

Molecular Biology of Prion Diseases

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Prions cause transmissible and genetic neurodegenerative diseases, including scrapie and bovine spongiform encephalopathy of animals and Creutzfeldt-Jakob and Gerstmann-Sträussler-Scheinker diseases of humans. Infectious prion particles are composed largely, if not entirely, of an abnormal isoform of the prion protein, which is encoded by a chromosomal gene. A posttranslational process, as yet unidentified, converts the cellular prion protein into an abnormal isoform. Scrapie incubation times, neuropathology, and prion synthesis in transgenic mice are controlled by the prion protein gene. Point mutations in the prion protein genes of animals and humans are genetically linked to development of neurodegeneration. Transgenic mice expressing mutant prion proteins spontaneously develop neurologic dysfunction and spongiform neuropathology. Understanding prion diseases may advance investigations of other neurodegenerative disorders and of the processes by which neurons differentiate, function for decades, and then grow senescent.

PRIONS ARE INFECTIOUS PATHOGENS THAT DIFFER FROM bacteria, fungi, parasites, viroids, and viruses, both with respect to their structure and with respect to the diseases that they cause (1). Molecular biological and structural studies of prions promise to open new vistas into fundamental mechanisms of cellular regulation and homeostasis not previously appreciated. Kuru, Creutzfeldt-Jakob disease (CJD), and Gerstmann-Sträussler-Scheinker syndrome (GSS) are all human neurodegenerative diseases that are caused by prions and are frequently transmissible to laboratory animals (2). Familial CJD and GSS are also genetic disorders. Individuals at risk can often be identified decades in advance of central nervous system (CNS) dysfunction (3, 4), yet no effective therapy exists to prevent these lethal disorders.

In addition to the three prion diseases of humans, four disorders of animals are included in the ensemble of prion diseases. Scrapie of sheep and goats is the most studied of the prion diseases. Bovine spongiform encephalopathy (BSE), transmissible mink encephalopathy, and chronic wasting disease of captive mule deer and elk are all thought to result from the ingestion of scrapie-infected animal products. BSE threatens the beef industry of Great Britain (5) and possibly other countries; the production of pharmaceuticals (6) involving cattle is also of concern. Control of sheep scrapie in many countries is a persistent and vexing problem (7).

Since 1986, more than 28,500 cattle have died of BSE in Great Britain (5). Many investigators contend that BSE, often referred to as "mad cow disease," resulted from the feeding of dietary protein supplements derived from rendered sheep offal infected with scrapie to cattle, a practice banned since 1988 (5). It is thought that BSE will disappear with the cessation of feeding rendered meat and bone meal, as has been the case in kuru of humans, confined to the Fore region of New Guinea and once the most common cause of death among women and children. Kuru has almost disappeared with the cessation of ritualistic cannibalism, suggesting that kuru was transmitted orally, as proposed for BSE.

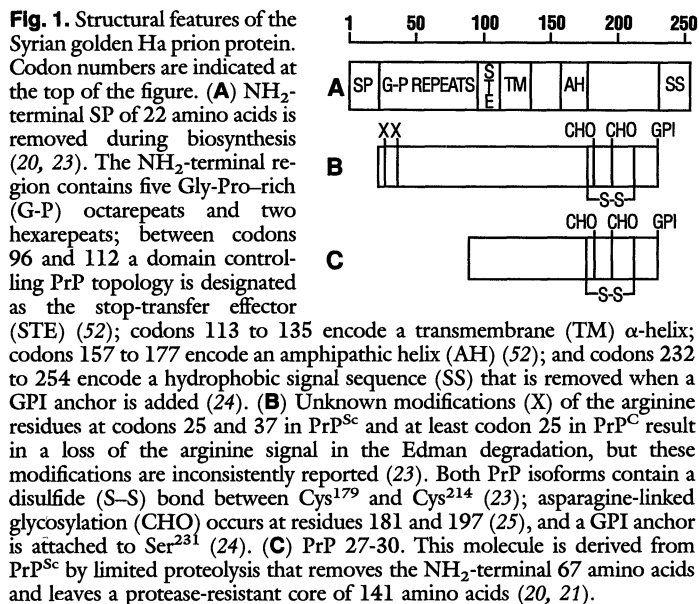
The Prion Hypothesis

The unusual biological properties of the scrapie agent were first recognized in studies with sheep (8). The experimental transmission of scrapie to mice (9) gave investigators a convenient laboratory model that provided information on the nature of the unusual infectious pathogen that causes scrapie (10, 11). Yet progress was slow because quantitation of infectivity in a single sample required housing 60 mice for 1 year before accurate scoring could be accomplished (9).

The development of a more rapid and economical bioassay for the scrapie agent in Syrian golden hamsters accelerated purification of the infectious particles (12, 13). Partial purification led to the discovery that a protein is required for infectivity (14), in agreement with earlier studies that raised the possibility that protein might be necessary (15). Procedures that modify nucleic acids did not alter scrapie infectivity (1). Other investigators found that scrapie infectivity resisted inactivation by both ultraviolet and ionizing radiation (10); these results prompted speculation that the scrapie pathogen might be devoid of nucleic acid—a postulate dismissed by most scientists. In addition to ultraviolet irradiation, reagents specifically modifying or damaging nucleic acids, such as nucleases, psoralens, hydroxylamine, and Zn^{2+} ions, do not alter scrapie infectivity in homogenates (1), microsomal fractions (1), purified prion rod preparations, or detergent-lipid-protein complexes (16, 17).

On the basis of these findings, I introduced the term "prion" to distinguish the proteinaceous infectious particles that cause scrapie, CJD, GSS, and kuru from both viroids and viruses (1). Hypotheses for the structure of the infectious prion particle included the following: (i) proteins surrounding a nucleic acid that encodes the proteins (a virus), (ii) proteins associated with a small polynucleotide, and (iii) proteins devoid of nucleic acid. Mechanisms postulated for the replication of infectious prion particles included those used by viruses, the synthesis of polypeptides in the absence of nucleic acid template, and posttranslational modifications of cellular proteins. Subsequent discoveries have narrowed hypotheses for both prion structure and the mechanism of replication.

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Discovery of the Prion Protein

Progress in the study of prions and the degenerative diseases of the CNS that they cause was accelerated by the discovery of a protein designated prion protein (PrP) (18). In subcellular fractions from hamster (Ha) brain enriched for scrapie infectivity, a protease-resistant protein of 27 to 30 kD, designated PrP 27-30, was identified; it was absent from controls. Purification of PrP 27-30 to homogeneity allowed determination of its NH₂-terminal amino acid sequence (19), which in turn permitted the synthesis of isocoding mixtures of oligonucleotides that researchers used to identify PrP complementary DNA (cDNA) clones (20, 21). PrP is encoded by a chromosomal gene and not by a nucleic acid in the infectious scrapie prion particle (20). Levels of PrP messenger RNA (mRNA) remain unchanged throughout the course of scrapie infection—an observation that led to the identification of the normal PrP gene product, a protein of 33 to 35 kD, designated PrP^C (20). PrP^C is protease-sensitive, whereas PrP 27-30 is the protease-resistant core of a 33- to 35-kD disease-specific protein, designated PrP^{Sc}.

Sequencing of molecular clones recovered from cDNA libraries that had been constructed from mRNA isolated from scrapie-infected Syrian Ha and mouse (Mo) brains showed that the Ha and MoPrP cDNAs encode proteins of 254 amino acids (Fig. 1) (20, 21). Identical sequences were deduced from genomic clones derived from DNA of uninfected, control animals (20). Human PrP consists of 253 amino acids (22). Signal peptides (SPs) of 22 amino acids at the NH₂-terminus are cleaved during the biosynthesis of Ha and MoPrPs in the rough endoplasmic reticulum (23). Twenty-three amino acids are removed from COOH-terminus of HaPrP on addition of a glycoinositol phospholipid (GPI) anchor (24). Two asparagine-linked oligosaccharides are attached to sites in a loop formed by a disulfide bond (23, 25). Limited proteolysis of PrP^{Sc} removes ~67 amino acids from its NH₂-terminus to produce PrP 27-30 (19, 20). Neither gas-phase sequencing nor mass spectrometric analysis of PrP 27-30 have revealed any amino acid differences between the sequences thus determined and that deduced from the translated sequence of molecular clones (26). The covalent structure of PrP^{Sc} remains uncertain because purified fractions contain ~10⁵ PrP 27-30 molecules per ID₅₀ unit (18). (One ID₅₀ unit is the infectious dose at which 50% of the animals develop scrapie.) If <1% of the PrP^{Sc}

molecules contained an amino acid substitution or posttranslational modification that conferred scrapie infectivity, our methods would not detect such a change (27).

Infectious Prion Particles

Information on PrP^{Sc} in prion diseases indicates that prions are composed of PrP^{Sc} molecules (Table 1). Although some investigators contend that PrP^{Sc} is merely a pathologic product of scrapie infection and that PrP^{Sc} coincidentally purifies with the “scrapie virus” (28), there are few data to support this view. No infective fractions containing <1 PrP^{Sc} molecule per ID₅₀ unit have been found; such a result would indicate that PrP^{Sc} is not required for infectivity. Some investigators report that PrP^{Sc} accumulation in hamsters occurs after the synthesis of many infective units (29), but these results have been refuted (30). The discrepancy appears to be due to comparisons of infectivity in crude homogenates with PrP^{Sc} concentrations measured in purified fractions.

The search for a component in the prion particle other than PrP has focused on a nucleic acid because the existence of such a component would readily explain different isolates or strains of infectivity (31). Specific scrapie isolates characterized by distinct incubation times retain this property when repeatedly passaged in mice or hamsters (31). Other factors modulating scrapie incubation times include PrP gene expression, murine genes linked to PrP (*Prn-i* and *Sinc*), dose of inoculum, route of inoculation, and the genetic origin of the prion inoculum. A scrapie-specific nucleic acid has not been found with reagents that modify or hydrolyze polynucleotides, with molecular cloning procedures, or with physicochemical techniques (16, 17, 32). Although available data do not permit exclusion of a scrapie-specific polynucleotide (27), its existence seems unlikely. That prions might contain noncovalently bound cofactors, such as peptides, oligosaccharides, fatty acids, sterols, or inorganic compounds, deserves consideration.

Table 1. Evidence that PrP^{Sc} is a major and necessary component of the infectious prion.

- 1) Copurification of PrP 27-30 and scrapie infectivity by biochemical methods. Concentration of PrP 27-30 is proportional to prion titer (18, 23).
- 2) Kinetics of proteolytic digestion of PrP 27-30 and infectivity are similar (18).
- 3) Copurification of PrP^{Sc} and infectivity by immunoaffinity chromatography. α -PrP antisera neutralization of infectivity (38).
- 4) PrP^{Sc} detected only in clones of cultured cells producing infectivity (50a).
- 5) PrP amyloid plaques are specific for prion diseases of animals and humans (34). Deposition of PrP amyloid is controlled, at least in part, by the PrP sequence (71).
- 6) Correlation between PrP^{Sc} (or PrP^{CJD}) in brain tissue and prion diseases in animals and humans (82).
- 7) Genetic linkage between MoPrP gene and scrapie incubation times (55, 56). PrP gene of mice with long incubation times encodes amino acid substitutions at codons 108 and 189, as compared to mice with short or intermediate incubation times (41).
- 8) Syrian HaPrP transgene and scrapie PrP^{Sc} in the inoculum govern the “species barrier,” scrapie incubation times, neuropathology, and prion synthesis in mice (71, 72).
- 9) Genetic linkage between human PrP gene mutation at codon 102 and development of GSS (3). Association between codon 200 point mutation or codon 53 insertion of six additional octarepeats and familial CJD (4, 62).
- 10) Mice expressing MoPrP transgenes with the point mutation of GSS spontaneously develop neurologic dysfunction, spongiform brain degeneration, and astrocytic gliosis (61).

PrP Polymers and Amyloid

The discovery of PrP 27-30 in fractions enriched for scrapie infectivity was accompanied by the identification of rod-shaped particles (18, 33). The rods are ultrastructurally indistinguishable from many purified amyloids and display the tinctorial properties of amyloids (33). These findings were followed by the demonstration that amyloid plaques in prion diseases contain PrP, as determined by immunoreactivity and amino acid sequencing (34). Some investigators believe that scrapie-associated fibrils are synonymous with the prion rods and are composed of PrP, even though these fibrils can be distinguished ultrastructurally and tinctorially from amyloid polymers (35, 36).

The formation of prion rods requires limited proteolysis in the presence of detergent (37). Thus, the prion rods in fractions enriched for scrapie infectivity are largely, if not entirely, artifacts of the purification protocol. Solubilization of PrP 27-30 into liposomes with a retention of infectivity (17) demonstrated that large PrP polymers are not required for infectivity and permitted the copurification of PrP^{Sc} and infectivity by immunoaffinity chromatography (38).

PrP Gene Structure and Expression

Localization of PrP genes to the short arm of human chromosome 20 and the homologous region of Mo chromosome 2 suggests that PrP genes existed before the speciation of mammals (39). Hybridization studies demonstrated <0.004 PrP gene sequences per ID₅₀ unit in purified prion fractions, indicating that the gene encoding PrP^{Sc} is not a component of the infectious prion particle (20). This feature distinguishes prions from viruses, including those retroviruses that carry cellular oncogenes, and from satellite viruses that derive their coat proteins from other viruses that had previously infected plant cells.

The entire open reading frame of PrP genes is contained in a single exon, eliminating the possibility that variant forms of PrP arise from alternative RNA splicing (20, 40, 41), but not excluding such mechanisms as RNA editing or protein splicing (42). The two exons of the HaPrP gene are separated by a 10-kb intron: exon 1 encodes a portion of the 5' untranslated leader sequence, whereas exon 2 encodes PrP and the 3' untranslated region (20). The MoPrP gene is composed of three exons, with exon 3 analogous to exon 2 of the Ha gene (40). The promoters of both the Ha and MoPrP genes contain copies of G-C-rich nonamers that may function as a canonical binding site for the transcription factor Sp1 (43).

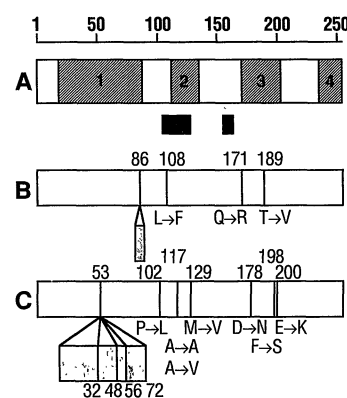
Although PrP mRNA is constitutively expressed in the brains of adult animals (20), it is regulated during development. In the septum; PrP mRNA and choline acetyl transferase were found to increase in parallel during development (44). In other brain regions, PrP gene expression occurred at an earlier age. The highest concentrations of PrP mRNA are found in neurons (45).

Four regions of the open reading frame of the mammalian PrP gene are conserved when the translated amino acid sequences are compared (Fig. 2) (20-22, 46, 47). Although the function of PrP^C is unknown, the MoPrP sequence is ~30% identical with a molecule found in fractions enriched for the acetylcholine receptor-inducing activity in chickens (48).

Synthesis of PrP Isoforms

Pulse-chase experiments with scrapie-infected cultured cells indicate that conversion of PrP^C is a posttranslational event (49). Although the synthesis and degradation of PrP^C are rapid (49, 50),

Fig. 2. Genetic map of prion protein open reading frames. Codon numbers are indicated at the top of the figure. (A) Four regions conserved among mammalian PrP molecules (hatched) (20-22, 46, 47); regions of MoPrP homologous to a molecule found in fractions containing acetylcholine receptor-inducing activity in chickens (black) (48). (B) Animal mutations and polymorphisms. Two alleles of bovine PrP identified, with one containing an additional octarepeat (stippled) at codon 86; a polymorphism at codon 171 in sheep PrP resulting in the substitution of arginine for glutamine (46). Mice with *Pm-p^b* genes have long scrapie incubation times and amino acid substitutions at codons 108 (Leu → Phe) and 189 (Thr → Val) (41). (C) Human PrP mutations and polymorphisms. Octarepeat inserts of 32, 48, 56, and 72 amino acids have been found (60, 62). Inserts of 48, 56, and 72 amino acids are associated with familial CJD. Point mutations at codons 102 (Pro → Leu), 117 (Ala → Val), and 198 (Phe → Ser) are found in patients with GSS (3, 60, 66). There are common polymorphisms at codons 117 (Ala → Ala) and 129 (Met → Val) (66, 83). Point mutation at codons 178 (Asp → Asn) and 200 (Glu → Lys) are found in patients with familial CJD (4, 64, 66). Single letter code for amino acids is as follows: A, Ala; D, Asp; E, Glu; F, Phe; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; and V, Val.



the accumulation of PrP^{Sc} is slow and confined to the chase period (Table 2) (49). These observations are in accord with studies that show that PrP^{Sc} accumulates in the brains of scrapie-infected animals, yet PrP mRNA concentrations remain unchanged (20).

Both PrP isoforms transit through the Golgi apparatus, where their asparagine-linked oligosaccharides are modified and sialic acid is added (25). PrP^C is presumably transported in secretory vesicles to the external cell surface, where it is anchored by a GPI moiety (24). In contrast, PrP^{Sc} accumulates in cells, where it is deposited in cytoplasmic vesicles, many of which appear to be secondary lysosomes (50a). Much of the mass added to PrP^{Sc} during posttranslational modification is due to asparagine-linked oligosaccharides, but they are not required for the synthesis of

Table 2. Properties of cellular and scrapie PrP isoforms. Numbers in parentheses after the properties indicate the reference source. PIPLC, phosphatidylinositol-specific phospholipase C. Half-time in hours, $t_{1/2}$.

Property	PrP ^C	PrP ^{Sc}
Concentration in normal Syrian Ha brain (71)	~1 to 5 μg/g	
Concentration in scrapie-infected Syrian Ha brain (71)	~1 to 5 μg/g	~5 to 10 μg/g
Presence in purified prions (18, 19, 33)	—	+*
Protease resistance (18-20, 33)	—	+†
Presence in amyloid rods (33, 34, 37)	—	+‡
Subcellular localization in cultured cells (24, 50a)	Cell surface	Cyto\$ vesicles
PIPLC release from membranes (24)	+	—
Synthesis ($t_{1/2}$) (49, 50)	<0.1	~1 to 3
Degradation ($t_{1/2}$) (49, 50)	~5	>>24

*Copurification of PrP^{Sc} and prion infectivity demonstrated by two protocols: (i) detergent extraction followed by sedimentation and protease digestion, and (ii) PrP 27-30 monoclonal antibody affinity chromatography. †Limited proteinase K digestion of HaPrP^{Sc} produces PrP 27-30. ‡After limited proteolysis of PrP^{Sc} (PrP 27-30 is produced) and detergent extraction, amyloid rods form; except for length, the rods are indistinguishable from amyloid filaments forming plaques. \$PrP^{Sc} is localized primarily in cytoplasmic vesicles. ||PrP^{Sc} de novo synthesis is a posttranslational process.

protease-resistant PrP in scrapie-infected cultured cells (51). This conclusion is based on results with the glycosylation inhibitor tunicamycin and with the expression of recombinant PrP with mutated asparagine-linked glycosylation sites. Experiments with transgenic mice may resolve whether unglycosylated PrP^{Sc} is associated with scrapie infectivity.

Two forms of PrP are found in cell-free translation studies: a transmembrane form that spans the bilayer twice (at the transmembrane and amphipathic helix domains) and a secretory form (Fig. 1) (52). The stop-transfer effector domain controls the topogenesis of PrP. That PrP contains a transmembrane domain as well as a GPI anchor poses a topologic conundrum. It seems likely that membrane-dependent events feature in the synthesis of PrP^{Sc}, especially because brefeldin A, which selectively destroys the Golgi stacks, prevents PrP^{Sc} synthesis in scrapie-infected cultured cells (53). The association of scrapie infectivity with membrane fractions has been appreciated for many years (11); hydrophobic interactions are thought to be responsible for the insolubility of infectious prion particles and for many of the difficulties encountered during attempts to characterize the particles (13, 17, 54).

Genetic Linkage of PrP with Scrapie Incubation Times

Studies of PrP genes (*Prn-p*) in mice with short and long scrapie incubation times demonstrated genetic linkage between a *Prn-p* restriction fragment length polymorphism (RFLP) and genes (*Prn-i* and *Sinc*) that modulate the incubation times of the disease (55–57). It remains to be established whether *Prn-p*, *Prn-i*, and *Sinc* are all allelic. The PrP sequences of NZW (*Prn-p^a*) and I/Ln (*Prn-p^b*) mice with short and long scrapie incubation times, respectively, differ at codons 108 and 189 (Fig. 2) (41). Although these amino acid substitutions suggest a congruency of *Prn-p* and *Prn-i*, experiments with *Prn-p^a* mice expressing *Prn-p^b* transgenes demonstrated a paradoxical shortening of incubation times (40), instead of the prolongation predicted from (*Prn-p^a* × *Prn-p^b*) F1 mice (long incubation times are dominant) (55–57). It is unknown whether this paradoxical shortening in transgenic (*Prn-p^b*) mice results from high levels of PrP^C expression.

Host genes also influence the development of scrapie in sheep. Parry argued that natural scrapie is a genetic disease that could be eradicated by proper breeding protocols (7). He considered its transmission by inoculation of importance primarily for laboratory studies and the communicable infection of little consequence in nature. Other investigators viewed natural scrapie as an infectious disease and argued that a host's genes modulate susceptibility to an endemic infectious agent (58). The dominant incubation time gene (*Sip*) for experimental scrapie in Cheviot sheep is thought to be linked to a PrP gene RFLP (59), a situation analogous to that for *Prn-i* and *Sinc* in mice. However, the data for genetic linkage in sheep are not convincing and further studies are needed, especially in view of earlier investigations in which susceptibility of sheep to scrapie was thought to be governed by a recessive gene (7). In Suffolk sheep, a polymorphism in PrP was found at codon 171 (Fig. 2B) (46); whether this polymorphism segregates with a *Sip* phenotype in Cheviot sheep is uncertain.

Human Familial Prion Diseases

CJD was believed to have a genetic basis when it was recognized that ~10% of CJD cases are familial (2). The discovery of the PrP gene (PRNP) in humans (22, 39) raised the possibility that muta-

tion might feature in the human prion diseases; a point mutation at PrP codon 102 was found to be genetically linked to GSS syndrome (Fig. 2C) (3). The codon 102 mutation has been found in American, British, German, Japanese, Canadian, Israeli, French, and Italian families, as well as in the Austrian family in which GSS was first described; these results suggest that the mutation may have arisen independently multiple times (60).

When the codon 102 point mutation was introduced into MoPrP in transgenic mice, spontaneous CNS degeneration occurred, characterized by clinical signs indistinguishable from experimental murine scrapie and neuropathology consisting of widespread spongiform morphology and astrocytic gliosis (61). By inference, these results suggest that PrP mutations cause GSS and familial CJD. It is unclear whether low levels of protease-resistant PrP in the brains of transgenic mice with the GSS mutation is PrP^{Sc} or residual PrP^C. Undetectable or low levels of PrP^{Sc} in the brains of these transgenic mice are consistent with the results of transmission experiments that suggest low titers of infectious prions. If brain extracts transmit CNS degeneration to inoculated recipients and the de novo synthesis of prions can be demonstrated by serial passage, then such observations would indicate that prions are devoid of foreign nucleic acid, in accord with studies that use other experimental approaches (10, 16, 28, 32).

An insert of 144 bp at codon 53 with six additional octarepeats has been described in individuals with CJD from four families that reside in southern England (Fig. 2C) (62); normal individuals have five octarepeats. Genealogical investigations have shown that all four families are related, suggesting that there was a single founder born more than two centuries ago. Seven or nine octarepeats (in addition to the normal five) were found in individuals with CJD, whereas deletion of one octarepeat or four additional octarepeats have been identified in individuals without the neurologic disease (62).

For many years the high incidence of CJD among Israeli Jews of Libyan origin was thought to be caused by the consumption of lightly cooked sheep brain or eyeballs (63). However, some Libyan and Tunisian Jews in families with CJD have a PrP gene point mutation at codon 200 (4, 64). One patient was homozygous for the mutation, but her clinical presentation was similar to that of heterozygotes (4); therefore, familial prion diseases are true autosomal dominant disorders like Huntington's disease (65). The codon 200 mutation also occurs in Slovaks originating from Orava in north central Czechoslovakia (60).

Other point mutations at codons 117, 178, and 198 also segregate with inherited prion diseases (66). Some patients once thought to have familial Alzheimer's disease are now known to have prion diseases on the basis of PrP immunostaining of amyloid plaques and PrP gene mutations (67). Patients with the codon 198 mutation have numerous neurofibrillary tangles that stain with antibodies to τ and have amyloid plaques (67) that are composed largely of a PrP fragment extending from residues 58 to 150 (68).

It has been suggested that PrP gene mutations render individuals susceptible to a virus (36). The putative scrapie virus is thought to persist in a worldwide reservoir of humans, animals, or insects without causing detectable illness. Yet one in 10⁶ individuals develop sporadic CJD and die from a lethal infection, whereas ~100% of people with PrP point mutations or inserts eventually develop neurologic dysfunction. That PrP gene germline mutations in patients and at-risk individuals cause familial prion diseases is supported by the experiments with transgenic mice described above. The transgenic mouse studies also suggest that sporadic CJD arises from the spontaneous conversion of PrP^C to PrP^{CJD} (a component of the prion that causes CJD) due either to a PrP gene somatic mutation or to a rare event involving modification of wild-type PrP^C.

Transgenic Animals and Species Barriers

The species barrier was discovered when scrapie prions were passed between species; this is a stochastic process characterized by prolonged incubation times (69). Prions synthesized *de novo* reflect the sequence of the host PrP gene and not that of the PrP^{Sc} molecules in the inoculum (70). On subsequent passage in a homologous host, the incubation time shortens to a constant length that is observed for all subsequent passages, and transmission becomes a nonstochastic process. The species barrier is of practical importance in assessing the risk for humans of acquiring CJD after consumption of scrapie-infected lamb or BSE-infected beef.

To test the hypothesis that differences in PrP gene sequences might be responsible for the species barrier, we constructed transgenic mice expressing HaPrP (71, 72). The PrP genes of Syrian hamsters and mice encode proteins differing at 14 residues. Incubation times in four lines of transgenic mice inoculated with Mo scrapie prions were prolonged, as compared to those observed for nontransgenic, control mice (Fig. 3A). Transgenic mice inoculated with Ha prions showed abbreviated incubation times in a nonstochastic process (Fig. 3B) (71, 72). The length of the incubation time after inoculation with Ha prions was inversely proportional to the level of HaPrP^C in the brains of the transgenic mice (Fig. 3, B and C) (71). HaPrP^{Sc} concentrations in the brains of clinically ill mice were similar in all four transgenic lines inoculated with Ha prions (Fig. 3D). Bioassays of brain extracts from clinically ill transgenic mice inoculated with Mo prions revealed that only Mo prions but no Ha prions were produced (Fig. 3E). Conversely, inoculation of transgenic mice with Ha prions led only to the synthesis of Ha prions (Fig. 3F). Thus, the *de novo* synthesis of prions in transgenic mice is species specific and reflects the genetic origin of the inoculated prions. Similarly, the neuropathology of transgenic mice is determined by the genetic origin of prion inoculum. Mo prions injected into transgenic mice produced neuropathology characteristic of mice with scrapie. A moderate degree of vacuolation in both the gray and white matter was found, whereas amyloid plaques were rarely detected (Fig. 3G). Inoculation of transgenic mice with Ha prions produced vacuolation of the gray matter, no vacuolation of

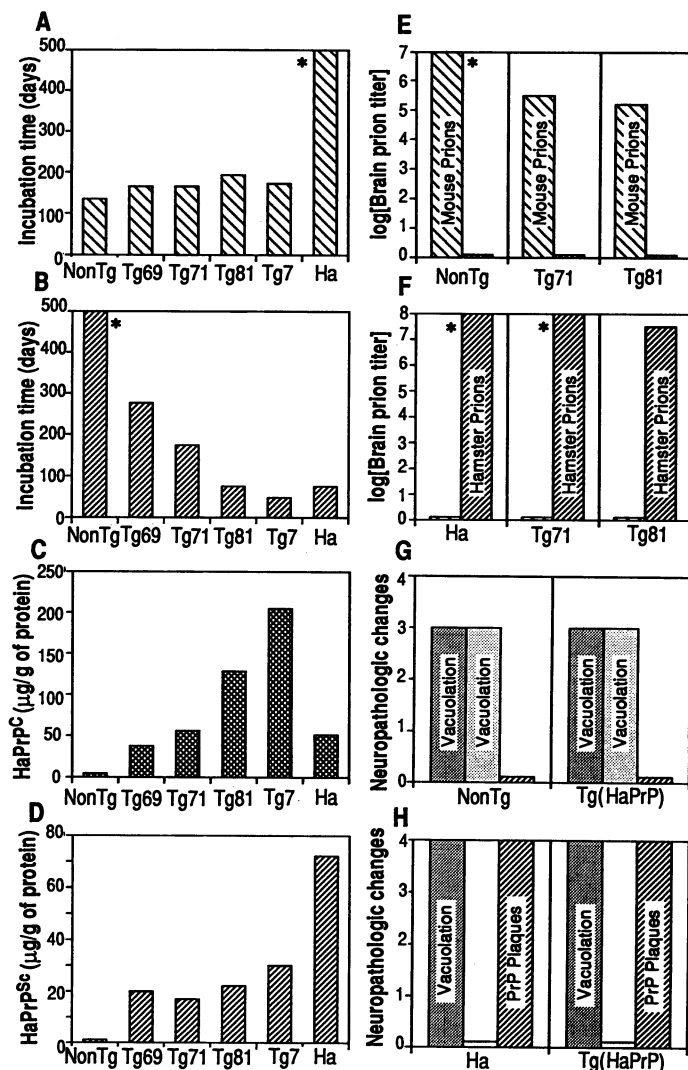
the white matter, and numerous HaPrP amyloid plaques, characteristic of Syrian hamsters with scrapie (Fig. 3H).

These studies with transgenic mice establish that the PrP gene influences all aspects of scrapie, including the species barrier, the replication of prions, the incubation times, the synthesis of PrP^{Sc}, and the neuropathologic changes.

Prion Multiplication

The mechanism by which prion infectivity increases is unknown. Some investigators believe that a scrapie-specific polynucleotide drives prion replication (28, 29, 31). If prions contain a scrapie-specific nucleic acid, then such a molecule would be expected to direct the multiplication of the scrapie agent by a strategy similar to that used by viruses (Fig. 4A). In the absence of any chemical or physical evidence for a scrapie-specific polynucleotide (16, 28, 32), it seems reasonable to consider alternative mechanisms that might be responsible for prion biosynthesis. The multiplication of prion infectivity is an exponential process in which the posttranslational conversion of PrP^C or a precursor to PrP^{Sc} appears to be obligatory (49). A PrP^{Sc} molecule might combine with a PrP^C molecule to produce a heterodimer that is subsequently transformed into two PrP^{Sc} molecules (Fig. 4B). In the next cycle, two PrP^{Sc} molecules combine with two PrP^C molecules, giving rise to four PrP^{Sc} molecules that combine with four PrP^C molecules, creating an

Fig. 3. Transgenic mice expressing Syrian Ha prion protein exhibit species-specific scrapie incubation times, infectious prion synthesis, and neuropathology (71). The number of mice used in each trial and the mean \pm SEM values can be found in (71) for (A) through (F). Asterisks indicate those values that exceed the scales in the y axes. (A) Scrapie incubation times in nontransgenic mice (NonTg) and four lines of transgenic mice expressing HaPrP and Syrian hamsters inoculated intracerebrally with $\sim 10^6$ ID₅₀ units of Chandler Mo prions serially passaged in Swiss mice. The four lines of transgenic mice have different numbers of transgene copies: Tg69 and Tg71 mice have 2 to 4 copies of the HaPrP transgene, whereas Tg81 mice have 30 to 50 and Tg7 mice have >60 . Incubation times are the number of days from inoculation to onset of neurologic dysfunction. (B) Scrapie incubation times in mice and hamsters inoculated with $\sim 10^7$ ID₅₀ units of Sc237 prions serially passaged in Syrian hamsters and as described in (A). (C) Brain HaPrP^C in transgenic mice and hamsters. HaPrP^C levels were quantitated by an enzyme-linked immunoassay. (D) Brain HaPrP^{Sc} in transgenic mice and hamsters. Animals were killed after exhibiting clinical signs of scrapie. HaPrP^{Sc} levels were determined by immunoassay. (E) Prion titers in brains of clinically ill animals after inoculation with Mo prions. Brain extracts from NonTg, Tg71, and Tg81 mice were bioassayed for prions in mice (left) and hamsters (right). (F) Prion titers in brains of clinically ill animals after inoculation with Ha prions. Brain extracts from Syrian hamsters as well as Tg71 and Tg81 mice were bioassayed for prions in mice (left) and hamsters (right). (G) Neuropathology in NonTg mice and Tg(HaPrP) mice with clinical signs of scrapie after inoculation with Mo prions. Vacuolation in gray (left) and white matter (center); PrP amyloid plaques (right). Vacuolation score: 0 = none, 1 = rare, 2 = modest, 3 = moderate, and 4 = intense. PrP amyloid plaque frequency: 0 = none, 1 = rare, 2 = few, 3 = many, and 4 = numerous. (H) Neuropathology in Syrian hamsters and transgenic mice inoculated with Ha prions. Degree of vacuolation and frequency of PrP amyloid plaques as in (G).



exponential process. Results from transgenic mice expressing Ha PrP transgenes show that the mice produce only those prions present in the inoculum (Fig. 3, E and F) (71). Presumably, PrP^{Sc} in the prion inoculum interacts with the homologous PrP^C substrate during replication to produce more of the same prions (Fig. 4C).

In the absence of any candidate posttranslational chemical modifications (26) that differentiate PrP^C from PrP^{Sc}, we must consider the possibility that conformation distinguishes these isoforms. Various isolates of scrapie prions (31) might result from multiple conformers that could act as templates for the folding of de novo synthesized PrP^{Sc} molecules during prion replication (Fig. 4D). Although this proposal is unorthodox, it is consistent with observations from transgenic mice studies that indicate that PrP^{Sc} in the inoculum binds to homologous PrP^C or a precursor to form a heterodimeric intermediate in the replication process (71). Presumably, "foldases," chaperones, or other macromolecules (73) feature in the conversion of the PrP^C-PrP^{Sc} heterodimer to PrP^{Sc} molecules. The number of PrP^{Sc} molecules composing a prion particle is unknown, but ionizing radiation studies indicate a target size of 55 kD, suggesting that a PrP^{Sc} dimer or possibly trimer is required for infectivity (74).

Two isolates of Ha prions inoculated into transgenic mice and different species of hamsters gave results indicating that the sequence and metabolism of PrP may profoundly influence the isolate phenotype. The Sc237 isolate of Ha prions produced incubation times of 77 ± 1 day ($n = 48$) in Syrian hamsters, whereas the 139H isolate yielded incubation times of 168 ± 7 day ($n = 54$) (31). HaPrP^C expression in Tg(HaPrP)7 mice is approximately fivefold higher than in Syrian hamsters (Fig. 3C). In Tg(HaPrP)7 mice, the Sc237 isolate produced incubation times of 48 ± 1 day ($n = 26$), whereas 139H gave incubation times of 40 ± 3 day ($n = 11$) (75). One interpretation of these observations is that Sc237 prions have a higher affinity for PrP^C than 139H prions that is only apparent at nonsaturating levels of substrate. Increased levels of PrP^C substrate in Tg(HaPrP)7 mice might saturate the PrP^{Sc} conversion process, thus resulting in a diminution of the incubation times for both prion isolates and eliminating the differences between them. In Chinese and Armenian hamsters with PrP gene sequences that differ from that of the Syrian at 7 and 8 codons, respectively (47), 139H produces incubation times that are either shorter or similar to those observed with Sc237. In this case, the amino acid sequence of PrP may modulate the affinities of PrP^{Sc} in the two isolates for PrP^C molecules; indeed, the formation of PrP^C-PrP^{Sc} heterodimers may be the rate-limiting step in the prion biosynthesis that determines scrapie incubation times (Fig. 4D).

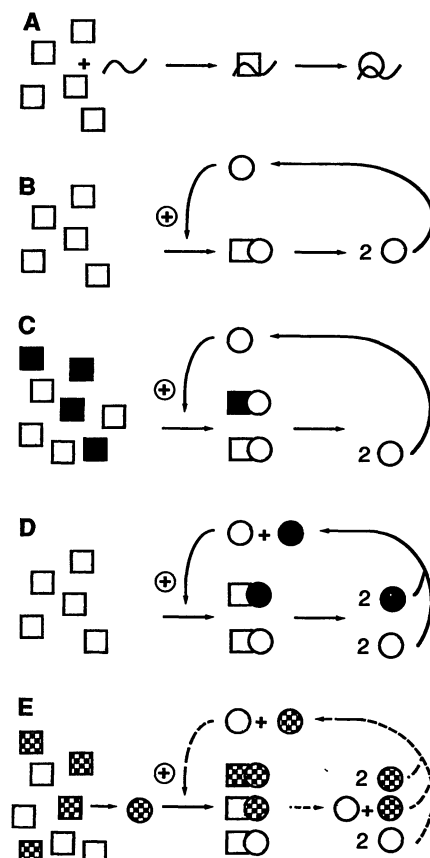
In humans carrying point mutations or inserts in their PrP genes, mutant PrP^C molecules might spontaneously convert into PrP^{Sc} (Fig. 4E). Although the initial stochastic event may be inefficient, once it happens the process would then become autocatalytic. The proposed mechanism explains the existence of individuals harboring germline mutations who do not develop CNS dysfunction for decades and is also consistent with results with transgenic mice that express the GSS mutation and spontaneously develop CNS degeneration (61). Whether all GSS and familial CJD cases are attributable to infectious prions or whether some represent inborn errors of PrP metabolism in which neither PrP^{Sc} nor prion infectivity accumulates is unknown.

Conformational changes in allosteric enzymes induced by phosphorylation or the binding of small ligands (76) might provide some precedent for the proposed models (Fig. 4, B through E). Consider the possibility that PrP^{Sc} acts as a ligand that induces a conformational change in PrP^C to produce a second PrP^{Sc} molecule. Noteworthy are five different crystalline allomorphs of mutant lysozyme from bacteriophage T4 (77); these are thought to represent a

continuous range of conformations that occur in solution. Rapid- and slow-folding populations of lysozyme have been observed; the latter are presumed to have arisen from *cis-trans* isomerization of peptide bonds preceding proline residues (78). Whether *cis-trans* proline isomerization is of significance in the conversion of PrP^C or a precursor to PrP^{Sc} is uncertain. Of interest are the folding and assembly of phage tail spike proteins into trimers that resist denaturation and proteolysis, properties reminiscent of those exhibited by PrP^{Sc} (79). In ciliates, the cytoplasmic inheritance of asymmetrical arrangements of surface structures (80) may also provide some insight into the mechanism by which PrP^C is converted to PrP^{Sc} during the propagation of distinct scrapie isolates.

Although results with transgenic mice argue for the interaction of PrP^{Sc} with PrP^C during scrapie prion multiplication, there are no data to support the proposal that prion multiplication proceeds through a crystallization process involving PrP amyloid formation (81). The absence or rarity of amyloid plaques in many prion diseases, as well as the inability to identify any amyloid-like polymers in cultured cells that synthesize prions, does not support this hypothesis (37, 71). Purified infectious preparations isolated from scrapie-infected Ha brains contain PrP^{Sc} molecules that exist as amorphous aggregates; only if PrP^{Sc} is exposed to detergents and limited proteolysis does it polymerize into prion rods with the ultrastructural and tinctorial features of amyloid (37). Furthermore,

Fig. 4. Some possible mechanisms of prion replication. (A) Two-component prion model. Prions contain a putative, as yet unidentified, nucleic acid or other second component (solid, thick wavy line) that binds to PrP^C (squares) and stimulates conversion of PrP^C or a precursor to PrP^{Sc} (circles). (B) One-component prion model—prions devoid of nucleic acid. PrP^{Sc} binds to PrP^C forming heterodimers that function as replication intermediates in the synthesis of PrP^{Sc}. Repeated cycles of this process result in an exponential increase in PrP^{Sc}. (C) Prion synthesis in transgenic mice (71). HaPrP^{Sc} (circles) binds to HaPrP^C (white squares), leading to the synthesis of PrP^{Sc}. Binding to MoPrP^C (black squares) does not produce PrP^{Sc}. Species barrier for scrapie between mice and hamsters represented by MoPrP^C-HaPrP^{Sc} heterodimer. (D) Scrapie isolates or strains in hamsters or mice. Multiple PrP^{Sc} conformers (circles) bind to PrP^C and constrain the conformational changes that PrP^C undergoes during its conversion into PrP^{Sc}. (E) Inherited prion diseases in humans and transgenic mice. Mutant PrP^C molecules (checked pattern in squares) might initiate the conversion of PrP^C to PrP^{Sc} (or PrP^{CJD}). If infectious prions are produced (dashed lines), then they stimulate the synthesis of more PrP^{CJD} in humans and PrP^{Sc} in experimental animals. Alternatively, prion infectivity is not generated, but the host develops neurologic dysfunction, spongiform degeneration, astrocytic gliosis, and possibly PrP amyloid plaques (2, 3, 60, 61).



dispersion of prion rods into liposomes results in a 10- to 100-fold increase in scrapie prion titer; no rods could be identified in these fractions by electron microscopy (17).

Future Challenges and New Approaches

Whether prions are composed entirely of PrP^{Sc} molecules or contain a second component needs to be resolved. Determining the crystal structures of PrP^C and PrP^{Sc}, as well as the structures of these molecules in solution, is important. Understanding the molecular events that feature in prion replication should help decipher the structural basis for the scrapie isolates or strains that have different incubation times in the same host. Whether distinct conformations of PrP^{Sc} correspond to different prion isolates is unknown. Elucidating the function of PrP^C might extend our understanding of the pathogenesis of prion diseases and point to other macromolecules that participate in a variety of human and animal diseases of unknown etiology. Lessons learned from prion diseases may give insights into the etiologies, as well as the pathogenic mechanisms, of such common CNS degenerative disorders as Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease.

The lack of effective therapies for the prion diseases, all of which are fatal, poses a significant challenge. Because the mechanism of prion replication appears unprecedented, it is not surprising that antibacterial, fungal, and viral therapeutics are of little value in the modification of the course of prion diseases. On the other hand, prenatal testing in families with prion diseases does present a method for controlling the genetic spread of these disorders.

Although the results of many studies indicate that prions are a new class of pathogens distinct from both viroids and viruses, it is unknown whether different types of prions exist. Are there prions that contain modified proteins other than PrP^{Sc}? Assessing how widespread prions are in nature and defining their subclasses are subjects for future investigation. Elucidation of the mechanism by which brain cells cease to function and die in prion diseases after a long delay may offer approaches to understanding how neurons develop, mature, and continue to transmit signals for decades.

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