Annu. Rev. Biochem. 2004. 73:617–56
doi: 10.1146/annurev.biochem.72.121801.161837

First published online as a Review in Advance on March 26, 2004

# EMERGING PRINCIPLES OF CONFORMATION-BASED PRION INHERITANCE

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**Key Words** PSI, amyloid, prion strains, species barrier, misfolding

■ **Abstract** The prion hypothesis proposes that proteins can act as infectious agents. Originally formulated to explain transmissible spongiform encephalopathies (TSEs), the prion hypothesis has been extended with the finding that several non-Mendelian traits in fungi are due to heritable changes in protein conformation, which may in some cases be beneficial. Although much remains to be learned about the specific role of cellular cofactors, mechanistic parallels between the mammalian and yeast prion phenomena point to universal features of conformation-based infection and inheritance involving propagation of ordered $\beta$-sheet-rich protein aggregates commonly referred to as amyloid. Here we focus on two such features and discuss recent efforts to explain them in terms of the physical properties of amyloid-like aggregates. The first is prion strains, wherein chemically identical infectious particles cause distinct phenotypes. The second is barriers that often prohibit prion transmission between different species. There is increasing evidence suggesting that both of these can be manifestations of the same phenomenon: the ability of a protein to misfold into multiple self-propagating conformations. Even single mutations can change the spectrum of favored misfolded conformations. In turn, changes in amyloid conformation can shift the specificity of propagation and alter strain phenotypes. This model helps explain many common and otherwise puzzling features of prion inheritance as well as aspects of noninfectious diseases involving toxic misfolded proteins.

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## INTRODUCTION

The idea that a protein conformation can replicate itself and therefore serve as a genetic element was first formalized by the prion hypothesis, which seeks to explain an unusual set of neurodegenerative diseases known as the transmissible spongiform encephalopathies (TSEs). These devastating diseases result in progressive cognitive and motor impairment and are characterized by the accumulation of proteinaceous brain lesions or plaques (1). Sheep scrapie was the first of these diseases to be recognized, but subsequently a set of human diseases, such as kuru and Creutzfeldt-Jacob Disease (CJD), was shown to have similar clinical and pathological features. TSEs have now been identified in a wide range of mammals, including cats, cows, mink, deer, and elk (2).

Though the TSEs can arise spontaneously or be inherited, they are also infectious (3). The earliest illustrations of infectivity were accidental; sheep scrapie was transmitted to an entire flock during routine vaccination, and kuru was transmitted through ritual cannibalism practiced by a tribe in New Guinea.

Subsequent experiments showed that human disease could be transmitted to primates and surprisingly indicated that the infectious agent was resistant to classic methods for inactivating nucleic acid. The purification of the infectious agent responsible for scrapie led to the remarkable discovery that it was composed primarily, if not entirely, of protein. On the basis of this observation, Stanley Prusiner proposed that a novel proteinaceous infectious agent, termed a "prion," was responsible for these diseases [reviewed in (4)]. It was later found that the infectious protein is a ubiquitous endogenous cellular protein, termed "PrP" for prion protein.

How might an endogenous protein be infectious? In a prescient argument prompted by the need to reconcile the failure to detect nucleic acids in the infectious agent responsible for scrapie with the newly emerging central dogma of molecular biology, Griffith (5) described three general mechanisms for replication of a protein so that "the occurrence of a protein agent would not necessarily be embarassing." In the first mechanism, a transcriptional activator could be infectious if it were to turn on a normally quiescent gene that participated in a positive feedback loop driving its own production. The second mechanism postulated a change in either protein conformation or multimeric state that cannot occur without a catalyst, such as a preformed multimeric nucleus. The final mechanism invoked an immune response feedback loop. Another mechanism has recently been described by Wickner (6): A zymogen, or self-activating enzyme, can be infectious if an active form is introduced into a pool of otherwise stably inactive proteins.

One of above mechanisms, propagation of conformational change, appears to underlie the mammalian TSEs. During purification of the infectious scrapie agent, a $\beta$-sheet-rich insoluble protease-resistant fragment of PrP was associated with highly infectious preparations. Surprisingly this form is covalently identical to the normal cellular form of PrP, but in uninfected animals PrP is alpha-helical, soluble, and protease sensitive (Figure 1). A variety of observations now support a model where the scrapie-associated form of PrP, termed "$PrP^{SC}$," is transmitted by conformational conversion of the normal cellular form, called $PrP^{C}$. Though two Nobel prizes have been awarded for research on TSEs, the biology of mammalian prion diseases is still hotly contested (7). Specifically, the size and nature of the infectious particle remain unresolved due to experimental limitations. First, the specific activity of purified material is extremely low (8). Second, infectious preparations without protease-resistant $PrP^{SC}$ have been found (9). Third, and most challenging, recombinant infectious material has not yet been produced in vitro to provide the formal proof of the protein-only hypothesis. Together, these technical limitations have left lingering questions whether other components, such as chaperones, small molecules, or even RNAs (10) could play a role in prion infection.

Despite these questions about the mechanisms of mammalian TSEs, it has become clear that proteins can serve as genetic elements and that prions are more widespread in biology than previously thought. In 1994, Wickner (11) proposed

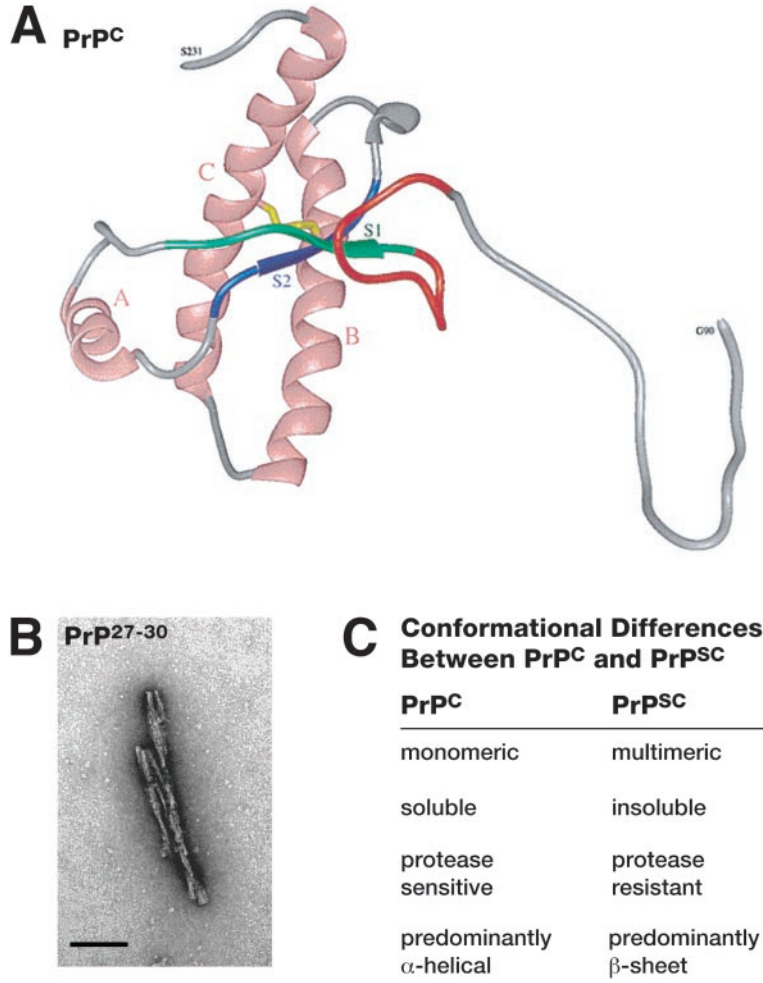


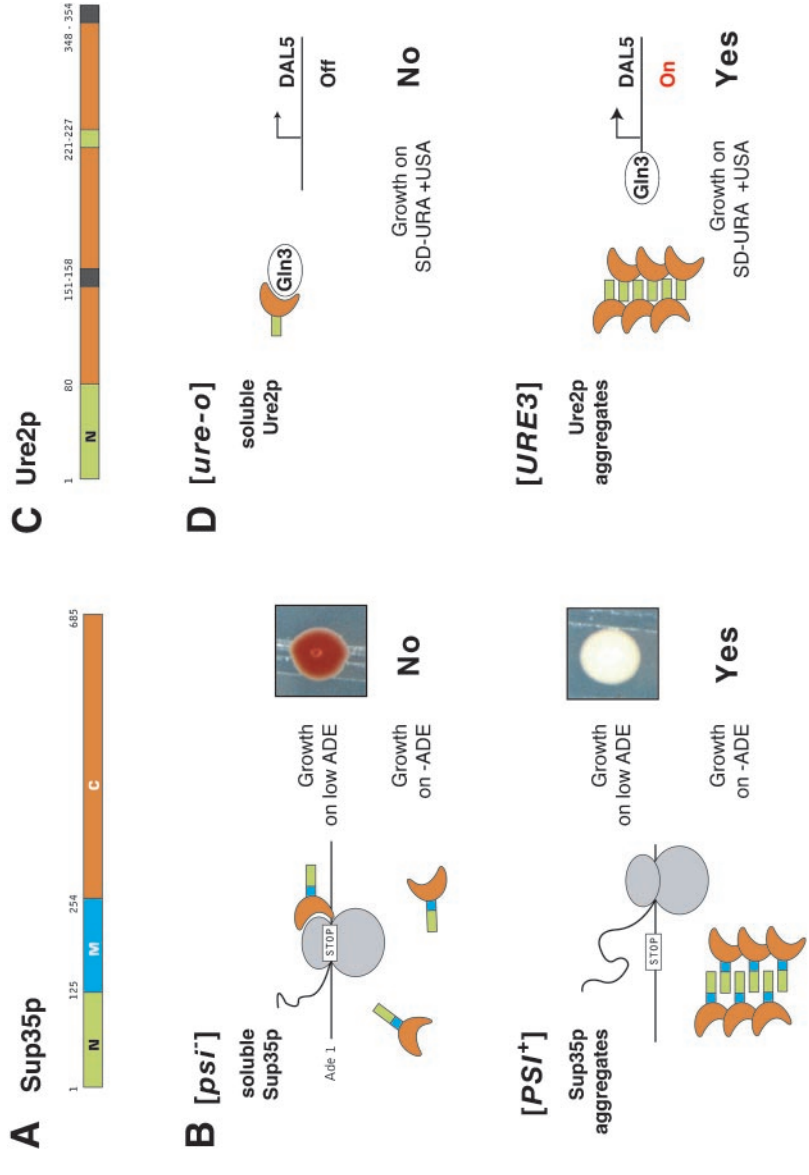

**Figure 1** $PrP^{C}$ and $PrP^{SC}$ are conformationally distinct. (*A*) Solution NMR structure of Syrian hamster $PrP^{C}$, residues 90–231. The structure is predominantly alpha helical with an unstructured amino terminus (199). (*B*) Negative stain EM of Syrian hamster $PrP^{27–30}$ (Sc237 strain), stained with uranyl acetate. The material is in insoluble protease-resistant high molecular weight aggregates that are predominantly $\beta$-sheet. Scale bar is 100 nm. Image courtesy of Dr. Holger Wille (unpublished material). (*C*) Summary of differences between $PrP^{C}$ and $PrP^{SC}$.

that the behavior of two non-Mendelian cytoplasmically inherited traits in *Saccharomyces cerevisiae*, [*PSI*$^{+}$] and [*URE3*], could be explained by a prion-like mechanism where an alternate protein form does not cause disease but does "infect" daughter cells as they bud from the mother. This model was based on

three remarkable features shared by [*URE3*] and [*PSI*$^+$]. One, propagation of [*URE3*] and [*PSI*$^+$] is dependent on the continuous expression of an associated gene, *URE2* and *SUP35*, respectively, yet their phenotypes mimic loss-of-function mutations in these genes. Two, [*URE3*] and [*PSI*$^+$] can be cured by growth on guanidine hydrochloride and can return to the prion state without any changes in the genome. Three, overexpression of Ure2p and Sup35p increases the frequency of de novo [*URE3*] and [*PSI*$^+$] appearance. Wickner's model elegantly explained these observations by postulating that overexpression results in a novel prion form of the protein. The prion form is self-propagating, which allows inheritance, and inactivating, which results in the apparent loss-of-function phenotype. This model has been confirmed and expanded by the work of multiple labs, and it is now established that both [*PSI*$^+$] and [*URE3*] are due to the self-propagating aggregation of Sup35p and Ure2p, respectively (12, 13). More fungal prion domains were subsequently discovered, including [*RNQ*$^+$], also known as [*PIN*$^+$], [*NU*$^+$], and [*Het-s*]. These have been comprehensively reviewed elsewhere (14, 15).

The yeast prions have provided genetically and biochemically tractable systems for studying prion behavior, greatly facilitating studies on the mechanism of conformation-based inheritance and infection (12, 13). [*URE3*] and [*PSI*$^+$] are the best characterized, and both offer accessible in vivo and in vitro experimental systems. In vivo, genetic screens can exploit the nitrogen uptake phenotype of [*URE3*] yeast or the nonsense suppression phenotype of [*PSI*$^+$] yeast (Figure 2). In vitro, propagation of [*URE3*] and [*PSI*$^+$] are modeled by the formation of amyloid fibers. Both Ure2p and Sup35p are modular proteins with their prion activity localized to an amino-terminal glutamine/asparagine-rich domain separable from domains responsible for their normal cellular function (Figure 2). These purified prion domains spontaneously form amyloid fibers only after a characteristic lag phase that can be eliminated by the addition of preformed seeds, mimicking propagation in vivo. Importantly, formal proof of the prion hypothesis has come from studies with [*PSI*$^+$] and [Het-s]. When introduced into cells, amyloid seeds generated in vitro from purified recombinant Sup35p, or Het-s* are able to cause de novo formation of the [*PSI*$^+$] and [Het-s] states, respectively (16, 17).

Although there are critical differences in the cellular location and phenotypic consequences between mammalian and yeast prions, they share a remarkable number of common mechanistic features. In this review, we compare and contrast these systems in an effort to build a general model for conformation-based infection and inheritance. We first consider that both mammalian and yeast prions appear to be due to the propagation of $\beta$-sheet-rich aggregates that resemble amyloid fibers. In contrast to disordered amorphous aggregates, amyloids are highly ordered fibrillar structures, formed by a wide variety of polypeptides with no homology in either their native structures or in their amino acid sequence (Figure 3). In many cases these amyloids are self-propagating; in


A Sup35p
1
125
254
685
N
M
C
C Ure2p
1
80
151-158
221-227
348 - 354
N
B [psi⁻]
soluble Sup35p
Ade 1
STOP
Growth on low ADE
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Sup35p aggregates
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Growth on SD-URA +USA
No
[URE3]
Ure2p aggregates
Gln3
DAL5
On
Growth on SD-URA +USA
Yes

general, however, amyloids are not infectious. Thus, an unresolved question is—what distinguishes prions from this larger class of misfolded proteins?

We next consider that both mammalian and yeast prions display multiple strains in which infectious particles composed of the same protein give rise to distinct phenotypes. This strain phenomenon has been difficult to reconcile with the protein-only hypothesis, but evidence is accumulating from both the prion and amyloid fields that a single polypeptide can form multiple distinct conformations, which may provide the structural basis for strain diversity. We then examine the sequence specificity of prion propagation, which manifests in both the mammalian and yeast systems as a "species barrier," inhibiting transmission between even highly related species. Finally, we review the evidence that strains and species barriers can result from the same underlying process, namely that a single polypeptide can form multiple self-propagating states. These different conformations can lead to distinct strain phenotypes and can determine the sequence specificity of prion propagation.

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**Figure 2** The yeast prions [*PSI*$^+$] and [*URE3*] are the result of self-propagating protein conformations. (*A*) Sup35p is a modular protein involved in translation termination; self-propagating aggregation is responsible for the [*PSI*$^+$] phenotype. The amino-terminal prion-forming domain, N (*green*), is glutamine- and asparagine-rich. The middle domain, M (*blue*), is rich in charged residues. The carboxy-terminal domain, C (*orange*), contains the essential translation-termination function of the protein. (*B*) Sup35p is soluble in [*psi*$^-$] yeast and able to facilitate translation termination while in [*PSI*$^+$] yeast; Sup35p is aggregated, resulting in suppression of nonsense codons. Translation termination can be monitored using an *ADE1* reporter harboring a premature stop codon. [*PSI*$^+$] cells are white and capable of growth on media lacking adenine, whereas [*psi*$^-$] yeast accumulate a red pigment caused by lack of Ade1p and are incapable of growth on adenine-less media. (*C*) Ure2p is a modular protein involved in regulation of nitrogen catabolism; self-propagating aggregation of Ure2p is responsible for the [*URE3*] phenotype. In addition to the glutamine/asparagine-rich amino terminus (*green*), Ure2p also contains another region that facilitates prion behavor (*green*) and portions that antagonize prion formation (*black*). The remainder of the protein (*orange*) resembles glutathione S-transferase and is necessary for Ure2p signaling of the presence of high-quality nitrogen sources through Gln3p. (*D*) Normally Ure2p binds the transcription factor Gln3p, preventing the upregulation of genes, such as *DAL5*, required for uptake of poor nitrogen sources. Serendipitously, Dal5p imports not only the poor nitrogen source allantoate, but also USA (*n*-carbamyl aspartate), an intermediate in uracil biosynthesis. Thus [*ure-o*] yeast cannot grow on ureidosuccinate (USA) medium lacking uracil. In [*URE3*] yeast, Ure2p is aggregated and inactive, leading to constitutive activation of Dal5p and enabling growth on USA media lacking uracil.

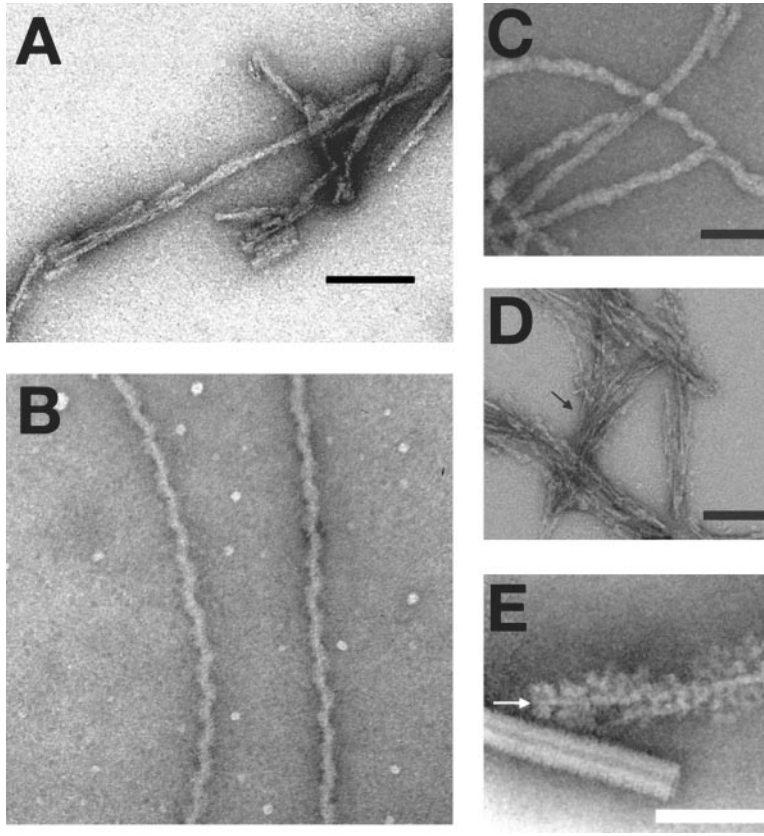


**Figure 3** Amyloid-like fibers are formed by a variety of prion proteins. (*A*) EM of Syrian hamster $PrP^{27–30}$ (Sc237 strain), stained with uranyl acetate. Bar = 100 nm. Image courtesy of Dr. Holger Wille (unpublished material). (*B*) Amyloid fibers formed by Sup35NM, stained with uranyl acetate. Sup35NM fibers are on average 5–10 nm in diameter. (*C*, *D*) EM of full-length Ure2p fibers stained with uranyl acetate before (*C*) and after (*D*) digestion with proteinase K. Arrow in *D* indicates position of a single fiber. Bar = 100 nm (89). (*E*) Amyloid fibers formed by full-length Ure2p, stained with vanadate and visualized by dark-field scanning transmission electron microscopy (STEM). Arrow indicates the core of the fiber. Bar = 50 nm (89).

## AMYLOID-LIKE SELF-PROPAGATING PROTEIN AGGREGATES UNDERLIE PRION INHERITANCE

The fundamental requirement of the prion hypothesis is that a protein be capable of adopting a state that can initiate and sustain its own replication. Although the cellular machinery for transcription and translation are used to generate new

polypeptides, the infectious protein must contain enough information to direct production of the prion rather than the normal cellular form. Abundant evidence has accumulated that both mammalian and yeast prions accomplish this by directing conformational change of a normal cellular host protein into an alternate prion conformation. These alternate conformations are $\beta$-sheet-rich multimers and resemble a broader class of ordered protein aggregates termed amyloids. Amyloids are associated with a variety of noninfectious neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, as well as a range of systemic amyloidoses (18). Here we review the evidence that prions operate by directing conformational change of a host protein and what is known about the formation and structure of these alternate conformations. Finally, we explore the steps of prion replication to explain why prions are an infectious subset of the larger class of proteins that misfold into amyloid. Specifically, we will focus on the infectivity requirements beyond simple self-propagating protein structures, a feature shared by many amyloids.

## Evidence for Conformational Changes in Mammalian Prions

The first evidence indicating that conformational change was involved in prion diseases arose during the purification of the infectious scrapie agent. A protease resistant protein fragment was found to copurify along with infectivity (8, 19). Subsequent cloning of this fragment revealed that it was part of a larger 33–35 kDa host glycoprotein, encoded by the *PRNP* gene (20–22). The normal cellular version of this protein, $PrP^{C}$, is distributed throughout many visceral tissues and is both soluble and highly sensitive to proteinase K digestion. However, in infected animals, an insoluble form of PrP is also present, $PrP^{SC}$. $PrP^{SC}$ accumulates in aggregates and plaques in the brain. Digestion with proteinase K cleaves only its first 66 amino-terminal residues, leaving a fragment referred to as $PrP^{SC27–30}$ with an SDS-PAGE mobility of 27–30 kDa.

The difference between $PrP^{C}$ and $PrP^{SC}$ appears to reside completely in their conformations. Though mutations in the nucleic acid genome can increase rates of spontaneous disease (2), the infectious disease occurs in the absence of such mutations. Moreover, systematic analysis of posttranslational modifications have failed to find any evidence that covalent modifications underlie formation of the infectious form (23, 24). By contrast, extensive evidence argues that $PrP^{C}$ and $PrP^{SC}$ adopt distinct conformations. For example, in addition to the protease resistance and solubility mentioned above, the two conformers vary in the exposure of a number of different epitopes (25) and have dramatically different thermodynamic stabilities (26) and secondary structure content. The structures of human, hamster, bovine, and mouse $PrP^{C}$ have been solved by NMR (27–30), and all are highly similar, predominantly alpha-helical folds. $PrP^{SC}$ on the other hand is predominantly $\beta$-sheet, as revealed by Fourier transform infrared spectroscopy studies (31).

Extensive evidence reviewed elsewhere implicates the conversion of $PrP^{C}$ to $PrP^{SC}$ in disease progression (32). In vivo, $PrP^{0/0}$ mice are not susceptible to prion infection, arguing that conversion of the endogenous protein is required to develop disease (33, 34). Furthermore, the lag time before developing disease is dependent on the concentration of PrP in the host (34–37). The infectious process can be recapitulated in cell culture using a neuroblastoma N2a cell line (38). In vitro extracts enriched in $PrP^{SC}$ can convert recombinant $PrP^{C}$ to a protease resistant form called $PrP^{RES}$, and this material exhibits similar specificity to that seen in vivo (39, 40). It has also been reported that shearing aggregates during the polymerization reaction increases the yield of protease-resistant material (41, 42). Nonetheless, to date de novo infectious material has failed to be created in vitro. A second caveat is that infectious prion diseases have been observed in the absence of detectable protease-resistant $PrP^{SC}$ aggregates (9, 43, 44). However, the question remains whether this absence is due to a titer of aggregates below detection limits, to a formation of an infectious conformation that is genuinely protease-sensitive, or to some more radical departure from the idea that conformational changes are necessary for generating infectivity.

## Evidence for Conformational Changes in Fungal Prions

Yeast prions, like mammalian prions, are characterized by the presence of an alternate conformation of a normal cellular protein. All fungal prion proteins identified have been shown using either differential sedimentation or size-exclusion chromatography to form high-molecular-weight complexes specifically in prion-containing cells (45–53). However, the degree of aggregation in vivo can vary with genetic background (54, 55) or with the expression level of the cognate prion protein (56). Aggregated protein can be visualized in intact cells by generating prion-GFP fusion proteins that are soluble and distributed evenly throughout the cytoplasm in wild-type cells, but they are organized into punctate foci stainable by the amyloid-specific dye, thioflavin-S, in prion-containing cells (46, 49, 50, 57–59). Ure2p has also been visualized by thin-section EM followed by immunogold staining and shown to form short cytoplasmic fibrils specifically in [*URE3*] yeast (60). In most cases, these aggregates have been shown to be highly stable and to have altered resistance to protease digestion (45–47, 51). Finally, de novo formation of these aggregates is slow, but once formed, they are stably inherited by daughter cells during mitosis.

The fungal prions have proven to be far more amenable to reconstitution in vitro than the mammalian prion system. Extracts from [$PSI^{+}$] yeast can catalyze conversion of soluble Sup35p, whereas extracts from [$psi^{-}$] yeast do not have this activity (61). Moreover, for Ure2p, Sup35p and HET-s, inheritance can be modeled using purified protein. Following a characteristic lag phase, these proteins spontaneously form amyloid-like aggregates. Importantly, the lag phase can be eliminated by the addition of small amounts of preformed fiber seed (62–64). A number of lines of experiments argue that this seeding effect underlies prion inheritance in vivo. For example, mutations in Sup35, which

affect aggregation in vivo, have parallel effects on the in vitro reaction (46, 65, 66). More directly for Sup35p and HET-s, it has been possible to create aggregates in vitro from recombinant protein and use these to convert wild-type cells to the prion state (16, 17, 66a). These experiments have provided the most complete evidence to date for the protein-only hypothesis.

Beyond supporting the prion hypothesis, this facile in vitro system allows more detailed mechanistic studies of prion conversion. Three questions stand out. One, what is the aggregation state of the infectious material? Specifically, are fibers necessary for infection or are they merely an assembly by-product of conformational conversion? What is the minimum size of an infectious particle? Two, when does conformational conversion occur? Monomers or oligomers could undergo spontaneous conformational conversions in solution that are subsequently stabilized by assembly into polymers, or conformational conversion could be driven by the assembly process itself. Three, what is the rate-limiting step in prion formation? Nucleated polymerization models argue that the formation of a multimeric nucleus is the slow step, whereas templated assembly models argue that conformational conversion is rate-limiting, though these two are not necessarily mutually exclusive. Detailed coverage of the literature addressing the conversion reaction is beyond the scope of this review, but we encourage interested readers to consult recent reviews (67–69) and research papers addressing the subject (70–74).

## Prion Aggregates Resemble Amyloid Fibers

Recently it has become clear that a wide range of unrelated proteins form structurally similar $\beta$-sheet-rich aggregates, often referred to as amyloids. Amyloids have received an enormous amount of attention caused by their association with a wide variety of protein misfolding disorders, including neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's (75). They are also found in a number of systemic amyloidoses, characterized by peripheral deposition of a number of aggregated proteins, such as lysozyme, transthyretin (TTR), immunoglobulin light chain, $\beta$2-microglobulin, and islet amyloid protein or amylin (76). Furthermore, a number of nondisease-associated proteins, for example acylphosphatase and the SH3 domain from $PIP_3$ kinase, have been shown to form amyloid under mildly denaturing conditions (77, 78). The ability of such diverse polypeptides to form amyloid argues that this fold is generally accessible to polymers of amino acids, perhaps because it is stabilized by main chain rather than side chain interactions (18).

Despite the variety in amyloid-prone proteins and their aggregated states, amyloids share similarities that make it useful to discuss them as a family of related structures (Figure 3). Amyloid fibers are characterized by a set of fiber diffraction reflections indicating that the $\beta$-sheets are organized in a cross-$\beta$ fold where the strands of the sheets run perpendicular to the fiber axis while the sheets run parallel to it (79–81). The repeating $\beta$-sheet structure allows the binding of the hydrophobic dyes thioflavin-T and Congo Red, both of which are commonly

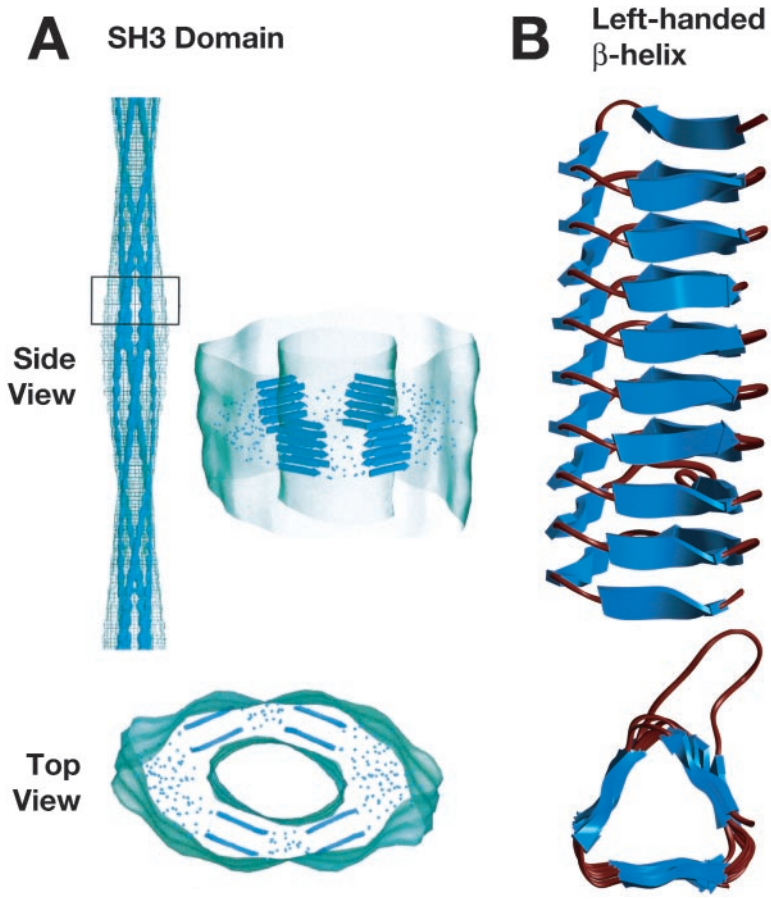


**Figure 4** Two models for amyloid structure. Both fulfill the requirements of the cross-$\beta$ fold in which individual $\beta$-strands are oriented perpendicular to the fiber axis, whereas $\beta$-sheets are oriented parallel to it. (*A*) Model from cryo-EM studies of amyloid formed by the SH3 domain from $PIP_3$ kinase (83). (*B*) An example of a left-handed $\beta$-helix (from UDP-N-acetylglucosamine pyrophosphorylase of *Streptococcus pneumoniae*, PDB ID 1G97), which has been proposed to resemble $PrP^{SC}$ (86). Image is courtesy of Dr. Cedric Govaerts (unpublished material).

used to monitor amyloid formation in vitro. Amyloids are often composed of multiple thin protofilaments that can associate in a variety of ways to create mature fibers with a range of diameters and helical twists [reviewed in (82)]. Currently, cryo-electron microscopy has yielded the most detailed structural model of an amyloid and indicates that protofilaments can be arranged around a hollow core (83) (Figure 4). Multiple folds can satisfy the constraints of a cross-$\beta$-sheet structure (81), such as the $\beta$-helix shown in Figure 4. Most

generally, amyloids are uncapped β-sheets that can incorporate new protein on their edges, leading to a fiber of defined diameter but unlimited length. In fact, well-behaved β-sheet-rich proteins appear to avoid aggregation by protecting their β-sheet edges with a variety of strategies (84).

Though the heterogeneity and insolubility of prion aggregates have made high-resolution structural studies difficult, they are known to share many features with amyloid (62–64, 85). Recently, reconstruction from electron micrographs of two-dimensional crystals of $PrP^{SC}$ present in infectious preparations has provided enough constraints to propose structural models (86). Additionally, a crystal structure of a PrP dimer has been solved, and these findings suggest how subunits might assemble into a fiber (87). Sup35p fibers give rise to the stereotypical amyloid cross-β diffraction pattern when subjected to fiber diffraction (71), and Ure2p fibers give rise to this pattern after being subjected to heat, though Ure2p may assemble into native-like filaments under physiological conditions (88). Both Sup35p and Ure2p fibers appear to consist of a central core made up of the prion domain with globular domains corresponding to the remainder of the protein decorating the periphery (62, 89) (Figure 3). Taken together with the self-propagating behavior of amyloid, the structural similarities between amyloid and prion aggregates suggest that propagation of β-sheet-rich amyloid-like core could provide the molecular mechanism responsible for prion growth.

## Self-Propagating Aggregates Are Not Sufficient for Infection/Inheritance

Amyloid fibers formed by many proteins are self-seeding (90–94), but few are infectious. For example, Aβ, the peptide whose aggregation is intimately correlated with Alzheimer's disease (95), exhibits stereotypical self-propagating behavior in vitro, forming an amyloid after a lag phase that can be eliminated by the addition of preformed fibers (67). Yet, Alzheimer's disease is not transmissible to primates or rodents (96, 97). What then is unique among the prion-associated amyloids that allows them to be infectious? We consider the steps of aggregation and transmission in Figure 5, comparing PrP and [$PSI^+$].

Initially, a self-propagating aggregate must form spontaneously. This is a step common to all of the amyloid diseases; in fact, most cases of Alzheimer's and Creutzfeldt-Jacob occur spontaneously in patients without any genetic predisposition to the disease (3). Yeast prions rarely occur spontaneously but are stable once formed (14). In mammals, mutations can accelerate the rate of spontaneous aggregation, as can overexpression of the aggregation-prone protein (34–37). In the case of yeast, truncations and expansions can accelerate the rate of spontaneous occurrence (66), and overexpression greatly increases the rate of prion formation (98). Finally, exposure to environmental factors, such as metals and pesticides, may also facilitate protein aggregation (99, 100).

Next, the newly formed prion must replicate itself. This involves two separable steps: growth of the infectious particle by addition to the aggregate and amplification of the number of infectious particles. Growth of the infectious

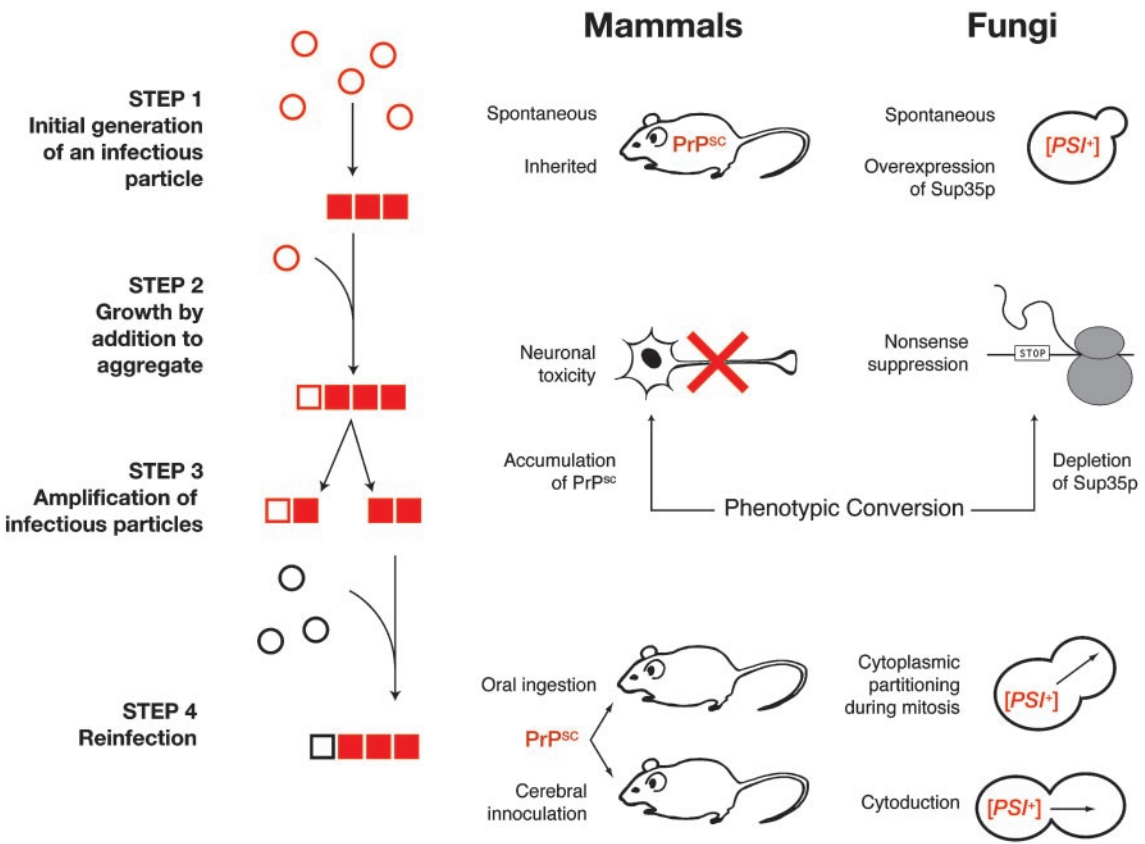


**Figure 5** Steps in prion transmission. (*left*) A general replication cycle for self-propagating conformationally based prion protein is shown. (*right*) Corresponding steps during prion infection in mammals and prion inheritance in fungi are shown.

particle comes about through recruitment and assembly of new protein onto the prion. However, this process alone would only lead to an increase in mass of protein in the prion form without net increase in the number of catalytic surfaces. Therefore, new infectious particles must somehow be released from the aggregate, either by spontaneous shedding or division by a cellular factor. Though how division is accomplished by mammalian prions is unclear, division of [*PSI*$^+$] aggregates appears to require the chaperone Hsp104p. A key piece of evidence for this model is the peculiar relationship between [*PSI*$^+$] and Hsp104p in which both deletion and overexpression of *HSP104* interferes with [*PSI*$^+$] propagation (101). Hsp104p is normally involved in the rescue of aggregated protein with the help of Hsp70p and Hsp40p (102), and indeed it may play a general role in prion propagation because deletion of *HSP104* cures all known yeast prions (14, 15). Division of aggregates could be another step differentiating transmissible and nontransmissible aggregates; if aggregates are too stable to either release small units or to be degraded by chaperones, they would never exponentially amplify during infection or inheritance.

Finally, these aggregates must be transmitted into a naive host and reach a pool of substrate protein. Mammalian prions can be ingested orally, as evidenced by the Mad Cow epidemic and by the transmission of kuru through ritualistic cannibalism. The prion infection then reaches the central nervous system (CNS) apparently through the lymphoid tissue. Once in the CNS, prions are able to spread from one cell to another presumably due to the presence of $PrP^C$, which is exposed on the surface of the cell (103). Yeast prions are transmitted naturally during cell division or experimentally using cytoplasmic mixing. This is clearly a critical step in differentiating between infectious and nontransmissible amyloids (104). Protein aggregates could vary in their ability to circumvent the body's defenses by being differentially susceptible to degradation and/or transport. There is recent evidence that systemic amyloids can also be transmissible when administered either orally or intraveneously after an inflammatory stimulus, arguing that under the right conditions more aggregates may prove to be infectious (105).

In addition to this growth and replication cycle, prion aggegates also cause a phenotypic change in their hosts. The need to distinguish phenotypic output from prion replication is emphasized by recent work that shows high titers of prions can exist without development of clinical symptoms (106). In the case of amyloid-related neurodegenerative diseases, the mechanism of toxicity and their tissue specificity must also be determined (107). In systemic amyloidoses, disease may be caused by mechanical disruption due to enormous amyloid burden because simple removal of amyloid deposits alleviates symptoms (108). In some cases, it may be that amyloid fibers are not toxic but instead are an inert repository for improperly folded proteins. In this case, the intermediates along the pathway to amyloid formation would be the neurotoxic species (107). Indeed, partially unstructured oligomers of both A$\beta$ and $\alpha$-synuclein are toxic to cells (109, 110), as are partially unstructured oligomers of an SH3 domain, which is

not associated with any known disease (111). For [*URE3*] and [*PSI*$^+$], the relationship to phenotype is more straightforward; sequestration of the prion protein in aggregates leads to a state similar to a loss-of-function phenotype (11, 112). In fact, the prion domain from Sup35p can be fused to other proteins to create novel prion elements with phenotypes caused by inactivation of the fusion protein (49, 66). However, a simple inactivation model is not sufficient to explain all fungal prions because [Het-s] and [*PIN*$^+$] lead to a gain-of-function phenotypes (51, 113, 114). More recently it has been shown that a neuronal member of the CPEB family shows prion-like properties in yeast and it is the prion-like form that has the greatest capacity to enhance translation of CPEB-regulated mRNA (114a). These results suggest a remarkable model in which conversion of CPEB to a prion-like state in stimulated synapses helps to maintain long-term synaptic changes associated with memory storage.

## PRION STRAIN VARIATION

One of the most fascinating and perplexing features of prion biology is the existence of multiple prion strains, wherein infectious particles composed of the same protein give rise to a range of prion states that vary in incubation time, pathology, and other phenotypic aspects. Observation of strain variability preceded the prion hypothesis, and in fact, it was originally used as evidence for the existence of a nucleic acid genome in the infectious particle. Strain variation was postulated to be caused by mutations in this genome. In the context of the proposal that transmissible encephalopathies result from propagating conformational changes in a prion protein, one must postulate that a single polypeptide can misfold into multiple infectious conformations, at least one for each phenotype. As disconcerting as this idea may be, there is increasing evidence from studies of both fungal and mammalian prions that it is indeed true. Nonetheless, there remain many unresolved questions regarding the origin of prion strains and their relationship to phenotype. For instance, what roles do cellular factors play (10, 115)? Do prion conformational differences lead to strain variation or simply reflect some other mechanism that actually encodes strain diversity? Formal proof of the conformational basis for prion strains has very recently been provided for the yeast [*PSI*$^+$] prion. Here it has been possible to fold the Sup35p prion protein into distinct infectious conformations (66a, 115a). Remarkably, infection of yeast with these different Sup35p conformations leads to distinct and heritable differences in [*PSI*$^+$] prion strains.

### Strain Variability in Mammalian Prions

Strain variability has always been closely associated with transmissible spongiform encephalopathies. Classic experiments in transmission of sheep scrapie to goats led researchers to group isolates according to clinical syndromes, such as

"drowsy" and "scratchy" strains (116). Material derived from these animals could infect mice in which these strains would propagate with distinct clinical and pathological parameters, such as patterns of brain lesions (117) and lag in incubation times. Use of isogenic mouse models made it unlikely that this variation arose from host genome polymorphisms (118).

With the identification of the PrP protein as the core component of the infectious particle, classification of strains could focus on molecular analysis of differences in the prion protein. Differences in secondary structure content (119), thermal stability (26, 120), and epitope exposure (121) of $PrP^{sc}$ isolates can be used to distinguish prion strains. Posttranslational modifications, such as glycosylation and attachment of GPI anchors, also show differences among known prion strains (2, 122, 123). Whether these covalent modifications modulate prion strains or reflect an inherent diversity among strains is still unknown.

## Conformational Differences Distinguish Mammalian Prion Strains

In light of the hypothesis that prions result from propagation of an infectious conformation, much of the effort in analyzing strains has focused on identifying strain-specific conformational differences in the prion protein. Initial evidence for such differences came from strains of transmissible mink encepholopathy (TME). $PrP^{SC}$ accumulated within the brains of infected minks and showed distinct proteolysis patterns and glycosylation profiles that correlated with different strain types. Upon injection of this material into naive hosts, not only did the newly infected animals exhibit strain-specific brain lesions and incubation times, but the converted $PrP^{SC}$ retained the proteolytic digestion pattern of the inoculum (124). Similarly, transmission of human-derived infectious material into transgenic mice expressing a human-mouse chimera produces $PrP^{SC}$ with hallmarks of the original strain, including protease sensitivity and glycosylation patterns (125).

A series of cell-free experiments have provided evidence that these protein conformations are sufficient to mediate their own propagation. Caughey and coworkers (39) developed an in vitro system in which brain-derived $PrP^{SC}$ mixed with $PrP^{C}$ converts $PrP^{C}$ to a protease-resistant $PrP^{SC}$-like state, called $PrP^{RES}$ (42). Paralleling the in vivo experiments, TME prion strains convert $PrP^{C}$ to a $PrP^{SC}$ similar to the initial strain, as defined by proteolysis and extent of glycosylation (126). Although other cellular factors, such as chaperones or a potential Protein X (127, 128), may be required for robust propagation of strain differences in vivo, the above observations suggest that the particular prion conformation can mediate strain-specific conversion of $PrP^{C}$.

## Strain Variability in Yeast Prions

The existence of strains appears to be a ubiquitous feature of prions, independent of the specific prion protein, the types of posttranslational modifications, or the

cellular site of conversion. Strain variability in fungal prions affects a range of different properties, including the strength of the associated phenotype, mitotic stability, and the dependence on molecular chaperones. Fungal prion strains were discovered during analysis of de novo induction of [*PSI*$^+$] by overexpression of Sup35p. Remarkably, inductants showed clear and heritable differences in color phenotype, caused by differences in the strength of nonsense suppression (98, 113). Genomic mutations cannot account for these differences; once a particular [*PSI*$^+$] variant was cured, the full spectrum of strains was reproduced upon reinduction. This variation among prion states has also been documented in Sup35p derived from other species. For instance, [*PSI*$^+$] elements arising in *S. cerevisiae* expressing the *Pichia methanolica SUP35* showed phenotypic variation, and they can be distinguished by their differential sensitivity to a host of chaperones, such as Hsp70p and Hsp40p family members (53, 129, 130). Chaperone discrimination is also seen with a chimeric prion domain derived from *Candida albicans* and *S. cerevisiae* Sup35p; overexpression of Hsp104p results in differential curing of these prion variants (P. Chien and J. Weissman, unpublished information).

Although [*PSI*$^+$] prion variants are the best characterized, similar variants have been seen in all yeast prions examined so far. De novo induction of the [*URE3*] prion results in variants distinguished by the strength of the associated phenotype and their susceptibility to curing by expression of an inhibitory fragment of Ure2p (48). The [*PIN*$^+$] element, mediated by self-propagating aggregates of the Rnq1p protein, also shows phenotypic variation. Unlike [*PSI*$^+$] and [*URE3*], the [*PIN*$^+$] phenotype is caused by a gain of function of the protein aggregate. [*PIN*$^+$] is required for efficient induction of [*PSI*$^+$] by overexpression of Sup35p (15, 49, 131, 132), and deletion of *RNQ1* does not mimic this phenotype. Strong [*PIN*$^+$] elements can generate high numbers of [*PSI*$^+$] cells upon overexpression of Sup35p, whereas weak variants are not as efficient at conversion (114).

## Yeast Prion Strains Modulate Solubility of the Prion Protein

An important link between yeast prion strain phenotypes and the conformation of the prion protein came from studies of variant-specific differences in the solubility of the endogenous prion protein (113, 133, 134). This was first shown using [*ETA*], a non-Mendelian genetic element isolated through synthetic lethality with particular alleles of translation release factors (135). Elegant experiments by Zhou et al. (133) showed that [*ETA*] was a weak variant of [*PSI*$^+$] and was distinguished primarily by a reduced level of Sup35p aggregation relative to strong [*PSI*$^+$] strains. Other [*PSI*$^+$] variants have now also been characterized and been shown to have similar differences in the degree of aggregation of Sup35p. Importantly, these variants propagate faithfully and are largely independent of the yeast genetic background (134). The variants of [*PIN*$^+$], which show differential ability to promote [*PSI*$^+$], also show differences in the amount of

aggregated Rnq1p, but there is no clear correlation between that phenotype and the degree of aggregation (114). Therefore changes in the relative fraction of aggregated protein can result in prion variants, but it is not the only possible mechanism for phenotypic diversity.

## In Vitro Analysis of Yeast Prion Strains

Further evidence that [*PSI*$^+$] variants are encoded by different prion conformations came from two lines of experiments. The first took advantage of an extract-based system in which [*PSI*$^+$] extracts containing aggregated Sup35p were mixed with [*psi*$^-$] extracts containing only soluble Sup35p that was converted to an insoluble form after incubation (61). Conformational differences between variants could be propagated in a cell-free system. When extracts from [*PSI*$^+$] variants showing differential sedimentation profiles of Sup35p were used to seed [*psi*$^-$] extracts, the newly aggregated material showed the same sedimentation as the original variants (134). Further experiments validate this notion because Sup35p aggregate-containing extracts generated from either strong or weak [*PSI*$^+$] variants showed different seeding efficiencies in in vitro polymerization reactions. However, this difference was lost when the newly polymerized material was used as seeds for secondary rounds of in vitro reactions, raising the possibility that faithful propagation of different Sup35p conformations in vivo depends on host factors (136).

## Yeast Prion Proteins Adopt Multiple Self-Propagating Forms

Work with pure protein has established that both Ure2p and Sup35p are able to adopt multiple self-propagating conformations. Spontaneous polymerization of either Sup35NM (the amino-terminal domain of Sup35p) (62) or Ure2p protein produces a range of amyloid fiber types (137). Even though the specific conformational differences between fiber types have not yet been determined, characteristics correlated with strain phenotypes, such as kinetics and seeding specificity, have been measured for Sup35NM fibers. When an atomic force microscopy (AFM)-based assay was used to measure growth from individual Sup35NM fibers, it was found that the purified polypeptide spontaneously forms multiple kinetically distinguishable fiber types. These could be sorted into a discrete number of classes on the basis of their growth polarity and elongation rate (Figure 6). Both the number of distinguishable fiber types and their relationship to protein aggregation rates suggest that these differences are well suited to account for [*PSI*$^+$] strain variation in vivo (138). Other in vitro work with a chimeric Sup35p system demonstrates that a single protein can form multiple biochemically distinguishable conformations with properties that reflect their in vivo strain phenotypes (Figure 7) (139, 140).

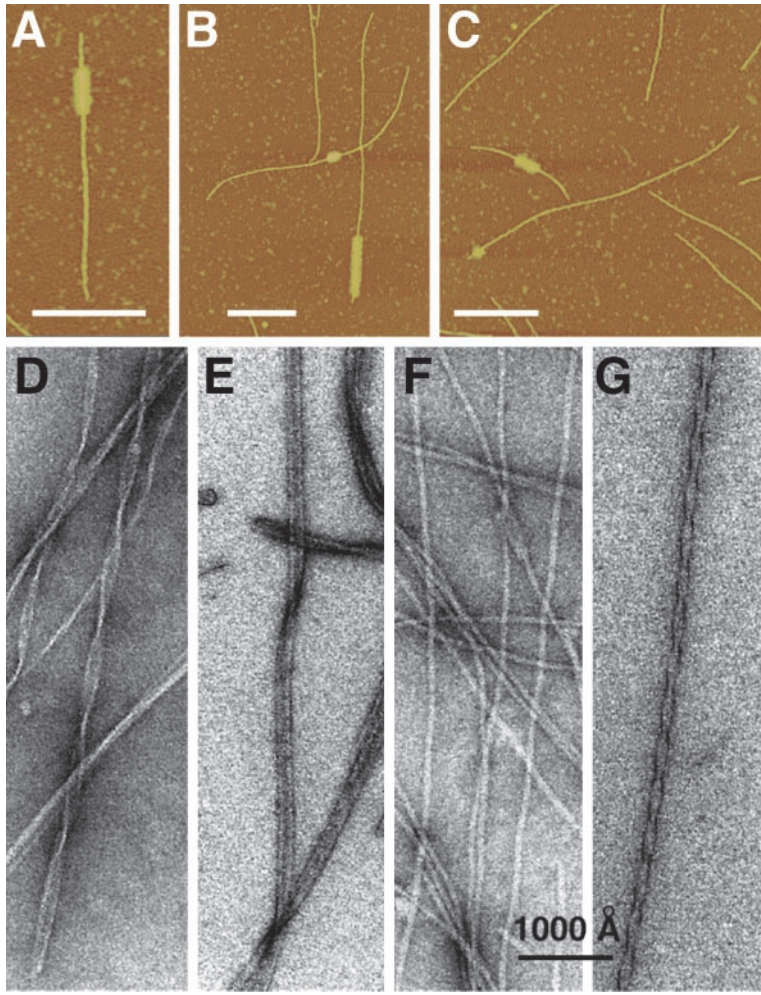


**Figure 6** Amyloid fibers adopt multiple distinguishable structures. (*A, B, C*) Amyloid fibers formed spontaneously by Sup35NM vary in their growth patterns, including overall rate and polarity of growth (138). Four kinetic fiber types visualized by an AFM single fiber growth assay are shown. The original seed is labeled with antibody and is therefore wider than the new growth extending from its ends. Note the presence of long and short symmetric and asymmetric fibers. Scale bar is 500 nm. (*D, E, F, G*) Negative stain EM of amyloid fibers formed spontaneously by the SH3 domain from $PIP_3$ kinase illustrates that they vary in the number of protofilaments and helical pitch (83). Scale bar is 100 nm.

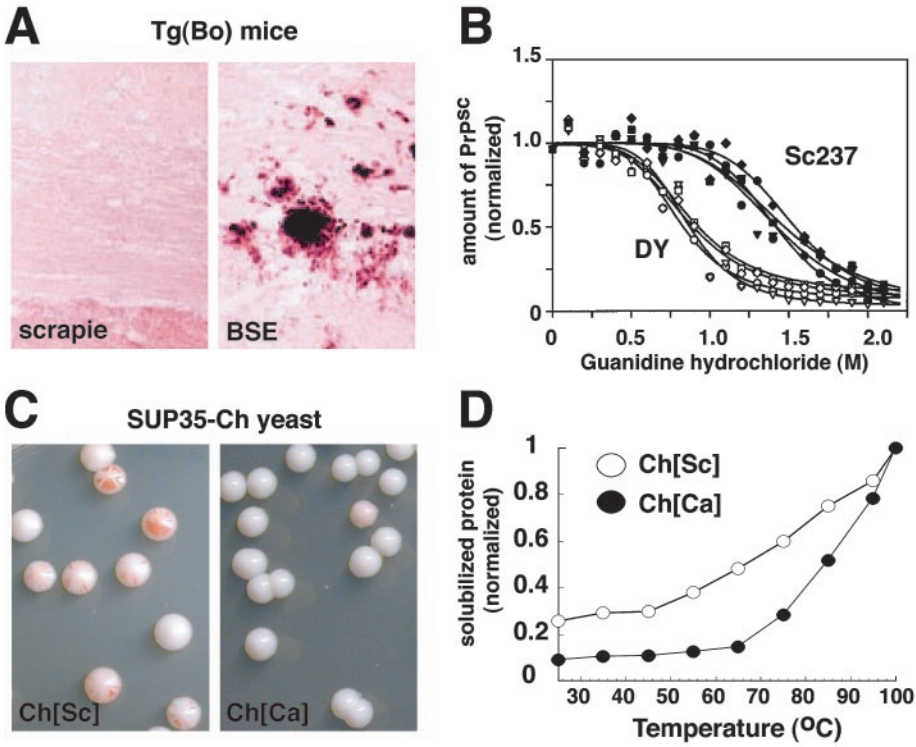


**Figure 7** Strain phenotypes in vivo correlate with in vitro differences in prion protein. (*A*) Subcallosal plaques caused in transgenic mice expressing bovine *PRNP* characteristic of infection by the indicated prion strain (200). (*B*) Denaturation profile of indicated strains of $PrP^{SC}$ showing stability differences (26). (*C*) Yeast harboring *SUP35* with Ch prion domain, induced to prion state by either Sc (Ch[Sc]) or Ca overexpression (Ch[Ca]) (139) with associated differences in phenotype. (*D*) Thermal denaturation of Ch fibers seeded by either Sc or Ca fibers (140).

## Structural Polymorphism Is a Common Feature of Amyloid Aggregates

Although the notion that prion strains are due to multiple infectious conformations was radical when first proposed, it is now clear that many proteins misfold into a variety of aggregates. This is especially true of amyloids; in this case, even during the same polymerization reaction a single polypeptide can adopt multiple fiber types, distinguishable by their ultrastructural properties, such as number of protofilaments and helical pitch as shown in Figure 6 (141–145). Just as crystal growth is intimately dependent on the nature of the solution, changing reaction conditions can shift the relative populations of morphologically distinct fibers. For example, amyloid forms of an SH3 domain were shown to be highly sensitive

to pH (83, 146). Aggregation of an Alzheimer's A$\beta$-derived peptide also showed strong dependence on pH, forming thicker ribbon-like fibers at higher pH (147). Changes in temperature can also alter the range of fiber morphologies as illustrated by work with polyglutamine peptide aggregates (74) and yeast prion amyloid fibers (140).

In addition to reaction conditions, covalent changes in the polypeptide, such as mutations or chemical modifications, can also modulate the spectrum of misfolded protein conformations. Mutations in A$\beta$ affect the ultrastructural packing of amyloid protofilaments and overall length of fibers when compared to wildtype (141, 148–150). Mutations in light chain domains also alter the morphology of amyloid fibers (151). Formation of nonfibrillar intermediates along the pathway to amyloid formation can also be influenced by changes in primary structure. For example, mutations in $\alpha$-synuclein correlating with early onset of Parkinson's disease have been linked to accelerated formation of toxic protofilament structures that are normally not present in wild-type polymerization reactions (152–154). Finally, the propagating form of yeast prion proteins can also be influenced by mutations (140), resulting in a shift in aggregate stability and in species-specific seeding processes (see below for discussion).

How do even relatively small changes in environment or in primary structure cause shifts in final aggregate morphology? Models for amyloid structure may shed light on the origin of this effect. The core of the amyloid structure is thought to consist of multiple layers of closely packed $\beta$-sheets. Morphological variants could arise from differences in side chain packing, register or topology of $\beta$-sheets, or quartenary structures. Though amyloid fibers are notoriously difficult to study using classical methods such as X-ray crystallography or NMR, high-resolution structural studies are clearly needed.

## SEQUENCE-DEPENDENT PRION TRANSMISSION: THE SPECIES BARRIER

Passage of transmissible spongiform encephalopathies between species has long been known to be limited by species barriers (155) and analogous barriers to propagation exist in the yeast prion systems (52, 53, 129, 156, 157). The primary structure of the prion protein is a critical determinant of the specificity of propagation because the inhibition of cross-species infectivity is intimately dependent on the degree of similarity between the sequences of the two prion proteins (2, 158, 159). Indeed, even point mutations or allelic variants can have dramatic effects on the specificity of prion propagation (160–164). Although host factors may play an important role, increasing evidence from in vitro studies argues that the growth of amyloid-like aggregates can account for much of the observed specificity. In general, prion infectivity is also highly dependent on the prion strain in question; we address this feature in the following section but focus now on primary structure differences.

## Mammalian Species Barriers In Vivo

Species barriers are common among the TSEs. Sheep scrapie isolates are delayed in transmission to goats (155), and human TSEs do not easily infect laboratory mice (165). Systematic exploration of this phenomenon has been greatly facilitated by the establishment of scrapie in transgenic mice in which a species barrier greatly slows prion transmission between Syrian hamsters and mice. From these studies, the sequence of the PrP protein has emerged as a critical determinant of cross-species transmission (158, 159). When $PrP^{SC}$ isolated from Syrian hamsters was intracerebrally injected into hamster hosts, the animals rapidly came down with disease, whereas mouse hosts showed no clinical symptoms after inoculation with the same material. A transgenic mouse expressing a copy of the Syrian hamster *PRNP* gene in addition to the endogenous copy was now highly susceptible to both hamster and mouse innoculum (158). Strikingly, inoculation with mouse-derived prions resulted in formation of exclusively mouse prions, and innoculation with hamster prions resulted in exclusive formation of hamster prions (159). However, interpretation of these results is complicated somewhat by the recent finding that high prion titers can exist in the absence of clinical disease features (106).

Since these classic studies, several transgenic experiments have confirmed the intimate relationship between the sequence of the prion protein and specificity of transmission (2, 166, 167). Nonetheless, other studies establish that in some contexts it is not the sole determinant. For example, transgenic mice expressing a human copy of *PRNP* in addition to their endogenous mouse copy [Tg(Hu) mice] are immune to human prions (127). Ablation of the mouse *PRNP* gene in Tg(Hu) mice makes them susceptible to human prions, whereas mice expressing a mouse-human chimera PrP [Tg(MH2 M) mice] are susceptible to human prions independent of the presence of the endogenous mouse copy (128). These data led to the suggestion that a species-specific factor (known as protein X) is necessary for prion susceptibility. In Tg(Hu) mice, this factor would bind selectively to the wild-type mouse $PrP^{C}$ protein, preventing proper conversion of the human $PrP^{C}$. On the other hand, Tg(MH2 M) mice were postulated to have both the human-derived sequence necessary for conversion and the recognition epitopes required for binding the prion-promoting factor (128).

## Role of Polymorphisms in Prion Transmission

Even within a single species, allelic variants of PrP affect mammalian prion transmission. The effects of genetic background on scrapie susceptibility were observed as early as 1959 by Gordon (see 128a) who found that some breeds of sheep were particularly sensitive to scrapie. In humans, familial forms of prion diseases are often associated with particular alleles of *PRNP* (2). Although mutations can result in general acceleration of prion onset and transmission, there is also a potential role for *PRNP* alleles to modulate prion transmission specificity. These observations have now been more extensively studied using transgenic mice (161, 168), cell culture (169), and cell extract systems (164), which

showed that single substitutions in primary structure can determine susceptibility and specificity to prion infection.

## Sequence-Specific Mammalian Prion Replication In Vitro

Conversion experiments in cell extract systems have helped define the molecular nature of species specificity in prion transmission (40, 42, 170). Incubation of mouse or hamster $PrP^{SC}$ extracts with recombinant $PrP^{C}$ protein from the same species resulted in conversion of the $PrP^{C}$ to a protease resistant form remiscent of $PrP^{SC}$. However, hamster $PrP^{SC}$ could not convert mouse $PrP^{C}$, suggesting that the specificity of prion propagation resulted from the ability of the infectious particle to bind to and convert soluble $PrP^{C}$(40). A similar in vitro result was also described for species-specific transmission of chronic wasting disease from cervids to other mammals (170). Although other factors necessary for prion replication (127, 128) may be present in these extracts, it seems likely that sequence-specific and direct interactions between $PrP^{SC}$ and $PrP^{C}$ underlie much of the prion species barrier.

## Transmission of [*PSI*$^{+}$] Is Highly Sequence Specific

Barriers inhibiting yeast prion transmission have been extensively studied using the yeast prion [*PSI*$^{+}$]. Cloning of *SUP35* genes from a broad range of budding yeast revealed that although the exact sequence of the amino-terminal domain varies, the features thought to be important for prion propagation, such as high glutamine/asparagine content, are preserved (52, 53, 129, 156, 157). Moreover, these domains can support prion states when expressed in a heterologous *S. cerevisiae* system (52, 53, 129) and, in one case examined, in the original yeast species (*Kluyveromyces lactis*) from which it was derived (156). The conservation of the prion-forming abilities of Sup35p together with the observation that presence of the prion can provide a selective advantage in certain conditions (15, 171, 172) suggests that rather than being a pathogen, [*PSI*$^{+}$] may represent a beneficial and conserved epigenetic mechanism for regulating protein function.

Analogous to the mammalian species barrier that limits induction and transmission, [*PSI*$^{+}$] prions are typically species specific (52, 53, 129, 156, 157). A particularly robust barrier exists between [*PSI*$^{+}$] prions formed from *S. cerevisiae*– and *C. albicans*–derived *SUP35* prion domains. Although these organisms would not naturally interact, species specificity can be studied using genetically manipulated yeast. Overexpression of *S. cerevisiae* Sup35p induces [*PSI*$^{+}$] in wild-type *S. cerevisiae* but not in yeast where the *SUP35* gene encodes for the *C. albicans* prion domain and vice versa (52). Even a single point mutation within the *S. cerevisiae SUP35* sequence is sufficient to confer specificity (65). However, in other cases, cross transmission between different *SUP35* sequences is possible albeit with reduced efficiency (53, 156). Such cross transmission could arise directly from some propensity of those Sup35p to be recruited into heterologous prions or indirectly through interactions with cellular machinery, such as chaperones.

## Specificity of Transmission in Other Yeast Prions

Barriers to transmission between different yeast prions have also been observed. For example overexpression of New1p induces [$NU^+$] but not [$PSI^+$], whereas overexpression of Sup35p induces [$PSI^+$] but not [$NU^+$] (49) or [*URE3*]. Finally, transient expression of heterologous species of Ure2p rarely induced [*URE3*] formation in *S. cerevisiae* even though similar expression of the *S. cerevisiae* Ure2p generated [*URE3*]-containing cells (173, 174). However, an important caveat is that it has not yet been shown that these alternate species of Ure2p can even form self-propagating prion states. If they cannot, then the lack of induction can be easily explained by the inability to form any type of infectious particles, rather than reflecting a specific transmission barrier between prions.

## Antagonism and Cooperation Between Yeast Prions

Even when a barrier prevents transmission of prion states between two different prion proteins, the presence of one prion can strongly influence both induction and propagation of a second. This influence can be positive, such as in the well-characterized [$PSI^+$]-inducibility ([$PIN^+$]) effect , where de novo induction of [$PSI^+$] by Sup35p overexpression only occurs in yeast harboring a second prion (49, 131, 132). Alternatively, prions can interfere with each other's propagation. For example, the [*URE3*] state is not inherited stably in [$PSI^+$] cells and vice versa (132, 175). The molecular bases of the above phenomena are poorly understood. In particular, a major open question is the extent to which this represents mixed polymers or an indirect effect, such as modulation of aggregation by chaperones.

On a related note, this effect of protein aggregates affecting de novo appearance of other aggregates seems to be a general effect, at least in yeast. Recent experiments demonstrated that aggregation of polyglutamine proteins is sensitive to the presence of other yeast prions (49), even though the polyglutamine proteins themselves cannot support prion inheritance in yeast. A mutant allele of the Machado-Joseph Disease (MJD) protein, containing an expanded polyglutamine tract fused to GFP, was used as a fluorescent reporter of aggregation. Aggregates of the Rnq1p or New1p prion domain were sufficient to promote aggregation of the mutant MJD protein, though in the absence of these aggregates, the reporter construct remained soluble (49).

## In Vitro Evidence for a Molecular Basis of Yeast Prion Specificity

Complementing in vivo observations of yeast prion specificity, it has been possible to recapitulate the sequence-specific propagation of the [$PSI^+$] prion in vitro. Extracts of [$PSI^+$] cells expressing *S. cerevisae* Sup35p can induce aggregation of Sup35p present in [$psi^-$] extracts from cells expressing *S. cerevisiae* but not *Pichia methanolica* Sup35p (129). An obligatory role for other

cellular factors can be eliminated using an in vitro polymerization reaction with only purified recombinant prion domains (62, 176). Both *S. cerevisiae*– and *C. albicans*–derived prion domains form amyloid fibers after characteristic lag times; addition of preformed fibers of *S. cerevisiae* Sup35p prion domains efficiently seeds polymerization of *S. cerevisiae* Sup35p prion domains but not domains derived from *C. albicans* and vice versa. Remarkably, even when present together in a mixture, these two species of prion domains show exquisite sequence specificity and form homopolymeric fibers (52).

### Sequence-Specific Amyloid Propagation

The sequence specificity seen in the purified Sup35p amyloid system is a common property of amyloid fibers, even of those not involved in prion phenomenon. Recent work with polyglutamine-containing proteins showed that formation of detergent-resistant amyloid aggregates is highly protein specific with coaggregation limited to proteins that share sequence homology outside the polyglutamine tract region (177). Peptides derived from the PrP protein also show preferential formation of homogeneous amyloids (178, 179), and polymers of A$\beta$ have stereochemical specificity for aggregate formation (180). Quantitative analyses of seeding efficacy also revealed that A$\beta$ was poorly seeded by fibers composed of completely unrelated peptides as well by fibers made up of a short amyloidogenic peptide, islet amyloid polypeptide (IAPP), despite the high degree of sequence similarity between A$\beta$ and IAPP (180a). Moreover, as was seen with the Sup35 prion protein, A$\beta$ appeared to be able to misfold into more than one amyloidogenic conformation as detected by seeding efficiencies. Finally, in vivo specificity is also seen during inclusion body formation and aggresome assembly (181, 182).

## RELATIONSHIP BETWEEN PRION STRAINS AND SPECIES BARRIERS

The phenomenological connection between strains and species barriers has long been appreciated. Even before the identification of an infectious agent responsible for TSEs, it was known that scrapie strains played a strong role in determining specificity of transmission (155, 160, 183). Indeed, these observations led to the concept of a transmission barrier, rather than a species barrier, to reflect the role of features other than simple sequence homology in determining prion infectivity (2). Understanding this relationship between prion strains and interspecies transmission has become especially relevant with the finding that the recent appearance of new variant CJD (nvCJD) seems to have resulted from the transmission of the prion strain responsible for Mad Cow Disease or bovine spongiform encephalopathy (BSE). BSE appears to be an especially promiscuous prion type, capable of crossing the species barrier that normally prevents transmis-

sion of animal prions, such as scrapie, to humans (2, 32, 184–186). Remarkably, the link between strains and species barriers seems to be general because strain variants of yeast prions can differ widely in specificity of transmission (139, 140, 187, 188). Below, we review the evidence linking strains, species barriers, and protein conformational changes in various prion systems. A synthesis of these observations suggests a model in which prion strains and transmission barriers are in large part manifestations of the same phenomenon: the ability of proteins to misfold into multiple amyloid-like conformations. This model helps explain several characteristic features of prion strains and species barriers.

## Prion Strains Affect Interspecies Mammalian Prion Transmission

During early studies of TSE infectivity, isolates of sheep scrapie were found to vary in their ability to infect goats, mice, and other laboratory animals (189). In one case, two sheep prion strains were investigated, a clinical isolate of naturally occurring sheep scrapie and a strain generated through experimental passage of BSE through sheep. The results were striking: Successful transmission of sheep scrapie to laboratory mice took ~800 days, whereas the mice inoculated with sheep-passaged BSE strains showed clinical signs in half that time (160). Later these experiments were refined through the use of isogenic transgenic mice. For instance, a transgenic mouse expressing a chimeric human-mouse PrP showed different susceptibility to two hamster-derived prion strains, even though they were composed of the same prion protein (190). Altogether these data argue that the nature of the prion strain is a key component of determining transmission across a species barrier.

## Passage Through a Species Barrier Modulates Prion Strains

The relationship between strains and species barrier is reciprocal: Just as strains show differing ability to cross between species, crossing a species barrier can result in a shift in strain characteristics. For example, clinical features and pathological hallmarks of scrapie were altered upon inoculation of goats with sheep scrapie. However, the infection of other sheep did not show this shift in scrapie disease profile (191). In studies of transmissible mink encephalopathies, researchers found that transmission of mink-derived drowsy prion strains into hamsters resulted in formation of both drowsy and hyper prion strains in a titer-dependent fashion (191a). Polymorphisms in the PrP gene present in a single species can also modulate the transmission of prion strains. Passage of BSE through transgenic mice expressing human PrP homozygous for valine at codon 129 does not affect strain type (165, 185). In contrast, BSE transmission to mice expressing human PrP homozygous for methionine at codon 129 resulted in mixture of both the parental BSE/nvCJD strain and new types similar to spontaneous CJD (192). An important caveat is that the existence of a species barrier does not necessitate a change in strain type. For example, in one study, large species barrier effects were observed upon mouse-to-hamster and upon

mouse-to-rat transmission when using a particular mouse-derived prion strain. When the material was then inoculated into mice, the mouse-to-hamster passaged strain showed significantly different properties as compared to the parent, but the mouse-to-rat isolate appeared unchanged (191).

## Emergence of Prion Strains Is Accompanied by a Change in Conformation

Experiments by Peretz and colleagues (190) helped define a molecular mechanism for the link between species barriers and strains by showing that emergence of new prion strains following interspecies transmission is accompanied by changes in prion conformations. These studies used two hamster prion strains, drowsy (DY) and Sc237, which could be distinguished by relative stability as measured by chemical denaturation (Figure 7) (26). The prion strains were administered to a line of transgenic mice in which a chimeric hamster/mouse PrP gene replaced the wild-type mouse allele. Inoculation with the DY strain resulted in rapid onset of disease and a characteristic DY-specific clinical phenotype. Consistent with this observation, the newly converted host $PrP^{SC}$ retained the conformational stability associated with the parent strain. In contrast, the Sc237 hamster prion strain exhibited a delayed transmission characteristic of a species barrier. The passage resulted in the emergence of a strain with significantly different clinical features and conformational stability than the original Sc237 strain. The new strain propagated faithfully in transgenic mice with a fixed period of latency distinct from the Sc237 strain (190). Thus both the clinical features and conformational hallmarks of a prion strain can change upon transmission across a species barrier.

## Yeast Prion Strains and Sequence-Dependent Transmission

A number of in vivo and in vitro experiments have pointed to an intimate link among strains, sequence, and conformational differences in the [$PSI^+$] prion systems. An early example of this came from studies of a Sup35p mutant [glycine at residue 58 to aspartic acid—known as PNM2 (193, 194)], which in some contexts is defective in yeast prion propagation (188). As mentioned previously, weak [$PSI^+$] variants exhibit mitotic instability and have lower levels of termination suppression when compared to strong [$PSI^+$] variants. Paradoxically, expression of PNM2 interfered with the suppression phenotype of strong [$PSI^+$] strains, whereas expression of PNM2 in a weak [$PSI^+$] strain actually enhanced the suppression phenotype (188). Because both weak and strong [$PSI^+$] variants were in genetically identical backgrounds, the clearest interpretation was that the variants consisted of distinct propagating forms of Sup35p that could be differentially influenced by expression of the mutant protein.

Elegant experiments by King (187) further investigated this link between yeast prion strains and sequence specificity. King explored the ability of three different [$PSI^+$] variants to recruit a panel of Sup35p mutants, as monitored by both suppression phenotype and by recruitment of GFP fusions. He found that

certain mutants could be preferentially recruited by some of the variants, but other mutants could not be recruited by any of the variants. Furthermore, coexpression of mutant prion domains cured the [*PSI*$^+$] variants to different degrees. These data led to the conclusion that the [*PSI*$^+$] strain variants were caused by structurally different Sup35p aggregates, each exposing different regions of the polypeptide. The ability to interact with a mutant would then be determined by the surface presented by a particular aggregate.

Work from our own lab, using a combination of in vivo and in vitro studies, has directly established that a single polypeptide can form more than one self-propagating amyloid conformation and that these conformations can determine the specificity of prion propagation. Moreover, we demonstrated that point mutations in a prion protein, by changing the spectrum of favored conformations, generate a de novo species barrier (139, 140). These experiments used a chimeric prion domain (known as Ch) composed of the first 40 amino acids of the *S. cerevisiae* Sup35p fused to the remainder of the prion domain from *C. albicans*. Whereas a barrier normally inhibits transmission between *S. cerevisiae* and *C. albicans* SUP35, the Ch prion domain is able to bridge this barrier. In vivo, Ch formed distinct prion strains with markedly different strengths and specificities upon induction by different Sup35p species (Figure 7). Similarly, when seeded with different species of Sup35p fibers in vitro, the purified Ch protein forms two distinct self-propagating amyloid forms. These conformations dictate seeding specificity: Ch seeded by *S. cerevisiae* Sup35p fibers efficiently catalyzes conversion of *S. cerevisiae* Sup35p (Sc) but not *C. albicans* Sup35p (Ca), and vice versa (139). These observations indicated that Ch bridges the species barrier by adopting two conformations (Figure 8), one that is specific for Sc (Ch[Sc]) and the other specific for Ca (Ch[Ca]).

This work was extended by looking at the effect of mutations in the Ch protein that were chosen to specifically disfavor Ch[Sc] or Ch[Ca]. Mutations that inhibited formation of the Ch[Ca] state, both in vivo and in vitro, prevented transmission between Ch and Ca without disrupting transmission to Sc. Conversely, mutants disfavoring Ch[Sc] were incapable of transmitting to Sc but remained susceptible to Ca. Interestingly, modulation of temperature also strongly influenced the preference for forming Ch[Sc] and Ch[Ca] (140). These observations indicate how changes in the sequence of a prion or changes in the environment can affect the specificity of a prion by modulating conformations.

## MODEL INTEGRATING PRION STRAINS, SPECIES BARRIERS, AND PRINCIPLES OF AMYLOID FORMATION

### Tenets of the Model

A synthesis of the experimental observations above suggests the following tenets linking prion strains, species barriers, and the physical principles that govern

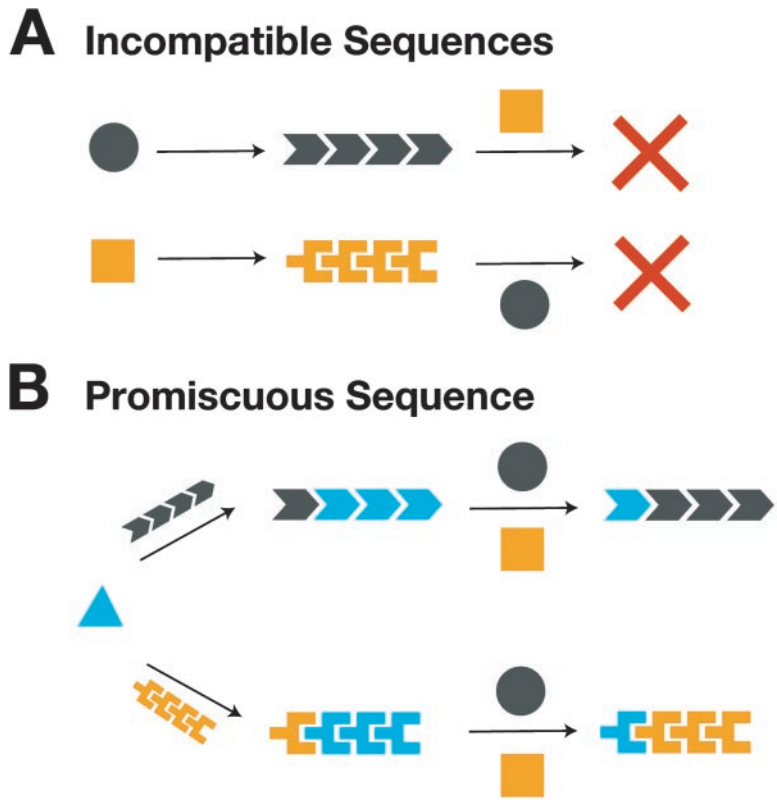


**Figure 8** Models depicting the relationship between transmission specificity and conformation. (*A*) Robust species barrier between two variants of prion protein that do not form compatible conformations and thus do not cross seed. (*B*) A single polypeptide, which can adopt two distinct conformations that allow assembly onto two otherwise incompatible prions.

protein misfolding. For the most part, these tenets have substantial experimental support and can also serve to guide the direction of future experiments.

1. Self-propagation of amyloid-like protein aggregates underlies prion growth.
2. A single protein can often misfold into multiple different amyloid conformations.
3. The phenotypic consequences resulting from an aggregated protein are highly dependent on the specific amyloid conformation.
4. The particular amyloid conformation that a protein adopts determines the specificity of growth.

5. Changes in protein sequence can modulate the spectrum of favored amyloid conformations.

## Relationship Between Conformation, Strains and Species Barriers

Based on these tenets, a model emerges in which prion strains and transmission barriers are in large part two different manifestations of the same phenomenon, the ability of a protein to misfold into multiple amyloid conformations. These conformations in turn determine both the specificity of growth and the phenotypic consequences of harboring a prion. Changes in sequence alter the range of preferred amyloid conformations thereby modulating transmission barriers and strain phenotypes.

Though this model, based on the propagation of amyloid-like structures, can account for many observed prion phenomena, it is clear that host cellular factors, such as chaperones or degradative machinery, can play a significant role in both the phenotype and propagation of prions. These factors are also likely to contribute to strains and species barriers by mechanisms other than changes in conformation. Furthermore, the simple ability to form a self-propagating aggregated state does not guarantee that a protein will be infectious. Although amyloid-like aggregation forms a physical basis for the propagation of prions, true understanding of what makes a prion more than an aggregated protein remains a central challenge. Nonetheless, our model suggests explanations for several features of prion inheritance.

STRAINS ARE A COMMON FEATURE OF PRION INHERITANCE Extensive evidence from both mammalian and yeast prion systems show that different propagating amyloid-like conformations are strongly correlated with distinct prion strains. This may be a specific case of the more general ability of amyloid fibers to form a range of self-propagating conformations. The ability of each conformation to robustly propagate differences, such as distinct fiber morphologies and assembly kinetics, could lead directly to heritable variation in phenotypes. For example, if the phenotype is due to the amount of soluble protein, variations in the aggregation rate will directly influence phenotype. Alternately, cellular factors may interact differently with the various conformations, leading to a distinct physiological outcome for different prion aggregates. A major goal is to elucidate the mechanism by which alternate prion conformations can cause different strain phenotypes.

TRANSMISSION BARRIERS ARE COMMON AND APPARENTLY EASY TO GENERATE Not only are species-specific transmission barriers a ubiquitous feature of prion propagation, they also arise rapidly, as evidenced by the small number of amino acid changes required to inhibit transmission between prions. This phenomenon can be explained by the ability of changes in polypeptide primary structure to alter the range of preferred amyloid fiber conformations. Even single point

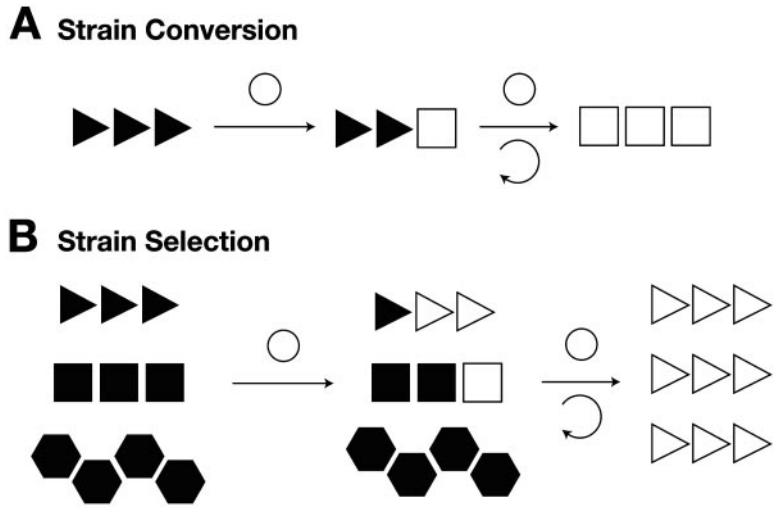


**Figure 9** Two models for strain switching upon passage through a species barrier. (*A*) In strain conversion, heterologous protein adopts a new conformation upon incorporation into prion seeds. (*B*) In strain selection, host protein selects a compatible conformation from a heterogeneous innoculum. Over multiple rounds of prion replication, the distribution of conformations changes.

mutations can in some cases shift the fiber conformation, resulting in a novel self-specific aggregate that is incompatible with the original parent sequence.

STRAINS DETERMINE TRANSMISSION SPECIFICITY Because amyloid conformations vary in their ability to recruit heterologous proteins, prion particles composed of the same protein but differing in their strain conformation will differ in their compatibility with the corresponding prion protein from another species. Variation in conformational compatibility between prion proteins thereby contributes to strain-specific transmission across a species barrier.

STRAINS SWITCH UPON TRANSMISSION ACROSS A SPECIES BARRIER Crossing a species barrier, though inefficient, is possible if a compatible prion conformation can be found and amplified. Observed switches in strains therefore may result from such an amplification of a conformation compatible with cross-species transmission. Although there are a variety of models that may explain this effect, two major possibilities stand out (2, 190) (Figure 9). In the first model, upon interspecies passage, assembly of the new polypeptide onto the infectious seed results in a new conformation. In the second model, new protein selectively grows on the subset of compatible seeds. In this interpretation, the transmission barrier acts as a sieve by selectively amplifying one component of a pool of conformations. This model demands that the initial strain actually consists

of a number of subtypes and that the biological strain phenotype reflects this collection.

## PERSPECTIVE

The ability of proteins to adopt multiple amyloid forms indicates a fundamental difference between the rules of protein folding and misfolding. Globular folds are stabilized by multiple cooperative interactions between specific side chains, resulting in unique well-defined structures. By contrast, recent studies indicate that amyloid formation is driven predominantly by main chain interactions, which can be locally favored or disfavored by specific side chains (195). As a consequence, a polypeptide can adopt multiple amyloid forms differing in their quaternary or possibly tertiary structures with specific side chains disfavoring a particular subset of structures without preventing amyloid formation altogether. Small differences in the rates of forming the various conformations will be reinforced by the self-propagating nature of amyloid formation; once a stable nucleus of a given conformation is formed, it rapidly dominates the reaction as it grows exponentially. Because of this, the reaction will be under kinetic control with the final conformation choice determined by the specific conditions of polymerization rather than the global thermodynamic minimum.

The fact that one polypeptide can misfold into multiple self-propagating forms helps explain a range of observations regarding prion inheritance. For example, the existence of transmission barriers between highly related species can be explained by the fact that the infectious conformation is sensitive to small changes in primary structure. Mutations affect the initial choice of conformation during de novo prion formation, and in turn, the conformation of the prion will determine which sequences can be recruited. A robust transmission barrier will therefore arise when the range of conformations adopted by two sequences are incompatible (2, 52, 53, 129, 156). Similarly, if the conformation affects phenotype as well as specificity, then changes in the primary structure of a prion protein could also alter the strain phenotype by shifting the infectious conformations (161, 168). It has been reported that crossing a transmission barrier can result in a change in the prion strain. Because conformations vary in their specificity, the transmission barrier could act as a sieve, selectively amplifying infectious forms compatible with the recipient prion sequence (2, 190). Finally, the failure to create transmissible forms of the mammalian prion protein (PrP) in vitro, despite a number of reports demonstrating production of self-propagating or protease-resistant PrP states (196–198), could be due to the preferential formation of noninfectious conformations outside the normal cellular context.

The degeneracy of amyloid formation may be important for understanding a range of protein misfolding disorders. There is increasing evidence that the toxicity of the different misfolded forms varies greatly, with some species being highly pathogenic, though others might even be protective (107, 109, 111). Given

the strong propensity of nonnative proteins to aggregate, therapeutic strategies designed to promote formation of nontoxic conformations rather than preventing amyloid formation altogether may be more tractable. In addition to selective pressure for function, this variability suggests that polypeptide sequences that form less toxic conformations when they do misfold will be preferred. More generally, any analysis of formation and consequences of amyloid-like aggregates needs to be tempered with the knowledge that there exists a range of conformationally distinct subtypes that will influence their biological effects.

## ACKNOWLEDGMENTS

We acknowledge Drs. Reed Wickner, Helen Saibil, Holger Willie, and Cedric Govaerts for providing images and Dr. Holly Field, Kim Tipton, Sean Collins, Anna Weissman, and members of the Weissman lab for helpful comments. P.C., now at Harvard University, was supported by a National Science Foundation Graduate Fellowship and was a Scholar of the Achievement Rewards for College Scientists (ARCS) Foundation. A.H.D., now at the University of California, Berkeley, was supported by a Howard Hughes Medical Institute Predoctoral Fellowship and funding from the National Institutes of Aging. J.S.W. was funded through the Howard Hughes Medical Institute, the Packard Foundation, the Searle Scholars Program, and the NIH.

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