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Prions: proteins as genes and infectious entities

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Infectious proteins (prions) include the transmissible spongiform encephalopathies (TSEs) of mammals, the amyloidoses [URE3], [PSI], and [PIN] of Saccharomyces cerevisiae and [Het-s] of the filamentous fungus Podospora anserina, and the self-activating in trans vacuolar protease B of yeast, called [β]. [Het-s] and [β] carry out cellular functions, namely, heterokaryon incompatibility and protein degradation, whereas the TSEs, [URE3], and apparently [PSI] are diseases. [PIN] appears to be neutral. We review the means of discovering prions, the interactions of these “autonomous” entities with their hosts (particularly chaperones), and the relation of prions to other nonnucleic acid heritable traits. That most amyloidoses are not infectious poses a conundrum.

We use the term “prion” (Prusiner 1982) to mean “infectious protein” in any organism, whatever the mechanism, and to imply the absence of a nucleic acid necessary for the infectivity. Although the word “prion” began its life as simply another name for the causative agent of the mammalian transmissible spongiform encephalopathies (TSEs), its usage changed with the discovery of the yeast infectious proteins [URE3] and [PSI] (Wickner 1994). These and the more recently discovered prions, [Het-s] of Podospora anserina (Coustou et al. 1997) and [PIN+ ] of yeast (Derkatch et al. 2001), are all based on self-propagating amyloid forms of chromosomally encoded proteins. The latest prion, [β] of Saccharomyces, is simply the active form of the vacuolar protease B, an enzyme that in certain conditions can be required in trans for activation of its own precursor protein (Roberts and Wickner 2003). In contrast to other prions, [β] is not a self-propagating amyloid. Other reviews also deal with a selection of these subjects (Wickner et al. 2004).

Transmissible spongiform encephalopathies

Scrapie is a uniformly fatal neurological disease of sheep known even in the 1700s (Parry 1983), but whose infectious nature was only recognized in the 1930s (Cuille and Chelle 1936). The similarity of the brain pathology of scrapie and that of the newly discovered human disease Kuru suggested that these diseases might be related (Hadlow 1959), and led to the demonstration of infectivity of the human material for monkeys (Gibbs et al. 1968). The spongy appearance of the brain, due to accumulation of large vesicles, is described by the term “transmissible spongiform encephalopathy” (TSE) for these diseases. Using mice as a host (Chandler 1961), it was found that the scrapie agent was markedly resistant to UV light, even compared with small viruses and bacteriophage (Alper et al. 1966, 1967), suggesting that the scrapie agent might not have an essential nucleic acid component (Alper et al. 1967). To account for this UV-resistance, without abandoning the tenets of molecular biology, it was suggested that an altered protein in an oligomer with unaltered protein subunits could drive the conversion of unaltered to altered form (Griffith 1967), essentially the modern prion model for scrapie, without the term “prion.” In fact, the first identification of PrP, the protein now believed responsible for scrapie, was to come soon after as a gene (Sinc) affecting scrapie incubation period in mice (Dickinson et al. 1968). However, Sinc was then viewed as a host factor helping replication of a virus or similar agent, not the structural gene of an infectious protein.

The purification of the scrapie agent (Bolton et al. 1982; Diringer et al. 1983) showed that the main component was a protein of 27–30 kD, which Prusiner dubbed PrP [for prion protein; Prusiner 1982]. The inability to find a characteristic nucleic acid component then (or now) further motivated the idea that the PrP protein itself might be the infectious agent (the protein-only model).

PrP is encoded by a chromosomal gene (Chesebro et al. 1985; Oesch et al. 1985) that is necessary for infection (Bueler et al. 1993). PrP is high in β-sheet structure and protease-resistant in infectious material compared with its normal state in the cell (Caughley et al. 1991; Pan et
addition to PrPSc. But given that a virus coat protein is doubt whether there is another essential component in its structure intimately controls the process. The only finding supporting the prion model for the TSEs. sen, to the same PrP-res form (Kocisko et al. 1994), fur- ther strengthening the prion model for the TSEs.

The absence of [PSI] in wild-type strains (Chernoff et al. 1993). Moreover, the protease-resistant, scrapie-associated form of PrP, called PrPSc or PrP-res, can catalyze the limited conversion of the normal form, PrPc or PrPsen, to the same PrP-res form [Kocisko et al. 1994], further supporting the prion model for the TSEs.

Thus, PrP is clearly necessary for TSE propagation, and its structure intimately controls the process. The only finding supporting the prion model for the TSEs. The only finding supporting the prion model for the TSEs.

One way in which this could be done is if recombinant PrP [made in Escherichia coli or yeast] could be folded in vitro into a form that, when injected into mice, could initiate a scrapie infection. Because the mouse assay is exquisitely sensitive, this refolding process need not be very efficient. This type of experiment has yet to succeed. Although recombinant PrP can be made relatively protease-resistant, such material has never been found to be infectious. If overproduction of normal or mutant PrP could induce the de novo appearance of TSE infectious for normal animals, this would also be a strong argument in favor of the sufficiency of PrP and the protein-only model.

How to find a prion

[See boxed text.] The means of detecting prions depends on the characteristics of the prion and on the system in which it is being expressed. Prions in microbiological systems will be detectable by means not available in animal populations. However, the attention given to individual humans is unlikely to be lavished on single yeast cells.

One way that a prion can arise is if a chromosomally encoded protein can undergo a change such that the altered form of the protein is necessary for the generation of the same altered form from the unaltered form [boxed text]. The consequences of such an event will depend on the effects of the prion form. If the prion form is toxic to the cells, then death of cells or individuals may be the result, such as is observed in the TSEs. If the prion form is simply an inactive form of a normally active protein, then a phenotype similar to that of mutation in the gene for the protein may be observed. This is the case for the yeast prions [URE3] and [PSI], which are inactive forms of Ure2p [a regulator of nitrogen catabolism] and Sup35p [a translation termination factor], respectively. If the prion is the active form of the protein, and carries out a function for the cell, then the phenotype conferred by the prion will be the wild type and be the opposite of that of a mutation in the gene for the protein. The [Het-s] prion of the HET-s protein was the first of this type. The recently described [β] prion of yeast is the active form of the vacuolar protease B. Prions of this type carrying out normal cellular functions are expected to be found in most wild strains, and this is the case for [Het-s] and [β]. The absence of [PSI] in wild-type strains (Chernoff et al. 2000, Resende et al. 2003] suggests that [PSI] is disadvantageous to yeast. In a mating of [PSI+] and [psi−] strains, all offspring are [PSI−], so even if [PSI] is neutral, it should spread through the population.

Three genetic criteria for a prion

A prion is an infectious protein, and horizontal transmission of infectious entities in yeast and filamentous fungi occurs by cytoplasmic mixing. Mating of yeast results in mixing of the cytoplasm of the parental strains even if the nuclei do not fuse. When two colonies of a filamentous fungus grow toward each other, their cellular processes (called hyphae) fuse to allow cytoplasmic mixing. In these cytoplasmic mixing events, viruses pass from one cell or colony to another. Thus, infectious proteins should likewise appear as nonchromosomal genes [Wickner 1994].

To distinguish prions from viruses or plasmids, three genetic properties were given that are expected to be found for self-inactivating prions and should not be found for nucleic acid replicons [Fig. 1; Wickner 1994].

1) Reversible curability. Curing of a nonchromosomal gene means its efficient elimination by some treatment, and is presumed to be caused by elimination of the nonchromosomal genome (the nucleic acid replicon if that is what encodes the gene). If a prion can be cured from a strain, it should nonetheless be able to arise again de novo in the cured strain because the protein capable of undergoing the prion change is still present in the cells and could still (at some low frequency) undergo that change. In contrast, when one of the yeast viruses is cured, it will not be found again unless it is reintroduced from outside. This is to be distinguished from reversible mutations, which arise rarely and revert rarely. For example, reversible mutations of mitochondrial DNA are well known.
(2) Overproduction of the protein increases the frequency with which the prion arises. Simply because there is more of the protein capable of undergoing the prion change, one expects the frequency with which the prion arises de novo to increase. In contrast, there is no chromosomally encoded protein whose overproduction will increase the frequency with which a nucleic acid replicon arises de novo; in fact, it will not arise de novo at all.

(3) The gene for the protein is required for propagation of the prion, and the phenotype of the presence of the prion is similar to that of mutation of the gene for the protein. The first half of this criterion does not distinguish prion from virus or plasmid: all will require some chromosomal gene for their propagation. For those prions that simply inactivate the protein when it assumes the prion form, the similarity of the phenotype to that of mutation of the gene will be expected. Of course, this is not true of scrapie, or of prions in which the prion form is the active form; therefore, for these elements, this feature is not evidence that they are prions. As discussed below, [URE3] and [PSI] satisfy all three criteria as prions of Ure2p and Sup35p, respectively [Wickner 1994]. The identification of [Het-s] and [PIN] as prions involved a variant of these criteria as well [see below; Coustou et al. 1997; Derkatch et al. 2001].

There is a critical difference between evidence for a prion and evidence concerning its mechanism. Finding that a protein is aggregated in cells carrying a non-Mendelian gene and not in cells without it does not point to the non-Mendelian gene being the prion. This could simply be a consequence of carrying the non-Mendelian gene [e.g., the mitochondrial DNA]. Moreover, there are numerous mammalian amyloids that are not prions. However, if one knows that the non-Mendelian gene is a prion of the protein in question, then this is important information pointing to the mechanism of prion propagation.

Showing that the progeny of a cell with a trait inherit that trait does not establish that the trait is infectious—a key part of being an “infectious protein.” Infection is horizontal transfer; inheritance by progeny is vertical transfer. Yeast and filamentous fungi accomplish horizontal transfer (of viruses and prions) by the cytoplasmic transfer that occurs during mating without nuclear fusion.

Clinical criteria for a mammalian prion

The TSEs have not been shown to follow any of the genetic criteria obeyed by [URE3] and [PSI] (and equivalent properties for [Het-s] and [PIN]). However, human TSE shows an array of traits that are unique among clinical conditions and, if all are present, should raise suspicions if seen in another disease. The clinical symptoms, course, and pathological findings of the human TSEs vary significantly, although all are central nervous system diseases that progress remorselessly to death. These include Creuzfeldt-Jakob disease (CJD), Gerstmann-Strausler-Schienker disease (GSS), Fatal Familial Insomnia (FFI), and others. Because all involve PrP we shall, in a convenient abuse of nomenclature, refer to them collectively as CJD.

CJD can be a spontaneous disease, an infectious disease, or an inherited disease. Although any two of these modes of transmission would fail to suggest a prion, the presence of all three is unknown outside of prion diseases. “Spontaneous disease” means that it is not associated with any other known exposure, genetic background, geographic area, or other evident factor. This
property may recall the de novo generation of the yeast prions, but it is, of course, impossible in a human population to ensure that transmission of an unknown virus is not occurring. More than 85% of CJD cases are in this category, and epidemiologic efforts to identify associations with other factors were long unsuccessful. Heterozygosity of the common 129M/V polymorphism in the gene for PrP is associated with immunity to CJD of this type [Palmer et al. 1991].

Inherited CJD, accounting for ~10% of total cases, is an autosomal dominant trait whose mapping to the PrP locus was one of the key findings that promoted the “protein-only” model [Hsiao et al. 1989; Owen et al. 1989]. Since that initial finding, a wide array of mutations nearly spanning the PrP open reading frame have been identified as the basis for inherited forms of CJD in various families. Different mutations are associated with somewhat different presenting symptoms, clinical course, and pathological findings, but there is at present no detailed understanding of the relation of mutation and these findings.

The infectious form of CJD has perhaps the most striking history. The dramatic stories of Gajdusek and Zigas [Zigas and Gajdusek 1957] in the jungles of New Guinea tracking down Kuru, the mysterious disease that was decimating the primitive Fore tribe in the highlands, has been the centerpiece of more than one popular account. Following Hadlow’s suggestion [Hadlow 1959], the infection of monkeys with material from Kuru patients proved that CJD could be infectious [Gajdusek et al. 1966]. Remarkably, both the inherited form and the spontaneous form could also be transmitted. In retrospect, a spontaneous case of CJD in the tribe was apparently transmitted to those participating in the ritual cannibalism of their funeral ceremonies.

Tragically, transmission by infection has also occurred after dura mater transplants or corneal transplants or following administration of growth hormone purified from human cadavers in the prerecombinant DNA era [Prusiner 1999]. Of course, the epidemic of bovine spongiform encephalopathy [Mad Cow disease; Wilesmith et al. 1988] and its spillover into the human population was also another infectious TSE [Will et al. 1996]. Fortunately, timely action by the British government to eradicate infected herds and block the feeding of rendered bovine material to herds prevented an extensive spread of BSE in humans. The epidemic in cattle peaked in 1992 and is nearly over, whereas the following human cases seem to be waning. The recent appearance of a single BSE cow in the United States has stimulated a strengthening of regulations that already limited refeeding.

Self-modifying proteins have the potential to be prions

The most recently described prion, [β], is simply the vacuolar protease B, which can be necessary for its own activation in the absence of protease A [pep4 mutants; Roberts and Wickner 2003]. In fact, any enzyme or protein whose modified form is necessary for its own modification is a candidate to be a prion. If a strain starts off without the active form, it cannot carry out the maturation and all of its progeny will likewise have only the inactive form. However, a cell with the active form will continue to carry out the maturation of the protein and pass on active enzyme to its offspring and transmit it horizontally as well. Thus, the active (modified) protein can be an infectious protein.

Although it is no surprise that proteases can carry out their own activation by processing their precursor form, it is novel to find such a protease acting as a nonchromosomal gene. More important, there are countless protein-modifying enzymes known, including protein kinases, protein methylases, protein acetylases, protein acylases, protein glycosyl transferases, and many others. If any of these enzymes mistakenly self-modified, and if the self-modified protein was then more inclined to self-modify, but did not properly modify other proteins, then it might be a prion.

Amyloid-based prions of yeast and filamentous fungi: [URE3], [PSI], [PIN], and [Het-s]

The [URE3] prion

When yeast has a good nitrogen source, such as ammonia or glutamine, it turns off the synthesis of enzymes and transporters needed for assimilating poor nitrogen sources, such as proline or allantoate. Ure2 mediates this “nitrogen catabolite repression” by binding the transcription factors Gln3p and Gat1p/Nil1p in the cytoplasm, preventing their entry into the nucleus, where they would promote transcription of many genes, including DAL5. Dal5p is the allantoate transporter, but also transports ureidosuccinate (USA), an intermediate in uracil biosynthesis (Fig. 2). A ure2 mutant can use USA in spite of the presence of ammonia, a normally repressing nitrogen source.

[URE3] is a nonchromosomal mutation isolated by Lacroute [1971] in the same screen that gave rise to ure2 mutations. [URE3] has all three of the properties expected of a prion of Ure2p [Wickner 1994]. [URE3] is efficiently cured by millimolar concentrations of guanidine [M. Aigle, cited in Wickner 1994], but from the cured clones can be isolated rare subclones that have cured the self-modified protein was then more inclined to self-modify, but did not properly modify other proteins, then it might be a prion.
cally in [URE3] strains, and this aggregation is determined by the prion domain [Edskes et al. 1999a]. That is, only molecules containing the Ure2p N-terminal prion domain show this aggregation.

**Ure2p prion domain.** The N-terminal 65 amino acid residues are sufficient when overproduced to induce the appearance of [URE3] at rates 100-fold higher than similar overproduction of the full-length protein [Fig. 5; Masison and Wickner 1995]. The same N-terminal domain is necessary for a Ure2p molecule to be inactivated by the prion change. In contrast, the C-terminal domain [residues 66–354] is sufficient to carry out nitrogen regulation. In addition, the prion domain propagates the [URE3] prion in the complete absence of the C-terminal nitrogen regulation domain [Masison et al. 1997]. This suggests that Ure2p molecules interact via their prion domains, and that this interaction inactivates the C-terminal nitrogen regulation domains [Wickner et al. 1996; Wickner et al. 1996]. The Ure2p prion domain is 40% asparagine, a feature also found in the Sup35p and Rnq1 prion domains (see below). Deletion of any of the runs of asparagine markedly diminishes the ability of the overexpressed Ure2p to induce prion formation [Maddelein and Wickner 1999].

**Amyloid of Ure2p is the [URE3] prion.** Amyloid is a special protein form defined by four properties [Fig. 4]: filamentous form, protease resistance, high β-sheet content, and yellow-green birefringence on staining with Congo Red [Kirschner et al. 2000]. Amyloids in humans are generally associated with disease, but adaptive amyloids are also known [Podrabsky et al. 2001; Chapman et al. 2002]. A synthetic Ure2p prion domain [residues 1–65] spontaneously and rapidly (seconds to minutes) forms filaments in vitro with all of the properties of amyloid (see Fig. 4; Taylor et al. 1999). The full-length native Ure2p purified from yeast is a stably soluble dimer, which only forms amyloid after prolonged incubation. Just as the overexpression of the prion domain increases the frequency with which [URE3] arises by 100- to 10,000-fold, addition of the prion domain peptide to the full-length Ure2p induces its formation of amyloid in vitro [Taylor et al. 1999]. These “cofilaments” promote the formation of amyloid by further added native Ure2p. Moreover, the pattern of protease-resistant fragments found for Ure2p amyloid formed in vitro is the same as that of Ure2p in extracts of [URE3] cells [Masison and Wickner 1995; Taylor et al. 1999; Speransky et al. 2001]. Thus, Ure2p amyloid formation is an autocatalytic process whose product resembles the aggregated protease-resistant Ure2p found in [URE3] prion-containing cells. Electron microscopy of thin sections of [URE3] cells reveals the presence of a network of filaments that can be specifically decorated by gold particles linked to antibody to the Ure2p C-terminal domain [Speransky et al. 2001]. The aggregated state of the Ure2p filaments in [URE3] cells is similar to the strong tendency of Ure2p amyloid filaments to aggregate in vitro, and makes plausible the importance in [URE3] prion propagation of the disaggregating chaperones that make multiple seeds ensuring stable transmission to daughter cells [see below].

**Architecture of Ure2p amyloid.** Exhaustive Proteinase K digestion of Ure2p amyloid filaments converts them from a diameter of 25 nm to ∼4 nm, consisting of Ure2p residues 1–70 [with some fraying of the ends in individual molecules, Baxa et al. 2003]. Thus, the core of the amyloid filaments is the prion domain. Because digestion of the soluble Ure2p does not leave any filaments or detectable fragments, it is clear that the connection between Ure2p molecules in the filaments is an interaction between one prion domain and the next. Dramatic support for this “amyloid core model” of Ure2p amyloid structure comes from scanning transmission electron micrographs of vanadate-stained filaments. These micrographs clearly show a central straight core in each filament with surrounding appended masses distributed in a less ordered manner [Fig. 6; Baxa et al. 2003].

The amyloid core model is further confirmed by the finding that fusions of the Ure2p prion domain to any of several soluble enzymes readily form amyloid, although the enzymes without the Ure2p prion domain do not form amyloid under physiological conditions [Baxa et al. 2002]. Remarkably, the activity of Ure2p1–65-glutathione-S-transferase or Ure2p1–80–green fluorescent protein is essentially the same in the soluble form and the filament form [Baxa et al. 2002]. This result is particularly notable for Ure2p1–65–GST because the Ure2p C-terminal domain is similar in both sequence and structure to glutathione-S-transferases [Coschigano and Magasanik 1991; Bouset et al. 2001; Umland et al. 2001].

One explanation for Ure2p inactivation in [URE3] cells is that the binding of Gln3p to Ure2p is sterically blocked in the filaments. Another possible explanation would be that Gln3p binding to Ure2p is in competition with its entry into the nucleus, and that Gln3p–Ure2p binding is a diffusion-limited process [Baxa et al. 2002]. Ure2p in the filamentous state would then be more difficult to find target for Gln3p, thus it would often diffuse into the nucleus before it found a Ure2p molecule to which to bind [Baxa et al. 2002].

**Mks1p and the RAS–CAMP pathway are necessary for [URE3] prion generation.** Overproduction of Mks1p, or a large central fragment of the protein, derepresses the nitrogen regulation system in a manner similar to the [URE3] prion, although most cells have not developed the prion [Edskes et al. 1999b]. Overexpression of Ure2p counters this effect. Similarly, deletion of RTG2 allows growth of cells on ureidosuccinate in spite of the presence of a repressing nitrogen source, and this effect depends on the MKS1 gene, suggesting that the latter is downstream in this pathway [Pierce et al. 2001]. Rtg2p is a known component of the “retrograde” regulation system that stimulates glutamate biosynthesis in response to defects in the mitochondrial genome [Liao and Butow 1993; Feller et al. 1997; Sekito et al. 2002]. Mks1p has now also been implicated in the retrograde system as well, and is believed here too to be acting between Rtg2p and the downstream components, Rtg1p and Rtg3p [Dilova et al. 2002; Sekito et al. 2002].

The effect of Mks1p on nitrogen regulation suggested that the same protein might affect the [URE3] prion. In-
deed, mks1 deletion mutants show a dramatic decrease in the frequency with which the [URE3] prion arises de novo [Edskes and Wickner 2000]. Even induction of [URE3] by overproduction of Ure2p or the prion domain is reduced to near background levels (although there is no effect on the level of Ure2p in the cells). Likewise, overproduction of Mks1p results in an increased frequency of appearance of [URE3], an effect distinct from its effect [in all cells] on derepressing nitrogen regulation [Edskes and Wickner 2000].

MKS1 was first found as a growth inhibitor that is antagonized by the RAS–cAMP pathway [Matsuura and Anraku 1993]. The growth-inhibition is specific for the nitrogen source, with growth nearly completely inhibited on rich nitrogen sources such as ammonia or glutamine, and a negligible effect on poor sources such as proline [Edskes and Wickner 2000]. The inhibition of Mks1p action by RAS suggested that RAS might also affect [URE3] generation. Indeed, expression of the activated Ras2Val19 protein has a depressing effect on [URE3] generation similar to that of an mks1 mutation [Edskes and Wickner 2000]. Whether Mks1p interacts directly with Ure2p or influences [URE3] generation through some indirect means remains to be determined.

[PSI], a prion of Sup35p

Brian Cox discovered a nonchromosomal genetic element [PSI] that made a very weak nonsense suppressor [SUQ5] into a strong suppressor [Cox 1965], and a strong suppressor become lethal [Cox 1971]. SUQ5 proved to be a serine-inserting tRNA mutant, and [PSI] has low-level suppressing activity even in the absence of SUQ5 [Liebman et al. 1975; Liebman and Sherman 1979].

[PSI] has all three of the genetic properties diagnostic of a prion of Sup35p [Wickner 1994]. Propagation of [PSI] requires SUP35, encoding a subunit of the translation termination factor, whose mutant phenotype resembles that of the presence of [PSI] [Young and Cox 1971; Doel et al. 1994; Frolova et al. 1994; TerAvanesyan et al. 1994; Stansfield et al. 1995]. [PSI] can be efficiently cured by growth in high-osmotic-strength medium [Singh et al. 1979], but [PSI] clones can be again isolated from the cured cells [Lund and Cox 1981]. Overproduction of Sup35p increases the frequency with which [PSI] arises de novo [Chernoff et al. 1993; Derkatch et al. 1996].

The prion domain of Sup35p, like that of Ure2p, is a glutamine–asparagine-rich N-terminal part of the molecule [Fig. 4] that is not essential for the translational role of the protein [TerAvanesyan et al. 1993, 1994; Derkatch et al. 1996]. Moreover, the glutamine residues are critical for prion propagation [DePace et al. 1998]. As shown in Figure 4, the Sup35p prion domain includes five imperfect repeats of an octapeptide sequence, YQQYNPQGG, similar to the octapeptide repeat, PHGGGWGQ, of PrP. Deletion of repeats interferes with [PSI] propagation [TerAvanesyan et al. 1994], and addition of repeats increases the frequency with which [PSI] arises [Liu and Lindquist 1999]. In analogy, added repeats in PrP cause inherited CJD [Owen et al. 1989], although the repeats are not needed for scrapie propagation [Flechsig et al. 2000].

The first hint of the mechanism of [PSI] prion propagation was the finding that the protein disaggregating chaperone Hsp104 was critical for [PSI] [Chernoff and Ono 1992; Chernoff et al. 1995]. Either depletion or overproduction of Hsp104 resulted in the loss of [PSI] from the cell, suggesting that a conformational change of Sup35p, presumably an aggregation, was involved in [PSI] prion propagation. Indeed, aggregation of Sup35p is associated with the presence of [PSI] [Paushkin et al. 1996]. The synthetic Sup35p prion domain formed amyloid in vitro, suggesting that this aggregation is amyloid formation [King et al. 1997]. Furthermore, full-length Sup35p can form amyloid, and this process is specifically
seeded by extracts of [PSI+] cells, and not by extracts of [psi−] cells (Glover et al. 1997).

As is seen below, studies of [PSI] have played a large role in examining the species barrier, prion strains, and in the discovery of the [PIN] prion.

Although overexpression of Sup35p induces the de novo formation of the [PSI] prion (Chernoff et al. 1993), it was noted that this was a strain-dependent phenomenon (Derkatch et al. 1997). The ability of Sup35p overexpression to induce [PSI] appearance proved to be dependent on a second nonchromosomal genetic element, dubbed [PIN] for “[PSI]-inducibility” (Derkatch et al. 1997). [PIN] is cured by growth in the presence of guanidine, like [URE3] and [PSI], and can arise again in the cured strain, suggesting that [PIN] might also be a prion (Derkatch et al. 2000).

Meanwhile, examination of proteins with N/Q-rich domains for possible prions resulted in the discovery that one such yeast protein, Rnq1p, could be found in an aggregated state in some strains and a soluble state in others (Sondheimer and Lindquist 2000). This difference proved to show cytoplasmic inheritance (Sondheimer and Lindquist 2000), again suggesting that it might be a prion.

Overproduction of any of several proteins having N/Q-rich domains is capable of having a [PIN]-like action, but most do so without being a nonchromosomal genetic element (Derkatch et al. 2001). The original [PIN] proved to be a prion form of Rnq1p (Derkatch et al. 2001); propagation of [PIN] requires the RNQ1 gene, and overproduction of Rnq1p increases the frequency with which [PIN] arises de novo (Derkatch et al. 2001). In addition, aggregation of Rnq1 correlated perfectly with the de novo formation of [PIN].

[PIN] might act by a cross-seeding mechanism: the prion domain of Rnq1p is N/Q-rich, all of the proteins that act like [PIN] have N/Q-rich regions, and the [URE3] prion (with its N/Q-rich prion domain) can mimic [PIN] (Bradley et al. 2002). Alternatively, these proteins might act by binding a cellular “anti-prion” component or by increasing the expression of a protein that facilitates prion formation.

A colony of a filamentous fungus is composed of cells with cytoplasmic connections allowing movement of cytoplasm (or even nuclei) throughout the colony. When two colonies contact, they will fuse if they are genetically identical. Genetic identity is tested by examining identity at a dozen polymorphic chromosomal loci, with a single non-identity-blocking fusion. In Podospora, this “heterokaryon incompatibility reaction” requires, among other things, identity at the het-s locus (Saupe 2000). The two common alleles of this locus, called het-s and het-S, encode proteins that differ in only 13 of their 289 residues (Turcq et al. 1990, 1991).

The het-S allele shows no unusual genetic features, but strains with the het-s allele can be in either of two phenotypic states, determined by the presence ([Het-s]) or absence ([Het-s∗]) of a nonchromosomal genetic element (Rizet 1952; Beisson-Schecroun 1962). Cells with [Het-s] show the incompatibility reaction with an het-s colony, whereas [Het-s∗] colonies are indifferent to the het-s/het-s status of their fusion partner. The [Het-s] gene is eliminated frequently by meiotic spore formation, but arises again at low frequency. Overproduction of the HET-s protein increases the frequency with which [Het-s] arises de novo (Coustou et al. 1997). The het-s gene is necessary for the propagation of [Het-s], but in
In this case the prion form is the active form of the protein, whereas a deletion of the het-s gene produces the same neutral phenotype as does the absence of the prion [Coustou et al. 1997]. Further evidence that [Het-s] is a prion form of the HET-s protein is the finding that the protein is protease-resistant in extracts of [Het-s] cells [Coustou et al. 1997].

As in Ure2p and Sup35p, a limited prion domain, sufficient to induce prion formation when overexpressed and capable of propagating the prion by itself, is found in the HET-s protein [Dos Reis et al. 2002; Balguerie et al. 2003]. As with Ure2p and Sup35p, deletion of the non-prion domain part of the HET-s molecule increases the efficiency with which it induces the [Het-s] prion [Balguerie et al. 2003]. However, unlike the N-terminal prion domains of Ure2p and Sup35p, the HET-s prion domain comprises the C-terminal 72 residues. Moreover, unlike Sup35p and Ure2p, this prion domain is not rich in asparagine or glutamine residues (Fig. 4).

In vitro, the HET-s protein can form amyloid that is partially resistant to digestion by proteinase K [Dos Reis et al. 2002]. The protease-resistant part of the amyloid is essentially the prion domain, namely, the C-terminal 72 residues of the molecule [Balguerie et al. 2003]. However, in the soluble form the prion domain is highly sensitive to protease digestion, suggesting that it is unstructured [Balguerie et al. 2003]. This view was supported also using hydrogen–deuterium exchange as a measure of solvent-accessibility [Nazabal et al. 2003]. The prion domain was fully solvent-accessible in the soluble form of the protein, but largely inaccessible in the amyloid form. In contrast, the N-terminal part of the protein did not change on amyloid formation. NMR studies led to the same conclusion [Balguerie et al. 2003].

**Amyloid of recombinant HET-s protein is infectious.** If in vitro manipulation of a recombinant protein makes it able to transmit a phenotype or disease, then this both constitutes proof that the protein is indeed a prion and provides critical information about the mechanism by which it is a prion. This type of experiment has so far only succeeded for the [Het-s] prion [Maddelein et al. 2002]. In this experiment, Maddelein et al. introduced recombinant HET-s protein into colonies of *Podospora* initially lacking the prion, using bombardment with metal particles coated with the protein. Colonies were assessed for the presence of the prion after several days of further culture. They found that if the particles were coated with the HET-s protein in the form of amyloid filaments formed in vitro, then nearly all of the colonies acquired the prion. In contrast, introduction of HET-s protein aggregated by heat or acid denaturation had almost no effect, as did the soluble form of the protein. These controls are, of course, critical because the authors had already shown that overproducing the HET-s protein increases the frequency with which [Het-s] arises de novo. If these experiments were to go beyond that result, it was essential to show that the form of the protein was important, not just its amount.

This experiment both confirmed that [Het-s] is a prion, and showed that the amyloid form of HET-s is the infectious form [Maddelein et al. 2002]. The infectious material sedimented rapidly, showing that the infectious material was not found in small oligomers, but was present in the amyloid material. In addition, extracts of [Het-s] cells could seed amyloid formation by recombinant HET-s protein, again confirming the nature of the infectious material [Maddelein et al. 2002].

**Sequence determinants of prion behavior in HET-s.** The prion domain of HET-s has no remarkable features, having neither repeats nor N/Q-rich parts. Although the prion domain of the HET-s protein is the C-terminal residues 218–289, the critical differences between the HET-s and HET-S proteins are at residues 23 and 33 [Deleu et al. 1993; Coustou et al. 1999]. The HET-s D23A P33H mutant of HET-s no longer propagates [Het-s] even though its prion domain remains intact. Likewise, HET-S H33P behaves like HET-s in adopting a prion form and supporting heterokaryon incompatibility. A single amino acid residue outside the prion domain is thus controlling the behavior of the full-length protein.

**Figure 4.** The four defining features of amyloid. Protein with all four of the features shown is said to be in amyloid form. In the known prions, only part of the protein assumes a β-sheet structure. The circular dichroism spectrum shown is just a theoretical example of the difference between an α helix and a β sheet, not an actual spectrum.
The increase of prion-inducing activity of each of Ure2p, Sup35p, and HET-s deletion or mutation of the non-prion-inducing regions of the molecules recalls the wide distribution of TSE-inducing mutations across the human PrP molecule. It is likely that the non-prion domain part of each molecule stabilizes the prion domain in a non-prion form, either by interacting with the prion domain, or by bringing in another component that interacts with the prion domain.

Meiotic drive and the [HET-s] prion. All known microbial prions are genetic cheaters in the sense that they are nonchromosomal genetic elements. Even though only one of the parents of a mating initially had the element, all or most of the offspring have it. As a result, a prion conferring no selective disadvantage should spread through the population. However, the [Het-s] prion takes this approach to an extreme. In a cross of female het-s [Het-s] with male het-S, those het-S progeny spores that receive [Het-s] are lost if the cross is done at low temperature (Dalstra et al. 2003). This extreme form of meiotic cheating, called “meiotic drive,” favors inheritance of the het-s allele, and thus the [Het-s] prion.

If, in spite of the genetic cheating, a prion is not widely distributed in the population, it is likely that carrying the prion is a disadvantage to the organism under most conditions.

How amyloid-based prions propagate: the key role of chaperones

Prion propagation involves more than just elongation of amyloid filaments. In animals, the progression of the infection through tissues and from one compartment to another is a complicated and as yet poorly understood process apparently involving lymphocytes as carriers. Within the nervous system, elegant experiments in the 1960s showed that infection follows neural pathways (Fraser 1982).

In Saccharomyces cerevisiae, the process of prion propagation involves chaperone activities, including Hsp104, Hsp70s, Hsp40s, and the TPR-containing co-chaperones. Chaperones are proteins that promote the proper folding of other proteins (for review, see Hartl and Hayer-Hartl 2002). Some chaperones, notably Hsp70s, bind to exposed hydrophobic and unstructured parts of nascent proteins, preventing their aggregation as the protein folds. Others, such as the Hsp60 barrels, denature misfolded proteins, giving them a second chance to refold. Still others, including yeast Hsp104, actually aggregate proteins.

Chernoff and coworkers found that either depletion or overexpression of Hsp104 impaired [PSI] propagation (Chernoff and Ono 1992; Chernoff et al. 1995). Low con-
Concentrations of guanidine efficiently cure all of the known yeast prions (Aigle 1979; Tuite et al. 1981; Wickner 1994; Derkatch et al. 1997). These low levels of guanidine (1–3 mM) are insufficient to denature proteins, but they are sufficient to inactivate Hsp104 (Ferreira et al. 2001; Jung and Masison 2001). Indeed, the D184S mutation of Hsp104 makes cells completely resistant to guanidine curing of prions (Jung et al. 2002), showing that guanidine cures prions by inactivating Hsp104.

As first proposed by Paushkin et al. (1996) for the role of Hsp104, guanidine cures the [PSI] prion by blocking the production of new prion seeds, presumably amyloid fragments (Ness et al. 2002; Cox et al. 2003). Blocking Hsp104 genetically or with guanidine results in a smaller number of prion seeds each of which is larger than in an unblocked strain (Wegrzyn et al. 2001; Kryndushkin et al. 2003). The accumulation of newly synthesized Sup35p in aggregates (presumed to be amyloid filaments) is not blocked by guanidine (Ness et al. 2002), showing that Hsp104’s role is not to unfold the normal Sup35p.

Other chaperones also are critical in prion propagation. Hsp70s can either stimulate or inhibit curing of [PSI] by overproduction of Hsp104 (Chernoff et al. 1999; Newnam et al. 1999). Mutation of the cytoplasmic Hsp70 Ssa1p can result in loss of [PSI] (Jung et al. 2000; Jones and Masison 2003), and Ssa2p is critical for [URE3] (Roberts et al. 2003). Although Ssa1p and Ssa2p are 97% identical, overproduction of Ssa1p cures [URE3], but Ssa2p does not (Schwimmer and Masison 2002).

A detailed analysis of the role of Ssa1p in [PSI] propagation indicates that mutant Ssa1ps that bind amyloid too tightly prevent [PSI] propagation, perhaps by blocking access of Hsp104 to the amyloid filaments and preventing Hsp104s breaking large filament to small ones (Jones and Masison 2003). Thus, defective Ssa1p results in a decrease in the number of prion seeds (Jones and Masison 2003). The role of Hsp70s and Hsp40s in prion propagation may also be related to their ability to cooperate with Hsp104 in solubilization of aggregates (Glover and Lindquist 1998).

This analysis also led to identification of the TPR-containing cochaperones as candidates for participation in prion propagation (Kryndushkin et al. 2002, Jones and Masison 2003). These proteins are known from studies in human systems to interact with Hsp70s and Hsp90s. Mutations in residues of Ssa1p known to be involved in interaction with Hsp40s and TPR-containing cochaperones affected [PSI] propagation (Jones and Masison 2003). Moreover, overexpression of the TPR-containing cochaperone Sti1p destabilized [PSI] (Kryndushkin et al. 2002).

The importance of chaperones has been confirmed by studies in other prion systems. Hsp104 is essential for the propagation of [URE3] (Moriyama et al. 2000), whereas [PIN] requires both Hsp104 (Derkatch et al. 1997) and Sis1p (an Hsp40; Sondheimer et al. 2001). Overproduction of the Hsp40 family member Ydj1p cures [URE3] (Moriyama et al. 2000). Ssa1p, Ssb1p, or Ydj1p could cure a weak strain of [PSI] as well as a hybrid prion made by fusing the prion domain of Pichia Sup35 to the functional domain of S. cerevisiae Sup35 (Kushnirov et al. 2000b). Possible roles of chaperones in prion propagation are diagrammed in Figure 7.

The species barrier
Transmission of scrapie within species is more efficient and has a shorter incubation period than between species.
suppressing [PSI]+ [Derkatch et al. 1996]. Analogous strains of [URE3] [Schlumberger et al. 2001] and [PIN] [Bradley et al. 2002] have also been found. Using a panel of SUP35 mutants, [PSI] isolates were grouped into three strains based on their response to the different sup35 alleles [King 2001]. Thus, different Sup35p sequences respond differently to different strains of the [PSI] prion [King 2001; Chien et al. 2003].

A prion based on an enzyme self-activating in trans

Given that the word “prion” means simply an infectious protein, prions need not be based on infectious amyloid. Any enzyme whose modification (or activation) requires the already modified (activated) form could be a prion. Cells initially lacking the modified form would be unable to generate it, and their offspring would be similar. Cells initially carrying the modified form would continue to modify the newly synthesized unmodified form, as would their offspring. Moreover, transfer of just the modified form to an individual lacking the modified form would convert the latter to the former, and this change would be heritable. Thus, the modified enzyme would be infectious. Of course, realization of this possibility requires that each state (“active” and “inactive”) be stable and viable and that the active protein be transmissible.

Indeed, a recently described prion of S. cerevisiae called [β]) is simply vacuolar protease B, whose active form, in pep4 strains, is essential for the proteolytic activation of its precursor form [Roberts and Wickner 2003]. The activation of protease B occurs in several steps in the endoplasmic reticulum and the vacuole, the latter catalyzed by either protease A [PEP4] or less efficiently by the mature active protease B itself [Jones 1991]. Zubenko and Jones noted that germination of pep4 meiotic segregants from a pep4/+ heterozygous diploid did not lose protease B activity immediately, but showed a “phenotypic lag” lasting many generations [Zubenko et al. 1982]. Their experiments were done on dextrose medium, which largely represses protease B transcription [Moehle and Jones 1990]. On nonrepressing glycerol medium, the self-activation of protease B proceeded indefinitely [Roberts and Wickner 2003]. Transfer of cytoplasm carrying the active protease B transferred the self-activation activity; therefore, active protease B is an infectious protein, a prion.

The [β] prion serves important functions for the cell. Cells lacking [β] are unable to sporulate and die more rapidly upon starvation compared with those carrying the prion [Roberts and Wickner 2003]. Thus [β], like [Het-s], is a prion that functions for the cell, rather than being a disease.

Whereas self-activating proteases have been long known, their serving as infectious proteins is new. The importance of this finding is that there are many protein-modifying enzymes that have the potential to become prions. Protein kinases, acetylases, methylases, glycosylases, fatty acid acylases, and the like could well modify themselves. The modified form might be better

Prion strains

Just as a virus can have distinct strains, producing different symptoms in the genetically identical host, so may a prion have strains. This phenomenon has long been recognized in studies of scrapie, and has been used as an argument against the protein-only model [Bruce et al. 1992]. However, a single protein can crystallize in several distinct forms and, likewise, one would expect that the linear crystal that is amyloid could have several structural forms. Prion strains are associated with detectable differences in the structure of PrP-res, with slightly different endpoints of protease-resistance or a different Fourier transform infrared (FTIR) spectrum [Bessen and Marsh 1992; Cauhey et al. 1998]. As noted above, prion strains are differently affected by the species barrier [Bruce et al. 1992], a fact particularly crucial in the BSE epidemic.

Distinct strains of [PSI] were first recognized based on their differing strength of nonsense suppression and stability [Derkatch et al. 1996]. The weak-suppressing [PSI] isolates were less stable, and more easily cured by guanidine or overproduction of Hsp104 than were strong-
at self-modification and altered in its ability to modify other proteins.

**Some other nonnucleic acid inheritance phenomena**

*Cortical inheritance in Paramecium aurelia: a prequel to prions*

The single-celled protozoan *Paramecium* has a cell surface covered with well-ordered rows of cilia. Mating of two cells involves fusion at the mouth, exchange of haploid micronuclei and separation. Following separation, some individuals were found to have alterations in the patterns of cilia around the mouth, and surprisingly, this altered pattern was passed on to the offspring (Beisson and Sonneborn 1965). Alteration of the pattern of cilia by microsurgery in *Tetrahymena*, another ciliate, likewise results in heritable changes (Grimes and Auferheide 1991). The overall morphology of *Paramecium* and the pattern of cilia in particular do not have left–right symmetry, and mirror-image individuals occasionally arise [Nelsen et al. 1989]. This change is propagated to the offspring, but is not due to any change in the chromosomal genome.

These phenomena can be viewed as organellar inheritance, with the mother cell’s cortex acting as a template in the production of the cortex of each of the daughter cells. The parallel with prions is obvious.

**Chromatin states and most amyloidoses are first cousins of prions**

The classical epigenetic phenomena based on “chromatin states” have recently been explained as self-propagating states of histone modification [for review, see Felsenfeld and Groudine 2003]. Histone-modifying enzymes bind specifically to already modified histones, providing the site-specific self-propagation mechanism. Although this is not an infectious entity, the mechanism is related to that of prions in that it is a form of self-propagating information that is fundamentally protein-based. The fact that the site of these events is on the chromosomal DNA and that the site-specificity of the alteration is ultimately determined by DNA sequences also distinguishes them from prions.

It is striking that the TSEs, which arise spontaneously in only one in $10^6$ people (or other animals), have been discovered as infectious entities, but the wide variety of amyloidoses of humans [Table 1], many arising spontaneously in a substantial fraction of the population, have not. Although efforts to make these amyloidoses infectious in experimental animals have been largely frustrated, these diseases are generally viewed as spreading within one individual by a self-seeding mechanism. Is the difference simply one of mobility of the amyloid, or is there a fundamental structural difference thatunderlies this dichotomy? The intimate relation of yeast prion transmission to chaperones suggests that this is at least part of the answer.

**Self-catalyzed assembly**

If the assembled form of a protein is necessary for its own assembly, then, although there may be no covalent changes involved, alternative heritable states may be possible. Evidence of this type of mechanism has been obtained for the mitochondrial Hsp60 chaperonin [Cheng et al. 1990]. Whereas *Saccharomyces* lacking mitochondrial DNA can grow if supplied with glucose, most mitochondrial proteins are encoded by the nucleus and the complete absence of the mitochondrion is lethal. Hsp60 has 14 subunits, each 60 kD, arranged in two layers of seven each. Located in the mitochondrial matrix (inside both membranes), Hsp60 is necessary for the assembly of many matrix proteins and is essential for growth [for review, see Langer and Neupert 1994].

An hsp60 ts mutant can be complemented by the wild-type protein if its expression is begun before the ts protein is inactivated, but not if the ts protein is inactivated first [Cheng et al. 1990]. This failure of complementation is true even though the cells can recover by allowing the inactivated ts protein to reactivate by shifting the temperature down. Biochemical data confirmed that the basis of this finding was that Hsp60 is necessary for its own assembly. It assists the folding into active form of nascent Hsp60 molecules newly imported into the mitochondria. Because Hsp60 is essential for growth, two alternative growth states cannot be found, and one cannot say this is a prion, but this type of mechanism might be the basis for a prion of a nonessential protein.

**Table 1. Disease-related amyloid proteins**

| Disease                          | Amyloid-forming protein |
|----------------------------------|-------------------------|
| Alzheimer’s disease              | Aβ peptide              |
| Diabetes                         | Amylin (IAPP)           |
| Amyotrophic lateral sclerosis    | Superoxide dismutase    |
| Multiple myeloma                 | Immunoglobulin light    |
| Chronic inflammations/ infections| Serum amyloid A protein |
| Familial senile amyloidosis      | Transthyretin           |
| Familial amyloidotic polyneuropathy | β2-microglobulin     |
| Chronic hemodialysis             | Apolipoprotein A1       |
| Parkinson’s disease              | α-synuclein             |
| Creutzfeldt-Jakob disease        | PrP                     |
| Huntington’s disease             | Huntington              |
likely that the yeast and fungal systems will continue to be “morphed” into prions, and why are they not prions to begin with? With these and other questions, it is likely that the yeast and fungal systems will continue to provide systems to generate answers.

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