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CHAPTER

9

Subviral Agents

INTRODUCTION

This chapter considers a number of infectious agents that are subcellular, but that are not viruses in the strict sense of the term. Some of these are not capable of independent replication but require a helper virus, in which case the agent is effectively a parasite of a parasite. Others replicate independently but use unconventional means to achieve their replication and spread. Many of the agents discussed here cause important diseases in plants or animals, including humans.

The agents to be considered include ***d***efective ***i***nterfering (DI) viruses that arise by deletions and rearrangements in the genome of a virus. DIs require coinfection by a helper virus to replicate. They may play an important role in modulation of viral disease or they may simply be artifacts that arise in laboratory studies. Related to DIs, at least conceptually, are satellites of viruses, which can replicate only in the presence of a helper. Satellites are known for many plant viruses and are known to influence the virulence of the helper viruses. Completely different are agents called viroids. Viroids consist of small, naked RNA molecules that are capable of directing their own replication. They do not encode protein, but instead contain promoter elements that cause cellular enzymes to replicate them. Many are important plant pathogens. Related to viroids are virusoids, which are satellites of viruses that resemble packaged viroids. There are also agents that combine the attributes of both satellites and viroids, such as hepatitis delta virus, which is an important human pathogen. Finally, prion diseases, caused by infectious agents whose identity is controversial but which may consist only of protein, are discussed.

DEFECTIVE INTERFERING VIRUSES

Defective interfering viruses are a special class of defective viruses that arise by recombination and rearrangement of viral genomes during replication. DIs are *defective* because they have lost essential functions required for replication. Thus, they require the simultaneous infection of a cell by a helper virus, which is normally the parental wild-type virus from which the DI arose. They *interfere* with the replication of the parental virus by competition for resources within the cell. These resources include the machinery that replicates the viral nucleic acid, which is in part encoded by the helper virus, and the proteins that encapsidate the viral genome to form virions.

DIs of many RNA viruses have been the best studied. Because DI RNAs must retain all *cis*-acting sequences required for the replication of the RNA and its encapsidation into progeny particles, sequencing of such DI RNAs can provide clues as to the identity of these sequences. Identification of *cis*-acting sequences is important for the construction of virus vectors used to express a particular gene of interest, whether in a laboratory experiment or for gene therapy.

The most highly evolved DI RNAs are often not translated and consist of deleted and rearranged versions of the parental genome. In the case of alphaviruses, whose genome is about 12 kb (Chapter 3), DI RNAs have been described that are about 2 kb in length. However, they have a sequence complexity of only 600 nucleotides, because sequences are repeated one or more times. The sequences of two such DI RNAs of Semliki Forest virus (SFV) are illustrated schematically in Fig. 9.1. From the sequences of these DIs as well as DIs of other alphaviruses, specific functions for the elements found in these DIs have been proposed. Other approaches

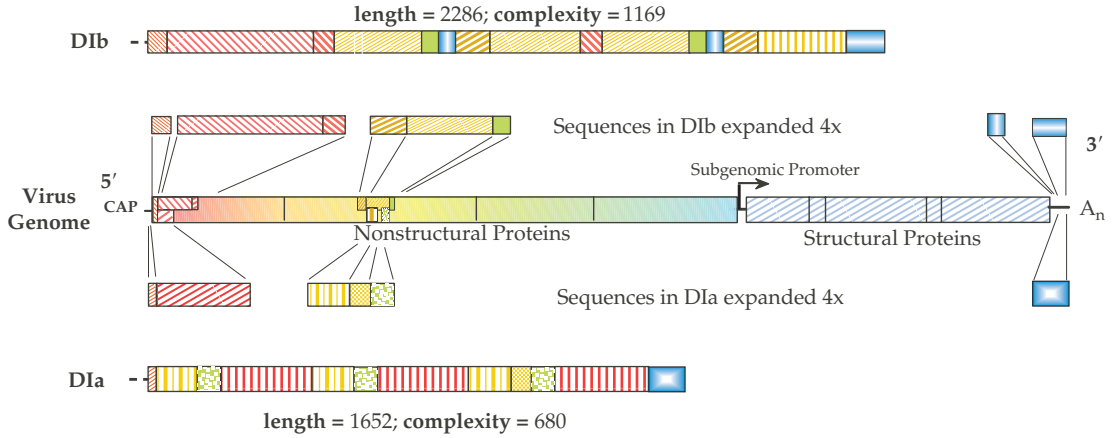

FIGURE 9.1 Schematic representation of DIs (defective interfering particles) found after high multiplicity infection of Semliki Forest virus. The central block shows the genome of the nondefective virus, with vertical lines demarking the four nonstructural and five structural polypeptides. The blocks of sequence found in two different DIs are expanded fourfold below and above. Their location in the DI genome is illustrated with blocks of identical shading. Note that some blocks of unique sequence are repeated three times in DIa and one block is repeated four times in DIb. Adapted from Strauss and Strauss (1997), Figure 1.

have then been used to confirm the hypotheses derived from such sequence studies. Thus, the 3′ end of the parental RNA, which is retained in all alphavirus DI RNAs, forms a promoter for the initiation of minus-strand RNA synthesis from the plus-strand genome. The 5′ end of the RNA is also preserved in many DI RNAs, such as those illustrated in Fig. 9.1. Surprisingly, however, it has been replaced by a cellular tRNA in some DI RNAs. The complement of this sequence is present at the 3′ end of the minus strand, where it forms a promoter for initiation of genomic RNA synthesis. The finding that the DI RNAs with the tRNA as the 5′ terminus have a selective advantage over the parental genome during RNA replication suggests that this promoter is a structural element recognized by the viral replicase. It also suggests that the element present in the genomic RNA is suboptimal, perhaps because the genomic RNA must be translated as well as replicated. Finally, repeated sequences from two regions of the genome are present in all alphavirus DI RNAs. It is thought that one sequence (shown as red patterned blocks in Fig. 9.1) is an enhancer element for RNA replication and the second (shown as yellow and green patterned blocks) is a packaging signal. Repetition of these elements may increase the efficiency of replication and packaging of the DI RNA.

Vesicular stomatitis virus (VSV) (Chapter 4) DI RNAs vary in size from a third to half the length of the virion RNA. Some DI RNAs are simply deleted RNA genomes, but others have rearrangements at the ends of the RNAs. Representative examples are illustrated in Fig. 9.2A. During replication of the RNA, the sequences at the ends must contain promoter elements for initiation of RNA synthesis. More genomic RNA (minus strand that is packaged in virions) is made than antigenomic RNA (which functions only as a template for genomic RNA synthesis) and therefore the promoter at the 3′ end of the antigenomic RNA is stronger than the promoter at the 3′ end of the genomic RNA. Thus, it is not surprising that some DI RNAs have the stronger promoter at the 3′ ends of both (+) and (−)RNA (as in Class II DIs), ensuring more rapid replication of the DI RNAs. The DI RNAs may have the luxury of doing this because they are not translated nor do they serve as templates for the synthesis of mRNAs.

The well-studied alphavirus DI RNAs and the VSV DI RNAs are not translated. For many DI RNAs, however, translation is required for efficient DI RNA replication. The best studied examples of this are DIs of poliovirus and of coronaviruses (these viruses are described in Chapter 3). DI RNAs of poliovirus are uncommon and contain deletions in the structural protein region. It has been suggested that in this case it is the translation product that is required for efficient replication of the RNA (the replicase translated from the RNA may preferentially use as a template for replication the RNA from which it was translated). In contrast, for at least one well-studied DI of a coronavirus, translation of the RNA is required for efficient replication, but the translation product is not important. In this case, translation may stabilize the DI RNA, since there appears to be a cellular pathway to rid the cell of mRNAs that are not translatable. If so, it is uncertain how DI RNAs that are not translated avoid this pathway. Some representative naturally occurring DIs of mouse hepatitis virus, a murine coronavirus, are illustrated in Fig. 9.2B.

Because DI RNAs are replicated by the helper virus machinery and encapsidated by the capsid proteins of the helper virus, they interfere with the parental virus by diverting these resources to the production of DI particles rather than to the production of infectious virus particles. It was the first noted by von Magnus in the early 1950s that

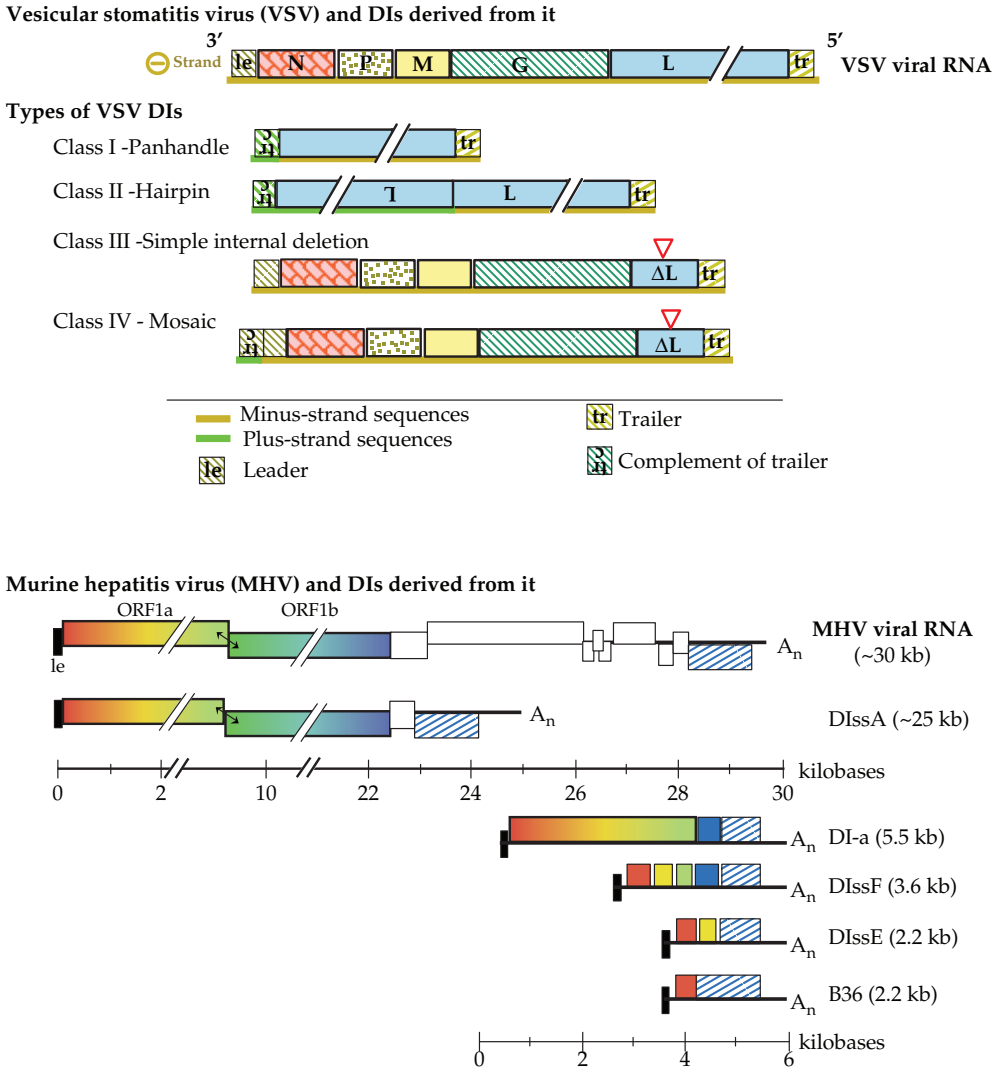

FIGURE 9.2 Types of DIs generated from a rhabdovirus and a coronavirus. Upper panel: diagrammatic representation of the VSV genome and members of the four classes of DI particles. The leader and trailer are shown as patterned blocks. The genome is shown 3′ to 5′ for the minus strand (ochre underline). The parts of the DIs corresponding to the complement of the minus strand are underlined in green. A red triangle marks the internal deletion in the L gene, which is found in Class III and Class IV DIs. Adapted from Whelan and Wertz (1997). Lower panel: structures of naturally occurring DI RNAs of MHV (a murine coronavirus). DIssA, DI-a, etc. were isolated from MHV-infected cells. The bottom line shows a synthetic DI replicon called B36. Sequences in the DIs are color coded by their region of origin in the parental virus genome. Adapted from Brian and Spaan (1997) Figure 1.

influenza virus, passed at high multiplicity for many passages, produced yields that cycled between high and low. This effect is illustrated schematically in Fig. 9.3A. We now know that this is due to the presence of DI particles. In early passages virus yields are high. When DIs arise, they depress the yield of virus. Because high multiplicities of infection are required to maintain DI replication, so that cells are infected with both the helper and the DI, low yields of virus lead to a reduction in DI replication in the next passage or two. Reduced DI replication leads to higher yields of virus. Thus, the yield of infectious virus continues to fluctuate.

In a laboratory setting, at least, DIs can drive the evolution of the wild-type virus. This is shown schematically in Fig. 9.3B. When virus is passed at high multiplicity for very many generations, mutants often arise that have altered promoters that are recognized by mutant replication proteins. Such mutants are resistant to the DIs that are in the population at the time, because the mutant replication proteins do not recognize the promoters in the DIs. The mutant virus

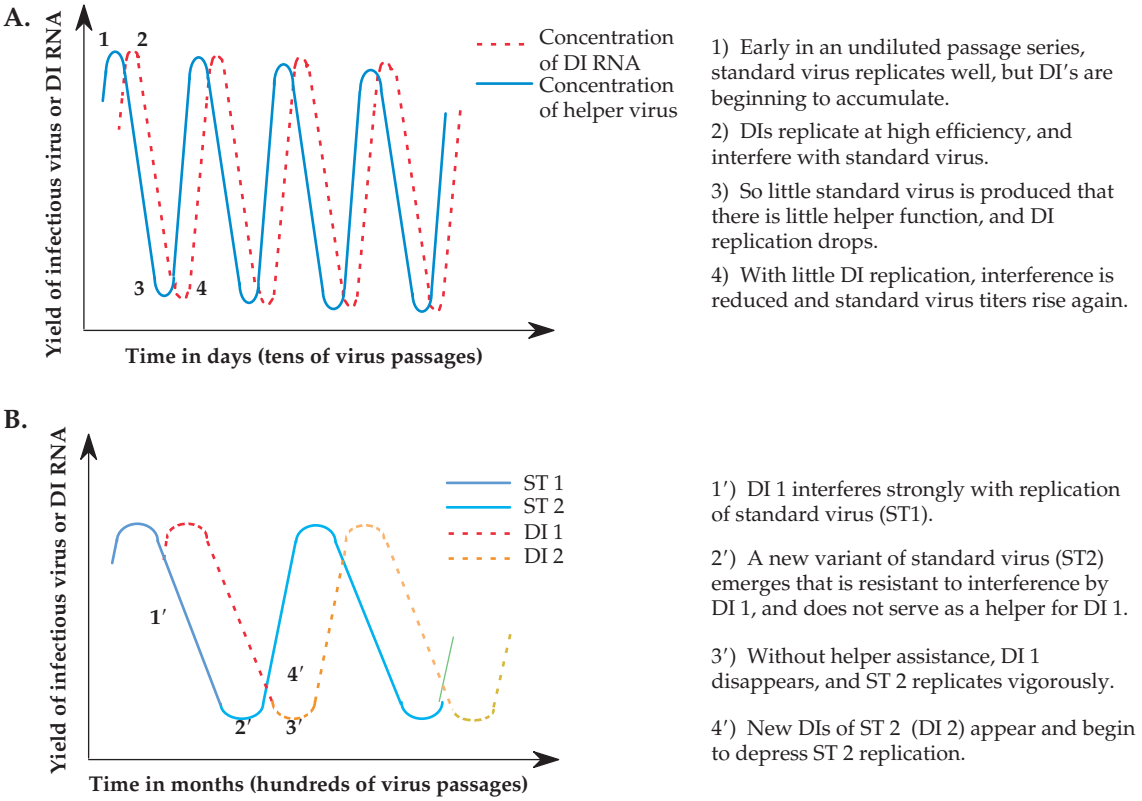

FIGURE 9.3 Stylized illustration of the influence of defective interfering particles on viral evolution. (A) Short-term generation of DI particles during undiluted passage and the cyclical fluctuations in infectious virus yield and concentration of DIs. This effect was first described by von Magnus in 1946 during repeated passage of influenza virus at high multiplicity. (B) Role of DI particles in driving long-term evolution of viruses. The net result of hundreds of passages is that variant 2 and its DIs completely replace the original wild type or standard virus 1. Adapted from *Encyclopedia of Viruses,* Figure 2 on p. 373.

rapidly takes over the population of virus because of its selective advantage. However, new DIs then arise that will interfere with the mutant virus, and the cycle repeats.

It is unclear whether DI particles serve a biological role in nature or whether they are artifacts of abortive recombination that appear in the laboratory because of the high multiplicities of infection that are often used. It has been argued that DIs may arise late in the infection of an animal by a virulent virus and lead to attenuation of symptoms by reducing the yield of infectious virulent virus. As described in earlier chapters, such attenuation could be important for the persistence of a virus in nature. Hepatitis delta virus, described later, is a satellite that replicates only in a cell infected by HBV. Thus, it is clear that it is possible to achieve the multiplicities required to maintain a defective virus in at least some circumstances. Reconstruction experiments in mice have shown that it is possible to attenuate the virulence of lymphocytic choriomeningitis virus by injecting DIs along with the virus. However, it is not clear that DIs will arise in an acute infection in time to ameliorate symptoms. Thus, it has not been possible to provide firm evidence that DIs actually modulate the virulence of their parents in nature, and the question remains open.

SATELLITES AND SATELLITE VIRUSES

A number of satellites and satellite viruses are listed in Table 9.1. The dependoviruses, a genus in the family *Parvoviridae*, were considered in Chapter 7. Although the dependoviruses require a helper, the helper is only needed in order to stimulate the cell to enter a stage in which the dependovirus can replicate. The helper does not provide any function directly related to dependovirus replication or packaging. Satellites and satellite viruses, however, are usually more intimately dependent on the presence of a helper virus to furnish functions directly required for the replication of the satellite genome or for its encapsidation into progeny particles.

Many plant viruses have satellites associated with them. When a satellite encodes its own coat protein, it is sometimes referred to as a satellite virus. Otherwise it is simply called a

TABLE 9.1 Satellites and Satellite Viruses

Group	Genome size	Helper virus	Host(s)	Comments
dsDNA satellite				
Bacteriophage P4	11.5 kb (10–15 genes)	P2 bacteriophage	Bacteria	All structural proteins from P2
ssDNA satellite ciruses[a]				
Dependovirus (AAV)	4.7 kb	Adenovirus herpesvirus	Vertebrates	See Table 7.16
dsRNA satellites				
M satellites of yeast	1 to 1.8 kb	*Totiviridae*	Yeast	Encode "killer" proteins; encapsidated in helper coat protein
ssRNA satellite viruses				
Chronic bee-paralysis virus associated satellite	3 RNAs, each 1 kb	Chronic bee-paralysis virus	Bees	
Tobacco necrosis virus satellite	1239 nt	Tobacco necrosis virus	Plants	
ssRNA satellites				
Hepatitis delta virus	1.7 kb	Hepatitis B virus	Humans	Encode two forms of δ antigen encapsidated by helper proteins
B-type mRNA satellites	0.8 to 1.5 kb	Various plant viruses	Plants	Encode nonstructural proteins, rarely modify disease syndrome
C-type linear RNA satellites	<0.7 kb	Various plant viruses	Plants	Commonly modify disease caused by helper
D-type circular RNA satellites "virusoids"	~350 nt	Various plant viruses	Plants	Self-cleaving molecules

[a] When a satellite encodes its own coat protein, it is known as a satellite virus.

satellite. One of the best studied satellite systems is tobacco necrosis virus (TNV) and its satellite, tobacco necrosis virus satellite (TNVS). TNV has a plus-strand RNA genome of about 3.8 kb. The TNV virion is icosahedral with T=3, and contains 180 copies of a single-coat protein species of about 30 kDa. Associated with many isolates of TNV in nature is TNVS. TNVS has an RNA genome of 1239 nucleotides. The TNVS virion is a T=1 icosahedral structure formed by 60 molecules of a single species of capsid protein encoded by the satellite RNA. The satellite RNA encodes only this single protein that encapsidates its own RNA. All of the functions required to replicate the RNA are provided by the helper TNV.

Some satellites of RNA plant viruses encode only a nonstructural protein required for RNA replication, and the RNA is encapsidated by the capsid protein of the helper virus. In other cases, the satellite is not translated into protein and depends on the helper for all of its functions, in which case it is functionally analogous to DI RNAs. A distinct class of satellite RNAs, called virusoids, consist of viroid-like RNAs that are encapsidated in the capsid protein of the helper virus. These are discussed in the next section.

Although satellites are quite common among plant viruses, they are almost unknown among animal viruses. It is very common among plant viruses to have the genome divided among two or more segments that are separately encapsidated into different particles, a situation that does not occur among animal viruses. Evidently, the mechanisms by which plant viruses are transmitted allow the infection of a plant, and of individual cells within a plant, by multiple particles that together constitute a virus or that constitute a virus and its satellites. Transmission of animal viruses between hosts or among the cells of a host does not appear to allow multiple infections with sufficient frequency to maintain virus systems that are constituted by multiple particles, with the exceptions of hepatitis δ virus, described later, and dependoviruses, which have evolved ways to persist within a cell until the helper virus comes along (Chapter 7). The defense mechanisms of the animal host may also play a role in this restriction.

VIROIDS AND VIRUSOIDS

Viroids are small, circular RNA molecules that do not encode any protein and that are infectious as naked RNA molecules. Sequenced viroids range from 246 to 375 nucleotides and possess extensive internal base pairing that results in the RNA being rodlike and about 15 nm long. A partial listing of viroids is given in Table 9.2. All known viroids

TABLE 9.2 Viroids

Family/genera	Type species	Genome size	Host(s)	Comments
Popsiviroidae[a]				
Popsiviroid	PSTVd	356 to 375 nt	Plants	Presence of central conserved region and lack of self-cleavage mediated by hammerhead ribozyme; replicate by an asymmetric rolling circle strategy in nucleus of infected cells, probably using RNA polymerase II
Hostuviroid	HpSVd	295 to 303 nt		
Cocaviroid	CCCVd	246 to 301 nt		
Apscaviroid	ASSVd	306 to 369 nt		
Coleviroid	CbVd-1	248 to 361 nt		
Avsunviroidae[b]				
Avsunviroid	ASBVd	246 to 250 nt	Plants	Lack central conserved region; replicate by a symmetric strategy in chloroplasts of infected plants using chloroplastic RNA polymerase; can form self-cleaving hammerhead ribozymes in both plus and minus strands
Pelamoviroid	PLMVd	337 to 399 nt		

[a] Formerly known as the Group B viroids.
[b] Formerly known as the Group A viroids.

infect plants. However, hepatitis δ, which infects humans, has many viroid-like properties and may be related to viroids. Many viroids are important agricultural pathogens, whereas others replicate without causing symptoms. Viroids are often transmitted through vegetative propagation of plants, but can also be transmitted during agricultural or horticultural practices in which contaminated instruments are used. Some viroids can be transmitted through seeds and at least one viroid is transmitted by an aphid.

On infection of a plant cell, viroid RNA is transported to the nucleus. The circular RNA appears to be copied by host-cell RNA polymerase II, using a rolling circle mechanism in which multimeric antigenomic sense RNA molecules are produced. The multimeric antigenome sense RNA can then be used as a template to make multimeric genomic sense RNA. This synthesis may also be performed by RNA polymerase II. The concatenated RNAs are cleaved and cyclized to produce the progeny viroid RNA molecules. In an infected cell, as many as 10^4 viroid RNAs can accumulate, most of them in the nucleus.

Some viroids are capable of self-cleavage by the concatenated RNAs to produce genome-length RNAs, followed by self-ligation to cyclize the unit-length molecule. Other viroids are not capable of self-cleavage and ligation. There are five groups of non-self-cleaving viroids, classified by the sequences in the central conserved region. The structures of these five groups are shown in Fig. 9.4. The conserved domains highlighted in the figure are thought to be important for the replication of the viroid (i.e., to form promoters recognized by RNA polymerase II) and for its cleavage to produce unit-length molecules. A pathogenesis domain is also highlighted. Changes in this domain affect the virulence of the viroid on infection of its plant host. For non-self-cleaving viroids, it is assumed that the concatenated RNAs are cleaved and ligated by host-cell enzymes.

The self-cleaving viroids possess a hammerhead ribozyme structure, illustrated in Fig. 9.5. The ribozyme activity cleaves the concatenated RNA at the points indicated by the arrows and ligates the ends to form circular molecules. The viroid RNA is very compact in its structure, with extensive secondary structure, including pseudoknots.

There also exist a large number of satellites called virusoids. Virusoid RNAs are about 350 nt in length, and the RNA is a single-stranded, covalently closed circle. The mechanisms by which virusoid RNA is replicated have not been precisely determined, but they appear to be viroid-like and may replicate by the same mechanisms as viroids. At least some virusoid RNAs are capable of self-cleavage. Virusoid RNAs are encapsidated by the capsid protein of the helper virus of which the virus is a satellite. Thus, transmission occurs by conventional virus-like means, and virusoids may have arisen from viroids that evolved a mechanism for packaging using a helper virus.

HEPATITIS δ

The hepatitis delta (δ) agent or virus (HDV) is a satellite of hepatitis B virus (HBV). It has a worldwide distribution, although strains isolated from different regions of the world differ by up to 40% in their nucleotide sequence. The distribution of HDV is not uniform around the world. Regions of particularly high prevalence include the Mediterranean basin, the Middle East, Central Asia, West Africa, the Amazon

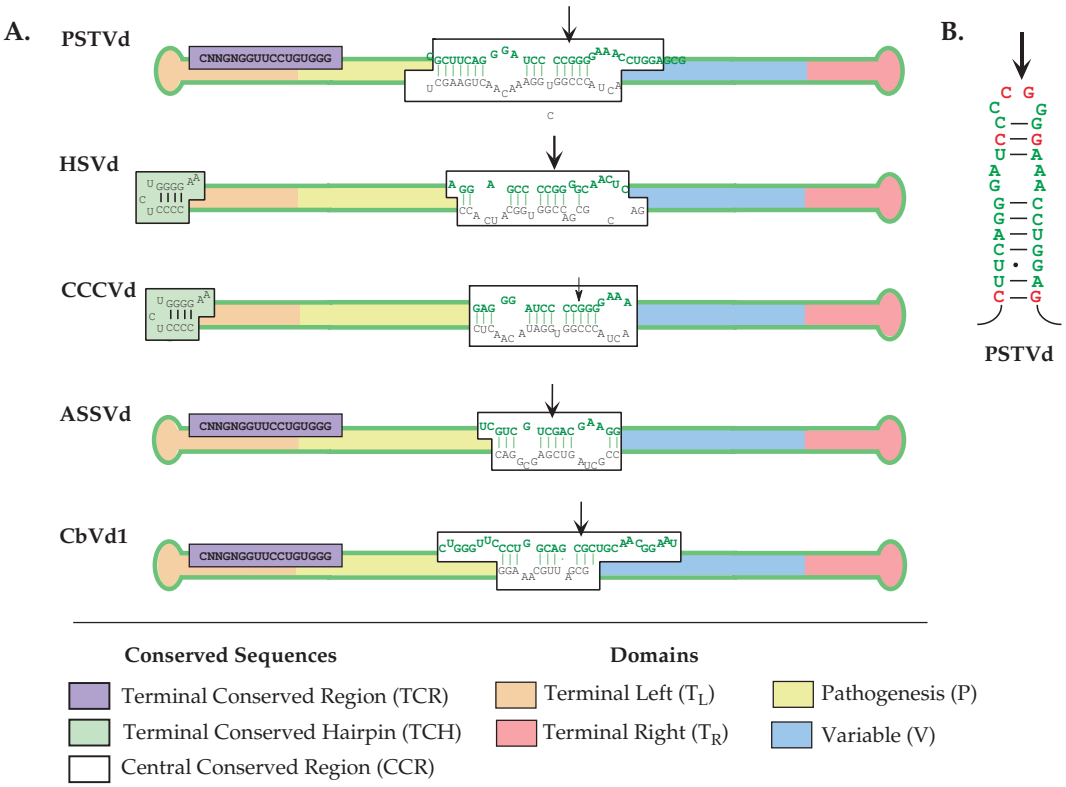

FIGURE 9.4 Models for the genomes of the type species of the five genera of non-self-cleaving viroids. They are as follows: Popsiviroid: PSTVd, potato spindle tuber viroid; Hostuviroid: HSVd, hop stunt viroid; Cocadviroid: CCCVd, coconut cadang-cadang viroid; Apscaviroid: ASSVd, apple scar skin viroid; Coleviroid: CbVd-1, Coleus blumei viroid-1. (A) The RNA strand is shown as a green closed loop. Four functional domains (T_L, T_R, P, and V) are indicated with different colors of shading. Three conserved sequences are boxed. The ***c***entral ***c***onserved ***r***egion (CCR) is a white box, the ***t***erminal ***c***onserved ***r***egion (TCR) is a lavender box, and the ***t***erminal ***c***onserved ***h***airpin (TCH) is a green box. The nucleotides in the upper strand of the CCR (dark green) can in each case form a stable stem and loop structure with the top of the loop at the black arrow, as shown for PSTVd in (B). In this alternative configuration, the nucleotides that are invariant within all five groups are shown in red. Adapted from Flores *et al*. (1997).

Basin, and certain islands in the South Pacific. HDV will only replicate in cells that are simultaneously infected with HBV and its distribution is thus dependent in part upon the distribution of HBV, which was shown in Fig. 6.29. However, as shown in Fig. 9.6, HDV is not uniformly distributed throughout the range of HBV. The percentage of hepatitis B patients that are also infected by infection with HDV ranges from 5% to more than 60% in different geographic areas.

Infection of humans by HDV can either occur by simultaneous infection with both HBV and HDV, or by superinfection with HDV of a person who is chronically infected with HBV. In the case of coinfection, a chronic infection by HDV, which requires that HBV also establish a chronic infection, is established only 1–3% of the time. Most often the infection is completely resolved and recovery occurs. In contrast, superinfection of chronically infected HBV patients with HDV leads to chronic infection by HDV in 70–80% of patients. The different outcomes following infection with HDV are illustrated schematically in Fig. 9.7, in which the symptomology at different times after infection is indicated.

The illness caused by HDV is usually more serious than that caused by HBV alone. The mortality rate from HDV infection is 2–20%, 10-fold higher than the rate for HBV infection alone, which is the next most severe form of viral hepatitis. Most cases of HDV infection are probably clinically important. It is estimated that 460 million people in the world are chronically infected with HBV of whom perhaps 20 million are also chronically infected by HDV. All persons chronically infected with HBV that are not already infected by HDV are at risk for contracting HDV and suffering a more severe form of hepatitis.

The mechanisms by which HDV is transmitted are not understood. It is conjectured that poor hygiene together with intimate contact among people who are infected with the

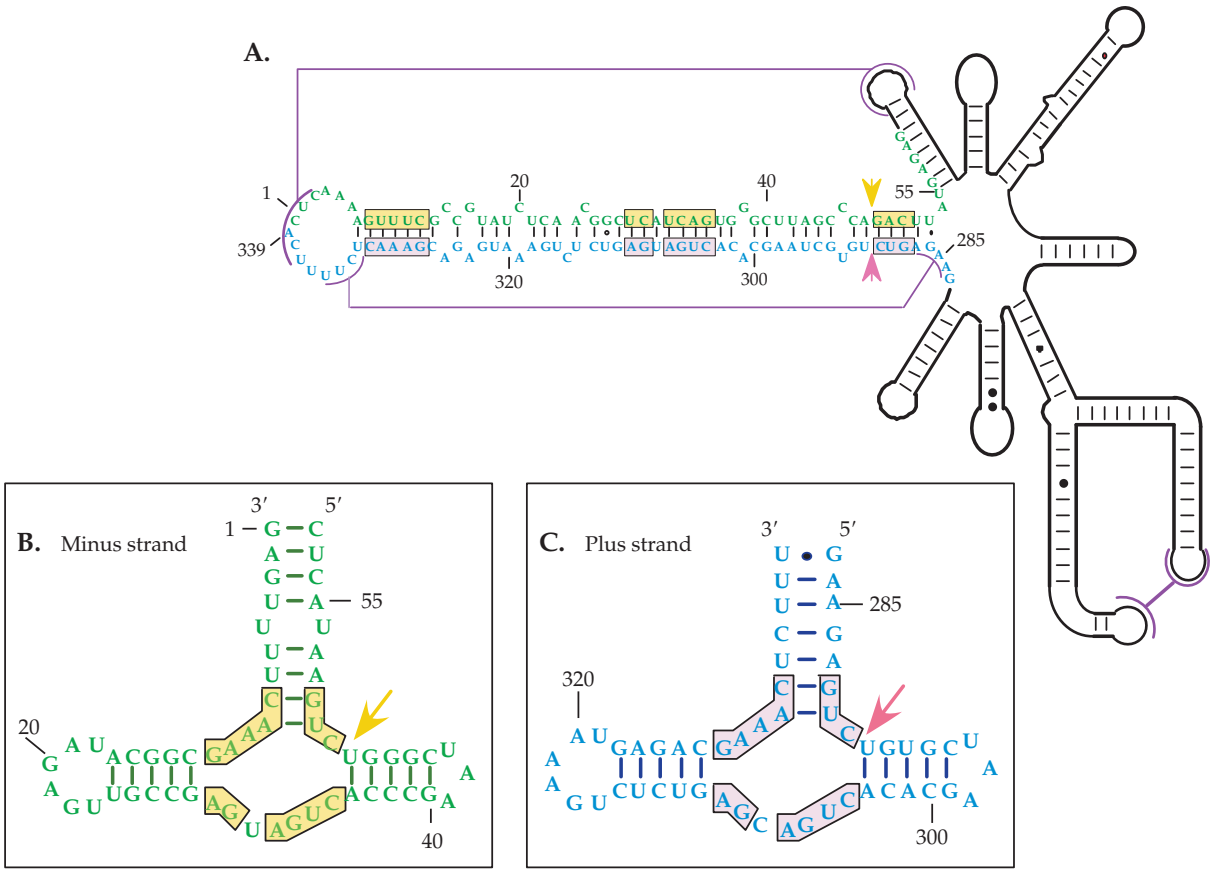

FIGURE 9.5 Predicted secondary structure of peach latent mosaic viroid (PLMV) (Family *Avsunviroidae*, Genus *Pelamoviroid*) RNA in solution. (A) The entire viroid. For most of the molecule, only the backbone is shown, with hydrogen bonds indicated by bars and G–U pairs indicated with dots. The numbering of the nucleotides is arbitrary. The structure is predicted to be even more compact due to pseudoknots formed by the regions joined by purple lines. The nucleotides making up the minus-strand and plus-strand hammerhead ribozymes are shown in green and blue letters, respectively. The nucleotides conserved in all hammerheads in *Avsunviroidae* are boxed and shaded, and the sites of cleavage are marked by arrows. (B) and (C) The structures of the two hammerhead ribozymes, using the same color conventions. The minus-strand hammerhead is made up of the complement of the sequence shown in green in (A). Adapted from Flores *et al*. (1997) and Pelchat *et al*. (2000).

virus may be an important source of transmission in many parts of the world. In developed countries, contaminated blood products and sharing of needles by drug users are important in the spread of the virus, but these are not important modes of transmission worldwide. Sexual transmission may occur, but again this does not appear to be an important component of the transmission of the virus on a worldwide basis.

Since HDV depends on HBV for its propagation, control of HDV is dependent on control of HBV. Current HBV vaccines are highly effective at preventing HBV infection and the increasing levels of vaccination against HBV, together with increased screening for the presence of HBV in blood products, has resulted in a dramatic reduction in recent years of new infections by HDV.

Replication of the HDV Genome and Synthesis of mRNA

The HDV genome is a single-stranded, covalently closed circular RNA molecule of 1.7 kb. The HDV genome can be thought of as a viroid into which has been inserted a gene encoding a single polypeptide, the hepatitis δ antigen (HDAg). As is the case for viroid RNA, HDV RNA has a high degree of secondary structure, with about 70% of the molecule being base paired internally so that it forms a rod-like structure. The HDV genome has minus-sense polarity, that is, it is complementary to the sequence that is translated into the HDAg. The structures of the genomic RNA, the mRNA for the HDAg, and of the antigenomic RNA are illustrated in Fig. 9.8A. The 0.8-kb mRNA is capped and

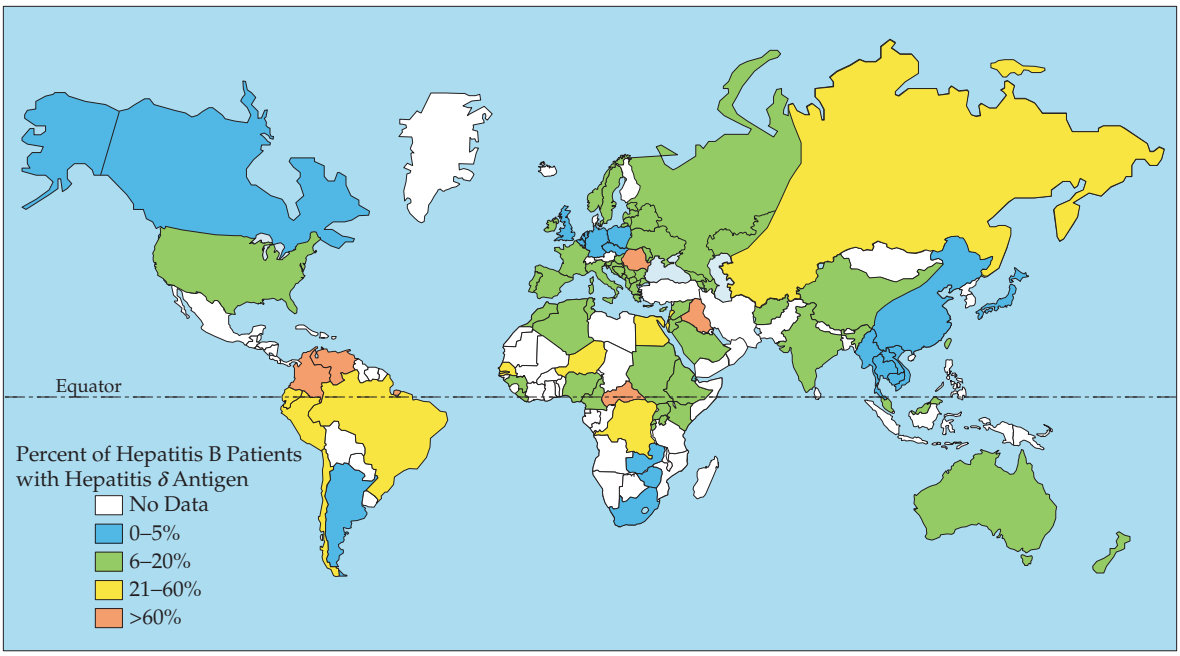

FIGURE 9.6 Worldwide distribution of hepatitis δ infection as measured by the presence of hepatitis δ antigen in the serum of hepatitis B infected patients with hepatitis. Adapted from Fields *et al.* (1996) p. 2826.

polyadenylated as is common for mRNAs. The 1.7-kb antigenome is an exact complement of the genomic RNA and, like genomic RNA, is also circular.

Following infection by the agent, the RNA is transferred to the nucleus. The HDAg, of which there are about 70 copies in the virion, is required for this. In the nucleus, the RNA is replicated by mechanisms related to those used by viroid RNA. However, there is the added complication that an mRNA for HDAg must also be produced. Thus, there are three elements to the replication of RNA: the production of an antigenomic RNA template from genomic RNA, the production of mRNA for HDAg from genomic RNA, and the production of genomic RNA from antigenomic RNA templates. It is believed that synthesis of genomes from antigenomic templates and synthesis of the mRNA from genomic templates are carried out by RNA polymerase II. However, synthesis of antigenomes from genomic templates may utilize another polymerase, perhaps RNA polymerase I. Other, currently unknown host factors also participate, and the HDAg, of which there are two kinds, S-HDAg and L-HDAg, as described later, both of which can be modified in various ways, is absolutely required. These replication steps are illustrated schematically in Fig. 9.9A and B.

Replication of the RNA, whether production of genomes or antigenomes, is thought to utilize a rolling circle mechanism in which concatenated RNAs are produced that are cleaved to unit length by the self-cleavage activity present in both genomic and antigenomic molecules. The resulting unit-length molecules are then cyclized. Although HDV RNA is capable of self-ligation, this process appears to be inefficient and it is thought that a cellular ligase is responsible for most cyclization of HDV RNA monomers.

There are differences in the synthesis of genomic and antigenomic RNA, including differences in their rates of synthesis, different sensitivities of their synthesis to drugs, in the requirement for different forms of HDAg (described later) for genomic and antigenomic RNA synthesis, and in the transport of the RNAs to different places within the cell after synthesis. Furthermore, synthesis of genomic and antigenomic RNA may occur in different places in the nucleus. Thus, synthesis of genomes is sensitive to inhibition by amanitin, requires S-HDAg that has been phosphorylated, methylated, and acetylated, and the RNA product is immediately exported to the cytoplasm. Synthesis may occur in the nucleoplasm. In contrast, synthesis of antigenomes is resistant to amanitin, requires L-HDAg but does not require its phosphorylation or acetylation, and the RNA product is retained in the nucleus. Synthesis perhaps occurs in the nucleolus. Approximately 10 times as much genomic RNA is produced as antigenomic RNA.

The genomic RNA is used as a template to produce the mRNA for the HDAg. The mechanism by which this RNA is produced is not fully understood. It may resemble the process for production of antigenomic RNA but occurs in a different place in the nucleus utilizing a different RNA polymerase and different forms of HDAg. Synthesis of mRNA is also

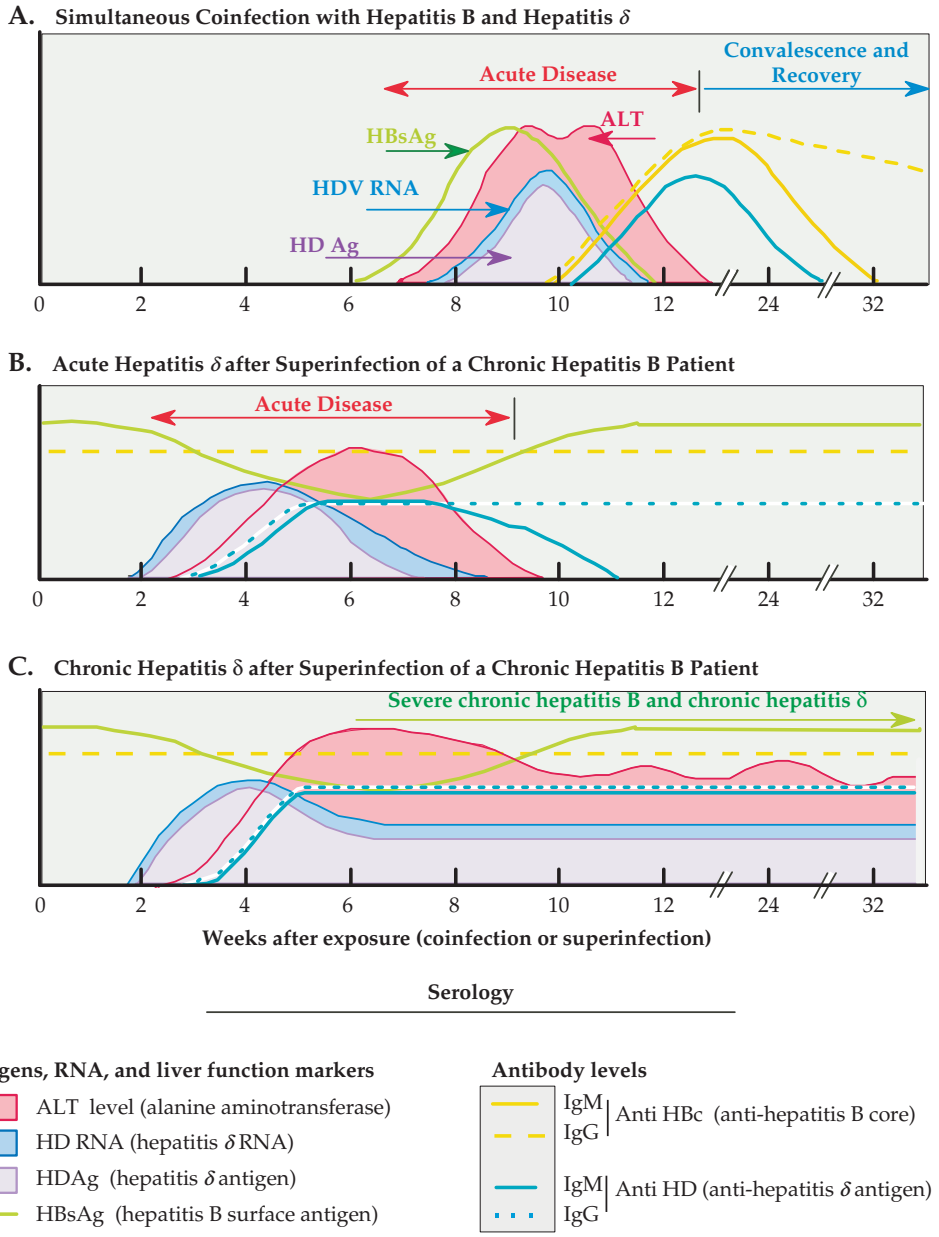

FIGURE 9.7 Patterns of anti-hepatitis B core antigen antibody, hepatitis B antigen, hepatitis δ RNA, hepatitis δ antigen, and ALT in patient serum during different types of coinfection with hepatitis δ and B. (A) Simultaneous infection by both types. (B) and (C) Superinfection by hepatitis δ of a patient with chronic hepatitis B infection. Many infections start with acute hepatitis δ as in (B). Some proportion of superinfections progress to chronic hepatitis with elevated liver enzymes and sustained production of hepatitis δ RNA and protein as in (C). Adapted from Fields *et al*. (1996) p. 2825.

sensitive to amanitin, suggesting that RNA Pol II is involved and synthesis may occur in the nucleoplasm. There is a polyadenylation site following the open reading frame (ORF) for HDAg, and cellular enzymes are assumed to cut the pre-mRNA and polyadenylate it similar to what happens with cellular mRNAs. The fact that the mRNA is also capped suggests that the origin of synthesis may differ from that used for RNA replication so that the process resembles cellular production of mRNA rather than the replication of HDV RNA.

HDV Delta Antigen

The mRNA for HDAg is exported to the cytoplasm and translated into a polypeptide of 195 amino acids, referred to as the small δ antigen or S-HDAg. This protein is required for RNA replication. Thus, for example, *in vitro* systems to study the replication of HDV RNA must be supplemented with S-HDAg for replication to occur. S-HDAg is a component of the infecting particle and is therefore present in the infecting

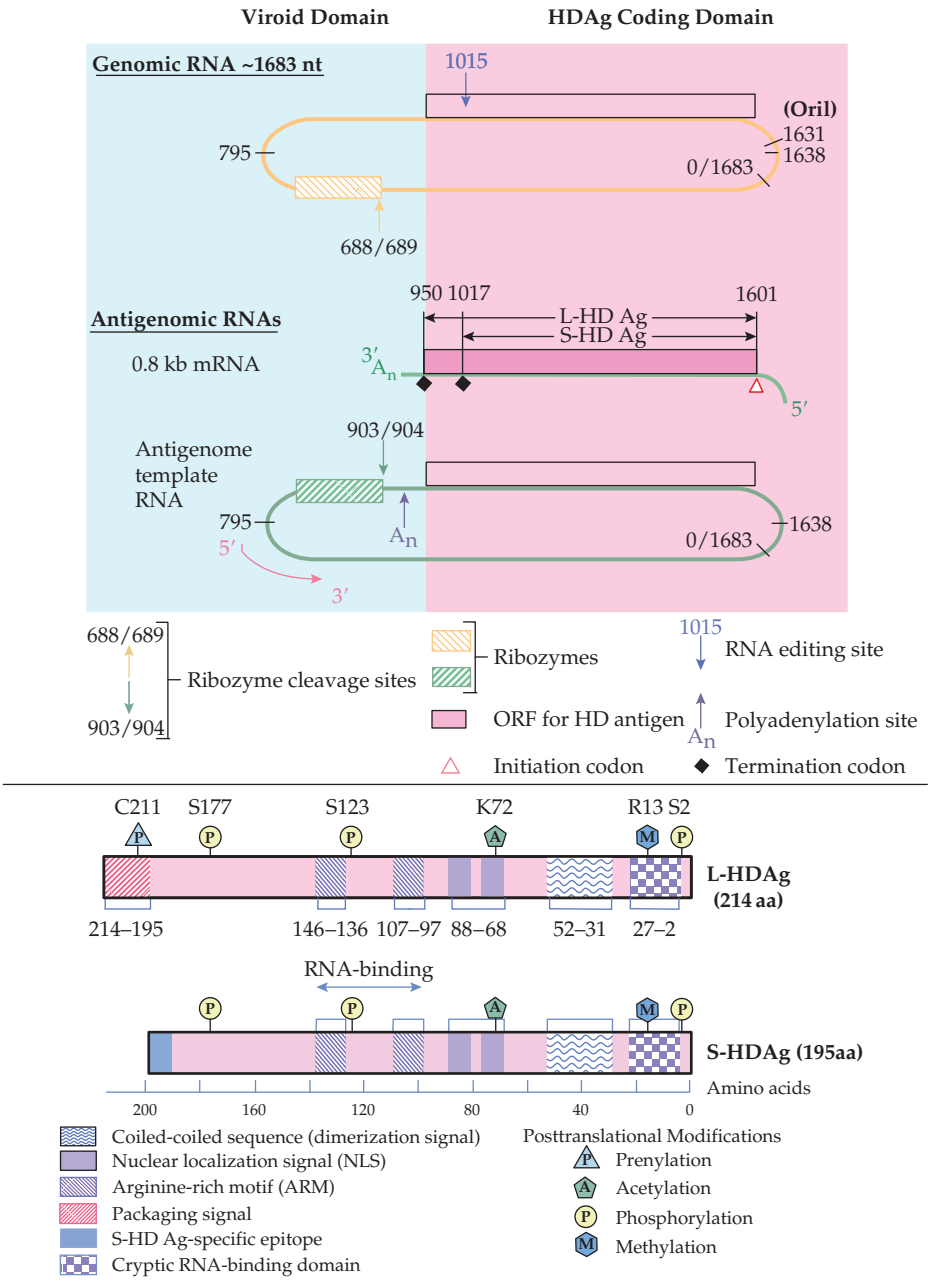

FIGURE 9.8 Structures of HDV RNA and of the HDAg. Above horizontal line: schematic diagram of the structure of HDV RNA. Nucleotides are numbered from the unique *Hind* III site in the cDNA clone of the prototype HDV. Numbering is 5′ to 3′ in the genomic RNA. Nucleotides 795 and 1638 represent the ends of the rodlike structure. Below line: schematic diagram of the structural and functional domains of the hepatitis delta antigen (HDAg). The protein is shown in the same orientation as the mRNA in part (A), with the amino acids numbered from right to left. Other features are described in the key. Adapted from Modahl and Lai (2000), and Lai (2005).

cell when RNA replication first begins. Production of new protein after infection enables RNA replication to accelerate.

A second form of δ antigen is also produced during infection. An RNA-editing event occurs in about one-third of the antigenomic templates, in which the termination codon UAG, at position 196 of the ORF for the δ antigen is changed to UGG, encoding tryptophan (see Fig. 9.8). This change is thought to be effected by deamination, in the antigenome, of the adenosine in the UAG codon to produce inosine. A cellular adenosine deaminase has been described that probably performs this function. Inosine pairs as guanosine, and continued replication of the RNA will lead to the substitution of G for A. This RNA editing site is specific and requires specific sequences within the antigenomic RNA for it to occur.

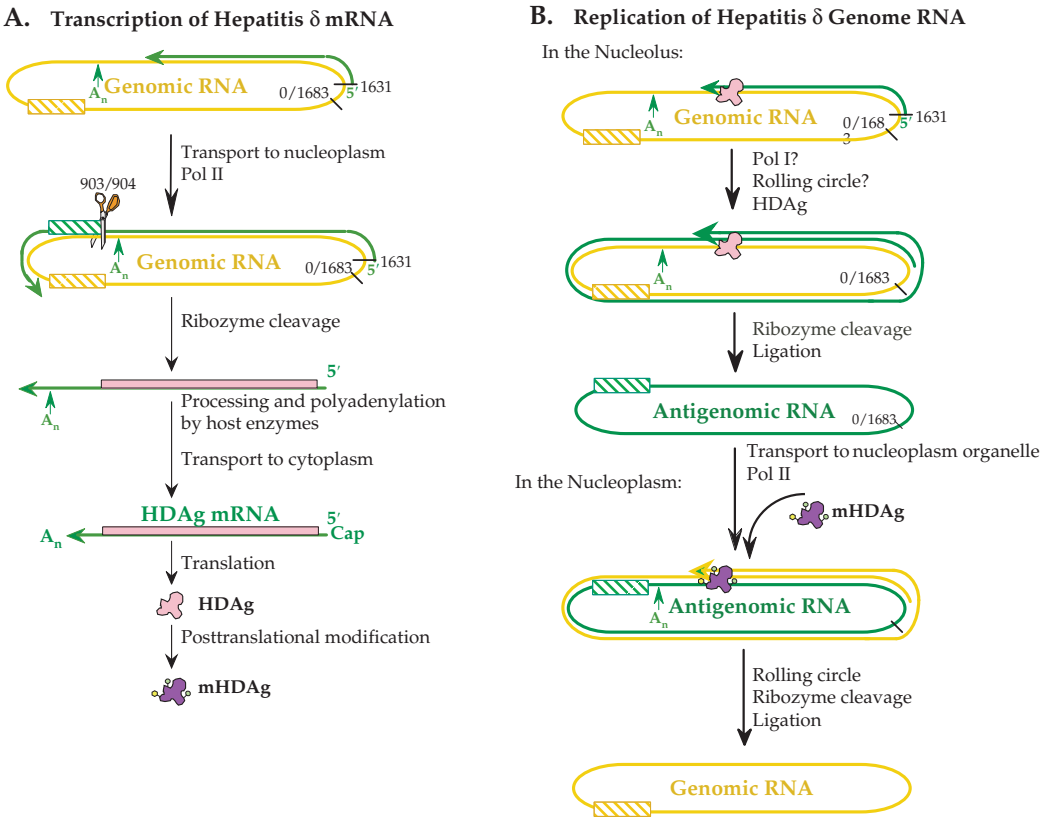

FIGURE 9.9 Transcription and replication of hepatitis δ RNA. (A) Transcription of hepatitis δ mRNA. RNA synthesis by Pol II begins about 30 nucleotides upstream of the HDAg ORF (at nt 1631). Plus-strand synthesis proceeds through the ORF. The nascent strand is cleaved by the ribozyme at nt 903/904 and the mRNA is subsequently processed and polyadenylated. HDAg mRNA is translated in the cytoplasm and some HDAg is posttranslationally modified (mHDAg). (B) Replication of hepatitis δ genome RNA. Genome RNA is transported to the nucleolus where it is replicated by Pol I in the presence of HDAg as a rolling circle. After ribozyme cleavage and ligation the antigenome RNA is transported out of the nucleolus with mHDAg and genome-sense RNA is synthesized by Pol II in a second rolling circle, then cleaved and ligated as before. Adapted from Lai (2005).

Change of the termination codon to a tryptophan codon leads to the production of a polypeptide that is 19 residues longer, for a total length of 214 amino acids, referred to as the large δ antigen or L-HDAg. Because editing is required, it is only produced later in the infection cycle. The extent of editing is controlled, perhaps by S-HDAg. Obviously, only genomes that are not edited can give rise to infectious virions. S-HDAg is required for replication, and only nonedited genomes encode it.

HDAg can be phosphorylated on Ser-2, Ser-177, and Ser-123, methylated on Arg-13, and acetylated on Lys-72. These modifications change the activities of the protein as well as its subcellular localization. As stated, S-HDAg is required for RNA replication. L-HDAg can be isoprenylated on a cysteine four residues from the C terminus that is therefore not present in S-HDAg. L-HDAg suppresses RNA replication and leads to a shift from replication of RNA to encapsidation of RNA into progeny virions. It is specifically required for virus assembly, and isoprenylation is required for this activity. A map of functional domains of the L- and S-HDAgs is shown in Fig. 9.8B.

Assembly of Virus

Assembly of HDV virions begins with the formation of a nucleocapsid or core that contains the HDV genome and both the L and S forms of the δ antigen. The core is 19 nm in diameter and matures by budding, using the HBV surface antigens. Budding appears to be the same as for HBV (Chapter 6), and the three surface antigens of HBV form the protein component of the outer envelope surrounding the HDV capsid. Thus, although the RNA of HDV can replicate independently of HBV, assembly of progeny virions requires the simultaneous infection of the cell by HBV to supply the surface glycoproteins needed to produce infectious particles.

HDV is extremely prolific. The serum of an infected individual can contain up to 10^{12} RNA-containing HDV particles per milliliter.

Host Range of HDV

The only known natural hosts for HDV are humans, but HDV can be experimentally transmitted to chimpanzees and to woodchucks. Infection of chimps requires coinfection with HBV, and this provides a useful primate model for the study of the agent. A second model system is furnished by woodchucks. Woodchucks can be chronically infected with woodchuck hepatitis virus (WHV, Chapter 6), a relative of HBV, and WHV can provide helper activity for HDV. Chronically infected animals can be infected with HDV, and in this case the surface properties of the HDV virion are determined by the helper WHV rather than by HBV.

PRIONS AND PRION DISEASES

Transmissible spongiform encephalopathies (TSEs), now often referred to as prion diseases, are progressive, fatal diseases of humans and of other animals. A listing of TSEs is given in Table 9.2. TSEs of humans include kuru, Creutzfeldt-Jakob disease (CJD), Gerstmann-Straüssler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). TSEs are characterized by neuronal loss that appears as a spongiform degeneration in sections of brain tissue, often accompanied by amyloid plaques or fibrils. The most prominent symptoms of disease are usually dementia (loss of intellectual abilities) or ataxia (loss of muscle control during voluntary movement) that results from the progressive loss of brain function. The disease always has a fatal outcome. In humans, death usually occurs within 6 months to 1 year of the first appearance of symptoms.

TSEs can be contracted by inoculation with or ingestion of brain tissue or other tissues containing the infectious agent, and thus they can be transmitted as an infectious disease. Kuru first came to light as an infectious disease and many cases of CJD in humans have been acquired by infection. However, TSEs can also occur as sporadic diseases for which there is no evidence of infection by an outside agent. In humans, CJD occurs sporadically with a frequency of about 10^{-6}. Finally, TSEs can appear as inherited diseases. GSS, most FFI, and some cases of CJD occur as dominant inherited diseases, associated with mutations in the gene for the prion protein. Inheritance of the mutant gene dramatically increases the probability of developing TSE, such that the probability of acquiring the disease over a lifetime may approach 100%. In most cases of sporadic or inherited TSE, the disease is transmissible as an infectious disease once it occurs.

There is now considerable evidence that all TSEs are related and result from defects in the metabolism of the prion protein. The pattern of symptoms associated with a particular TSE may vary, however, depending in part on how the disease was contracted; on the source of the infecting agent; and on the nature of mutations in the prion protein. Thus, although the prion protein is central to disease in every case, symptomology can differ, in part because the particular area of the brain most affected can vary.

There is no immune response associated with any TSE. No antibodies are formed and no inflammation marked by the infiltration of mononuclear cells is present. As stated, it is relentlessly progressive and always results in death.

Kuru

Kuru was a disease of epidemic proportions among the Fore people of New Guinea that reached a prevalence of about 1% of the population. The disease was characterized by progressive ataxia that led to total incapacitation and death, normally in 12–18 months after the appearance of symptoms in adults or 3–12 months in children. The demonstration that kuru was transmissible to primates by inoculation of brain tissue from people dying of the disease was the first demonstration of the transmissibility of a TSE in humans. These transmission studies and other studies of kuru resulted in a Nobel prize for Carleton Gajdusek in 1976, the first of two prizes for work with TSEs (Chapter 1).

Kuru is believed to have been spread among the Fore people by cannibalism in which the bodies of relatives who had died were eaten in a ritualistic feast. Women and children were more often affected than men, and it is thought this was because they prepared the body for the feast and they ate the brains of deceased relatives. Men were less often affected, it is conjectured, because they ate primarily other body parts. It has been postulated that the epidemic began when a member of the tribe died of a sporadic case of CJD, and the disease was then spread to others through cannibalism. Through the efforts of missionaries, cannibalism ceased many years ago and the disease has become progressively rarer. Now only older people who contracted the infectious agent during the time of cannibalism continue to develop the illness. From studies of the continuing development of kuru in older Fore people, it is known that the disease can appear as long as 40 years after the event that resulted in infection with the agent.

Sporadic and Iatrogenic CJD

CJD in man is usually a sporadic illness that occurs with a frequency of about 10^{-6} that is uniform around the world. However, once the disease has arisen it is transmissible by inoculation of infected material into experimental animals such as primates and transgenic mice. CJD has also been

transmitted iatrogenically to humans. Iatrogenic cases have occurred in recipients of pituitary-derived human growth hormone obtained from cadavers, some of whom died of CJD; in recipients of homographs of dura mater derived from cadavers; through implantation into epilepsy patients of contaminated silver electrodes that had been incompletely sterilized; and through corneal transplants. The infectious agents of TSEs are extremely difficult to inactivate and require extraordinary sterilization techniques in order to destroy their infectivity. Better methods of sterilization have been introduced, and human growth hormone is now produced in bacteria from recombinant DNA plasmids, so that the iatrogenic spread of CJD has been greatly reduced.

Sporadic FFI has also been described. No case of sporadic GSS is known, however.

Inherited Forms of Human TSE

About 5% of CJD cases arise in a familial, autosomal dominant fashion and are associated with mutations in the gene for the prion protein. GSS and most cases of FFI are also inherited forms of TSE associated with mutations in the prion protein. Many of the responsible mutations are illustrated in Fig. 9.10, in which a schematic diagram of the human prion protein is presented.

A dozen single amino acid substitutions in the prion protein have been found to be associated with inherited CJD, GSS, or FFI. Additionally, an element normally containing five repeats of a 24-nucleotide sequence (encoding an 8-amino-acid repeat, P-Q/H-G-G-G-W-C-Q) has been found to contain one to nine extra repeats, probably originating from unequal crossing over, in some cases of inherited CJD or GSS. These three diseases are distinguished on the basis of symptomology, which is overlapping. CJD is characterized by ataxia, dementia, and behavioral disturbances. GSS is usually characterized by cerebellar disorders accompanied by a decline in cognitive ability. FFI, as its name suggests, is characterized by abnormal sleep patterns, including intractable insomnia.

The penetrance of the different mutations varies but is usually very high. For example, CJD caused by the change from glutamic acid-200 to lysine (E200K), when residue 129 is homozygous for methionine, has been estimated to have a penetrance of 0.45 by age 60 and a penetrance of more than 0.96 above age 80. Thus, a person with this mutation is almost certain to develop CJD if he or she lives long enough.

Attempts have been made in many cases of inherited TSEs to transmit the disease to subhuman primates or to mice. Transmission has been achieved in most cases tested. Thus once the disease arises, it is transmissible to animals that do not contain the mutation.

In addition to mutations associated with inherited TSEs, several polymorphisms in the prion protein are known that are not associated with disease (Fig. 9.10). The polymorphism at residue 129 is of particular importance. Homozygosity at this position affects the probability of contracting TSE.

TSEs in Other Animals

Naturally occurring TSEs of a number of other mammals are known. The oldest known TSE, in fact, is that of sheep, and is called scrapie. Scrapie has been known for more than 200

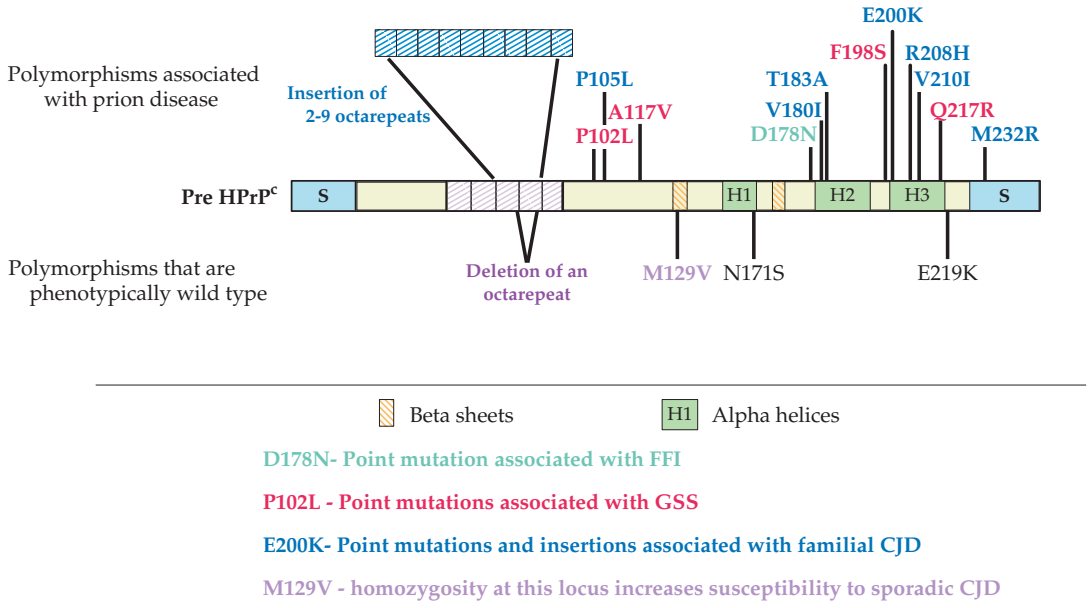

FIGURE 9.10 Mutations found in the human prion protein gene. Polymorphisms that are phenotypically wild type are shown below the schematic of the gene; mutations that segregate with inherited prion diseases are shown above the gene. GSS, FFI, and CJD are defined in Table 9.3. Adapted from Prusiner (1998) and Riek *et al.* (1996).

TABLE 9.3 Prion Diseases

Disease (abbreviation)	Natural host	Experimental hosts	Cause of disease
Scrapie	Sheep and goats	Mice, hamsters, rats	Infection in genetically susceptible sheep
Transmissible mink encephalopathy (TME)	Mink	Hamsters, ferrets	Infection with prions from sheep or cattle
Chronic wasting disease	Mule deer, white tail deer, and elk	Ferrets, mice	Unknown
Bovine spongiform encephalopathy (BSE)	Cattle	Mice	Infection with prion-contaminated meat and bonemeal
Feline spongiform encephalopathy (FSE)	Cats	Mice	Infection with prion-contaminated beef
Exotic ungulate encephalopathy (EUE)	Nyala, oryx, and greater kudu	Mice	Infection with prion-contaminated meat and bonemeal
Kuru	Humans	Primates, mice	Infection through ritual cannibalism
Creutzfeldt-Jakob disease	Humans	Primates, mice	
iCJD (iatrogenic)	Humans		Infection from prion-contaminated human growth hormone, dura mater grafts, etc.
sCJD (sporadic)	Humans		Somatic mutation or spontaneous conversion of PrP^{c} to PrP^{Sc}
nvCJD (new variant)	Humans		Ingestion of bovine prions?
fCJD (familial)	Humans		Germ line mutation in PrP gene
Gerstmann-Straüssler-Scheinker syndrome (GSS)	Humans		Germ line mutation in PrP gene
Fatal familia insomnia (FFI)	Humans	Primates, mice	Germ line mutation in PrP gene (D178N, M129)
Fatal sporadic insomnia (FSI)	Humans		Somatic mutation or spontaneous conversion of PrP^{c} to PrP^{Sc}

Source: Adapted from Granoff and Webster (1999), p. 1389.

years and is widely distributed in Europe, Asia, and America. The name comes from the tendency of animals to rub themselves against upright posts, apparently because of intense itching that arises from this neurological disease. Scrapie appears to be transmitted horizontally in sheep flocks, but the mechanism by which it is transmitted is not understood. The infectious agent is very resistant to inactivation and may persist in pastures for a long time. It may be ingested, but other mechanisms for persistence have also been proposed.

Scrapie appears to have been transmitted to a number of other mammals. In some cases the spread has been to animals that share pasturage with infected sheep, such as white-tailed deer, mule deer, and elk (where the disease is called chronic wasting disease). In these cases, it is thought that infection occurs by the same mechanisms that maintain scrapie in sheep flocks. In other cases, spread has occurred via food derived from infected sheep that was fed to mink (transmissible mink encephalopathy), domestic cats or exotic cats in zoos (feline spongiform encephalopathy), ungulates in zoos (exotic ungulate encephalopathy), and perhaps to cattle. However, there is no evidence that scrapie has ever spread to humans, despite the long history of human consumption of scrapie-infected sheep.

Bovine spongiform encephalopathy (BSE), also called mad cow disease, is a TSE of cattle that was recently an epidemic in Britain. The epidemic was maintained by feeding to cattle the processed offal from cattle and other animals, that is, by a form of animal cannibalism as happened with kuru in humans. Although this practice was of long standing, it did not cause trouble until recently, when a change in the rendering process was introduced. It is believed that this change allowed the BSE agent to survive the processing steps, whereas formerly it had been killed during rendering. The result was an epidemic of BSE that spread across all of Britain (Fig. 9.11). At the height of the epidemic, there were more than 35,000 cases of BSE per year in Britain (Fig. 9.12).

The original source of the BSE that led to the epidemic is uncertain. It may have arisen from a spontaneous case of BSE, similar to spontaneous CJD in humans, although spontaneous BSE in cattle appears to be rare or nonexistent. A second possibility is that it may have arisen from infection with scrapie from infected sheep, since sheep offal was included in the rendered offal.

Once the epidemic of BSE in cattle in Britain was recognized, legislation was introduced that banned the feeding of any ruminant-derived protein to ruminants. Also introduced was legislation to make BSE a notifiable disease and to prohibit the use of brain, spinal cord, and certain other offals from any bovine animal in human food. These initial bans were subsequently enlarged and extended in various ways

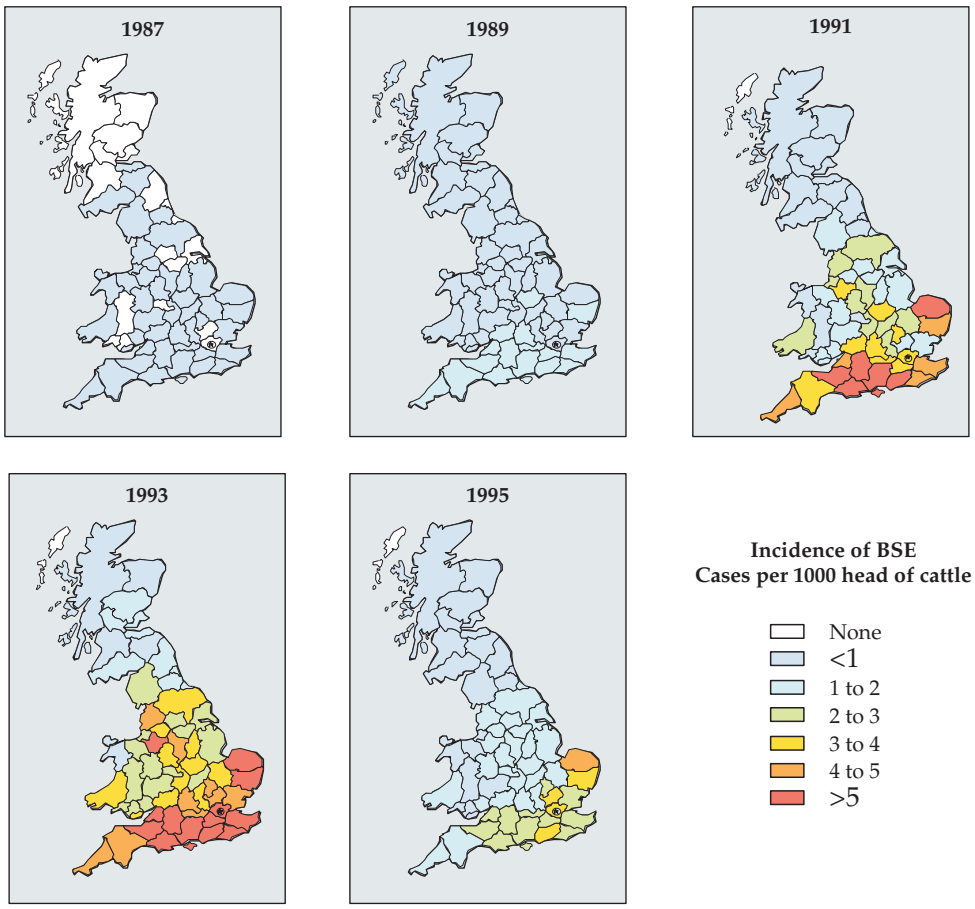

FIGURE 9.11 Spread of the BSE epidemic in the British Isles. Geographic distribution of the incidence of BSE per head of cattle by county from 1989 to 1995. Adapted from Anderson *et al*. (1996).

(see Fig. 9.12 and its legend). The ruminant feed ban resulted in the waning of the epidemic in cattle in Britain, but new cases continued to arise, whether the result of a long latent period of the infectious agent, or from contaminated ruminant feed that continued to enter the system, or from alternative modes of transmission, such as passing the infection from mother to calf. With the recognition of new variant CJD in people, the issue of eradicating BSE became more pressing and culling of cattle was undertaken. This culling of herds containing BSE-infected cattle together with the subsequent culling of herds infected with foot-and-mouth disease virus, as well as the continued enforcement of the ruminant feed bans, have resulted in a marked reduction in the incidence of BSE, although not to its total eradication (Fig. 9.12).

New Variant CJD in Humans

At the beginning of the BSE epidemic, public health officials in Britain had little fear that the epidemic might pose a threat to human health. There is a species barrier to the transmission of the TSE from any particular animal to another animal. Even in cases where transmission does occur in experimental systems, there is a requirement for an adaptation event before the agent can be readily transmitted. Humans were thought not to be sensitive to animal TSE agents because of this species barrier. In particular, no evidence for the transmission of scrapie to humans has ever been found despite the fact that people all over the world, but especially in Britain, have eaten sheep infected with scrapie for 2 centuries.

In 1995 and 1996, however, 12 cases of a variant form of human CJD occurred in Britain. These new variant CJD cases (nvCJD) were characterized by an unusually early age of onset, with some cases in their teens, and by a different symptomology. A comparison of the ages at which people in Britain contracted sporadic CJD during the last 25 years with that of the ages of the first 21 cases of nvCJD is shown in Fig. 9.13A. Sporadic CJD is primarily a disease of people in their 50s, 60s, and 70s, with a peak of occurrence in the early 60s. Cases in people under 40 are rare. Variant CJD to date has been a disease of young people, primarily people in their teens, 20s, and early 30s. Symptomology also differs. Sporadic CJD is characterized by dementia as an early symptom, whereas variant CJD

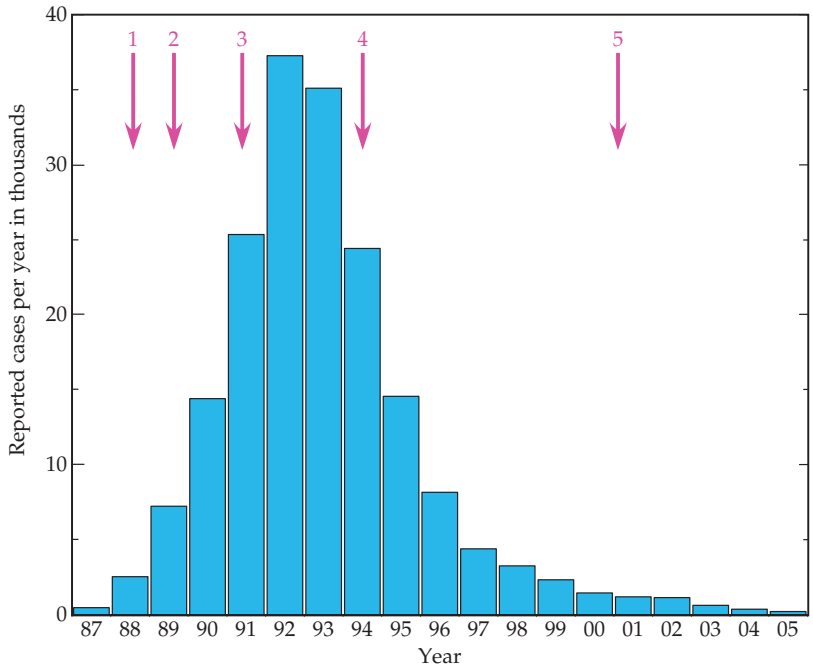

FIGURE 9.12 Confirmed cases of BSE (bovine spongiform encephalopathy) in British cattle per year between 1987 and 2005. Arrows indicate (**1**) ruminant feed ban (1988); (**2**) specified offals ban, to prevent offals proteins from entering the human food chain (1989); (**3**) extended specified offals ban [prohibiting feeding of offal proteins to pigs and poultry (1991)]; and (**4**) offals ban futher extended to include offals from bovines < 6 months old (1994). (**5**) Prohibit the use of any animal protein (excluding milk and fish meal) from feed for any farmed animal species (2001). Note that data after 2002 could be biased by the large number of cattle slaughtered during the foot-and-mouth-disease virus (FMDV) epidemic in 2001, which must have contained some infected animals. Adapted from Anderson *et al*. (1996), the "2004 Institute of Food Science and Technology Information Statement on BSE," and data from http://www.oie.int/fr/info/fr_esbru.htm.

is characterized by psychiatric symptoms, usually depression, and the patient is often first seen by a psychiatrist. Third, time to death averages somewhat longer in variant CJD than in sporadic CJD. The number of cases of nvCJD rose for several years, plateaued in the year 2000, and then declined, as shown in Fig. 9.13B. Also shown in this figure for comparison are the number of cases of sporadic CJD each year in Britain; the rise in the number of cases of sporadic CJD reported over this time frame is probably due to increased recognition of CJD disease, catalyzed in part by the nvCJD epidemic. Through 2005 there had been a total of about 150 cases of nvCJD.

There is now a considerable body of evidence that nvCJD is caused by infection with BSE and results from eating BSE-contaminated meat. For one, the BSE prion and the human nvCJD prion are closely related and differ from other CJD prions (see later). For another, the nvCJD epidemic closely parallels the BSE epidemic with an 8-year lag. It appears, therefore, that the incubation period of nvCJD, at least to date, averages about 8 years. It is not clear how many cases may ultimately arise. As described earlier, kuru has a long latent period, with disease developing as long as 40 years after infection. The decline in the incidence of nvCJD following control of BSE, however, suggests that the dynamics of nvCJD disease are different and that only small numbers of disease will continue to arise, perhaps the result of the species barrier that exists for the transmission of BSE to humans. Further, it is not understood why the young are so much more sensitive to nvCJD than are the old. A sensationalized and gripping account of kuru, CJD, and BSE is found in the book *Deadly Feasts* by Richard Rhodes.

Prion Protein

The nature of the infectious agents responsible for scrapie and other TSEs has been controversial, in part because the study of these agents has presented enormous technical difficulties. The kuru agent was shown to be transmissible to other primates many years ago, but the incubation period is very long in these animals (more than 10 years in some cases) and they are expen-

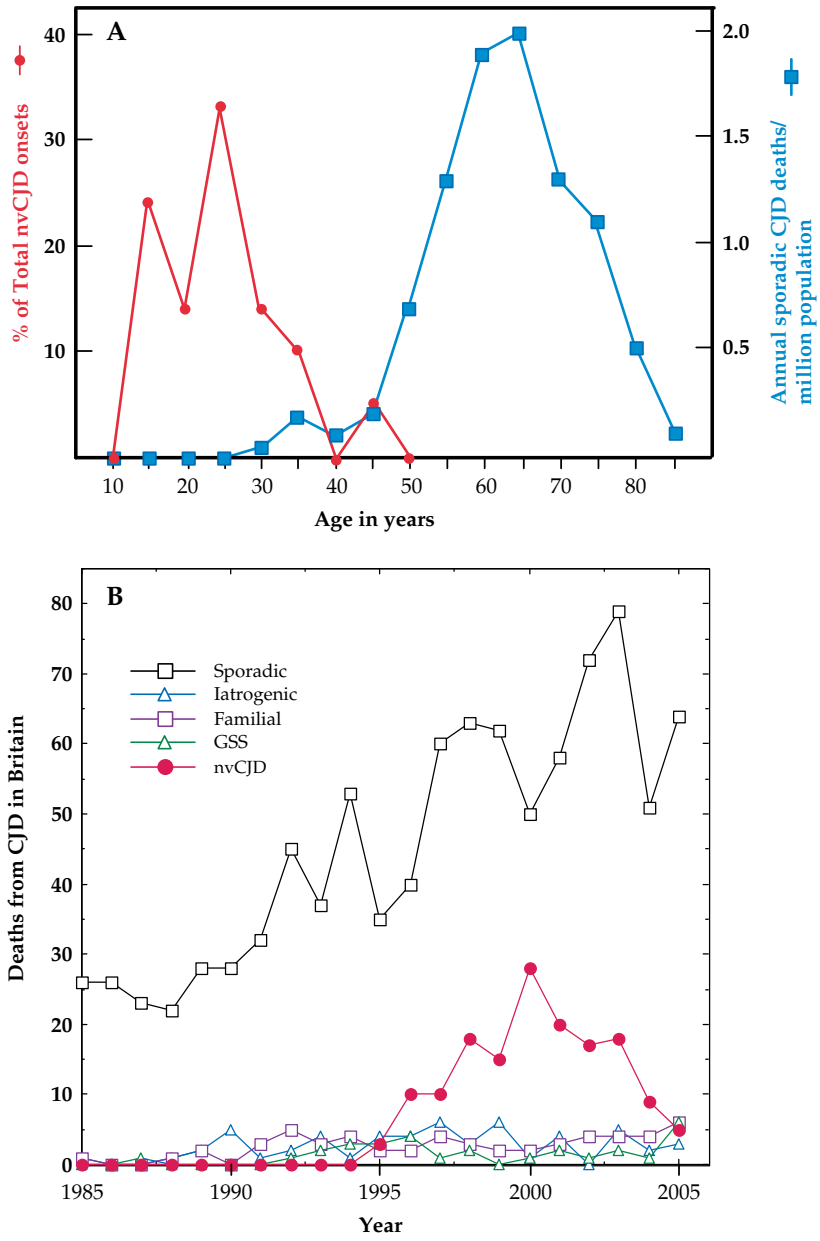

FIGURE 9.13 Creutzfeldt-Jakob disease (CJD) in Britain. (A) Age distribution of the first 21 cases of new variant CJD (nvCJD) in 1995 and 1996, compared with the annual age-specific death rates for sporadic CJD (573 cases) in Britain between 1970 and 1994. The scales have been chosen to optimize comparison of the age distributions. Adapted from Nathanson (1999) Figure 7 on p. 449. (B) Cases of CJD of various etiologies and Gerstmann-Straüssler-Scheinker syndrome (GSS) in Britain from 1985 to 2005. The rise in the number of cases of sporadic CJD reported is probably due to increased recognition of the disease. Data are Monthly CJD Statistics, from the Department of Health of the United Kingdom.

sive to maintain, which limited early progress in the study of the molecular biology of the agents. The subsequent discovery that many TSEs could be transmitted to mice and hamsters, in which the incubation period was much shorter, as short as 60 days in some instances, speeded up progress. Transgenic mice, in particular, have been very useful because the genetic background can be controlled. However, such studies remain slow and tedious because an infectivity assay often takes more than 1 year.

Studies in mice and other animals, as well as the finding that mutations in the prion protein are associated with

inherited TSEs in humans, have made clear that the prion protein, abbreviated PrP, is intimately involved in the transmission of TSE and in the disease process. The normal cellular protein is referred to as PrP^c. The structure in solution of the C-terminal half of the mouse version of this protein (residues 121–231) is illustrated in Fig. 9.14. The protein has a high content of α helix. In this half of the protein, there are three α-helical domains of 11, 15, and 18 residues, and only a short (four residues in each strand) two-stranded antiparallel β sheet. The N-terminal 98 residues of this protein form a flexible random coil in solution, as determined by nuclear magnetic resonance imaging.

The prion protein is synthesized as a larger precursor of 254 amino acids that contains both N-terminal and C-terminal extensions (Fig. 9.15). The N-terminal extension is a signal sequence that leads to the translocation of PrP into the lumen of the endoplasmic reticulum. It is removed by signal peptidase, as are most N-terminal signal sequences. The C-terminal extension is removed by another cellular protease and the protein is attached to a phosphoinositol glycolipid anchor that anchors the protein in the membrane. The protein is N-glycosylated on two sites. The processed protein is transported to the plasma membrane and transiently

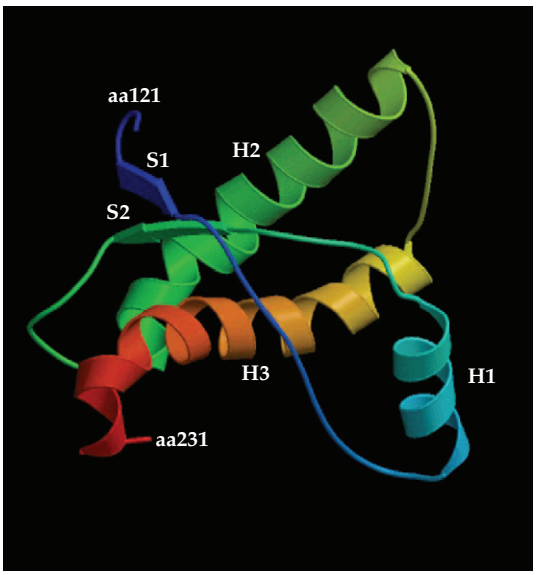

FIGURE 9.14 The structure of the prion protein. The structure of residues 121–231 of the mouse prion protein in solution as determined by NMR is shown. The protein is color coded from blue at residue 121 to red at residue 231, with β sheets shown as flat arrows and α helices as coils. The second and third helices are linked with a disulfide bond (not shown). (Compare with Figures 9.10 and 9.15.) Adapted from Riek *et al*. (1996).

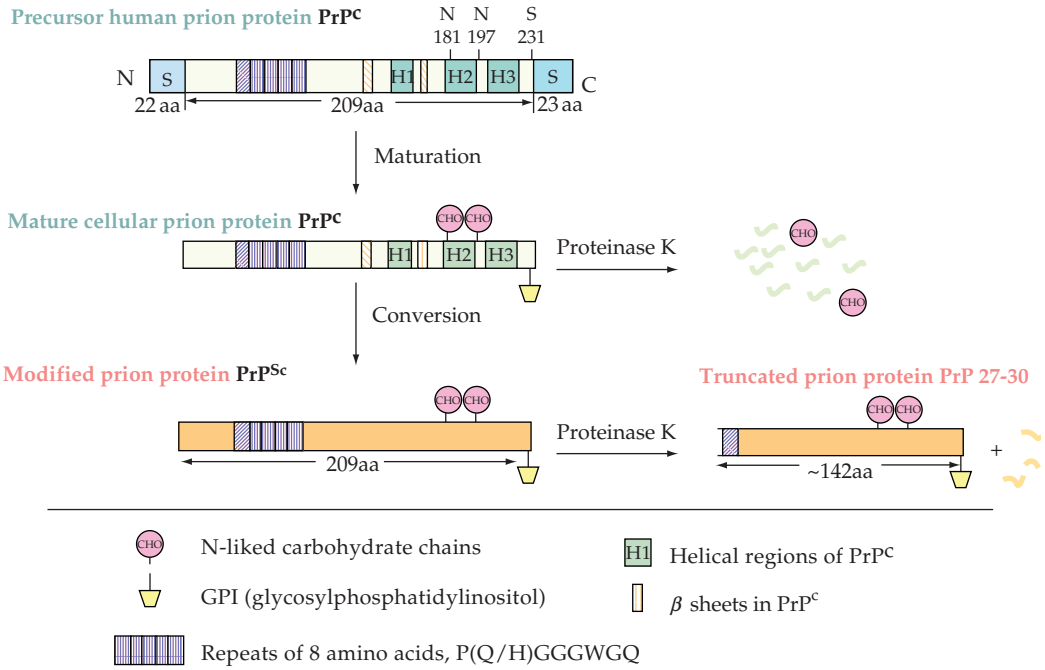

FIGURE 9.15 Isoforms of the human prion protein. The precursor protein is 254 amino acids long. Maturation involves removal of the N-terminal signal sequence and the C-terminal 23 amino acids (two boxes marked S) N-linked glycosylation at Asn181 and Asn197, and linkage of GPI near the new C terminus. After exposure to scrapie prions, the protein is converted to PrP^{Sc}, which is partially resistant to proteinase K. This conversion involves loss of some helical regions (H's) in the cellular form, and formation of new β sheets. Adapted from Weissmann (1996), Riek *et al*. (1996), and Prusiner (1998).

displayed on the surface of the cell with a half-life of about 5 hours. It is then recycled into endosomal compartments and eventually into lysosomes, where it is degraded.

The function of PrP^c is unknown. It is expressed by a number of different cells, including neurons, hematopoietic stem cells, and follicular dendritic cells. Many knockout mice that lack the gene for PrP have been constructed and most appear normal. The conservation of this protein indicates that it must perform some important function, but apparently its functions can be replaced by other proteins through the redundancy of many mammalian pathways. Recent studies have suggested that the protein is important for the renewal of hematopoietic stem cells and for the development of neurons, and that when an organism is stressed this developmental function becomes critical.

The brains of most humans or experimental animals exhibiting TSEs contain a conformational variant of PrP^c called PrP^{Sc} (Sc for scrapie) or PrP^{res} (res for resistant to protease). PrP^{Sc} is found in aggregates that are largely resistant to digestion with proteases. Treatment of PrP^{Sc} with proteases and subsequent disaggregation of the proteolyzed PrP^{Sc} give rise to a molecule that is truncated by about 80 amino acids at its amino terminus (Fig. 9.15). In contrast, PrP^c is completely destroyed by such protease treatment, and the normal PrP is also referred to as PrP^{sen} (for sensitive to protease). Circular dichroism and infrared spectroscopy indicate that PrP^{Sc} has a much higher content of β sheet than does PrP^c, 43 versus 3%, and a lower content of α helix, 30 versus 42%, suggesting a profound conformational rearrangement of the prion protein in the process of conversion from PrP^c to PrP^{Sc}.

Studies with Mice

Transgenic mice have been useful in the study of TSEs. Mice have been made that lack the gene for the prion protein, or that express wild-type or mutant prion proteins at levels from less than normal to several times normal. Most mice that make no PrP^c are normal, as described. However, such mice are resistant to scrapie infection. They do not become ill, and no infectious material is produced in the brain after inoculation of scrapie. In contrast, mice that overexpress PrP are more sensitive to infection with scrapie. The incubation period is shorter, and the animals die more quickly after inoculation with scrapie.

Thus, the presence of PrP is essential for the development of TSE in mice. It has also been found that individual neurons must be able to produce PrP if they are to be sensitive to scrapie-induced death. Neurografts from a donor mouse that expresses PrP have been implanted into mice that lack the PrP gene. Upon inoculation of scrapie into the brain, the neurons in the graft develop a typical scrapie-induced disease pathology. However, neurons outside the graft remain healthy.

Ex Vivo and *in Vitro* Studies

Cell lines have been established that are persistently infected with scrapie. These cells continuously produce PrP^{Sc}, which allows biochemical studies to be performed over a shorter time frame. The infected cells produce infectious material that causes scrapie when inoculated into mice. Of great interest has been the development of an *in vitro* system for the conversion of PrP^c to PrP^{Sc}. In this system, radioactive PrP^c is mixed with unlabeled PrP^{Sc}, and the conversion of the labeled PrP^c to PrP^{Sc} is followed by its becoming resistant to protease. These studies make clear that PrP^c can be converted to PrP^{Sc} by exposure to PrP^{Sc} in a process that does not require the activities of intact cells. However, so much infectivity is associated with the PrP^{Sc} added to the reaction mixture that no increase in infectivity can be demonstrated. Thus, these studies do not address the question of the nature of the infectious agent. These studies have also been useful in the study of the species barrier, which can be quantitatively examined in such reactions.

Protein-Only Hypothesis

It is clear that PrP is important in the development of TSEs. There are two unresolved questions about PrP and the disease process, however. First, is the infectious agent that leads to TSE PrP^{Sc} itself or is it another entity, such as a virus? Second, does PrP^{Sc}, or some other modified form of PrP, cause the symptoms of the disease, or is it simply a side effect of the disease process?

Preparations of the infectious agent purified from scrapie-infected mouse brain consist largely of PrP^{Sc}. There is very little nucleic acid in infectious preparations of PrP^{Sc}. In particular, there is no homogeneous DNA or RNA molecule that might arise from a virus, for example. This has led to the hypothesis that PrP^{Sc} is itself the infectious agent. In this model, "infectious" PrP^{Sc} induces PrP^c to assume the PrP^{Sc} conformation, and the accumulation of PrP^{Sc} in the brain leads to the pathology associated with TSEs. Most of the experimental data are compatible with such a model. PrP^{Sc} does induce PrP^c to assume the PrP^{Sc} conformation, as described earlier. Mutations in PrP^c could make it easier for the protein to assume the PrP^{Sc} conformation, compatible with the observation that some mutations result in inheritance of TSEs. The species barrier could result from lowered interaction affinities between proteins of different sequence. However, it has not been possible to prove this hypothesis. PrP^{Sc} preparations have a very low specific infectivity, with at least 10^5 molecules of PrP^{Sc} required for infection. Thus it remains possible that contaminants in the preparation might be required for infectivity. It has not been possible to demonstrate an increase in infectivity associated with the conversion of PrP^c to PrP^{Sc}, as described before, which would provide solid evidence that PrP^{Sc} is infectious.

In addition to the inability to prove the protein-only hypothesis, which could be due to the technical difficulties associated with this system, there are specific conceptual difficulties with PrP^{Sc} as the infectious agent. One of the major criticisms of the protein-only hypothesis is the fact that as many as 20 different strains of scrapie exist as assayed in mice. These strains of scrapie differ in properties such as the length of the incubation period following infection before disease becomes apparent, the areas of the brain affected, and the symptoms of the disease, but these properties do not vary within a strain. Such properties are expected for an infectious entity with a nucleic acid genome, but are difficult to reconcile with the properties of an infectious protein. If the protein-only hypothesis is true, these differences in properties could only result from differences in the conformation of PrP^{Sc} in the different strains. How is it that a single, fairly small protein can take up so many different conformations and that each can induce the production of more protein having the same conformation?

Supporters of the protein-only hypothesis suggest that a limited number of conformational states of the prion protein would be sufficient to explain the multiple strains of scrapie that exist. They point to experimental data that show that at least two demonstrably different conformational states of the prion proteins of two different mammals exist that "breed true." Two different strains of transmissible mink encephalopathy that produced different disease characteristics in mink were passaged in hamsters. The PrP^{Sc} from the two strains, isolated from infected brain, are differently truncated at the amino terminus on treatment with proteases *in vitro*. Thus the conformations of the PrP^{Sc} in these two strains, both derived from hamster PrP, must be different. Furthermore, this difference can be reproduced in an *in vitro* reaction in which PrP^{c} is mixed with the two different types of PrP^{Sc}. Each type of PrP^{Sc} induces PrP^{c} to assume its own distinct conformation, as shown by the protease resistant fragment that is produced from the PrP^{c} on it conversion to PrP^{Sc}.

In a second example of demonstrably different prion conformations, human prions isolated from two different cases of TSE, one FFI and the second CJD, were found to be differently truncated after protease treatment. Passage of these TSEs in transgenic mice that expressed a chimeric mouse–human prion protein gave rise to prions in infected brain that reproduced the differences in truncation. Thus, these conformational differences breed true when passed in mice.

These studies demonstrate that PrP^{Sc} can exist in at least two conformational forms, that the different conformational forms can produce different symptoms, and that the different forms are capable of propagation by inducing PrP^{c} to take up their own particular conformation. Thus, the experimental data are consistent with the protein-only hypothesis, although it has not been proven conclusively and many still doubt its validity. The hypothesis received a vote of confidence when its most outspoken and passionate advocate, Dr. Stanley Prusiner, was awarded the 1997 Nobel Prize for his "discovery of prions."

Transport of Infectivity to the Brain

Related to the conceptual problem of an infectious protein is how it might be transported to the brain after ingestion with food. This problem has been addressed in studies that ascertain in which tissues PrP^{Sc} is present following ingestion of PrP^{Sc}, and studies with transgenic mice that express PrP only in certain tissues. These various studies are compatible with a model in which infectivity is transported via axons following direct neuroinvasion of peripheral nerves. In the case of infection with only low doses of infectious material, amplification in follicular dendritic cells in lymphoid tissue may be required before neuroinvasion occurs. Thus, in terms of the protein-only model, PrP^{Sc} might induce the conversion of PrP^{c} to PrP^{Sc} in cells in Peyer's patches, which then spreads via lymphatic tissue to peripheral nerves by sequential conversion of PrP.

Formation of the PrP^{Sc} Seed

If PrP^{Sc} can transmit the disease to a new susceptible host, how is it formed in the first place? Current models propose that the conformational change resulting in PrP^{Sc} occurs rarely, but that once PrP^{Sc} is formed, it acts as a seed to induce the formation of more PrP^{Sc}. Two models to explain the conversion of PrP^{c} to PrP^{Sc} by PrP^{Sc} have been proposed. In one, PrP^{Sc} (which may be present in an aggregate) and PrP^{c} form a complex, and the PrP^{Sc} induces the conformational change in PrP^{c}. In the second model, PrP^{c} undergoes spontaneous transitions to different conformational states that are short lived and revert quickly to the native PrP^{c} conformational state. These conformational variants, however, can be locked into place by interaction with PrP^{Sc}. In either case, the altered PrP joins the aggregated PrP^{Sc} to form a larger aggregate. Since the aggregated PrP^{Sc} is insoluble, the reaction is essentially irreversible. Such a process could also explain the species barrier. The PrP proteins of different animals differ slightly in sequence. PrP^{c} that is identical in sequence to the PrP^{Sc} seed could interact with such a seed more readily than with a PrP^{Sc} seed that differs in sequence.

The protein-only hypothesis still requires a seed of PrP^{Sc} to begin the reaction. One possibility is that it can form spontaneously with a very low probability. Perhaps spontaneous changes in the conformation of PrP^{c} to the PrP^{Sc} conformation might be fixed if this change occurred simultaneously in a number of adjacent or interacting molecules. The effect of mutations in PrP^{c} might be to increase the probability of change to the PrP^{Sc} conformation, with the result that disease occurs more frequently. Such a model is compatible with data for human TSEs, where sporadic CJD occurs, albeit infrequently. However, sporadic disease has not been seen in shorter-lived animals. No sporadic BSE has been described,

and in countries where scrapie in sheep has been eradicated, such as New Zealand and Australia, no recurrence of disease has been observed.

Does PrP^{Sc} Cause the Disease?

If PrP^{Sc} is responsible for the pathology of TSE disease, and not simply a by-product of disease, the mechanism by which it causes disease is uncertain. An early model suggested that PrP^{Sc} itself is neurotoxic. However, it has been shown that a neuron must be able to express PrP^{c} before it can be killed by exposure to PrP^{Sc}. Thus simple neurotoxicity of PrP^{Sc} is not the cause of neuronal death. However, it is possible that conversion of PrP^{c} to PrP^{Sc} at the surface of the cell, which is known to occur, followed by accumulation of PrP^{Sc} in lysosomes as the neuron attempts to recycle it, could be toxic. In this model, it is the resistance of PrP^{Sc} to proteases in the lysosome that results in toxicity.

Recent findings have suggested another possibility. PrP can be expressed as a membrane-spanning protein as well as a protein anchored by a glycolipid anchor (Fig. 9.16). One membrane-spanning form, called ^{Ctm}PrP, has its C terminus

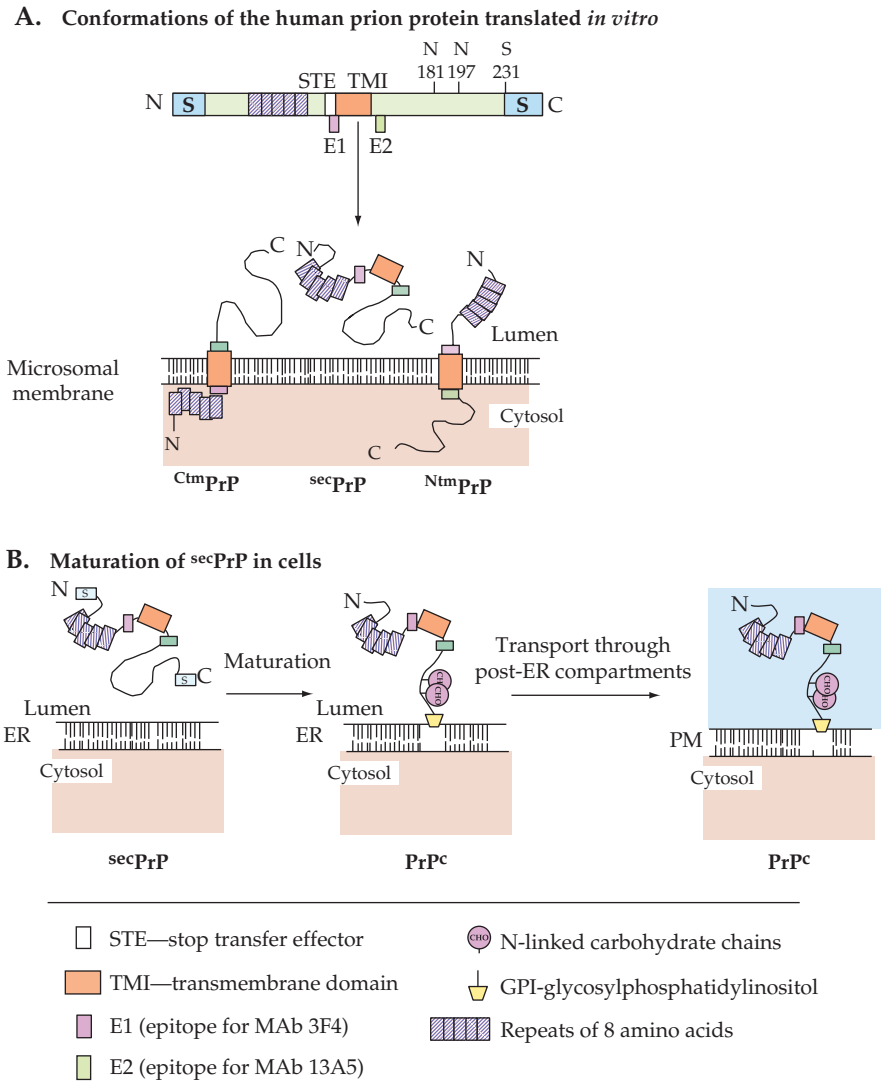

FIGURE 9.16 Postulated topology of PrP proteins in membranes. (A) Topology of PrP proteins in membranes after translation in a cell-free system supplemented with microsomes. The topology was determined by a combination of protease digestion from the cytosolic compartment and identification of the domains protected within the lumen using the two MAbs, 3F4 and 13A5. Mutations have been shown to affect the ratio of the three forms shown, and greater concentrations of ^{Ctm}PrP are associated with neurodegenerative disease in mice. (B) Model for maturation and association with membranes of ^{Sec}PrP in cells. ER, endoplasmic reticulum; PM, plasma membrane. Adapted from Hegde *et al*. (1998).

outside the cell and the N terminus inside, with a transmembrane domain near the middle of the molecule. Preliminary data suggest that this form of PrP is neurotoxic: ^{Ctm}PrP has been found in brains of animals, including humans, suffering from TSE but not in normal brains. This has led to a model in which ^{Ctm}PrP is regularly produced at some frequency, but the normal cell has a mechanism to eliminate it. Overproduction of ^{Ctm}PrP, either by mutation or by a failure to eliminate it, leads to the symptoms of TSE. In this model, production of PrP^{Sc} might somehow result in the accumulation of ^{Ctm}PrP, perhaps by overwhelming the ability of the cell to eliminate it.

PRIONS OF YEAST

Prions, defined as agents that possess two (or more) conformational forms, a soluble "normal" form and an aggregated form that can induce the conversion of the normal form to more of itself, have also been found in fungi. Two prions have been found in yeast (*Saccharomyces cerevisiae*) and a third in *Podospora* spp. The yeast prions have the characteristics of disease but the *Podospora* prion performs a normal cell function (controlling heterokaryon compatibility). The yeast prions are called [URE3], which affects nitrogen catabolism, and [PSI], which affects the termination of polypeptide chains during translation. A diagram of these proteins is shown in Fig. 9.17. [PSI] is a prion form of Sup35p, which is a translation release factor. In the [PSI] state, Sup35p assumes an altered conformation and aggregates, like PrP^{Sc}. The [PSI] state is dominant and can be transmitted to other yeast cells by transfer of cytoplasm containing [PSI]. Thus, the prion state induces the normal cell protein to assume the prion state, as with the model for PrP^{Sc}. The effect of the [PSI] state on the cell is to render Sup35p nonfunctional, and thus has the same effect as deletion of the gene encoding the protein. Loss of Sup35p activity leads to increased readthrough of stop codons during translation, and renders nonsense suppressor tRNAs much more active. [URE3] is the prion state of Ure2p, a protein involved in nitrogen catabolism. Like [PSI], [URE3] is an aggregated form of a conformational variant of Ure2p, and is dominant and transmissible. Loss of Ure2p by the cell affects the metabolism of nitrogen. Normal cells can assume the prion state with a low frequency, but once assumed the prion state is retained. Cells in the prion state can be cured by certain treatments that break up the protein aggregates and cause the protein to assume a normal conformation. Studies of yeast prions have shown that Sup35p produced in bacterial cells can be converted to [PSI] *in vitro* by introduction of a small seed of [PSI]. The [PSI] produced *in vitro* can be used in turn to convert more Sup35p to [PSI]. The [PSI] produced *in vitro* is infectious—when introduced into yeast it induces the assumption of the prion state. Thus, these studies clearly show that yeast prions are infectious and that only protein is required for infectivity, providing further support for the protein-only hypothesis of mammalian TSEs.

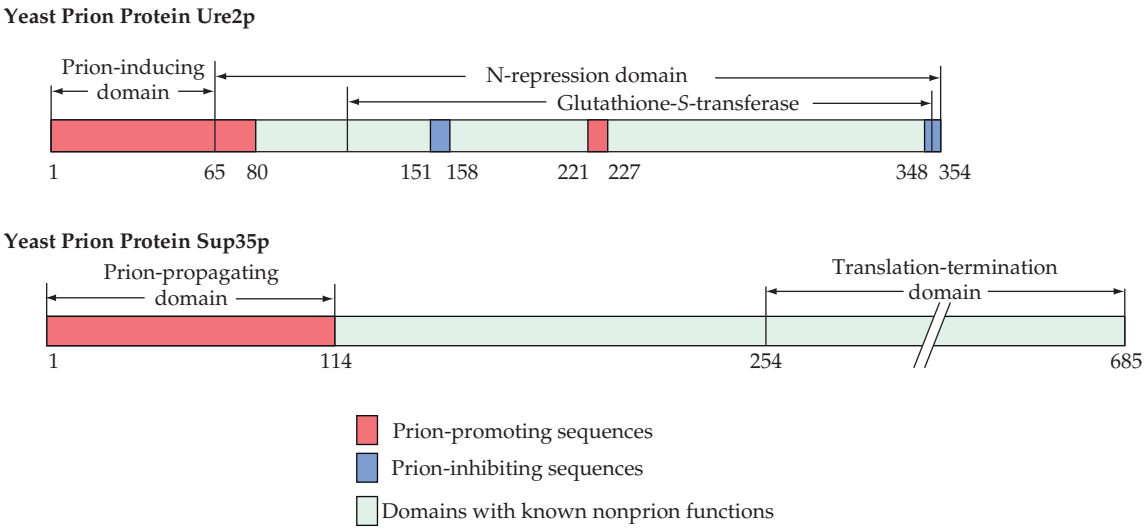

FIGURE 9.17 Comparison of two yeast prion proteins. The prion domains (red) of Ure2p and Sup35p are rich in Asn and Gln residues, which are important for prion generation and propagation. Adapted from Figure 10 of Wickner *et al.* (1999).

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