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# Prions Affect the Appearance of Other Prions: The Story of *[PIN⁺]*

**Irina L. Derkatch,[2] Michael E. Bradley,[2] Joo Y. Hong, and Susan W. Liebman[1]**
Laboratory for Molecular Biology
Department of Biological Sciences
University of Illinois at Chicago
900 South Ashland Avenue
Chicago, Illinois 60607

## Summary

**Prions are self-propagating protein conformations. Recent research brought insight into prion propagation, but how they first appear is unknown. We previously established that the yeast non-Mendelian trait *[PIN⁺]* is required for the de novo appearance of the *[PSI⁺]* prion. Here, we show that the presence of prions formed by Rnq1 or Ure2 is sufficient to make cells *[PIN⁺]*. Thus, *[PIN⁺]* can be caused by more than one prion. Furthermore, an unbiased functional screen for *[PIN⁺]* prions uncovered the known prion gene, *URE2*, the proposed prion gene, *NEW1*, and nine novel candidate prion genes all carrying prion domains. Importantly, the de novo appearance of Rnq1::GFP prion aggregates also requires the presence of other prions, suggesting the existence of a general mechanism by which the appearance of prions is enhanced by heterologous prion aggregates.**

## Introduction

Prions are the causative agents of transmissible spongiform encephalopathies (TSEs) such as mad cow disease in cattle and Creutzfeldt-Jakob disease (CJD) in humans, as well as of the yeast *[PSI⁺]* and *[URE3]* and the *P. anserina [Het-s]* non-Mendelian traits (reviewed in Prusiner, 1998; Cox, 1994; Liebman and Derkatch, 1999; Wickner et al., 1999). Prions result from a conformational change in a protein that makes it "infectious", i.e., capable of transmitting its abnormal conformation to newly synthesized or native protein molecules of the same primary structure. Both yeast and mammalian prions can be viewed as heritable amyloidoses (Wickner et al., 2000): they form insoluble protease-resistant aggregates (Oesch et al., 1985; Masison and Wickner, 1995; Patino et al., 1996; Paushkin et al., 1996) that appear to act as seeds during prion propagation (Caughey et al., 1995; Paushkin et al., 1997). The realization that humans can acquire CJD from infected cattle has stimulated tremendous public concern. Nonetheless, the most common cause of CJD is not due to infection, but rather to the spontaneous formation of PrP prions. Thus, factors that influence the spontaneous appearance of prions are of great importance.

It is not known how prions form de novo. They are presumed to arise either through the spontaneous folding of a nonprion molecule into the prion shape or by the chance interaction of two or more nonprion molecules. Either event is more likely when the protein is present at higher concentration. Thus, one of the criteria for prions is that they should appear more frequently upon overproduction of their proteins (Wickner, 1994, Wickner et al., 1995). Indeed, amplification of the *SUP35* and *URE2* genes (Chernoff et al., 1993; Wickner, 1994) and, more specifically, overproduction of the Sup35 and Ure2 proteins (Derkatch et al., 1996; Masison et al., 1997), respectively, induce the de novo appearance of *[PSI⁺]* and *[URE3]*. Prion domains of Sup35 and Ure2 were identified because overproduction of just these domains is sufficient to induce the appearance of the prion (Masison and Wickner, 1995; Derkatch et al., 1996), and because the uninterrupted production of these domains is required for prion maintenance (Ter-Avanesyan et al., 1994; Masison et al., 1997). The concentration of the prospective prion protein relative to ligands is also crucial for de novo prion formation: overproduction of Sup45, a Sup35 ligand, inhibits *[PSI⁺]* induction (Derkatch et al., 1998), and expression of prion domains on truncated proteins incapable of efficient interaction with ligands increases prion induction (Masison and Wickner, 1995; Derkatch et al., 1996). Finally, depletion of the Ssb chaperone enhances the appearance of *[PSI⁺]* (Chernoff et al., 1999).

Overall similarity with the Sup35 and Ure2 prion domains, and, in particular, high Gln/Asn contents (reviewed in Tuite, 2000), were used to find the potential yeast prion proteins, Rnq1 and New1 (Sondheimer and Lindquist, 2000; Santoso et al., 2000). Since no phenotypic changes were associated with Rnq1 or New1 inactivation, their propensity to aggregate following the overproduction of their prion domains was used to support the hypothesis that these proteins can form prions. Also, Rnq1 was shown to be aggregated in some, but soluble in other yeast strains, and the aggregated state was transmitted by cytoplasmic mixing and required the presence of the Hsp104 chaperone (Sondheimer and Lindquist, 2000), traits shared by *[PSI⁺]* and *[URE3]*.

We recently showed that *[PSI⁺]* appears de novo in some but not other yeast strains. The presence of an epigenetic element named *[PIN⁺]*, for *[PSI⁺] in*ducibility, is necessary for *[PSI⁺]* to arise either spontaneously or following induction by excess Sup35 (Derkatch et al., 1997). *[PIN⁺]* is required at the step of *[PSI⁺]* appearance (but is not needed for the propagation of *[PSI⁺]*) and exerts its influence on *[PSI⁺]* induction through the Sup35 prion domain (but is not located there). *[PIN⁺]* appears to be a prion because, like the established yeast prions *[PSI⁺]* and *[URE3]*, it is inherited in a non-Mendelian manner, depends upon Hsp104 for propagation, is cured by growth in the presence of low levels of guanidine hydrochloride (GuHCl), and reappears in cured strains (Derkatch et al., 2000). Also, like *[PSI⁺]* (Derkatch et al., 2000) and *[URE3]* (Fernandez-Bellot et al., 2000), newly appearing *[PIN⁺]* elements are frequently unstable (Derkatch et al., 2000). Two models were proposed to explain how the presence of one prion, *[PIN⁺]*, could be

[1] Correspondence: suel@uic.edu
[2] These authors contributed equally to this work.

essential for the appearance of another, *[PSI⁺]* (Derkatch et al., 2000). The "Pin as an inhibitor" model predicts that soluble Pin protein in the nonprion conformation inhibits the de novo formation of *[PSI⁺]*. This is the classic model for a prion: phenotypes associated with *[PSI⁺]* and *[URE3]* result from inactivation of Sup35 or Ure2, respectively, and mimic the phenotypes caused by mutations in *SUP35* or *URE2*. The alternative "seeding" model predicts that *[PIN⁺]* prion aggregates facilitate *[PSI⁺]* appearance.

The data presented indicate that the presence of any one of several prions can make cells *[PIN⁺]*. We also show that, like *[PSI⁺]*, the de novo aggregation of Rnq1 depends upon the presence of other prions. We disprove the "Pin as an inhibitor" model for *[PIN⁺]* and discuss how the presence of heterologous prion aggregates could enhance the de novo formation of another prion.

## Results

### Identification of High-Copy Library Plasmids that Eliminate the *[PIN⁺]* Requirement for the Induction of *[PSI⁺]*

To identify the gene encoding the *[PIN⁺]* prion, we screened for genes that in high copy induce *[pin⁻]* cells to become *[PIN⁺]*. The rationale is that overproduction of a protein that can take on a prion shape should facilitate the de novo appearance of that protein's prion form. To detect the induction of *[PIN⁺]*, we used a fusion of the N-terminal part of Sup35 containing the prion domain, Sup35NM, with green fluorescent protein, GFP. This fusion, called Sup35NM::GFP, efficiently induces the de novo appearance of *[PSI⁺]* in *[PIN⁺]* but not *[pin⁻]* cells (Zhou et al., 2001). A *[psi⁻][pin⁻]* 74-D694 strain carrying unexpressed *SUP35NM::GFP* was transformed with a high-copy yeast genomic library (Nehlin et al., 1989). Transformants were grown for about 35 generations in an attempt to induce *[PIN⁺]*, then Sup35NM::GFP was overproduced in order to induce *[PSI⁺]*. The appearance of *[PSI⁺]* should reveal transformants in which *[PIN⁺]* was either induced or appeared spontaneously (the latter occurs in 74-D694, but very infrequently; Derkatch et al., 2000).

Transformants were screened for the appearance of *[PSI⁺]* using both suppression and aggregation assays (Figure 1). The suppression assay is based on the fact that in *[PSI⁺]* cells, the translational termination factor, Sup35, forms self-propagating prion aggregates and is therefore unavailable for translational termination. This results in enhanced nonsense suppression, which is estimated using the *ade1-14* nonsense mutation: suppression of *ade1-14* allows *[PSI⁺]* cells to grow on media lacking adenine (−Ade) (Inge-Vechtomov et al., 1988).

Of 15,000 transformants screened, 600 showed the growth on −Ade expected of *[PSI⁺]* cells. However, many of these candidates could be due to library plasmids that cause nonsense suppression but do not affect *[PSI⁺]* appearance. To identify plasmids that allow for *[PSI⁺]* appearance, we used the aggregation assay based on the assumption that only suppression resulting from the de novo appearance of *[PSI⁺]* should be accompanied by the formation of large dot and ring-like fluorescent aggregates following overproduction of Sup35NM::GFP. Of the 600 candidates examined, 38 passed the aggregation test. Plasmids were isolated from these candidates and, upon retransformation, 30 again passed the screen for the *[PIN⁺]* gene. The suppression induced was verified to be *[PSI⁺]* because it was cured by growth on +GuHCl that efficiently eliminates *[PSI⁺]* (Tuite et al., 1981). Both weak and strong *[PSI⁺]* strains (Derkatch et al., 1996) were induced in transformants carrying the same library plasmid (data not shown). Importantly, none of these library plasmids caused the de novo appearance of *[PSI⁺]* when introduced into the *[psi⁻][pin⁻]* strain alone, confirming that the induction of *[PSI⁺]* still required overproduction of the Sup35 prion domain.

### Identification of Genes with Prion-like Domains on the Plasmids that Passed the Screen for *[PIN⁺]*

Following sequence analyses of the inserts, the 30 plasmids that passed the screen were grouped into 11 sets (Figures 2A–2K). Inserts within each set overlapped. Plasmids in sets A, C, D, E, G, and H allowed for the most efficient induction of *[PSI⁺]* (approximately 20%–50% of that seen in the *[PIN⁺]* control) whereas plasmids from the other sets had a more modest effect (approximately 2%–20%; see Figure 1). Set A was composed of three clones each carrying *NEW1*; set B of two clones both carrying *URE2*. The finding of these two genes in our screen suggested that overexpression of more than one prion gene could promote the induction of *[PSI⁺]* in a *[pin⁻]* background. Likewise, the accompanying paper shows that overproduction of the New1 prion domain promotes the induction of *[PSI⁺]* in a *[pin⁻]* background and that mutations in this domain can inhibit this activity (Osherovich and Weissman, 2001 [this issue of *Cell*]).

Using prion domains of Sup35, Ure2, Rnq1, or New1 as baits, we performed searches (WU-BLAST 2.0; gapped alignment; Altschul and Gish, 1996) against the proteins in the *Saccharomyces* Genome Database (http://genome-www.stanford.edu). Each of the overlapping regions in sets C–J encoded sequences with a high degree of similarity to one or more of these prion baits. The same searches revealed that only 108 of the ∼6,000 *S. cerevisiae* genes had regions with equal or greater homology to the prion domain baits than the sequences found in our inserts. The probability that by chance, 10 of the 11 plasmids sets would encode a protein belonging to this group of 108 in their overlapping regions of three genes or less is extremely low ($P < 2 \times 10^{-12}$). It therefore appears that overproduction of numerous proteins containing prion-like domains can promote the induction of *[PSI⁺]* in a *[pin⁻]* background. Furthermore, the remaining plasmid set (K) encoded Ste18, a protein with a Gln-rich region. Such regions are present in most of the genes identified in our screen, and a Gln-rich stretch is an important component of the Sup35 prion domain (DePace et al., 1998).

### *[PIN⁺]* as a Prion Form of Rnq1

The screen for the gene encoding the *[PIN⁺]* prion uncovered 11 candidates, all with presumptive prion domains. To identify the gene that encodes the *[PIN⁺]*

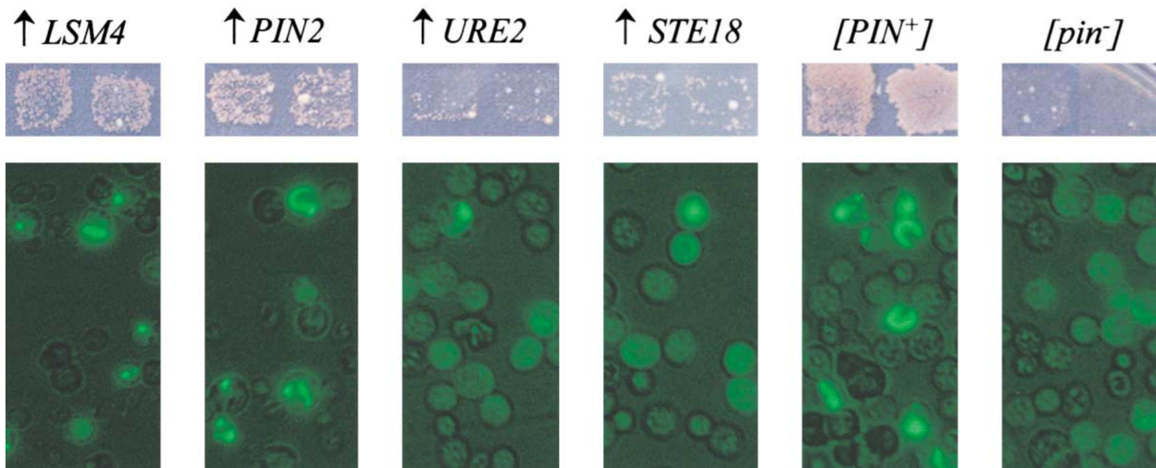



Figure 1. Screen for Gene Encoding *[PIN+]*

Shown are transformants of *[psi−][pin−]* 74-D694 carrying pSUP35NM::GFP-HIS3 and representative library plasmids that passed the screen. Genes shown were on the library plasmids and were implicated in the induction of *[PSI+]* (see Figure 2). Growth on −Ade (top), and aggregation of Sup35NM::GFP (bottom) was analyzed following library plasmid amplification and induction of *SUP35NM::GFP*. The controls are *[psi−][PIN+]* 74-D694 (*[PIN+]*) and *[psi−][pin−]* 74-D694 (*[pin−]*) carrying pSUP35NM::GFP-HIS3 and the vector used to construct the library, pHR81.

present in our strains, we asked if any of several genes identified in the screen, *URE2*, *NEW1*, and *PIN2*, as well as the only other known yeast prion gene, *RNQ1*, were required for the maintenance of *[PIN+]*.

A deletion of *RNQ1* in a *[psi−][PIN+]* BY4741 strain caused the loss of the Pin+ phenotype, while deletions of *URE2*, *NEW1*, and *PIN2* in BY4741 didn't cure *[PIN+]* (Figure 3, top row). Western blot analysis of Rnq1 (Figure 3) and in vivo Rnq1::GFP aggregation analyses (not shown) revealed that Rnq1 was aggregated in BY4741 *[PIN+]* and each deletion derivative that retained *[PIN+]*.

We also used cytoduction, a cytoplasmic mixing technique routinely used to transmit yeast prions, to ask if the addition of cytoplasmic elements from another *[PIN+]* strain, 74-D694, could cause the Δ*rnq1* strain to become *[PIN+]*. For these experiments, a *[pin−] kar1-1* strain, c10B-H49, was first mated with the *[PIN+]* 74-D694 donor and then with the Δ*rnq1* recipient strain. Because *kar1-1* causes a deficiency in karyogamy, progeny containing only the recipient nucleus, but a mixture of the donor and recipient cytoplasms (cytoductants), could be selected. We found that *[PIN+]* could not be transmitted to the Δ*rnq1* strain, although it was efficiently transmitted to the isogenic wild-type (WT) strain BY4741 and its Δ*ure2*, Δ*new1*, and Δ*pin2* deletion mutants that were cured of *[PIN+]* by growth on +GuHCl (Figure 3, bottom row). Note, while Rnq1 became soluble following curing on +GuHCl, it became aggregated again upon the addition of 74-D694 *[PIN+]* cytoplasm.

The finding that transmission of the Pin+ phenotype to recipient strains requires *RNQ1* indicates that deletion of *RNQ1* either causes the loss of *[PIN+]*, or masks the Pin+ phenotype. The latter possibility was eliminated by showing that *[PIN+]* was not recovered following transmission of cytoplasm from Δ*rnq1* donor strains (the Δ*rnq1* derivative of BY4741 and five independent cytoductants of this Δ*rnq1* strain exposed to 74-D694 *[PIN+]* cytoplasm) into a *[pin−]* recipient that does not have a deletion of *RNQ1* (c10B-H49). In controls where *RNQ1* *[PIN+]* strains were used as donors, *[PIN+]* was transmitted to the recipient. Thus, *RNQ1* is required for maintenance of the *[PIN+]* prion found in yeast strains BY4741 and 74-D694.

Because other genes, e.g., *HSP104*, that do not encode the Pin protein are required for the maintenance of the *[PIN+]* prion (Derkatch et al., 1997), these results fall short of proving that *[PIN+]* is a prion form of Rnq1. To prove this connection it was necessary to show the coappearance of Rnq1 aggregates and *[PIN+]*. We previously described the isolation of spontaneous *[PIN+]*s in a *[pin−]* 74-D694 strain (Derkatch et al., 2000; see also Experimental Procedures). We now show that while the *[pin−]* parent strain contains only soluble Rnq1, all eight spontaneous derivatives selected for being *[PIN+]* have simultaneously acquired Rnq1 aggregates (Figure 4). Aggregation of Rnq1 was scored using both an Rnq1::GFP assay (not shown) and Western analyses of fractionated lysates. In addition, like the original *[PIN+]* found in 74-D694, these spontaneous *[PIN+]*s could be cytoduced into GuHCl-cured *RNQ1* strains but not into Δ*rnq1* strains. Thus, the spontaneous *[PIN+]*s were caused by Rnq1 prion aggregates.

To show that the prion domain of Rnq1 is sufficient to cause *[PIN+]*, a 74-D694 Δ*rnq1* strain carrying a fusion of just the Rnq1 prion domain to a reporter construct (Sondheimer and Lindquist, 2000) was used. Cells bearing the prion form of this fusion, called *[RPS+]*, were *[PIN+]*, while cells with the nonprion form of the fusion, called *[rps−]*, were *[pin−]*. Furthermore, all attempts to cure cells selectively of *[RPS+]* while retaining *[PIN+]* failed (data not shown).

Thus, since deleting *RNQ1* cured *[PIN+]* and the spontaneous appearance of *[PIN+]* was always accompanied by the appearance of Rnq1 aggregates, the presence of Rnq1 aggregates is clearly sufficient to make strains *[PIN+]*. Why then wasn't *RNQ1* cloned in the screen for *[PIN+]*? *RNQ1* may have been missed because the search was not exhaustive. To test if *RNQ1* would pass

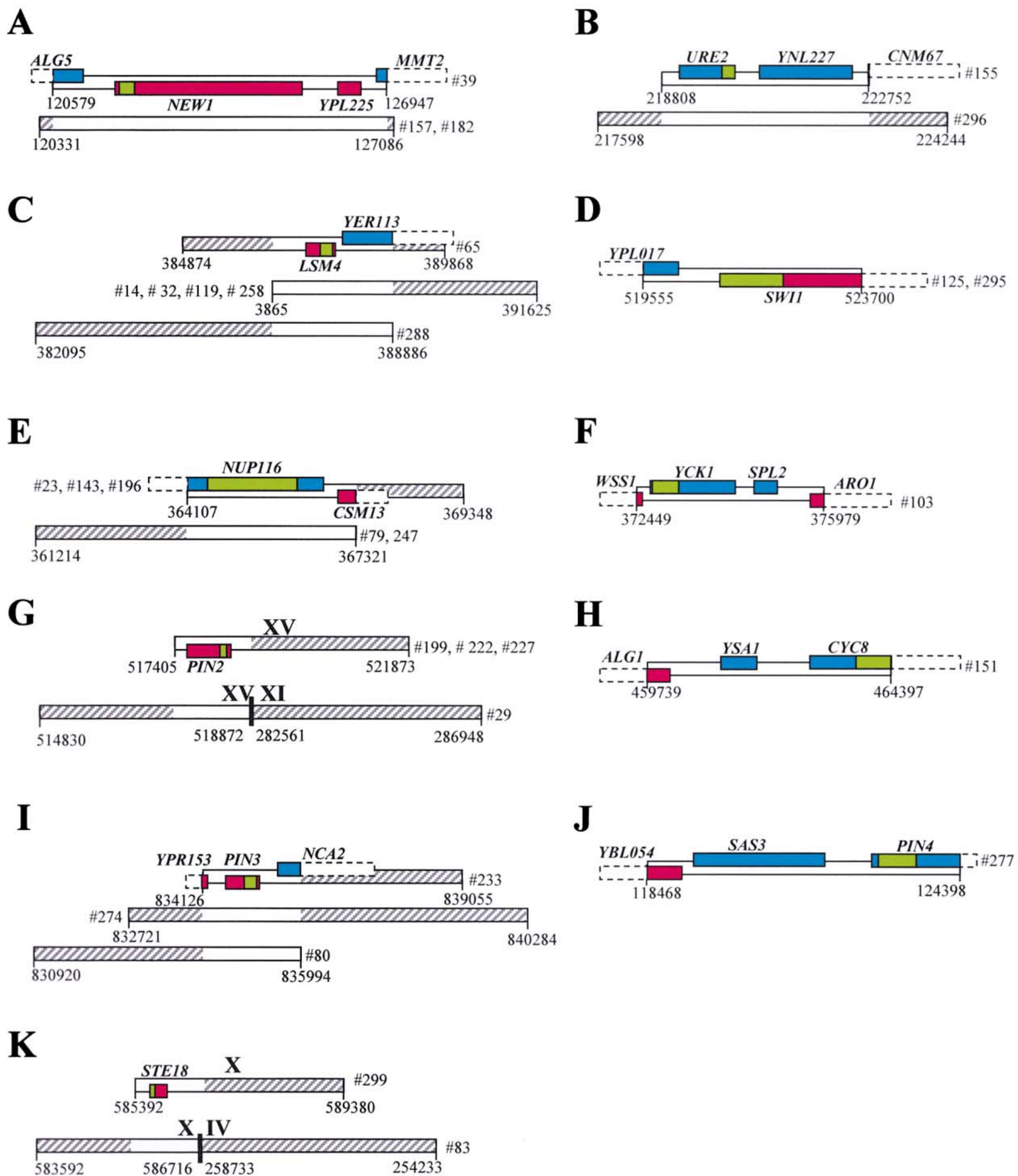



Figure 2. Maps of Inserts from Plasmids Identified in the Screen for the *[PIN⁺]* Gene

Inserts of the 30 library plasmids that overcame the requirement for *[PIN⁺]* were placed into 11 nonoverlapping sets. The chromosomal locations and genetic composition of the inserts are shown using data and nomenclature from the *Saccharomyces* Genome Database (http://genome-www.stanford.edu). Insert sequences outside the overlapping regions are hatched. Genes in overlapping regions are in blue if transcribed from right to left and in red if transcribed from left to right. Hypothetical short ORFs for which expression has not been established are not shown if they overlap better-characterized genes since such ORFs are not likely to encode proteins. Regions encoding prion or prion-like domains (shown in green) were chosen on the basis of homology with the most similar known prion domain or, for Ste18, on the basis of high Gln content. When only a portion of a gene is in the overlap region or insert, the remainder of the gene is shown as a dashed box. The identification number of each plasmid is shown. Occasionally, plasmids cloned had identical inserts (e.g., in [A], #157 and #182). Note, while #151 (H) and #277 (J) contain only the 3′ portions of *CYC8* and *PIN4*, respectively, in-frame AUG codons on both plasmids could initiate translation products containing the prion domains. The uncharacterized ORFs *YOR104W*, *YPR154W*, and *YBL051C*, have been named *PIN2*, *PIN3*, and *PIN4*, respectively.

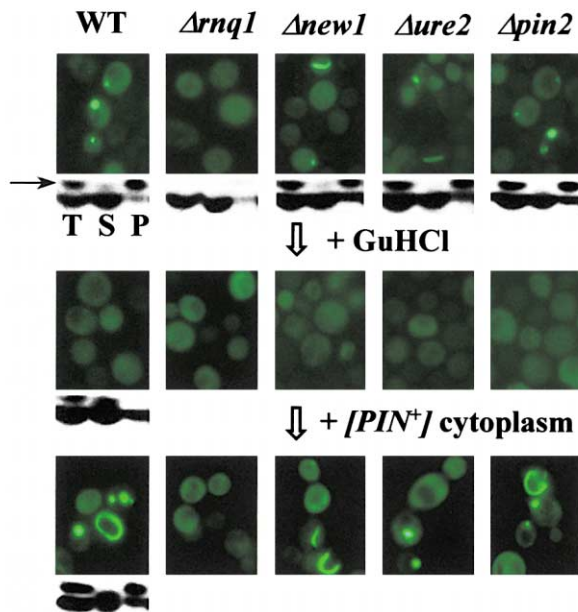



Figure 3. *RNQ1*, but Not Other Prion Genes, Is Required for the Maintenance of the Pin$^+$ Phenotype in BY4741 and 74-D694

The top row shows wild-type (WT) *[psi$^-$][PIN$^+$]* strain BY4741 and its deletion derivatives. The middle row shows GuHCl-cured *[pin$^-$]* derivatives of the strains shown above. The bottom row shows strains from the middle row after the addition of *[PIN$^+$]* donor cytoplasm. Shown is fluorescent microscopy of cells transformed with pSUP35NM::GFP-URA3 and grown on +Cu for 3 days to express the fusion. The presence of *[PIN$^+$]* was scored by the appearance of Sup35NM::GFP dot- and ring-like aggregates. Where shown, Western blots of lysates (T) separated into supernatant (S) and pellet (P) fractions were used to determine the presence or absence of Rnq1 aggregates. An arrow marks the band that corresponds to Rnq1. The lower band is due to cross-reactivity of the antibody. The presence of Rnq1 in the pellet indicates that it is in the aggregated prion form. The aggregation state of Rnq1 was also scored in all cultures using the in vivo Rnq1::GFP assay (data not shown).

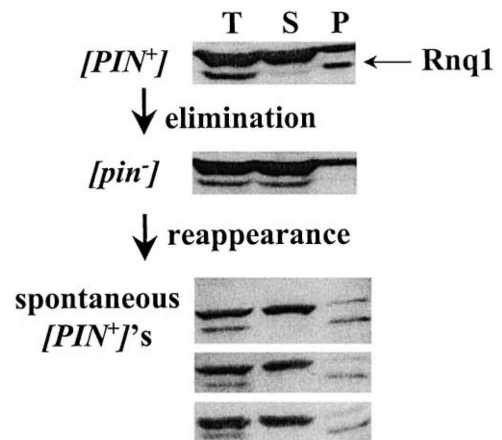



Figure 4. Spontaneous Appearance of *[PIN$^+$]* Is Accompanied by the Appearance of Rnq1 Aggregates

Shown are Western analyses of Rnq1 in supernatant (S) and pellet (P) fractions of lysates (T) of the original *[PIN$^+$]* version of 74-D694, its GuHCl cured *[pin$^-$]* derivative, and three representative independent spontaneous *[PIN$^+$]* derivatives that arose in the cured strain. The band that corresponds to Rnq1 is marked. The band above is due to cross-reactivity of the antibody. The presence of Rnq1 in the pellet indicates that it is in the aggregated prion form.

the screen, the *SUP35NM::GFP* and the *RNQ1::GFP* fusions were overexpressed in *[psi$^-$][pin$^-$]* 74-D694. We found that although the overproduction of Rnq1::GFP facilitated the de novo formation of *[PSI$^+$]* aggregates, it was rather inefficient and would probably have failed the screen (data not shown).

### *[PIN$^+$]* as a Prion Form of Ure2

Although prion aggregates of Rnq1 were shown to cause *[PIN$^+$]* in strains 74-D694 and BY4741, the screen for the gene encoding *[PIN$^+$]* identified 11 different known and candidate prion genes. Thus, we tested whether *[URE3]*, a prion encoded by one of these genes, *URE2*, could also cause the Pin$^+$ phenotype. Cytoplasm from a *[URE3]* strain, 4184, was transferred into BY4741 Δ*rnq1*. Following cytoduction, overproduction of Sup35NM::GFP caused the formation of dot- and ring-like aggregates diagnostic of *[PSI$^+$]* induction (Figure 5, left). Because the *RNQ1* deletion precluded the recipient from bearing Rnq1 prion aggregates, this result suggests that *[URE3]* causes *[PIN$^+$]*. To prove this, we demonstrated that the *[PIN$^+$]* from the Δ*rnq1* cytoductants could be transferred into wild-type *[pin$^-$]* or Δ*rnq1* strains, but not into an isogenic Δ*ure2* strain. Thus, more than one prion can make cells *[PIN$^+$]*. Regardless of which prion element is responsible, we refer to all strains harboring a non-Mendelian element that allows *[PSI$^+$]* to be induced de novo as *[PIN$^+$]*.

### *[URE3]* Facilitates the De Novo Formation of Rnq1 Prion Aggregates

The fact that *RNQ1* was not identified in the screen for the *[PIN$^+$]* gene could be explained if Rnq1 aggregates were not efficiently induced in strains lacking prions. Indeed, when BY4741 was cured of prion aggregates either by growth on +GuHCl or by disrupting *RNQ1*, Rnq1::GFP overproduction essentially failed to induce aggregates (Figure 6A). Rare ($<$0.1%) cells had dots.

To ask if the *[URE3]* prion could facilitate the de novo formation of Rnq1 prion aggregates, we cytoduced *[URE3]* into Δ*rnq1* and GuHCl-cured *[pin$^-$]* derivatives of BY4741 (Figure 6A). In these cytoductants, about 5% of the cells produced dot- or rod-like Rnq1::GFP aggregates. The rod-like aggregates are reminiscent of the ring-like aggregates seen upon the de novo induction of *[PSI$^+$]* (Zhou et al., 2001), and are diagnostic for the de novo appearance of Rnq1 aggregates because only dots were seen in cells with preexisting Rnq1 aggregates, e.g., in BY4741 (Figure 6A). As expected, the ability to form such aggregates was GuHCl-curable. Thus, *[PSI$^+$]* is not the only prion that requires another prion to facilitate its induction; the de novo appearance of Rnq1 prion aggregates also requires the presence of a prion.

### *[PSI$^+$]* Also Facilitates the De Novo Formation of Rnq1 Prion Aggregates

Because *[URE3]* facilitates the appearance of Rnq1 aggregates, we asked if *[PSI$^+$]* would have a similar effect.

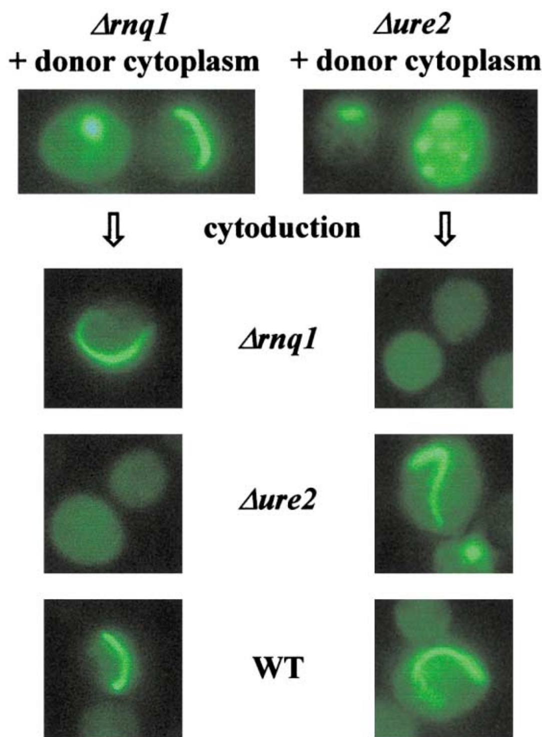



Figure 5. Prions Formed by Either Ure2 (left) or Rnq1 (right) Facilitate the De Novo Induction of *[PSI⁺]*

(Left) Cytoplasm from strain 4184 was cytoduced into a Δ*rnq1* derivative of BY4741 (Δ*rnq1* + donor cytoplasm) which was then used as a donor to transfer cytoplasm into Δ*rnq1,* Δ*ure2*, or wild-type *[pin⁻]* derivatives of BY4741. (The second cytoduction was performed using c10B-H49 *[pin⁻]* as an intermediate.) The appearance of Sup35NM::GFP aggregates indicative of *[PIN⁺]* was visualized by crossing to a *[psi⁻][pin⁻]* tester carrying *SUP35NM::GFP* and by growing the diploids for 3 days on +Cu. Following the first cytoduction, about 5% of the cells contained ring-like or dot aggregates. Following the second cytoduction, a similar level and type of aggregation was observed in Δ*rnq1* and wild-type cells, but no aggregates were seen in Δ*ure2* cytoductants. This shows that *[URE3]* can make cells *[PIN⁺]*. The results of the reciprocal experiment where cytoplasm from 4148 was first cytoduced into *[pin⁻]* BY4741 Δ*ure2* (Δ*ure2* + donor cytoplasm) are shown at right. Following the first cytoduction, Δ*ure2* cells became *[PIN⁺]*, although BY4741 Δ*ure2* cannot maintain *[URE3]*. In this case, the Pin⁺ phenotype is due to the presence of Rnq1 prion aggregates (that were also present in 4184) and thus is dependent upon *RNQ1*. This figure demonstrates that Ure2 and Rnq1 prion aggregates have similar effects on *[PSI⁺]* formation.

Isogenic *[pin⁻][psi⁻]* and *[pin⁻][PSI⁺]* strains were transformed with inducible *RNQ1::GFP*. Although *[PSI⁺]* did not increase Rnq1::GFP aggregate formation in freshly grown transformants, storage for 21 days at 4°C dramatically increased the number of cells with dot-shaped Rnq1 aggregates in *[PSI⁺]* but not *[psi⁻]* cultures (Table 1, storage experiment 1). The fact that only dot- and not rod-shaped aggregates were detected, and that they could be seen without prolonged Rnq1::GFP overproduction, indicates that the Rnq1 aggregates were produced during storage. When cultures from storage experiment 1 were colony purified, most of the freshly grown colonies displayed essentially no aggregates in the presence or absence of *[PSI⁺]* (Table 1, no storage). However, when stored again, *[PSI⁺]* but not *[psi⁻]* cultures exhibited a dramatic increase in dot-shaped Rnq1::GFP aggregates (Table 1, storage experiment 2).

Occasional freshly grown colonies from previously stored *[PSI⁺]* cultures contained cells with aggregated Rnq1 (Table 1). Out of the 18 such colonies found, only one had Rnq1::GFP aggregates in essentially every cell, whereas in the remaining colonies, Rnq1::GFP aggregates were found in 20%–60% of the cells. Taken together, this demonstrates that heritable Rnq1 prion aggregates appear during storage of *[PSI⁺]* cells, and that the newly arising Rnq1 prions are unstable, thus resembling newly arising *[PIN⁺]*, *[PSI⁺]*, and *[URE3]*.

**Overexpression of Genes that Eliminate the *[PIN⁺]* Requirement for *[PSI⁺]* Induction Enhances the De Novo Appearance of Rnq1::GFP Aggregates**

We next asked if the 11 genes implicated above for the ability to enhance the induction of *[PSI⁺]*, could also enhance the induction of other prions. We chose to examine the de novo formation of Rnq1 prion aggregates because, like *[PSI⁺]*, Rnq1 aggregation was not induced efficiently in strains cured of prions by growth on +GuHCl, but was facilitated by the presence of *[PSI⁺]* or *[URE3]*.

The *[psi⁻][pin⁻]* 74-D694 strain carrying unexpressed *RNQ1::GFP* was transformed with 11 of the plasmids identified in the *[PIN⁺]* screen, each carrying a different candidate gene. Following amplification of the library plasmids, *RNQ1::GFP* expression was induced and cells were examined for the appearance of Rnq1::GFP aggregates. Frequent fluorescent aggregates were found in cultures overexpressing plasmids from sets A–E and G–I (Figure 6B). Furthermore, plasmids that had the best effect on Sup35NM::GFP aggregation had the best effect on Rnq1::GFP aggregation. As in the case of the de novo appearance of Rnq1::GFP aggregates in the presence of *[URE3]* (see Figure 6A), the Rnq1::GFP aggregates induced in this experiment were not limited to punctate dot shapes, but included rod-shaped aggregates, indicating that the prion aggregates were appearing de novo.

## Discussion

To investigate how the prion-like *[PIN⁺]* trait influences the de novo appearance of *[PSI⁺]*, we set out to identify what we believed would be a single gene encoding the *[PIN⁺]* prion. We screened a high-copy library assuming that overexpression of the *PIN* gene would induce *[PIN⁺]*, and uncovered 11 known or presumptive-prion proteins that participate in diverse cellular processes such as regulation of transcription (Ure2, Swi1, and Cyc8), signal transduction (Yck1 and Ste18), nuclear transport (Nup116), and mRNA processing (Lsm4). These results suggest that more than one prion can make cells *[PIN⁺]*. Notably, all known yeast prions were either retrieved in the screen or were otherwise shown to act as *[PIN⁺]*. We directly showed that the presence of either Rnq1 or Ure2 prion aggregates makes yeast strains *[PIN⁺]* and that the spontaneous appearance of *[PIN⁺]* is accompanied by the appearance of Rnq1 aggregates. Moreover, *[PSI⁺]* is not the only prion that

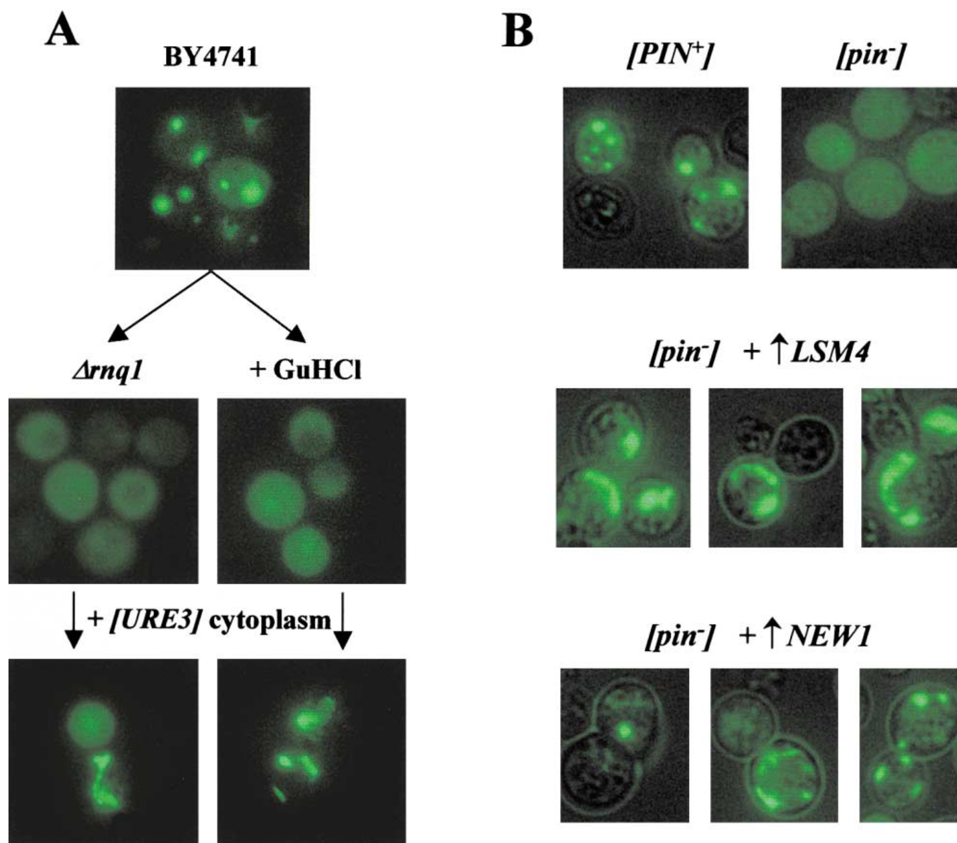



Figure 6. The De Novo Formation of Rnq1::GFP Prion Aggregates Is Enhanced by *[URE3]* or Overexpression of Genes Identified in the *[PIN⁺]* Screen

(A) The effect of *[URE3]* on Rnq1::GFP aggregation was visualized by crossing the strains described below to a *[psi⁻][pin⁻]* tester carrying pRNQ1::GFP-URA3, and by growing the diploids for 3 days on +Cu. The original BY4741 strain is shown in the top row. Also shown are the BY4741 Δ*rnq1* mutant and the GuHCl-cured *[pin⁻]* derivative before (second row) and after (third row) they were cytoduced with *[URE3]*. *[URE3]* originates from 4184 but was passaged through a Δ*rnq1* strain in order to eliminate Rnq1 prion aggregates that were also found in 4184. The fact that it was *[URE3]* and not another unidentified prion that facilitated the formation of Rnq1::GFP aggregates was proved in an experiment analogous to the one in Figure 5: additional passaging through a Δ*ure2* strain made cytoplasm inefficient in facilitating Rnq1 aggregation (data not shown).

(B) Shown are transformants of *[psi⁻][pin⁻]* 74-D694 carrying pRNQ1::GFP-HIS3 and representative library plasmids that passed the screen. The genes indicated were on the library plasmids and were implicated in the induction of *[PSI⁺]* (see Figure 2). Aggregation of Rnq1::GFP was analyzed following amplification of library plasmids and induction of Rnq1::GFP. The controls are *[psi⁻][PIN⁺]* 74-D694 (*[PIN⁺]*) and *[psi⁻][pin⁻]* 74-D694 (*[pin⁻]*) carrying pRNQ1::GFP-HIS3 and the vector used to construct the library, pHR81.

requires the presence of other prions for its de novo appearance: aggregates of Rnq1::GFP are not induced in strains cured of prions, but do appear in strains that either carry other prions (*[PSI⁺]* or *[URE3]*) or overproduce prion-like proteins.

Prions are of interest not only because the PrP prion represents a serious threat to the health of humans and livestock, but also because prions provide a novel mechanism for the inheritance of traits and the modulation of protein function. Furthermore, the conundrum that the human genome is composed of fewer genes than expected (Int. Human Genome Seq. Cons., 2001) may be in part explained because conversion into a prion can modulate the activity of a protein encoded by the same allele. It is thus important to determine how many different proteins can exist as prions and the traits they affect. Our search for the *[PIN⁺]* gene allowed us to address two important issues of prion research: the identification of novel prions, and the mechanisms governing the de novo formation of prions.

### Identification of Novel Prions

Prions were first identified by their unusual properties: PrP as infectious material that did not contain nucleic acid (Griffith, 1967; Prusiner, 1982); *[URE3]* and *[PSI⁺]* as reappearing epigenetic factors (Wickner, 1994). Using the genetic criteria for a prion established by Wickner et al. (1995) the *P. anserina* mating-type incompatibility epigenetic factor *[Het-s]* (Coustou et al., 1997) and the yeast *[PIN⁺]* element (Derkatch et al., 1997, 2000) were also proposed to be prions.

Another approach has been to search databases for presumptive prion domains by looking either for sequences that are homologous to known prion domains or are unusually Gln/Asn-rich. Using the criterion of at least 30 Gln/Asn's in 80 consecutive residues, 107 *S.*

Table 1. Effect of *[PSI⁺]* on De Novo Formation of Rnq1 Aggregates

| Strain[a] | % cells with aggregated Rnq1::GFP[b] No storage[c] | Following storage[d] Exp. 1 | Exp. 2 | Colonies with aggregated Rnq1::GFP[b,e] |
|---|---|---|---|---|
| *[psi⁻]* | 0.02 ± 0.01 | 0.14 ± 0.08 | 0.04 ± 0.02 | 0/245 |
| Weak *[PSI⁺]* | 0.06 ± 0.00 | 0.96 ± 0.51 | 2.81 ± 1.50 | 7/275 |
| Strong *[PSI⁺]* | 0.10 ± 0.01 | 4.60 ± 1.58 | 5.04 ± 1.84 | 11/260 |

[a] *[PSI⁺] [pin⁻]* 74-D694 variants were obtained by transiently overproducing Sup35 in a *[psi⁻] [PIN⁺]* derivative to induce *[PSI⁺]* (weak #21, strong #8, Derkatch et al., 1996) and then selecting for colonies that retained *[PSI⁺]* but lost *[PIN⁺]* following growth on +GuHC1 (Derkatch et al., 2000).
[b] Expression of Rnq1::GFP was maintained at a low-level on SD-Ura (which contains 0.25 μM $CuSO_4$).
[c] ~45,000 exponentially growing cells of the indicated strain variants carrying the Rnq1::GFP fusion were scored.
[d] ~9,000 exponentially growing cells previously stored at 4°C for 21 (Exp. 1) or 14 (Exp. 2) days were scored.
[e] Cultures from "Following storage, Exp. 1" were colony purified and ~250 colonies were scored.

*cerevisiae* proteins were identified as potential novel prions (Michelitsch and Weissman, 2000). Two proteins identified from database searches, Rnq1 and New1, have been shown to contain domains that, when fused to reporter constructs, can create artificial prions (Sondheimer and Lindquist, 2000; Santoso et al., 2000). While a stable New1 prion has not yet been isolated, the wild-type Rnq1 was shown to exist in a stable prion form.

An unexpected dividend of our screen for the *[PIN⁺]* gene was that it identified potential prions, and thus is an in vivo screen for prions. In addition to Ure2 and New1, we retrieved nine potential *[PIN⁺]* prions. All but one of these exhibited obvious homology to the prion domains of Sup35, Ure2, New1, and/or Rnq1 ($P < 0.0037$) and eight appeared on the list of 107 *S. cerevisiae* proteins rich in Gln/Asn. However, many proteins showing higher homology to the prion domain baits (e.g., Ybr016w and Nup100) or having a higher Gln/Asn content (e.g., Snf5 and Gpr1), were not retrieved. Since it is unlikely that all the proteins identified as prospective prions from database searches are indeed prions, the functional assay is useful to identify a subset that are more likely to be prions. It also identifies presumptive prions that do not satisfy the criteria used in the database searches. For example, Pin3 and Pin2 were not identified in the search using the Gln/Asn criterion. Nonetheless, Pin3 had 29 Gln/Asn's in 81 consecutive residues; Pin2 had 15 in 47. Finally, Ste18 was not identified by either of the previous database screens because it is very short (110 aa), but its N terminus is very Gln/Asn rich (16 in 35). Limitations of our screen are that it may not identify proteins that do not become prions in the absence of another prion or that fail to enhance the de novo appearance of *[PSI⁺]*.

Whether all candidate *[PIN⁺]* proteins can form stable prions is unknown. Because newly appearing prions are unstable, it is difficult to determine if transient overexpression of a candidate gene can induce a stable prion phenotype. However, aligning Yck1, Lsm4, and Ste18 with other members of their respective protein families reveals a chimeric structure similar to that displayed by Sup35 (see Supplemental Data at *Cell* website [http://www.cell.com/cgi/content/full/106/2/DC1]), supporting the hypothesis that these are also prion genes.

## How Is the De Novo Formation of Prions Enhanced by Other Prions?

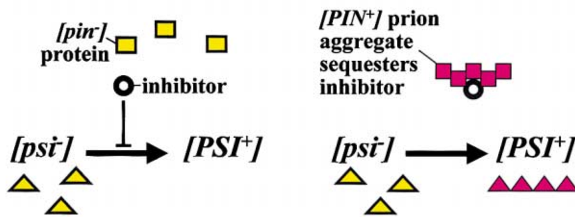
We have shown that Rnq1 prion aggregates and *[PSI⁺]* reciprocally affect the de novo appearance of one another. Furthermore, the de novo formation of both of these prions is enhanced by the presence of *[URE3]*. Apparently, once one prion appears in a cell, other prions are more likely to form. Such a cascade of prion formation may provide a selective advantage under certain conditions. While the de novo formation of some prions may almost exclusively depend upon heterologous prions, formation of other prions may occur in the absence of such aggregates. Indeed, two degradation-prone artificial constructs carrying the Sup35 prion domain were capable of inducing *[PSI⁺]* in the absence of *[PIN⁺]* (Derkatch et al., 2000). However, even in these cases, *[PIN⁺]* enhanced the frequency of *[PSI⁺]* induction. Apparently, enhancement of prion formation by other prions is a widespread phenomenon and may be a general principle with broad ramifications.

How do *[PIN⁺]* prions enhance the formation of other prions? Nonprion protein isomers in *[pin⁻]* cells might actively inhibit the de novo formation of *[PSI⁺]* (Derkatch et al., 2000). If this were true, deletion of the *[PIN⁺]* gene would inactivate the inhibitor protein and cause cells to be phenotypically Pin⁺. We show this is not the case because cells cured of prions and carrying deletions of ***RNQ1***, ***URE2***, or ***NEW1*** retain a Pin⁻ phenotype, indicating that these genes do not encode a unique inhibitor. We also disprove that there is more than one inhibitor because the nonprion forms of these proteins fail to inhibit *[PSI⁺]* induction in the presence of another prion.

Alternatively, the presence of *[PIN⁺]* prion aggregates may enable the de novo formation of *[PSI⁺]* (Derkatch et al., 2000). We consider two possible mechanisms: titration of an aggregation inhibitor and direct seeding (Figure 7). Whichever mechanism is correct, it may not require heritable prions, but might also occur upon the formation of temporary prion-like aggregates that form when proteins with prion-like domains are overproduced.

According to the titration model, a protein (e.g., a chaperone) normally inhibits the de novo formation of prion aggregates by disaggregating initial prion seeds. The presence of *[PIN⁺]* prion aggregates would sequester this protein, allowing initial prion seeds of another protein a chance to develop. The finding that Sup35, Ure2, Rnq1, and PrP all form fibers in vitro without any seeding (Glover et al., 1997; King et al., 1997; Taylor et al., 1999; Thual et al., 1999; Schlumpberger et al., 2000; Sondheimer and Lindquist, 2000; Jackson et al., 1999) seems consistent with the titration model, because of

TITRATION MODEL FOR *[PIN$^+$]*




SEEDING MODEL FOR *[PIN$^+$]*

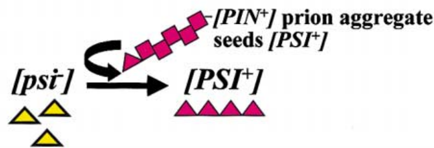



Figure 7. Titration and Heterologous Seeding Models to Explain How Prion Aggregates Enhance the De Novo Formation of Other Prions

See text.

the absence, in vitro, of an inhibitor protein normally sequestered by *[PIN$^+$]*. However, the in vitro conditions may be too different from the cellular environment to be compared to *[PIN$^+$]* cells due to the absence of cellular ligands that normally retard the spontaneous appearance of prions but are not likely to be sequestered by *[PIN$^+$]* aggregates (e.g., Sup45 for *[PSI$^+$]*; Derkatch et al., 1998). The following observations argue against the titration hypothesis. While the model predicts the possibility of compromising the activity of the inhibitory protein by mutation, thereby causing a Pin$^+$ phenotype in the absence of prion-like aggregates, EMS mutagenesis of a *[psi$^-$][pin$^-$]* strain failed to uncover a stable Pin$^+$ mutant in a screen that had a >97% chance of hitting any given yeast gene (I.L.D., S. Masse, and S.W.L., unpublished data). Thus, if there is a titratable inhibitor, there is likely to be more than one. Also, extreme overproduction of Sup35, which might be expected to overcome the inhibition, failed to do so (Derkatch et al., 1997). Finally, Hsp104, the primary candidate for a titratable inhibitor because of its participation in disassembling protein aggregates and in prion maintenance (Parsell et al., 1994; Chernoff et al., 1995; Moriyama et al., 2000), is apparently not inhibiting prion de novo appearance, since overexpression of Hsp104 does not prevent an excess of Sup35 from inducing *[PSI$^+$]* (Zhou et al., 2001).

The direct seeding model proposes that *[PIN$^+$]* aggregates provide a nidus on which the first seeds of a different prion can form (Figure 7). Since prion propagation is thought to involve a homologous prion seed that either templates conversion to the prion conformation, or stabilizes molecules that spontaneously fold in the prion shape (Caughey, 2000; Serio et al., 2000), the process by which *[PIN$^+$]* prion aggregates occasionally provide a nidus for the de novo formation of a heterologous prion protein could be analogous to, but orders of magnitude lower than, the efficient propagation of a homologous prion seed. A similar mechanism has been suggested to explain how injecting mice with synthetic amyloid-like fibrils or modified silk in a β sheet conformation enhances the formation of AA-amyloid fibrils (Johan et al., 1998; Kisilevsky et al., 1999). Furthermore, interactions between heterologous prion or prion-like domains have been reported: the Sup35 prion domain interacts with a prion-like domain in Sla1 (Bailleul et al., 1999). Also, the Lsm4 prion-like domain is required for interaction with the product of the human spinal muscular atrophy disease gene, *SMN* (Friesen and Dreyfuss, 2000), that itself must form oligomers in order to interact with Sm proteins (Pellizzoni et al., 1999).

The seeding model doesn't require that different prions in the cell aggregate together. Indeed, this is unlikely because distinct separate prion aggregates were formed when Sup35 prion domains from different species of yeast were coexpressed in a single cell (Santoso et al., 2000), and because *[PIN$^+$]* does not affect *[PSI$^+$]* phenotypes or stability (Derkatch et al., 2000). Rather, the seeding model proposes that occasionally, a single heterologous prion aggregate can seed the de novo formation of another prion, which then proceeds to seed its own rapid and separate aggregation. The inefficiency of this process is consistent with the species barrier for prion infection, while providing an explanation for how the barrier can be occasionally breached resulting in TSE transmission from one species to another.

## Experimental Procedures

### Yeast Genetics

Standard methods were used (Sherman et al., 1986; Rose et al., 1990). Unless mentioned, yeast were grown at 30°C on YPD. YPD containing 5 mM GuHCl (+GuHCl) or 0.05 mg/ml ethidium bromide (+EtBr) was used to cure prions (Tuite et al., 1981) or *[RHO$^+$]*, respectively. Transformants were grown on SD selective for the plasmid (e.g., −Ura or −Ura, His). SD lacking adenine (−Ade) was used to select for *[PSI$^+$]* and for suppression analyses (at 20°C and 30°C). SD selective for the plasmid and containing 50–70 μM $CuSO_4$ (e.g., −His+Cu) was used to express *CUP1*-driven constructs.

In cytoduction experiments, c10B-H49 was used as a donor or recipient. The *kar1-1* mutation in this strain reduces the efficiency of nuclear fusion following mating. Donor strains were *[RHO$^+$]* and recipients *[rho$^0$]*. When c10B-H49 was used as a recipient, the cytoductants with a recipient nucleus and *[RHO$^+$]* cytoplasm from the donor were selected directly on synthetic glycerol medium containing 3 mg/l cycloheximide (SG+Cyh) because *cyh2* in c10B-H49 allows selection against both diploids and donors. Otherwise cytoductants were selected on SG and checked for recessive recipient markers.

### Strains

74-D694 (*MAT*a *ade1-14 leu2-3,112 his3-Δ200 trp1-289 ura3-52 [psi$^-$][PIN$^+$]*) and its derivatives were described previously (Chernoff et al., 1995; Derkatch et al., 1996, 1997, 2000). The *[psi$^-$][pin$^-$]* derivative was obtained on +GuHCl. Spontaneous *[PIN$^+$]* derivatives were obtained following prolonged incubation that facilitated the appearance of *[PIN$^+$]* (Derkatch et al., 2000). Briefly, rare *[PSI$^+$]* cells were selected, cured of *[PSI$^+$]*, and the resulting *[psi$^-$]* cells were tested for the presence of *[PIN$^+$]*. Δ*rnq1* derivatives of 74-D694 with *SUP35* replaced by *RMC*, were kindly supplied by Sondheimer and Lindquist (2000). The protein encoded by *RMC* is a fusion of the prion domain from Rnq1 and the portion of Sup35 lacking the prion domain but functional in translational termination.

BY4741 (*MAT*a *his3-Δ1 leu2-Δ met15-Δ ura3-Δ [psi$^-$][PIN$^+$]*) and its Δ*rnq1*, Δ*ure2*, Δ*new1*, and Δ*pin2* deletion mutants are from Research Genetics; *[psi$^-$][pin$^-$]* derivatives were obtained on +GuHCl; *[rho$^0$]* mutants on +EtBr. Strain 4184 (*MATα kar1-1 arg1 ura2 [URE3] [PSI$^+$][PIN$^+$]*) was kindly provided by R. Wickner. Note, *[PSI$^+$]* from

4184 was often not transferred to cytoductants: 9 out of 58 cytoductants were *[psi⁻]*, and only these were scored for *[PIN⁺]*. The *[psi⁻][pin⁻]* derivatives of c10B-H49 (*MATα SUQ5 kar1-1 ade2-1 lys1-1 his3 leu1 cyh2 [rho⁰]*, kindly provided by V. Kushnirov and M. Ter-Avanesyan), SL1010-1A (*MATα ade1-14 met8-1 leu2-1 his5-2 trp1-1 ura3-52*; Zhou et al., 1999) and 64-D697 (*MATα ade1-14 trp1-289 lys9-A21 ura3-52 leu2-3,112*; Derkatch et al., 1997) were obtained on +GuHCl.

**Plasmids**

Centromeric pGFP-URA3 (kindly provided by S. Lindquist; listed as pCUP::GFP in Zhou et al., 2001), pSUP35NM::GFP-URA3 (listed as pSUP35NM-GFP), and pRNQ1::GFP-URA3 (kindly provided by Sondheimer and Lindquist, 2000) carry, respectively, GFP and fusions of amino acids 1–254 of Sup35 and full-length Rnq1 with GFP driven by the *CUP1* promoter. pSUP35NM::GFP-HIS3 and pRNQ1::GFP-HIS3 are identical to, respectively, pSUP35NM::GFP-URA3 and pRNQ1::GFP-URA3 but are pRS413-based. pHR81 is a 2 μg *URA3 leu2-d* vector used to construct the genomic library (Nehlin et al., 1989; kindly provided H. Ronne).

**Scoring for *[PSI]*, *[PIN]*, and the Rnq1 prion**

Suppression assays for *[PSI]* and *[PIN]* were as described (IngeVechtomov et al., 1988; Chernoff et al., 1995; Derkatch et al., 1996, 1997): the *ade1-14* nonsense mutation in 74-D694 prevents *[psi⁻]* derivatives from growing on −Ade, while suppression of *ade1-14* in *[PSI⁺]* permits growth. *[PIN⁺]* was detected by the appearance of Ade⁺ colonies (indicative of the de novo appearance of *[PSI⁺]*) following overexpression of *SUP35NM::GFP* in *[psi⁻]* strains into which pSUP35NM::GFP-URA3 or pSUP35NM::GFP-HIS3 were introduced by transformation or by mating to *[psi⁻][pin⁻]* tester strains 64-D697 or SL1010-1A already harboring the plasmids. Expression of *SUP35NM::GFP* was induced by replica-plating the transformants (or diploids) to +Cu, and cultures were then replica-plated to −Ade. GuHCl curing was used to distinguish Ade⁺ colonies that were due to *[PSI⁺]* from Ade⁺ revertants or other translational suppressors.

In vivo assays for *[PSI⁺]* and Rnq1 prion aggregates were as described previously (Patino et al., 1996; Zhou et al., 2001; Sondheimer and Lindquist, 2000). Aggregates were visualized under a fluorescence microscope (Axioskop; Carl Zeiss, Inc) in cells expressing, respectively, Sup35 prion domain or Rnq1, fused to GFP. *[psi⁻][PIN⁺]* and *[PSI⁺]* cultures are easily distinguished by the shape, frequency, and time of appearance of Sup35 aggregates (Zhou et al., 2001). Dot aggregates appear in most *[PSI⁺]* cells (*[PIN⁺]* or *[pin⁻]*) following even brief (1 hr) expression of *SUP35NM::GFP*. The presence of *[PIN⁺]* (scored only in *[psi⁻]* derivatives) results in the appearance of both dot and ring (Zhou et al., 2001) aggregates in about 20% of cells following prolonged (36–100 hr) expression of *SUP35NM::GFP*. The presence of Rnq1 prion aggregates results in the appearance of dot aggregates in most cells following brief expression of *RNQ::GFP*.

Western blot assays also scored for the presence of Rnq1 prion aggregates (Sondheimer and Lindquist, 2000). Cleared lysates (10 min at 3,000 g) of cells broken with glass beads in 50 mM Tris-HCl, 10 mM KCl, 100 mM EDTA, 1 mM DTT, 1.0% Triton X-100, and 0.2% SDS were fractionated at 280,000 g for 30 min. Polyclonal anti-Rnq1 antibody was a kind gift of N. Sondheimer and S. Lindquist.

**Screen for *[PIN⁺]***

*[pin⁻][psi⁻]* 74-D694 carrying pSUP35NM::GFP-HIS3 was transformed with a library of 4–6 kb Sau3A yeast genomic inserts in the BamHI site of the 2 μg *URA3 leu2-d* vector, pHR81 (Nehlin et al., 1989; kindly provided by H. Ronne). To allow for the de novo induction of the *[PIN⁺]* prion in transformants carrying the *[PIN⁺]* gene, transformants selected on −Ura,His were picked and replica-plated twice to −Ura,His where the library plasmids were at about 10–20 copies/cell, and in parallel to −Leu,Ura,His, where the library plasmids were amplified to about 100 copies/cell because of the poorly expressed *leu2-d* allele. To screen for transformants that acquired *[PIN⁺]* cells, we examined their ability to become *[PSI⁺]* by replica-plating to −His+Cu to induce *SUP35NM::GFP* and then to −Ade to select for *[PSI⁺]*. For candidates that grew on −Ade, cells from −His+Cu were examined under the microscope for the presence of Sup35NM::GFP aggregates diagnostic for *[PIN⁺]*. The Pin⁺ phenotype was stronger in cultures passed on −Leu,Ura,His than on −Ura,His, possibly because the higher amplification of *PIN* genes enhanced *[PIN⁺]* induction (as in the case of Sup35 and *[PSI⁺]*; Derkatch et al., 1996). However, overamplificaton of 5% of the library plasmids (including #79 and #247) inhibited growth.

**DNA Manipulations**

Standard protocols were used (Sambrook et al., 1989; Rose et al., 1990). To purify candidate library plasmids, DNA isolated from yeast carrying library plasmids as well as pSUP35NM::GFP-HIS3 was transformed into KC8 *E. coli* (*R-M+ lacX74 strA galUK leuB600 trpC9830 pyrF::Tn5 hisB463*; kindly provided by K. Struhl). Cells with library plasmids were selected on M9 +His +Leu +Trp −Ura. Plasmid from *E. coli* was used to transform yeast and for restriction analyses and sequencing (University of Chicago CRC DNA Sequencing Facility). Primers homologous to sequences flanking the BamHI site in pHR81, 5′ GTAAAACGACGGCCAGT 3′ and 5′ AGTCTCATCC TTCAATGCTATC 3′, were used to sequence the ends of inserts. In 28 of 30 plasmids, this identified 3.5–7.6 kb inserts of contiguous yeast genomic DNA, in agreement with estimates by restriction analyses. Two plasmids, #29 and #83 (Figure 2), contained tandem inserts of yeast genomic DNA, so sequencing was continued to identify the junctions.

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