CHAPTER 16

Replication Strategies of RNA Viruses Requiring RNA-directed mRNA Transcription as the First Step in Viral Gene Expression

A significant number of single-stranded RNA viruses contain a genome that has a sense *opposite* to mRNA (i.e., the viral genome is *negative-sense RNA*). To date, no such viruses have been found to infect bacteria and only one type infects plants. But many of the most important and most feared human pathogens, including the causative agents for flu, mumps, rabies, and a number of hemorrhagic fevers, are negative-sense RNA viruses.

The negative-sense RNA viruses generally can be classified according to the number of segments that their genomes contain. Viruses with **monopartite** genomes contain a single piece of virion negative-sense RNA, a situation equivalent to that described for the positive-sense RNA viruses in the last chapter. A number of groups of negative-sense RNA viruses have **multipartite** (i.e., *segmented*) genomes. Viral genes are encoded in separate RNA fragments, ranging from two for the arenaviruses to eight for the orthomyxoviruses (influenza viruses). As long as all RNA fragments enter the cell in the same virion, there are no special problems for replication, although the packaging process during which individual segments must all fit into a single infectious virion can be inefficient.

It is well to remember that there is a fundamental difference in the replication strategy of a negative-sense RNA virus as compared to a positive-sense RNA virus. Since the virus must have the infected cell translate its genetic information into proteins, it must be able to express mRNA in the infected cell. With a negative-sense RNA virus, this will require a *transcription* step: Genetic information of the viral genome must be transcribed into mRNA. This presents a major obstacle because the cell has no mechanism for transcription of mRNA from an RNA template.

The negative-sense RNA viruses have overcome this problem by evolving means of carrying a special virus-encoded enzyme—an **RNA-dependent transcriptase**—in the virion. Thus, viral structural proteins include a few molecules of an enzyme along with the proteins important for structural integrity of the virion and for mediation of its entrance into a suitable host cell. Clearly, the isolated genome of negative-sense RNA viruses cannot initiate an infection, in contrast to the positive-sense RNA viruses discussed in Chapter 15. Other groups of viruses (notably retroviruses, discussed in Chapter 20) include enzymes important to mRNA expression in their virion structures, but focusing on negative-sense RNA viruses' replication strategies provides useful general considerations.

One of the more interesting general questions concerning these viruses is: How did they originate? Sequence analyses of replicating enzymes encoded by different viruses often demonstrate similarities to cellular enzymes, implying a common function and suggesting a common origin. While the cellular origin of most viral enzymes can be established by sophisticated sequence analysis, this has yet to be accomplished with RNA-directed RNA transcriptases. The sequence and characterization of more cellular genes are becoming available daily, and eventually a good candidate for a progenitor enzyme will be identified. When this is done, more definitive statements can be made concerning origins of these viruses.

The fact that no bacterial viruses with this replication strategy have been identified is at least consistent with the possibility that negative-sense RNA viruses are of recent origin. A recent origin would imply that all the negative-sense RNA viruses are fairly closely related to each other, and there is some evidence that this is the case.

REPLICATION OF NEGATIVE-SENSE RNA VIRUSES WITH A MONOPARTITE GENOME

There are four "families" of negative-sense RNA viruses that package their genomes as a single piece of RNA: Rhabdoviridae, Paramyxoviridae, Filoviridae, and Bornaviridae. They all share some similarities of gene order and appear to belong to a common "superfamily" or order: Mononegavirales.

Interestingly, despite genetic relatedness of these viruses, they do not share a common shape, although all are enveloped. Also, the rhabdovirus family contains several members that infect plants. Is this a "recent" radiation to a new set of hosts? Whatever the answer to this question, there is no doubt that the Mononegavirales viruses are a successful group with significant pathologic implications for humans and other vertebrates.

Human diseases caused by the viruses of this order include relatively mild flu-like respiratory disease (parainfluenza) caused by a paramyxovirus. More severe diseases include mumps, measles, hemorrhagic fevers with high mortality rates caused by Marburg and Ebola virus (filoviruses), and neurological diseases ranging from relatively mild ones caused by bornavirus to the invariably fatal encephalitis caused by rabies virus (a rhabdovirus). The diseases characterized by high mortality rates are not maintained in human reservoirs but rather are zoonoses — diseases of other vertebrates transmissible to humans (see Chapter 3).

The replication of vesicular stomatitis virus – a model for Mononegavirales

Infection of humans with naturally occurring strains of rabies virus leads to fatal diseases. This and other factors make this virus difficult and dangerous to work with — indeed, much of the work on it is carried out in a few very isolated laboratories in the United States, including Plum Island in Long Island Sound. In contrast, the closely related rhabdovirus vesicular stomatitis virus (VSV) is one of the most carefully studied extant viruses. Its replication strategy forms a valid model for the replication of all Mononegavirales viruses and provides important insights for the study of replication of other viruses with negative-sense RNA genomes. Remember that negative-sense viruses must have some way to turn the viral genome (virion RNA) into mRNA before infection can proceed.

The vesicular stomatitis virus virion and genome

The VSV virion and genetic map are shown in Fig. 16.1. Like most rhabdoviruses, it has a distinctive bullet-shaped structure. The VSV genome encodes five proteins, all present in the virion in different amounts. The viral genome is about 11,000 bases long. Since individual mRNAs are generated from the virion negative-sense RNA, viral genes in the genome have an order opposite to the order in which mRNAs appear in the cell. Locations of the viral genes are shown in the genetic map of Fig. 16.1. The L and P proteins function together to cap mRNA, generate mRNA, polyadenylate positive-sense viral mRNA, *and* replicate the viral genome. (Remember, the virus must bring its own replication enzymes into the cell because the cell cannot deal with single-stranded RNA that is not like mRNA.)

Generation, capping, and polyadenylation of mRNA

The first part of the VSV replication cycle is outlined in Fig. 16.2. Virus attachment and internalization occur by receptor-mediated endocytosis. The virion does not fully disassemble in the infected cell. The intact ribonucleoprotein (RNP) nucleocapsid contains the genomic positive-sense template and transcription/replication enzymes. The virion-associated transcription/RNA replication enzyme initiates and caps each of the five discrete positive-sense mRNAs within this transcription complex. Like the positive-sense RNA viruses expressing capped mRNA, this capping takes place in the cytoplasm.

Interestingly, while the cap structure is identical to that found on cellular mRNA, the specific phosphodiester bond cleaved in the cap nucleoside triphosphate is different from that cleaved in the nucleus by cellular enzymes. This difference suggests that this enzymatic activity was probably

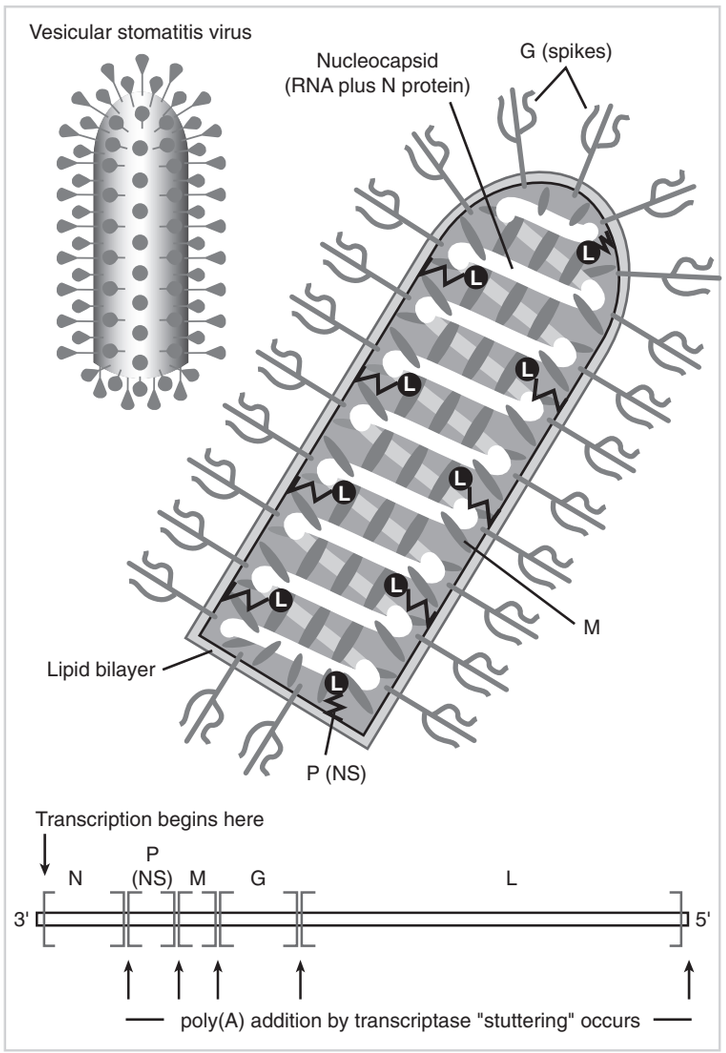


Fig. 16.1 The vesicular stomatitis virus (VSV) virion. All rhabdoviruses have this characteristic bullet shape that appears to be due to the P (formally called NS) and L proteins interacting with the envelope in a specific way. The 70 × 180 nm VSV virion contains enzymes for RNA transcription that can be activated by mild detergent treatment and incubation with nucleoside triphosphates in vitro. The genetic map of VSV is also shown. The 11,000-nucleotide (nt) virion negative-sense strand RNA encodes five individual mRNAs; each is capped and polyadenylated by virion enzymes. Note, because the genomic RNA serves as a template for mRNA synthesis, it is shown in 3′ to 5′ orientation instead of the conventional 5′ to 3′ orientation. (N, nucleocapsid; M, matrix; G, envelope glycoprotein; L, part of the replication enzyme; P (or NS), also part of the replication enzyme.)

not simply "borrowed" by the virus from an existing cellular capping enzyme, but was derived from some other enzymatic activity of the cell.

The polyA tails of the mRNA species are generated at specific sites on the negative-sense genome by a "rocking" mechanism in which the enzyme complex "stutters" and generates a long polyA tail and releases mRNA. The enzyme can then release, start over, or continue on. The process is outlined in Fig. 16.3; this biochemical "decision" has resulted in a polarity of abundance of viral mRNA and the proteins encoded: nucleocapsid (N) protein > P (NS) protein > matrix (M) protein > envelope glycoprotein (G) > L protein.

The generation of new negative-sense virion RNA

Negative-sense virion RNA can only be generated from a full-length positive-sense template in an RI-2 complex. But the partially disrupted virion generates mRNA-sized pieces of positive-sense strand. As shown in Fig. 16.3, full-length negative-sense strand is only generated when levels of N

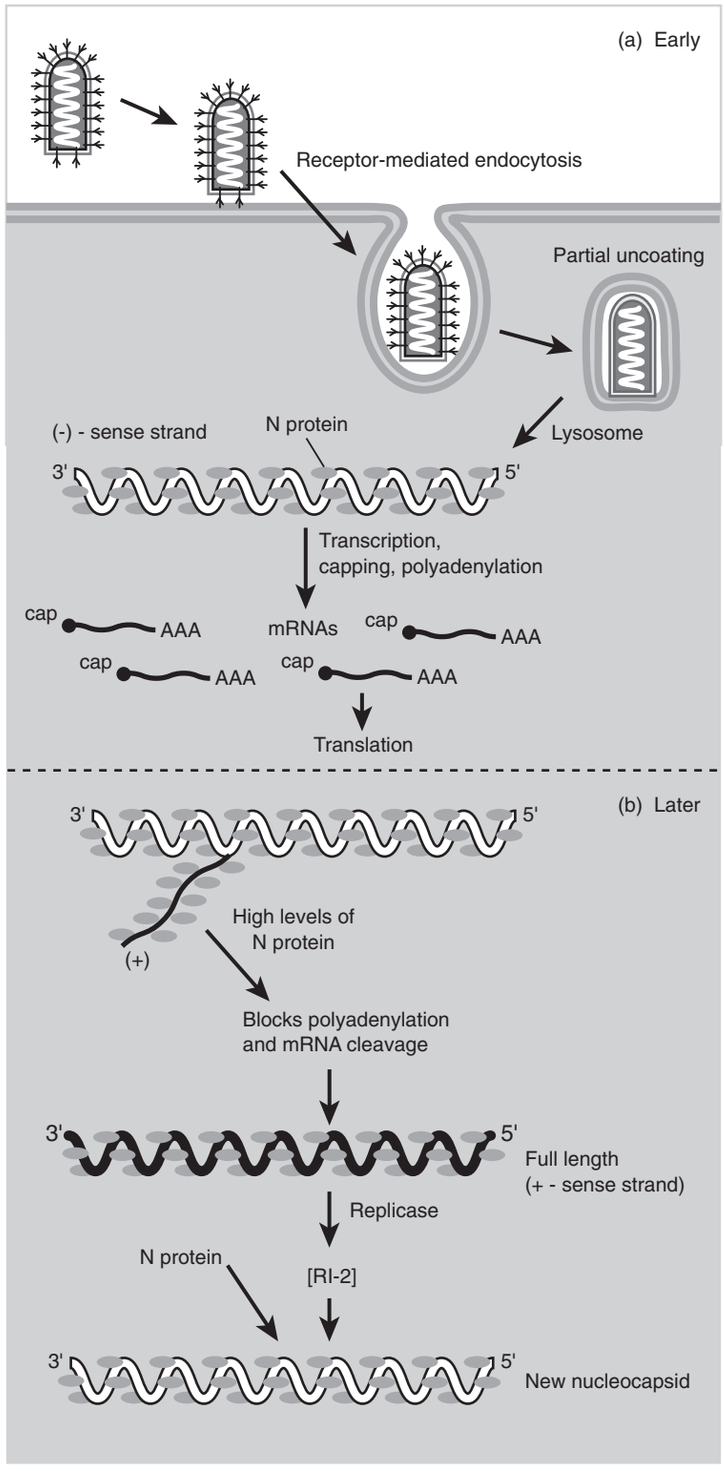


Fig. 16.2 The VSV replication cycle. *a.* Early events in infection begin with virus attachment to the receptor followed by receptor-mediated endocytosis and partial uncoating to virion ribonucleoprotein (RNP). This is transcribed into mRNAs that are translated in the cytoplasm. *b.* Later, as protein synthesis proceeds, levels of the N (nucleocapsid) protein increase, and some nascent positive-sense strand from RI-1 associates with it. This association with N protein blocks the polyadenylation and cleavage of individual mRNAs, and the growing positive-sense strand becomes a full-length positive-sense strand complement to the viral genome that serves as a template for negative-sense RNA synthesis via RI-2. *c.* At still later times in the replication cycle, viral proteins associate with the nucleocapsids made up of newly synthesized negative-sense genomic RNA and N protein. These migrate to the surface of the infected cell membrane, which has been modified by the insertion of viral G protein translated on membrane-bound polyribosomes. M protein aids the association of the nucleocapsid with the surface envelope and virions form by budding from the infected cell surface.

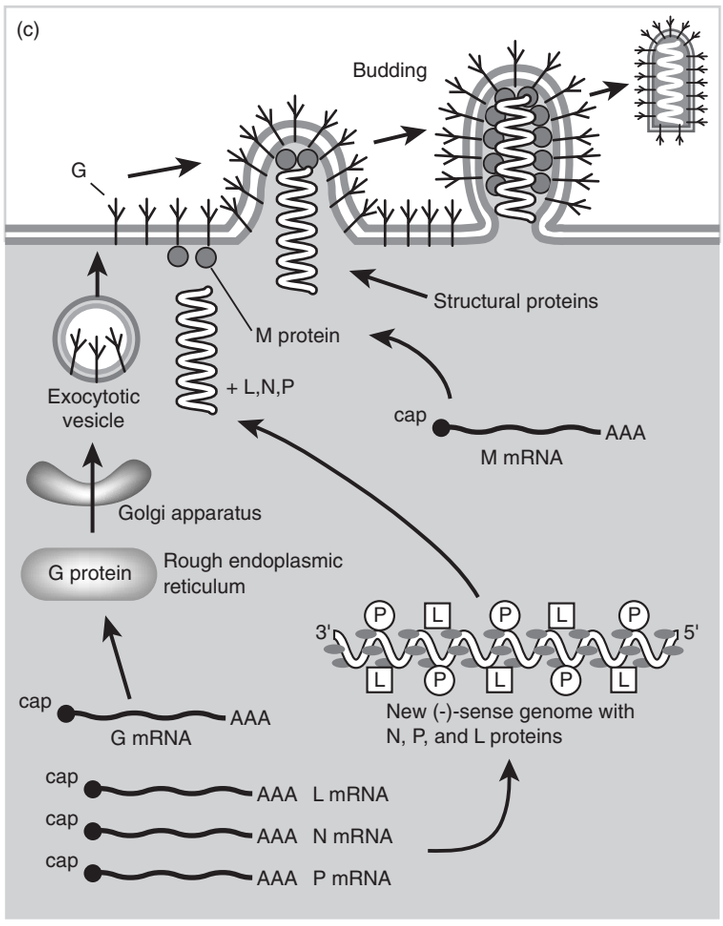


Fig. 16.2 *Continued*

protein become high enough in the cell so that newly synthesized positive-sense RNA can associate with it. This association prevents the rocking-polyadenylation-cleavage-reinitiation process used in the generation of mRNA and allows the formation of full-length template. This genome-length positive-sense template serves as template for new virion negative-sense strand, which also associates with N, and the other structural proteins encoded by the virus.

While the process and biochemical "choice" between production of mRNA and full-length positive-sense template RNA are best characterized in the replication of VSV, it appears that very similar mechanisms exist for other viruses in the Mononegavirales order. Further, other negative-sense viruses that have multipartite genomes probably utilize equivalent mechanisms since (where characterized) their positive-sense genome templates are larger than the positive-sense mRNA expressed during infection.

The details of VSV infection and morphogenesis are generally similar to those discussed for positive-sense enveloped viruses, which are described in some detail in Chapter 15. The process of mRNA synthesis, template synthesis, and new negative-sense genome formation continues for an extended period until sufficient levels of the viral structural proteins are attained to form the virion RNPs. Virion RNP then buds through the plasma membrane and is released. These late events are outlined in Fig. 16.2c.

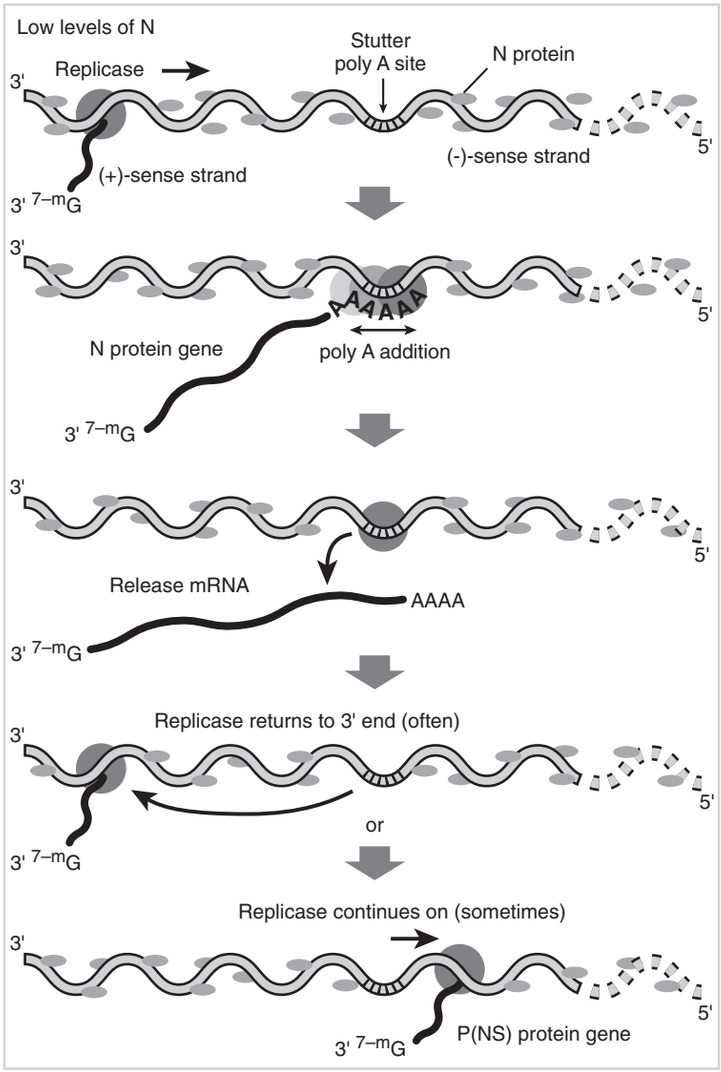


Fig. 16.3 A higher-resolution schematic of the generation of positive-sense strand mRNA from genomic negative-sense strand RNA template in the absence of N protein. Polymerase associates with the template at the extreme 3′ end, and "tunnels" or "burrows" under the N protein. Transcription begins with capping of the nascent mRNA, and proceeds through the first gene on the template (the N protein) gene. At the end of this gene, the transcriptase encounters an intergenic "pause" or stutter site. The enzyme pauses here and adds a number of A residues to the nascent mRNA, which is then released. The transcriptase then either dissociates from the template and begins the process over again at the extreme 3′ end, or continues on to synthesize a transcript encoding the next gene on the genomic template. At the end of this gene, the same process occurs. Since the transcriptase has a higher probability of returning to the extreme 3′ end of the template, the mRNAs are synthesized in decreasing amounts, with those encoding N protein > P (NS) protein > M protein > G protein > L protein.

The mechanism of host shutoff by vesicular stomatitis virus

As noted, many virus infections are characterized by virus-mediated inhibition of host mRNA and protein synthesis. The mechanism of this shutoff varies with the virus in question. For example, poliovirus, which does not utilize a capped mRNA, actually inhibits the ability of capped mRNA to be translated by modification of a translation initiation factor following infection. Obviously, this mechanism cannot work with viruses that express capped mRNA.

Since VSV does not utilize the cell's nucleus during its replication, it essentially "enucleates" the host cell following infection. This enucleation is another function carried out by the viral N protein. In this role, the protein specifically interferes with the transport of proteins into and out of the nucleus by inhibiting the nuclear transport proteins of the cell (see Chapter 13). Since some negative-sense RNA viruses, such as bornaviruses and flu viruses, utilize the nucleus for replication, this mechanism cannot be universal for negative-sense RNA viruses.

The cytopathology and diseases caused by rhabdoviruses

The disease caused by VSV involves formation of characteristic lesions in the mouth of many vertebrates (hence the name, vesicular stomatitis). Although humans can be infected by VSV, this virus is primarily a disease of cattle, horses, and pigs. Such a wide host range seems to be a common feature of rhabdovirus infection. VSV-induced disease can be severe in animals because they cannot eat during the acute phase of infection. The course is generally self-limiting and mortality rates are not significant, provided proper care is given the affected animal. Such is obviously not possible with free-ranging cattle, and VSV outbreaks can have severe economic consequences if not properly managed.

The disease caused by the related rabies virus demonstrates a completely different strategy for virus pathogenesis and spread. The essentially 100% mortality rate for rabies is in distinct contrast to mortality rates for most viral diseases. The pathogenesis of rabies is briefly described in Chapter 4. It should be remembered that since rabies is spread by animal bites, the behavioral changes induced by the virus are important for its spread. Except under the stress of mating or in territorial disputes, vertebrates (especially carnivores, the general host for rabies) do not randomly attack and bite other members of their own species. The high replication of rabies virus in the salivary glands of the rabid host, along with excitability and other induced behavior changes, makes the infected animal a walking "time bomb." This is an excellent example of how a virus of submicroscopic proportions and encoding only a few genes can direct the billions of cells of its host animal to a single purpose: propagation of the virus.

Paramyxoviruses

Paramyxoviruses have large genomes (approximately 15 kb) and their replication cycle is reminiscent of that described for rhabdoviruses. One notable exception is that several (including mumps) generate mRNA that has been edited by the addition of extra G nucleotides as the mRNAs for specific genes are expressed. The addition of these nucleotides is apparently accomplished by a stuttering step similar to that involved in the addition of polyA residues at the end of transcripts. This editing results in several variant mRNAs being expressed from a single viral gene.

Paramyxoviruses can be subdivided further into paramyxovirus proper, parainfluenza virus, mumps virus (*Rubulavirus*), measles virus (*Morbillivirus*), and pneumoviruses such as respiratory syncytial virus. The structure of Sendai virus, a typical paramyxovirus that causes respiratory disease in mice, and its genetic map are shown in Fig. 16.4.

The pathogenesis of paramyxoviruses

Mumps, measles, canine distemper, and rinderpest are all caused by paramyxoviruses. Mumps is classified as a relatively benign "childhood" disease; the infection usually occurs in children just when they begin to socialize in preschool or day-care facilities. The virus spreads rapidly, generally causes a mild inflammation of glandular tissue in the head and neck, and leads to lifelong immunity. Since the symptoms are generally forgotten and do not lead to any notable physiological consequences, the disease is considered mild.

Infection of postpubescent children or adults, however, can be a significantly different story. Here, the virus can infect gonadal tissue and lead to major discomfort, and occasionally, to permanent reproductive damage.

The pathology of respiratory syncytial virus also is quite different for infants and adults. This virus establishes a mild, cold-like infection in an adult's nasopharynx. Following recovery, the virus can persist in the throat as a relatively normal member of the microbe population that coexists in this moist, warm environment. Since it is not invasive, the persistent infection is usually asympto-

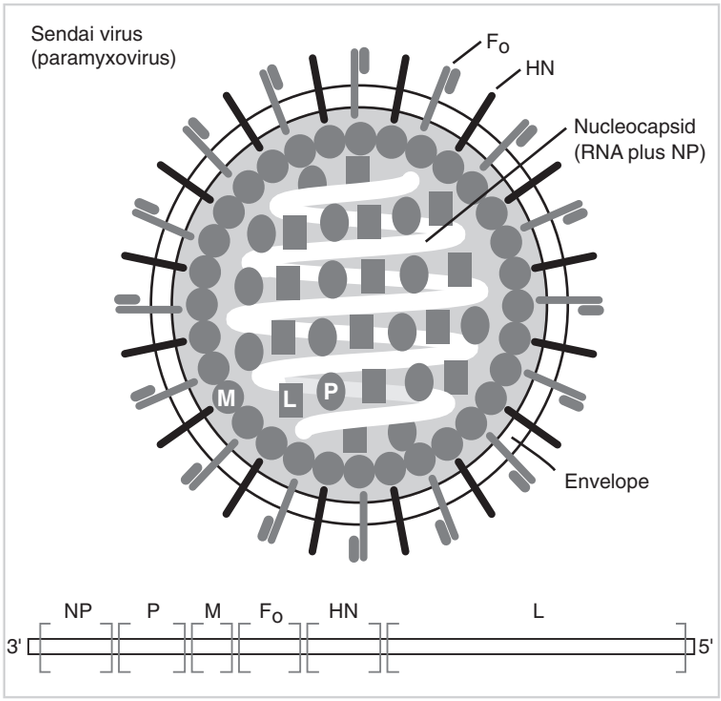


Fig. 16.4 The genetic map and virion structure of Sendai virus, a typical paramyxovirus. The Sendai virion is a flexible, helical nucleocapsid that contains the 15,000-nucleotide (nt) genome and is about 18 nm in diameter and 1000 nm in length. The roughly spherical enveloped virion is about 150 to 200 nm in diameter. The gene marker "HN" is a membrane protein that contains both neuraminidase and hemagglutination activity. The replication strategy is similar to that outlined for VSV. Also like VSV, the negative-sense strand genomic RNA is shown 3′ to 5′ instead of in the conventional 5′ to 3′ orientation.

matic unless there is a complicating environmental factor. Such a factor can be very dry air in heated buildings during winter in temperate zones throughout the world. This dry air can lead to chronic respiratory irritation and mild infections by respiratory syncytial virus, as well as other pathogens. Unfortunately, the virus can spread from adults to children and infants. In hospitals, an active infection in nursery health care workers can lead to fatal epidemics in newborns whose undeveloped immune systems cannot cope with the infection.

Measles, as described in Chapter 4, although often termed a childhood disease, can cause major neurological damage to infected children, and its introduction into unprotected populations has resulted in high mortality.

Measles and the closely related distemper and rinderpest viruses cause serious and often fatal diseases at all ages. Distemper infections cause high mortalities in domestic and wild animals, and the broad host range and easy transmission of canine distemper has resulted in its being a major infectious agent in marine mammals. Another related virus, Rinderpest, is a serious disease of domestic cattle that has spread to wild ungulates in sub-Saharan Africa. Indeed, it is considered a greater threat than human habitat encroachment to the survival of much African wildlife, both because of its pathology and because of human efforts to stop the natural and necessary seasonal migration of wild ungulates that harbor the virus to prevent reinfection of domestic cattle. This is a prime example of human habitat disruption leading to ecological distress. Such disruption can be a major factor in evolution of viral disease as discussed in Chapters 1 and 22.

Filoviruses and their pathogenesis

In 1967 some medical researchers working with Ugandan African Green monkeys (an important experimental animal and source of cultured cells) in Marburg, Germany, and in Yugoslavia contracted a severe hemorrhagic fever that was highly infectious to clinical staff via blood contamina-

tion. A total of 7 of 25 of these workers subsequently died of the infection. Since its first appearance, the infectious agent, termed Marburg virus, has caused several outbreaks of hemorrhagic fevers with similar mortality rates in sub-Saharan Africa, notably in Zimbabwe, South Africa, and Kenya.

In 1976, an outbreak of a similar disease with a significantly higher mortality rate (50%–90%) occurred in Zaire and Sudan. Eventually, over 500 individuals were infected. A virus related to Marburg virus, named Ebola virus, was proved to be the infectious agent by identification of specific antibodies in the blood of victims and survivors. Several sporadic outbreaks of this disease have been reported in Africa since then.

The high mortality rate of Ebola virus infection and its proclivity for spread to hospital workers via contaminated blood, respiratory aerosols, and body fluid contamination have made it a favorite subject for doomsayers and sensationalists in the media. Hollywood entered the scene with the recent movie "Outbreak," which was generally inaccurate and misleading. Still, the properties of the disease and its ease of spread have served as a warning to public health workers and epidemiologists that acute infectious disease is a continuing threat to human society. This threat is generally discussed in Chapter 1. A major source of concern in assessing the risk posed by filoviruses is that the natural reservoir for these viruses has yet to be identified; surveys of antibody titers in a number of wild monkey populations argue against these monkeys being a reservoir. In addition, bats have been investigated and, while they can be infected with the virus in laboratory settings, no virus has been recovered from bats in the endemic areas.

These two viruses, along with a third, Reston virus, which infects humans but causes no marked disease, are members of a group of nonsegmented, negative-sense RNA viruses called filoviruses. These viruses are characterized by a very flexible virion that assumes characteristic comma and semicircular shapes in the electron microscope. The viral genome is about 19 kb long and encodes a polymerase (Pol), a glycoprotein (G), nucleoprotein (NP), and four other structural proteins (VP40, VP35, VP30, and VP24) in the following order: 5′-Pol-VP24-VP30-G-VP40-VP35-NP-3′.

This gene order and the general structure of the genome are quite reminiscent of those seen with other viruses of the Mononegavirales superfamily, and while there is little known about the details of the replication cycle, it can be assumed to be similar to the cycles described for rhabdoviruses and paramyxoviruses. Indeed, workers in Germany recently showed that the mRNA for a variant of the viral glycoprotein is modified by an editing reaction similar to that described for mumps virus.

Bornaviruses

The bornaviruses are a fourth member of the Mononegavirales superfamily. They have only recently been subjected to careful molecular biological study, but the following facts are known. They cause a variety of neurological symptoms in all warm-blooded vertebrates infected by them. Infection can also lead to behavioral modifications ranging from minor to severe, although the aggressive frenzy seen in the late stages of rabies is not seen.

The bornavirus genome is approximately 9 kb long and encodes six genes, including envelope proteins, other structural proteins, and a viral polymerase. Bornavirus mRNAs are capped and polyadenylated, and are the only nonsegmented negative-sense RNA viruses that use the nucleus of the infected cell as a site of replication. The best-characterized group of negative-sense RNA viruses that do this are the orthomyxoviruses described later—these have segmented genomes. Like mRNA expression by these viruses, some bornavirus positive-sense RNAs generated from genomic negative-sense strand are spliced in the nucleus, but in contrast, bornavirus mRNAs are capped by the viral-encoded polymerase instead of utilizing cellular caps.

Interest in further characterization of these viruses has been heightened by the finding that they can infect humans. Since horses, sheep, and cattle are frequent reservoirs, this puts agri-

cultural workers at risk. Recently, bornavirus infections of nomadic horsemen of central Asia were suggested to be a factor in certain prevalent forms of mental illness. If this suggestion is supported by firm evidence, it would be the first clear indication of a viral source of mental disease in humans.

INFLUENZA VIRUSES – NEGATIVE-SENSE RNA VIRUSES WITH A MULTIPARTITE GENOME

The negative-sense RNA viruses with monopartite genomes share enough similarities to allow their grouping into a superfamily, the Mononegavirales. In contrast, the three major groups (orthomyxoviruses, bunyaviruses, and arenaviruses) have not been convincingly grouped into a single superfamily. Despite this, the negative-sense RNA viruses with multipartite genomes also share some features in replication strategies and genomic sequence.

Due to periodic and frequent spread through the human population, influenza (flu) virus infections are almost as familiar to the human population as are colds. Influenza virus is the prototype of the orthomyxovirus group. There are three distinct types of influenza virus: types A, B, and C. Type A is usually responsible for the periodic flu epidemics that spread through the world, although type B can also be an agent. Influenza types A and B have eight genomic segments, and type C has seven.

The suffix-*myxovirus* was originally coined to group these viruses with the paramyxoviruses since both were associated with respiratory infections and both are enveloped and therefore, readily inactivated with lipid solvents. While these two groups share some general features of structural organization and proteins of related sequence, they are not at all closely related. Similarities and differences between these two groups of viruses are shown in Table 16.1.

The influenza A (flu virus) virion, which is shown along with the genes encoded in its eight genomic negative-sense RNA segments in Fig. 16.5, looks somewhat like a small version of a paramyxovirus virion. As noted in Table 16.1, several of the membrane envelope proteins in these two virus groups clearly are related. Despite this, the replication details are quite different. Flu virus mRNA is generated from transcription of separate and individual flu RNPs in the infected cell's nucleus.

Involvement of the nucleus in flu virus replication

Despite some general similarities with VSV in transcription of the genomic negative-sense strands of influenza virus to generate mRNA, there are important differences in the overall replication process. A major difference is that influenza virus mRNA synthesis and genome replication require the cell's nucleus. There are two readily apparent reasons for this. First, flu replicase cannot cap

Table 16.1 Similarities and differences of orthomyxovirus and paramyxovirus.

Similarities	Differences
RNA genome is single stranded, negative sense.	Orthomyxovirus mRNA can be spliced.
They both have a helical nucleocapsid.	Orthomyxoviruses have a segmented genome.
They both have virion-associated transcriptase.	Orthomyxoviruses require a nucleus for replication.
Virion buds from the cell surface.	Orthomyxovirus mRNA requires cellular caps (cap stealing).
They both have two related glycoproteins: neuraminidase and hemagglutinin.	

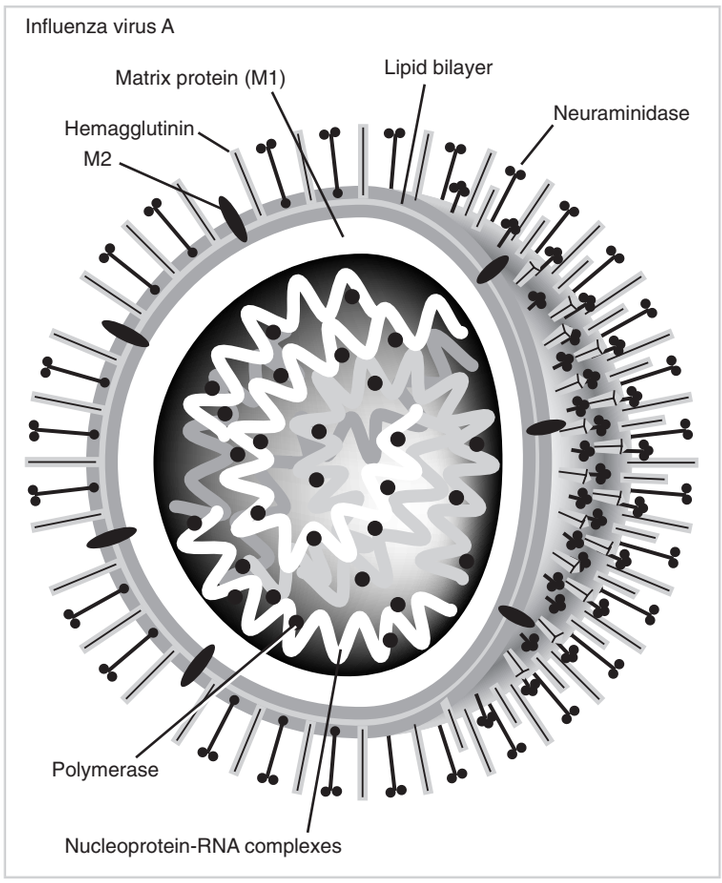


Fig. 16.5 The structure of influenza virus A. The virion is about 120 nm in diameter, and the genome is made up of eight helical nucleocapsid segments that total about 13,600 nucleotides of negative-sense strand RNA. The virus requires the nucleus for replication. Although these virions also exhibit neuraminidase and hemagglutinin, the glycoproteins responsible are separate.

mRNA; therefore, each flu virus mRNA generated has to use a cellular mRNA cap as a "primer." Synthesis of each flu virus mRNA begins with a short stretch of cellular mRNA with its 5′ methylated cap. This **cap snatching or stealing** is a form of intermolecular splicing, and is accomplished by the flu virus replication-transcription complex as it associates with actively transcribed cellular mRNA. Thus, the virus inhibits cellular mRNA transport and protein synthesis, but not initiation of transcription.

Second, influenza A virus utilizes the intramolecular splicing machinery of the host cell's nucleus. Two of the RNPs of the flu virus express mRNA precursors that are spliced in the nucleus. Each of these gene segments, then, can encode two related proteins. This splicing takes place via cellular spliceosomes in a manner identical to that described in Chapter 13. The result of the splices is that two segments of the viral genome actually generate four distinct mRNAs. Thus, with influenza A, the eight flu virus negative-sense genomic segments encode 10 specific mRNAs that are translated into distinct viral proteins.

Generation of new flu nucleocapsids and maturation of the virus

An abbreviated schematic of the influenza A virus replication cycle in a susceptible cell is shown in Fig. 16.6. Infection is initiated by virus attachment to cellular receptors followed by receptor-mediated endocytosis. The separate RNPs with their negative-sense genome segments are transported to the nucleus where viral mRNA synthesis begins.

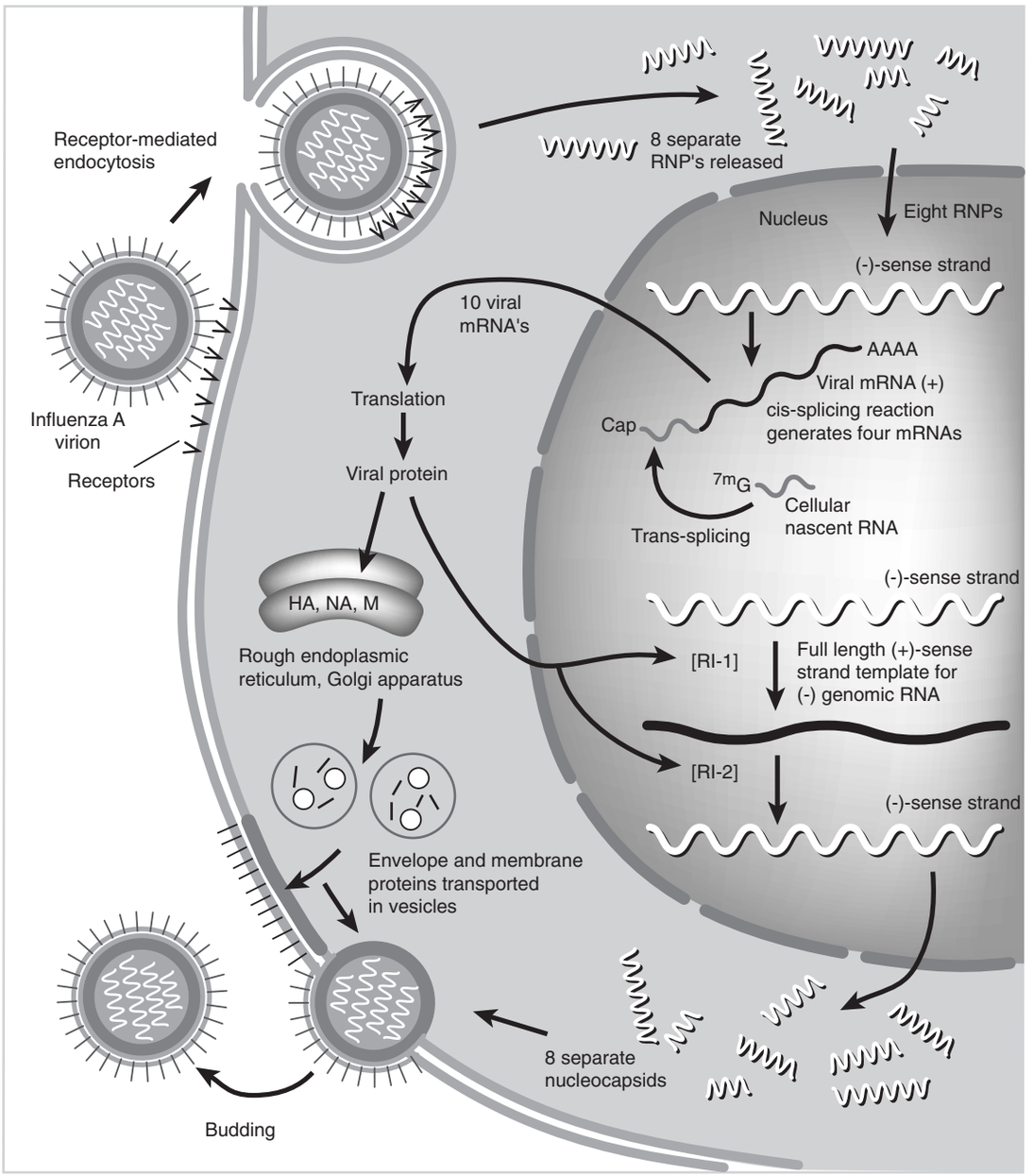


Fig. 16.6 An outline of the replication cycle of influenza. Following virus attachment to its cellular receptor(s) and endocytosis, the envelope fuses with vesicular membrane. The released ribonucleoprotein (RNP) capsid segments, each containing a specific negative-sense genomic segment, migrate to the nucleus where transcription of positive-sense RNA takes place using virion-associated transcriptase. The transcription and formation of mRNA require the "snatching" or "stealing" of caps of nascent cellular mRNA by a *trans*-splicing mechanism. Two of the pre-mRNAs generated in this way are further subjected to one of two alternative *cis*-splicing reactions using cellular machinery, so that each generates two separate mRNAs. Translation of viral proteins leads to proteins that modify the cell and its plasma membrane. The viral proteins associated with the nucleocapsid RNPs migrate to the nucleus where they mediate the synthesis of full-length positive-sense template and synthesis of negative-sense strand genomic RNA. Viral membrane-associated proteins are translated on the rough endoplasmic reticulum and processed in the Golgi apparatus. New virions form by the association of the nucleocapsids with virus-modified membrane and budding. Influenza A virus does not control this aspect of packaging; therefore, phenotypic mixing is frequent following mixed infection. (NA, neuraminidase; HA, hemagglutinin; M, matrix protein.)

Viral mRNA synthesis requires the activity of at least two influenza virus polymerase subunits; PB1 and PB2. PB1 has active sites that bind the conserved 3′ and 5′ sequences of vRNA, as well as the endonuclease activity necessary to cleave the host cap sequence. In addition, PB1 has the polymerizing activity of the complex. PB2 has cap binding activity and it is to this subunit that the host pre-mRNA binds. Cleaving of the small (1 to 13 nucleotide) cap structure from the host begins the process of mRNA synthesis, during which a capped, subgenomic copy of the vRNA is produced. Synthesis stops about 15 to 22 nucleotides short of the 5′ end of the vRNA, where a small (4 to 7 nucleotide) U region serves to causes stuttering or reiterative synthesis, producing a poly[A] tail, a mechanism similar to the one we saw earlier for the rhabdoviruses.

At some point, viral RNA synthesis must switch from making mRNA to making full length template RNA and then new vRNAs. This switch requires the presence of multiple copies of the viral protein NP, as well as the polymerase subunit PA. A complete model of this change to full length synthesis has not yet been worked out. However, the synthesis would require the formation of RI-1 and RI-2 intermediates.

Since all viral RNA synthesis takes place in the nucleus, it is necessary that newly replicated genomes be transported to the cytoplasm for maturation of new virus particles. This transport takes place when new vRNA molecules complex with two viral proteins: M1 and NS2. The NS2 protein contains a nuclear export signal that interacts with a cellular nuclear export protein (an exportin) and likely also overrides the nuclear localization signals present on the NP and polymerase proteins.

Flu nucleocapsids that have been assembled in the nucleus and transported into the cytoplasm migrate to the cell's surface where virions bud off. Control of the number of segments getting into each flu virion is sloppy. Many virions are generated with multiple copies of small segments and lacking one or more large segment.

Influenza A epidemics

Flu is generally considered to be a mild disease, but influenza can be a major killer of the aged and the immune compromised. Even though the body mounts a strong and effective immune reaction to influenza infections, and the individual is immune from reinfection upon recovery, the virus is able to mount periodic epidemics in which prior immunity is no protection. The solution to this apparent enigma is found in the broad host range of influenza A and the unique ability of influenza A (but not B or C) genomic segments to be independently packaged into individual virion particles during infection. Such a situation leads to a very inefficient packaging process, but allows for rapid dissemination of a favorable mutation. If there is a mixed infection of two different influenza A virus strains in the same cell, significant genetic changes can arise and will provide a significant evolutionary advantage to the progeny.

Since most immune protection against a viral infection is directed against surface components of any virus (the membrane glycoproteins in the case of influenza virus), one can predict that the antigenic properties of these surface proteins will change or "drift" over time. This drift is due to the random accumulation of amino acid changes (mutations), along with the slight selective advantage of a virus that has a surface protein not as efficiently recognized by the immune system as those of the virus that induced immunity in the first place. Such drift is found in many viruses and other pathogens.

With influenza A, however, independent packaging of the individual RNPs in the infected cell provides a more rapid means of antigenic variation. There is always the possibility that an individual can be infected at the same time with two different influenza A viruses. This will not happen very often, but if it does, one result of the mixed infection will be the generation of a new hybrid virus that might have, say, a hemagglutinin membrane glycoprotein from one parent and all the other components from the human virus. To add to this, swine influenza virus strains recognize

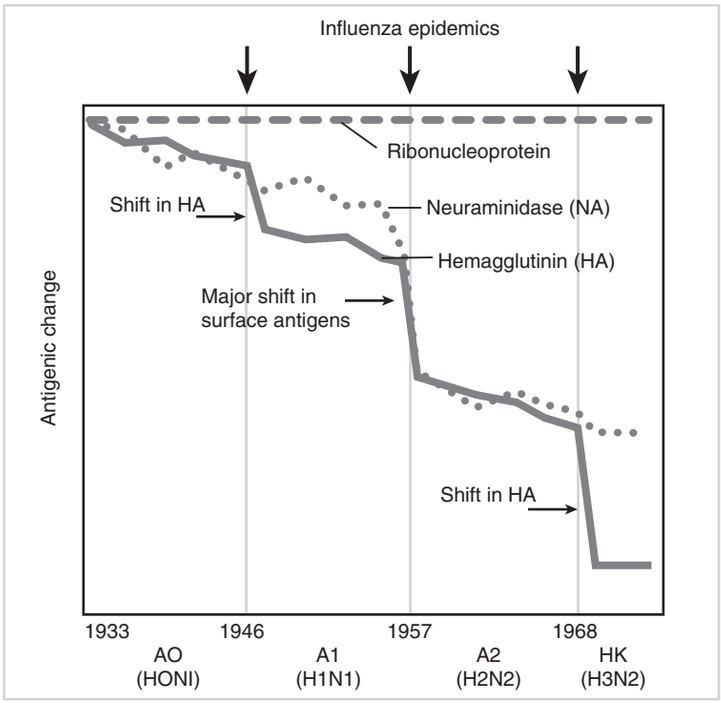


Fig. 16.7 Antigenic changes in the surface glycoproteins of influenza A virus between 1933 and 1968. Abrupt changes in these antigens (antigenic shifts) are the result of mixed infections and random assortment of nucleocapsids to generate novel genotypes. Such shifts, which occur with random frequency, lead to epidemics worldwide. Strain designations at the bottom of the figure indicate hemagglutinin (H) and neuraminidase (N) genotypes.

some of the human cell receptors utilized by their human influenza A virus counterparts. This means that in a farm where pigs are intensely cultivated, a multiple infection could involve a swine virus as well as a human virus. This abrupt change in antigenic nature of the membrane protein is termed **antigenic shift**.

The problem with antigenic shift is complicated by the fact that pigs (but not humans) have efficient receptors for avian influenza viruses. Therefore, a multiple infection in pigs with different avian strains or avian and porcine strains can lead to a very significant reassortment of different markers. This can happen with some frequency in areas in which there is very intense farming and animal husbandry in relatively limited spaces, which is typical of many small farms in East Asia where pigs, ducks, chickens, and other animals are all tended together.

Upon antigenic shift, the resulting successful virus is essentially a "new" virus, and is relatively unaffected by the immune defenses mounted against earlier forms of virus. Thus, the new virus can spread throughout the population despite the high level of immunity to prior forms of influenza A. The timing of the occurrence of such new viruses cannot be predicted, but can be readily quantified by measuring the antigenic reactivity of viral components to various standard immune reagents generated against earlier forms of the virus. When such a new virus is seen, an epidemic can be predicted.

The immunological variation of various flu virus proteins from virus isolated over a considerable period of time is shown in Fig. 16.7. When both the hemagglutinin and neuraminidase components change together (as in generation of the influenza A2 virus in 1957), a major worldwide epidemic (pandemic) can occur. Note that the interior RNP is antigenically stable. One reason for this stability is that there is little humeral immune reaction to these components of the virus because they are not efficiently presented at the infected cell surface by MHC class I; therefore, there is little or no pressure to change. Indeed, it is the antigenic stability of the RNP that defines the major influenza types. Another factor contributing to the stability of the sequence of the capsid

proteins forming the RNP is that most changes to these interior proteins would interfere with their function, and thus lead to a virus with impaired ability to replicate.

Recently, this process of antigenic variation in influenza A was observed to be even more complicated. In the fall of 1997, avian influenza was diagnosed in humans in Hong Kong. The virus did not pass through a swine intermediary, which makes this the first documentation of direct infection of humans by avian influenza virus. The direct infection is rare as the avian virus appears to be transmitted very inefficiently between humans; despite this, however, several humans died. Notwithstanding the inefficient passage between humans, the high concentration of people in Hong Kong, along with their proclivity for purchasing live poultry for home butchering, led to a worrisome outbreak of the disease. The draconian measures of wholesale slaughtering of all live poultry within the confines of the former British colony appear to have been effective in stopping this outbreak, but the process could well repeat in the future with more ominous results.

OTHER NEGATIVE-SENSE RNA VIRUSES WITH MULTIPARTITE GENOMES

Bunyaviruses

In terms of the number of members, the bunyavirus family (Bunyaviridae) is one of the largest known, with well over 300 serologically distinct viruses. The family itself consists of five separate genera, as listed in Table 16.2. Most members of this diverse family are arboviruses, being transmitted by mosquitoes, ticks, sandflies, or thrips. The hantaviruses, however, are vectored by rodents.

Virus structure and replication

Bunyaviruses all have tripartite, negative-sense RNA genomes. As outlined in Fig. 16.8, the enveloped virions are about 90 to 110 nm in diameter. The membrane contains two viral glycoproteins: G1 and G2. Within the particle are three size classes of circular nucleocapsids, each consisting of one of the genomic RNAs in a helically symmetric complex with the nucleocapsid (N) protein and the viral polymerase (L). Genome sizes and gene products for each of the genera are shown in Table 16.3.

Since these are negative-sense viruses, the first event after infection is transcription. For La Crosse virus, a typical bunyavirus, viral mRNAs are produced from each genome segment, as also shown in Fig. 16.8. Viral messages have 5′ capped termini and 3′ ends with no polyA. The cap structures are derived from cytoplasmic host mRNA by endonucleolytic cleavage. This cap-snatching reaction, although similar to that described for influenza virus, takes place *outside* the nucleus.

Table 16.2 The bunyaviruses.

Genus	Vector	Examples
Bunyavirus	Mosquito	La Crosse encephalitis virus, Bunyamwera virus
Nairovirus	Tick	Dugbe virus, Nairobi sheep disease virus
Phlebovirus	Sandfly	Rift Valley fever virus, Uukuniemi virus
Hantavirus	Rodent	Hantaan virus, Sin Nombre virus
Tospovirus	Thrip	Tomato spotted wilt virus

Table 16.3 Genome sizes of gene products of the bunyaviridae.

Gene or Protein	*Bunyavirus*	*Nairovirus*	*Phlebovirus*	*Hantavirus*	*Tospovirus*
L RNA	6.4–6.7 kb	12 kb	6.4–6.7 kb	6.4–6.7 kb	8.9 kb
L protein	240–260 kd	460 kd	240–260 kd	240–260 kd	331 kd
M RNA	4.5 kb	4.9 kb	3.2–3.9 kb	3.6 kb	4.8–4.9 kb*
G1	108–120 kd	68–76 kd	55–70 kd	68–76 kd	78 kd
G2	29–41 kd	30–45 kd	50–60 kd	52–58 kd	52–58 kd
NS_M	10–16 kd	None	78 kd, 14 kd	None	34 kd
S RNA	0.98 kb	1.8 kb	1.7–1.9 kb*	1.8 kb	2.9 kb*
N	19–25 kd	48–54 kd	24–30 kd	48–54 kd	28.8 kd
NS_S	10–13 kd	None	29–37 kd	None	52.4 kd

* Genes are ambisense.

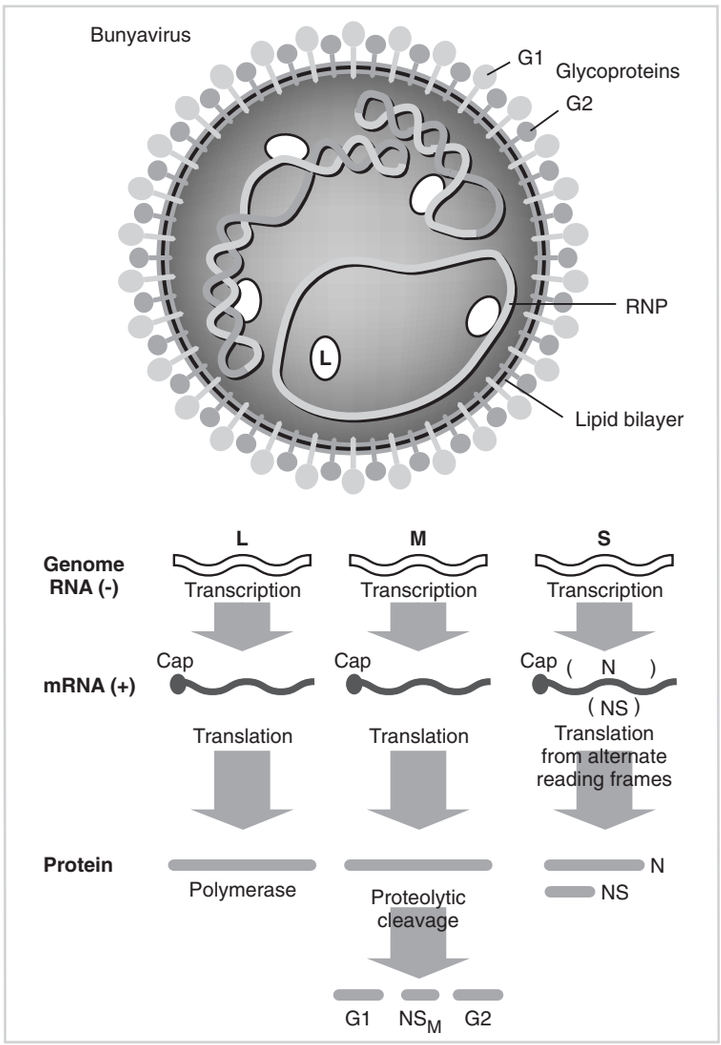


Fig. 16.8 The bunyavirus virion. The three ribonucleoprotein (RNP) segments, each associated with both L and N protein, are contained within a well-defined envelope made up of two glycoproteins. The virion diameter ranges from 80 to 120 nm. The size of the RNPs as determined by their sedimentation rates (see Chapter 11) and the size of the RNA genomes and the proteins encoded by the various members of the Bunyaviridae are shown in Table 16.3. The general scheme of gene expression and genome replication of La Crosse virus is also shown. Expression and replication take place in the cytoplasm, but have many similarities to the process outlined for influenza virus. The positive-sense strand mRNA expressed from the S genomic segment contains two partially overlapping translational reading frames that are out of phase with each other. Alternative recognition of one or the other translation initiation codons by the cellular ribosomes leads to the expression of two proteins with a completely different amino acid sequence.

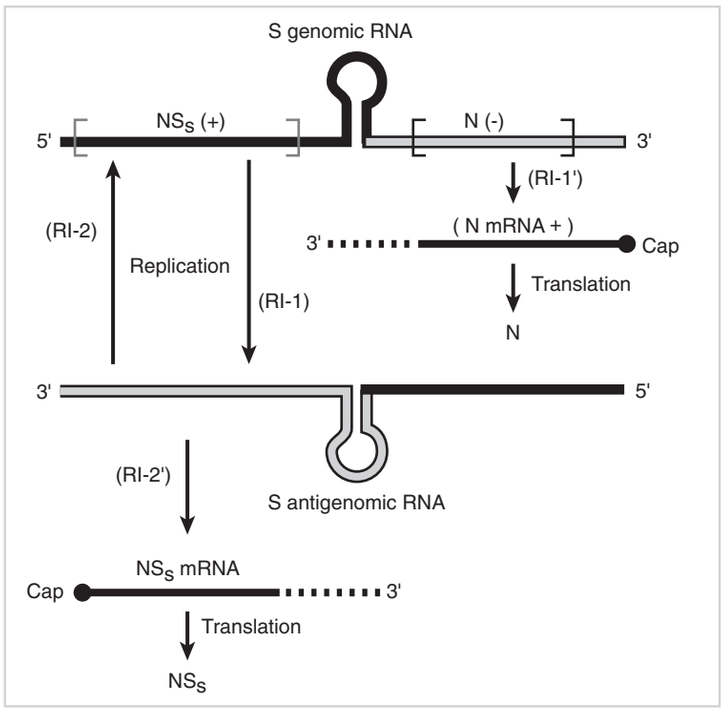


Fig. 16.9 The ambisense strategy of gene expression exhibited by some bunyaviruses and by arenaviruses. The expression of the small genomic segment of a tospovirus as phlebovirus is shown. With these viruses, full gene expression requires the generation of a subgenomic mRNA of same sense as the genomic RNA. Thus, even though the genomic RNA is nominally negative sense, it has regions of positive-sense information in it! This strategy is referred to as *ambisense* since both senses are present in the genome.

The viral mRNAs are subgenomic, as with influenza. Replication of the bunyavirus genomic (and antigenomic) RNA occurs in the cytoplasm. These RNAs have 3′ and 5′ inverted complementary sequences of about 10 to 14 nucleotides that may play a role in the replication event. The nucleocapsids themselves have a circular form that may reflect base pairing of these sequences.

The three genome segments demonstrate a variety of expression strategies; some of these are also shown in Fig. 16.8. The gene products expressed are shown in Table 16.3. The largest segment expresses a single protein, the viral polymerase (L). The middle-sized segment encodes two or three proteins, depending on the specific virus in question. Expressed proteins are the two glycoproteins G1 and G2, along with—where present—a nonstructural protein NS_M. These proteins are translated as a precursor polyprotein that is posttranslationally cleaved.

The smallest RNA genome segment encodes one or two viral proteins. For the nairoviruses and hantaviruses, this segment expresses mRNA for the N protein. In *Bunyavirus* genus, the subgenomic RNA from this segment can be translated into the N protein or, using a separate, alternate reading frame, into another nonstructural protein, NS. Apparently the "decision" as to which reading frame is utilized in this small mRNA is entirely random. Sometimes the ribosome starts at one AUG and sometimes at the other.

The small genomic segments of the phleboviruses and the tospoviruses are **ambisense genomes**; i.e., they contain both positive- and negative-sense genes. The term "ambisense" refers to the fact that the open reading frames defining the two proteins are oriented in opposite directions in the genome RNA, and their expression requires a strategy that is vaguely reminiscent of that utilized in the expression of Sindbis virus subgenomic RNA. This is shown in Fig. 16.9. The small (S) virion-genomic RNP is transcribed into a positive-sense mRNA that is translated as the N protein encoded within the negative-sense portion of the ambisense virion genomic segment. The genomic ambisense RNA also serves as the template for the transcription of a separate ambisense antigenomic RNA that acts as a template for the transcription of capped mRNA encoding the NS_S (nonstructural S) protein. This RNA is the same sense as the virion RNA; thus, even though

Phlebo- and Tospoviruses are negative-strand RNA viruses, a portion of their genome is mRNA (i.e., positive) sense.

Pathogenesis

Members of the bunyavirus family infecting vertebrates cause four kinds of disease in humans and other animals: encephalitis, hemorrhagic fever, hemorrhagic fever with renal involvement, and hemorrhagic fever with pulmonary involvement. La Crosse encephalitis virus is transmitted by mosquitoes and is one of the main causes of viral encephalitis during spring and summer in the upper Midwest. Rift Valley fever virus, transmitted by the sandfly, causes recurring zoonoses and epidemics of hemorrhagic fever in sub-Saharan Africa. Hantaan virus, transmitted by rats, is the prototype of the *Hantavirus* genus and causes Korean hemorrhagic fever, a disease complicated by renal failure.

Recently, Sin Nombre virus, another member of the *Hantavirus* genus, was identified as the causative agent of outbreaks of a relatively fatal hemorrhagic fever with pulmonary involvement, termed *hantavirus adult respiratory distress syndrome (HARDS).* This and related viruses, transmitted by aerosols from fecal pellets of small rodents such as the deer mouse, are found distributed throughout the United States, although localized epidemics of HARDS have occurred in areas such as the Southwest. Epidemiological investigations of these outbreaks suggest that increases in the rodent vector population (aided by sporadic mild wet winters that increase forage for the rodents) result in increasing likelihood of transmission to humans.

Arenaviruses

Arenaviruses have bipartite, single-strand, negative-sense RNA genomes contained as helical nucleocapsids within an enveloped particle 90 to 100 nm in diameter. The virions also contain a number of host cell ribosomes accidentally packaged with the finished particles. These ribosomes play no role in the virus infectious cycle. The presence of these ribosomes gives the virus particles a "sandy" appearance in electron micrographs, leading to the name of the family (*arena* is the Latin word for "sand").

Virus gene expression

The largest genome segment (7.2 kb) encodes two proteins: the viral polymerase, L, and a smaller regulatory protein, Z. The small genome segment encodes the glycoprotein precursor, ultimately cleaved into the two membrane proteins, GP1 and GP2, as well as the nucleocapsid protein, NP. In each case, the two open reading frames contained within the genome segment are arranged in an ambisense fashion. In each case, there is a stretch of RNA between the two genes that consists of a hairpin loop structure that may play a role in regulating the termination of mRNAs transcription.

Primary transcription of the genome produces subgenomic mRNAs for the L and NP proteins. This is followed by transcription from the antigenome RNAs to yield the subgenomic mRNAs for Z protein and the glycoprotein precursor. The virus's mRNAs have methylated 5′ caps that may be derived from host messages. The 3′ ends of viral mRNAs are not polyadenylated. Replication of viral genomes may involve inverted terminal complementary sequences, as described for the bunyaviruses.

Pathogenesis

Lymphocytic choriomeningitis virus (LCMV) causes a mild, influenza-like disease in mice and humans, although rare and severe encephalomyelitis has been observed. At the other end of the

spectrum are severe and often fatal diseases caused by agents such as Lassa fever virus in West Africa and agents of the South American hemorrhagic fevers: Junin virus (Argentina), Machupo virus (Bolivia), and Guanarito virus (Venezuela).

A very interesting aspect of these viruses' pathogenesis (as outlined in Chapter 7) is that infection of infant animals (whose immune system is still developing) generally leads to persistent infections. If, however, the virus infects an adult animal with a fully functioning immune system, rapid death follows. Wild populations harboring the virus can secrete large amounts of virus that can be lethal to humans or other animals interacting with them. This is one of the reasons why habitat destruction in Africa with its accompanying disruption of native rodent populations that are chronic carriers of the virus has led to periodic outbreaks of arenavirus-induced fatal disease.

VIRUSES WITH DOUBLE-STRANDED RNA GENOMES

The family Reoviridae contains nine distinct genera with infectious agents specific for vertebrates (reoviruses and rotaviruses), invertebrates (cytoplasmic polyhedrosis virus) and plants (wound tumor virus). Members of this family have genomes consisting of 10, 11, or 12 segments of double-stranded (ds) RNA. There is a group of bacterial viruses, many infecting *Bacillus subtilis*, which also contains segmented, double-stranded genomes. The replication strategy employed by these viruses must take into account that the genome is dsRNA, which is extremely stable, and consequently difficult to dissociate into a form exposing a single-stranded template for RNA-directed mRNA transcription.

Reovirus structure

Reovirus contains 10 dsRNA segments. A schematic of the virion and the protein coding strategy of the genomic segments is shown in Fig. 16.10. These genome segments of the reoviruses are packaged into an icosahedral capsid that consists of two — or in some members, three — concentric shells, each having icosahedral symmetry. The capsid is made up of three major structural proteins, as well as a number of low-abundance structural proteins, including virion-associated transcriptase, as the virions contain all of the enzymatic machinery necessary for the production of viral mRNA, including activities involved in capping and methylation. Genome segments range in size from about 4 kbp to about 1 kbp. The genomic RNAs have 5′ methylated caps on the positive-sense strand of the duplex and a 5′ triphosphate on the negative-sense strand. Neither strand is polyadenylated.

The reovirus replication cycle

Some features of the replication of reovirus in the infected cell are shown in Fig. 16.11. After attachment and entry into the host cell cytoplasm via receptor-mediated endocytosis, reovirus particles are partially uncoated, leaving behind an inner-core subviral particle. This subviral particle contains the 10 genome segments and transcriptional enzymes. Production of mRNAs occurs by the copying of one strand of each duplex genome into a full-length strand. The mRNAs are capped and methylated by viral enzymes but do not have polyadenylated 3′ termini. These transcriptional events require six viral enzymes, including a polymerase, a helicase, an RNA triphosphatase, a guanyltransferase, and two distinct methyltransferases. The latter three enzymes are all involved in the capping reaction.

Each of the genome segments encodes a single transcript that is translated into a single protein, except for one of the smaller segments (S1) of the *Orthoreovirus* genus. This segment encodes two proteins encoded in two nonoverlapping translational reading frames. Both proteins are encoded by the same mRNA by virtue of random recognition of either of the two translation initiation

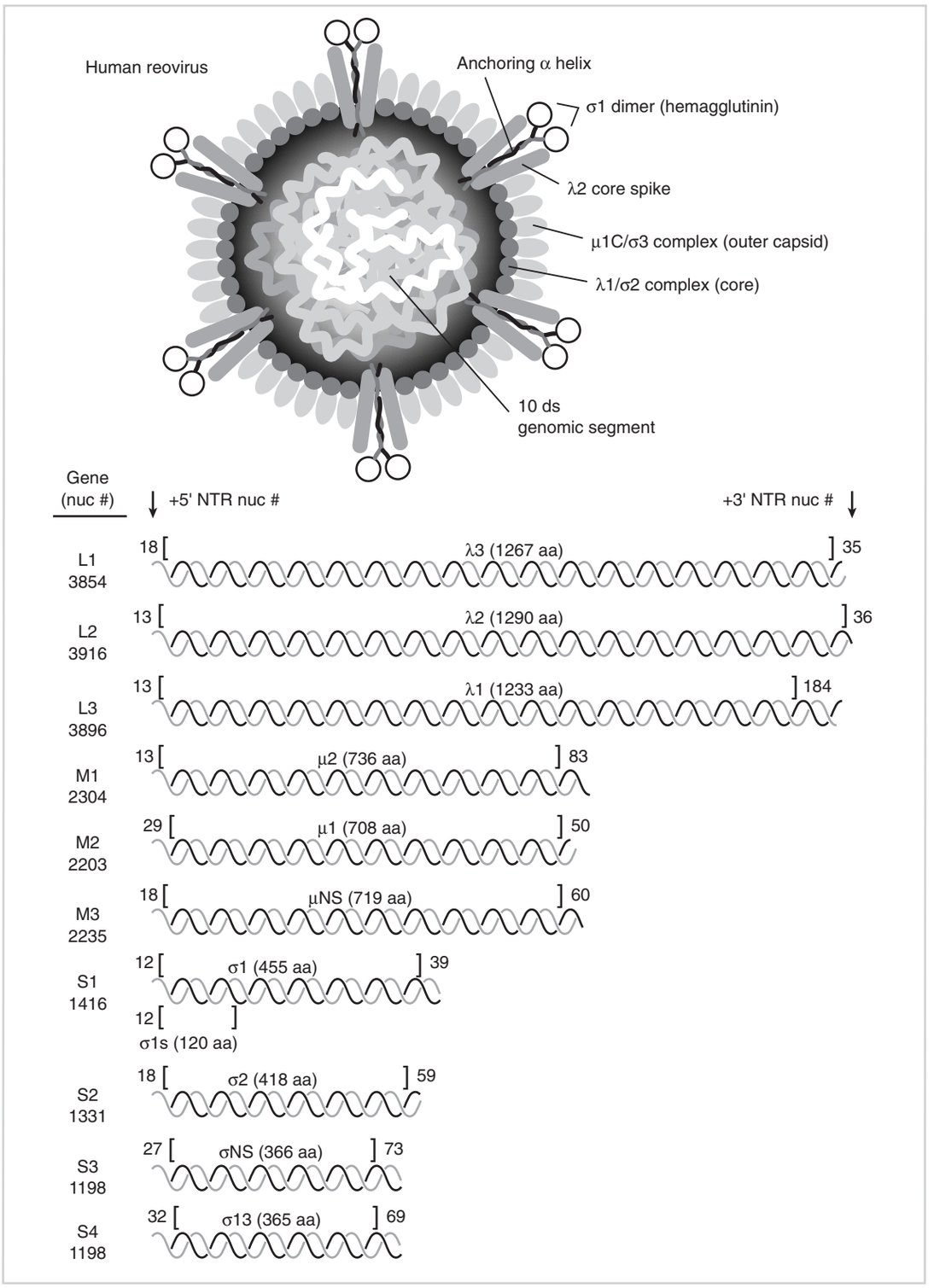


Fig. 16.10 The 60 nm-diameter human reovirus with its double shell. The 10 segments of the reovirus genome and the proteins encoded are shown. Note that the S1 segment encodes two overlapping translation frames. Like the situation with the La Crosse virus mRNA encoded by the S genomic fragment, these proteins are expressed by alternate initiation sites for translation. Thus, the virus encodes 11 proteins. The total size of the genome is 23,549 base pairs.

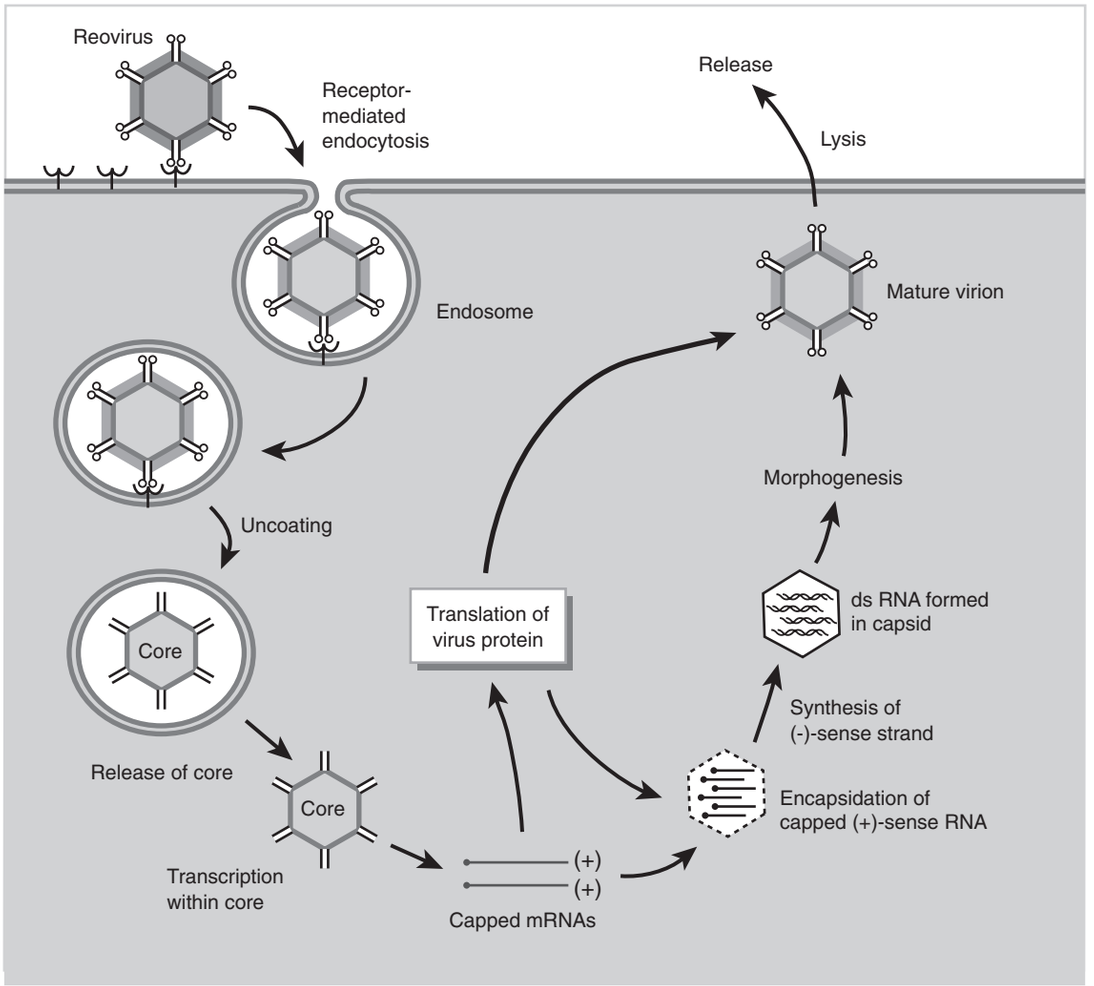


Fig. 16.11 The reovirus replication cycle. Virus attachment is followed by receptor-mediated endocytosis. Virion "core" particles are formed by the degradation of the outer shell in the endosome, and this core particle expresses capped mRNA using a virion transcriptase. Various viral proteins are translated and structural proteins assemble around newly synthesized viral mRNA. This process is apparently random, since random assortment of genetic markers following mixed infection is readily observed (see Chapter 3). The complementary strand of the double-stranded genomic RNAs is synthesized in the immature capsid while morphogenesis proceeds. Virus release is by cell lysis.

codons by cellular ribosomes. Most of the gene products are structural, either forming one of the multiple capsids or comprising the transcriptional complex of enzymes found within the core.

Replication of the double-stranded genomes and final assembly of progeny virions is not completely understood. It is thought that 10 unique mRNAs associate to form a core progeny virion, associating with the appropriate capsid proteins. These positive-sense RNAs then serve as templates for the synthesis of negative-sense strand, leading to the production of progeny double-stranded genomes within the nascent particle.

This rather convoluted means of generating the double-stranded genome is a consequence of the fact that dsRNA will not readily serve as a template for its own synthesis because of its very great stability. The environment inside the capsid is apparently relatively nonaqueous, and in this non-polar space, the dsRNA is more readily denatured due to charge repulsion between the phosphate backbones of the two RNA strands. Thus, the double-stranded genome is able to partially denature

to serve as a template to generate large quantities of positive-sense mRNA that is extruded from the inner core.

Replication of reovirus RNA, then, does not involve RI-1 or RI-2 intermediates. Further, ideally, no free dsRNA is formed inside the cytoplasm of the infected cell, precluding the induction of interferon. In practice, however, this situation is not realized, and many cells infected with reovirus produce significant interferon. While the yield of virus is quite sensitive to the interferon-mediated antiviral state in cells, apparently the major induction occurs rather late in the replication cycle where cellular organization is deteriorating. Thus, the virus is able to keep ahead of the response for a period of time sufficient for efficient replication in the host.

Pathogenesis

The prototype viruses of this family (now grouped in the genus *Orthoreovirus*), although originally isolated from human sources, are not known to cause clinical disease in humans. The name *reovirus* stands for "respiratory enteric orphan virus," an orphan virus being one for which no disease is known.

In contrast, members of the *Rotavirus* genus are perhaps the most common cause of gastroenteritis with accompanying diarrhea in infants and remain among the leading causes of early childhood death worldwide. Other significant pathogens of humans and domestic animals found in this family include Colorado tick fever virus (*Coltivirus* genus) and bluetongue virus of sheep.

SUBVIRAL PATHOGENS

As touched on in Chapter 1, viruses, as efficient and compact as they may be, are not in fact the simplest infectious agents. A number of other entities that are smaller than viruses can cause disease in animals and plants. These agents can be collectively considered to be subviral pathogens. They may contain genetic information for the expression of a protein, or they may express no gene products at all. A number of them may not even be contained within a capsid, and one group, the prions, while able to replicate themselves, does not appear to contain nucleic acid.

Subviral pathogens are parasitic on cellular processes, but if viruses parasitize the ability of a cell to express protein from information contained in nucleic acids, subviral pathogens can be considered to be parasitic on other macromolecular process in the cell, including transcription and protein assembly and folding.

A large number of subviral pathogens lacking capsids are parasitic on plants, and many can cause plant pathology without expressing protein. These agents can be differentiated by a detailed characterization of their modes of replication, but only the viroids are considered in this text because of this group's relationship to the human pathogen hepatitis delta virus (HDV).

Hepatitis delta virus

As briefly outlined in Chapter 4, HDV appears to be absolutely dependent on coinfection with hepatitis B virus (HBV) for spread. Despite this, there are a significant number of cases where it can be inferred that an individual was infected with HDV without any evidence of active or prior HBV infection.

The HDV genome, shown in Fig. 16.12, has very significant similarities with plant viroid RNAs! It is difficult to come up with a convincing scenario that explains how a plant pathogen could become associated with a human hepatitis virus that has certain important similarities to retroviruses (see Chapter 21). The HDV particles are enveloped with a membrane containing the three envelope glycoproteins of HBV. Within the envelope is the HDV nucleocapsid containing a

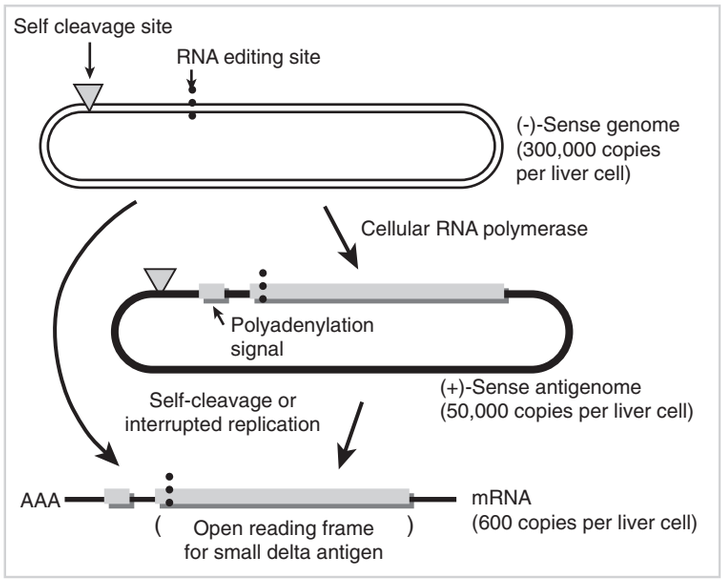


Fig. 16.12 The three RNAs of hepatitis delta virus found in infected liver cells. The genomic negative-sense RNA, which is replicated by means of RNA polymerase II, encodes the antigenomic positive-sense RNA, which is the template for genomes, and a subgenomic positive-sense mRNA. This mRNA is cleaved from the antigenomic RNA by RNA self-cleavage. Further, the RNA can be edited by cellular enzymes so that the first translational terminator can be altered. With such edited RNA, a protein 19 amino acids larger than that expressed from unedited RNA is encoded.

covalently closed, circular, single-stranded 1.7 kb RNA molecule of negative-sense orientation complexed with multiple copies of the major gene product of this RNA, the *delta antigen*.

The circular RNA can form base pairs within itself, forming a rodlike structure reminiscent of plant viroid agents (see below). The delta antigen contains three major structural domains. There are two RNA-binding domains, a nuclear localization signal, and a multimerization domain characteristic of members of proteins in the **leucine zipper** family. Many of these proteins are known to have a role in regulating transcription.

After entry and uncoating, the genome and associated delta antigen are transported to the nucleus of the cell where the replicative cycle begins. The delta virus genome is transcribed and replicated by *host cell RNA* polymerase II! This is truly unique in animal virus systems, and is a major exception to the rule that cells cannot copy RNA into RNA. Somehow this agent has evolved to co-opt one of the three host RNA polymerases for this job.

RNA is transcribed into an antigenome that is positive sense and also a covalently closed circle. Transcription also generates a subgenomic mRNA that is capped and polyadenylated and is translated into the delta antigen. The generation of the subgenomic mRNA may occur by transcription that does not continue to generate the full antigenomic template for transcription of further genomic RNA. Alternatively, it may be generated by the circular RNA acting as a **ribozyme** that autocatalytically cleaves itself into a linear form. This latter, rather bizarre mechanism is known to be the way that unit-length genomic RNA is generated from circular intermediates generated during the replication process. The term *ribozyme* was invented by Thomas Cech to explain the fact that in splicing of fungal pre-mRNAs, the RNA molecules can assume a structure so that they can hydrolyze an internal phosphodiester bond without the mediation of any protein at all. He was awarded the Nobel Prize for this discovery.

The delta antigen comes in two forms, a small version (195 amino acids) and a somewhat larger version (214 amino acids). The two forms differ by 19 amino acids, and translation of the larger form results from an RNA editing reaction that changes a UAG stop codon into a UGG. This editing suppresses the termination codon and allows continued translation. The short form of the delta antigen is required for genome replication while the long form suppresses replication and promotes virus assembly.

HDV is spread by blood contamination and causes a pathology much like that of other hepatitis viruses, resulting in liver damage. The severity of this disease results from coinfection with HBV or superinfection of an HBV-positive patient with HDV. In this latter situation, fatality rates can be as high as 20% and virtually all survivors have chronic hepatitis.

While HDV pathology requires coinfection with HBV, this does not explain occurrence and spread of the virus. The virus is found in indigenous populations of South America and is prevalent in Europe, Africa, and the Middle East, but is relatively uncommon in Asia, where there is a high frequency of endemic HBV infections. There may be some way the virus can be maintained and spread without HBV, or it may be able to replicate asymptomatically in some hosts who are also asymptomatically infected with HBV.

Viroids

Plant viroids are infectious agents that have no capsid and have an RNA genome that encodes no gene product; they do not require a helper for infectivity. Potato spindle tuber viroid is the prototype of this class of agents. The viroids are covalently closed, circular, single-stranded RNAs, 246 to 375 nucleotides long, whose sequence is such that base pairing occurs across the circle, as shown in Fig. 16.13. As a result, these agents have the form of a dsRNA rod with regions of unpaired loops. Their replication is carried out by plant RNA polymerase, and likely proceeds through an antigenome. Large multimeric structures can be observed in infected plant nuclei, and self-cleavage of such multimers into unit-length RNA molecules is involved in "maturation" of the infectious form.

Viroids spread from plant to plant through mechanical damage caused by insects or by cultivation. They are also spread by propagation of cuttings from infected plants. Viroids may also be present in seeds. Very often, viroids are transmitted during the manipulation of crop plants for harvest, as is the case with the coconut Cadang-Cadang viroid, transmitted from tree to tree on the metal spikes harvesters wear on their shoes to climb the trunk.

More than 20 viroids have been described infecting a wide variety of plant species. Many of these have great agricultural significance and are known to destroy fields of economically important crops. The actual mechanism of their pathogenesis is obscure but it clearly involves specific sequences within the viroid RNA, as there are examples where a viroid RNA with sequence very similar to a pathogenic one is not pathogenic and can provide some protection to the host plant.

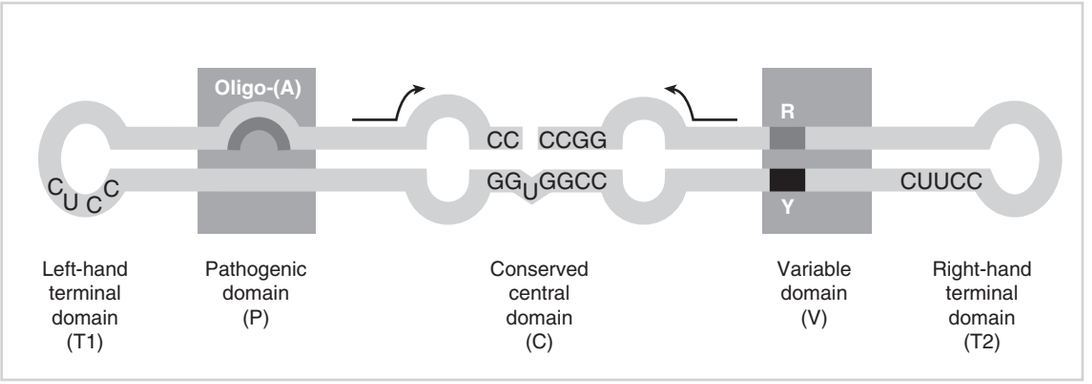


Fig. 16.13 The potato spindle tuber viroid genome. Various pathogenic strains range from 250 to 360 nucleotides in length. This circular RNA does not encode a protein, but the sequences indicated as pathogenic are required to cause the disease. Modification of these sequences leads to a viroid that is nonpathogenic and can protect the plant from pathogenesis by the original viroid. Viroid RNA is replicated with cellular RNA polymerase, forming large multimeric structures of both positive and negative sense. Individual viroid RNA is released by RNA self-cleavage.

It has been postulated that pathogenic regions of the viroid RNA interact with one or more host factors, but this has not been demonstrated.

Prions

As noted earlier, HDV utilizes an envelope borrowed from a helper virus, and itself encodes only one gene product. Pathogenic and nonpathogenic plant viroids are able to propagate their genomes without encoding capsid or any other protein. Prions form a logical limit to how simple a pathogen's structure can be. Prions are infectious agents that do not appear to have nucleic acid genomes!

Unfortunately, this simplicity does not mean that investigation of the problem of prion pathogenicity is itself simple. Prion-based diseases have a very long incubation time, and the biological assay is slow and expensive. Further, the fact that prion-induced disease is mediated by protein means that the infectious agent is extremely difficult to inactivate. Most methods for sterilization of infectious agents are ineffective for prions.

The name *prion* was coined by Stanley Prusiner (who won the 1997 Nobel Prize in medicine for his studies) as an acronym for *proteinaceous infectious particle*. Prions are the causative agents of a series of spongiform encephalopathies, including scrapie disease of sheep, Kuru and Creutzfeld-Jakob disease (CJD) of humans, and bovine spongiform encephalopathy (BSE), popularly termed "mad cow disease."

It is fair to argue that these infectious agents are not viruses in any real sense of the word. Still, the fact remains that many techniques for the study of their structure, propagation, and pathogenesis are based on the study of viruses, and prions, perhaps arbitrarily, are included in most compendiums describing virus replication and virus-induced disease.

Prions are most consistently characterized simply as copies of a single host protein that can assume more than one structure (or **isoform**) upon folding after translation. Thus, the DNA sequence that originally encodes the prion is a part of the host genome itself. One isoform is benign while the other induces cytopathology.

Scrapie, the prion-based disease of sheep, has been investigated most thoroughly, but it is assumed that the agents of all the other diseases are similar if not identical. The protein in question, called PrP, is a normal gene product found in the brain where it is synthesized and degraded in a manner similar to many other proteins characterized by dynamic turnover in the cell. When PrP is changed to the infectious form, called PrP_{Sc} (in the case of scrapie) or PrP_{CJD} (in the case of Creutzfeld-Jakob disease), the protein is converted into the pathogenic isoform.

Whereas PrP is normally stable in its benign configuration, certain alterations in a single amino acid caused by a heritable mutation can lead to an unstable protein. This unstable protein can spontaneously convert to the pathogenic form with some low frequency. The properties of this converted protein differ in many ways from those of the normal form (for instance, in solubility and protease resistance). It is thought that accumulation of this abnormal form in the brain leads to cell death and the characteristic neurological symptoms of prion-based disease.

What is most important to spread of the disease is that the abnormal PrP_{Sc} protein is able to catalyze the conversion of normal PrP to the disease isoform. While this conversion is most efficient in the original animal, the protein can also induce the conversion when introduced into another animal, especially if it, too, contains the critical amino acid.

Although the exact mechanism of this conversion is not clearly understood, models to explain the phenomenon suggest that interaction between the normal and disease forms of the proteins can result in replication of the abnormal form through an intermediate that may normally be part of this protein's degradation pathway.

Transmission of these infectious agents has been clearly demonstrated. For instance, on mink farms, animals given feed that contains waste material from sheep slaughter may contract a prion

disease called "transmissible encephalopathy." Likewise, Creutzfeld-Jakob disease is transmittable from patient to patient by an iatrogenic route, due to contaminated instruments.

As predicted from this model, susceptibility to prion-based diseases in humans and animals is a genetic trait. Still, given a high-enough inoculum, conversion of benign PrP to the pathogenic form can take place even when the original protein substrate does not contain the critical amino acid. Transmission via contamination of neurological probes that have been sterilized normally has been well documented, and occurs with enough frequency to excite real concern.

Recently, in Great Britain, an outbreak of BSE (mad cow disease) resulted from feeding dairy and beef cows with dietary supplements synthesized from the offal and carcasses of scrapie-infected sheep. The practice of using slaughterhouse renderings as a feed supplement has been widespread in animal husbandry, and since scrapie is a relatively common disease in some herds of sheep in Great Britain, the use of contaminated carcasses was well established. The problem arose because of the way this material was rendered. In the past, the offal was rendered by extensive heat treatment, which apparently was sufficient to destroy PrP_{Sc}. In the 1980s, however, the high cost of fossil fuel led English suppliers to use a chemical method of rendering the carcasses that ineffectively inactivated the prion material. The very long incubation period of prion-induced BSE resulted in a long delay before symptoms appeared in British herds.

As damaging as this has been to the English cattle industry, there is an even more serious possibility. There is good documentation that the disease can be transmitted to domestic and zoo cats, and recently, a number of young people in Britain have developed Creutzfeld-Jakob disease. This was never reported to occur in young adults in England previously, and it has been suggested that the cattle disease is transmissible to humans. This possibility has been difficult to substantiate because while the normal incidence of spontaneous Creutzfeld-Jakob disease is very low, the number of new cases does not represent a statistically significant increase. Disturbingly, however, the disease was formerly confined to the elderly, and the occurrence of the disease in young people is worrisome. This concern is enhanced by the fact that the form of prion isolated from young patients has a glycosylation pattern similar to the PrP_{BSE} found in cattle and is significantly different from the glycosylation pattern of PrP_{CJD} isolated from older victims of the disease.

For this reason, the British beef-processing industry has been sorely tested. New national policies concerning the feeding of cows were implemented, and it is currently illegal to purchase certain cuts of beef in England that are considered to be potential carriers of the disease, including cuts with large amounts of bone marrow and nerve tissue. Other countries have banned the importation of British beef.

The rate of occurrence of youth-associated Creutzfeld-Jakob disease has not increased since public health officials have become aware of the problem. But the measures were only implemented after a fairly long period of potential exposure, and the incubation period of the disease may vary greatly in individuals according to their genetic background. Therefore, the actual impact of the introduction of a prion-based disease to cattle is still unknown and a matter of some controversy.

QUESTIONS FOR CHAPTER 16

1 What features of the viral replication cycle are shared by measles virus, vesicular stomatitis virus, and influenza virus?

2 When the *genomes* of negative-sense RNA viruses are *purified* and introduced into cells that are permissive to the original intact virus, what will occur?

3 The Rhabdoviridae are typical negative-sense RNA viruses and must carry out two types of RNA synthesis during infection: transcription and replication. *Briefly* describe each of these modes of viral RNA synthesis.

4 Sin Nombre virus is the causative agent of the outbreak of hantavirus-associated disease that was first identified in a cluster of cases originating in the Four Corners area of the southwestern United States.

a To which virus family does this virus belong?
b Which animal is the vector for transmission of this virus to humans?
c What feature of the disease caused by this virus makes it different from other members of its genus?

5 Bunyavirus gene expression includes three different solutions to the problem of presenting the host cell with a "monocistronic" mRNA. For each of the genome segments (L, M, and S), describe in a simple drawing or in one sentence how this problem is solved.

6 Your laboratory has now become the world leader in research on the spring fever virus (SpFV), especially the debilitating variant SpFV-4 that causes senioritis. Your team has determined that these viruses are members of the family Orthomyxoviridae, but an international commission on virus nomenclature has suggested that they be assigned to a subgenus of the influenza viruses. While you agree with the family designation, you are convinced that they belong to a new genus that you have tentatively called the *Procrastinoviruses*.

The following Table list properties of SpFV strains that your laboratory has investigated.

Viral function		Results for SpFV
A	Virion membrane glycoproteins	Two major proteins, one with hemagglutinin activity and the other with neuraminidase activity
B	Matrix proteins in virion	One matrix protein
C	Genome segments	Eight single-stranded RNA molecules
D	Viral mRNA synthesis	Nuclear location, with cap scavenging from host mRNA precursors and RNA splicing to produce some species of viral mRNA
E	Nonstructural (NS) proteins in infected cells	Three NS proteins, two encoded by RNA segment 8 and one encoded by RNA segment 6
F	Site of infection	Generalized neuromuscular locations, ultimately targeting higher neural functions associated with memory and motivation

a Which of these features justify inclusion of SpFV in the Orthomyxoviridae family?
b Which of these features justify your proposal that SpFV should be considered a new genus of this family?
c You have just received an isolate of SpFV-4 obtained from a severe outbreak of senioritis at a large East Coast university. The epidemic began among a group of students who had just returned from a semester abroad in Paris. As an expert virologist, which viral proteins do you predict are most likely to distinguish this isolate of SpFV-4 from those you have investigated in your laboratory?
d What phenomenon could account for these differences?

Continued

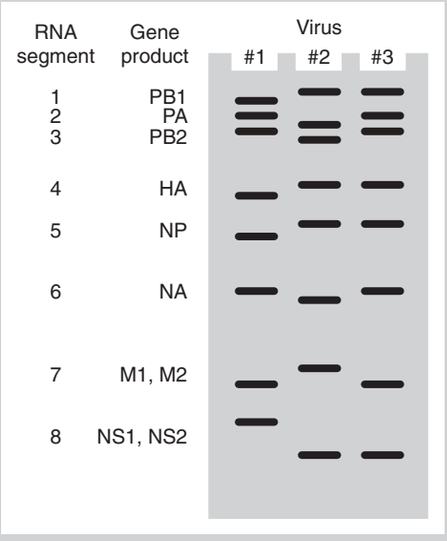
7 What are two differences between the members of the *Hantavirus* genus and members of the other genera of the family Bunyaviridae?

8 Influenza virus will *not* grow in a cell from which the nucleus has been removed. Although influenza virus does not have a DNA intermediate in its life cycle, there is still a requirement for nuclear functions.

a List two molecular events during the influenza virus life cycle that require something provided by the host cell nucleus.

b For which of these events is the *physical presence* of the nucleus in the cell absolutely required? Why?

9 The data shown in the figure below were obtained for three different isolates of influenza type A virus. The three viruses (designated 1, 2, and 3) were grown in cell culture in the presence of radioactive RNA precursors. The radiolabeled RNA genome segments were then separated by electrophoresis through a polyacrylamide gel. The drawing below shows the relative migration in this gel of each of the genome segments. In addition, the segment number and the viral gene product or products produced by that segment are shown.



a In isolates of influenza virus H and N numbers refer to the genotypes of the hemagglutinin and neuraminidase, respectively. Suppose that virus 1 is found to be H1N1 and virus 2 is found to be H2N3. What would be the designation for virus 3?

b The antiviral drug amantidine is used to stop or slow down an influenza virus infection. Virus 1 is sensitive to amantidine, while virus 2 is resistant to this antiviral agent. Your mentor predicts that you will find virus 3 to be sensitive to amantidine. What evidence in this electropherogram leads your mentor to suggest this?

c By what genetic mechanism (typical for the Orthomyxoviridae) did virus 3 arise?

10 Reovirus is the prototype member of the family Reoviridae. Describe the features of this virus that make it different from other RNA genome viruses.

11 Hepatitis delta virus (HDV) is classed as a subviral entity. What is a unique feature of the genome replication of this agent?

12 Viroids are infectious agents of plants and are circular, single-stranded RNA molecules. Describe the features of infection of a plant with this kind of agent.

13 In what sense can a prion be described as a "self-replicating entity?"