008). Gene silencing is also called posttranscriptional gene silencing (PTGS) in plants or RNA interference (RNAi) in animals. Gene silencing has dramatic but unexplored ecological implications; as a hypothetical example, a bison-borne virus could silence genes for antigrazing defenses, thus facilitating its transmission (Wren et al., 2006; Glick et al., 2008). Thus, it is important to understand the mutualistic interactions between viruses and their hosts and the mechanisms involved in manifestation of these interactions. The recovery of a virus-infected plant from the disease symptoms in most cases has been attributed to gene silencing, and the cases where the recovery phenotype is not observed despite the production of siRNAs have been attributed to the presence of strong virus-encoded silencing suppressors (Szittya et al., 2003; Chellappan et al., 2004; Vanitharani et al., 2005). In addition, virus–host interactions are modified by environmental factors (Figures 11.4 and 11.5), particularly temperature and light conditions through alteration in the efficiency of gene silencing and its suppression (Chellappan et al., 2004; Glick et al., 2008).

In response to gene silencing, viruses have evolved suppressor proteins that can counteract gene silencing, and in the case of geminiviruses, AC2, AC4, and AV2 proteins have been recognized as the silencing suppressors

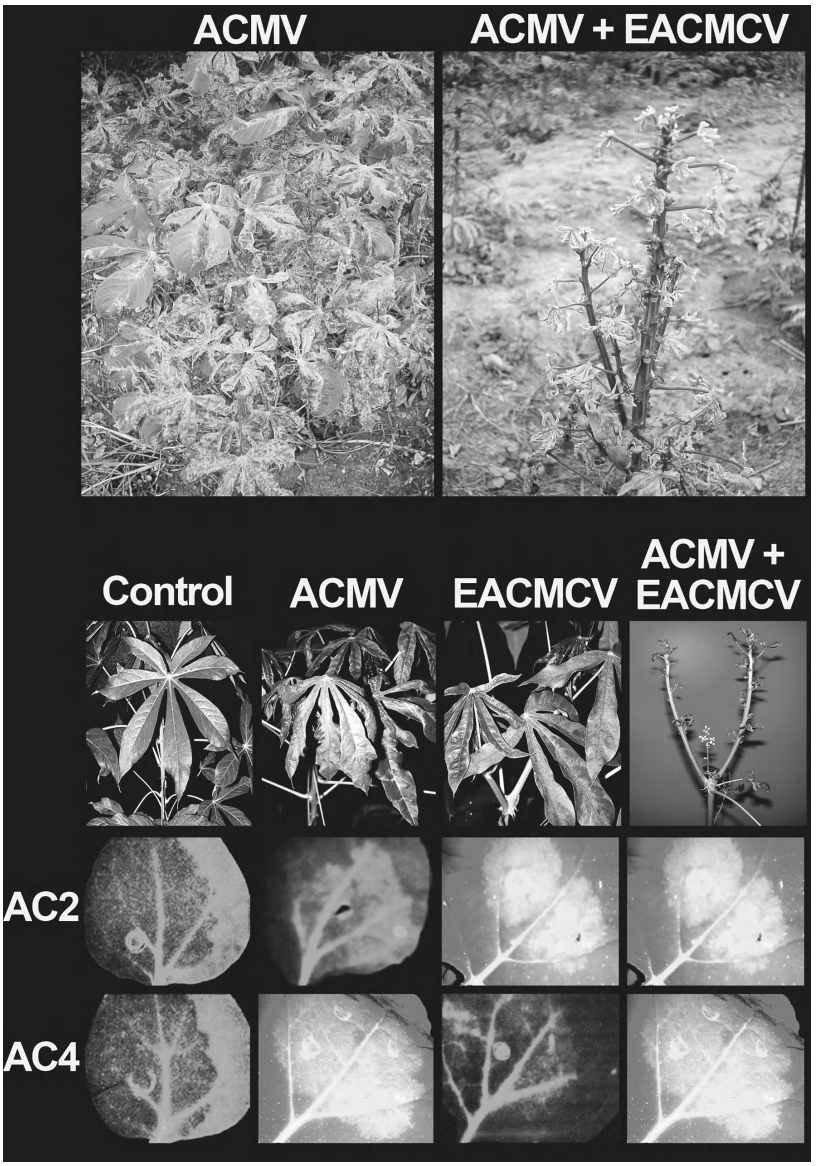


FIGURE 11.4 Example of synergism between two geminiviruses, ACMV and East African cassava mosaic Cameroon virus (EACMCV). Top panel shows a single infection of cassava with ACMV (left) and a dual infection of cassava with ACMV and EACMCV (right) in Ghana. Bottom panel: reproduction of synergistic interaction between ACMV and EACMCV in the lab; from left to right: control cassava plant, ACMV, EACMCV, and dual infection of cassava. Lower panel: picture of the effect of gene silencing suppression of the geminivirus AC2 and AC4 proteins of both viruses on tobacco. Green color indicates no PTGS suppression, while yellow color indicates PTGS suppression. Dual PTGS suppression by AC2 and AC4 from two geminiviruses corresponds to the collapse of cassava (top right). (*See the color version of this figure in Color Plate section.*)

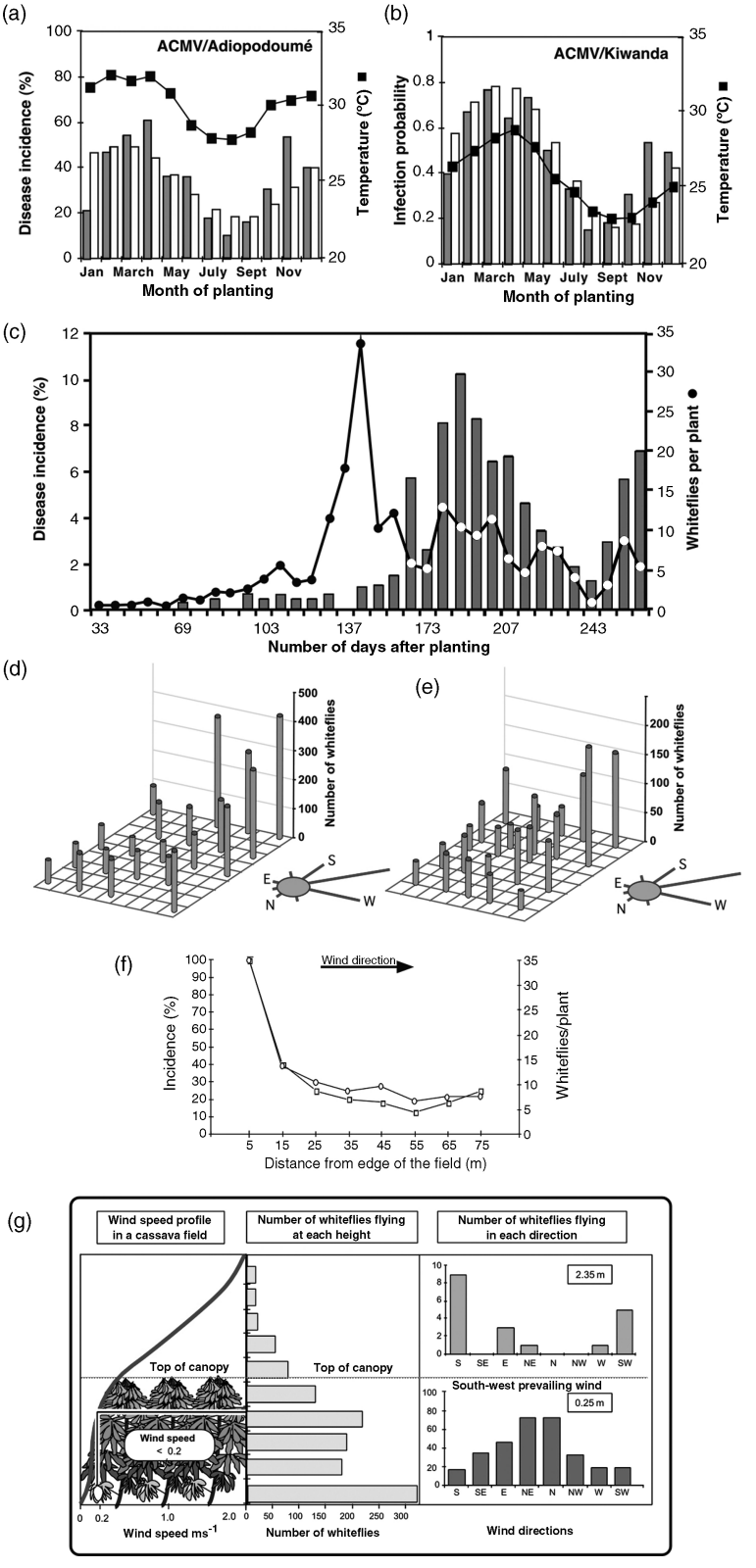
(Vanitharani et al., 2005; Bisaro, 2006; Glick et al., 2008). In the case of mixed infection where two or more viruses are present, it can lead to synergism, which refers to greatly increased disease symptoms and virus accumulation (Figure 11.4) (Fondong et al., 2000; Latham and Wilson, 2008). Molecular studies have shown that the presence of two or more different PTGS suppressor proteins encoded by different virus species, acting at different steps along the gene silencing pathway, can more effectively inhibit antiviral silencing, ultimately resulting in synergism (Figure 11.4) (Vanitharani et al., 2005; Mlotshwa et al., 2008). Therefore, having more than one type of PTGS suppressor gives an advantage to viruses, synergistically interacting in mixed infections, and thus helps in establishing a severe/successful disease. In addition to silencing suppression, the synergistic interactions can occur at different levels of the virus cycle, that is, gene expression, replication, movement, or encapsidation (Guevara-González et al., 1999).

11.2 VIRUS–VECTOR–PLANT ECOSYSTEMS

Since 1980, attention has been focused on virus survival systems as ecological systems (Figure 11.3) (Thresh, 1980; Harrison, 1981). In the case of soil-inhabiting vectors such as nematodes or fungi, a well-developed ability of the viral vector to survive at a site seems to compensate for a vector having limited ability to spread to new sites. In contrast, some viruses transmitted by aerial vectors spread readily to new sites, but perennate (survive under adverse conditions) inefficiently at existing ones. Possession of effective means both for spreading and for perennating is a feature of some of the most consistently prevalent viruses (Harrison, 1983, 1985). Because viruses in the same taxonomic entity tend to have similar survival systems, and different types of plant communities support different kinds of survival systems, it is to be expected that specific groups of viruses will be best adapted to specific types of plant communities. Mechanically transmitted viruses such as potexviruses and tobamoviruses occur in high concentrations and are very stable in their hosts, features that allow them to perpetuate under adverse conditions. Other plant viruses, such as the ilarviruses, which are mainly found in woody species, or pollen- and fungi-transmitted viruses that persist in the resting spores of their vectors, are favored by monoculture. Viruses such as tobra-, nepo-, gemini-, and luteoviruses can survive in a variety of wild plants because they have wide host ranges and long persistence in their vectors, features that make them fit to survive in communities that contain many plant species (Harrison, 1983). The pandemic of cassava mosaic disease (CMD) that began in Uganda in the 1990s and later spread to the neighboring African countries is an outcome of three major players: (i) synergistic interactions among the mixed infections of different cassava-infecting geminiviruses (ACMV: African cassava mosaic virus and EACMV: East African cassava mosaic virus; Figure 11.4); (ii) appearance of a more cassava-adapted whitefly biotype; and (iii) the presence of farmer-preferred highly susceptible cultivars and loss of genetic diversity in the cultivated cassava genotypes (Fargette et al., 2006). In addition to all these factors, the entire process could be driven by climate change and exacerbated by issues such as the fact that in geographic regions with rapid growth of human population, global trade increasingly displaces plants from their actual centers of domestication/cultivation, thus dispersing previously localized viruses and their vectors (Garrett et al., 2006).

11.2.1 Ecological Factors Affecting Virus Survival and Spread

Plant virus diseases are not simply an outcome of interplay among the host, the pathogen, and the environment. The interactions observed are instead complex and involve a multitude of factors. Figure 11.3 provides a comprehensive picture of the major factors involved in the


(a)
ACMV/Adiopodoumé
Disease incidence (%)
Temperature (°C)
Jan March May July Sept Nov
Month of planting
(b)
ACMV/Kiwanda
Infection probability
Temperature (°C)
Jan March May July Sept Nov
Month of planting
(c)
Disease incidence (%)
Whiteflies per plant
Number of days after planting
(d)
Number of whiteflies
S
E
N
W
(e)
Number of whiteflies
S
E
N
W
(f)
Wind direction
Incidence (%)
Whiteflies/plant
Distance from edge of the field (m)
(g)
Wind speed profile in a cassava field
Number of whiteflies flying at each height
Number of whiteflies flying in each direction
Top of canopy
Top of canopy
Wind speed < 0.2
2.35 m
South-west prevailing wind
0.25 m
S SE E NE N NW W SW
Wind speed ms-1
Number of whiteflies
Wind directions

development, spread, and survival of a plant viral disease. Evidently, the key components involved include the crops and the conditions under which they grow, the availability of vectors that spread infection, the sources of infection, the types of soil and water, and the climate. Whether or not a crop will suffer from a virus disease depends on its susceptibility, on the presence of sources of infection, and certainly on the availability and behavior of vectors.

For viruses that are transmitted by airborne vectors such as the aphids, leafhoppers, or whiteflies, several factors such as weather conditions, wind speed and direction, and the presence of barrier crops may play a role in the eventual spread and survival of a virus (Figure 11.5). Both the crop plants themselves and the adjacent weeds may act as reservoirs for spread of infection. The growing conditions that plants are subjected to can also greatly influence crop susceptibility and the sensitivity of crops to viral infection, as well as affect vector behavior. The opportunity for disease spread within crops and epidemic development also depends on the type of planting pattern. A homogeneous crop may yield a uniform product, but if the genotype is susceptible, the effects of virus infection can be dramatic (Bos, 1983). Introduction of new plant genotypes or an increase in the cropping area can also lead to either a sudden outbreak of new diseases or allow a tremendous increase in the spread of an existing one.

Among the biological factors that affect the general survival and spread of a virus in the field and help to determine potential success of the virus under adverse conditions are the physicochemical properties of the virus itself. Its physical characteristics influence viral stability in the plant or soil, the concentrations that the virus can reach in plants, and their rate of movement and distribution within host plants (Figure 11.3). Viruses that can go systemic rapidly or can move into plant seeds have a far greater chance of survival than do those viruses that either spread slowly or cannot cross the outer barrier of the developing ovule. Furthermore, a virus that can mutate, recombine, or otherwise quickly adapt itself in response to changes in the environment will be selected naturally for better survival and dispersal. When combined with the potential to infect a diverse host range, such responsive viruses have a better opportunity to maintain themselves and spread efficiently. The pattern of spread within the crop and the rate and extent of spread may depend on available sources of viral inoculum, the size or concentration of inoculum load, the availability of vector species, and the persistence of the virus in the vector. In the case of whitefly-transmitted geminiviruses (WTGs), the spread of viruses is by winged adults. Therefore, factors that affect the survival and behavior of adult whiteflies can have an effect on virus spread. Several examples of geminivirus diseases have provided evidence for a positive correlation between *B. tabaci* population size and the spread of persistent and semipersistent viruses. Examples of these diseases are the horsegram yellow mosaic virus in India (Muniyappa and Reddy, 1983), the ACMV in Ivory Coast (Fauquet and Fargette, 1990), and the tomato yellow leaf curl virus in Israel (Cohen et al., 1988).

FIGURE 11.5 Correlation between ACMV incidence and monthly mean temperatures in Ivory Coast (a) and Tanzania (b). The observed disease incidence is represented by filled histograms, and the calculated disease incidence values are shown by the open histograms. (c) Relationship between the incidence of ACMV and number of adult whiteflies per cassava plant at different stages of cassava growth in Ivory Coast. Reprinted with permission from Fargette et al. (1994). Distribution of adult whiteflies caught in yellow trap, in a cassava field during the first 2 months (d) and the third month (e) of cassava growth. The two directional symbols on the right side of (d) and (e) indicate the relative frequency of winds from each direction. (f) ACMV incidence (open diamonds) and number of whiteflies per plant (open squares) at different distances in meters from the edge of cassava fields. Reprinted with permission from Fauquet and Fargette (1990). (g) The effect of wind speed, direction, and the impact of cassava canopy on the number of adult whiteflies as estimated by the number of vector whiteflies trapped. Reprinted with permission from Fauquet and Fargette (1990). (*See the color version of this figure in Color Plate section.*)

11.2.1.1 Temperature, Light, and Relative Humidity The population of adult whiteflies can vary significantly between different seasons, primarily in association with changes in temperature, rainfall, and relative humidity (Figure 11.5a). Higher temperatures and increased relative humidity favor whitefly population buildup, while lower temperatures and heavy rainfall can lead to a decline in the whitefly population (Fargette et al., 1993, 1994). The development time of *B. tabaci* varies greatly between different host plant species, and the rate of development is positively correlated with temperature, with its maximum at 28°C. The longevity of *B. tabaci* adults is 10–15 days in the field during summer and 30–60 days during winter.

Temperature can also directly influence virus proliferation by manipulating virus-induced gene silencing (Chellappan et al., 2005; Vanitharani et al., 2005). Higher temperature is known to trigger virus-induced gene silencing and thus induce recovery phenotypes in virus-infected plants, in contrast to nonrecovery phenotypes obtained at lower temperature. In addition to temperature, both light and humidity have also been shown to manifest the viral symptoms through virus-induced gene silencing (Patil and Fauquet, unpublished; Fu et al., 2006).

11.2.1.2 Wind Speed and Direction Whiteflies are not uniformly distributed within the cassava fields, but are mostly concentrated at the margins of the crops, being especially prevalent on the upwind borders (Fargette et al., 1985, 1993). Correspondingly, the number of whiteflies is relatively small within crop fields irrespective of the average density of the whitefly population or the overall size of the field. In Ivory Coast, a higher incidence of ACMV was measured at the upwind edges of the cassava fields and this corresponded to a measurable vector distribution gradient (Figure 11.5f) (Fargette et al., 1985). The mean number of whiteflies correlated with an increase in the incidence of disease until the crops were about 3–5 months old. Subsequent increases in the incidence of viral disease to a maximum at crop maturity were not related to whitefly numbers because with age the nutritional quality of the plants decreases to the point that the older plants are no longer suitable for whitefly feeding.

11.2.1.3 Movement of the Insect Vector Spread of infection in time and space is determined by the movement of adult whiteflies and is positively correlated with the size of the vector population (Figure 11.5c). The homopteran vectors take a series of steps before they actually land on an appropriate host plant and start feeding (Powell et al., 2006; Fereres and Moreno, 2009). There are two main ways by which *B. tabaci* moves: a short active movement over distances measured in meters (Fauquet et al., 1986) and long-distance passive movement controlled mainly by the wind (Youngman et al., 1986). The major flight hours for the vector are during the morning, but sometimes a short peak is observed in the afternoon (Fauquet et al., 1986). Short-range migration takes place regularly between cultivated and weed hosts, and this constant movement greatly contributes to persistence of the flies in cropping systems and to their ability as vectors. The adult whiteflies are reported to be relatively shortsighted and, in contrast to aphids, most of them fly close to the ground (Gerling et al., 1986; Byrne and Bellows, 1991). Movement between crop and weed hosts is accomplished by those whitefly populations that tumble along very close to the ground whose direction of movement is determined by the wind. The average flight speed of an adult whitefly within the canopy is estimated to be about $0.2\,m\,s^{-1}$ (Figure 11.5g) (Yao et al., 1987).

11.2.1.4 Feeding Behavior of the Insect Vector The host plants can modify the behavior of their insect vectors by triggering physiological changes in the plants that ultimately affect their attractiveness and in turn the vectors' feeding preference. Vector distribution, virus concentration, and susceptibility of

plants to virus inoculation are all related to leaf age. Up to 95% of all the adult whiteflies in cassava fields are found to be concentrated on the lower surface of the youngest leaves of the shoot apices (Fauquet and Fargette, 1990). It is important to note that *B. tabaci* adults prefer to feed on young leaves, and this preferential feeding practice of whiteflies has increased their chances to acquire and transmit these viruses. ELISA results have demonstrated that the highest viral titer may be found in the young leaves of cassava plants in comparison with older leaves. And, in fact, virus particles could not be detected serologically in older leaves even though the leaves were symptomatic (Fargette et al., 1987). Young cassava leaves are also the ones most susceptible to infection. A significantly higher rate of transmission was achieved by whiteflies that had previously fed on young leaves of *Datura stramonium* infected with TYLCV than by those whiteflies fed on mature leaves. Czosnek et al. (1988) have shown that the highest concentration of TYLCV viral genomic DNA is located in the shoot apex. The relatively high efficiency of whiteflies to acquire viruses from young leaves is probably due to their better feeding behavior on younger leaves, which results from the relatively large amounts of soluble nitrogenous compounds available in the younger leaves (Mound, 1983).

The virus spread in the field is affected both by the "feeding preference" and "orientation preference" of the vector. When the vector orients toward infected plants, the virus spread is faster, but slows down once most of the plants are infected. In the case of many virus–vector combinations, the vectors benefit by feeding on virus-infected plants and show a higher intrinsic rate of natural increase (Srinivasan and Alvarez, 2007) or enhance their potential to migrate through increased number of alates (Blua and Perring, 1992). Also, the vectors can gain additional benefits by feeding on diseased plants, such as the one that viruliferous insect vectors may in turn disrupt the natural development and survival of their own natural predators, as observed in the case of aphids carrying bean yellow dwarf virus (BYDV) that are poor hosts to the parasitoid *Aphidius ervi* (Fereres and Moreno, 2009).

11.2.1.5 Host Plant Genotype Besides the above-mentioned physical and biological factors that determine vector buildup in a field, host plant differences most often determine whether the vector will colonize a particular field. The plant viruses have highly variable host ranges: some infect only one or a few related species (termed as specialists), whereas others can infect a wide range of hosts from different taxonomic groups (termed as generalists) (Woolhouse et al., 2001). It is well accepted that adaptation to a specific environment is often coupled with loss of fitness in alternative environments, as the mutations that are beneficial in the first case might be deleterious in the alternative environment (Kawecki, 1994). There are several reports of viruses expanding their host range from susceptible to resistant plant genotypes. The convergent evolution of Pelargonium flower break virus (PFBV) populations adapted to *Chenopodium quinoa* is the most remarkable example (Rico et al., 2006). Studies have also shown that the amount of virus genetic diversity is driven by the constraints imposed by the host plant rather than by the virus' own evolving genomic makeup (Schneider and Roossinck, 2001).

In Uganda, mean whitefly numbers were significantly greater on the susceptible plant varieties than on ACMV-resistant varieties (Fauquet et al., 1987; Otim-Nape et al., 1994, 1995), but this also depends on the resistance to whitefly of each genotype. The cassava-infecting geminiviruses are transmitted in a persistent manner. The minimum acquisition access, inoculation access, and latent periods for a successful transmission are 3–5 h, 10 min, and 3–4 h, respectively (Thresh et al., 1998). Adult whiteflies retain the virus for at least 9 days where it persists throughout molting and is transmitted transovarially. Up to 1.7% of adult whiteflies were shown to be infective when collected in heavily infected cassava fields (Fargette et al., 1990).

11.2.1.6 Impact of Host Reservoirs on Virus Infection and Spread

Wild plants and weeds greatly assist in virus survival through adverse periods (Thresh, 1982). Several crops are short-lived and absent from the field during winters or dry summers, or during crop rotations. At these times, wild alternative hosts may be essential for virus survival. Viruses may also perennate in annual weeds if they can pass through the seeds of such weed hosts (Duffus, 1971). It is likely that viruses that infect embryos remain infective in seeds for as long as the seeds remain viable and that this infection may not reduce seed viability and vitality (Bos, 1981). When transmitted by weed seeds, a virus has tremendous potential to survive in the soil for long periods of time. Virus-infected seedlings of weeds act as major reservoirs of infection for efficient short-distance spread by vectors to the crop and other plants. Virus infection in the wild hosts is often symptomless. Aside from harboring crop viruses and other pathogens, wild plants act as important reservoirs and sources of insects, mites, and nematodes. Certain wild plant species may be indispensable to a vector as its alternative host, acting as an essential intermediary in the ecology of the virus. Arthropod vectors often have diverse food plants, including several wild ones, and may probe many species on which they happen to alight. Wild plants and weeds thus may often also serve as reservoirs of both virus and vector. Most recently, alternative hosts have been identified for ACMV and EACMV species in Nigeria, thus supporting the theories discussed above (Alabi et al., 2008). Although ACMV is transmitted from cassava to cassava alone and not from the wild hosts, studies have shown the presence of cassava mosaic geminiviruses in their wild relative *Manihot glaziovii* in Uganda (Sserubombwe et al., 2008).

The prevalence and distribution of sources of infection also affect the spread of virus in the field. In Uganda, high population densities of adult whiteflies were positively correlated with increased incidence of African cassava mosaic disease, suggesting that differences in rates of disease spread were mainly due to differences in vector population (Otim-Nape et al., 1995). It has also been demonstrated that whiteflies are better adapted to transmit the local viruses than the exotic viruses (Fargette and Fauquet, 1988; Fargette et al., 1993; Maruthi et al., 2002).

11.2.2 The Role of Weed Plants

While weeds undoubtedly play an important role in maintaining whitefly populations in agroecosystems, cultivated hosts are often equally or more important (Duffus, 1971; Norris and Kogan, 2005). This complex of continuous availability of host plants and the movement of *B. tabaci* among them is a vital link in the dissemination of several viruses. Populations of *B. tabaci* are maintained, albeit at relatively low levels, on a series of cultivated and weed hosts through the winter and spring months. They subsequently migrate into cotton during the summer, where their population builds up exponentially (Byrne et al., 1996). In every situation where whiteflies are a serious problem, wild and cultivated hosts grow in proximity to one another, so that whiteflies have little difficulty in finding new sites when existing conditions on any individual plants become less hospitable (Gerling, 1984). Host preference has also been observed among those whiteflies that prefer cucumbers to tomatoes and in turn prefer tomatoes to either corn or eggplants. As a host, cucumber is preferred to eggplant, bean, tobacco, tomato, squash, pepper, and watermelon (Al-Hitty and Sharrif, 1987).

In Ivory Coast, two *B. tabaci* biotypes were distinguished using isozyme analyses, one of which was restricted to cassava, while the other colonized a range of crop and weed hosts (Burban et al., 1992). Whiteflies on cassava were found to be adapted to cassava; and their only other host was found to be eggplant and this might have an implication for why some viruses seem to bypass some hosts because of vector behavior (Burban et al., 1992).

11.3 CONTROL OF VIRUS DISEASES

Understanding the factors involved in the ecology and epidemiology of certain virus diseases can help to design effective ways to manage those diseases. Crops can be protected from damage by viruses through (a) avoiding or removing sources of infection, (b) preventing or reducing virus spread, and (c) improving crop resistance or through integrated disease management approaches (Jones, 2004). Thus, in most of the whitefly-transmitted geminivirus diseases, different cultural practices such as roughing, manipulation of planting dates, and removal of reservoir plants and weeds within and around the fields may limit the spread of virus infection (Harrison, 1981). Besides these agronomic practices, there are many other strategies used to control plant viral diseases that have been extensively reviewed and thus they are only briefly discussed here. These include improved cultural practices, breeding of natural resistant varieties, and improved biotechnological strategies (Thresh and Cooter, 2005; Thresh, 2006; Sudarshana et al., 2007; Vanderschuren et al., 2007).

11.3.1 Roughing of Wild and Cultivated Hosts

Wild plants are widely recognized as important direct sources of viruses and virus vectors. Their removal both eliminates active sources of infection and subsequent virus spread in the seeds and prevents vectors from having breeding sites. Studies showing the effect of reservoirs in initiation and subsequent establishment of virus infection in the field have been conducted in roughed (weeded) and unroughed fields. Fargette et al. (1990) reported that a larger source of infection resulted in an increase in the aerial spread of cassava mosaic disease spread over greater distances compared to smaller plots. For those fields where the diseased plants were periodically removed, the spread of infection was checked compared to that occurring in unroughed fields, signifying the need to adopt good cultural practices as a means to contain virus spread (Robertson, 1987).

WTGs are not seed transmitted, but for other viruses that are, seed transmission is another important source of infection as it introduces the disease at a very early stage of plant growth, allowing infection to spread to neighboring plants while they are still young. In such cases, it may be prudent to start with a virus-free stock as an effective means to control virus diseases. This same prevention concept holds true for many vegetatively propagated plants where the main source of infection is the plant itself, so that development, propagation, and maintenance of virus-free stock is essential. Now, there are several suppliers of virus-free stocks for a variety of agricultural and horticultural plants making rapid initial multiplication of virus-free material possible. Maintaining general hygiene in the field to ensure that all tools, knives, clothing, and hands are clean can prevent the spread of a large number of viruses that are mechanically transmitted.

11.3.2 Manipulation of Planting and Harvesting Dates

In areas where the same crops or a group of related crops are grown repeatedly and there are chances that these may act as potential volunteers for introducing infection to the next crop, it may be possible to limit virus infection by introducing a break in the cycle where no susceptible plants are grown. For those viruses that are transmitted by airborne vectors, a change in the sowing cycle may influence the time and amount of disease incidence. The best time for planting will depend on the time of maximum vector influx. If the vector influx is a late migration, early sowing may allow the plants to be past their vulnerable stages of infection by the time the vectors invade the field. Altering the spacing between plants or plant density can be a useful strategy to reduce the movement of a vector population within and around the crop canopy (Narasimhan and Arjunan, 1976; Fargette and Fauquet, 1988; Fargette et al., 1990).

11.3.3 Avoidance of Vectors

Coupled with a reliable disease forecasting system, a judicious use of systemic insecticides can lower the population of vectors within the crop. Good control of the spread of tomato leaf curl and the yellow vein mosaic of okra has been achieved through control of whitefly vector by the use of insecticides (Shastri and Singh, 1973; Singh et al., 1973, 1979). For whiteflies, beside chemical control, biological control can be achieved through the use of their natural predators, and parasitoids can help reduce the whitefly population to a great extent (Gerling, 1990).

11.3.4 Plant Resistance

One of the most effective means of controlling virus infections is to grow either resistant cultivars, that is, crop varieties that are resistant to pathogens, or use crop varieties that do not allow vector populations to build up. For most WTG diseases, a combination of phytosanitation (treatment of the above-ground parts of the crop plants) and use of resistant varieties is encouraged. Improved varieties of cassava raised at the International Institute for Tropical Agriculture (IITA) show some degree of resistance to ACMV and have now been widely adopted in Nigeria, Uganda, and other African countries (Mahangu et al., 1994). Virus-free plant stocks have been obtained by rigorous selection and through the use of meristem tip culture. These stocks have been shown to be free of cassava mosaic disease in India, and a substantial increase in yield has been achieved by their use (Nair, 1990). Similarly, tomato cultivars with improved tolerance to tomato leaf curl virus have been selected (Moustafa and Nakhla, 1990), and some of these cultivars have been released for commercial use (Pilowsky et al., 1989). Crop resistance to insect vectors is likely to alter the population size, activity, and probing and feeding behavior of the vectors, thereby influencing the pattern of virus spread. Whitefly resistance has been used to minimize virus transmission in various diseases, such as those caused by cowpea golden mosaic virus, bean golden mosaic virus, and cotton leaf curl virus (Vetten and Allen, 1983). Considering the potential for devastation due to whitefly-transmitted geminiviruses, and the ineffectiveness of alternative controls, resistance breeding is a very useful strategy to control geminiviruses whenever available.

11.4 ROLE OF MAN AND CLIMATE CHANGE IN VIRUS ECOLOGY

At present, the world is experiencing accelerated climate change, accompanied by rapid expansion in human activity, which are significantly impacting plants, vectors, and viruses, causing increasing instability within the virus–plant pathosystem. There are several examples of agricultural attempts by humans that inadvertently have resulted in the appearance and spread of new diseases either by introduction of new germplasm or by introduction of new vector species. The rapid expansion in human activities includes adopting more intensive, extensive, and diverse agronomic practices such as monocropping system leading to loss of genetic diversity; increased fragmentation and disturbance of indigenous vegetation; unscrupulous use of chemical control measures; and irrigation and protected cropping, all of which favor emergence of viruses and development of epidemics (Jones, 2009). For instance, cotton leaf curl virus has attained epidemic proportions in Pakistan by the introduction of new host genotypes that turned out to be extremely susceptible to the local virus, resulting in a severe outbreak of the viral disease and wiping out most of the cotton crop (Mansoor et al., 2006). Other examples of outbreaks include those of turnip mosaic virus and cauliflower mosaic virus in Britain caused by the introduction of new, highly vulnerable genotypes of Brussels sprouts (Tomlinson and Ward, 1982), of lettuce yellow vein virus in lettuce in California (Zink and Duffus, 1975), and of rice yellow mottle virus in Africa as

a result of introducing high-yielding rice cultivars (Bakker, 1974; Raymundo and Buddenhagen, 1976). In addition, introduction of new crops over lame areas can have dramatic influence over vector population and spread of diseases, as observed for whitefly-transmitted golden yellow mosaics of legumes in Brazil as a result of planting soybean, which is a preferred host of whiteflies, over extensive areas (Costa, 1976). The spread of the sweet potato whitefly *B. tabaci* (B biotype) to Europe and the Americas has been associated with human transport of plant materials (e.g., soybean, okra, and eggplant) into the New World (Cock, 1986). A new population of *B. tabaci*, named B biotype to distinguish it from the A biotype indigenous to North America, is now dispersed worldwide (Brown and Bird, 1992, 1995), primarily because of its extraordinary ability to adapt to an extremely broad host range of plant species (Bedford et al., 1994).

Furthermore, the role of man in the dissemination of virus diseases is exemplified by free trade and trafficking of plant material. These activities resulted in the appearance and spread of East African cassava mosaic virus in coastal and inland countries of East and Southern Africa because of considerable movement of cuttings along the roads, railways, and other transportation routes (Thresh et al., 1998). Such risks are obvious when considering vegetatively propagated plant material or seed-borne viruses that are introduced in new areas because of exchange or import of plant material. Similarly, tomato yellow leaf curl virus from Israel was apparently imported into the Dominican Republic a few years ago along with tomato seedlings and it has been reported in other Caribbean countries (e.g., Cuba) and in Florida (Polston and Anderson, 1997; Polston et al., 1999).

The shift in spatial and temporal climate patterns occurring due to the changes in temperature, rainfall, and wind patterns can significantly alter the range of introduced crops grown and areas cultivated, result in the introduction of new weed species, and increase both the abundance and the distribution activity of insect vectors responsible for transmitting the viral diseases (Harrington et al., 2001).

11.5 EMERGENCE OF NEW RECOMBINANT GEMINIVIRUSES

Natural selection of the fittest variant is a basic concept of evolution; thus, every change in the environment or replicative niche of the virus may imply different fitness requirements. This becomes especially important in many plant viruses that have a broad host range or that can use several different vector species for transmission. Those viruses that replicate both in plants and in insect vectors have dramatically different selection pressures (Roossinck, 1997). Natural transmission of some plant viruses involves a vector, which variously may be an insect, a fungus, or a nematode, and considerations of natural fitness must include the constraints of the vector. The uptake of virus by the vector always involves some degree of specificity, and the viral proteins must provide an appropriate fit in order for transmission to occur (Gray, 1996).

Considering that viruses are extremely adaptable and capable of rapid change, it is not surprising that most of the recently emerging viruses are recombinants (Padidam et al., 1999). Since geminiviruses appear to exhibit a considerable degree of sequence variation, both within and between populations, and appear to have a rapidly increasing host range, DNA recombination is likely to be responsible for some of the variation seen in the geminiviruses, allowing related viruses to exchange genes or parts thereof in mixed infections, potentially generating ever more fit variants (Lefeuvre et al., 2007, 2009; Varsani et al., 2008). Figure 11.6 shows the global distribution of the 59 isolates of the tomato yellow leaf curl-like viruses (TYLCVs) belonging to 6 different begomovirus species causing tomato yellow leaf curl disease (Abhary et al., 2007). Evidence has been found for both homologous and nonhomologous recombinations. This evidence includes the release of

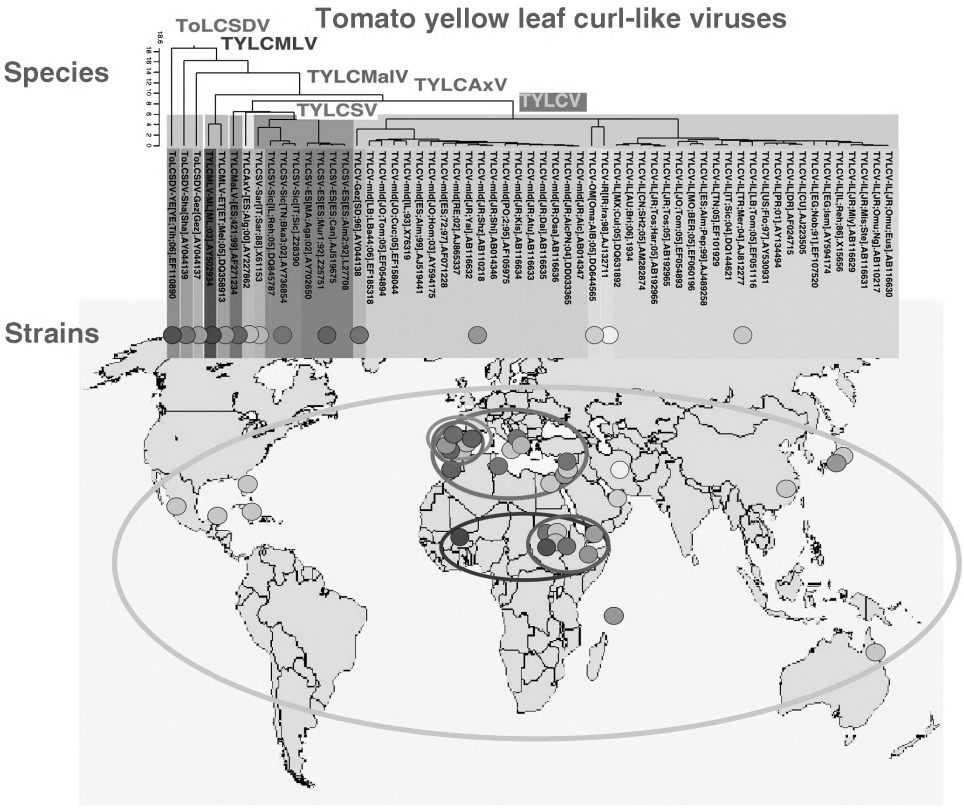


FIGURE 11.6 World map on which each of the 59 members represents 6 begomovirus species of the TYLCV cluster. The upper part of the diagram shows a phylogenetic tree of these 59 viruses using their complete A component sequence. The Clustal V algorithm of the program MegAlign from DNAStar has been used and distances in percentage difference are indicated on the left. The tree shows a partition in six major clusters, one for each of the six designated species, TYLCV, TYLCSV, TYLCAxV, TYLCMalV, TYLCMLV, and ToLCSDV, respectively, in yellow, green, gray, red, blue, and purple colors. These six species constitute the so-called TYLCV cluster of the OW begomoviruses. The individual viruses composing these clusters are positioned on the world map, as dots of various colors representing their pertaining to one of the 15 specific strains of the 6 species, as indicated in the colored boxes at the bottom of the tree. On the world map, the individuals pertaining to the same species are circled with the same color as indicated by the name of the species of the boxes on the phylogenetic tree. Adapted from Abhary et al. (2007). (*See the color version of this figure in Color Plate section.*)

infectious viral DNA from monomer-containing recombinant plasmids, deletion of foreign sequences, reversion of deletion mutants to generate a wild-type genome size, and production of defective subgenomic DNA molecules (Patil and Dasgupta, 2006). Nonhomologous recombination may result in deletions, insertions, and repetitions of viral sequences (Bisaro, 1994).

Whenever the hosts of two or more whitefly-transmitted geminiviruses are present together in the same area (Brown and Bird, 1992), intergenetic viral recombination provides a mechanism for the production of new forms, and this process may already have played a key role in the genesis of the forms that exist today (Zhou et al., 1997). A novel type of recombinant virus, EACMV-Ug (Uganda strain), has been associated with a recent epidemic on cassava in Uganda (Patil and Fauquet, 2009). This recombinant virus typically has severe effects and is an example of interspecific

recombination between ACMV and EACMV, leading to the emergence of a new geminivirus pathogen (Patil and Fauquet, 2009). Another example of recombination-related emergence of viral variants is represented by the Pakistani isolates of cotton leaf curl virus and okra yellow vein mosaic virus (OYVMV) (Zhou et al., 1998). The cotton leaf curl epidemic in Pakistan is caused by several distinct viral species, with recombination events involving OYVMV and other unspecified geminiviruses probably having been involved in their evolution (Fauquet et al., 1998; Zhou et al., 1998). Infections by potato yellow mosaic virus in Trinidad and Tobago (Umaharan et al., 1998) and tomato yellow leaf curl virus in Italy and Spain not only caused severe damage but also spread throughout these countries (Noris et al., 1994). Like the other examples, these viruses are recombinants that presumably evolved in response to changes in the ecosystem.

11.6 CONCLUSIONS

It is obvious that one of the best approaches to understanding and solving virus problems is ecological. While humankind is responsible for inducing several virus epidemics by interfering with crop ecosystems, man has continued to develop various cultural control measures to minimize the spread of viruses. Finally, it is in our interest to realize that virus ecosystems are very dynamic and that we will need to continually improve the phytosanitary methods used to contain viral diseases. The role of humans in the spread of new diseases, the ability of the vector to adapt itself to harsh environments, and, not in the least, the extraordinary ability of the viruses to mutate, recombine, and transcomplement each other in a race to survive offer a very real challenge. With an integrated approach based on the factors considered here, these problems can be managed to some extent by advanced techniques of detection and diagnosis, and prediction models for disease advent and spread.

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CHAPTER 12

VIROIDS AND VIROID DISEASES OF PLANTS

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12.1 INTRODUCTION

Viroids, in spite of their name that hints at a relationship with viruses, differ from viruses in fundamental aspects that include structure, function, and evolutionary origin. Viroids are the smallest replicons described so far, being exclusively composed by a small (in the range of 250–400 nt) circular RNA (Diener, 2003; Flores et al., 2005; Tsagris et al., 2008; Ding, 2009), whereas the genome of a typical plant virus such as *Tobacco mosaic virus* (TMV) is a linear RNA of about 6000 nt (Goelet et al., 1982). Moreover, virus genomes (DNA or RNA) encode at least one and most frequently several proteins that mediate their replication, movement, and suppression of the host antiviral response, while viroids are nonprotein-coding RNAs; consequently, RNA viruses and viroids need to parasitize primarily the translation and transcription apparatus of their hosts, respectively. Finally, RNA viruses and viroids have independent evolutionary lineages, with the latter being considered remnants of the "RNA world" that presumably preceded our present world based on DNA and proteins (Diener, 1989; Flores and Owens, 2008).

From an ecological standpoint, viruses are found infecting all cell types, from mycoplasmas and bacteria to eukaryotic cells, in contrast to viroids that have been described so far only in higher plants (in monocots and dicots), where they frequently incite symptoms similar to those characteristic of virus infections. Actually, *Potato spindle tuber viroid* (PSTVd), the first viroid reported (Diener and Raymer, 1967; Diener, 1972; Gross et al., 1978), was identified when searching for the virus presumed to cause a potato disease. The similar phenotypic effects induced by viruses and viroids may just reflect a lack of specificity in the ultimate macroscopic response of their hosts, although the possibility that they may affect the same host defensive response, particularly RNA silencing (see below), cannot be dismissed. In this chapter, we will first present the molecular properties of viroids and their diversity and then move on to describe the interactions of these unique biological entities with their environment and, principally, with their hosts.

12.2 STRUCTURE AND CLASSIFICATION

12.2.1 Structural Domains and Conserved Motifs

Although PSTVd and the first several viroids characterized immediately thereafter exhibit certain common structural properties, noteworthy among which is a rod-like secondary structure with a central conserved region (CCR) (Keese and Symons, 1985), the discovery of *Avocado sunblotch viroid* (ASBVd) (Symons, 1981) revealed the existence of a second group of viroids. The initial doubts that rather than a true viroid, ASBVd might belong to the group of viroid-like satellite RNAs—which share with viroids the small size and circularity but are functionally dependent on a helper virus for completing their infectious cycle—disappeared when additional viroids were subsequently found to also display the most striking feature of ASBVd, namely, self-cleavage of strands of both polarities via hammerhead ribozymes (Hutchins et al., 1986; Hernández and Flores, 1992; Flores et al., 2000), a feature with deep implications for viroid replication and evolution. Therefore, from a structural perspective, there are two major viroid groups represented by PSTVd and ASBVd that eventually gave rise to two taxonomic families.í

12.2.2 Taxonomy: Families, Genera, Species

The International Committee on Taxonomy of Viruses (ICTV)—regardless of their differences, viroids and viruses are grouped together for practical reasons—currently recognizes approximately 30 viroid species (Table 12.1). Initially, the only demarcating criterion was sequence similarity, with an arbitrary limit (less than 90%) separating different viroid species from variants of the same species. However, to avoid unnecessary prolifera-

TABLE 12.1 Classification of Viroids

Family[a]	Genus[a]	Species[a]	Abbreviation	Nucleotides[b]
Pospiviroidae	*Pospiviroid*	*Chrysanthemum stunt*	CSVd	354–356
		Citrus exocortis	CEVd	368–375 (463–467)
		Columnea latent	CLVd	370–373
		Iresine	IrVd	370
		Mexican papita	MPVd	359–360
		Pepper chat fruit	PCFVd	348
		Potato spindle tuber	PSTVd	356–361 (341)
		Tomato apical stunt	TASVd	360–363
		Tomato chlorotic dwarf	TCDVd	360
		Tomato planta macho	TPMVd	359–360
	Cocadviroid	*Citrus bark cracking*[c]	CBCVd	284
		Coconut cadang-cadang	CCCVd	246–247 (287–301)
		Coconut tinangaja	CTiVd	254
		Hop latent	HLVd	256
	Hostuviroid	*Hop stunt*[d]	HSVd	294–303
	Apscaviroid	*Apple dimple fruit*	ADFVd	306, 307
		Apple scar skin[e]	ASSVd	329–334
		Australian grapevine	AGVd	369
		Citrus bent leaf	CBLVd	315, 318
		Citrus dwarfing[f]	CDVd	294, 297
		Citrus viroid V	CVd-V	284
		Citrus viroid VI[g]	CVd-VI	330
		Grapevine yellow speckle 1	GVYSVd-1	366–368
		Grapevine yellow speckle 2[h]	GYSVd-2	363
		Pear blister canker	PBCVd	315, 316
	Coleviroid	*Coleus blumei 1*	CbVd-1	248–251
		Coleus blumei 2	CbVd-2	301, 302
		Coleus blumei 3	CbVd-3	361–364
Avsunviroidae				
	Avsunviroid	*Avocado sunblotch*	ASBVd	246–251
	Pelamoviroid	*Chrysanthemum chlorotic mottle*	CChMVd	398–401
		Peach latent mosaic	PLMVd	335–339 (348–351)
	Elaviroid	*Eggplant latent*	ELVd	332–335

[a]Classification follows scheme proposed in the VIIIth Report of the International Committee on Taxonomy of Viruses with some modifications.
[b]Sizes of variants containing insertions or deletions arising *in vivo* are shown in parentheses.
[c]Formerly termed citrus viroid IV.
[d]Includes cucumber pale fruit, citrus cachexia, peach dapple, and plum dapple viroids.
[e]Includes pear rusty skin and dapple apple viroids.
[f]Formerly termed citrus viroid III.
[g]Formerly termed citrus viroid original source.
[h]Formerly termed grapevine viroid 1B.

tion of species, the ICTV later introduced the need for a second independent criterion that for viroids refers in most instances to host range and symptom expression. Candidates not fulfilling the two independent criteria are regarded as tentative viroid species.

As indicated above, there are two major groups of viroids epitomized by PSTVd (with CCR and without hammerhead structures) and ASBVd (without CCR and with hammerhead structures). These structural differences have functional implications: PSTVd and related

species (family *Pospiviroidae*) replicate in the nucleus through an asymmetric rolling-circle mechanism, while ASBVd and related species (family *Avsunviroidae*) replicate in plastids through a symmetric rolling-circle mechanism (see next section). Therefore, the criteria demarcating both families are very clear. Family *Pospiviroidae* is subdivided into five genera according to the type of CCR and the presence (or absence because apparently they are mutually exclusive) of a terminal conserved region (TCR) and a terminal conserved hairpin (TCH) (Figure 12.1): *Pospiviroid* (type species PSTVd), *Hostuviroid* (type species *hop stunt viroid* (HSVd)), *Cocadviroid* (type species *Coconut cadang-cadang viroid* (CCCVd)), *Apscaviroid* (type species *Apple scar skin viroid* (ASSVd)), and *Coleviroid* (type species *Coleus blumei viroid 1* (CbVd-1)). Within the family *Avsunviroidae*, ASBVd forms its own genus (*Avsunviroid*) because of properties that include a low G + C content (38%, which is unique given that this content is higher than 50% for other viroids), thermodynamically unstable single-hammerhead structures (Figure 12.2), and a lowest free energy secondary structure that is quasi-rod-like. In contrast, *Peach latent mosaic viroid* (PLMVd) and *Chrysanthemum chlorotic mottle viroid* (CChMVd) have typical G + C contents (slightly higher than 50%), predicted most stable secondary structures that are branched and stabilized by a kissing-loop interaction (Bussière et al., 2000; Gago et al., 2005) (Figure 12.1), and thermodynamically stable single-hammerhead structures (Figure 12.2). Moreover, these two viroids are

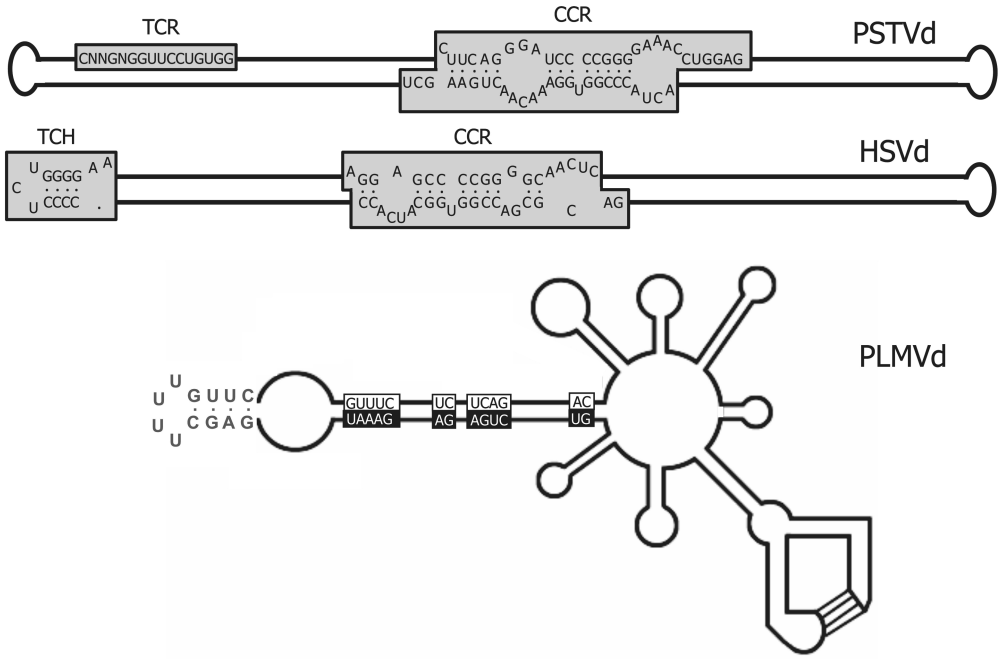

FIGURE 12.1 Structure of viroids. *Upper and middle panels*: schemes of the characteristic rod-like secondary structures of the genomic RNAs of *Potato spindle tuber viroid* (PSTVd) and *Hop stunt viroid* (HSVd), respectively (family *Pospiviroidae*), with the central conserved region (CCR), the terminal conserved region (TCR), and the terminal conserved hairpin (TCH). *Lower panel*: scheme of the branched secondary structure of the genomic RNA of *Peach latent mosaic viroid* (PLMVd) (family *Avsunviroidae*), in which the sequences conserved in the (+) and (–) polarities of most natural hammerhead ribozymes are boxed with black and white backgrounds, respectively; the kissing-loop interaction is indicated with lines, and the characteristic 12 nt hairpin insertion of the reference variant containing the pathogenicity determinant of an extreme chlorosis (peach calico) is highlighted with blue color. (*See the color version of this figure in Color Plate section.*)

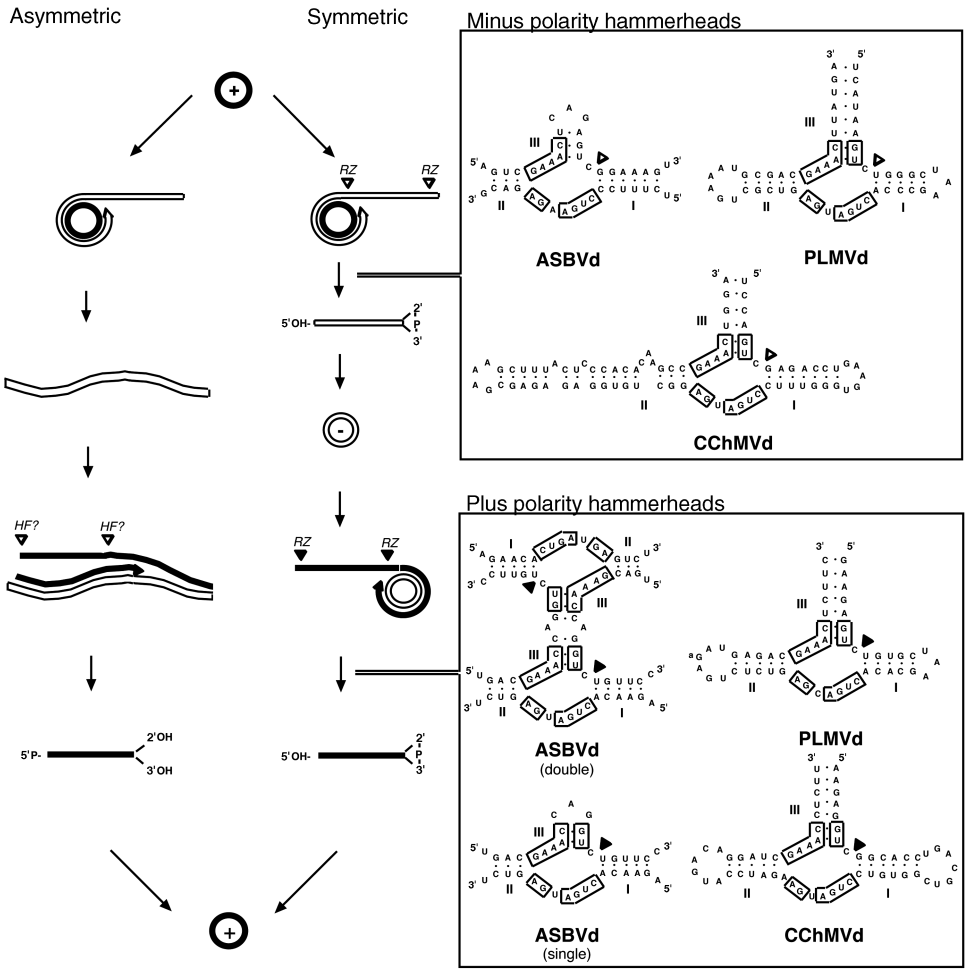

FIGURE 12.2 Rolling-circle mechanism for viroid replication. The (+) polarity (solid lines) is assigned by convention to the most abundant infectious RNA and the (–) polarity (open lines) to its complementary strand. The alternative asymmetric and symmetric pathways involve one and two rolling circles, respectively. In the symmetric pathway, cleavage of (+) and (–) multimeric strands is mediated by hammerhead ribozymes (RZ), which generate linear monomeric RNAs with 5′-hydroxyl and 2′-3′-cyclic phosphodiester termini. Arrowheads denote the self-cleavage sites. The hammerhead structures that can be formed by *Avocado sunblotch viroid* (ASBVd), *Peach latent mosaic viroid* (PLMVd), and *Chrysanthemum chlorotic mottle viroid* (CChMVd) RNAs are shown on the right, with conserved nucleotides boxed; the single-hammerhead structure of the ASBVd (+) strand is thermodynamically unstable and self-cleavage is most likely mediated by a double-hammerhead structure. In the asymmetric variant, cleavage of the multimeric (+) strands is catalyzed by a host factor (HF, probably a member of the RNase III family) that generates linear monomeric RNA with 5′-phosphomonoester and 2′- and 3′-hydroxyl termini. How ligation occurs is still uncertain. Adapted from Flores et al. (1997).

insoluble in 2 M LiCl (Navarro and Flores, 1997), a singular property that might be related to their unusual branched conformation. On this basis, PLMVd and CChMVd are grouped within the genus *Pelamoviroid* (type species PLMVd). Finally, a third genus (*Elaviroid*) has been created for *Eggplant latent viroid* whose properties are intermediate between those of the members of the two other genera in the family *Avsunviroidae* (Fadda et al., 2003).

12.3 REPLICATION AND MOVEMENT

12.3.1 Asymmetric and Symmetric Rolling-Circle Mechanism

On the basis of the circular structure of the genomic RNA and the detection of low levels of oligomeric RNAs, presumed to be replicative intermediates (Grill and Semancik, 1978) in different viroid-infected tissues, several groups have proposed that viroid replication proceeds via a rolling-circle mechanism (Branch et al., 1981; Owens and Diener, 1982; Branch and Robertson, 1984) (Figure 12.2). Although the RNA nature of both the incoming genomic RNA and replicative intermediates might suggest, as with most RNA viruses, the involvement of RNA-dependent RNA polymerases, viroids are strikingly transcribed by host DNA-dependent RNA polymerases reprogrammed to accept RNA templates. These enzymes reside in specific subcellular compartments that include the nucleus and plastids, wherein PSTVd and ASBVd (and their replicative intermediates) are, respectively, located (Flores et al., 2005).

In brief, the rolling-circle mechanism proposes that the infecting circular genomic RNA, to which the (+) polarity is arbitrarily assigned, is reiteratively transcribed into oligomeric (–) and subsequently (+) strand RNAs that are cleaved by an RNase and ligated by an RNA ligase to produce monomeric (+) circular progeny. Accordingly, there are two RNA–RNA transcription steps and depending on the specific template for the second step, the rolling-circle mechanism is described as "asymmetric" or "symmetric." Because the oligomeric (–) strands, but not their monomeric circular derivatives, have been detected in tomato infected by PSTVd, this and other members of the family *Pospiviroidae* are assumed to follow an asymmetric pathway with a single rolling circle (Branch et al., 1988; Feldstein et al., 1998). Conversely, because monomeric circular (–) RNA, most likely resulting from processing of the oligomeric (–) strands, has been identified in avocado infected by ASBVd, this and other members of the family *Avsunviroidae* presumably replicate by a symmetric pathway with two rolling circles (Daròs et al., 1994). Moreover, the finding that oligomeric (+) and (–) RNAs of members of this family self-cleave through hammerhead ribozymes (see below) provides additional evidence against their possible role as templates (Figure 12.2).

In the family *Pospiviroidae*, the enzyme catalyzing elongation of viroid strands is nuclear RNA polymerase II (Pol II), as inferred from experiments using the inhibitor α-amanitin or a monoclonal antibody against the major subunit of Pol II (Mühlbach and Sänger, 1979; Warrilow and Symons, 1999). Initiation of PSTVd (–) strand synthesis is mapped to a specific position in the left terminal loop (Kolonko et al., 2006), but the corresponding initiation site for (+) strand synthesis remains unidentified. Cleavage of oligomeric (+) strand RNAs is proposed to be directed by a specific RNA conformation, either a GAAA-capped loop (Baumstark et al., 1997) or a double-stranded structure (Gas et al., 2007), which destabilizes a single phosphodiester bond. The second alternative invokes the participation of a class III RNase that typically acts on double- or highly structured single-stranded RNA and generates products with 5′-P and 3′-OH termini. Following a conformational shift presumably facilitated by an element of tertiary structure (the loop E present in PSTVd and closely related viroids), these termini are brought into close proximity and then joined by a specific RNA ligase. Mutational analysis of PSTVd has identified additional RNA motifs critical for replication (and systemic trafficking, see below) (Zhong et al., 2008).

In the family *Avsunviroidae*, elongation of viroid RNAs is catalyzed by a nuclear-encoded RNA polymerase (NEP) located in plastids. This conclusion is supported by two lines of evidence: first, the effects of the inhibitor tagetitoxin on RNA synthesis *in vitro* by chloroplast preparations from ASBVd-infected tissue (Navarro et al., 2000); second, the active synthesis of PLMVd in peach leaves displaying

a PLMVd-induced albinism in which transcription in plastids is essentially NEP dependent (Rodio et al., 2007). Taking advantage of the fact that primary RNA transcripts in the chloroplast contain a characteristic 5′-triphosphorylated group, initiation of (+) and (–) strands has been mapped to equivalent structural positions in ASBVd (the right terminal A + U-rich loops of the proposed quasi-rod-like secondary structures) (Navarro and Flores, 2000) and PLMVd (a short double-stranded RNA motif that also contains the self-cleavage sites for RNAs of both polarities) (Delgado et al., 2005). Remarkably, cleavage of the oligomeric RNA intermediates to their corresponding unit-length counterparts is mediated by hammerhead ribozymes embedded in RNA strands of both polarities (Hutchins et al., 1986; Flores et al., 2000) (Figure 12.2). This reaction most likely occurs cotranscriptionally *in vivo* (Carbonell et al., 2006) and is catalyzed by the central conserved core of a small RNA motif (the hammerhead structure), as well as finely tuned by interactions between loops flanking this core and proteins (Daròs and Flores, 2002; De la Peña et al., 2003; Khvorova et al., 2003; Dufour et al., 2009). The resulting monomeric RNAs containing 5′-OH and 2′,3′-cyclic phosphodiester either self-ligate spontaneously (generating a 2′,5′-bond) (Côté et al., 2001) or are joined by an unidentified chloroplastic RNA ligase (Flores et al., 2005).

12.3.2 Intracellular, Cell-to-Cell, and Long-Distance Movement

After entering a susceptible host cell, an incoming viroid must pass through the cytoplasm prior to entering the nucleus (PSTVd and related viroids) or chloroplast (ASBVd and related viroids) and initiating replication. Following replication, newly synthesized progeny reverses this process, first moving to adjacent cells via intercellular connections known as plasmodesmata, then entering the vascular system where they move from metabolic source to sink via the phloem, and finally exiting the vascular system and reentering uninfected (usually younger) portions of the plant. Figure 12.3 shows the different cells and tissues encountered by viroids as they move systemically throughout an infected plant.

Our current understanding of viroid transport is based largely on studies carried out with PSTVd and two experimental hosts (*Nicotiana benthamiana* and tomato), as well as HSVd and its natural/experimental host cucumber. Beginning at the intracellular level, transport of fluorescently labeled PSTVd RNAs from the cytoplasm into the nucleus is a saturable, sequence-specific process that, unlike the import and export of many cellular proteins and RNAs, does not appear to involve GTP hydrolysis. Disruption of the cytoskeleton with oryzalin or cytochalasin D does not inhibit PSTVd transport into the nucleus (Woo et al., 1999). Only a small portion of the entire molecule is required for nuclear targeting (Abraitiene et al., 2008), and the addition of this signal (a short palindromic sequence located in the upper portion of the central domain) to a nonviroid mRNA synthesized in the cytoplasm allows that molecule to enter the nucleus. This palindromic sequence can fold in several different ways, and it is not yet clear which conformation provides the actual signal that targets PSTVd to the nucleus. PSTVd presumably enters the nucleus in the form of an RNA–protein complex, but the identity of the host proteins involved in nuclear import/export remains unknown.

PSTVd also contains multiple signals regulating its ability to move from cell to cell via the plasmodesmata. Early microinjection studies that followed the movement of fluorescently labeled RNA transcripts in symplasmically connected leaf mesophyll cells (Ding et al., 1997) revealed that infectious PSTVd RNAs (but not nonviroid control RNAs of similar size) moved rapidly from cell to cell. Site-directed mutagenesis of PSTVd has produced a number of variants that can replicate in protoplasts (i.e., single cells) but are unable to spread systemically in whole plants following mechanical inoculation (Figure 12.4). Such variants are presumably defective in some

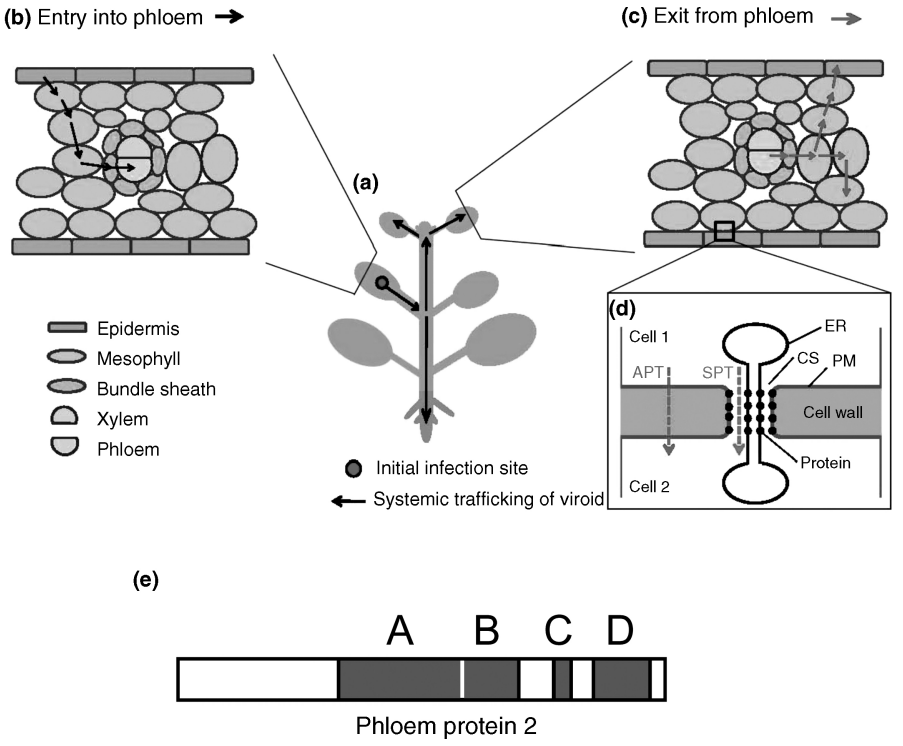

FIGURE 12.3 Viroid movement pathways. (a) Schematic drawing illustrating the movement of viroid progeny from the inoculated leaf (photosynthetic source) to the upper leaves and roots (metabolic sinks). (b) Cross section of an inoculated leaf showing cell-to-cell trafficking from an initially infected epidermal cell to the phloem prior to long-distance transport to other organs. For simplicity, not all cell types in the mesophyll, xylem, and phloem tissues are illustrated. (c) In a systemically infected leaf, the viroid exits the phloem and traffics into the surrounding nonvascular cells. (d) Intercellular movement of viroids occurs via plasmodesmata, specialized structures located in the cell walls of adjacent cells that are equivalent to the gap junctions connecting certain types of mammalian cells and allow direct cell-to-cell symplasmic transport (SPT). The plasma membrane (PM) also permits exchange of certain molecules across the cell wall via apoplastic transport (APT). ER, endoplasmic reticulum; CS, cytoplasmic sleeve. Adapted from Takeda and Ding (2009). (e) Schematic diagram showing the relative positions of four conserved motifs (A–D) in PP2 and PP2-like proteins. The N-terminal portions of these proteins vary in length and may contain additional AIG1, F-box, or Toll domains. (*See the color version of this figure in Color Plate section.*)

aspect of either cell-to-cell or long-distance movement. Using *in situ* hybridization techniques, Ding and colleagues have shown that several naturally occurring variants of PSTVd are unable to cross specific cellular boundaries and have identified a motif that potentiates its efficient trafficking from the bundle sheath into mesophyll but not in the reverse direction (Qi et al., 2004). As yet, little is known about the host components (presumably proteins) regulating cell-to-cell movement, but VirP1, a tomato protein isolated on the basis of its ability to bind PSTVd, has been shown to interact specifically *in vitro* with a 71 nt bulged hairpin that includes loops 23–26 (Figure 12.4) (Maniataki et al., 2003). Sequence changes in the nearby right terminal loop disrupting one of the two VirP1 binding motifs also interfere with systemic movement (Hammond, 1992). Unlike PLMVd (Rodio et al., 2007), PSTVd is not able to invade the rapidly dividing cells in the shoot apical meristem (Zhu et al., 2001).

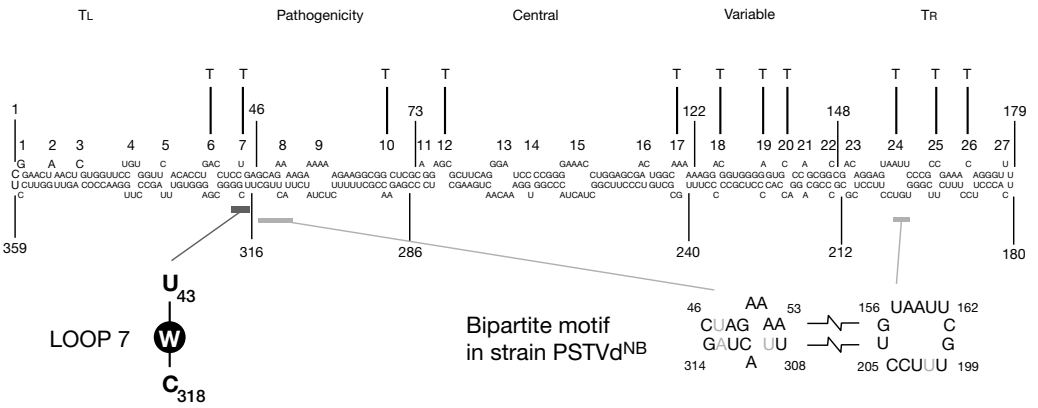

FIGURE 12.4 Secondary structure of PSTVd showing the relative locations of cell-to-cell and long-distance trafficking motifs identified by site-directed mutagenesis (Zhong et al., 2007), as well as the five structural domains proposed by Keese and Symons (1985). Loop 7 contains a U/C *cis*-WC/WC base pair with water insertion that is required for trafficking from bundle sheath to phloem. Trafficking in the opposite direction (i.e., from bundle sheath to mesophyll) involves a bipartite motif involving loops 8 and 24. Intracellular transport of PSTVd from the cytoplasm into the nucleus requires a palindromic sequence that is located in the upper portion of the central region and encompasses loops 12–16 (Abraitiene et al., 2008). Adapted from Takeda and Ding (2009). (*See the color version of this figure in Color Plate section.*)

Using dot-blot hybridization to follow PSTVd movement in infected tomato seedlings, Palukaitis (1987) reported a source-to-sink pattern of movement indistinguishable from that of most plant viruses. Newly synthesized viroid progeny begin to leave the inoculated leaf several days after inoculation and are transported to the shoot and root apices via the phloem. Although less widely appreciated, the role of the phloem as an "information superhighway" integrating many diverse aspects of plant growth and development via trafficking of much larger molecules, for example, noncell-autonomous proteins and mRNAs, as well as small regulatory RNAs has been long studied because of its role in the bulk transport of sucrose and other low molecular metabolites (Lough and Lucas, 2006). Most viruses that infect plants require coat protein for long-distance movement, but whether or not particle formation is required for movement in the phloem is uncertain. A number of other virus-encoded proteins (including the viral movement protein(s) responsible for cell-to-cell movement) also play important roles in long-distance movement.

Long-distance movement of HSVd appears to involve formation of a complex with a host protein known as PP2 (phloem protein 2), one of the most abundant proteins in phloem sap. Originally characterized as a chitin (i.e., *N*-acetyl glucosamine) binding lectin, PP2 is a multifunctional 26 kDa protein that is synthesized in companion cells, transported into sieve elements via the plasmodesmata, and translocated throughout the plant. Studies by Gómez et al. (2004, 2005) have shown that PP2 isolated from cucumber possesses all the properties expected for a host protein mediating long-distance viroid movement, namely, (i) the ability to form a ribonucleoprotein (RNP) complex with viroid RNA, (ii) the ability to interact with the plasmodesmata and increase their size exclusion limit (to permit the RNP complex to exit infected cells), and (iii) the ability to move long distances through the sieve elements (to distribute the viroid RNA throughout the whole plant). The genes encoding PP2 belong to small multigene families that are highly conserved among species within the genus *Cucurbita*, but two observations (i.e, their wide distribution in the plant kingdom and the

presence of additional functional domains in the amino terminal extension found in certain family members, see Figure 12.3e) suggest that these proteins may also play important roles outside the phloem. In addition to PP2, phloem exudate from melon also contains two smaller RNA binding proteins (Gómez et al., 2005). One of these smaller species (i.e, a 17 kDa protein) is closely related in sequence to PP2, and like PP2, is able to move in the phloem. Consistent with their proposed role in long-distance viroid transport, all three proteins were able to bind a second highly structured viroid RNA, that is, ASBVd.

12.4 HOST RANGE, SPECIFICITY, AND DEFENSE

12.4.1 Differences in Host Range Among Viroid Species and Families

For reasons that may be related to their replication and accumulation in plastids, members of the family *Avsunviroidae* have a very restricted host range and infect only the species in which they were initially reported and, in some instances, closely related species. Several members of the family *Pospiviroidae* (e.g., those forming the genera *Coleoviroid* and *Cocadviroid*) also have narrow host ranges, but other members of the family *Pospiviroidae* having have relatively broad host ranges include including viroids in the genus *Apscaviroid* that infect woody species, or in the genera *Pospiviroid* and *Hostuviroid* infecting both herbaceous and woody species. The ability to infect specific hosts may depend on only slight changes in primary structure, as illustrated by the finding that a single-nucleotide substitution can convert PSTVd from noninfectious to infectious for *Nicotiana tabacum* (Wassenegger et al., 1996).

Recent studies with citrus viroids have shown that viroid replication/accumulation in certain hosts is extremely inefficient. When such hosts are grafted or topworked on susceptible species, however, the viroid moves downward and upward to grafted tissues, wherein viroid replication/accumulation occurs efficiently (Bani-Hashemian et al., 2010).

12.4.2 Tissue- and Host-Specific Variants

As a result of two factors, the high mutation rates of the RNA polymerases involved in their replication (see Section 12.8.3) and selective pressures imposed by the host, viroids replicate within their hosts as populations composed of closely related variants. The first evidence that different hosts impose different selective pressures was obtained with CEVd, where viroid populations recovered after serial transmission to different hosts exhibited differences in nucleotide sequence, biological properties, and titer (Semancik et al., 1993). This finding received further support from a phylogenetic analysis of HSVd variants showing that such variants were clustered into several groups corresponding to specific hosts (Kofalvi et al., 1997; Amari et al., 2001).

Additional long-term assays carried out in different citrus hosts emphasize the role of the host in shaping CEVd population structure (Bernad et al., 2009). Examination of viroid populations recovered from two hosts inoculated with the same CEVd source showed that the resulting genetic diversity was host dependent and remarkably different from that of the original source used as inoculum. Moreover, these two populations reacquired the ancestral structure and genetic composition of the source inoculum upon return to the initial host species (Bernad et al., 2009). A final example illustrating the influence of the host is provided by an unusually heterogeneous isolate of CEVd recovered from symptomless *Vicia faba*. In this case, the population of CEVd variants became more homogeneous after transmission to tomato, but, in contrast with the situation observed in citrus, back transmission from tomato to *V. faba* did not restore the original population structure (Gandía et al., 2007).

Viroids are found in virtually all plant tissues and organs of infected plants (Singh et al., 2003). With the exception of PLMVd (Rodio et al., 2007), the available evidence indicates that viroids do not invade the shoot apical meristem, a situation that facilitates recovery of viroid-free plants from infected sources by *in vitro* culture of small shoot tips (containing only the meristematic dome and 2–3 leaf primordia). The lack of vascular connections between the meristem and other plant tissues and organs is considered to be the cause of the absence of viroids (and viruses) in this plant compartment, although RNA silencing may play an important role (see Section 12.4.3).

In spite of the evidence showing that viroids can easily invade most plant parts, recent studies indicate that infection is essentially restricted to the phloem at least in certain viroid/host combinations and that a barrier preventing trafficking from the bundle sheath to adjacent tissues may exist (Bani-Hashemian, 2009) (see also Section 12.3.2). Different tissues may also impose selective pressures on the structure of viroid populations; for example, specific variants of ASBVd were found associated with the distinct symptoms characteristic of the avocado sunbloth disease (Semancik and Szychowski, 1994), suggesting a relationship between selection pressure(s) and symptom expression.

12.4.3 Role of RNA Silencing

Eukaryotic cells have developed several RNA-based mechanisms to regulate gene expression and counteract invading nucleic acids such as transposons, viruses, and transgenes. By targeting DNA or RNA in a sequence-specific manner, these regulatory networks selectively inhibit RNA expression at the transcriptional or posttranscriptional level (RNA silencing) (Chen, 2009). Key elicitors of RNA silencing pathways are double-stranded or highly structured single-stranded RNAs that are processed by RNase III enzymes (Dicer or Dicer-like (DCL) in plants) to generate small RNAs (sRNAs) containing 18–25 nt (Carthew and Sontheimer, 2009; Chen, 2009). Four DCLs, generating different size classes of sRNAs, have been identified in Arabidopsis. DCL1 produces mostly 21 nt sRNAs, and it is involved in processing highly structured endogenous transcripts into microRNAs (miRNAs) that regulate developmental pathways. DCL4 and DCL2 act hierarchically to generate 21 and 22 nt small interfering RNAs (siRNAs) from double-stranded RNAs that may result from transcription of host DNA or the activity of RNA-dependent RNA polymerases (RDRs). These enzymes, acting upon aberrant transcripts, may activate an amplification step that ultimately leads to synthesis of secondary siRNAs and activation of the RNA silencing machinery in a noncell autonomous mode (Voinnet, 2008; Dunoyer and Voinnet, 2009). *Trans*-acting siRNAs (tasiRNAs), a special class of secondary siRNAs involved in controlling developmental phase changes and organ polarity, are derived from dsRNAs generated by RDR6 acting upon nonprotein-coding transcripts targeted by a miRNA cleavage. After incorporation into the RNA-induced silencing complex (RISC), both miRNAs and siRNAs guide its Argonaute (AGO) component to specific complementary RNAs for degradation or translation arrest (Vaucheret, 2008). Finally, DCL3 is required for the production of the heterochromatin-associated 24 nt siRNAs that mediate RNA-dependent methylation (RdDM) at specific DNA loci (Verdel et al., 2009).

RNA silencing also provides an immune-type response, at least in plants and invertebrates, against virus infection. Highly structured regions of viral genomic RNAs and the double-stranded RNAs generated during replication can be targeted by host DCLs to generate virus-derived sRNAs (vsRNAs) that then drive RISC-mediated degradation of the invading virus RNA. Host RDRs generate secondary vsRNAs that elicit synthesis of a systemic signal activating RNA silencing in uninfected cells before virus arrival. Viruses counterattack this defense mechanism by encoding proteins

that suppress RNA silencing pathways at several steps (Csorba et al., 2009), thereby interfering with RNA silencing-based regulation of host genes and inducing developmental defects similar to the characteristic symptoms of viral infections (Kasschau et al., 2003).

Involvement of RNA silencing in viroid–-host interaction was revealed when several groups showed that viroid-derived sRNAs (vd-sRNAs) similar to miRNAs and siRNAs accumulate in plants infected by both nuclear- and chloroplast-replicating viroids (Itaya et al., 2001; Papaefthimiou et al., 2001; Martínez de Alba et al., 2002). Whether viroids actually resemble RNA viruses, being both triggers and targets of RNA silencing, remains controversial, however. On the one hand, it has been shown that while artificial sensor RNAs are targeted by RISC complexes loaded with vd-sRNAs (Vogt et al., 2004; Itaya et al., 2007), viroid RNAs themselves resist degradation, presumably as a consequence of their compact secondary structure (Wang et al., 2004; Itaya et al., 2007; Gómez and Pallás, 2007). On the contrary, an active role of vd-sRNAs in antiviroid defense is supported by (i) the reduced infectivity and delay in symptom expression observed when mature viroids are coinoculated with homologous double-stranded RNAs or vd-sRNAs (Carbonell et al., 2008), (ii) the resistance against PSTVd infection of transgenic plants expressing inverted repeats of an almost full-length viroid RNA and accumulating high levels of vd-sRNAs (Schwind et al., 2009), and (iii) the "cross-protection" phenomenon observed following challenge inoculation of infected plants with a related viroid (Niblett et al., 1978). Cross-protection is sequence specific and easily explained in the context of RNA silencing (see Section 12.6.2).

The apparent conflict among these various results most likely stems from the different experimental approaches used. More recently, the importance of RNA silencing in antiviroid defense has been highlighted by a reverse genetic approach: early in infection, PSTVd accumulates to very high levels in transgenic *N. benthamiana* wherein the expression of RDR6, involved in amplification and systemic spread of RNA silencing, is knocked down by RNA interference (Di Serio et al., 2010). Moreover, PSTVd, which is excluded from meristems in wild-type *N. benthamiana* (Zhu et al., 2001), is able to invade floral and vegetative meristems in the RDR6-deficient line, thereby showing that an RNA silencing-based defense system may restrict viroid trafficking in infected hosts (Di Serio et al., 2010). In this respect, viroids resemble viruses, whose entry into the shoot apical compartment is regulated by an RNA surveillance system (Foster et al., 2002; Qu et al., 2005; Schwach et al., 2005).

How do viroids escape RNA silencing? PSTVd is not a strong suppressor of silencing, being unable to impair RNA silencing of a reporter gene in transgenic *N. benthamiana* (Itaya et al., 2007). However, it cannot be excluded that viroids may have some suppressor activity not detectable with this experimental system and that this activity, coupled with efficient viroid replication and subcellular localization in silencing-free cell compartment(s), would cooperatively enable viroids to cope with the plant defense system. In support of this view, previous findings indicate that suppression of RNA silencing can be elicited by replication of a viral RNA without the direct involvement of a specific RNA silencing suppressor protein (Takeda et al., 2005).

Deep sequencing of vd-sRNAs in several viroid–host combinations has provided insight into the origin and the possible role of vd-sRNAs, expanding upon conclusions from previous low-scale sequencing that pointed to the mature circular viroid RNAs as the main substrate for vd-sRNAs genesis (Itaya et al., 2007; Martín et al., 2007; Machida et al., 2007). Contrastingly, high-throughput sequencing has shown that vd-sRNAs of both polarities accumulate to comparable levels in tissues infected by nuclear or chloroplastic viroids and derive mostly from specific regions (hot spots) in the respective full-length RNAs. Interestingly, vd-sRNAs of different sizes map to each hot spot, suggesting that several DCLs access to the same viroid RNA regions (Navarro et al., 2009;

Di Serio et al., 2009, 2010; Martínez et al., 2010; Bolduc et al., 2010). These data, together with the lack of correlation between hot spots and structured regions of the genomic viroid RNAs, suggest that dsRNAs are the preferred targets for DCL. Recent sequencing of vd-sRNAs from HSVd-infected cucumber plants revealed a size bias in vd-sRNAs from leaves and phloem, with the latter showing a preference for 22 nt vd-sRNAs containing a conserved sequence motif that suggests selective trafficking of vd-sRNAs (Martínez et al., 2010).

Owing to their potential to function as miRNAs or tasiRNAs by loading RISC and inactivating endogenous mRNAs, vd-sRNAs are proposed to be key effectors of viroid pathogenesis (Papaefthimiou et al., 2001; Wang et al., 2004; Gómez et al., 2008, 2009). Other data (e.g., Schwind et al., 2009; Di Serio et al., 2010) are inconsistent with this view, however. Alternatively, viroids may induce symptoms by competing for host enzymes involved in the synthesis of miRNAs and siRNAs, thus affecting the pathways regulated by these molecules. For nuclear viroids, the possibility that vd-sRNAs could interfere with host methylation is consistent with the finding that PSTVd replication induces *de novo* methylation of homologous transgenic DNA sequence, a finding that, incidentally, provided the first evidence for RdDM (Wassenegger et al., 1994).

12.5 PATHOGENESIS

12.5.1 Symptom Expression and Symptomless Hosts

Most viroids were initially identified because of their ability to induce symptoms and are therefore considered to be plant pathogens. However, available information now indicates that many viroids can infect and replicate in certain hosts without causing any visible effect. These latent infections are particularly well documented in citrus, which may harbor seven different viroids that generally remain unnoticed until the infected budwood is grafted on sensitive rootstocks (Vernière et al., 2004). Similarly, grapevines may harbor as many as five different viroids that very seldom—and only under specific environmental conditions or in coinfections with *Grapevine fanleaf virus* (GFLV)—induce disease symptoms (Flores et al., 1985; Koltunow and Rezaian, 1988, 1989; Rezaian, 1990). In the case of pospiviroids and hostuviroids, which have wide host ranges, the number of known tolerant natural and experimental hosts continues to increase, primarily as a result of recent reports showing that PSTVd, CEVd, *Tomato apical stunt viroid* (TASVd), and *Tomato chlorotic dwarf viroid* (TCDVd) are widespread in solanaceous ornamentals (see Section 12.8.1). Symptomless hosts enable viroid survival by acting as reservoirs from where viroids can readily move to other susceptible hosts. A good example is broad bean naturally infected with a highly heterogeneous CEVd population that accumulates to only a very low titer; owing to its sequence diversity, this population has the potential to infect a wide range of alternative hosts (Gandía et al., 2007).

The symptoms induced by viroids in sensitive hosts can affect both the whole plant (stunting) and specific organs including leaves (epinasty, rugosity, mosaic, chlorosis, mottling, browning), stems (shortening, thickening), bark (scaling, pitting, gumming), flowers (variegation), fruits (size, color, deformation), seeds (abortion), and reserve organs (tuber malformation). Perhaps the only family-specific symptom is the extreme chlorosis incited by certain variants of ASBVd and PLMVd (Semancik and Szychowski, 1994; Malfitano et al., 2003). Symptom expression may vary from extremely mild to severe and even lethal, depending on the presence or absence of disease-specific variants in the infected plants (see Section 12.5.3). High temperature and light intensity favor symptom expression (and viroid accumulation) (Sänger and Ramm, 1975; Carbonell et al., 2008), thus possibly explaining why viroids are pathogens that predominantly affect subtropical and glasshouse crops.

12.5.2 Cytopathological Effects

As noted previously, the visible symptoms associated with viroid infection often resemble quite closely the symptoms induced by conventional RNA or DNA viruses. These similarities also hold true at the cellular level, where changes in the structure of cell walls, chloroplasts, and membranous structures in the cytoplasm known as "plasmalemmasomes" or "paramural bodies" and the accumulation of electron-dense deposits are all detected in viroid-infected tissues (Diener, 1987). Many of the symptoms such as stunting and epinasty associated with viroid infection are indicative of altered hormone metabolism, but how these metabolic and regulatory changes are connected to visible changes in cell structure remains to be determined.

Infection of *Gynura aurantiaca* with CEVd (Semancik and Vanderwoude, 1976) or tomato with PSTVd (Hari, 1980) has been reported to lead to the appearance of "plasmalemmasomes" or "paramural bodies," some of which are located near the cell wall. As discussed by Diener (1987), opinions differ concerning the relative abundance of these structures in healthy and infected cells, and their function(s) remain unknown. Wahn et al. (1980) were the first to describe irregular thickening and other cell wall abnormalities associated with CEVd infection in *G. aurantiaca*; later studies (e.g., Momma and Takahashi, 1983) revealed similar changes with several other viroid–host combinations. Interestingly, no changes were visible in the apical dome and first two pairs of leaf primordia of HSVd-infected hop plants; undulations and variable thickening were first observed in the third leaf primordium. As discussed above (see Section 12.3.2), *in situ* hybridization revealed that a second pospiviroid (i.e., PSTVd) is unable to enter or efficiently replicate in the shoot apical meristem of tomato (Zhu et al., 2001). The stunted growth of PSTVd-infected tomato plants is the result of restricted cell growth rather than inhibition of cell division or differentiation, and this stunting is positively correlated with downregulation of LeExp2, an expansin gene encoding a protein known to play an important role in the expansion of young cells via a "loosening" in the structure of their cell walls (Qi and Ding, 2003).

Perhaps the most intriguing cytopathology associated with viroid infection are disturbances in chloroplast structure, particularly abnormalities in thylakoid membranes and disruption of grana. For viroids such as PLMVd that replicate in the chloroplast, such effects are not unexpected. A closer examination of this phenomenon by Rodio et al. (2007) has revealed that despite the profound disruption of plastid gene expression in the albino portions of the infected leaf, PLMVd replication is still possible. Such a result is consistent with the proposed role of a nuclear-encoded chloroplastic RNA polymerase in replication of members of the family *Avsunviroidae* (see Section 12.3.1). Interestingly, similar chloroplast abnormalities are also reported for several pospiviroid–host combinations, where viroid replication is confined to the nucleus and effects on chloroplast structure are likely to be indirect. Recent evidence reviewed by Seay et al. (2009) indicates that the plant immune system utilizes the chloroplast as the primary site for the regulation of cell death programs that are an important part of the plant defense response. As many important structural proteins of chloroplast are encoded by nuclear genes and translated on cytoplasmic (rather than plastid) ribosomes, it is not difficult to imagine how viroid replication in the nucleus could trigger chloroplast-based signaling pathways leading to alterations in these key organelles.

12.5.3 Molecular Determinants of Pathogenicity

The molecular mechanisms that enable an infectious nonprotein-coding RNA to elicit symptoms in the host plant represent one of the most fascinating and still unanswered questions in plant biology. Viroid symptoms

generally consist of developmental defects (see Section 12.5.1) that must derive from the ability of the infecting RNA to subvert, directly or indirectly, host developmental programs. The information content of viroids, when it is deciphered, should contribute to a better understanding of RNA-based regulatory networks in eukaryotic cells.

Apart from promoting the accumulation of pathogenesis-related proteins (see, e.g., Tornero et al., 1994), the ability of viroid RNAs to interfere with host gene expression is examined by macroarray analysis, with the result that a mild and a severe strain of PSTVd were seen to induce and repress a large number of both common and certain specific tomato genes (Itaya et al., 2002). Similar results have been obtained by differential display of citron leaves infected by *Citrus dwarfing viroid* (CDVd) (Tessitori et al., 2007). Although the primary event modulating host gene expression remains unknown, available evidence indicates that viroid pathogenesis results from specific interactions between the infecting RNA and certain host factors. Viroid accumulation is not necessarily associated with symptom expression, as exemplified by *Columnea latent viroid* (CLVd) (Hammond et al., 1989) and ELVd (Fadda et al., 2003); moreover, variants of the same viroid differing minimally in sequence and accumulating at similar levels may incite very different pathogenic responses in a common host (Gross et al., 1981; De la Peña et al., 1999; Malfitano et al., 2003), indicating the involvement of specific regions of the viroid genome in symptom expression.

The existence of structural domains functionally related to pathogenesis was first reported for PSTVd (Dickson et al., 1978; Gross et al., 1981), in which variants causing mild, intermediate, severe, and lethal effects were found to differ by only a few nucleotide changes located within a "virulence modulating region" within the P domain (Schnölzer et al., 1985; Herold et al., 1992). Naturally occurring severe and mild strains of CEVd differ by as many as 26 nucleotide changes in the P and V domains, but biological assays of *in vitro* constructs revealed that only changes in the P domain were associated with virulence (Visvader and Symons, 1986; Bernad and Duran-Vila, unpublished results). Attempts to correlate these results obtained using experimental hosts (tomato and *G. aurantiaca*) with others from citrus hosts have failed, indicating that the pathogenicity determinants are host specific. In support of this notion, characterization of a representative CEVd isolate together with additional site-directed mutagenesis experiments has recently shown that (i) virulence in citron can be altered by as few as two nucleotide changes in the P domain and (ii) variants behaving as latent in citron induce severe symptoms in herbaceous experimental hosts (Murcia et al., 2011).

Symptom severity may also be modified by determinants mapping outside the P domain (Sano et al., 1992; Rodriguez and Randles, 1993). For example, a single U/A substitution at position 257 in the C domain of PSTVd has been reported to dramatically increase symptom severity in tomato without altering the accumulation level or trafficking ability of the viroid (Qi and Ding, 2003). Interestingly, although position 257 is located within the PSTVd loop E motif (Branch et al., 1985; Eiras et al., 2007; Wang et al., 2007), this substitution does not affect its secondary (Qi and Ding, 2003) or tertiary structure (Zhong et al., 2006). As described previously (see Section 12.4.1), this loop E motif appears to modulate several key steps in the infection cycle, for example, host specificity (Wassenegger et al., 1996), transcription (Zhong et al., 2006), and ligation (Gas et al., 2007). A second illustrative example is provided by HSVd, whose pathogenicity in citrus is determined by a 5–6 nucleotide motif located in the V domain (Reanwarakorn and Semancik, 1998; Palacio-Bielsa et al., 2004). All HSVd strains inducing citrus cachexia exhibit a strict conservation of this motif, which affects the organization of a short helical region and two flanking loops (Palacio-Bielsa et al., 2004). A single-nucleotide change is sufficient to suppress symptom expression

(Serra et al., 2008b), but characterization of additional HSVd sources has revealed that classification into just two groups (cachexia inducing with the conserved motif of 5–6 nucleotides and noncachexia inducing lacking this motif) may be inadequate (Mohamed et al., 2009). Symptom expression in citrus may change when pathogenic and nonpathogenic HSVd variants coexist in a field isolate.

Pathogenicity determinants have also been identified in several chloroplast-replicating viroids. For example, ASBVd infections in avocado can be latent (symptomless carriers) or associated with a variety of different symptoms on leaves, stems, and fruits. Distinct leaf symptoms such as severe chlorosis associated with vascular tissues (bleaching), variegations expressed throughout the whole blade, and latency are associated with specific ASBVd variants (Semancik and Szychowski, 1994). The lack of an amenable bioassay for ASBVd has hindered efforts to investigate the proposed association of a poly(A) loop in the right terminal domain with leaf bleaching and variegation (Semancik and Szychowski, 1994; Schnell et al., 2001). CChMVd and PLMVd have similar branched secondary structures, and site-directed mutagenesis and bioassays carried out with infectious cloned cDNAs have mapped the determinants for leaf chlorosis to a single U-rich tetraloop (De la Peña et al., 1999; Malfitano et al., 2003). More specifically, the PLMVd pathogenicity determinant maps to a 12–13 nt hairpin insertion present in variants inducing an extreme chlorosis known as "peach calico" (PC) (Figure 12.1). Most PLMVd variants unable to induce visible symptoms lack this insertion (Malfitano et al., 2003), whereas others may contain a hairpin capped by a GA-rich instead of a U-rich loop (Rodio et al., 2006). Interestingly, these insertions can spontaneously appear and disappear during infection, suggesting that latent variants can evolve into pathogenic ones and *vice versa* (Malfitano et al., 2003; Rodio et al., 2006). Further dissection of the PC-inducing hairpin has revealed that not only the loop but also the stem, in particular its size and nucleotide composition, determines the phenotype induced by the infecting variants (Navarro, Delgado, Flores, and Di Serio, unpublished results). Similar to PSTVd, infected tissues contain comparable titers of symptomatic and non-symptomatic CChMVd and PLMVd variants (De la Peña et al., 1999; Malfitano et al., 2003; Rodio et al., 2006). Whether these pathogenicity determinants interact directly with cellular components or induce alternative conformations in the genomic viroid RNA, thereby making it competent for specific interaction(s) that eventually incite symptom production, is not known.

For some specific viroid–host combinations, details of the mechanism responsible for symptom production have been partially elucidated. PLMVd variants that induce PC block chloroplast differentiation at an early developmental stage (most likely in the shoot apical meristem) by impairing maturation of the plastid ribosomal RNA (rRNA). This impedes translation of plastid-encoded proteins and ultimately produces ultrastructural malformations in chloroplasts, histological alterations in leaves, and the macroscopic albino phenotype (calico) resembling that of certain variegated mutants in which plastid rRNA maturation is also impaired (Rodio et al., 2007). For PSTVd, the variant carrying an U/A substitution at position 257 inhibits cell growth but not cell division or differentiation in tomato, causing a severe stunting that has been correlated with the downregulation of an expansin gene (Qi and Ding, 2003) (see Section 12.5.2). These studies suggest mechanistic models for pathogenesis but do not identify the primary molecular event(s).

The nature of this initial molecular event remains elusive. When additional data failed to support an early model correlating PSTVd severity with the thermodynamic stability of a "virulence modulating" region in the P domain (Schnölzer et al., 1985), Owens et al. (1996) proposed an alternative model in which the ability of the mature viroid RNA to interact with unidentified host factors is controlled by the degree of bending of the P

domain (Owens et al., 1996; Schmitz and Riesner, 1998). Protein kinases that elicit a signaling cascade in the host are activated or induced differentially by mild and severe PSTVd strains (Hiddinga et al., 1988; Diener et al., 1993; Hammond and Zhao, 2000), but evidence of a direct interaction with the pathogenic domain of the viroid is still lacking. More recently, pathogenesis has been linked to RNA silencing (see Section 12.4.3). Identification of the primary event eliciting viroid pathogenesis should help to decide between these alternatives and it remains a major task for future studies.

12.6 INTERACTIONS BETWEEN VIROIDS AND VIRUSES

12.6.1 Viroid Coinfection: The Case of Citrus and Grapevine

The number of viroids described so far is relatively small when compared to other plant pathogens; nevertheless, at least two crops, citrus and grapevine, are found to be natural hosts of several viroids. Seven viroids in citrus and five in grapevine have been described thus far, all belonging to the family *Pospiviroidae*. Their prevalence in commercial cultivars is most likely favored by vegetative propagation. Commercial citrus is nowadays generally graft propagated on seedling rootstocks and because citrus viroids do not appear to be seed transmissible, they must have been perpetuated and disseminated in the grafted cultivars through the international exchange of germplasm. Commercial grapevines are also graft propagated on rootstocks, which were previously propagated as rooted cuttings, a sequential practice that favors the prevalence of infecting viroids in both the scion and the rootstock.

The seven viroids described in citrus (CEVd, *Citrus bent leaf viroid* (CBLVd), HSVd, CDVd, CBCVd, *Citrus viroid V* (CVd-V), and *Citrus viroid VI* (CVd-VI)) belong to three different genera in the family *Pospiviroidae*, and four of them (CEVd, HSVd, CDVd, and CBLVd) appear to be widespread in all the citrus growing areas where they are found as mixtures of two, three, or four viroids coinfecting the same plant. The five viroids isolated from grapevine (*Grapevine yellow speckle viroid 1* and *2* (GYSVd-1 and GYSVd-2), *Australian grapevine viroid* (AGVd), HSVd, and CEVd) belong to the same three genera, and while three of them (GYSVd-1, GYSVd-2, and HSVd) appear to be widespread in table grape cultivars, only two (GYSVd-1 and HSVd) are widespread in wine cultivars. Selection of table grape cultivars infected with GYSVd-2 may reflect a desirable effect of this viroid on quality parameters (Semancik et al., 1989).

12.6.2 Interference: Cross-Protection

Interference between plant viruses is often referred to as "cross-protection," thus reflecting its most relevant practical implication (the other being for testing virus relatedness). Briefly, infection with a mild or latent virus strain protects the infected plant against later challenge inoculation with a severe strain of the same or of a closely related virus. The titer of the challenging virus and the intensity of its associated symptoms are temporarily diminished or even abolished. Because this concept was coined before viroids were discovered and their key differences with viruses recognized, cross-protection between viroids was also inadvertently described, specifically between mild and severe strains of members of the two different families of viroids, PSTVd (Fernow, 1967) and PLMVd (Desvignes, 1976). Later, cross-protection was reported between different members of the genus *Pospiviroid* (Niblett et al., 1978), two strains of the same viroid (Horst, 1975; Duran-Vila and Semancik, 1990), and, more recently, between viroids of different genera that share common domains (Vernière et al., 2006). Assuming that a common mechanism (with variations) operates for both viruses and viroids, the mechanism is likely to be RNA based, given that viroids do not encode any protein. RNA

silencing (see Section 12.4.3) offers a very attractive framework to explain why cross-protection is observed only between closely related viroids (or viruses).

During the recent years, it has been shown that protection against a virus can be afforded to plants by expressing transgenically nonprotein-coding viral RNA sequences and that this RNA-mediated cross-protection is mechanistically analogous to posttranscriptional gene silencing (Ratcliff et al., 1997, 1999). This scheme can be easily extended to viroids by assuming that the vd-sRNAs, which result from the DCL action on the RNA of the preinoculated latent or mild strain, program RISC against the invading RNA of the challenging severe strain and promote its inactivation (Flores et al., 2005). Therefore, the specificity of cross-protection between viroids would be a consequence of the sequence specificity of RISC. Additional experiments should clarify whether cross-protection is indeed another manifestation of RNA-mediated gene silencing. In the meantime, the alternative view that cross-protection between viroids could result from competition for a limiting host factor (i.e., a transcription factor) needed for completing their infectious cycle must also be considered.

12.6.3 Synergism

The clearest examples of synergistic interaction among viroids coinfecting a single plant have been reported in citrus, where naturally infected plants may contain as many as seven viroid species. The first evidence indicating that mixed infections modulate symptom expression was obtained in bioassays of field trees coinfected with several viroids: the citron indicator expressed symptoms more severe than those expected from additive effects of the viroids present in the inocula (Duran-Vila et al., 1986, 1988). Moreover, when grafted onto a viroid-sensitive trifoliate orange rootstock, citrus coinfected with several viroids induced exocortis-like symptoms in the absence of CEVd (Ito et al., 2002). Synergistic effects have also been observed in long-term field assays (Vernière et al., 2006). Recent experiments under more controlled conditions have confirmed that coinfection with specific pairs of citrus viroids of the genus *Apscaviroid* (with sequence similarity below 70%) leads to symptom exacerbation; compared to the respective single infections, viroid titers remain unaltered in coinfected plants (Serra et al., 2008a).

Synergistic interactions between distantly related viruses have long been known, but only recently has the involvement of viral-encoded suppressors of RNA silencing in such interactions been recognized. In addition to antiviral defense, RNA silencing also regulates certain key steps in plant development. Recognizing a significant degree of overlap between these two pathways, it is not difficult to envision how coinfection by two unrelated viruses could result in enhanced symptom expression. If their silencing suppressors affect different steps in the RNA silencing pathway, accumulation of one of the coinfecting viruses may also increase (Pruss et al., 1997; MacDiarmid, 2005). This mechanism is not directly applicable to viroids because their genomes do not encode any proteins (including suppressors of RNA silencing). Nevertheless, viroids might divert for their own replication enzymes of the host RNA silencing machinery: synergistic effects of two coinfecting viroids would then result from effects on distinct components of this machinery (see Section 12.4.3). Alternatively, synergism between viroids could operate through a mechanism different from gene silencing, especially considering that viroid titers are not modified in contrast to the situation observed in virus synergism.

12.6.4 Viroid–Virus Interplay

The preceding sections have dealt with interference and synergism between viroids. Considering that similar phenomena have been described for viruses and that coinfections by viroids and viruses are common, the question of possible virus–viroid interaction immediately

arises. To our knowledge, no examples of interference between coinfecting viruses and viroids have been reported. Any data of this sort would be difficult to interpret because the effects might well be secondary: infection by a viroid (or virus) may induce a general weakening of the host plant and, indirectly, reduce its ability to support the replication of the coinfecting pathogen. In contrast, there are some results on synergism between viroids and viruses that may be explained, at least tentatively, within the framework of RNA silencing.

The vein banding disease of grapes is characterized by yellow mottling or chrome yellow bands along the principal leaf veins of affected vines (Goheen and Hewitt, 1962). In California, this syndrome has been observed only in vines infected with the three more common grapevine viroids, GYSVd-1, GYSVd-2, and HSVd, as well as with the nepovirus GFLV (Szychowski et al., 1995). Moreover, vines containing only HSVd and GFLV were nonsymptomatic, indicating that HSVd is not involved in the vein banding disease. In contrast, the severity of vein banding was directly correlated with enhanced titers of GYSVd-1 and GYSVd-2, suggesting that their increased replication and/or accumulation is linked to the disease. Supporting this notion, vein banding and yellow speckle symptomatic and nonsymptomatic vines from Italy contained GYSVd-1 and HSVd, but vein banding symptomatic vines displayed a higher titer of GYSVd-1 and were GFLV infected. Altogether, these data show that vein banding disease is induced by a synergistic reaction between a viroid (GYSVd-1 and possibly GYSVd-2) and a virus (GFLV) (Szychowski et al., 1995). One plausible explanation is that a silencing suppressor presumably encoded by GFLV attenuates the host defensive response and induces the increased accumulation of GYSVd-1, thus resembling the situation reported in synergism between coinfecting viruses (see preceding section). Unfortunately, while this is an attractive hypothesis, the existence of silencing suppressors associated with nepoviruses has not yet been reported (Csorba et al., 2009).

A second example of synergistic interactions between viroids and viruses is noted in citrus. Most, if not all, species of this plant genus can be infected by *Citrus tristeza virus* (CTV), a member of the family *Closteroviridae* (Bar-Joseph and Dawson, 2008), as well as by up to seven members of the family *Pospiviroidae*, with multiple coinfections occurring frequently under natural conditions. Recently, it was observed that the presence of CTV enhances the titer of CDVd in coinfected Mexican lime, thereby mimicking to some extent the synergism between coinfecting viruses. Symptom expression is not intensified in plants coinfected by CTV and CDVd, however (Serra, Peña, and Duran-Vila, unpublished results). In principle, this effect might be caused by one or more of the three silencing suppressors encoded by the long 19.3 kb genome of CTV, namely, p25, p20, and p23 (Lu et al., 2004). To further characterize their possible roles, three transgenic lines of Mexican lime ectopically expressing one of these proteins were inoculated with CDVd. Although p20 and p25 produced no or just a moderate increase in viroid titer with respect to that of the nontransgenic control, the presence of p23 resulted in an increase in the viroid titer similar to that observed in the nontransgenic control infected by CTV. Thus, p23 seems to be a major player in the synergistic interaction between CTV and CDVd (Serra, Peña, and Durán-Vila, unpublished results). Information regarding the specific mode of action of p23 is limited, but in addition to its role as an intracellular suppressor of RNA silencing, p23 also acts as a pathogenicity determinant in citrus (Ghorbel et al., 2001; Fagoaga et al., 2005).

12.7 TRANSMISSION

12.7.1 Insect Vectors

For many years, the role of insect vectors in the perpetuation and spread of viroids was overlooked, masked by their efficient dissemination via vegetative propagation of infected plant

material. Early attempts to determine whether or not PSTVd was insect transmissible yielded inconsistent and contradictory results; for example, reports of transmission by the aphids *Myzus persicae* and *Macrosiphum euphorbiae* (Kennedy et al., 1962; Smith, 1972) were not confirmed in later studies (Schuman et al., 1980; De Bokx and Piron, 1981). The first compelling evidence that viroids might be insect transmissible involved *Tomato planta macho viroid* (TPMVd), where the viroid was shown to be naturally transmitted by the aphid *M. persicae* after acquisition from wild hosts such as *Physalis aff. foetens* and *Solanum rostratum* (Galindo et al., 1989). Following an acquisition period of 24 h, transmission was persistent and depending on the source plants, transmission rates as high as 97% were observed.

Occasional contamination of *Potato leafroll virus* (PLRV) isolates maintained at the International Potato Center with PSTVd provided the first indication that PLRV might facilitate aphid transmission of PSTVd (Salazar et al., 1995). Additional surveys confirmed that most PSTVd-infected field-grown potatoes were also infected with PLRV (Querci et al., 1997). Because PLRV, a member of the genus *Polerovirus*, is readily aphid transmitted and *M. persicae* is the most efficient vector, this aphid species was chosen for further transmission assays. *M. persicae* was able to transmit PSTVd in a persistent manner but only when the aphids were allowed to acquire the viroid from source plants doubly infected with PSTVd and PLRV (Querci et al., 1997). Similar results were obtained with transmission assays conducted with other hosts of PSTVd and PLRV, for example, tomato, *P. floridana*, and *Datura stramonium* (Syller et al., 1997).

Even though only small amounts of PSTVd were associated with purified PLRV virions, its resistance to digestion by micrococcal nuclease indicated that the viroid was actually encapsidated within the PLRV particles (Querci et al., 1997). PLRV is a phloem-limited virus, but not all of the viroid molecules released when the particles open and virus replication begins would be expected to be reencapsidated in the progeny virions. After release, these PSTVd molecules would be free to follow the normal pattern of long-distance viroid movement in the phloem and transport to adjacent cells and tissues able for viroid replication. Support for this scenario comes from the results of transmission assays using a potato cultivar that is highly resistant to PLRV infection. Plants on which the viruliferous aphids were allowed to feed became infected only with PSTVd, demonstrating that PLRV acted only as viroid carrier (Syller and Marczewski, 2001). Unfortunately, no additional examples of viroid transmission involving insect transmitted viruses have been reported, and it is not yet clear whether or not transencapsidation is absolutely required. Early experiments conducted with *Velvet tobacco mottle virus* (VTMoV) demonstrated that PLRV is not the only virus able to encapsidate PSTVd (Francki et al., 1986); thus, it is possible that other virus–viroid–vector combinations could play a similar role in natural viroid transmission.

Finally, the phenomenon of epidemiological data describing CCCVd spread and the distribution pattern of infected coconut palms in the field is most easily explained by invoking the presence of one or more insect vectors (Hanold and Randles, 1991). Efforts by Zelazny and Pacumbaba (1982) to identify the vector(s) among phytophagous insects collected from infected coconut palm trees were unsuccessful, but the possibility that the presence of a virus might be required for successful transmission was not considered. Bumblebees (*Bombus ignitus*) have recently been shown to transmit two different pospiviroids under greenhouse conditions, but the exact mode of TASVd (Antignus et al., 2007) and TCDVd (Matsuura et al., 2010) transmission remains to be determined.

12.7.2 Seed and Pollen

Transmission of PSTVd and ASBVd, type species of their respective viroid families, through seed and pollen has long been known (Singh, 1970; Kryczynski et al., 1988; Singh

et al., 1992; Wallace and Drake, 1962). Transmission efficiencies vary greatly depending on the specific viroid–host combination, and unfortunately, no generalizations are possible. Among the family *Pospiviroidae*, high and low rates of seed transmission are reported for CbVd-1 (Singh et al., 1991) and CCCVd (Manalo et al., 2000), respectively, with data about other viroids being conflicting: seed transmission is reported for *Chrysanthemum stunt viroid* (CSVd) infecting chrysanthemum (Monsion et al., 1973) and tomato (Kryczynski et al., 1988), but no evidence for seed transmission in chrysanthemum was found by Hollings and Stone (1973). Although early results indicated that HSVd is not transmissible by cucumber seeds (Van Dorst and Peters, 1974), later results have shown that this viroid is seed transmitted in tomato (Kryczynski et al., 1988).

Improved sensitivity of detection methods has contributed to clarifying the role of seed transmission in the epidemiology of certain viroids. Seed transmission of grapevine viroids was neglected until CEVd, GYSVd-1, GYSVd-2, and AGVd were detected in grapevine seedlings by RT-PCR and molecular hybridization (Wan Chow Wah and Symons, 1999). TCDVd, a viroid closely related to PSTVd, was originally considered non-seed transmissible in tomato (Singh et al., 1999), but more recently it has been detected by RT-PCR in tomato seeds and seedlings (Singh and Dilworth, 2009). In the past few years, vertical transmission of other pospiviroids closely related to PSTVd has been shown: TASVd in tomato (Antignus et al., 2007) and CEVd in *Verbena* and *Impatiens*, two valuable ornamental species (Singh et al., 2009), suggesting that seed transmission of pospiviroids is more frequent than initially thought.

Viroid identification in seeds does not necessarily imply transmission to seedlings, as shown by ASSVd, which has been detected in the coat and subcoat portions of apple seeds (Hadidi et al., 1991), but not in the resulting seedlings (Howell et al., 1995; Desvignes et al., 1999). Similarly, although PLMVd is detected in the tegument and peeled kernels of seeds from infected peach trees, seedlings from these seeds are viroid-free (Barba et al., 2007), indicating that the viroid is seed-borne but not seed transmissible, likely because it does not enter the embryo. Infected pollen can also transmit a viroid to healthy plants, as shown for PLMVd by experimental pollination (Barba et al., 2007). Pertinent in this context is the finding that some viroids are eliminated during pollen development: *Hop latent viroid* (HLVd) is removed from uninucleate pollen after the first pollen mitosis, an observation that has been correlated with the expression of pollen-specific nucleases (Matousek et al., 2008).

The mechanisms underlying seed and pollen transmission are not known. Recently, it was proposed that an RNA silencing mechanism may block the invasion of developing flower and vegetative meristems in *N. benthamiana* by the nuclear-replicating viroid PSTVd (Di Serio et al., 2010), but not by the chloroplast-replicating viroid PLMVd in peach (Rodio et al., 2007). Since PSTVd is both pollen and seed transmitted, just how and when this defense mechanism is overcome by the viroid remains to be clarified.

12.7.3 Role of Modern Agriculture in Viroid Ecology

Although their origins are often unknown, many viroid diseases appear to be an unintended consequence of modern agricultural practices. Noting that their often striking symptoms would make these diseases difficult to overlook, Diener (1979) has drawn attention to the fact that not a single viroid disease is known to have existed before the twentieth century. Descriptions of several viral diseases affecting cultivated plants, in contrast, date back to several centuries. According to this view, chance transfer of viroids from wild plants to cultivated crop species has occurred repeatedly. What has changed during the past 100 years or so are the genetic characteristics of the crops under cultivation and the techniques used for propagation. Small plantings of locally adapted (and genetically variable) local varieties have been replaced by large-scale

monoculture of genetically identical plants that are often mass produced in a central location. Viroid diseases are thus essentially iatrogenic.

PSTVd has never been isolated from any of the wild potato species growing in the Andes, center of origin for the cultivated potato (*S. tuberosum* L.), and the absence of any known source of genetic resistance to PSTVd infection in cultivated potatoes indicates that this viroid and its host did not coevolve (Diener, 1987). How and when this viroid was introduced into cultivated potatoes is not clear, but studies reported by Martínez-Soriano et al. (1996) suggest one possible scenario. In this work, several wild *S. cardiophyllum* plants growing in Mexico were shown to be latently infected with *Mexican papita viroid* (MPVd), a previously unknown viroid most closely related to TPMVd. A variety of wild solanaceous species were introduced into the United States from Mexico in the late nineteenth century as part of an early effort to identify genetic resistance to *Phytophthora infestans*, the potato late blight fungus. It is possible that commercial potatoes being grown in the United States became infected by chance transfer of MPVd (or other related viroid) from this germplasm material.

In the case of PSTVd, transfer to the cultivated species appears to have been a comparatively recent event. Quite a different situation may exist for certain "old line" varieties of citrus where a single variety is shown to harbor as many as 4–5 different viroids and this association between viroid and host may date back 1500 years or more (see Section 12.6.1; Bar-Joseph, 1996). Mosaics from a sixth century C.E. synagogue in the Northern Negev region of Israel depict Etrog citron fruits exhibiting typical viroid-induced malformations. Etrog citron, a native of the Himalayan foothills in India, was the first variety of citrus to reach the Mediterranean basin—probably as fruits imported from the Near East in 200–300 C. E. Because viroids are not seed transmissible in citrus, Bar-Joseph suggests that viroid-infected grapevines provide the most likely source of inoculum for the newly arrived citrus. Long before the introduction of citrus, grapevines were vegetatively propagated throughout the Mediterranean basin and Near East. In addition to CEVd and HSVd, grapevines are known to support the replication of at least three other apscaviroids similar to those found in citrus—often as symptomless carriers. Contaminated pruning and grafting tools provide the most likely route of viroid transfer.

In recent years, increasing amounts of tomatoes and other vegetable and flower crops are being grown in greenhouses or under plastic. Productions schedules often involve overlapping production of ornamental and vegetable species, sometimes in the same greenhouse compartments. If one of these species (often an ornamental) is a symptomless host, an outbreak of viroid disease in the vegetable crop is often just a matter of time. A series of recent publications have documented several such situations (see next section).

Because viroids such as PSTVd are mechanically transmissible, they can be introduced into potential host plants via the hands, clothes, or equipment used by workers or visitors to the greenhouse. If a viroid infection is identified in a greenhouse-grown crop, all parts of the greenhouse should be thoroughly cleaned, preferably using a steam cleaner and a scrub brush for parts that are difficult to clean. A regular acid treatment can be used for watering tubes and drippers. After cleaning the greenhouse and associated equipment, application of a disinfectant completes the eradication procedure. When cultivation of crops susceptible to infection resumes, extra monitoring for symptoms and/or diagnostic testing is advisable. Ideally, growers should grow only a single crop or they should separate different crops and lots, preferably in different compartments.

12.8 VIROID EPIDEMIOLOGY AND CONTROL

12.8.1 Recent Detection of Pospiviroids in Ornamentals: A Latent Threat

PSTVd may cause losses in potato (Pfannenstiel and Slack, 1980) and is also a risk for

tomato, a natural (Elliot et al., 2001; Verhoeven et al., 2004) and experimental host (Raymer and O'Brien, 1962). As a consequence, PSTVd is considered a quarantine pest in the EU, United States, and several other countries. Despite the restrictive measures imposed by quarantine status, repeated outbreaks of PSTVd and other pospiviroids have occurred in tomatoes growing in several European countries since 1988. The origin of these infections remains elusive (Verhoeven et al., 2004), but one of the pospiviroids detected in symptomatic tomato plants was CLVd, a viroid initially reported to naturally infect only one ornamental species without inciting symptoms (Hammond et al., 1989). These findings highlight the risk posed by viroids that latently infect certain ornamentals but are able to move into other susceptible crops via physical contact or pollen transmission (see Sections 12.7.2 and 12.7.3). Adding to these concerns is a report by Nie et al. (2005) describing symptomless infections of other ornamental species (*Verbena* spp., *Impatiens* spp., and *Vinca minor*) growing in Canada by three additional pospiviroids (*Iresine viroid* (IrVd), CEVd, and CSVd).

These findings stimulated a large-scale survey in the Netherlands that identified frequent symptomless infections of *Brugmansia* spp. and *S. jasminoides* plants by PSTVd (Verhoeven et al., 2008a). The same survey also identified a number of healthy-looking *Verbena* and *Cestrum* sp. plants that were infected by CEVd and TASVd, respectively. Following this report, PSTVd-infected *S. jasminoides* or *Brugmansia* spp. are reported in several EU countries, as well as additional symptomless ornamentals naturally infected with PSTVd, including *S. rantonetti* (syn. *Lyciantus rantonetti*) (Di Serio, 2007) and *Streptosolen jamesonii* (Verhoeven et al., 2008b). Owing to the quarantine status of PSTVd, eradication measures were undertaken whenever the viroid was identified in ornamentals. In the Netherlands, these actions resulted in losses (€3–5 million in 2007) to growers that were considerably higher than the costs needed to ensure the permanent exclusion of PSTVd (De Hoop et al., 2008). These efforts, together with the use of a viroid-free propagation material, are further vindicated by the recent finding in Italy of PSTVd-infected tomato growing in close proximity to *S. jasminoides* infected by the same viroid (Navarro et al., 2009). Molecular and biological assays support the view that PSTVd was accidentally transmitted to tomato from the neighboring infected *S. jasminoides* plants, thus providing the first evidence of PSTVd spread from a symptomless ornamental solanaceous host into a susceptible horticultural crop. Together with phylogenetic analyses (Verhoeven et al., 2010), these data support the view that ornamental solanaceous hosts have also been the source of past PSTVd infections of tomato.

Whether additional reports of diseased tomato plants infected by other pospiviroids, including TASVd (Antignus et al., 2007) and CEVd, CLVd, TASVd, and TCDVd (Verhoeven et al., 2004), are epidemiologically related to infections of other symptomless ornamentals is not known. We note, however, that several healthy-looking ornamentals have been reported to harbor most of these viroids: examples include *Verbena* spp. (Bostan et al., 2004; Singh et al., 2006), *V. minor* (Singh et al., 2009), and *Petunia* spp. (Verhoeven et al., 2007) for TCDVd; *Verbena* spp. and *Impatiens* spp. for CEVd (Singh et al., 2006; Verhoeven et al., 2008a); *S. jasminoides* for CEVd and TASVd (Verhoeven et al., 2008a); and *S. pseudocapsicum* and *Cestrum* for TASVd (Spieker et al., 1996; Verhoeven et al., 2008b).

How are pospiviroids transmitted from ornamentals to horticultural crops? A possible role for insects cannot be excluded (Section 12.7.1) and requires further study, but transmission efficiency between different botanical species under field conditions is presumably quite low. Human transmission during crop handling seems a more plausible alternative, and consecutive/repeated manipulation of infected and healthy plants in the absence of proper hygienic measures would favor viroid transmission between susceptible hosts (see Section 12.7.3). Moreover, seed transmission of these viroids, as shown by the identification of CEVd in commercially distributed seeds and

seedlings of *Verbena* and *Impatiens* spp. (Singh et al., 2009), would favor rapid dispersion to uncontaminated areas, with the viroid remaining unnoticed due to the absence of symptoms in the infected ornamentals.

12.8.2 Sequence Variability Among HSVd Isolates from Different Hosts: The Origin of Hop Stunt Epidemics

In Section 12.7.3, we discussed circumstantial evidence suggesting that grapevine may act as a symptomless reservoir for several viroids commonly recovered from citrus. One such viroid is HSVd, and quite recently, direct experimental evidence has appeared linking symptomless infections of grapevine by HSVd with outbreaks of "hop stunt" in cultivated hops (Kawaguchi-Ito et al., 2009).

Hop is a perennial herb that originated in the Near East–Caucasus region. Hop cultivation began in Germany in the middle of eighth century, and foreign cultivars arrived in Japan only in the late nineteenth century. The disease now known as hop stunt was first recognized in Japan in the 1940s. At the time when the viroid nature of this disease was established (i.e., 1977), HSVd was thought to occur only in certain hop varieties cultivated in Japan. Over the next decade or more, studies carried out in a number of laboratories revealed that the host range of HSVd is actually quite broad; in addition to hop, other natural hosts include cucumber, citrus, grapevine, and a number of other pome and stone fruit species (Sano, 2003). Infections of grapevine by HSVd are asymptomatic, but infection of sensitive citrus cultivars with specific HSVd sequence variants results in a disease known as cachexia (see Section 12.5.3). Other diseases such as dapple fruit of plum and peach have also been described in association with HSVd. Phylogenetic analysis of HSVd variants isolated from these various hosts indicates that they can be divided into five host-related clusters (Amari et al., 2001; Sano et al., 2001), one of which contains isolates from grapevine and hop.

As described by Kawaguchi-Ito et al. (2009), sequence variations among Japanese isolates of HSVd from hop are concentrated at just eight positions, that is, positions 25, 26, 32, 54, 193, 265, and 282. Japanese isolates from grapevine, in contrast, show major variations at positions 26, 32, 44/45, 46, 47, 180, 256, and 257. To examine the possible role of grapevine and other potential viroid reservoirs in the origin of hop stunt disease, blocks of viroid-free hop plants were inoculated with natural HSVd isolates derived from hop, grapevine, plum, or citrus. Viroid populations in infected plants were then monitored over a 15-year period for the appearance of new sequence variants. Figure 12.5 compares the evolution of two naturally occurring isolates of HSVd, one derived from hop and the other from grapevine. Although initially quite homogeneous, both isolates soon began to accumulate various combinations of changes at positions 25, 26, 54, 193, and 281 that, upon prolonged infection, underwent convergent evolution to yield a small number of "adapted" variants. In the isolate from grapevine (Figure 12.5b), some of these adapted variants were identical in sequence to isolates such as hKFKi responsible for hop stunt disease outbreaks in Japan, China, and the United States. In all three countries, hops and grapevines can often be found growing in the same regions. By successfully reproducing the process by which a naturally occurring HSVd sequence variant can mutate into other variants currently present in commercial hops, these investigators have firmly established the role of grapevine as a symptomless reservoir in which HSVd can evolve and be transmitted to other susceptible crop species.

12.8.3 Emergence and Recombination

As indicated in the introduction to this chapter, viroids are presumed to have emerged in the early history of life on our planet (the so-called "RNA world"). The strongest evidence in support of such an ancient evolutionary

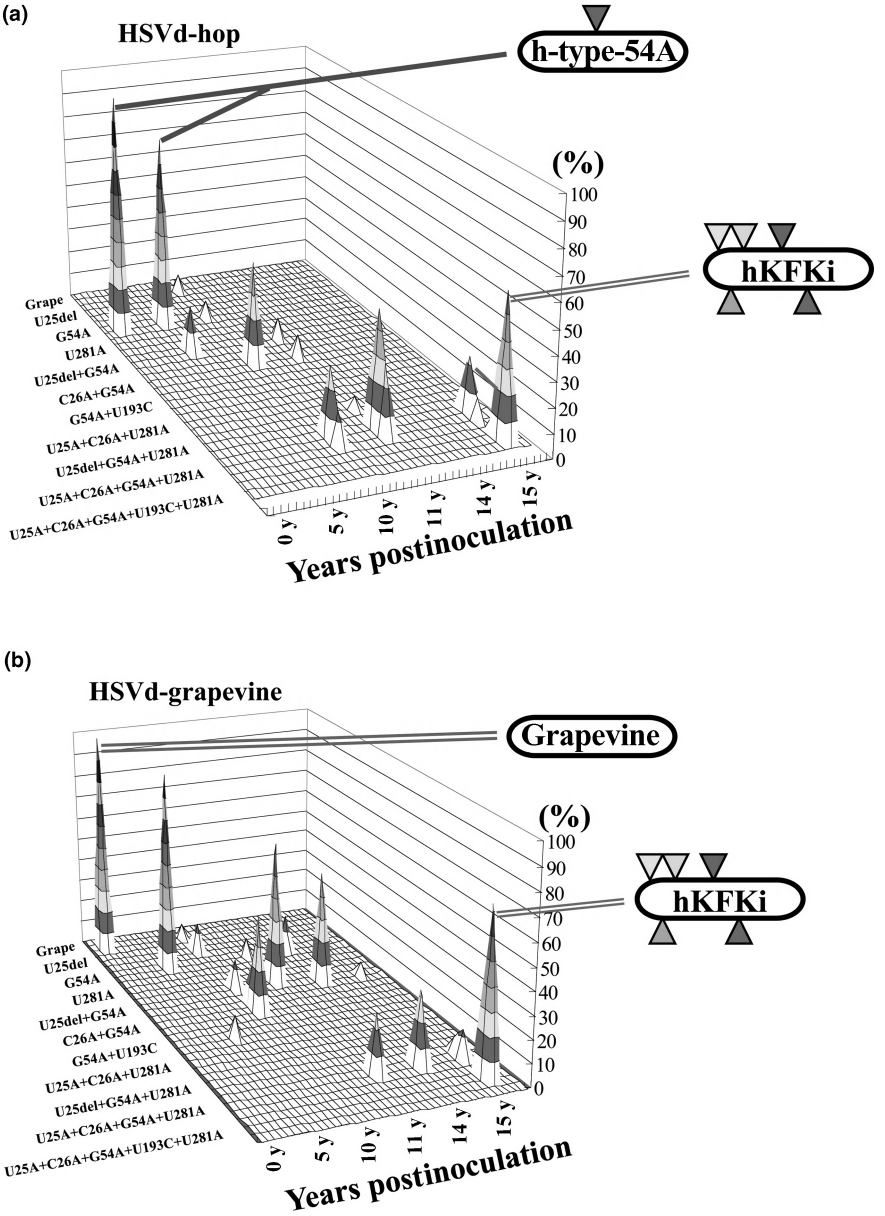

FIGURE 12.5 Convergent evolution of two natural isolates of HSVd during prolonged passage in hop. The upper (a) and lower (b) panels summarize data for HSVd-hop and HSVd-grapevine, respectively. The left side of each panel contains a schematic of the sequence changes and frequency of the resulting variants over a 15-year period. Names of selected mutants are arranged along the *x*-axis. The *y*-axis shows the frequency (%) of each mutant in the population. The *z*-axis indicates the number of years postinoculation. On the right side of each panel are schematic of the predominant HSVd variants detected at the beginning and end of the trial. Sequence changes present in the different variants are color coded: U26A (yellow triangle), C26A (blue triangle), G54A (red triangle), U193C (purple triangle), and U281A (green triangle). Adapted from Kawaguchi-Ito et al. (2009). (*See the color version of this figure in Color Plate section.*)

origin for viroids is their small size, lack of protein-coding capacity, and, particularly, the presence of hammerhead ribozymes in members of the family *Avsunviroidae* (Diener, 1989; Flores et al., 2000). Recent data add further support to this view. Specifically, the mutation rate for CChMVd (a hammerhead viroid) is one mutation per 400 nucleotides transcribed, the highest reported for any biological entity (Gago et al., 2009). Mutation rates of this magnitude are reminiscent of those postulated for primitive replicons in the "RNA world," for which an error-prone replication has been postulated, and most likely results from transcription by a proofreading-deficient chloroplastic DNA-dependent RNA polymerase redirected to use RNA rather than its native DNA as template. Whether a similar situation exists for members of the family *Pospiviroidae* remains to be determined.

The frequent recombination events associated with viroid replication are believed to result from the tendency of RNA polymerases that have been forced to transcribe a nonnative and highly structured RNA template to "jump." After pausing at a highly structured region, the RNA polymerase, together with its bound nascent strand, detaches from the template and reinitiates synthesis on either a different region of the same template or on a different template. The fact that several viroids appear to be natural mosaics of sequences also found in other viroids (Hammond et al., 1989; Rezaian, 1990) is consistent with this view.

Natural chimeric viroids have been identified in both grapevine (Rezaian, 1990) and citrus (Puchta et al., 1991), in which the combination of vegetative propagation and a long production life under field conditions where plants are constantly exposed to new viroid infections would be expected to facilitate recombination. However, the lack of an appropriate assay system to experimentally tackle this question—no chimeric viroid is generated after coinfecting a single host with multiple viroids—has limited further progress. The closest approximation to such an experimental system involves certain coleus plants naturally infected by *Coleus blumei viroids* 1, 2, and 3 (CbVd-1, CbVd-2, and CbVd-3). Comparison of their respective nucleotide sequences revealed that CbVd-2 is composed of two blocks of sequences, one identical to the right-hand side of CbVd-1 and the other identical to the left-hand side of CbV-3. Boundaries between these two blocks of sequence are sharp; thus, CbVd-1 and CbVd-3 are considered to be "ancestors" of CbVd-2 (Spieker, 1996; Sänger and Spieker, 1997). Unfortunately, this system in which recombinant events appear particularly frequent has not been further explored, apart from the discovery of new chimeric viroids in this genus.

Alternatively, artificial viroid chimeras that are infectious and genetically stable have been constructed *in vitro*. These include chimeras between variants of the same viroid species (Visvader and Symons, 1986), between species in the same genus (Owens et al., 1990; Sano et al., 1992), and even between species in different genera (Sano and Ishiguro, 1998). Only partial success was obtained in the latter case, however, highlighting the limitations of this approach as the phylogenetic distance between the parental species becomes wider. Because genetic information in viroids is very condensed, with a significant fraction of individual nucleotides being most likely involved in more than one biological role and interacting with other nucleotides separated in the primary structure, it is not easy to predict which artificial recombinants will be ultimately viable.

12.9 CONCLUSIONS

As described above, modern agriculture has profoundly modified the natural environment, thereby playing a major role in viroid (and virus) ecology. Constant interchange of propagative material has clearly contributed to the current widespread distribution of viroids in economically relevant crops (and to significant economic losses), but alternative routes for viroid transmission as well as their interactions

with viruses and other viroids remain poorly explored. Thanks to available detection methods, most of the viroids responsible for diseases affecting crops of agronomic interest may have already been identified. Nevertheless, the recent discovery of a new viroid in diseased pepper (Verhoeven et al., 2009) indicates importance of continuing close surveillance, particularly considering the broad range of symptomless ornamentals harboring viroids potentially harmful to horticultural crops.

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