Title

The role of the prion determining domain of Sup35p in formation, propagation, and manifestation of a species barrier of the yeast prion [PSI$^+$]

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The Role of the Prion Determining Domain of Sup35p in Formation, Propagation, and Manifestation of a Species Barrier of the Yeast Prion [PSI^+]

by

Alex Santoso

DISSERTATION

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ACKNOWLEDGEMENT

This thesis is dedicated to my family: my father Teguh, my mother Shintawati, my brother Steve, and to the love of my life Tiffany. Without their boundless love, absolute support, and words of encouragement in my times of need, or without them to share my joys and discoveries, perhaps this thesis would never have been written. Therefore it is my single greatest pleasure to dedicate my work and accomplishments in their names.

It is somewhat unfair that my limited ability to describe in great depths the contributions of my colleagues and friends to this work has forced me to convey gratitude in a condensed manner. First and foremost, I would like to thank Dr. Jonathan Weissman for his support of the goal of my work and his tolerance for the fashion by which I carried out that work. It is my good fortune to have done my graduate work in his laboratory, for he granted me not only support for but also independence in my scientific education. I would also like to thank members of my thesis committee, Dr. Keith Yamamoto and Dr. Wendell Lim, for their scientific guidance and more importantly, for their moral support during my graduate studies.

A thesis, written solely by a single person, belie an unfortunate connotation that the work described within involve solely that single person. Nothing could be further from the truth. I am fortunate to have known and collaborated with Angela DePace, Peter Chien and Lev Osherovich, which resulted in two publications described in this thesis. I am especially indebted to Peter for his infectious, not to mention tireless, enthusiasm for experiments, and to Lev for his insights and uncanny knowledge of trivia, scientific and otherwise, as well as for his friendship outside of lab.

Lastly, it is my pleasure to have worked side by side, bantered with, and confided in, my labmates and friends: Siew Ho Schleyer, Ben Tu, Jue Wang, Kevin Travers, Helmut Sparrer, Melissa Michelitch and Vladimir Denic. It is my sorrow to know that no longer will my days be filled by laughing with, or sometimes at them and likewise them at me, by listening to their good selection of music and otherwise, or simply by talking about science or other less ethereal subjects with them. I am, however, confident that they all will undoubtedly succeed in their pursuits, and that our paths will one day cross again. To these people I wished to convey my appreciation for making failed experiments seemed tolerable, and the hard days and long nights spent in lab enjoyable.

The Role of the Prion Determining Domain of Sup35p in Formation, Propagation, and Manifestation of a Species Barrier of the Yeast Prion [PSI$^+$].

by

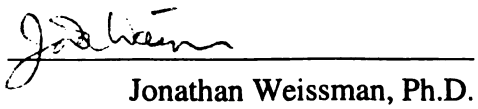
Alex Santoso

Abstract

The molecular basis for the propagation of the yeast [PSI$^+$] factor involve a self-propagating prion conformation of Sup35p, which is mediated by its amino terminal domain termed PrD. We have identified mutations in this domain that result in poor recruitment of these molecules into, or cause curing of, the wildtype Sup35 prion aggregates in vivo. Consistent with their in vivo phenotypes, these purified proteins carrying these mutations show markedly decreased rates of in vitro amyloid formation. These mutants map exclusively to a short amino-terminal region in the PrD, which is especially rich in glutamines and asparagines. Furthermore, this region can be replaced by a tract of polyglutamine without deleterious effect on the ability of Sup35p to form prion aggregates, suggesting that a similar mechanism underlies the formation of yeast prion amyloid and the aggregates implicated in polyglutamine-mediated neuropathologies.

A survey of a broad spectrum of budding yeast species reveals the conservation of the sequence and the ability of PrD from Sup35p homologs to form prions. A novel two-plasmid genetic system identifies a species barrier, reminiscent to that found in mammalian prions, which inhibits prion conversion between Sup35p of different yeast species. This barrier can be reproduced in vitro using pure proteins, where ongoing polymerization of one Sup35p species does not affect conversion of another. Surprisingly, chimeric analysis identifies the same amino terminal PrD region implicated in the formation and propagation of the [PSI$^+$] factor as being responsible for mediating species-specificity in prion conversion.

The ability of the PrD to confer prion-based regulation of protein activity is modular and transferable. We have constructed a novel prion-based regulation of a cytoplasmic enzyme and nuclear transcriptional activator by fusion of these proteins to the Sup35 PrD. Together with the conservation of prion function across distantly related yeast species, the modularity of the PrD prion function suggests that prions may be evolutionarily selected for their ability to regulate protein activities reversibly and to propagate this knowledge in form of a novel protein-based genetic inheritance.



Jonathan Weissman, Ph.D.
Advisor

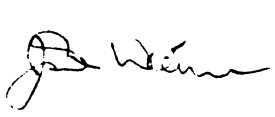
Contributions Acknowledgement

Part of the text and figures of this thesis is reproduced with permission from material previously published in Cell. Chapter 2, titled A Critical Role for Amino-Terminal Glutamine/Asparagine Repeats in the Formation and Propagation of a Yeast Prion, is the result of a collaboration with Ms. Angela DePace and Dr. Paul Hillner. This chapter is reproduced from Cell, Vol. 93, p.1241-1252, June 26, 1998. The mutagenesis, isolation and identification of Sup35 PrD mutants and their in vivo characterizations, presented in Figures 1 to 4 were performed by Ms. DePace, under supervision of Dr. Jonathan S. Weissman. Atomic Force Microscopy on the purified fibrils, presented in Figure 5B, was performed by Dr. Hillner, under supervision of Dr. Ronald D. Vale. Lastly, construction and characterization of PrD variants containing polyglutamine tract, presented in Figure 7, were performed by Dr. Weissman.

Chapter 3, titled Molecular Basis of a Yeast Prion Species Barrier is the result of collaboration with Mr. Peter Chien and Mr. Lev Z. Osherovich, both under supervision of Dr. Weissman. This chapter is reproduced from Cell, Vol. 100, p.277-288, January 21, 2000. The cloning and sequencing of Sup35 PrD homologs from various yeast budding yeast species, presented in Figure 1, and the fluorescence of co-expressed *Saccharomyces cerevisiae* and *Candida albicans* PrDs, presented in Figure 3C, were performed by Mr.

Chien. The construction of yeast strain deleted for *SUP35*, identification, cloning and characterization of New1p, presented in Figure 7, were performed by Mr. Osherovich. The construction of and analyses of the ability of, chimeric PrDs, to cause prion conversion, presented in Figure 6A and 6B, were performed by Dr. Weissman.

Chapter 4, titled Prion-Based Switch of Biological Functions, details the unpublished construction of Sup35 PrD to a cytoplasmic enzyme and a nuclear transcriptional activator, and analyses of the effects of these fusions on protein functions. Subcloning of the *ADH1* promoter driving the expression of the PrD fusion with the transcriptional activator was performed by Mr. Osherovich. With the exceptions listed above, the rest of the work was performed by the author of this thesis, Alex Santoso, under supervision of Dr. Weissman.



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Chapter 1

Introduction

The formation of amyloid aggregates is the result of the failure of amyloidogenic proteins to fold into their correct tertiary structures. In the past couple of decades, an increasing number of human diseases are suspected to be caused by or to involve the formation of these specific types of protein aggregates. To date, more than twenty proteins have been implicated in amyloid diseases, which affect different organs or tissues and thus fall into diverse symptomatic groupings. These diseases include inherited or age-related systemic amyloidoses, as well as inherited, spontaneous and infectious neuropathologies.

The later collective group itself represents a diverse group of neurodegenerative diseases in which amyloid aggregation is predominantly manifested as abnormal protein depositions in specific brain tissues. For example, in either the familial or spontaneous Alzheimer's disease, neurodegeneration is linked to the formation of extraneuronal amyloid plaques composed primarily of Aβ aggregates and the formation of intracellular neurofibrillary tangles composed largely of tau protein aggregates (see Price et al., 1998 for review). In Parkinson's disease, neurodegeneration is linked to the formation of intracellular inclusions called Lewy bodies, which seem to be composed primarily of α-synuclein aggregates. Mutations of α-synuclein implicated in the inherited form of the disease are found to cause acceleration of amyloid formation in vitro (see Olanow and Tatton, 1999 for review). In the familial Huntington's disease and in other CAG repeat expansion-mediated ataxias, the expansion of polyglutamine (polyQ) tracts in the respective proteins leads the formation of neuronal intranuclear inclusion bodies in vivo, as well as decreased protein solubilities and increased amyloid formations in vitro (see

Robitaille et al., 1997 for review). Lastly, in the inherited, infectious or spontaneous transmissible spongiform encephalopathies (TSEs), an altered conformation of the mammalian prion protein PrP is implicated as the causative agent (see below, also see Prusiner 1998 for review). TSEs are particularly important to this thesis, as these prion diseases are caused novel protein-based mechanism of disease infectivity and pathogenesis. Furthermore, the causative PrP prion serves as an important model for the topic of this thesis, i.e. the yeast prion Sup35p.

Structural studies reveal that in contrast to amorphous aggregation, the aggregations of amyloidogenic proteins form ordered rod-like structures termed amyloid fibrils. Though fibrils from different proteins differ in detail, they share general ultra-structural characteristics: they are approximately 100 angstrom wide, cross β-sheet structures composed of interwoven protofilaments, with individual β strands perpendicular to the long fibril axis and the component β sheets parallel to it (Lansbury et al., 1995; Sunde and Blake, 1997). Histological studies indicated that upon binding to the dye Congo red and Thioflavin T at regular intervals, these fibrils exhibit red-green birefringence under polarized light and increased fluorescence, respectively (Klunk et al., 1989; Naiki and Nakakuki, 1996; Puchtler et al., 1962). Both of these characteristics have been used commonly as distinguishing hallmarks of amyloid fibrils. However, despite these shared features, comparison of amino acid residues of these amyloidogenic proteins fails to reveal any obvious sequence similarities or motifs.

The ability of some amyloids to catalyze the conversion of soluble proteins to the insoluble aggregates is thought to be central to their ability to self-propagate. The hypothesis that amyloidogenic prion proteins can exist in two stable conformations (soluble monomers or small oligomers and large, insoluble amyloid fibrils) is proposed to explain the infectivity of mammalian PrP in TSE diseases (Griffith, 1967; Prusiner, 1982; 1998). Though controversial when first introduced, this prion hypothesis since has been supported by a wealth of data from much research efforts, and thus has gained broad albeit not universal scientific acceptance. Outlined below is a brief description of the discoveries and biological characterizations of both mammalian and yeast prions that are relevant to this thesis. As this introductory chapter is by no means exhaustive, readers interested in greater details are referred to more complete reviews (e.g. Horwich and Weissman, 1997; Prusiner, 1998 for mammalian prion reviews and Cox et al., 1988; Lindquist, 1997; Serio and Lindquist, 1999; Wickner, 1996 for reviews on yeast prions) and the references therein.

Prion Diseases are Caused by Pathogenic Conformation of PrP

Mammalian and human prion diseases comprise a set of invariably fatal neurodegenerative pathologies, in which mode of transmission and pathogenesis involve a novel protein-based mechanism. Different forms of the diseases involve the destruction of different brain tissues of the host and can be manifested in either familial, infectious or sporadic forms. It is thus remarkable that the causative agent is determined to be an

altered conformation of one protein, mammalian PrP. In a healthy or unaffected neuron, this protein exists in a normal α-helical conformation called PrP^{C}. In a diseased neuron, however, PrP is found to be in an abnormal, β-sheet rich prion conformation called PrP^{SC}. Mutations that predisposes PrP to adopt the prion conformation are thought to be the molecular basis of the familial form of these diseases, whereas spontaneous appearance of PrP^{SC} is thought to be responsible for the spontaneous form. More intriguing, however, is the ability of PrP^{SC} to convert an endogenous PrP^{C} to the prion form, which is thought to be the basis of prion infectivity (see below).

The proposal that a pathogenic protein conformation was the causative agent of prion diseases emerged from much research efforts that were initially focused on scrapie. A fatal neurodegenerative disease that affected sheep and goats, scrapie was the first prion disease identified. Sheep afflicted by scrapie showed sign of progressive ataxia (the term "scrapie" was derived from the effort of these animals to remain standing by leaning or scraping against a fence or a post) before they succumbed to the disease. Autopsies of the scrapie brain revealed spongiform neuronal tissue degeneration and depositions of protein plaques. As scrapie afflicted mostly sheep of the Suffolk breed, it was initially thought as a purely genetic disease resulting from improper breeding (Parry, 1962; Parry, 1983; however, see also Hunter et al., 1997). Transmissibility of a causative pathogen, however, was strikingly demonstrated when vaccination of a healthy population of sheep against a common virus, with an inoculum inadvertently derived from a scrapie-infected animal, caused scrapie infection and the subsequent decimation of the herd (Gordon, 1946).

The similarities between scrapie and kuru, a disease affecting the cannibalistic Fore tribe of New Guinea highlands, led Gajdusek and coworkers to propose a hypothesis that both diseases might be caused by the same or the same class of pathogens (Gajdusek and Zigas, 1959; Hadlow, 1959). Similar to scrapie-infected sheep, people afflicted by kuru showed symptoms of ataxia, dementia, spongiform encephalopathies, and extracellular protein depositions. Transmissibility of this human disease was experimentally demonstrated through intracerebral inoculation of homogenized kuru brain extracts to chimpanzees and subsequently to mice and hamsters (Gajdusek et al., 1966). These results suggested that cannibalistic practice of the tribe was responsible for the transmission of kuru in New Guinea. In this light, governmental actions directed the elimination of this practice which presumably led to the eventual eradication of this disease (Gajdusek, 1977).

Other human neurodegenerative diseases, including Creutzfeld-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker (GSS) syndrome and fatal familial insomnia (FFI), were subsequently shown to be prion diseases by their similarities to scrapie and kuru (Gerstmann et al., 1936; Goldfarb et al., 1992). Patients afflicted by these diseases showed symptoms of progressive ataxia or dementia. Additionally, post-mortem analyses of their brain tissues showed remarkable spongiform degenerations and plaque depositions. Furthermore, these diseases were shown to be transmissible to primates through intracerebral inoculation experiments (Gibbs et al., 1968), or even iatrogenically to patients through neurosurgeries, brain tissue or cornea graftings, and hormone treatments with materials derived from diseased cadavers. (Brown, 1993).

The prolonged incubation time between the initial infection and the appearance of the symptoms of the prion diseases, which could span a period of months to years, had initially focused the search for the causative pathogen on viral agents termed "slow viruses" (Gajdusek, 1977, 1985; Sigurdsson, 1954). The viral nature of the infectious agents was also suggested when it was discovered that, consistent with the behavior of viruses, disease infectivity could be filtered (Gordon, 1946). A surprising result from radiation studies, however, suggested that nucleic acids were not part of the infectious agent. Unlike viruses, scrapie and subsequently CJD infectivities were shown to be extremely resistant to inactivation by ultraviolet and ionizing radiation (Alper et al., 1966, 1967). Alternative explanations on the nature of the infectious agent were then proposed, and included toxic lipids, polysaccharides, and proteins (Gibbons and Hunter, 1967; Griffith, 1967; Hunter et al., 1968).

The discovery that serial transmissions of prion infectivities to mice and hamsters shorten the disease incubation time greatly aided the effort to purify the infectious fraction from scrapie infected brain tissues. Biochemical purification of prion infectivity, however, proved to be very difficult and thus the molecular nature of the infectious agent remained unanswered for more than a decade since the various unorthodox hypotheses were proposed. In 1982, Prusiner and coworkers were able to achieve partial purification of the scrapie infectivity from diseased brain homogenates (Prusiner et al., 1980; Prusiner, 1982). In agreement with previous radiation data, the infectious fraction was found to be resistant to treatments that would degrade nucleic acids. It was, however, sensitive to treatments that could modify or hydrolyze proteins such as prolonged

protease digestion or incubation with protein denaturants and detergents (Prusiner et al., 1980; Prusiner et al., 1982).

Although purification of the prion agent to homogeneity proved to be difficult due to its insolubility, partial proteolytic digestion was successfully used to isolate and identify a protein that co-purified with the infectious activity. Partial digestion of a subcellular fraction of hamster brain enriched for scrapie infectivity by proteinase K identified a protease-resistant protein of the size 27-30 kDa, which was absent in negative control of healthy brain extract (Bolton et al., 1982; Prusiner et al., 1982). Further purification of this fragment to homogeneity, followed by protein sequencing and cDNA cloning identified the protein to be the mammalian PrP (Basler et al., 1986; Chesebro et al., 1985; Oesch et al, 1985; Prusiner et al., 1984). This association between the protease resistant PrP fragment, termed PrP27-30, and scrapie infectivity was subsequently further strengthened when it was discovered that immunoaffinity purification of detergent-solubilized scrapie brain resulted in enrichment or co-purification of this fragment with the infectious activity (Gabizon et al., 1988).

The identification the mammalian PrP revealed that the sequence of the endogenous protein encoded by the chromosomal *PRNP* gene, as well as the expression levels in healthy tissues were found to be identical to that found in infected brain tissues (Basler et al., 1986; Chesebro et al., 1985; Oesch et al., 1985). Furthermore, PrP was found not only in the brain, but also in visceral tissues that remained normal during the course of the prion diseases. Despite extensive research efforts, there was no convincing

evidence that there were covalent differences between PrPs from healthy and diseased tissues (Stahl et al., 1993). There was, however, a striking difference in their protease sensitivities, protein solubilities and secondary structure conformations (see Meyer et al., 1986). Therefore, to distinguish the two forms the normal cellular PrP was termed PrP^{C}, whereas the protein associated with scrapie-infected tissues was termed PrP^{SC}. The differences in their protease sensitivities were demonstrated when limited proteinase K digestion was found to degrade PrP^{C} completely. Similar digestion of PrP^{SC}, however, yielded a core of protease resistant fragment of 27-30 kDa, which constituted the PrP27-30 described above. The basis of the differing protease resistance could perhaps be explained by the next difference between the two forms of PrP: it was observed that whereas PrP^{C} was soluble, the PrP^{SC} isoform was very insoluble (and thus not accessible to the protease). PrP^{SC} was often found localized to amorphous aggregates in fractions enriched for prion infectivity. Interestingly, the brain fractions enriched for PrP27-30 often also contained rod-like structures reminiscent of the amyloid fibrils, which were shown to be highly infectious (Diringer et al., 1983; Prusiner et al., 1983). Lastly, spectroscopic studies found that the conformation of PrP^{C} was predominantly α-helical, whereas PrP^{SC} was mainly composed of β-sheet (Caughey et al., 1991; Pan et al., 1993; Safar et al., 1993).

Mammalian Prion Species Barrier and Strains

The molecular basis for scrapie infectivity was thought to involve the conversion of the endogenous PrP^{C} of the host into the PrP^{SC} isoform, which was catalyzed by the

prion form. This conversion of endogenous protein was first illustrated by the ability to serially inoculate healthy mice or hamsters with fractions enriched for PrP^{SC} (Chandler, 1963). It was discovered that PrP^{SC} could be purified from the brain of the inoculated hamsters without loss of infectivity. The newly isolated PrP^{SC} could then be subsequently inoculated to fresh healthy animals, and the infection cycle could be repeated indefinitely (Prusiner et al., 1980, 1982). Without conversion of the normal PrP^{C}, scrapie infectivity should have been diluted out during serial passage in the animal hosts. Moreover, it was discovered that mice with disrupted *PRNP* gene ($Prnp^{0/0}$) were resistant to scrapie infections. These mice were also incapable of producing new infectivity, as the brain homogenates of these mice after inoculated with PrP^{SC} were found have decreasing infectious activities over time (Büeler et al., 1993; Prusiner et al., 1993 see however Lasmézas et al., 1997; Race and Chesebro, 1998).

This prion-mediated conversion had also been used to explain the spontaneous form of the prion diseases. This form of human prion diseases was predominantly comprised of CJD and some of GSS cases, and afflicted patients without mutations in their PrP genes (Masters et al., 1978). Although it was not precisely known how prions arise in these patients, a number of hypotheses had been put forward. These included undetected infection either from other human or animal sources (Gajdusek, 1977), spontaneous conversion of PrP^{C} to PrP^{SC}, and somatic mutation of the *PRNP* gene which resulted in mutant PrP that was more prone to PrP^{SC} formation (Hsiao et al., 1991; Prusiner, 1989). Of these, the first proved to be the least attractive, as extensive efforts to link sporadic CJD to chance prion infections had been unfruitful (Cousens et al., 1990;

Malmgren et al., 1979). The most attractive remained that sporadic CJD cases resulted from spontaneous conversion of PrP^C to the prion form, which initiated an autocatalytic process of prion conversion. This hypothesis was supported by a transgenic mouse experiment, in which mice that expressed various level of wildtype hamster, sheep or mouse *PRNP* transgenes were constructed. It was observed that these animals spontaneously developed diseases clinically similar to that of prion diseases (Westaway et al., 1994). The onset of the disease was directly related to the transgene dosage or the PrP expression level. For example, mice homozygous for the hamster PrP transgene regularly developed the disease earlier than the hemizygous mice. Interestingly, PrP expression level was also shown to control the length of incubation time in cases of scrapie inoculation, suggesting that the both spontaneous and seeded conversion of PrP^C to PrP^{SC} were dependent on PrP concentration (Carlson et al., 1986, 1994; Dickinson et al., 1968).

Analyses of the *PRNP* genes from patients diagnosed with the inherited form of CJD, GSS and FFI revealed more than twenty non-conservative mutations that segregated with these prion diseases. For example, affected members of some families with inherited CJD had point mutations of E200K in their *PRNP* genes, or the expansion of the amino-terminal octapeptide repeats of PrP, whereas the point mutation of P102L was linked to familial GSS (Goldfarb et al., 1990, 1991; Goldgaber et al., 1989; Hsiao et al., 1989, 1991; Owen et al., 1989). Interestingly, single amino acid polymorphisms could affect the manifestation of the prion disease : in the background of V129, a point mutation of D178 was linked to familial CJD, whereas in the background of M129, the

same point mutation resulted in familial FFI (Goldfarb et al., 1992). Furthermore, this polymorphism appeared to influence disease susceptibility and the manifestation not only of the inherited, but also of the spontaneous or iatrogenic forms. This was illustrated by the susceptibility or predisposition of people homozygous for either M or V at position 129 to spontaneous or iatrogenic CJD (Palmer et al., 1991).

The link between PrP mutations and prion formation or infectivity was further strengthened by two additional experiments. In the first experiment, it was observed that the inherited form of prion diseases could also be transmitted to primates through injection of brain extracts from familial GSS patients (Masters et al., 1981). The second experiment involved transgenic expression of the P102L mutation in the background of mouse PrP. It was observed that mice with high expression level of this transgene spontaneously developed neuronal spongiform degeneration and PrP amyloid plaques, similar to those seen in the brains of GSS patients (Hsiao, 1994; Telling et al., 1996a). Although they contained no detectable protease-resistant PrP, brain homogenates of these diseased mice were found to be infectious when injected into mice expressing low level of this mutant PrP, which by themselves would not have developed the disease spontaneously. This suggested that the spontaneous disease due to high expression of PrP with the GSS mutation also involved the creation an infectious activity that could be transmitted serially to healthy mice expressing lower amount of the same mutant. Together, these experiments showed that the familial prion diseases involved the creation of infectious prion particles, thus explaining the perplexing etiological observation that the same prion diseases could be either spontaneous, inherited or infectious.

To date, PrP^{SC} mediated conversion of PrP^{C} to the prion form has only been inferred by data from human pathologies and transgenic mice experiments. Despite extensive effort, the in vitro creation of scrapie infectivity has yet to be demonstrated. Therefore, it was encouraging when Caughey, Lansbury, Chesebro and coworkers demonstrated the ability of PrP^{SC} to convert PrP^{C} to a protease-resistant form reminiscent to that of PrP^{SC} (Kocisko et al., 1994). Here, radiolabelled PrP^{C} immunoprecipitated from tissue culture cells was treated by the protein denaturant guanidine hydrochloride mixed with fifty-fold excess unlabelled PrP^{SC}, and was subjected to limited proteolysis by proteinase K. The use of radioactive metabolic labeling allowed the detection of a protease resistant protein that resembled PrP27-30. However, as the converted material comprised only a small fraction of the conversion reaction which occurred in the presence of excess PrP^{SC}, it was not possible to determine whether it was infectious. Interestingly, pre-treatment of the unlabelled PrP^{SC} with guanidine increased the efficiency of the conversion, which suggested that during the course of the conversion of PrP^{C} to the prion form, PrP^{SC} itself needed to undergo conformational changes as well.

The recent description of a new variant of CJD (vCJD) cases in Britain, with pathologies similar to that of bovine spongiform encephalopathy (BSE) or "mad cow" disease, underscored a growing concern that this new pathology resulted from the transmission of a cattle disease to human (Will et al., 1996). Autopsies of vCJD patients revealed the formation of a unique pattern of plaques that had become a central characteristic of this disease. Further characterizations of PrP^{SC} derived from vCJD-inoculated mice showed distinct properties compared to other forms of CJD, but similar

to that of BSE derived prion particles (Collinge et al., 1996). Moreover, intracerebral inoculation of BSE brain homogenates from cows resulted in a disease clinically similar to vCJD in primates (Lasmézas et al., 1996). These results suggested that infectious BSE prion derived from cows was able to overcome a "species barrier" to infect humans.

The existence of the prion species barrier, or resistance of one species to infection by prion particles generated in another species, was revealed by a series of transgenic mice experiments. It was first observed that although mice were generally resistant to infection by hamster-derived prions, transgenic mice expressing hamster PrP were susceptible (Scott et al., 1989). There seemed to be a requirement for species-specific interactions between PrP^{SC} and the endogenous PrP^{C} in the prion conversion process. As would be expected from a species-specific prion conversion, inoculation of these transgenic mice with hamster prions resulted in brain homogenates that could infect only hamsters. Conversely, inoculation with mouse prion particles resulted in homogenates that could infect only mice (Prusiner et al., 1990). These results suggested that in mice expressing both hamster and mouse PrP^{C}, inoculation with hamster PrP^{SC} resulted in the conversion of only the hamster PrP^{C} to the prion form, whereas inoculation with mouse PrP^{SC} resulted in the formation of only the mouse PrP^{SC}. Furthermore, if species specific interaction was necessary for prion conversion, absence of these interactions could be expected to confer immunity from prion infections. Indeed, if these transgenic mice also harbored disruption in the endogenous mouse *PRNP* gene in addition to expressing the hamster PrP gene, they were resistant to infection by mouse-derived, but not by hamster-derived prions (Büeler et al., 1993; Prusiner et al., 1993).

The recapitulation of the species barrier in vitro further strengthened the hypothesis that species-specific interactions between the prion particles during the conversion process were responsible for the species barrier (Kocisko et al., 1995; see however Telling et al., 1995 and below). In this experiment, it was observed that the addition of unlabelled hamster PrP^{SC} to metabolically labeled purified hamster PrP^{C} resulted in its conversion to a protease-resistant form. The addition of hamster PrP^{SC} to mouse PrP^{C}, however, did not result in conversion of the mouse PrP. Furthermore, construction of a chimeric protein composed of hamster and mouse PrP sequences, allowed the identification of amino acid residues that could mediate this species specificity.

In contrast to the findings above, however, transgenic studies of mice expressing transgenic human PrP suggested the involvement of a host factor in prion conversion. Unlike the susceptibility of mice expressing both endogenous mouse and transgenic hamster PrPs to infection by hamster PrP^{SC}, it was observed that mice expressing both mouse PrP and high level of transgenic human PrP were resistant to human PrP^{SC} infection (Telling et al., 1994, 1995). Ablation of the endogenous mouse *PRNP* gene or expression of a chimeric mouse/human PrP in with wildtype *PRNP* gene, however, rendered these mice susceptible to human PrP^{SC} infection. These results argued for the existence of a cellular host factor necessary for prion conversion, postulated to be a protein termed protein X. This protein was hypothesized to bind preferentially to PrP^{C} native to the host. Elimination of the endogenous chromosomal gene removed the competition for this factor, thus allowing the transgenic human PrP^{C} to be converted by

inoculation of human PrP^{SC}. Introduction of mouse sequence to the human PrP resulted in a chimeric PrP that could bind protein X sufficiently well as compared to the endogenous mouse PrP. Thus this rendered mice with even low expression of the chimeric mouse/human PrP to be susceptible to human PrP^{SC} infection.

First used as evidence for viral basis of scrapie infectivity, the existence of "prion strains" remains one of the most perplexing phenomena in prion biology. Strains refer to the distinct neuropathological symptoms, patterns of neurodegeneration, length of incubation times, and even patterns of PrP^{SC} proteolytic digestion produced by infectious materials from different sources (Fraser and Dickinson, 1973; Carp and Callahan, 1991; DeArmond, 1993). These properties are stably retained even after serial passages in isogenic mice, thus eliminating the possibility that these differences are due to differences in genetic background of the host. Two particularly telling examples of prion strain diversities are as follows.

The first was the observation that transmissible mink encephalopathy had two distinct symptomatic manifestation when transmitted to hamsters (Bessen and Marsh, 1992a). One strain, termed hyper (HY), caused hyperactivity in affected animals, whereas another termed drowsy (DY) caused scrapie-like drowsiness. PrP^{SC} particles derived from DY and HY hamsters showed different proteolytic sensitivities, as limited proteinase K digestion resulted in protease-resistant protein with different amino termini (Bessen and Marsh, 1992b, 1994). Furthermore, cell-free conversion reaction of PrP^{C} by

prion derived from either HY or DY strains produced two distinct protease-resistant PrP particles consistent with the PrP^{SC} strain used (Bessen et al., 1995).

The second example stemmed from the observation that PrP^{SC} derived from the brains of patients with familial CJD and FFI showed different protease resistance (Monari et al., 1994; Parchi et al., 1996). Given the differences in PrP sequence due to the disease-causing mutations, this was not surprising. The ability of this strain property to be transmitted to isogenic mice expressing chimeric mouse/human PrP transgene, however, was unexpected and was particularly revealing (Telling et al., 1996b). In this experiment, brain extract from deceased patients with either inherited CJD or FFI was injected intracerebrally to $Prnp^{0/0}$ mice expressing chimeric mouse/human PrP, which resulted the development of disease. When analyzed, it was discovered that brain extract of these mice contained chimeric PrP^{SC} with digestion pattern similar to that of the injected strain of PrP^{SC}. This strain was shown to be stably transmitted in an isogenic genetic background, as the mouse brain extracts were subsequently used to inoculate healthy transgenic mice, which then yielded PrP^{SC} particles with protease resistance patterns consistent with the original strains used.

The stable transmission of prion strains, through passages in hamsters or transgenic mice, was thought to involve different conformations of the PrP^{SC} particles. Such conformational differences could be represented in either different tertiary structures or quaternary association of PrP^{SC} molecules, and thus resulted in varying protease accessibility or sensitivities. The two experimental findings outlined above

argued that PrP^{SC} acted not only as catalyst, but also as a template for the conversion of PrP^{C} to the prion form.

Since its discovery, mammalian prion had been defined in context of an infectious pathogen. However, the concept of a prion protein that could exist in two stably inherited conformations, as described below, could be extended to factors not associated with transmissible infectious agents. Particularly revealing were the recent advances in the field of yeast prions, which led to the appreciation that the prion phenomena were more universal than originally thought and could also account for a novel mechanism of inheritance of genetic information.

The Discovery of Prion Phenomena in the Yeast *Saccharomyces cerevisiae*

In 1994, Reed Wickner put forth a hypothesis to explain a series of puzzling data concerning two non-Mendelian genetic elements found in *Saccharomyces cerevisiae* (Wickner, 1994). He proposed that the baffling and sometimes paradoxical genetic data that had been collected for more than thirty years on the inheritances of $[PSI^{+}]$ and [URE3] determinants (Cox, 1965; Lacroute, 1971) could be explained by one simple molecular mechanism: that these two elements were yeast prions. By this proposal, Wickner had expanded the original concept of the prion phenomena to include a novel mechanism of genetic inheritance, in addition to the accepted role of prions as novel infectious pathogens. The existence of prions in yeast provided researchers with the

ability to do classical yeast genetics, such as screens and selections for extragenic factors required for, or intragenic sequence requirements of, prion formation and propagation (e.g., Chernoff et al., 1995; DePace et al., 1998; Kockneva-Pervakhova, 1998; Patino et al., 1996). In addition, yeast prions also provided an additional reference points to determine general properties of prions, as opposed to that specific to the mammalian or yeast system (e.g., Kushnirov et al., 2000; Li and Lindquist, 2000; Santoso et al., 2000; Serio and Linquist, 1999; see also Horwich and Weissman, 1997).

These two elements, [PSI^+] and [URE3] fulfilled certain properties expected of a prion protein, described as follows (Wickner, 1994). First, similar to the infectious nature of the mammalian prion, a yeast prion was expected to be non-Mendelian, and could be transferred laterally to another yeast by mating or cytoduction. Second, the chromosomal gene of the yeast prion protein was essential for the production of new prion proteins. Analogous to the transgenic experiment that showed disruption of the endogenous *PRNP* gene resulted in mice that were not capable of supporting prion replication (Büeler et al., 1993; Prusiner et al., 1993), the absence of newly synthesized protein due to deletion of the chromosomal prion gene should eliminate the ability of the yeast to maintain prion propagation. Third, it could be reasonably expected that since it constituted a non-native conformation of the normal protein, the prion form was phenotypically similar to a recessive loss of function mutation in the chromosomal gene encoding for the prion protein. Fourth, overexpression of the normal protein increased the spontaneous appearance of the prion form. Similar to the increased frequency of the appearance of scrapie in transgenic mice expressing elevated level of PrP (Westaway et

al., 1994), overexpression of the yeast prion protein increased the spontaneous appearance of the prion form by increasing the number of protein molecules that could stochastically convert. Finally, unlike nucleic-acid-based inheritance elements, prions could arise again at some low but finite probability in a yeast cell cured of such elements, without its re-introduction from outside of the cell. This reversible curability was a direct result from the encoding of genetic information in the conformations of the prion proteins without any change in their primary sequences, and thus distinguished the prion determinants from nucleic acid based vectors such as viruses or non-Mendelian mitochondrial DNA elements (Wickner, 1996).

Although both [PSI$^+$] and [URE3] offer exciting and viable avenues for studying the prion phenomena in yeast, further discussion on the basic biology of yeast prions in this thesis is focused on studies done on [PSI$^+$]. There are two main reasons to do so: first, this thesis describes research performed on this element. As result of this work, mutational analyses have identified key residues involved in [PSI$^+$] formation and propagation (DePace et al., 1998). The analogy between yeast and mammalian prions also has been extended to include the effect of species-differences in protein sequences in prion specificity (Santoso et al., 2000). Second, experiments that characterized the properties of both [PSI$^+$] and [URE3] are very analogous. Thus, illustration of the basic biological properties of yeast prions can be achieved without incurring redundancies by using only one of the two genetic elements as an example. Readers interested in the biology of [URE3] are referred to able reviews of this prion (Tuite and Lindquist, 1996; Wickner et al., 1999; Wickner, 1996).

[PSI$^+$] is a non-Mendelian element that increased the efficiency of translational suppression of stop codons. In a cell harboring loss of function mutants due to premature termination of protein translation by nonsense mutations, the presence of this epigenetic element restores protein functions by "suppressing", or promoting readthrough of, the nonsense stop codons. First identified as an enhancer of a weak ochre tRNA suppressor SUQ5, [PSI$^+$] was initially characterized as an allosuppressor (Cox, 1965; for complete review on [PSI$^+$]-mediated translational suppression see Serio and Lindquist, 1999). Later, it was found to increase suppression efficiencies of other nonsense suppressor tRNAs or frameshift mutants (Cummins et al., 1980; Firoozan et al., 1991; Liebman et al., 1975; Ono et al., 1979a, 1979b). Eventually, [PSI$^+$] was shown to be an omnipotent suppressor as it was capable of acting alone as a translational suppressor of the UAA, UAG and UGA stop codons in absence of other suppressor mutants or suppression-enhancing drugs (Chernoff et al., 1995; Firoozan et al., 1991; Ishiguro et al., 1981; Liebman et al., 1975). Indeed, certain combinations of this element with strong translational suppressor mutants or drugs were synthetically lethal, as would be expected of the formation of too many abnormally read-through proteins (Cox, 1971, 1977; Liebman and All-Robyn, 1984).

In contrast to suppressor tRNA mutants, which suppressed only a specific stop codon (Sherman et al., 1979), the ability of the [PSI$^+$] element to suppress all three nonsense stop codons was similar to the phenotypes of mutations in the *SUP35* and *SUP45* genes. These genes encoded translational termination factors (Hawthorne and Leupold, 1974; see also Hinnebusch and Liebman, 1991). The *sup35* and *sup45* mutant

alleles, however, were recessive and were meiotically inherited in a 2 : 2 fashion as would be expected for Mendelian genes. Conversely, [PSI$^+$] was dominant and was inherited in a non-Mendelian fashion. Mating between [PSI$^+$] and [psi$^-$] haploid cells yielded a [PSI$^+$] diploid, sporulation of which resulted in meiotic progeniy that are all [PSI$^+$]. Furthermore, it was possible to transmit this epigenetic element by cytoduction by use of a *kar1* mutant (Conde and Fink, 1976; Cox, 1965). Here, the cytoplasmic contents of a [PSI$^+$] cell was mixed with that of a [psi$^-$] cell in absence of nuclear fusion, thus without any transfer of chromosomal genetic material between the two. Upon the ensuing cellular division, it was discovered that the "recipient" cell was converted to [PSI$^+$].

The experiments above outlined [PSI$^+$] characteristics that were consistent with known cytoplasmic elements. Thus it was not surprising that the initial effort to map this epigenetic factor focused on the conventional non-Mendelian genetic elements. Efforts to link [PSI$^+$] to plasmids, mitochondrial genomes or the known yeast viruses, however, proved to be unfruitful (Leibowitz and Wickner, 1978; Tuite et al., 1982; Young and Cox, 1972). To add further complexity, both [PSI$^+$] and [psi$^-$] states appeared to be metastable. Although they were inherited well by their mitotic progenies, interconversions between the two states occurred at rates higher than expected for chromosomal mutations.

In addition to the rare spontaneous reversion to the [psi$^-$] state, a [PSI$^+$] cell could be effectively "cured" of the prion element when subjected to growth in presence of

certain chemicals. First reported by Sherman and co-workers (Singh et al., 1979), this was re-discovered serendipitously by Tuite and co-workers when an inadvertent drop of methanol used to sterilize yeast spreading apparatus fell onto a media subsequently plated with [PSI$^+$] cells. After incubation, [psi$^-$] cells were found in the area containing methanol (Tuite et al., 1981; also M.F. Tuite, reported in Serio and Lindquist, 1999). Systematic screen of curing agents identified guanidine hydrochloride, ethylene glycol, dimethylsulfoxide and ethanol among others (Tuite et al., 1981). As one of the most effective curing agents, the protein denaturant guanidine hydrochloride was the most commonly used and studied. When supplemented to the growing media at 5mM, a concentration too low to cause direct protein unfolding, almost 100% of [PSI$^+$] cells were cured of the prion element (Tuite et al., 1988).

The effects of nucleic acid mutagens on the translational suppression of [PSI$^+$], as well as on [PSI$^+$] maintenance and propagation suggested that chromosomal mutations could eliminate this epigenetic factor, or suppress its phenotypes (Cox et al., 1980; Tuite and Cox, 1980). Most nuclear mutations, caused by mutagenic chemicals such as ethylmethane sulfonate or ultraviolet irradiation, resulted in the elimination of translational suppression, without elimination of the [PSI$^+$] factor. The UV irradiation induced mutations that did cause elimination of the [PSI$^+$] element followed linear kinetics, suggesting that mutation of a single genetic locus was sufficient. Furthermore, these mutations were suppressed by DNA proofreading mechanisms, which were located in the nucleus. This contrasted with the previous localization of the epigenetic element to the cytoplasm of the cell (Conde and Fink, 1976; Cox; 1965). Thus it was not surprising

that these result led to much confusion and the incorrect conclusion that the [PSI$^+$] factor was conferred by a nucleic acid determinant.

The link between the epigenetic factor [PSI$^+$] and its determinant *SUP35* provided much to the framework to explain molecular basis of the curious element, shown previously to be located in the cytoplasm, yet somehow was eliminated by a nuclear mutation. This link was established genetically in two separate experiments. In the first, it was observed that plasmid overexpression of Sup35p in [psi$^-$] cells caused suppression of nonsense codons, which continued even after the plasmid was lost (Chernoff et al., 1993). Indeed, these yeast cells were subsequently determined to be [PSI$^+$] (Derkatch et al., 1996; Ter-Avanesyan et al., 1994). Induction of this factor was dependent on the overproduction of Sup35p, as overexpression of a plasmid-borne *SUP35* gene containing a nonsense codon in [psi$^-$] failed to induce the conversion of the yeast to the [PSI$^+$] state.

In the second experiment, one of the nuclear mutants termed *PNM* mutants (*Psi No More*) that caused curing of the [PSI$^+$] element was cloned (Doel et al., 1994; Young and Cox, 1971). This dominant mutant, called *PNM2*$^-$, was determined to be a mutant allele of the *SUP35* gene. These two experiments outlined above linked the [PSI$^+$] cytoplasmic element to the *SUP35* nuclear gene by showing that transient overexpression of Sup35p could induce the appearance of [PSI$^+$] and that the propagation of this state, in turn, depended on the production of Sup35p.

Sup35p, along with its co-factor Sup45p, is responsible for polypeptide translational termination. Elimination of this gene is lethal to the cell, whereas partial loss of function mutations lead to decreased translational termination fidelity, as reflected in increased suppression of nonsense stop codons of marker genes (reviewed in Hinnebusch and Liebman, 1991). Similar to the phenotypes of [PSI^+], these mutants are able to suppress all three nonsense stop codons, they are classified as omnipotent suppressors. Sup35p is composed of 685 residues and is divided into three regions with distinct functions, amino acid compositions or homologies to other proteins (Kikuchi et al., 1988; Kushnirov et al., 1987, 1988; Wilson and Culbertson, 1988). The amino terminal domain of residues 1 to 123, termed the N domain or the PrD (Prion determining Domain, see below) is rich in glutamines and asparagines, and is relatively devoid of charged residues. The middle or M domain, composed of residues 124 to 253, is composed of many charged amino acids. These two domains have no significant homologies to proteins of known functions and are not essential for cell viability, yet they are evolutionarily conserved in Sup35 homologues from other species of budding yeast (Kushnirov et al., 1990b, 1990c; Resende et al., 1997; see also Chapter 3 or Santoso et al., 2000).

The carboxyl-terminal domain of residues 254 to 685, termed the C or EF domain, is homologous to the translational elongation factor EF-1α and contains four consensus GTP binding sites. In contrast to the PrD and M domains, this domain is essential for cell viability (Kushnirov et al., 1990a; Ter-Avanesyan et al., 1993b) and is highly conserved in eukaryotes (Hoshino et al., 1989; Ito et al., 1998; Kushnirov et al.,

1990b; Samsonova et al., 1991). Along with Sup45, the EF domain of Sup35 is responsible for the translational termination activity of the full length protein, as mutations that conferred the recessive suppression of nonsense stop codons localize to this domain (Cox, 1993; Doel et al., 1994; see also Hinnebusch and Liebman, 1991). Plasmid expression of this domain in [PSI^+] cells inhibits the nonsense codon suppression, but does not cure the [PSI^+] element, as suppression reappears after the plasmid is lost (Derkatch et al., 1996; Kushnirov et al., 1990a; Ter-Avanesyan et al., 1993b, 1994)

The localization of the region responsible for [PSI^+] formation and propagation to the PrD domain was demonstrated by overexpression studies using fragments of Sup35p and deletion analyses of this protein. Soon after it was discovered that overexpression of the full length Sup35p could induce de novo appearance of [PSI^+]-mediated nonsense suppression (Chernoff et al., 1993), overexpression studies of various fragments of this protein in [psi^-] identified the PrD as the domain responsible for [PSI^+] induction (Ter-Avanesyan et al., 1993). In a parallel experiment, the *SUP35* gene of haploid [PSI^+] yeast was replaced with a series of amino-terminal deletion mutants (Ter-Avanesyan, 1994). It was observed that Sup35p deleted for the PrD was incapable of maintaining the propagation of the [PSI^+] state, as yeast cells expressing only this construct were found to revert to [psi^-]. Subsequent research supported this findings, as natural and engineered mutations or deletions of residues in the PrD (thus classified as *PNMs*) were found to interfere with [PSI^+] propagation (DePace et al., 1998; Doel et al., 1994; Li and Lindquist, 1999). The experiments outlined above suggested that the Sup35 PrD was

required for not only [PSI$^+$] induction or formation, but also for its maintenance or propagation.

[PSI$^+$] Prion is An Alternate Conformation of Sup35p

Wickner's yeast prion hypothesis provides the conceptual framework to explain all the discrete, complex and sometimes confusing genetic data of the [PSI$^+$] inheritance. This hypothesis argues that the [PSI$^+$] and [psi$^-$] states of yeast can be explained by two conformations of Sup35p (Wickner, 1994). In a [psi$^-$] yeast, Sup35p exists in an active state that is capable of faithfully terminating polypeptide translations at stop codons. In a [psi$^-$] cell carrying an *ade1-14(UGA)* marker, faithful translational termination results in truncated and inactive Ade1p, which in turn results in red colony color on media containing low amount of adenine and the inability to grow in media lacking adenine. Conversely, in [PSI$^+$] cells, Sup35p exists in an inactive state, which results in a reduced availability of the cellular pool of translational termination activity. This is reflected in a low but significant rate of translational readthrough of stop codons in [PSI$^+$]. In a [PSI$^+$] yeast carrying the *ade1* marker, nonsense stop codon suppression causes a low but significant rate of readthrough of the stop codon, thus producing full length and active Ade1p. This results in white colony color on low adenine media and the ability to grow on media devoid of adenine. The [PSI$^+$] state of the Sup35p can either be induced by transient overexpression of the full length Sup35p or the PrD fragment, or arise de novo spontaneously, albeit rarely, by sporadic conversion of Sup35p to the prion form. Once

achieved, the [PSI$^+$] state predominates by catalyzing the conformational changes of the active Sup35p into the inactive prion form and the process becomes self-sustaining.

In this prion hypothesis framework, the role of the PrD was clear : it was required for or mediates the conformational changes of Sup35p to the prion form, but nature of the physical differences was unknown. Studies on the nature of the conformational changes between the normal and the prion forms of Sup35p did not detect covalent differences or protein expression levels between proteins from isogenic [psi$^-$] and [PSI$^+$] cells (Patino et al., 1996; Paushkin et al., 1996). Rather, when assayed by ultracentrifugation or size exclusion chromatography, Sup35p from [PSI$^+$] cells appeared to form large, insoluble aggregates whereas Sup35p from [psi$^-$] cells remained soluble. Expression of a PrD fused to green fluorescent protein (GFP) confirmed the existence of this prion aggregate in living cells: fluorescent foci were observed in [PSI$^+$] cells, whereas diffuse fluorescence was observed in [psi$^-$] cells. Furthermore, reminiscent of the differences in protease sensitivities between PrP^{SC} and PrP^{C} of the mammalian prion (Prusiner, 1998), it was observed that Sup35p isolated from [PSI$^+$] extract was more resistant to protease digestion than that isolated from [psi$^-$].

The findings of the insolubility or aggregation of the prion form allowed us to create a model of the propagation of the [PSI$^+$] prion (Figure 1). To take an example of the [PSI$^+$] induction experiment, episomal overexpression of the PrD first induced de novo formation of PrD aggregates, which then recruited the full length endogenous Sup35p. Once established, these PrD-full length protein aggregates could be supported

solely by the endogenous production of wildtype Sup35p, as the PrD expression plasmid could be subsequently lost (Chernoff et al., 1993; Derkatch et al., 1996; Ter-Avanesyan et al., 1994). Experiments using the fusion PrD-GFP protein lent further support, as it was observed that overexpression of the fusion protein in [psi$^-$] resulted in an initial diffuse fluorescence, with formation of fluorescent foci in these cells over time (Patino et al., 1996). The appearance of the aggregated fluorescent foci correlated well with the formation of the Sup35 prion, as assayed by plating onto selective media. In event of a cellular division or mitosis, this aggregated form of Sup35p would be passed to the daughter cell, which in turn would support the prion state by its endogenous production of Sup35p. Similarly, in the event of mating and meiosis, when a haploid [PSI$^+$] cell was mated to a [psi$^-$] cells, the aggregated Sup35p were able to recruit the soluble translational release factor into the growing prion aggregate, which resulted in translational suppression in the diploid cell. Sporulation of this diploid, in turn yielded four segregants, each of which contained the Sup35p aggregates.

Expression of Sup35p lacking the PrD in [PSI$^+$] cells resulted in a pool of soluble translational release factor in addition to the full length Sup35p aggregates. The soluble translational termination activity resulted in an antisuppression phenotype or the inhibition of nonsense stop codon suppression as proper translational termination could then occur. The propagation of the prion aggregate was not inhibited, although the [PSI$^+$]-mediated suppression phenotype was masked, as demonstrated by the following experiment. Here, an antisuppressed [PSI$^+$] diploid carrying one copy of wildtype *SUP35* and one copy lacking the PrD was constructed. Upon sporulation, half of the segregants

which contained the full length *SUP35* gene were [PSI$^+$] and had reverted back to the suppressed state, whereas the segregants that carried the copy of *SUP35* lacking the PrD were [psi$^-$] (Ter-Avanesyan et al., 1994).

A biochemical mechanism of [PSI$^+$] prion propagation was suggested by a series of in vitro experiments using purified recombinant Sup35 protein fragment (Glover et al., 1997; King et al., 1997; see also DePace et al., 1998 or Chapter 2). Purified PrD fragment was found to be very insoluble in aqueous buffer, but formed β-sheet rich fibrils in organic and even denaturing buffers, as observed by electron microscopy. A longer fragment of PrD-M domains was found to be more amenable to kinetic studies. It was observed that this fragment, purified under denaturing conditions, also formed β-sheet fibrils when diluted from denaturant. Importantly, fibril formation was preceded by a lag phase, which could be eliminated by addition of pre-formed fibril "seed". This seeding ability was not limited to purified recombinant protein, as it was observed that cell lysates from [PSI$^+$], but not from [psi$^-$] yeast, could accelerate fibril formation of purified PrD-M. Furthermore, it was observed that addition of small amount of [PSI$^+$] lysates caused the conversion reaction of Sup35p from [psi$^-$] lysates to the prion form, as assayed by ultracentrifugation and protease resistance (Paushkin et al., 1997). True to the prion-like inheritance of the [PSI$^+$] state, this seeding reaction could be repeated in several consecutive cycles. Taken together these experiments clearly suggested a mechanism of the [PSI$^+$] prion-catalyzed conversion of endogenous cellular Sup35p into the prion form through PrD-mediated protein-protein interactions.

Further evidence of the involvement of PrD-mediated aggregation or fibril formation in the prion inheritance was obtained when it was observed that the in vitro fibril formation kinetics of various PrD mutants mirrored their in vivo behaviors in formation and propagation of the [PSI^+] state. For example, in vivo inhibition of [PSI^+] propagation or reduced rate of [PSI^+] induction by PrD-M fragments containing a deletion or N-terminal repeat truncation was reflected in the extended lag phase of in vitro fibril formation assay using these fragments, even after addition of pre-formed fibril seed (Glover et al., 1997; Liu and Lindquist, 1999). Furthermore, PrD-M fragments with single point mutations that inhibited prion formation or propagation in vivo was also found to have decreased fibril formation propensity in vitro (DePace et al., 1998 or see Chapter 2). Conversely, PrD-M fragments containing repeat duplication was observed to have increased [PSI^+] induction in vivo and increased propensity to form fibrils in vitro (Liu and Lindquist, 1999).

These β-sheet rich Sup35p fibrils were found to bind the amyloid specific dye Congo red, and showed red-green birefringence under polarized light (Glover et al., 1997; King et al., 1997). These characteristics were reminiscent of amyloid fibrils and depositions associated with a number of human amyloid diseases, including prion and neurodegenerative diseases, as well as systemic amyloidoses (for review see Bellottti et al., 1999; Koo et al., 1999; Horwich and Weissman, 1997). The link between yeast prions and human polyQ-expansion mediated neurodegenerative diseases was further strengthened by the observation that replacement of an amino-terminal region of the Sup35 PrD with a polyQ tract of pathogenic length retained the ability of the PrD to form

amyloids (DePace et al., 1998). These findings suggested that the prion propagation of [PSI$^+$] element and polyQ-mediated pathogeneses might share similar underlying molecular mechanisms.

Further support for alternate phenotypes encoded in different conformational states of a protein came from a genetic screen for factors that could inhibit the translational suppression phenotype of [PSI$^+$], which identified a molecular chaperone Hsp104p (for Heat Shock Protein) as a cellular factor that played a large role in yeast prion maintenance or propagation. A member of the large ClpB/HSP100 chaperone family, Hsp104p functioned by promoting disaggregation of proteins aggregations (Glover and Lindquist, 1998; Parsell et al., 1991, 1994). This protein was required for cellular survival after a heat shock, which caused a high rate of protein unfolding and aggregation (Sanchez and Lindquist, 1990; Sanchez et al., 1992).

It was discovered that Hsp104p overexpression at modest level inhibited [PSI$^+$]-mediated nonsense codon suppression, but did not cause elimination of the prion factor. However, expression of Hsp104p to higher levels, even transiently, was effective in curing of the [PSI$^+$] determinant (Chernoff et al., 1995; Newnam et al., 1999; reviewed in Lindquist et al., 1995). Furthermore, active Hsp104 was necessary, as overexpression of mutant alleles of *HSP104* which contained defective ATP binding sites did not cause elimination of the prion factor. Since the only known function of Hsp104p was to cause conformational changes of proteins, including disaggregating proteins that had already aggregated, it was suggestive that the same mechanism was responsible for [PSI$^+$] prion

curing. Interestingly, deletion of *HSP104* also caused curing of the [PSI$^+$] element (Chernoff et al., 1995). Sporulation of a [PSI$^+$] diploid carrying only one chromosomal copy of *HSP104* resulted in 2 : 2 segregation of the [PSI$^+$]. The segregants that contained the wildtype *HSP104* were [PSI$^+$], whereas those that carried the *hsp104* deletion were [psi$^-$].

The findings that both overexpression and deletion of Hsp104p resulted in curing of the [PSI$^+$] factor led to two hypotheses on the role of this molecular chaperone in prion formation and propagation. The first, proposed by Chernoff, Lindquist and co-workers, suggested that an intermediate activity of Hsp104p was required for the conformational changes associated with the prion state (Chernoff et al., 1995). The second, proposed by Kushnirov and Ter-Avanesyan, suggested that Hsp104p was required in prion propagation through cell division by breaking up or severing the prion aggregates which could then segregate to the daughter cells (Kushnirov and Ter-Avanesyan, 1998). Deletion of the *HSP104* gene interfered with propagation of the prion aggregates through mitosis, whereas increased level of Hsp104p led to prion curing by severing the aggregates beyond their capability to recruit soluble monomeric Sup35p. However, experiment has yet to determine the correct mechanism of Hsp104p's role in prion formation and propagation.

Yeast Prion Strains and Species Barrier

Two additional aspects of [PSI$^+$] prion biology were analogous to that of the mammalian prions. The first was the existence of prion strains. As described above, in mammalian prion strains referred to the different disease symptoms, location of central nervous system neurodegeneration, and pattern of PrP^{SC} proteolysis (see Prusiner, 1998 for review). In the [PSI$^+$] system, different prion strains referred to the various strengths of nonsense codon suppression and mitotic or meiotic stabilities of prion propagation. In [psi$^-$] yeast carrying the *ade1-14* marker, induction of [PSI$^+$] prion by Sup35p overexpression resulted in colonies with pink, light pink or white colors, corresponding to weak, intermediate or strong strains as scored by nonsense suppression (Cox, 1965; Derkatch et al., 1996). Similar result was obtained in yeast marked with *lys2-87*, thus showing that the variable suppression strengths were not due to a peculiarity with the *ade1* mutant allele. Propagated colonies retained their respective levels of suppression, demonstrating that these strain properties were stably inherited. It should be noted that these differences were not due to polymorphisms in the genetic background of the host. Rather, they were caused by small variations of the [PSI$^+$] states, presumably encoded in different conformations of the Sup35p prion. This was remarkably demonstrated as curing of these various [PSI$^+$] strains yield [psi$^-$] derivatives, each of which could be re-induced to give rise to all these different prion strains.

Another strain of [PSI$^+$], called [ETA$^+$] before its connection to the Sup35 prion was appreciated, demonstrated another heritable variability of prion strains. Similar to [PSI$^+$], [ETA$^+$] was discovered as a non-Mendelian factor that caused weak suppression of nonsense stop codons, and was synthetically lethal with certain *sup35* and *sup45*

mutant alleles (All-Robyn et al., 1990; All-Robyn and Liebman, 1983; Liebman and All-Robyn, 1984). Unlike [PSI^+], however, [ETA^+] was meiotically less stable as it was passed to only approximately 70% of the haploid segregants. [ETA^+] was eventually identified as a strain of [PSI^+] when it was observed that Sup35 PrD was required in the formation and maintenance of this state (Zhou et al., 1999). Indeed, the aggregation state of Sup35p was found to be linked to this factor. Sup35p of [ETA^+] strains was found to be more soluble than that of [PSI^+] cells, which could account for its weak nonsense suppression and perhaps for its reduced meiotic stability as well.

The second additional analogy between the mammalian and yeast prions was the existence of a species barrier to prion infection or induction, respectively. In case of mammalian prion, this barrier referred to the resistance to infection of a host animal by inoculation of PrP^{SC} derived from a foreign animal species (Scott et al., 1989; Prusiner 1998). In case of yeast prion, species barrier referred to the lack of prion induction in *S. cerevisiae* by overexpression of Sup35 PrD homologues from foreign species of budding yeast (Chernoff et al., 2000; Kushnirov et al., 2000; Santoso et al., 2000). In brief, it was observed that Sup35 homologues from a large spectrum of budding yeast species contained amino-terminal domains with amino acid compositions reminiscent to the Sup35 PrD of *S. cerevisiae*. Furthermore, in addition to domain conservation, the ability of these domains to aggregate specifically with PrDs of their own species in a prion-like manner was evolutionarily conserved as well. This suggested an evolutionarily beneficial role for prions, and provided a possible mechanism of the maintenance of multiple prion-

based states in a single cell (see Chapter 3 or Santoso et al., 2000 for further details; see also Li and Lindquist, 2000).

Are There Biological Roles for Yeast Prions?

The significant similarities between the mammalian prion and the known yeast prions created a new avenue of viewing the prion mechanism not solely as a biological aberration that led to pathologies. Lindquist, Chernoff, Tuite and co-workers proposed that in case of [PSI^+], prion-based inheritance could be an epigenetic regulation of translational termination (Chernoff et al., 1998; Eaglestone et al, 1999; Lindquist, 1997). In this hypothesis, suppression of stop codons on certain genetic loci could result in carboxyl-terminal extension of proteins, thus creating novel domains with novel functions or modifying protein levels by altering the polypeptide turnover rates. In support of a biological function of yeast prions, it was discovered that certain physiological conditions favored the survival of yeast in either [PSI^+] or [psi^-] states. Although there was no difference in growth rates of isogenic [PSI^+] and [psi^-] cells grown in rich conditions, Tuite and co-workers observed that certain physiological stress conditions favored one state over the other (Eaglestone et al., 1999). It was discovered that [PSI^+] cells exhibited enhanced tolerance to heat and ethanol, whereas [psi^-] cells exhibited enhanced cold tolerance (Eaglestone et al., 1999; Y.O. Chernoff, personal communication; Santoso and Weissman, unpublished observation). Interestingly, a condition that favored one state also promoted the conversion to the other state. For example, growth in presence of ethanol, which favored the survival of [PSI^+] cells, was

also found to promote conversion of these cells to the [psi$^-$] state. Conversely, cold temperature, which favored survival of the [psi-] cells also, promoted their conversion to [PSI$^+$]. Presumably this could be a mechanism to keep both states of [PSI$^+$] and [psi$^-$] populated, to ensure survival of the yeast colonies in event of a drastic change in growing conditions. Although synthesis of novel protein domains seems an attractive and likely mechanism, the exact molecular basis of the modulation of cellular survival by Sup35 prion as well as the mechanism of the conversion between the two states, however, remain to be determined.

So far, the exact function of the Sup35 PrD has yet to be revealed, although it has been suggested that this domain mediates the interaction of the translational release factor with cytoskeletal components (Bailleul et al., 1999). However, if yeast prions represent a novel mechanism of regulating protein activities and of genetically inheriting this information, as hypothesized above, two additional general properties should emerge.

First, prions can control activities of other proteins fused to it through prion-dependent aggregation. This assumes that PrD evolved as a modular regulatory domain and its control of protein activity through prion aggregation is not limited to the translational termination functions. Indeed, deletion of the Sup35 PrD domain is not lethal to yeast and its effect is limited to the elimination of the prion factor or suppression of its phenotypes (Ter-Avanesyan, 1994). Moreover, as fusion of the PrD-M to proteins such as GFP or catalytic domain of Ure2p created [PSI$^+$]-dependent protein aggregation (Patino et al., 1996; Y.O. Chernoff, personal communication).

Recently, Lindquist and co-workers described an additional example of the modularity of the PrD by fusing this domain to glucocorticoid receptor (GR), a steroid-hormone regulated transcriptional factor (Li and Lindquist, 2000). The effect of the [PSI$^+$] state of the yeast was then assayed by a β-galactosidase reporter construct under control of GR. In [psi$^-$] cells, upon addition of steroids, β-galactosidase activity was observed, suggesting that the PrD-GR fusion proteins were soluble and active. Conversely in [PSI$^+$] cells, no β-galactosidase activity was observed, suggesting that the fusion protein was in an inactive state. This effect, however, was observed only at extremely high levels of fusion protein expression and had much higher rate of reversion than that of the original [PSI$^+$] prion (S.L. Lindquist, personal communication). Independently, we were able to achieve stable [PSI$^+$] state-dependent regulation of orthogonal protein activities through low level expression of the fusion of Sup35 PrD-M to a cytoplasmic enzyme HIS3 and a transcriptional activator fusion LexA-B42 (Santoso and Weissman, unpublished data). These results suggested that the Sup35 PrD-mediated prion aggregation evolved as a modular regulatory domain (see also Lindquist, 1997).

The second property is that protein domains that are able to regulate protein activities through prion-mediated aggregation are not limited to the two known yeast prions Sup35p and Ure2p. Indeed, various genetic results in other fungi might readily be explained by the prion hypothesis. For example, the unusual propagation of vegetative incompatibility between [Het-S] and [Het-s] strains of *Podospora anserina* was recently attributed to a prion mechanism (Coustou et al., 1997; see also Silar and Daboussi, 1999). Furthermore, inspection of yeast proteins that share homologies to Sup35 PrD identified novel putative prions and prion-forming domains (M. Michelitch and J.S. Weissman,

manuscript in preparation; Santoso et al., 2000; Sondheimer and Lindquist, 2000). Such sequence comparisons identified two proteins (New1p and Rnq1) with domains that conferred prion-like translational suppression phenotypes, when fused to the Sup35 EF domain, with inheritance similar to that of [PSI^+]. These two additional properties, supported by our findings that the Sup35 PrD sequence and ability to aggregate in a prion-like manner were strongly conserved in the evolution of budding yeast species, suggest that yeast prions confer novel regulation of protein activity and mediate a novel mechanism of genetic inheritance.

The Yeast Prion [PSI^+] Provides a Unique System to Study Properties General to Prions and other Amyloidogenic Proteins

Decades of research on the prion as well as other amyloid diseases had given us an immense wealth of valuable data and their corresponding interpretations. The once controversial hypothesis that prion proteins could exist in two stable conformations, one of which was associated with neuropathologies, and that conversion to the prion state could be catalyzed by the prion form had now gained scientific acceptance (Prusiner, 1998). Indeed the transformation of this prion hypothesis from being controversial to enjoying wide-support culminated when the 1998 Nobel Prize for Physiology or Medicine was awarded to Stanley Prusiner his discovery of prions as novel infectious agents.

Despite wealth of data from remarkable experiments to determine the mechanism of prion conversions, many questions remained unanswered. For example, although it was known that certain amino acids in mammalian PrP when mutated as in case of the inherited prion diseases, increased the propensity of prion formation, the sequence requirement for PrP^{SC} formation or the sensitivity of PrP^{SC} to changes in protein sequence was unknown. This was not surprising since mutations of the amino acid residues that are essential for PrP^{SC} would presumably resulted in healthy individuals or resistance to prion infection, a phenotype that was difficult to study clinically. Additionally, the mechanism by which specificities in PrP interactions, either with itself or with other factors, was manifested in the mammalian prion species barrier was unknown. Experiments using transgenic mice expressing mouse/human chimeric PrP suggested that differential binding to a host factor termed protein X was responsible for the species barrier (Telling et al., 1995). In contrast, in vitro reconstitution of the prion conversion reaction using purified mouse and hamster PrP proteins suggested species-specific interactions of PrP molecules were sufficient to create this barrier (Kocisko et al., 1995). The discovery of the prion phenomena in yeast provided us with a unique avenue and tools to examine these questions.

After Wickner's yeast prion hypothesis, researchers initially focused on the confirmation that these phenotypes are indeed the result of the ability of the yeast proteins to exist in the normal and prion isoforms. In a relatively short time this was accomplished: a large amount of genetic, cell biological and biochemical data had provided strong support for the prion model of [PSI^+] (and [URE3]) inheritance (for

review see Cox, 1988; Serio and Lindquist, 1999; Wickner, 1996). Undoubtedly the research on yeast prions benefited greatly from the published work on mammalian prion, which served as guideposts on studying the general behaviors and characteristics of prion proteins. Even so, there are two main advantages that the yeast [PSI^+] system offered over the mammalian system, as listed below.

The first advantage is the ability to exploit yeast as a genetically tractable organism. To this end, the ability to conduct genetic screens or selections for factors that modified the Sup35 prion inheritance was greatly facilitated both by the robust and stable phenotypes of [PSI^+] and [psi^-] cells, and the fast generational time of yeast. Results of such screens were the identification of *HSP104* and *SSA1* gene products and of *SUP35* mutants as extragenic factors and intragenic variants respectively, that were able to affect the formation and propagation of the [PSI^+] prion (Chernoff et al., 1995; Newnam et al., 1999). Furthermore, researchers were able to use additional cell and molecular biological tools such as fusion to GFP to visualize aggregation in vivo and ultracentrifugation analyses to test protein solubilities (Patino et al., 1996; Paushkin et al., 1996).

The second advantage is the ability to perform biochemical and kinetic studies using purified recombinant Sup35 proteins or fragments. The PrD-M fragment, purified under denaturing conditions, readily formed amyloids upon dilution to physiological buffer and was kinetically tractable by use of Congo red or Thioflavin T binding assay (DePace et al., 1998; Glover et al., 1997; King et al., 1997; Klunk et al., 1989; Paushkin et al., 1997). This de novo formation of amyloids occurs only after a lag phase, which

could be eliminated by the addition of small amount of pre-formed fibrils. A similar seeding effect was also seen by the addition of a small amount of [PSI$^+$], but not of [psi$^-$] lysates.

In the work detailed in the following chapters, we took advantage of the [PSI$^+$] system to investigate properties that seemed to be general to prions as well as those that seemed to be specific to the Sup35 prion. Chapter Two, titled "A Critical Role for Amino-Terminal Glutamine/Asparagine Repeats in the Formation and Propagation of a Yeast Prion", was the result of a collaboration with Angela DePace and Paul Hillner, and was published in the journal Cell in 1998. For this work we exploited facile genetic tools of *S. cerevisiae* to define the sequence requirement for the formation and propagation of the [PSI$^+$] prion. We identified Sup35p mutants that either were poorly recruited into, or cause curing of, the growing wildtype prion aggregates in vivo. Correspondingly, these mutants showed decreased rates of de novo amyloid formation in vitro. These mutations mapped exclusively to a short region highly enriched for glutamines and asparagines, in the amino-terminus of the PrD. These mutations were predominantly single amino acid substitutions of polar to charged residues. Kinetic analyses of the concentration-dependence of amyloid formation and the similarities between the prion-like [PSI$^+$] inheritance and polyglutamine-mediated aggregation involved in neurodegenerative diseases were also discussed.

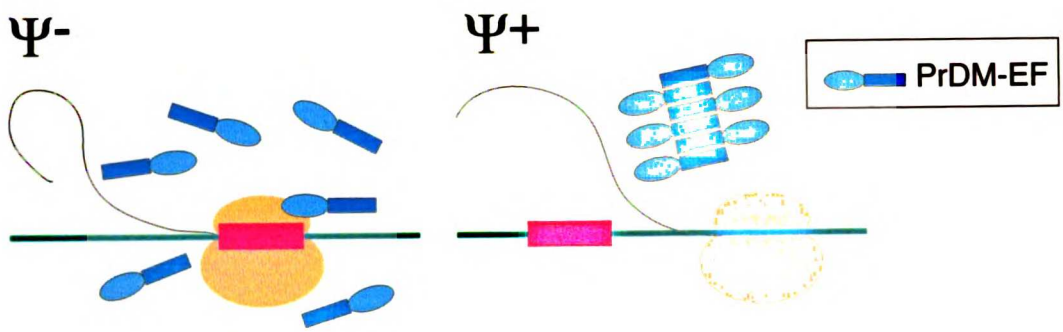
Chapter Three, titled "Molecular Basis of a Yeast Prion Species Barrier", was the result of a collaboration with Peter Chien and Lev Osherovich. In this work we examined

the conservation of the Sup35 PrD across a wide spectrum of budding yeast. Expression of the homologous PrD from foreign yeast species in *S. cerevisiae*, in context of a novel genetic system designed to monitor protein aggregation, revealed the existence of species specificity in the formation of Sup35p prion aggregates in vivo. Reminiscent of that discovered in the mammalian prion system, this specificity was manifested in a species barrier that inhibited prion induction between Sup35p from different yeast species. By construction of chimeric proteins containing sequences from *S. cerevisiae* and *Candida albicans* PrDs, we succeeded in isolating the region responsible for this species specificity to a short domain identified previously in the works described in Chapter 2. The evolutionary role of prion as a novel mechanism for inheritance of genetic information, as suggested by the conservation of homologous Sup35p PrD sequences and their ability to aggregate, as well as the identification of a novel protein domain that showed prion inheritance, were also discussed.

In Chapter Four, titled "Prion-Based Switch of Biological Functions", we briefly described the utilization of the Sup35 PrD-M fusion proteins to create a stable biological switch to control protein activity through a prion-based mechanism. We found that fusion of this fragment to a cytoplasmic enzyme HIS3 in a *his3*$^-$ genetic background, or to a nuclear transcription activator fusion of LexA-B42, imparted regulation of biological activities by the [PSI] state of the yeast. This result not only extended the available tools for yeast genetics, but also provided further support for the concept of prions as protein-based mechanism of inheritance of genetic information.

Figure 1. Schematic Model of Sup35p in [psi$^-$] and [PSI$^+$] Cells.

In [psi$^-$] cell, soluble Sup35p mediates translational termination at the nonsense stop codon (red bar) of the *ade1-14* gene. In contrast, in the suppressed [PSI$^+$] cell, the absence of soluble Sup35p results in translational readthrough of the nonsense mutation. Below, effects of the [PSI] state of the yeast on PrDM aggregation, translational termination, and Ade1p synthesis, as well as color and growth phenotypes of [psi$^-$] and [PSI$^+$] yeast.



	Ψ-	Ψ+
PrDM Aggregated?	No	Yes
Non-sense Stop Suppression	Unsuppressed	Suppressed
Adenine Synthesis	Truncated Ade1p	Full Length Ade1p
Color on Low Adenine Media	Red	White
Growth on Media Lacking Adenine	No Growth	Growth

Chapter 2

A Critical Role for Amino-terminal Glutamine/Asparagine Repeats in the Formation and Propagation of a Yeast Prion

A Critical Role for Amino-terminal Glutamine/Asparagine Repeats in the Formation and Propagation of a Yeast Prion

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Running title: Role of Gln/Asn repeats in a yeast prion

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Summary

The yeast [*PSI* $^{+}$] factor propagates by a prion-like mechanism involving self-replicating Sup35p amyloids. We identified multiple Sup35p mutants that either are poorly recruited into, or cause curing of, wild-type amyloids in vivo. In vitro, these mutants showed markedly decreased rates of amyloid formation, strongly supporting the protein-only prion hypothesis. Kinetic analysis suggests that the prion state replicates by accelerating slow conformational changes rather than by providing stable nuclei. Strikingly, our mutations map exclusively within a short glutamine/asparagine rich region of Sup35p, and all but one occur at polar residues. Even after replacement of this region with polyglutamine, Sup35p retains its ability to form amyloids. These and other considerations suggest similarities between the prion-like propagation of [*PSI* $^{+}$] and polyglutamine-mediated pathogenesis of several neurodegenerative diseases.

Introduction

Virtually all denatured proteins have a strong propensity to form amorphous aggregates. This aggregation is largely driven by the association of hydrophobic regions that are normally buried in the native structure (for review, see Jaenicke and Seckler, 1997). In contrast to these more frequent disordered aggregates, some proteins form ordered aggregates called amyloid fibrils. Amyloidogenic proteins show no obvious sequence similarity, nor do their native folds resemble one another. Yet despite this diversity amyloid fibrils appear to share a similar architecture.

Amyloid fibrils are cross ß-sheet structures in which the individual ß-strands run perpendicular to the long axis of the fibril, whereas the faces of the ß-sheets extend parallel to it (Lansbury et al., 1995; Sunde and Blake 1997). These fibrils are roughly 100Å in diameter and are typically composed of 4-6 interwoven protofilaments. Recent X-ray diffraction studies suggest that the ß-sheets composing the individual protofilaments are twisted, resulting in a helical conformation propagating along the amyloid axis (Sunde et al., 1997; see however Lazo and Downing, 1998). The ordered structure of amyloids allows them to bind the dye Congo red at regular intervals, leading to a characteristic red-green birefringence under polarized light. Despite these shared structural features, electron microscopy (EM) and atomic force microscopy (AFM) (Harper et al., 1997b) analyses indicate that there are ultrastructural variations (e.g., differences in the number and packing of the protofilaments) between the amyloids. It is also possible that individual protein monomers retain partial native structure within the amyloid (Liu et al., 1998).

To date, ~20 proteins have been found to form amyloids associated with human disease. These include the mammalian prion protein, PrP, the infectious protein implicated as the causative agent of transmissible spongiform encephalopathies (see Caughey and Chesebro, 1997; Prusiner et al, 1998). PrP forms amyloid fibrils which, at least under some conditions, are associated with the infectious agent. Similarly, some non-prion neurodegenerative diseases, such as Alzheimer's and Huntington's disease, also involve amyloid formation. In Huntington's, as well as several other neurodegenerative diseases, amyloid formation appears to be caused by expansion of a polyglutamine (polyGln) tract (e.g. Paulson et al., 1997; Scherzinger et al., 1997). Finally, systemic amyloidoses result from the aggregation of a number of proteins such as lysozyme and transthyretin (TTR) (Wetzel, 1997).

Despite active research, many basic questions about the conversion from native state to amyloid fibril remain unanswered. For example, little is known about what stabilizes amyloid structures. In particular, how sensitive is amyloid formation to changes in primary sequence? Are amyloids, like amorphous aggregates, principally stabilized by hydrophobic interactions? The mechanism of propagation of infectious prion diseases and its relationship to that of non-infectious amyloidoses is also poorly understood (Prusiner et al., 1998; Harper and Lansbury, 1997). For example, what is the nature of the rate-limiting step in de novo formation of amyloids and how do preformed fibrils accelerate this process? Lastly, our understanding of the role of cellular factors, such as molecular chaperones, in promoting formation of amyloids is incomplete (Chernoff et al., 1995; Kaneko et al., 1997). In particular, do in vitro conversion

reactions using purified proteins accurately reproduce the propagation of disease or infection in vivo?

The prion-like phenomenon [*PSI* $^{+}$] of the yeast *S. cerevisiae* provides a powerful model system to address these questions. [*PSI* $^{+}$] is a non-Mendelian trait that causes suppression of nonsense mutations (Cox et al., 1988). This nonsense suppression is thought to result from the conversion of the translation termination factor Sup35p from a soluble and functional state into insoluble and inactive amyloid fibrils (see Lindquist, 1997; Wickner et al., 1995). [*PSI* $^{+}$] appears to propagate by a prion-like mechanism in which the amyloid form of Sup35p promotes the conversion of newly made soluble Sup35p to insoluble fibrils. The increased nonsense suppression caused by the [*PSI* $^{+}$] factor can be readily monitored by using a strain carrying a marker gene with a nonsense mutation. Thus, the [*PSI* $^{+}$] phenomenon allows one to assess the microscopic aggregation state of the Sup35p protein in vivo by examining the macroscopic properties of a yeast colony.

Here we use the [*PSI* $^{+}$] system to define the sequence requirements for efficient formation and prion-like propagation of Sup35p amyloids in vivo. In addition, using an in vitro conversion reaction (Glover et al., 1997; King et al., 1997; Paushkin et al., 1997), we have exploited mutant Sup35p proteins that are defective in amyloid formation to help elucidate the mechanism of prion propagation.

Results

Mutagenesis of the Sup35p Reveals a Requirement for Glutamine/Asparagine Rich Sequences

Sup35p is composed of three domains (Ter-Avanesyan et al., 1993) (Figure 1A). The N-terminal prion determining domain (PrD) is necessary and sufficient for propagation of aggregates. Deletion of this domain allows Sup35p to remain soluble even in [*PSI* $^{+}$] cells (Paushkin et al., 1996). Conversely, fusion of the PrD to GFP confers aggregation of the fusion protein in a [*PSI* $^{+}$] dependent manner (Patino et al., 1996). The middle domain (M) of Sup35p is a highly charged region of unknown function. The C-terminal domain, referred to as EF because of its homology to elongation factors, encodes a translation termination activity (Stansfield et al., 1995). Expression of this domain without a PrD provides a pool of soluble, active EF domain, resulting in a loss of nonsense suppression even in cells containing wild type (WT) Sup35p aggregates (Ter-Avanesyan et al., 1993). Hereafter, [*PSI* $^{+}$] refers to the presence of heritable aggregates; [*PSI* $^{+}$] yeast that also contain soluble EF, and therefore no longer exhibit nonsense suppression, will be referred to as antisuppressed.

We have developed a genetic screen to identify PrD mutants that are defective in amyloid formation and propagation. This screen is based on the fact that such mutants will remain soluble even in a [*PSI* $^{+}$] yeast cell that contains WT Sup35p aggregates, thereby conferring an antisuppressor phenotype (Figure 1B). In order to track the suppression phenotype, a [*PSI* $^{+}$] strain harboring a nonsense mutation in the *ADE1* gene was used (Chernoff et al., 1995). In yeast retaining their nonsense suppression

phenotype, functional Ade1p is produced resulting in white colonies on complete medium (YPD) and an ability to grow on medium lacking adenine (SD-ADE). However, in either [*psi*$^-$] or antisuppressed yeast, Ade1p is truncated and nonfunctional, resulting in red colonies on YPD and failure to grow on SD-ADE. The PrD was uniformly mutagenized using PCR under conditions that favored single nucleotide changes. A library of sup35 genes with mutagenized PrDs was episomally expressed on a URA3 marked plasmid in a strain that contains chromosomally encoded WT Sup35p.

Yeast expressing compromised PrDs were readily identified because they formed red/white sectored colonies on YPD (Figure 1C). White sectors result from yeast that have lost the plasmid expressing mutant Sup35p but have retained WT aggregates. Conversely, red sectors result from either an antisuppressor phenotype caused by the presence of the mutant Sup35p or conversion to [*psi*$^-$] (i.e. permanent loss of all Sup35p aggregates). The antisuppressor (ASU) phenotype was confirmed by the lack of functional Ade1p under growth conditions that require retention of the mutant Sup35p plasmid (i.e., inability to grow on SD-ADE, -URA, Figure 1D). In contrast, the nonsense suppression phenotype returned following plasmid loss (i.e., growth on SD-ADE medium). The ASU mutants were then examined for their ability to cause permanent conversion to a [*psi*$^-$] state. Such "curing" alleles are referred to as Psi No More (PNM) mutants (Doel et al., 1994). Cured yeast were identified by the presence of red colonies on 5-FOA medium, which selects for yeast that have lost the plasmid expressing the mutant Sup35p. The fraction of cured cells caused by transient expression of PNM mutants ranged from ~1-50% and generally increased with expression time. We screened ~15,000 colonies for ASU phenotypes and ~30,000 colonies for PNM phenotypes. In

this pool, we estimate that every point mutant is represented 5-10 fold. In total, 28 ASU and 13 PNM mutants were sequenced. The observed redundancy in recovered alleles is in good agreement with this estimated overrepresentation (Figure 2A). The individual mutations are denoted by the first letter of the phenotype (A or P) followed by the amino acid changes.

Unexpectedly, all ASU and PNM mutants contained changes in a short region at the extreme N-terminus of the PrD between residues 8 and 24 (Figure 2A). The failure to identify mutations outside of this region was not due to lack of diversity in the original mutant pool, as a number of mutations that did not result in an ASU phenotype were found throughout the PrD. Nor was it because the residues outside this region were unnecessary for PrD function: deletion of residues 53-124 of the PrD resulted in an ASU phenotype (data not shown), and a previously described chromosomal PNM mutant maps outside this region (Doel et al., 1994). Rather, it appears that point mutations outside this region did not result in a sufficiently robust ASU phenotype to pass our screens. Although the entire PrD has a high Gln/Asn content, the subdomain identified by our mutagenesis shows a particularly strong enrichment for these amino acids (Figure 2B). Moreover, all of the mutations occurred in Gln or Asn residues with the exception of a single Gly and a single Ser mutant. Virtually all mutations resulted in a change to charged amino acids. Particularly striking is the predominance of Gln or Ser to Arg mutations. This is physically reasonable because the bulky and charged Arg sidechain is likely to be particularily disruptive to protein-protein interactions. Gly or Asn to Arg mutations are not observed, however, because they would require two nucleutide changes.

ASU and PNM Mutants Show Diminished Ability to be Recruited into WT Sup35p Aggregates In Vivo

The screen for ASU and PNM mutants was based on the hypothesis that the antisuppression phenotypes resulted from a decreased ability of mutant PrDs to be recruited into WT Sup35p aggregates. We tested this using a GFP based assay described by Lindquist and coworkers (Patino et al., 1996). WT PrD-GFP converts from a diffuse fluorescence pattern in [*psi* $^-$] cells (Figure 3A) to a punctate pattern in [*PSI* $^+$] (Figure 3B), making it possible to monitor the aggregation state of PrD fusion protein in vivo. Each of the ASU and PNM mutants was fused to GFP and put under control of the inducible CUP1 promoter. Use of an inducible promoter minimizes secondary effects that prolonged expression of mutants might have on the solubility of the WT Sup35p. In contrast to WT PrD-GFP, which had uniformly punctate patterns in [*PSI* $^+$] cells, each mutant led to a mixed population of fluorescent patterns: in individual cells, the fusion proteins either appeared entirely soluble (Figure 3C), in discrete foci (Figure 3D), or more commonly in both (Figure 3E).

We next examined the aggregation state of the mutant PrDs using a centrifugation assay that made it possible to determine the solubility of full length Sup35p protein expressed from its natural promoter (Patino et al., 1996; Paushkin et al., 1996). In [*PSI* $^+$] cells, Sup35p is in aggregates that are efficiently separated from soluble protein fractions by centrifugation. In contrast, Sup35p in [*psi* $^-$] cells remains predominately soluble following centrifugation (Figure 4A lanes 1-4). To distinguish mutant Sup35p from chromosomally encoded WT protein, the mutant proteins were tagged with the HA antigen. Following centrifugation, levels of soluble and aggregated

proteins were examined by western-blot analysis. We found that 9 of the 11 ASU and PNM mutants showed significant increases in soluble protein in [*PSI* $^{+}$] cells (Figure 4A, B). As might be expected from its comparatively weak ASU phenotype, the tagged version of AN8D showed little, if any, increase in soluble protein (Figure 4A lanes 5, 6). AQ14R was atypical, having a strong phenotype, yet little soluble protein and decreased protein levels (Figure 4A, lanes 7, 8). We also examined if expression of the ASU and PNM mutants led to solubilization of WT Sup35p in [*PSI* $^{+}$] yeast by co-expressing HA-tagged WT Sup35p and untagged mutant proteins. Though most ASU mutants did not result in an increase in soluble WT Sup35p, expression of AS17R led to significant solubilization of WT protein (Figure 4C lanes 3,4). The PNM mutants generally caused a modest increase in soluble WT protein with the strongest effect resulting from expression of PQ24R (Figure 4C lanes 5, 6).

Wildtype Sup35 PrD Rapidly Forms Amyloids In Vitro in a Reaction That Has Both Concentration Dependent and Independent Phases

Recently, three groups demonstrated that the PrD domain from Sup35p can form self-propagating aggregates in vitro (Glover et al., 1997; King et al., 1997; Paushkin et al., 1997) facilitating greatly detailed studies of the mechanism of fibril formation. Although in all of these studies amyloid formation was preceded by a lag phase that was eliminated by the addition of preformed fibrils, there were dramatic differences in the time scale of conversion. In particular, Ter-Avanesyan and coworkers (Paushkin et al., 1997) observed that in cell-free extracts undergoing a constant slow rotation, a protein containing the PrD and M domains (PrD-M) was recruited into preformed aggregates

within 2 hours. In contrast, Lindquist and coworkers found that pure PrD-M formed amyloids slowly: spontaneous conversion took days and seeded conversion took ~20 hours (Glover et al., 1997). Initially we observed that in unrotated reactions pure PrD-M converted slowly even in the presence of preformed fibrils although the conversion reaction was complicated by amorphous aggregation and settling of fibrils during long incubation times.

These difficulties could be minimized by slowly rotating the samples during the incubation. Rotation also caused a dramatic increase in the conversion kinetics, as detected by Congo red binding, with seeded reactions going to completion in as little as 45 minutes and unseeded reactions within 180 minutes (Figure 5A). As judged by gel filtration, all of the monomeric starting material appears to have converted to higher order oligomeric forms. We also observed structural changes on this time scale, such as increased ß-sheet content by circular dichroism (data not shown). Finally, EM and AFM analyses directly confirmed that the conversion results in fibril formation (Figure 5B and data not shown). Although amyloids observed by AFM were similar in dimensions and general features to those seen by EM (Glover et al., 1997; King et al., 1997), this analysis revealed several novel features. For example, we saw fibrils containing a prominent ~10 nm ridge along the filament axis as well as branching fibrils and fibrils emanating from globular structures (Figure 5B). Branching fibrils were also recently observed in AFM analysis of ß-amyloid protein filaments (Harper et al., 1997a) and could be important for creating new fibrils during amyloid propagation.

The rapid and reproducible kinetics of conversion allowed us to perform quantitative analyses of fibril formation. The conversion kinetics can be characterized by

two terms: a lag time (T_0), the time prior to the increase in Congo red binding, and a conversion time (T_c), the time between initiation of conversion and its completion. At 2.5μM, PrD-M has a lag time of 114 +/- 4 minutes and a conversion time of 53+/- 12 minutes (Figure 5A). To eliminate contaminating preformed fibrils, urea denatured PrD-M (30kDa) was passed through a 100kDa filter prior to initiation of conversion. This filtration step appeared to be sufficient as passage through a 50kDa filter immediately after dilution from denaturant did not effect the conversion kinetics (data not shown). Addition of increasing amounts of preformed fibrils accelerated conversion by decreasing T_0 without significantly changing T_c. For example, 0.7% (wt/wt) fibrils shortened the T_0 to 24 +/-1 minutes, and 3% (wt/wt) fibrils eliminated the lag time completely (Figure 5A).

Using the in vitro assay, we probed the nature of the rate-limiting step responsible for the lag phase. In particular, is this lag due to the cooperative formation of an ordered oligomeric nucleus as suggested by the nucleation-polymerization model (Jarrett and Lansbury, 1993)? Because the seed is oligomeric, the nucleation model predicts that the lag time will show a strong concentration dependence that approaches zero at high concentrations. We found that within a range of 1.25 to 6μM, increasing PrD-M concentration did lead to a modest (~2-fold) decrease in lag time (Figure 5c). Further increases in PrD-M concentration, however, did not lead to a further decrease of T_0 to below 65 minutes. Thus at concentrations above 6μM, the rate-limiting step in conversion is unaffected by protein concentration. Importantly, the molar fraction of Congo red bound also did not change significantly over the observed concentration range (1.25-12μM).

Purified Mutant ASU Proteins Show Decreased Rates of Amyloid Formation In Vitro

We next examined the kinetics of de novo amyloid formation for the ASU mutants. A representative conversion curve of an ASU mutant (AQ15R) reveals a marked decrease in conversion kinetics compared to WT PrD-M (Figure 6A). As with the other mutants, the slow rate of fibril formation of AQ15R was predominantly attributable to an increase in lag time (T_0 = 260 +/- 20 min vs. 114+/-4 min for WT) rather than a change in T_c. A summary of T_0 of all the mutants is shown in Figure 6B. Significantly, the mutants which showed the lowest solubility in vivo (AN8D and AQ14R), had T_0 values comparable to that of WT protein, whereas the stronger mutants (AS17R, AQ15R, and AG20D) showed significantly larger T_0 values.

To simulate amyloid propagation in vivo, we examined the ability of preformed WT PrD-M fibrils to promote the conversion of purified ASU mutant proteins. For 4 of the 6 ASU mutants, addition of WT fibrils did accelerate the conversion kinetics. However, the rate of seeded conversion was significantly slower for all of the ASU mutants than for WT (Figure 6B). For example, in the presence of 0.7% (wt/wt) WT fibrils, the T_0 of AQ15R was 83+/-5 minutes as compared to 24+/-1 minutes for WT. Interestingly, for two of the mutants with the strongest in vivo phenotype, AS17R and AQ22R, addition of WT fibrils did not increase the rate of amyloid formation even though AQ22R showed only a modest defect in de novo fibril formation. Intringuingly, we also found that AS17R mutant fibrils fail to seed the conversion of WT Sup35p (Figure 6C).

The N-terminal Region of the PrD Identified by Mutagenesis Can be Functionally Replaced with PolyGln.

Gln and Asn residues are chemically similar, differing solely by a single carbon atom separating the carboxyamide group from the backbone. Although the Ser sidechain does not have a hydrogen bond acceptor like a carboxyamide sidechain, it is a polar group with a hydrogen bond donor. To test the functional equivalence of these sidechains, we made a series of glutamine replacement variants (GRV) of the Sup35p PrD (Figure 7). In the first set of variants, termed GRV(+Gly) and GRV(-Gly), all of the residues in the N-terminal region of the PrD that were identified by mutagenesis, either including or not including the single Gly, respectively, were replaced with Gln. A second set of variants, termed GRV(polyQ+Gly) and GRV(polyQ-Gly) were made in which all of the residues (amino acids 8-24) in the N-terminal region, either including or not including the single Gly, were replaced with Gln. These domains were fused to GFP and put under the control of the CUP1 promoter.

We examined the aggregation state of the GRV-GFP fusion proteins in [*PSI*$^+$] or [*psi*$^-$] yeast as a function of expression time. The behavior of the GRV(-Gly) variant was indistinguishable from wild-type PrD. In [*PSI*$^+$] cells, GRV(-Gly)-GFP showed punctate fluorescence even at early points in the induction (3 hour) whereas only diffuse staining was observed in [*psi*$^-$] cells (Figure 7). For GRV(+Gly), at early times the fluorescent pattern in [*PSI*$^+$] cells appeared diffuse in a significant fraction of the cells. At later times, however, a uniformly punctate pattern was observed. This indicates that GRV(+Gly)-GFP was also capable of being recruited in WT aggregates although with

decreased efficiency compared to GRV(-Gly)-GFP. The behavior of the GRV[polyQ-Gly] and GRV[polyQ+Gly] was comparable to GRV[-Gly] and GRV[+Gly], respectively, indicating that replacement of the N-terminal residues not identified by mutagenesis had little effect on function of the PrD (Figure 7 and data not shown). Intriguingly, GRV(+Gly) retained its ability to form de novo aggregates, as even at early expression times a small fraction (~1%) of the [*psi* $^-$] cells showed a punctate fluorescence pattern. At longer incubation times, this fraction increased to ~25%. These aggregates are likely to represent, at least in part, genuine conversion events, as over-expression of either GRV-GFP fusion in [*psi* $^-$] cells resulted in a enhanced rate of conversion to [*PSI* $^+$] state. Taken together, these observations indicate that even after replacement of the N-terminal Gln/Asn region with a polyGln stretch, the PrD retained its ability both to form new aggregates and to be recruited into existing aggregates.

Discussion

Even though the association of amyloid fibrils with diseases has been appreciated for several decades, little is known about what makes a protein amyloidogenic. The cytoplasmically inherited [*PSI* $^{+}$] factor of yeast provides a powerful system for studying amyloid formation in vivo and in vitro. Here, we have exploited the PSI phenomenon to identify point mutations in the prion determining domain (PrD) of Sup35p that are defective in amyloid formation and propagation. This work differs fundamentally from previous genetic studies of mammalian amyloid disorders that have relied on identification of naturally occurring mutations which accelerate disease. Such mutations typically increase the propensity to form amyloids by destabilizing the native state and thus do not report directly on the requirements for amyloid formation (Kelly et al., 1997; Wetzel, 1997).

The mutants that we have identified allowed us both to define the sequence requirements for Sup35p amyloid formation in vivo and to test the relevance of in vitro conversion reactions. Two classes of mutants that lead to increased levels of soluble protein were identified: the first (ASU) inhibited incorporation into aggregates without irreversibly preventing propagation of WT amyloids, the second (PNM) resulted in curing of the [*PSI* $^{+}$] state in addition to its ASU phenotype. In vitro conversion reactions using pure ASU-PrD proteins indicate that the increased solubility of these mutants is caused directly by a defect in recruitment of soluble protein by WT prion-like amyloids. We are currently investigating the molecular basis of the curing by the PNM mutants.

Our studies reveal a critical role for Gln and Asn residues in stabilization of the amyloid state. First, all of the mutations cluster to the most Gln/Asn rich region of the Sup35p PrD. Second, all of the mutations were found in Gln or Asn residues, with the exception of a single Ser and a single Gly. Finally, when present in the context of full length Sup35p, this region can be replaced by a polyGln tract while retaining its ability both to form de novo amyloids and to be incorporated into pre-existing WT amyloids. These observations argue for the functional equivalence of the various polar residues (Gln, Asn, and Ser) in this critical region of the Sup35p PrD. A possible explanation for the stabilization of the amyloid state by Gln/Asn residues was suggested by Perutz and coworkers who pointed out that in ß-sheets carboxyamide side-chains could form a "polar zipper" involving a network of sidechain-mainchain hydrogen-bonds (Stott et al., 1995).

It is likely that Gln and Asn rich domains play an important role in stabilizing a significant subset of amyloid phenomena. The putative PrD region of the Sup35p homologue of *Pichia pinus* is rich in Asn and Gln despite the lack of linear sequence homology with the *S. cerevisiae* gene (Kushnirov et al., 1990). Even more striking is the recently sequenced *SUP35* gene from *Candida albicans* which contains ~55 Gln residues in its putative PrD domain, including long stretches of pure Gln repeats near the N-terminus. These observations suggest that the amino acid composition of a protein, as much as its exact primary sequence, determines its amyloidogenicity. Another epigenetic factor in yeast, [*URE3*], also appears to propagate by a prion-like mechanism (Wickner, 1994). The protein responsible for the [*URE3*] state, Ure2p, is extremely rich in Asn as well as other polar residues. Furthermore, Ure2p has been reported to form filaments in vitro (King et al., 1997). Perhaps most interestingly, a number of neurodegenerative

diseases have recently been found to result from expansion of polyGln repeats that leads to intracellular aggregates in vivo and amyloids in vitro (see below).

Implications for the Prion Hypothesis

The prion hypothesis argues that a protein alone can act as an infectious agent, leading to a permanent change in the phenotype of the infected organism (Prusiner et al., 1998). This infectivity is thought to result from an altered conformation that both causes the phenotypic change and promotes conversion of new protein, thereby allowing self replication. Studies on the mammalian prion protein PrP, the yeast prion Sup35p, and non-prion amyloids have reproduced self-propagating conformational changes in vitro (Glover et al., 1997; King et al., 1997; Paushkin et al., 1997; Harper and Lansbury, 1997). Connecting these conformational changes to the phenotypic change, however, has proven more elusive. Here, we show that pure PrD can propagate amyloids on a physiological time scale in vitro. Moreover, we used a series of point mutants that lead to a loss of the prion associated phenotype to test the relevance of the in vitro conversion reaction. These mutants cause solubilization of protein in vivo and decreased rates of de novo amyloid formation and recruitment into preformed fibrils in vitro. These observations provide perhaps the most compelling data to date linking an in vitro conversion reaction to the phenotypic change effected by a prion, and thus provide strong support for the prion hypothesis.

The studies also furnish insights into the mechanism by which the Sup35p prion propagates its altered conformation. In vitro characterizations of the kinetics of amyloid formation from purified amyloidogenic proteins or peptides have suggested that

conversion is a nucleation-dependent process (Jarrett and Lansbury, 1993; Harper and Lansbury, 1997). In such processes, the lag phase results from the thermodynamically unfavorable formation of competent nuclei, and is followed by a conversion phase in which the transition from monomer to fibril occurs in a rapid, cooperative fashion. The nucleation-polymerization model makes several predictions (Jarrett and Lansbury, 1993) that distinguish it from other conversion mechanisms (e.g. the template conversion model in which the catalyst acts by promoting a rate-limiting conformational change [Prusiner and al, 1998]). First, there is a critical monomer concentration below which no conversion occurs. Second, the length of the lag time is highly dependent on the protein concentration. Finally, the lag phase will approach zero at infinite concentration, and as this limit is approached, addition of seed no longer accelerates fiber formation.

Here we tested critically the nucleation-polymerization model by examining the kinetics of de novo amyloid formation of Sup35p. We find that within a range of 1.25-12 μM the conversion of monomer appears to go to completion. Thus the critical concentration, if it exists, is well below micromolar as compared to 10-40 μM for ß-amyloid peptide (Harper and Lansbury, 1997). We also find that the rate of amyloid formation shows only modest concentration dependence. In particular, between 1.25 and 6 μM the rate of spontaneous conversion is ~first order. At concentrations between 6 and 12 μM, the lag time has a constant value of ~65 min. Yet this lag phase is completely eliminated by addition of as little as 3% (wt/wt) preformed aggregate. It remains possible that at extremely low concentrations, the kinetics of conversion would be adequately described by the nucleation polymerization model. However, we find dramatic catalysis of fibril formation even at concentrations where oligomerization is not rate limiting.

These data argue that fibrils accelerate the conversion of Sup35p by promoting a conformational change (e.g., conversion of profibrils to fibrils [Harper et al, 1997b]) rather than solely by providing a multimeric nucleus.

Although pure Sup35p PrD-M forms amyloids rapidly in vitro, a variety of cellular factors are likely to play an important role in propagation of the Sup35p prion state. For example, the molecular chaperone HSP104 is required for maintenance of the [*PSI* $^{+}$] state; paradoxically, HSP104 also causes loss of [*PSI* $^{+}$] when overexpressed (Chernoff et al., 1995). We find that the kinetics of conversion are enhanced greatly by the addition of a slow rotation step. Similar enhancement in the kinetics of polymerization is observed when hemoglobin S is subjected to rotation forces (Briehl, 1980). Given that in the absence of such forces, conversion of Sup35p in vitro is far slower than the doubling time of yeast, it is an attractive hypothesis that in a growing cell amyloid fibrils undergo continuous disrupton. As noted by Ter-Avanesyan and coworkers, HSP104 might contribute to such an effect as it has been shown to break up other protein aggregates (Paushkin et al., 1996; see however, Patino et al., 1996). Using the in vitro assay, we can quantitatively assess the ability of HSP104 or other cellular components to increase the number of polymerizing ends in an amyloid fibril sample.

A molecular chaperone, termed protein X, has also been implicated in propagation of the prion form of the mammalian PrP protein (Kaneko et al., 1997). The identity of protein X is unknown but its existence has been inferred by the behavior of a number of dominant negative forms of PrP. Our dominant negative Sup35p alleles could also result from altered recognition by a molecular chaperone such a HSP104. Alternatively, our Sup35p mutants might form heteropolymers with WT protein that fail

to effectively promote conversion of either WT or mutant protein. Consistent with this proposal, GFP analysis reveals that the mutants can be recruited into WT aggregates in vivo. Moreover, in vitro WT and AS17R amyloids are mutually defective in seeding each other's conversion.

Implications for the Mechanism of CAG Repeat Mediated Pathogenesis: PolyGln Diseases as Intracellular Prions?

A number of inherited neurodegenerative diseases result from expansion of the CAG nucleotide triplet leading to the insertion of a polyGln tract at the protein level. Concurrent with our studies, polyGln insertions were shown to lead to formation of amyloid fibrils in vitro and in vivo (e.g., Paulson et al., 1997; Scherzinger et al., 1997; see however Kahlem et al., 1998). This polyGln mediated aggregation appears to be intimately associated with pathogenesis.

Our functional analysis of Sup35p amyloid formation raises the interesting possibility that [*PSI* $^{+}$] and polyGln mediated pathogenesis are related phenomena involving intracellular prion-like propagation of amyloid fibrils stabilized by a polar network of carboxyamide side chains. There are, however, important differences between the aggregation of Sup35p and polyGln mediated amyloid formation. Notably, while the total number of Gln/Asn residues in Sup35p is comparable to the number found in the pathogenic repeat disorders, the Gln/Asn tracts in Sup35p are not continuous. There is also currently no direct evidence to address whether polyGln amyloids facilitate their own conversion during pathogenesis. A prediction of the self-replicating amyloid model is that a cell will undergo a stochastic conversion event, after which newly made

protein will be recruited into amyloid fibrils. Consistent with this, we found in a related set of studies that huntingtin protein containing pathogenically expanded polyGln repeats fused to GFP is initially soluble when expressed in yeast, but eventually forms aggregates that appear to propagate stably (AS and JSW, unpublished data). We are currently using the in vitro assay to directly examine the propagation of huntingtin amyloids.

Regardless of the exact mechanism of propagation, yeast could provide a powerful genetic system for studying polyGln-mediated amyloid formation. In particular, yeast should facilitate efforts to find extragenic factors involved in the conversion process (e.g. molecular chaperones, transglutaminases, or proteolytic processing enzymes). Indeed, as with Sup35p, increased HSP104 levels slow somewhat the rate of huntingtin aggregation (AS and JSW, unpublished data). Finally, using yeast it should be possible to rapidly screen for dominant negative mutants or small molecules capable of inhibiting the conversion process in vivo.

Experimental Procedures

General Procedures and Reagents

Isogenic [*PSI*$^+$] and [*psi*$^-$] versions of strain 74D-694 [*MATa, ade1, his3, leu2, trp1, ura3*; suppressible marker *ade1-14(UGA)*] were used (Chernoff et al., 1995). All nucleic acid and yeast manipulations were performed according to standard laboratory protocols (Sherman, 1991; Ausubel et al., 1995). PCR primers were as follows: P1 GGCCCCCCTCGAGTGAGAGAACCGTTAAATTCCC; P2 CCCCGGATCCTGCTAGTGGGCAGATATAGAT; P3, GGGAGGCTCGAGCTTCAACGATTTCTATGATTC; P4, GGGGGATCCTATGTGATGATTGAT TGATTG; P5, GCGGGATCCACAATGTCGGATTCAAACCA; P6, CCGGCCGAATTCAGATCTATCGTTAAC ; P7, CCCCCCAGATCTGAATTCCCATGGATGTTTGGTGGTAAAGATCAC; P8, CCCCCGAGCTCACCTTGTTTATG GTATATGGT; P9, GGGGGGGCATATGTCGGATTCAAA CCAAGGC; P10, CCGGGACGCGTGAATTCTTAAT GGTGGTGATGGTGATGGTGATGATGATCG TTAACAACTTCGTCATC. *S. Cerevisiae* Genomic DNA was used as a PCR template (Stratagene).

Plasmid Construction

In order to facilitate exchange of promoters and protein domains, PCR was used to introduce restriction sites flanking the promoter, the PrD-M domain and the EF domain of *SUP35*, generating a set of interchangeable cassettes. The promoter cassette was defined by a 5' XhoI site and a 3' BamH1 site, the PrD-M cassette by a 5' BamH1 site and 3' BglII and EcoR1 sites and the EF cassette by a 5' EcoR1 site and a 3' SacI site. Two promoters were used: the endogenous *SUP35* promoter (Sp) (P1 and P2), and the *CUP1* promoter (Cp) (P3 and P4). PrD-M domains were generated by P5 and P6. The EF domain was generated using P7 and P8. This domain was exchanged with GFP where indicated; GFP containing the appropriate sites was derived from pRS316-Pho4-GFP (E.K. O'Shea, personal communication). Epitope tags were inserted between the M and

EF domains, using the 5' BglII site and the 3' EcoR1 site. All plasmids were derived by ligating the insert between the Xho1/Sac1 sites of the following vectors; pRS316 (low copy CEN/ARS, *URA3+*), pRS315 (low copy CEN/ARS, *LEU2+*), and pRS426 (high copy 2μm, *URA3+*) (Christianson et al., 1992). Constructs are denoted by the plasmid backbone, promoter, PrD allele, and whether the EF or GFP domain is present (Epitope tags are noted between the PrD-M and EF domains where relevant). For bacterial expression, the appropriate allele of PrD-M was amplified using P9 and P10, which introduces a His tag at the C-terminus. These products were subcloned into NdeI/EcoRI sites of a T7 expression vector. The sequence of all plasmids was confirmed by dye termination sequencing (Perkin Elmer).

Screen For ASU and PNM Mutants

The SUP35 PrD was mutagenized by 30 rounds of PCR amplification with Taq polymerase using the manufacturer's conditions and P1 and P6. Diversity in the mutant pool was maintained by keeping 3-6 separate PCR pools and 3-6 separate transformation pools. Based on the levels of incidental second site mutations as well as sequencing of phenotypically WT PrD alleles, we estimate that ~30% of the PCR products contain at least one point mutation. The mutagenized PrD was reintroduced into p316SpSUP-EF by co-transforming [*PSI*$^+$] yeast with the linear PCR product and 316SpSUP-EF gapped by digestion with BamHI and PflmI. Transformants were selected on SD-URA, harvested by washing in TE and plated at density of 500 cells/plate on YPD to test for sectoring. The sectoring colonies were patched onto SD-URA, retested for sectoring by growth on YPD,

tested for plasmid dependent antisuppression by growth on SD-URA, ADE and SD-URA, and tested for curing by growth on SD-URA supplemented with 5-FOA.

GFP Induction/Microscopy and Extract Preparation/High Speed Centrifugation

Yeast carrying p426CpSUP-GFP, p426CpASU/PNM-GFP (11 alleles), or p426CpGRV-GFP (4 alleles) were grown to early log phase in SD-URA. At the indicated time after induction with 50μM $CuSO_4$, samples were spun down, resuspended in TE and examined by fluorescence microscopy (Olympus BX60) and photographed by film or CCD camera (Photometrics).

To prepare extracts, 50 ml cultures of yeast carrying either p316SpSUP$_{(HA)3}$EF, p316SpASU/ PNM$_{(HA)3}$EF, or p315SpSUP$_{(HA)3}$EF and p316SpASU/PNM-EF were grown in appropriate selective medium to mid-log phase and harvested at room temp. Cell pellets were washed once in an equal volume of distilled water and resuspended in Buffer A (25mM Tris-HCl pH 7.5, 50mM KCl, 10mM $MgCl_2$, 1mM EDTA, 5% Glycerol, 1mM PMSF, 2 μg/ml pepstatin and leupeptin, and 100μg/ml ribonuclease A). Cell pellets were resuspended in approximately two volumes of Buffer A, 2 volumes of glass beads were added and cells were disrupted by vortexing for 3 minutes at 4°C. Centrifugation was performed as described (Patino et al., 1996) with minor modifications. The slurries were spun at 8000g for 3 minutes at 4°C to pellet unbroken cells and glass beads; the remaining supernatant was spun at 100,000g for 30 minutes at 4°C. Supernatant was removed and the pellet was resuspended in an equal volume of cold Buffer A. Samples (20μg of total protein) were analyzed by SDS-PAGE and

western blot analysis (Ausubel et al., 1995) using the 16B12 antibody (Babco) and goat anti-mouse IgG - HRP conjugate (Bio-Rad).

PrD-M Expression and Purification

Pure PrD-M was prepared largely as described previously (Glover et al., 1997). Briefly, cell pellets from 1-2 liters of growth were lysed in 30 ml of Buffer B (25 mM Tris pH 7.8, 300mM NaCl, 6M urea). Subsequent to centrifugation at 25,000g (20 min), the supernatant was filtered by 0.22μm filter (Millipore) and applied to 20 ml Ni-NTA agarose (Qiagen) column. The column was washed with 5 column volumes Buffer B and eluted with a pH gradient in same buffer without NaCl. Pooled fractions were applied to a 30 ml Source 15S column (Pharmacia) equilibrated in 50mM MES pH 6.0, 6M urea and eluted with 0-400 mM NaCl gradient in the same buffer. Pure PrD-M was concentrated with centricon-10 (Amicon), filtered by microcon-100 (Amicon), aliquoted and stored at -80° C. Protein concentration was determined by UV absorption at 275nm.

Congo Red Binding Assay

Congo red binding assays were carried out largely as described previously (Klunk et al., 1989). Congo red (Sigma) was dissolved in Buffer C (5mM potassium phosphate, 150mM NaCl), filtered with 0.22μm filter (Millipore) and adjusted to 100mM. To initiate conversion, concentrated PrD-M was diluted at least 100-fold into Buffer C in 2ml microcentrifuge tubes at room temperature. The solution was subjected to constant rotation (7.5 rpm) using a RKVS Rotamix (ATR, Inc.). At indicated times, protein was diluted to 2μM in the presence of 10μM Congo red in Buffer C. Absorbances at 540 and

477 nm were determined (either by Shimadzu UV160U or Aviv 14DS spectrophotometer). Congo red bound per mole of PrD-M was determined by the formula $(A_{540}/25292)-(A_{477}/46306)$. Quantitative measurement of the length of the lag phase (T_0) was performed by fitting the middle of the sigmoidal curves with a straight line and solving the time for which the amount of Congo red bound is that of baseline. Likewise, the length of the conversion phase (T_c) was calculated by subtracting T_0 from the time at which dye binding is maximal.

Atomic Force Microscopy

Protein samples were absorbed to freshly cleaved mica surface. AFM images were collected with a NanoScope IIa (Digital Instruments) operated in contact mode with a commercial NP-S silicon nitride tips.

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Figure Legends

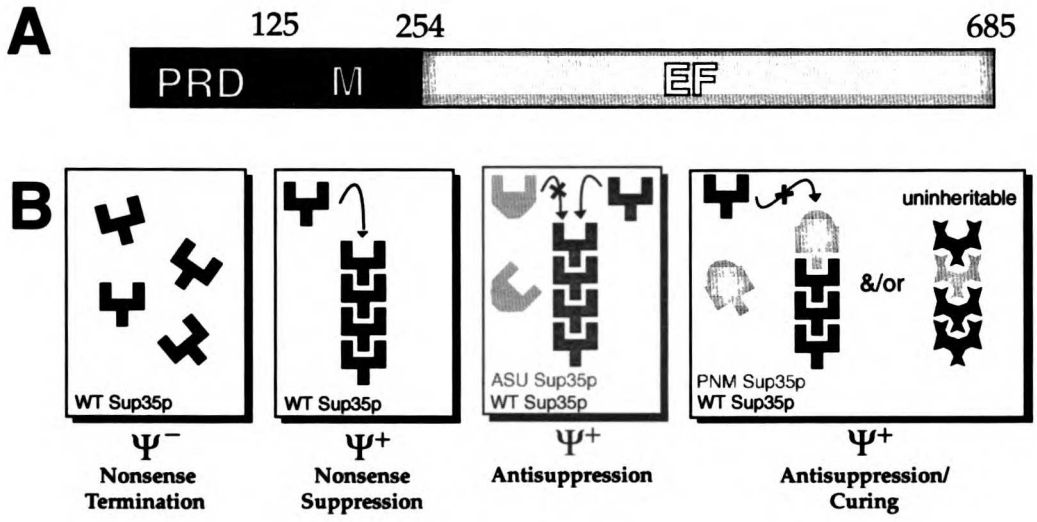
Figure 1. Illustration of the Screen for ASU and PNM Mutants

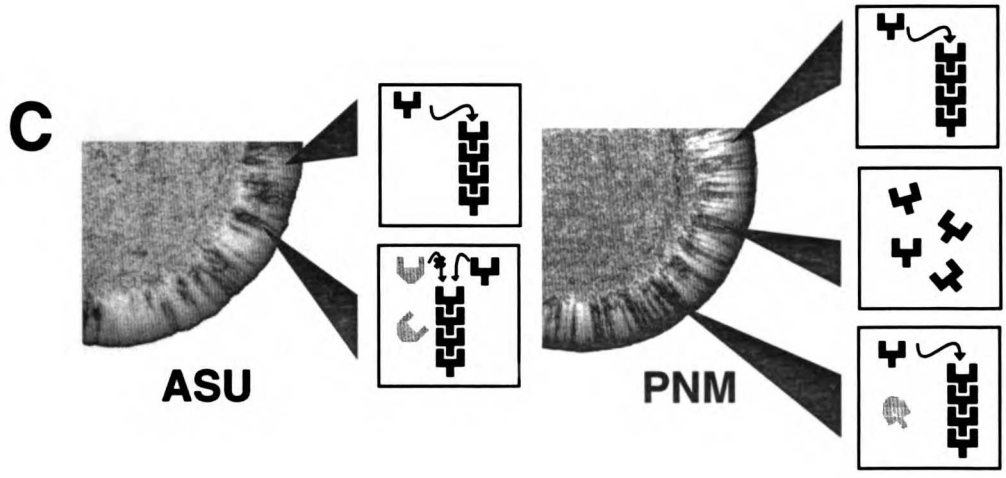
(A) Schematic of Sup35p domain structure. Residue numbers are indicated on the top of the schematic.

(B) Illustration of the logic underlying the screen for AntiSuppressor (ASU) and Psi-No-More (PNM) mutants. Throughout the figures, strain background is indicated by Ψ^+ for [*PSI* $^+$] and by Ψ^- for [*psi* $^-$]. The Sup35p alleles present are indicated in the lower left of each panel , and diagramatically with solid and gray shapes representing WT Sup35p, and mutant Sup35p, respectively. The phenotypes resulting from expression of the indicated Sup35p alleles are listed in the bottom row beneath each panel. Amyloids are denoted by stacked monomers, and soluble Sup35p by unattached subunits. Two hypothetical mechanisms for PNM mediated curing are illustrated in the final panel. These involve either capping of amyloids to prevent further monomer addition (left) or conformational change which renders the amyloids uninheritable (right).

(C) Examples of sectored colonies resulting from expression of ASU and PNM mutants. The solubilization states of the various alleles of Sup35p that could lead to the observed color changes are illustrated on the right of the colony (see text). Diagrams and symbols are as in (B).

(D) Sample data demonstrating plasmid dependent antisuppresion. Growth of a [*PSI* $^+$] strain initially bearing AQ15R on a URA3 marked plasmid on the indicated medium is shown. [*PSI* $^+$] and [*psi* $^-$] expressing WT protein are shown for comparison.


A
125
254
685
PRD
M
EF
B
WT Sup35p
WT Sup35p
ASU Sup35p
WT Sup35p
uninheritable
&/or
PNM Sup35p
WT Sup35p
Ψ−
Nonsense
Termination
Ψ+
Nonsense
Suppression
Ψ+
Antisuppression
Ψ+
Antisuppression/
Curing


C
ASU
PNM

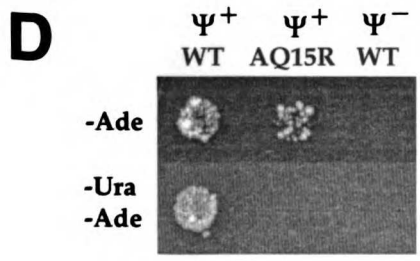

D
Ψ+
WT
Ψ+
AQ15R
Ψ−
WT
-Ade
-Ura
-Ade

Figure 2. Summary of ASU and PNM Mutants

(A) Bar graph of the frequency and phenotype of the PrD mutants as a function of residue position. Amino acid changes from the WT sequence (x-axis) are indicated within the bars. All mutants contain changes within residues 8-24 of the PrD. Double mutants are indicated by a superscript and contain the following changes: (1) PN9K, Q24R (2) PQ10R, Q91L (3) PQ15R, Q33R (4) PQ24R, N26D where the mutant is denoted by the first letter of the phenotype (A or P) followed by the amino acid changes. For double mutants, changes outside of residue 8-24 are not reported when they do not affect the phenotype.

(B) Average glutamine/asparagine content of the PrD as function of residue number. The color indicates the percent of glutamine and asparagine residues in a window spanning nine amino acids (scale is indicated on the right). The black bar above the graph marks the location of the isolated PrD mutations.

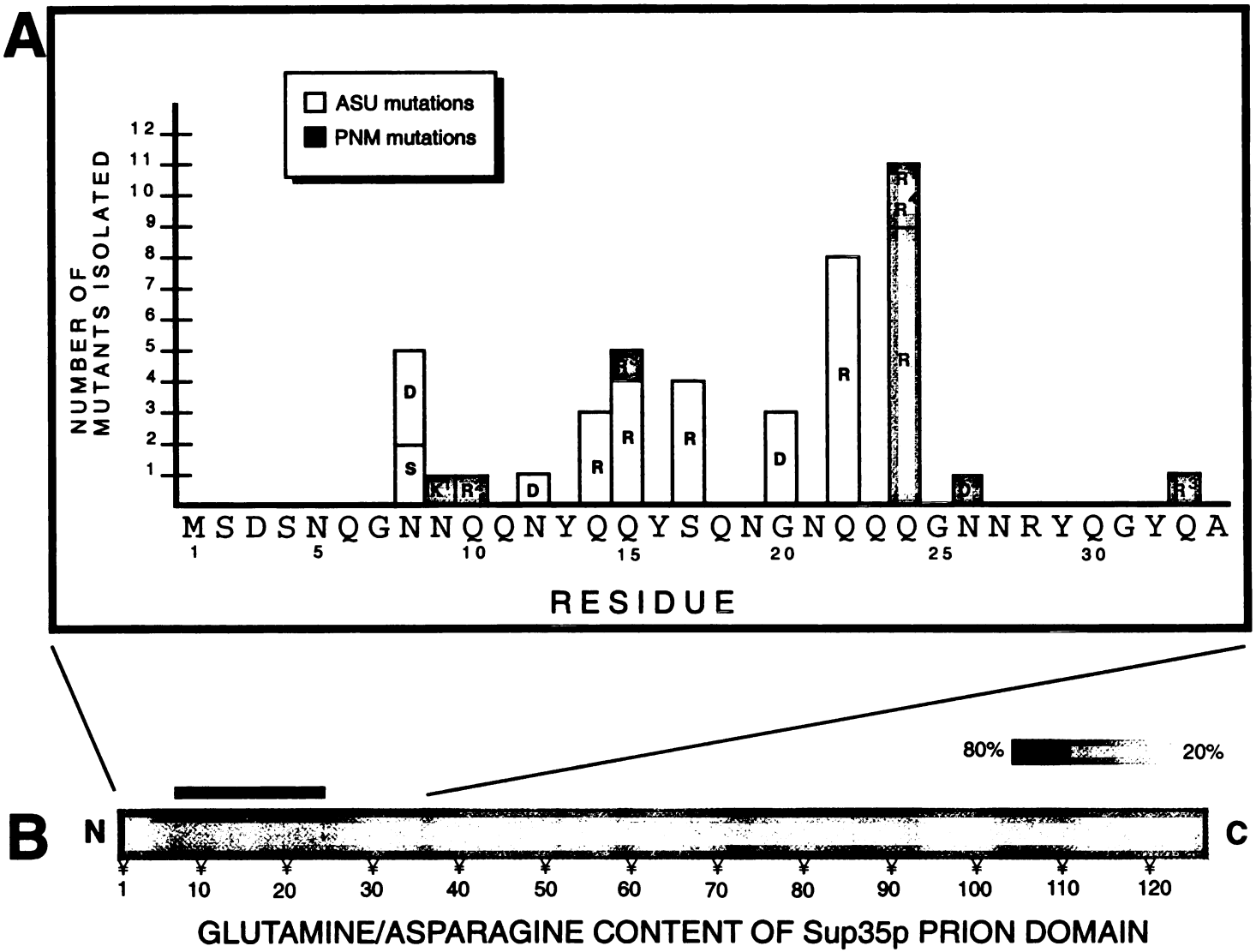

A
ASU mutations
PNM mutations
NUMBER OF MUTANTS ISOLATED
1 2 3 4 5 6 7 8 9 10 11 12
M S D S N Q G N N Q Q N Y Q Q Y S Q N G N Q Q Q G N N R Y Q G Y Q A
1 5 10 15 20 25 30
RESIDUE
80%
20%
B
N
C
1 10 20 30 40 50 60 70 80 90 100 110 120
GLUTAMINE/ASPARAGINE CONTENT OF Sup35p PRION DOMAIN

Figure 3. Aggregation State of Mutant PrD as assayed by GFP Fluorescence

Yeast expressing a PrD-GFP fusion construct were examined 2 hours post-induction. Diffuse fluorescence throughout the cytoplasm is indicative of soluble protein, while bright discrete foci are indicative of protein associated with prion-like Sup35p aggregates. Larger aggregates result in a halo of fluorescence which is not interpreted as soluble protein. The PSI state and PrD allele are indicated below.

(A) [*psi* $^{-}$] yeast expressing WT-PrD-GFP.

(B) [*PSI* $^{+}$] yeast expressing WT-PrD-GFP.

(C) A representative field in which an ASU mutant appears entirely soluble in presence of WT aggregates ([*PSI* $^{+}$] expressing AG20R-PrD-GFP).

(D) A representative field in which an ASU mutant appears to associate predominantly with WT aggregates ([*PSI* $^{+}$] expressing AQ22R-PrD-GFP).

(E) Representative fields in which ASU mutants appear to partition between soluble pools and discrete foci ([*PSI* $^{+}$]-yeast expressing AQ15R-PrD-GFP [top] and AN8D-PrD-GFP [bottom]).

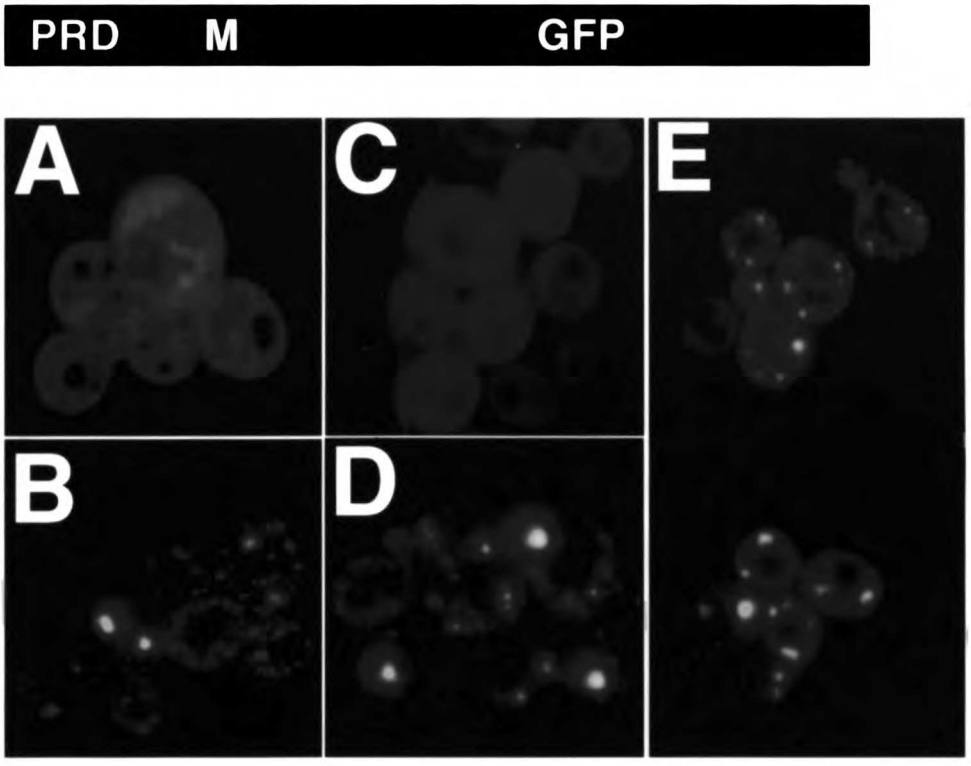

PRD
M
GFP
A
C
E
B
D

Figure 4. Aggregation State of Mutant PrD Alleles as Assayed by High Speed Centrifugation

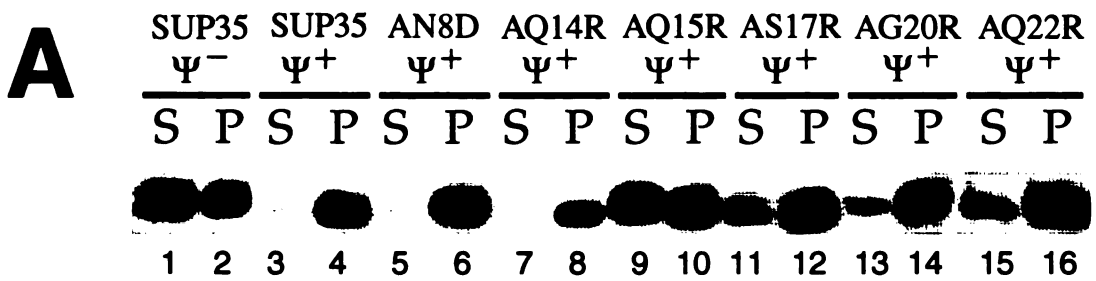
Mid-log phase cell extracts were prepared from yeast strains carrying a plasmid expressing the indicated allele of Sup35p. Extracts were centrifuged at 100,000g and soluble (S) or pelleted (P) fractions were assayed by western blot. Plasmid derived protein was distinguished from WT chromosomal Sup35p by the addition of a hemaglutanin (HA_3) tag between the M and EF domains (schematic at top). The PSI state of the cells and the expressed mutant protein allele are indicated above each set of lanes.

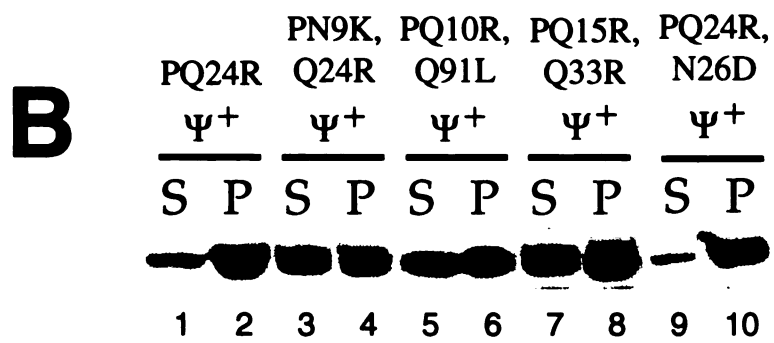
(A) Solubility of WT Sup35p compared to ASU mutant proteins in [*PSI* $^+$] yeast. For comparison, WT Sup35p in [*psi* $^-$] yeast is also shown. Note that the presence of Sup35p in [*psi* $^-$] pellet is not likely to represent prion-like Sup35p aggregates, thus the levels of soluble protein are an underestimate of the amount of functional Sup35p.

(B) Solubility of PNM mutants in [*PSI* $^+$] yeast.

(C) Solubility of WT Sup35p in [*PSI* $^+$] yeast co-expressing mutant PrD alleles. WT Sup35p was tagged with (HA_3) while mutant protein was untagged and therefore not recognized by western blot. The untagged mutant protein present is indicated in parentheses above each set of lanes.


PRD
M
HA3 tag insertion


A
SUP35 Ψ−
SUP35 Ψ+
AN8D Ψ+
AQ14R Ψ+
AQ15R Ψ+
AS17R Ψ+
AG20R Ψ+
AQ22R Ψ+
S P S P S P S P S P S P S P S P
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16


B
PQ24R Ψ+
PN9K, Q24R Ψ+
PQ10R, Q91L Ψ+
PQ15R, Q33R Ψ+
PQ24R, N26D Ψ+
S P S P S P S P S P
1 2 3 4 5 6 7 8 9 10

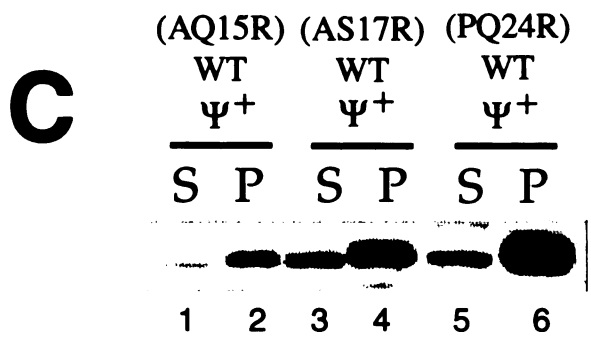

C
(AQ15R) WT Ψ+
(AS17R) WT Ψ+
(PQ24R) WT Ψ+
S P S P S P
1 2 3 4 5 6

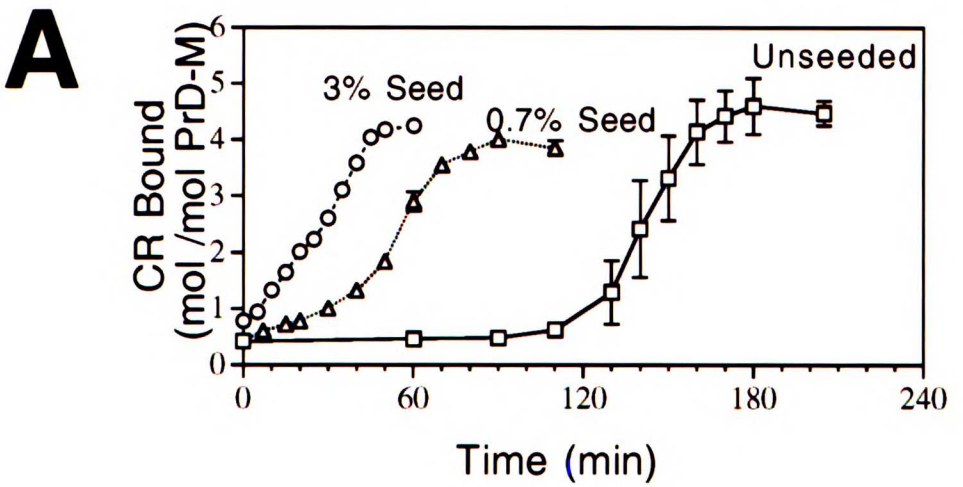
Figure 5. In Vitro Amyloid Fibril Formation of WT Sup35p

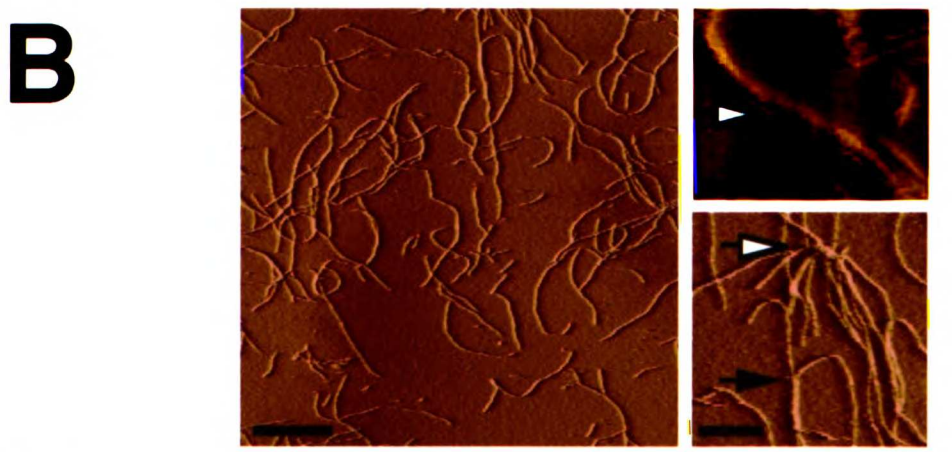
To initiate conversion, concentrated protein in urea was diluted at least 100-fold into buffer C, and subjected to continuous slow rotation. At the indicated times, the extent of fibril formation was assayed by Congo red (CR) binding. Each time curve is performed at least 3 times, unless noted otherwise. Errors larger than the size of symbols are indicated by bars.

(A) Kinetics of conversion of 2.5 μM WT PrD-M in absence (square) and presence of 0.7% (wt/wt) (triangle) and 3% (wt/wt) (circle) pre-formed WT fibril. The kinetics of WT seeded with 3 % (wt/wt) fibril were done in duplicate.

(B) Atomic Force Micrograph of WT PrD-M amyloids. Examples of a fibrillar ridge (open arrow) are seen in the top right panel. In the bottom right panel an amorphous structure from which many fibrils emanate (open arrow), and a branching fibrils (closed arrow) are highlighted. Scale bars are 1μm, except that of upper right figure (100 nm).

(C) Effect of concentration on the lag phase (T_0) of WT Sup35. T_0 is calculated as described in Experimental Procedures.


A
CR Bound
(mol /mol PrD-M)
3% Seed
0.7% Seed
Unseeded
0
1
2
3
4
5
6
0
60
120
180
240
Time (min)


B

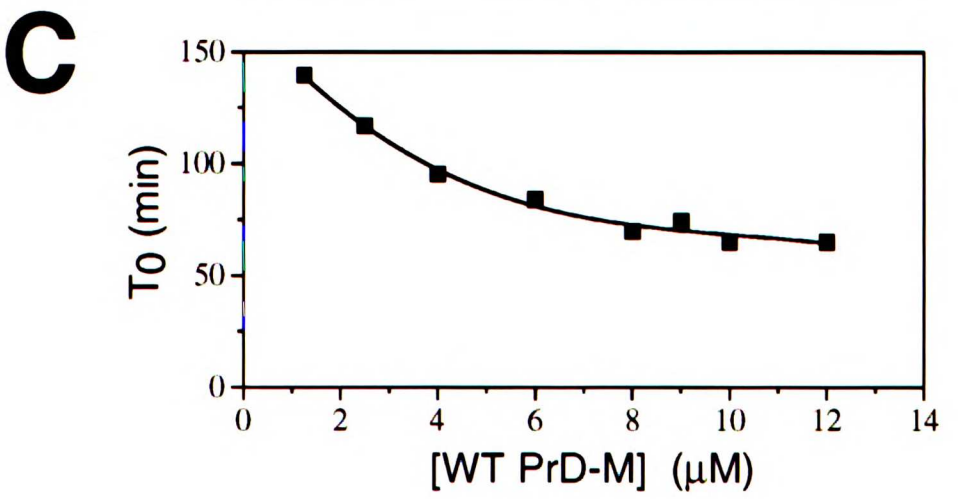

C
To (min)
0
50
100
150
0
2
4
6
8
10
12
14
[WT PrD-M] (μM)

Figure 6. Effects of ASU Mutations on the Kinetics of Amyloid Fibril Formation

Amyloid formation was monitored by examining Congo red (CR) binding as a function of conversion time. Errors larger than the size of symbols are indicated by bars.

(A) Comparison of amyloid fibril formation between a representative ASU mutant (AQ15R) and WT PrD-M either in the absence (upper panel) or presence (lower panel) of 0.7 % (wt/wt) WT fibril.

(B) Summary of the lag phases (T_0) in amyloid formation for the ASU mutants either in the absence (black bar) or presence (shaded bar) of 0.7 % (wt/wt) WT fibril. Note the failure of WT fibrils to accelerate conversions of AS17R and AQ22R.

(C) Conversion kinetics of WT PrD-M in the presence of 0 % (circle), 0.7 % (square), and 3 % (diamond) (wt/wt) AS17R fibrils.

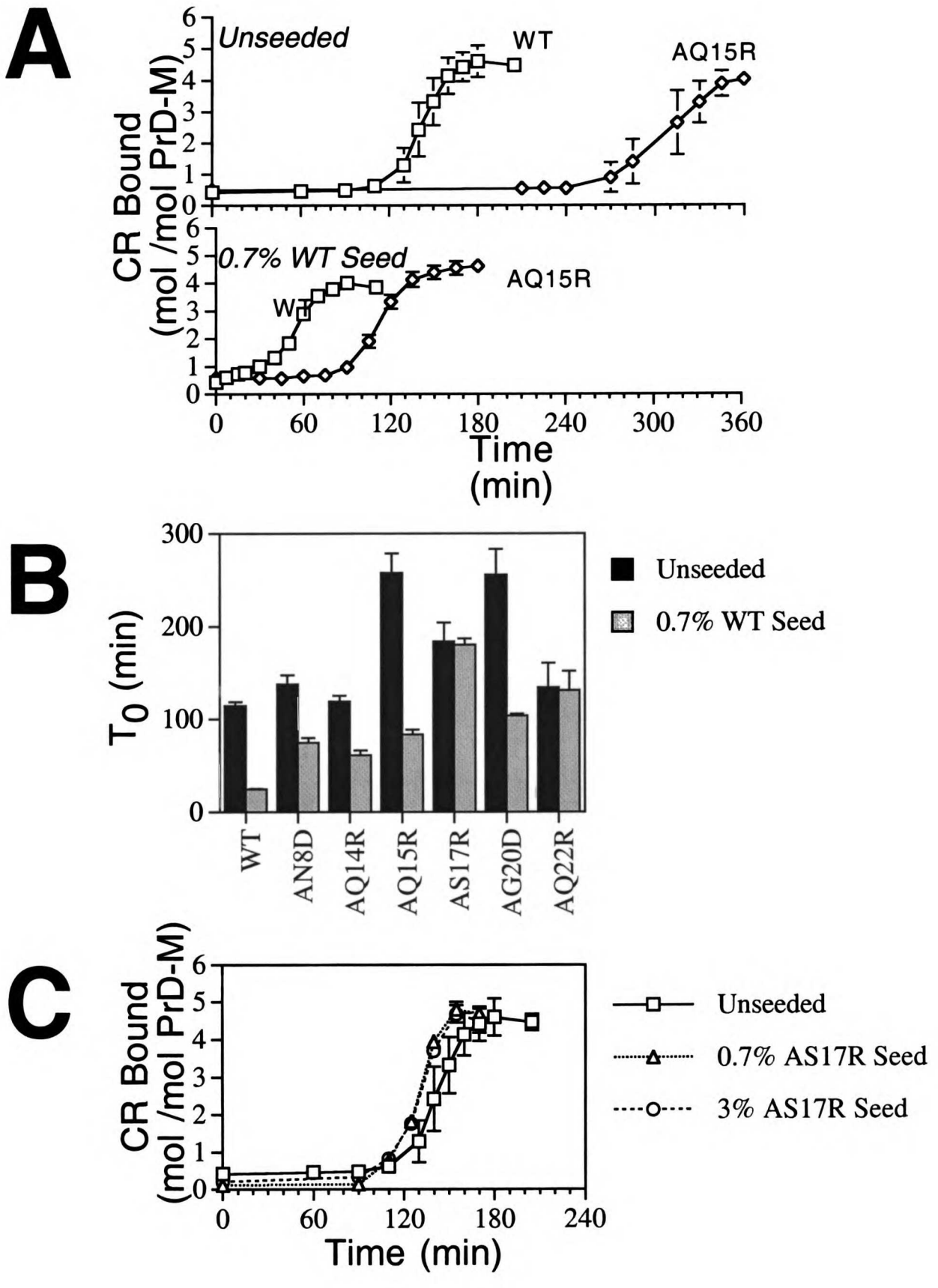
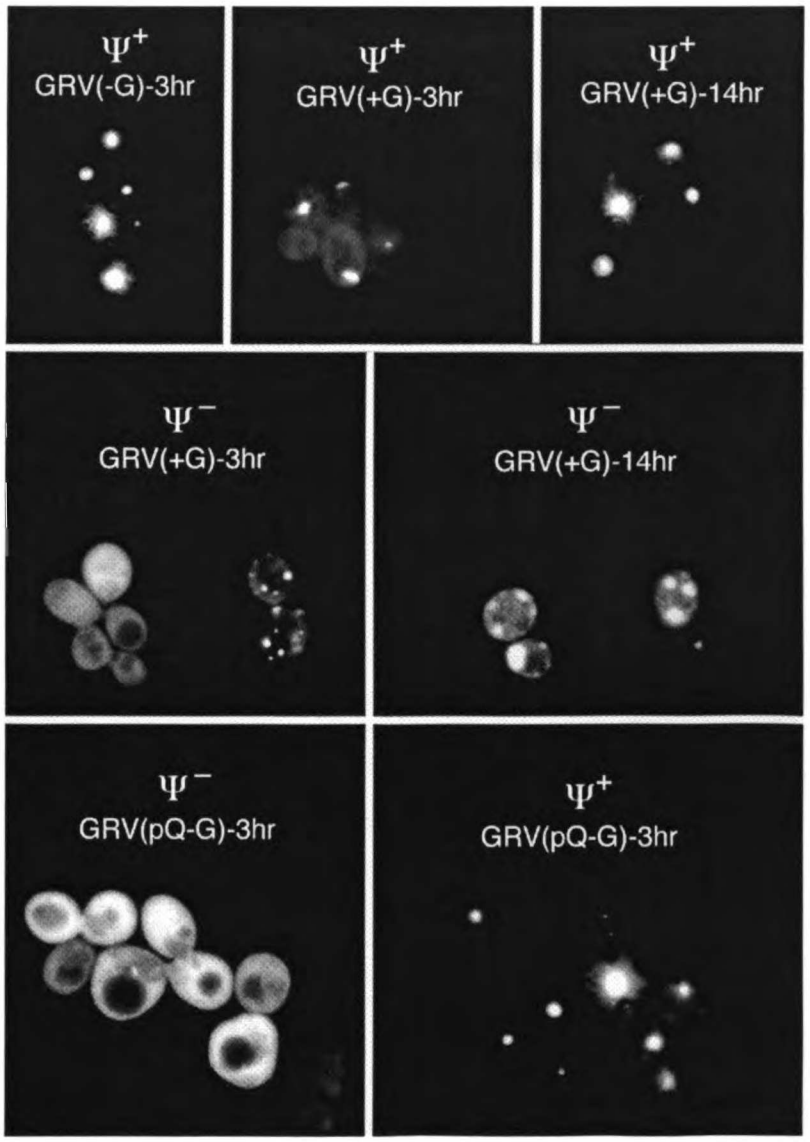

A
Unseeded
WT
AQ15R
CR Bound (mol /mol PrD-M)
0.7% WT Seed
WT
AQ15R
0
60
120
180
240
300
360
Time (min)
B
300
200
100
0
T_0 (min)
Unseeded
0.7% WT Seed
WT
AN8D
AQ14R
AQ15R
AS17R
AG20D
AQ22R
C
CR Bound (mol /mol PrD-M)
0
60
120
180
240
Time (min)
Unseeded
0.7% AS17R Seed
3% AS17R Seed

Figure 7. Recruitment of GRV Alleles of Sup35p by WT Aggregates

Yeast expressing GRV-GFP fusion proteins were examined at the indicated time after induction. The PSI state of the cells and the expressed GRV allele are as noted. The amino acid changes, relative to WT, in the various GRV alleles, are indicated in top panel. Amino acids altered in ASU or PNM mutants are denoted by an asterisk.

```
                          * * *  *  * *  * *  *  *  *
WT              M S D S N Q G N N Q Q N Y Q Q Y S Q N G N Q Q Q G
GRV(+G)         - - - - - - - Q Q Q Q Q - Q Q - Q Q - Q - Q Q Q -
GRV(-G)         - - - - - - - Q Q Q Q Q - Q Q - Q Q - G - Q Q Q -
GRV(pQ-G) - - - - - Q - Q Q Q Q Q Q Q Q Q Q Q G Q Q Q Q -
```



Chapter 3

Molecular Basis of Yeast Prion Species Barrier

Molecular Basis of a Yeast Prion Species Barrier

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and Jonathan S. Weissman

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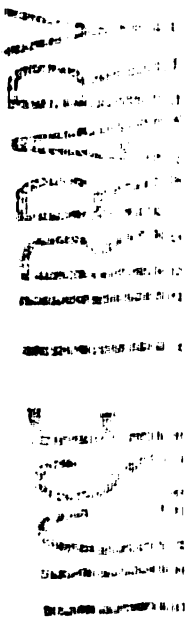
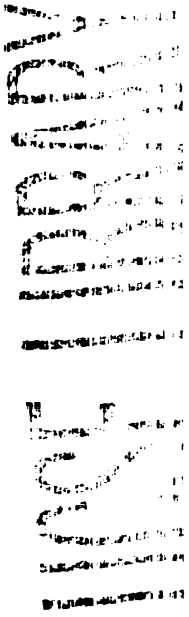
(email: jsw1@itsa.ucsf.edu).



Summary

The yeast [PSI$^+$] factor is inherited by a prion mechanism involving self-propagating Sup35p aggregates. We find that Sup35p prion function is conserved among distantly related yeasts. As with mammalian prions, a species barrier inhibits prion induction between Sup35p from different yeast species. This barrier is faithfully reproduced in vitro where, remarkably, ongoing polymerization of one Sup35p species does not affect conversion of another. Chimeric analysis identifies a short domain sufficient to allow foreign Sup35p to cross this barrier. These observations argue that the species barrier results from specificity in the growing aggregate, mediated by a well-defined epitope on the amyloid surface and, together with our identification of a new yeast prion domain, show that multiple prion-based heritable states can propagate independently within one cell.



Introduction

Amyloid protein aggregates have been increasingly implicated in human diseases, including prion-based encephalopathies, non-infectious neurodegenerative diseases, and systemic amyloidoses (Koo et al., 1999). Amyloids are β-sheet rich, ordered structures consisting of protofibrils (Sunde and Blake, 1997) that coalesce in vitro to form extended fibrils which bind the dye Congo red. Fibrils are also found under some conditions in vivo, although their role in pathogenesis remains unresolved (Lansbury, 1999). Despite having similar aggregated structures, sequence comparison of amyloidogenic proteins fails to reveal any obvious similarities.

A striking property of most amyloids is the ability to catalyze their own propagation. In prion diseases, this self-propagation is thought to be the basis of protein-mediated infectivity. Here, the abnormal β-sheet rich prion form (PrP^{SC}) can convert the normal cellular α-helical protein (PrP^{C}) into the prion isoform (reviewed in Prusiner et al., 1998). Even in the non-infectious amyloid diseases, such as Alzheimer's disease, amyloid self-propagation may be critical to disease progression (Lansbury, 1999).

Studies of the mammalian prion have highlighted the importance of specificity in amyloid propagation. Here, a species barrier limits PrP^{SC} derived from one species from infecting another preventing, for example, the transmission of scrapie from sheep to man (Prusiner et al., 1998). The recent description of variant Creutzfeld-Jacob disease, however, suggests that in rare instances bovine prions can cross the species barrier to infect humans. Extensive transgenic mouse and chimera analyses indicate that the species barrier is largely due to differences in sequence of the prion proteins. However,

the extent to which the species barrier is mediated by direct interaction between prion particles as opposed to species-specific interactions with cellular factors is unresolved (Kocisko et al., 1995; Telling et al., 1995). Even in non-prion amyloid diseases, the ability of amyloids to incorporate other types of protein has been implicated in the disease process (Han et al., 1995), although other studies have failed to observe cross-seeding between different amyloid forming peptides (Come et al., 1993).

The prion-like phenomenon [PSI^+] of *Saccharomyces cerevisiae* offers a powerful system to study the molecular basis of amyloid propagation and specificity. Identified as a non-Mendelian trait that confers suppression of nonsense mutations, [PSI^+] arises from conversion of the translational termination factor Sup35p from a soluble and active state into an insoluble and inactive amyloid (Wickner et al., 1995; Lindquist, 1997). The ability of Sup35p amyloids to incorporate newly made soluble proteins is thought to be the basis of [PSI^+] propagation. As Sup35p aggregation increases translational readthrough, the presence of the [PSI^+] prion can be readily monitored.

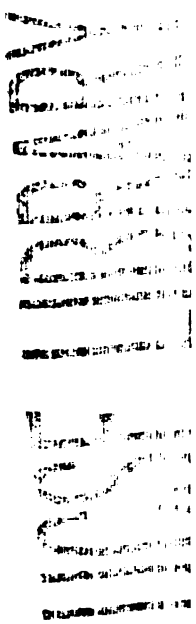
To investigate the requirements for prion formation and amyloid specificity and to examine if there is evolutionary pressure to retain prion function, we cloned and characterized Sup35p from a spectrum of Saccharomycetales (budding yeasts). Interestingly, we find that the ability to support a prion mechanism of inheritance is broadly conserved. Moreover, as with mammalian prions, a species barrier prevents cross-species prion induction. We have taken advantage of these phenomena to elucidate the requirements for prion formation and the molecular basis of this species barrier.

Results

The Prion Domain is Conserved in Yeast Evolution

The N-terminus of Sup35p is necessary and sufficient for prion formation and propagation. This prion domain (PrD) is connected to the C-terminal translation termination domain (EF) by a highly charged middle domain (M) of unknown function (Ter-Avanesyan et al., 1993). Deletion of the PrD allows Sup35p to remain soluble and functional even in [PSI^+] yeast, whereas transient overexpression of this domain induces conversion of [psi^-] yeast to [PSI^+] (Patino et al., 1996; Paushkin et al., 1996). Mutational analyses have begun to define the sequence requirements for prion formation and propagation (DePace et al., 1998; Liu and Lindquist, 1999). Although the PrD is generally tolerant to amino acid changes, several unusual features were found to be important. In particular it has a high glutamine (Gln) and asparagine (Asn) and low charge content. In addition, the PrD has a set of imperfect oligopeptide repeats, deletion and expansion of which modulate its ability to induce the conversion to [PSI^+].

To determine whether these features are conserved, we cloned and characterized Sup35p PrDs from a variety of yeasts. Taking advantage of the conservation of the EF domain, we used one-sided PCR (Frohman, 1993) as well as available *SUP35* sequences (Kushnirov et al., 1990) to clone sequences upstream of this region. In total, we examined *SUP35* genes from seven non-*S. cerevisiae* budding yeast species (*Candida albicans*, *Kluyveromyces lactis* and *marxianus*, *Pichia methanolica* and *pastoris*, *Saccharomycodes ludwigii* and *Zygosaccharomyces rouxi*) (Figure 1A).



Significantly, all of the Sup35 proteins examined have N-terminal regions similar in composition to the PrD and M domains of the *S. cerevisiae* protein (Figures 1B and C). Although there is little exact sequence homology, all of the PrDs have a high Gln/Asn (36 to 43 percent) and a low charge (2 to 10 percent) content. This composition resembles that of a modular prion domain from another *S. cerevisiae* prion protein, Ure2p (Edskes et al., 1999), but is very different from that of full length proteins from the *S. cerevisiae* genome which have on average 9 percent Gln/Asn and 23 percent charged residues (Figure 1B). Finally, the imperfect oligopeptide repeats of QGGYQQYN, although highly divergent, are clearly detectable (Figure 1C).

Foreign PrDs Aggregate, but do not Interact with *S. cerevisiae* Sup35p

The unusual sequence composition common to all Sup35p N-terminal domains prompted us to examine if the ability to support prion-based inheritance is also conserved. Focusing on *C. albicans*, *K. lactis* and *P. methanolica*, we asked whether the foreign Sup35p N-terminal domains could be recruited efficiently into the *S. cerevisiae* [PSI^+] aggregate using both functional and visual assays. In the functional assay, foreign PrDM fused to *S. cerevisiae* EF domain, termed PrDM-EF, is ectopically expressed in the [PSI^+] yeast. The PrDM-EF gene is under control of the *S. cerevisiae SUP35* promoter, resulting in a moderate, constitutive level of expression. If the foreign PrDs were not incorporated in the endogenous [PSI^+] aggregate, soluble fusion protein would provide functional translation termination activity, thereby leading to an antisuppressed phenotype. This can be phenotypically monitored by use of yeast harboring an *ade-1* marker with a suppressible nonsense mutation (Chernoff et al., 1995). In suppressed

yeast, functional Ade1p is produced, resulting in white colonies on low adenine medium and growth on adenine-free medium. By contrast, the lack of functional Ade1p in either [psi$^-$] or antisuppressed [PSI$^+$] yeast results in red colonies on low adenine and lack of growth on adenine-free media. As expected, when *S. cerevisiae* PrDM ($PrDM_{SC}$) was used in the fusion protein, [PSI$^+$] yeast retained the suppression phenotype (Figure 2A). In contrast, expression of fusion proteins containing PrDMs from *C. albicans*, *K. lactis* and *P. methanolica*, denoted $PrDM_{CA}$, $PrDM_{KL}$, and $PrDM_{PM}$ respectively, conferred antisuppression to [PSI$^+$] cells, suggesting that they are not inactivated by the endogenous *S. cerevisiae* aggregate.

In the visual assay, the in vivo aggregation state is observed directly using an inducible PrDM fused to GFP (Patino et al., 1996). As expected, upon $PrDM_{SC}$-GFP induction, punctate foci appear rapidly in the majority of [PSI$^+$] cells (Figures 2B and C). In contrast, [psi$^-$] cells show a prolonged diffuse cytoplasmic fluorescence, with foci forming slowly. Expression of the fusion of the N-terminal regions of *C. albicans*, *K. lactis* and *P. methanolica* to GFP also resulted in formation of foci. Indeed, de novo formation of foreign PrDM foci in [psi$^-$] cells, especially of $PrDM_{PM}$-GFP, is faster than that of *S. cerevisiae* PrDM. In contrast to $PrDM_{SC}$-GFP, however, the in vivo kinetics of foreign PrDM aggregation are similar in [psi$^-$] and [PSI$^+$] cells (Figure 2C). Together, these assays show that although the ability of these PrDs to aggregate is conserved, the foreign prion domains are not incorporated into the endogenous Sup35p aggregate present in [PSI$^+$] yeast.

Conversely, we asked if foreign PrD aggregates could incorporate soluble *S. cerevisiae* Sup35p present in [psi$^-$] yeast. Aggregates formed by transient overexpression

of $PrDM_{SC}$, either by itself or fused to GFP, incorporate native full length Sup35p, leading to a permanent conversion to [PSI^+] (Chernoff et al., 1993; Patino et al., 1996). After 24 hours of induction, ~1.5% of [psi$^-$] cells convert to [PSI^+] (Figure 2D). In contrast, similar levels of overexpression of foreign PrDM-GFPs, even at timepoints when foci are readily observable, fail to induce [PSI^+] conversion (Figure 2D and inset). Thus the foreign PrD aggregates are unable to seed the in vivo aggregation of *S. cerevisiae* Sup35p.

Hereafter, we designate the aggregation state of the foreign PrDs by [CHI]. For example, in [psi$^-$ CHI^+] yeast, endogenous Sup35p is soluble and the foreign PrD is aggregated, whereas in [psi$^-$ chi$^-$] yeast both the endogenous and foreign Sup35p are soluble.

Foreign PrDs Form Stable Prions that are Limited by a Species Barrier

Given the long evolutionary distance separating these yeast species, the failure of the foreign PrDs to interact with *S. cerevisiae* prions is not surprising, but leaves unresolved the question of whether the foreign PrDs behave as prions. To address this question, we devised a novel genetic system which allowed us to monitor the induction and propagation of [CHI^+] (Figure 3A). Here, two plasmids were introduced into yeast. The first, termed the maintainer plasmid, encodes an epitope-tagged PrDM fused to *S. cerevisiae* EF domain, termed PrDM-EF, under control of the *S. cerevisiae SUP35* promoter. The second inducer plasmid encodes a PrDM-GFP fusion protein under control of the inducible *CUP1* promoter. Transient overexpression of the inducer protein, either from the same (homotypic) or different (heterotypic) species as the maintainer PrD,

results in de novo formation of the GFP fusion aggregates, thus mimicking infection experiments used in studies of mammalian prions. The ability of the inducer aggregates to "infect" the maintainer PrD is monitored by the permanent change in suppression phenotype.

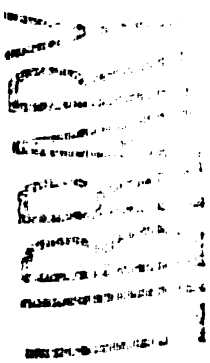
This system allowed us to test whether the foreign PrD aggregates behave as prions, and if so, whether divergence in sequences leads to a species barrier to prion propagation (Figure 3A). In support of the species barrier model, we found that the conversion of the maintainer PrD is induced only upon overexpression of the homotypic inducer PrD. For example, overexpression of $PrDM_{CA}$-GFP in the presence of $PrDM_{CA}$-EF maintainer, resulted in ~7.5% adenine-prototrophic [PSI^+ CHI_{CA}^+] colonies (Figure 3B). By contrast, less than 0.005% [chi_{CA}^-] cells were converted when an empty control plasmid or any of the heterotypic inducers were used. Likewise, in the presence of $PrDM_{KL}$-EF and $PrDM_{PM}$-EF maintainers, only the overexpression of the homotypic inducers caused efficient conversion to the [CHI^+] state. Finally, double fluorescence experiments using fusions between PrDM and two color variants of GFP showed that prion aggregates from two different PrD species do not co-localize (Figure 3C).

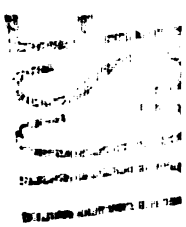
We next asked whether the [CHI^+] prion state could propagate stably. [PSI^+ CHI^+] yeast were sequentially patched onto medium which selects for the maintainer plasmid, but neither the inducer plasmid nor the prion state. Interestingly, some [PSI^+ CHI^+] isolates rapidly reverted back to unsuppressed [chi^-] state, whereas other strains propagated the [CHI^+] aggregate robustly. After three successive patches, corresponding to ~60 generations, a typical strong [PSI^+ CHI_{CA}^+] strain had lost the inducer plasmid but could still grew on medium lacking adenine. Even more remarkably,

no red sectors were observed on low adenine media, demonstrating that even in the absence of selection yeast uniformly retained the [CHI^+] prion (Figure 3D).

The reversible loss of the [PSI^+] factor, by overexpression of the molecular chaperone HSP104 or by exposure to guanidine hydrochloride, provided critical evidence that [PSI^+] inheritance is mediated by a change in protein conformation rather than by a DNA element (Wickner, 1994; Chernoff et al., 1995). HSP104 overexpression and guanidine caused solubilization of the [CHI_{CA}^+] aggregate, suggesting that both [CHI^+] and [PSI^+] prions are cured by similar mechanisms (Figure 3E and data not shown). Furthermore, the [CHI_{CA}^+] prion can propagate even in yeast lacking the endogenous sup35 gene (Figure 3F). As with [PSI^+ CHI^+] yeast, transient exposure to guanidine also cures the [CHI^+] state. Together, these data provide strong genetic evidence that foreign PrDs can support prion-based inheritance and that a barrier prevents cross-seeding between different species of PrDs.

Using a centrifugation assay (Patino et al., 1996; Paushkin et al., 1996), we confirmed biochemically that the [CHI^+] suppression phenotype results from a heritable aggregation of the foreign PrDM-EF protein. Aggregated Sup35p from [PSI^+] yeast extract fractionates to the pellet following high-speed centrifugation, whereas soluble Sup35p from [psi^-] yeast extract remains largely in the supernatant. When subjected to this centrifugation assay, $PrDM_{CA}$-EF protein from [CHI_{CA}^+] yeast fractionates to the pellet (Figure 3E). By contrast, it remains largely in the supernatant both in the unconverted and the guanidine or HSP104 cured [chi^-] strains (Figure 3E, and data not shown).





Selective Seeding of Fibril Formation Recapitulates the Species Barrier In Vitro

Purified Sup35p forms self-seeding amyloids in vitro, thereby providing a simple biochemical system to examine the molecular basis of the observed species barrier (Glover et al., 1997; King et al., 1997; Paushkin et al., 1997; DePace et al., 1998). After dilution of purified $PrDM_{SC}$ from denaturant, there is an initial lag phase of ~120 minutes, followed by a cooperative conversion from random coil to β-sheet rich amyloid fibrils. This conversion can be readily monitored by selective binding of the fibrils to the dye Congo red (Figure 4A). Importantly, the addition of pre-formed fibril catalyzes this conversion by eliminating the lag phase, thus recapitulating in vitro the self-propagation of the prion state.

We attempted to recapitulate [CHI^+] prion propagation in vitro using purified *C. albicans* PrDM proteins ($PrDM_{CA}$). Upon dilution from denaturant, $PrDM_{CA}$ exhibited a cooperative transition to fibrils following a lag phase of ~140 minutes. As with $PrDM_{SC}$, addition of a small amount (1%) of pre-formed $PrDM_{CA}$ fibrils eliminated the lag phase (Figure 4B). Strikingly, the addition of $PrDM_{CA}$ fibrils, even at amounts which could efficiently catalyze $PrDM_{CA}$ amyloid formation, did not convert $PrDM_{SC}$ (Figure 4A). Conversely, addition of $PrDM_{SC}$ seed did not alter the kinetics of $PrDM_{CA}$ fibril formation (Figure 4B). Thus our in vitro seeding experiments mirror a key aspect of the in vivo homotypic conversion experiments, in which prion formation is initiated only by homotypic PrD overexpression.

In the selective seeding experiments above, addition of a fibril seed failed to convert PrDM from another species. However, for yeast to stably exist in a [PSI^+ chi^-]

state, the foreign PrD must remain soluble in the presence of continuous aggregation of similar levels of the endogenous Sup35p. To better simulate this condition, we tested the effect of selective seeding of a solution containing an equal concentration of $PrDM_{SC}$ and $PrDM_{CA}$ monomers. Addition of a small amount of $PrDM_{SC}$ seed (5%) shortly after the initiation of the polymerization reaction caused an immediate conversion detected by Congo red binding (Figure 5A). Consistent with the notion that ongoing $PrDM_{SC}$ polymerization does not induce conversion of $PrDM_{CA}$, the curve plateaus at a level corresponding to conversion of half of the total protein. Subsequent addition of $PrDM_{CA}$ seed (5%) initiated the conversion of the remaining protein. In contrast, if no seed or more $PrDM_{SC}$ seed was added, there was no immediate conversion. Instead a slow rise in Congo red binding was observed with kinetics indistinguishable from that of the spontaneous conversion of $PrDM_{CA}$ (Figure 5B).

Immuno-electron microscopy confirmed that these two prions had a strong preference to form amyloid fibrils composed of a single species. We used a species-specific polyclonal antibody to label *S. cerevisiae* PrDM with 5-nm gold particles and a monoclonal epitope-tag specific antibody to label $PrDM_{CA}$ with 15-nm gold. In a reaction where equimolar amounts of $PrDM_{SC}$ and $PrDM_{CA}$ were polymerized simultaneously, individual fibrils were labeled with only one size of gold particles, suggesting that, as observed in vivo in the double fluorescence studies (Figure 3C), fibrils were composed exclusively of a single species of PrD (Figure 5C).

Prion Specificity is Encoded in a Short Region of the PrD Domain

Taking advantage of the visual and [PSI$^+$] conversion assays described above, we next asked whether a specific region of the PrD is responsible for the species barrier. We created two complementary chimeric proteins, the first of which ($PrDM_{SC1\text{-}39CA}$) contained residues 1 to 39 from *S. cerevisiae* with the remaining PrD sequence from *C. albicans*, and the second ($PrDM_{CA1\text{-}39SC}$), in which the first 39 residues of $PrDM_{SC}$ were replaced with the *C. albicans* sequence (Figure 6A). Microscopic examination of GFP fusions shows that both chimeras form aggregates (Figure 6C and data not shown). However, while over-expression of $PrDM_{SC1\text{-}39CA}$ was highly effective at converting [psi$^-$] yeast to [PSI$^+$], over-expression of $PrDM_{CA1\text{-}39SC}$ had no detectable effect (Figure 6B).

A previous study had found that mutations which cause poor incorporation into or curing of wild type [PSI$^+$] aggregates cluster to a short region composed of residues 8 to 26 (DePace et al., 1998). To test whether this epitope is sufficient to allow crossing of the species barrier, we constructed a third chimera consisting of residues 8 to 26 of *S. cerevisiae* replacing the corresponding *C. albicans* sequence, denoted $PrD_{SC8\text{-}26CA}$ (Figure 6A). This chimeric PrDM-GFP retains the ability to aggregate and is seeded by the endogenous Sup35p aggregate, as shown by the visual assay (Figure 6C). More remarkably, it induces conversion of [psi$^-$] yeast to [PSI$^+$] with only a modestly reduced efficiency (~ 2 fold) compared with wildtype $PrDM_{SC}$ (Figure 6B). These data indicate that a short peptide epitope composed of residues 8 to 26, presumably on the growing face of the amyloid, is sufficient to mediate specificity in the incorporation of monomers into the polymerizing fibril (Figure 6D, see Discussion).

Identification of a Novel Prion-forming Protein in *S. cerevisiae*

The observation that all known yeast prion proteins have high Gln/Asn content and few charged residues (Figure 1B) suggests that proteins with similar properties could form prions. A search of genomic databases revealed that several other proteins have domains with similar properties (Figure 7A and M. Michelitsch and J.S.W., in preparation). We experimentally examined the ability of one such domain from a protein encoded by the uncharacterized ORF *YPL226W* (*NEW1*) to form a prion using the two-plasmid assay described above. We fused the first 153 amino acids of New1p to an HA_3-epitope-tagged EF domain and expressed this fusion protein, termed New1p$_{1-153}$-HA_3-EF, driven by the *SUP35* promoter. Initially, this fusion protein complemented the deficiency of Sup35p activity associated with [PSI$^+$], indicating that it is not inactivated by the [PSI$^+$] prion; we termed this antisuppressed state [PSI$^+$ nu$^-$]. However, transient over-expression of the same fragment fused to GFP, but not over-expression of PrDM$_{SC}$-GFP or PrDM$_{CA}$-GFP, caused ~10 percent of [PSI$^+$ nu$^-$] cells to convert to a suppressed state termed [PSI$^+$ NU$^+$] (Figure 7B). Similarly to [CHI$^+$], we found significant variation among [NU$^+$] isolates, with some rapidly reverting to [nu$^-$] and others propagating stably (data not shown). Finally, centrifugation analysis directly demonstrated that the [NU$^+$] state results from aggregation of the New1p-EF fusion (Figure 7C). Taken together, these observations argue the Gln/Asn-rich N-terminal region of New1p can support a prion mechanism of inheritance.

Discussion

To investigate how prions can specifically propagate in the complex cellular milieu, we have cloned and characterized the N-terminal prion domain (PrD) from a range of budding yeasts. Despite the long evolutionary distances separating these species (Kurtzman, 1994), the Sup35p homologs examined contain PrDs capable of forming prions. In particular, upon induction of aggregates by overexpression, the foreign PrDs switch from an initially soluble [chi$^-$] state to an aggregated [CHI$^+$] prion state. This prion state can stably propagate until cured by guanidine treatment or HSP104 over-expression. As with mammalian prions (Prusiner et al., 1998), a species barrier prevents prion aggregates from one species from converting soluble PrDs of another.

Surprisingly, even in identical genetic backgrounds, different [CHI$^+$] isolates of the same foreign PrD species as well as different [NU$^+$] isolates show markedly different stability and levels of nonsense suppression. Analogous strain differences were found previously in [PSI$^+$] as well as in mammalian prions (Derkatch et al., 1996; Prusiner et al., 1998; Zhou et al., 1999). Initially discovered as differences in pathology between isolates of scrapie, mammalian prion strains were thought to result from nucleic acid variations, arguing against a protein-mediated mechanism of prion infectivity. It now appears that the presence of stable, distinct strains, possibly arising from different prion conformations, is an inherent property of prions.

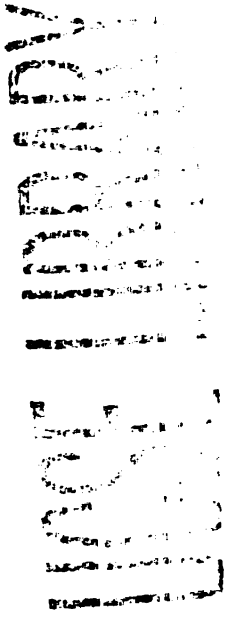
Insights into the Molecular Architecture of the Prion Domain: Specificity and Stability Domains Within the PrD

The mammalian prion species barrier, which prevents the spread of scrapie and bovine spongiform encephalopathy to man, has been the focus of intense research efforts (Prusiner et al., 1998). Many questions about the molecular basis of the prion species barrier remain. For example, in vitro experiments indicate that specific interactions between PrP^{SC} and PrP^{C} lead to a species barrier in generating the protease-resistant form (Kocisko et al., 1995; Horiuchi and Caughey, 1999). In vivo chimera analyses, however, suggest that species-dependent interactions between the prion particles and an unidentified host factor, termed Protein X, also contribute substantially to the species barrier (Telling et al., 1995). These issues have been difficult to resolve because of the inability thus far either to produce de novo infectious PrP^{SC} or to effectively recapitulate sustained prion propagation in vitro.

Our data show that for the yeast [PSI^{+}] prion, the species barrier results from a remarkable specificity in interaction between the prion protein itself, mediated by a well-defined epitope in the PrD (Figure 6D). First, for all of the species examined, only overexpression of homotypic PrD induces prion formation. Second, as with [PSI^{+}] (Tuite et al., 1981; Chernoff et al., 1995), transient overexpression of the molecular chaperone HSP104 or exposure to guanidine, which most likely acts by modulating cellular factors, cures the [CHI_{CA}^{+}] prion. Thus, at least for these components, the ability of the *S. cerevisiae* folding machinery to regulate the aggregation state of the *C. albicans* PrD is conserved. Third, in vitro selective seeding experiments faithfully recapitulate the species barrier. Strikingly, even in an equimolar solution of $PrDM_{SC}$ and $PrDM_{CA}$,

seeded polymerization of the *S. cerevisiae* protein leads to rapid formation of pure $PrDM_{SC}$ fibrils without affecting the polymerization kinetics of the *C. albicans* protein. Finally, chimeric analyses reveal that prion specificity of *S. cerevisiae* can be conferred to the PrD of another species by a substitution of 19 amino acid residues near the PrD N-terminus.

This localization of a species-determining region helps reconcile disparate results from previous efforts to dissect PrD function. A screen for mutant PrDs that either fail to interact with or cause curing of the endogenous [PSI^+] prion found that these mutations located between residues 8 and 26 (DePace et al., 1998). However, sequences C-terminal to this region have been shown to be critical for prion function (Doel et al., 1994; Liu and Lindquist, 1999). Moreover, expansion of the imperfect oligopeptide repeats, also located outside of this species-determining domain, dramatically enhances the rate of prion formation. These observations, together with our in vitro selective seeding experiments, suggest a model (Figure 6D) in which the N-terminus resides on the surface of the growing amyloid and contributes much of the specificity of PrD monomer recruitment. By contrast, the more C-terminal region might largely be involved in intra-molecular interactions that stabilize the prion form. Substitution of *S. cerevisiae* residues 8-26 into PrD_{CA} would thus change the surface of the amyloid, allowing it to incorporate PrD_{SC} monomers. By contrast, expansion or deletion of the imperfect oligopeptide repeats would lead to increased or decreased stabilization of the PrD, thereby modulating its tendency to form amyloids without altering prion specificity.



An Epigenetic Switch: Prion-based Protein Regulation?

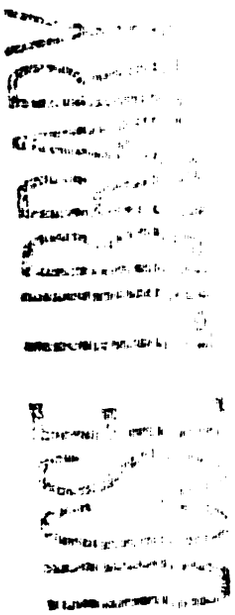
Alleles of the mammalian prion protein prone to prion formation are rare, as would be expected given the devastating effects of these diseases. By contrast, the ability of the N-terminal domain of Sup35p to form a prion is conserved across the budding yeasts. This functional conservation is remarkable since the PrD sequence is not strongly conserved and earlier mutational analyses showed that even single point mutations in the *S. cerevisiae* PrD can inhibit prion formation (DePace et al., 1998). These observations raise the intriguing possibility that rather than being pathogenic, [PSI$^+$] might be an evolutionarily beneficial state. However, we can not rule out the possibility that the retention of prion function is a by-product of conservation of an unidentified PrD function (Bailleul et al., 1999). Consistent with a beneficial role for the [PSI$^+$] prion, Tuite and coworkers found that following exposure to high temperature or ethanol some [PSI$^+$] yeasts show enhanced survival compared to isogenic [psi$^-$] yeasts (Eaglestone et al., 1999). In addition, we find that over-expression of $PrDM_{CA}$-GFP in *C. albicans* induces formation of punctate foci, although it remains to be seen whether these aggregates can propagate in a prion-like manner (unpublished observations)

As a mechanism of inheritance, prions provide a number of potentially advantageous features (Lindquist, 1997). Prion formation allows a cell to inhibit the activity of a specific protein and propagate this state indefinitely while retaining the potential to restore the original protein activity. Moreover, the rate of conversion to and from the prion state can be dramatically enhanced by changes in the environment (Tuite et al., 1981). Finally, because prion domains are modular (Ter-Avanesyan et al., 1993;

Patino et al., 1996), fusion to prion domains could potentially allow prion-based regulation of a broad range of proteins.

For a prion to serve as an epigenetic switch, it must propagate specifically without interfering with other proteins. Specificity of prion interactions, resulting from differences in primary sequence and manifested as a barrier to cross-species prion induction, could serve as mechanism to prevent such improper interactions. Consistent with the proposal that multiple prion states could propagate independently in the same cell, we have identified a new prion-forming domain in *S. cerevisiae* (the N-terminal portion of New1p) and have shown that its prion state propagates independently of [PSI^+]. Furthermore, we find that over-expression of a Ure2p fragment which efficiently induces the [URE3] prion (Edskes et al., 1999) does not induce [PSI^+] nor is Ure2p incorporated into [PSI^+] aggregates (data not shown), indicating that the existence of one prion within a cell does not promote the appearance of others.

If multiple different prions can exist independently within a single cell, then how many different prions are there? The spectrum of Sup35p PrD sequences, together with earlier mutational analyses, provide a wealth of data to search for novel prions. Despite little strict sequence conservation, all of the examined Sup35p PrDs as well as the Ure2p prion domain contain an extremely high Gln/Asn and low charge content (Figures 1B and 7A). Moreover, in some neurodegenerative diseases, expansion of polyGln repeats leads to intranuclear aggregates in vivo and self-propagating amyloids in vitro (for review, see Bates et al., 1998). A search of genomic databases for domains with amino acid content comparable to Sup35p PrDs revealed a handful of such domains in both yeast and nematodes (Figure 7A and M. Michelitsch and JSW, in preparation), at least one of



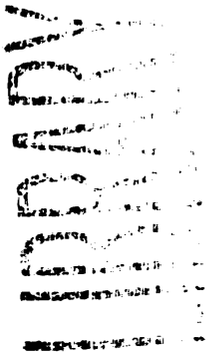
which (New1p) forms a prion in yeast. The challenge now is to determine how many other Gln/Asn rich domains can form stable, self-propagating prions, and what the physiological role for such novel prions may be.

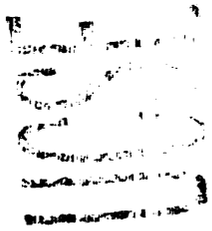
Experimental Procedures

General Procedures and Reagents

Isogenic *S. cerevisiae* [psi$^-$] and [PSI$^+$] strains 74-D694 [*Mat* **a**, *ade1-14*(UGA), *his3, leu2, trp1, ura3*] (Chernoff et al., 1995) were used for all experiments except the Sup35p deletion studies which used YJW541 [*Mat* **a** , *ade1-14, his3, leu2, trp1, ura3, sup35::TRP1*]. *C. albicans* SC5314, *K. marxianus* and *P. pastoris* were gifts from A. Johnson, E. Blackburn and C. Craik, respectively. *K. lactis* and *S. ludwigii* were gifts from I. Herskowitz. *P. methanolica* (56509) and *Z. rouxi* (48232) were obtained from ATCC. Nucleic acid, immunoblot and yeast manipulations were performed according to standard protocols (Ausubel, 1987). All plasmid sequences were confirmed by dye termination sequencing (Perkin-Elmer).

Oligonucleotide primers were as follows:
P1 GGGGGATCCGTCGACACTAGTACAATGTCTGACCAACAGAATACT;
P2 CCCAGATCTTCTAGAATCCTTGACAACTTCTTCGTC;
P3 GGGGGATCCGTCGACACTAGTACAATGTCTCAAGATCAACAGCAA;
P4 CCCAGATCTTCTAGAATCGTTGACAATGGAGGCATC;
P5 CGACGAGGATCCGTCGACATGTCAGACCAACAAAATCAAGACCAAGGG;
P6 CAAAGTGAATTCAGATCTATCTTTAACGACTTCTTC;
P7 GGGCGGCATATGTCTGACCAACAGAATACTCAG;
P8 GCCCGAATTCTTAGTGATGATGGTGATGGTGGTGATCCTTGACAACTTC
TTCGTC;
P9 CCGGAATTCTTAATGGTGATGATGGTGATGAGCGTAATCTGGAACGTCATA;
P10 AACGGTTGGGTCATCCATCTT;
P11 TTTGTTGGTATCCATGACCCATGACAAGTACCA;
P12 GGCCCAGTGAGCAGAGTGACGGAGGACTCGAGCTCAAGCTAATCCGGCGTGCA
TTGAC;
P13 GATCGTCAATGCACGCCGGATTTACGCC;
P14 CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT
P15 ACCTGCCC;
P16 GCGCGTCGACATGCCTCCAAAGAAGTTTAAGG;
P17 GCGCCGAATTCGGGAGATCTTTGATTTTTGCAATCAGTGATACTTTGACA
TTCAGG;
Q0 CCAGTGAGCAGAGTGACG;
Q1 GACTCGAGCTCAAGCTAA;
AP-1 CCATCCTAATACGACTCACTATAGGGC;
AP-2 ACTCACTATAGGGCTCGAGCGGC.



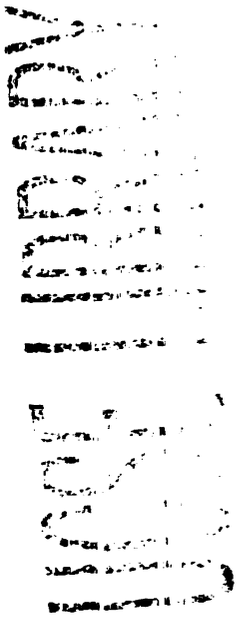


Cloning of foreign SUP35p PrDs

To clone Sup35 PrDM domains, we modified the RACE procedure for capturing cDNA 5' ends (Frohman, 1993) to allow analysis of genomic DNA. Yeast genomic DNA from the desired species was digested to completion by a restriction enzyme that results in either 5' GATC overhangs or blunt ends. Linkers compatible with either the GATC (annealed P12, P13) or blunt ends (annealed P14, P15) were then ligated onto the ends of the genomic fragments. PCR amplification was performed using the ligated fragments as templates, the EF specific primer P10 and linker primer Q0 or AP1 for the GATC or blunt ended fragments, respectively. Amplified products were used as templates in a second round of PCR using nested EF specific primer P11 and linker primer Q1 or AP2 for the GATC and blunt ended derived fragments, respectively. For reactions resulting in a single distinct band, PCR products were purified by agarose gel electrophoresis and sequenced.

Plasmid Construction

All yeast expression vectors used a previously described (DePace et al., 1998) modular insert composed of a promoter domain flanked by 5' Xho1 and 3' BamHI sites, a PrDM/New1p1-153 domain flanked by 5' BamHI/Sal1 and 3'BglII/EcoRI sites, and an EF/GFP/eCFP/eYFP domain flanked by 5'EcoRI and 3'SacI sites. For the maintainer plasmid, the insert was cloned into the XhoI-SacI sites of a URA3 marked CEN/ARS plasmid (pRS316), and a triple HA epitope was inserted between the BglII/EcoRI sites. For the inducer plasmids, the insert was cloned into the XhoI-SacI sites of a LEU2 marked 2μm plasmid (pRS425). To create the foreign PrDM inducer and maintainer



plasmids, $PrDM_{CA}$ (primers P1, P2), $PrDM_{PM}$ (P3, P4), or $PrDM_{KL}$ (P5, P6) domains were PCR amplified from genomic DNA and inserted into the BamHI/EcoRI sites of the appropriate $PrDM_{SC}$ encoding plasmid. To create the New1p inducer and maintainer plasmids, the first 153 codons of *NEW1* were PCR amplified (P16 and P17) and inserted into the Sal1/EcoR1 sites of the appropriate $PrDM_{SC}$ encoding plasmid. The chimeras $PrDM_{SC1\text{-}39CA}$ (encoding a protein in which residues 40-124 of $PrDM_{SC}$ were replaced with residue 44-140 from $PrDM_{CA}$), $PrDM_{CA1\text{-}39SC}$ (encoding a protein in which residue 1-39 of $PrDM_{SC}$ was replaced with residue 1-44 of $PrDM_{CA}$) and $PrDM_{SC8\text{-}26CA}$ (encoding a protein in which residue 1- 7 and 27-124 of $PrDM_{SC}$ were replaced with residues 1-7 and 30-140 from $PrDM_{CA}$, respectively) were all derived from the $PrDM_{SC}$ inducer plasmid by seamless cloning (Stratagene). Plasmids encoding eCFP and eYFP were obtained from Clontech. For bacterial expression, 6xHis tagged $PrDM_{CA}$ with (P7, P9) or without (P7, P8) an HA tag was PCR amplified and inserted into NdeI/EcoRI sites of a T7 expression vector.

In Vivo GFP Foci Formation

Yeast carrying the indicated species of PrDM-GFP inducer plasmid were grown to early-log phase in SD-LEU, and induced with 50 μM $CuSO_4$. At indicated times, cells were examined by fluorescent microscopy (Olympus BX60) and photographed by CCD camera (Photometrics). For quantitative measurement, random fields were chosen and percentage of fluorescent cells with punctate foci were calculated. Double fluorescence images were collected by wide-field 3D deconvolution microscopy (Agard et al., 1989) using filters optimized for CFP and YFP fluorescence (Chroma).

[PSI$^+$] Conversion Assay and Two-Plasmid Assay for Aggregation

For [PSI$^+$] conversion assay, [psi$^-$] yeast freshly transformed with the indicated inducer plasmids were grown in 10ml SD-LEU to early-log phase and induced with 50 μM $CuSO_4$. After 24 hours, samples were plated onto SD-ADE. For the two-plasmid [CHI$^+$] conversion assay, [PSI$^+$] yeast freshly transformed with the indicated species of maintainer and inducer plasmids were grown in 10 ml SD-URA-LEU to early-log phase and induced with 50 μM $CuSO_4$. At indicated times, samples were plated onto SD-URA-ADE. For both assays, after five days incubation at 30°C, visible colonies were counted. For the [CHI$^+$] induction experiments in the sup35 deletion strain, YJW541 initially carrying p316SpSupEF (Depace et al, 1998) and a HIS marked PrDMCA-EF maintainer plasmid were grown on 5-FOA to ensure loss of p316SpSupEF yielding the [psi° chi$^-$] strain. The [psi° CHI$^+$] was derived by transient overexpression of $PrDM_{CA}$ from an inducer plasmid, selection on medium lacking ADE and subsequent loss of inducer plasmid. For the [NU$^+$] induction experiments, [PSI$^+$] yeast freshly transformed with the New1p inducer and maintainer plasmids and treated as described above for the [CHI$^+$] experiments.

Immuno-Electron Microscopy

PrDM fibrils (8μg) produced from a conversion reaction containing equimolar $PrDM_{SC}$ and $PrDM\text{-}HA_{CA}$ were incubated with 50μg rabbit polyclonal antibody raised against $PrDM_{SC}$ (HTI Bio-products) and 100μg of the mouse monoclonal anti-HA antibody 16B12 (Babco) for 20 minutes at room temperature. 5 μl each of 5 nm gold conjugated to anti-rabbit and 15 nm gold conjugated anti-mouse secondary antibodies (Nanoprobes)

were added and incubated for additional 20 minutes. The solution was added to a glow discharged carbon coated nickel grid, washed extensively with water, and stained with 2% uranyl acetate. Electron micrographs were collected with EM400 Transmission Electron Microscope (Phillips).

Other Assays

Centrifugation assays were performed as previously described (DePace et al., 1998). Recombinant PrDM was purified under denaturing conditions as described previously (Glover et al., 1997; DePace et al., 1998). Congo red binding assays were carried out as described previously (DePace et al., 1998).

Acknowledgments

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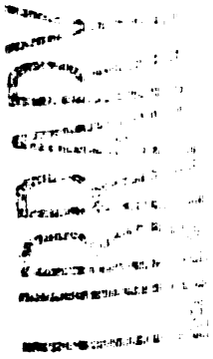
Figure legends

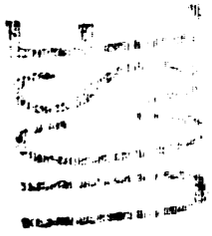
Figure 1. Evolutionary Analysis of Sup35p PrDs

(A) Phylogenetic relationship of yeast species based on 26S RNA sequences (Kurtzman, 1994). For comparison, the fission yeast *S. pombe* whose Sup35p does not have a PrD (Ito et al., 1998), is shown. Scale (percent divergence) is denoted on top. Resende et al. have previously submitted a full length sequence of *SUP35* from *C. albicans* (AF020554) to public databases.

(B) Plot of percent of charged (Arg, Lys, Asp, Glu) versus Gln/Asn residues for the Sup35 PrD homologs as well as the *S. cerevisiae* Ure2p prion domain. For comparison, the full-length *S. cerevisiae* ORFs including specifically the entire Sup35p and Ure2p proteins are also shown.

(C) Amino acid sequence comparison of PrD homologs. Amino acid identities and similarities are indicated by dark gray and light gray boxes, respectively. Sequences were aligned using the ClustalW algorithm (Higgins et al., 1996). The black bar denotes the approximate location of the oligopeptide repeats.





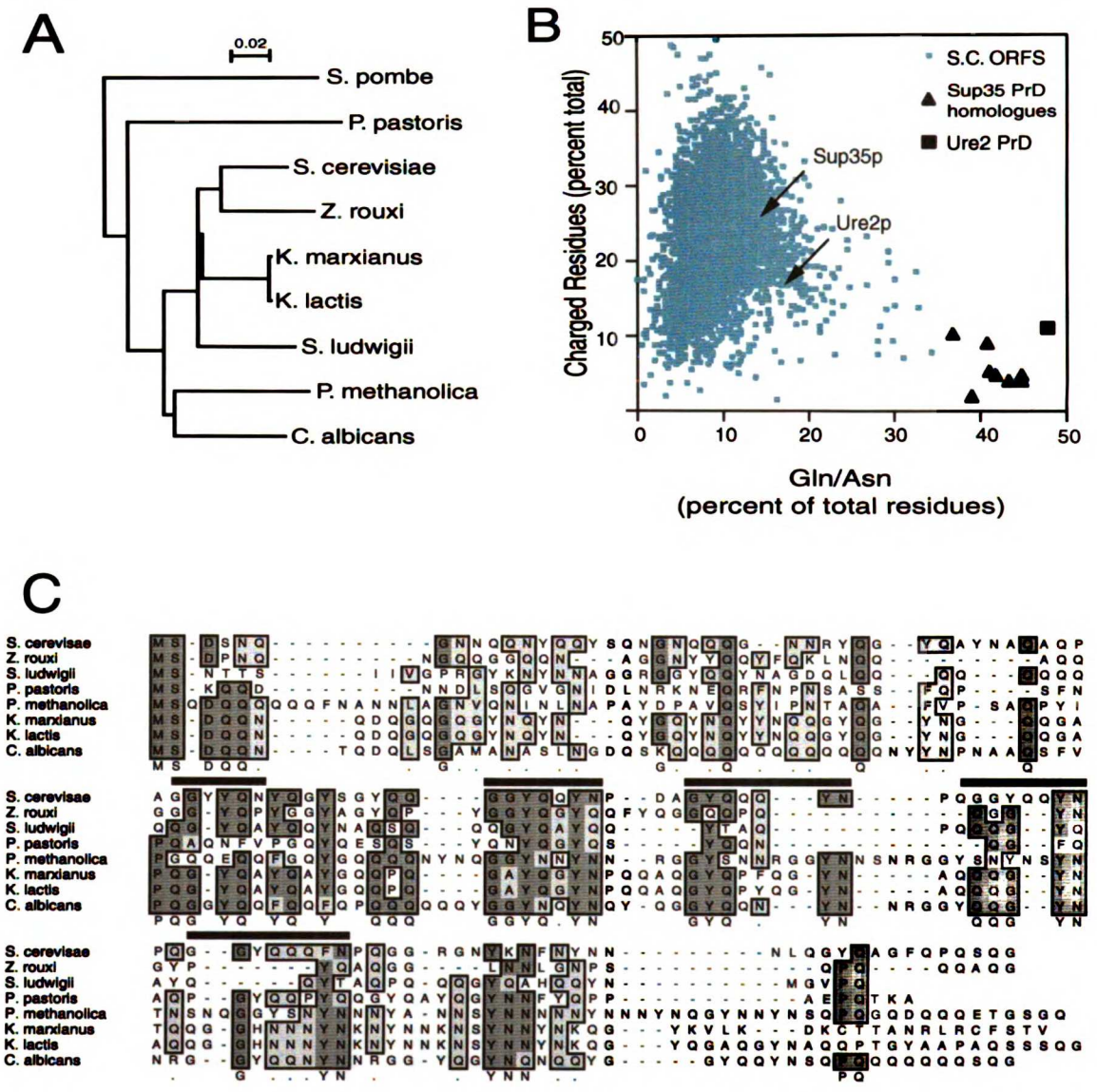

A
0.02
S. pombe
P. pastoris
S. cerevisiae
Z. rouxi
K. marxianus
K. lactis
S. ludwigii
P. methanolica
C. albicans
B
S.C. ORFS
Sup35 PrD homologues
Ure2 PrD
Sup35p
Ure2p
Charged Residues (percent total)
Gln/Asn
(percent of total residues)
C
S. cerevisiae
Z. rouxi
S. ludwigii
P. pastoris
P. methanolica
K. marxianus
K. lactis
C. albicans

Figure 2. Examination of the Ability of Foreign PrDs to Interact with *S. cerevisiae* PrD

Throughout the figures CA, KL, PM, and SC refer to $PrDM_{CA}$, $PrDM_{KL}$, $PrDM_{PM}$, and $PrDM_{SC}$, respectively. Ψ^+ and Ψ^- indicate the presence or absence of the *S. cerevisiae* PrD aggregate. χ^+ and χ^- indicate the presence or absence of the foreign PrDM aggregates. All quantitative experiments were conducted in triplicate and errors are indicated.

(A) Effect of foreign PrDM-EF fusion proteins on the [PSI^+] suppression phenotype. [PSI^+] yeast expressing the indicated species of PrDM-EF fusion were plated on low or no ADE medium. For comparison, [psi-] yeast are also shown. On top is shown a schematic model explaining the antisuppression phenotype (red colonies on low ADE and lack of growth on no ADE) resulting from expression of foreign PrDM. In [psi^-] cells, soluble Sup35p mediates translational termination at the ade1 nonsense mutation (red bar). In suppressed [PSI^+] yeast, absence of soluble Sup35p results in translational readthrough. Failure of foreign PrDM-EF to be incorporated into the [PSI^+] amyloid leads to soluble EF and antisuppression.

(B) Illustration of GFP visual assay. On top is shown a schematic of the copper inducible PrDM-GFP plasmid. From left to right are examples of diffuse GFP fluorescence in [psi^-] cells, discrete foci in [PSI^+] cells and de novo aggregates formed by overexpression of $PrDM_{CA}$-GFP, and $PrDM_{PM}$-GFP as observed by fluorescence microscopy.

(C) Quantitative foci formation kinetics. [PSI^+] and [psi^-] yeast, as indicated, containing either $PrDM_{SC}$-GFP (left) or the indicated foreign PrDM-GFP (right) were induced in

early-log phase, and results are plotted as the percentage of fluorescent cells with visible foci as a function of induction time.

(D) Induction of [PSI^+] by overexpression of PrDM-GFP. [psi^-] yeast containing a plasmid encoding the indicated species of PrDM-GFP or an empty control plasmid were plated on media lacking adenine either before (uninduced) or after 24 hours of copper induction. The number of [PSI^+] colonies (CFUs) per 10^5 cells is plotted. (Inset) Immunoblots of inducer PrDM expression following induction.

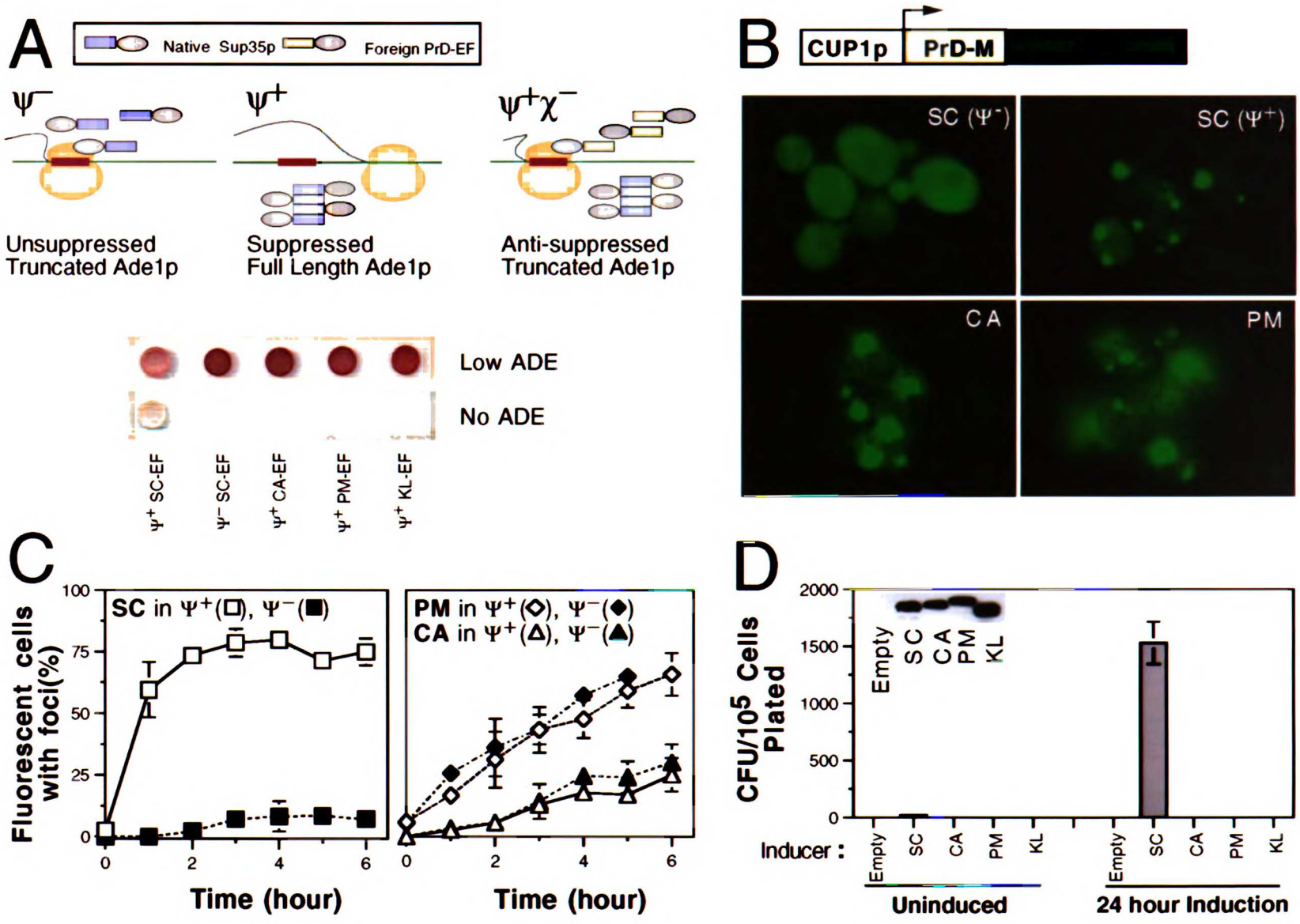

A
Native Sup35p
Foreign PrD-EF
Ψ⁻
Ψ⁺
Ψ⁺χ⁻
Unsuppressed
Truncated Ade1p
Suppressed
Full Length Ade1p
Anti-suppressed
Truncated Ade1p
Low ADE
No ADE
Ψ⁺ SC-EF
Ψ⁻ SC-EF
Ψ⁺ CA-EF
Ψ⁺ PM-EF
Ψ⁺ KL-EF
B
CUP1p
PrD-M
SC (Ψ⁻)
SC (Ψ⁺)
CA
PM
C
Fluorescent cells with foci(%)
SC in Ψ⁺(□), Ψ⁻(■)
PM in Ψ⁺(◇), Ψ⁻(◆)
CA in Ψ⁺(△), Ψ⁻(▲)
Time (hour)
Time (hour)
D
CFU/10⁵ Cells Plated
Empty
SC
CA
PM
KL
Inducer :
Uninduced
24 hour Induction

Figure 3. Examination of the Ability of Homotypic or Heterotypic PrD Overexpression to Induce [CHI$^+$] Prions

(A) Schematic of [CHI$^+$] two-plasmid induction assay. Expression of foreign PrDM-EF from the maintainer plasmid in [PSI$^+$ chi$^-$] cells results in soluble EF activity, leading to antisuppression. Aggregates are introduced by transient overexpression of the PrDM-GFP fusion either from the same (homotypic) or different (heterotypic) species as the maintainer. The ability of the introduced aggregates to convert the PrDM-EF fusion yielding [PSI$^+$ CHI$^+$] yeast can be monitored by a persistent change in suppression phenotype. Below, for each of the three indicated models, the predicted effect of homotypic and heterotypic inducers on [CHI$^+$] conversion is shown.

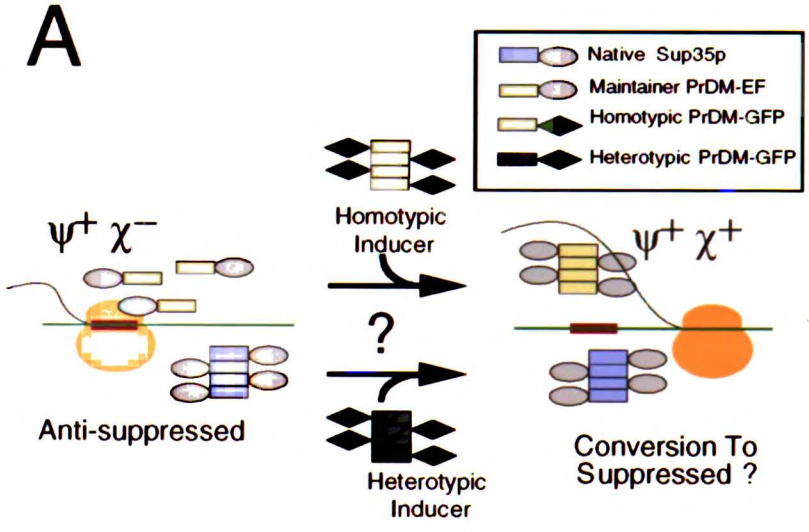
(B) Induction of [CHI$^+$] by overexpression of homotypic and heterotypic PrDM-GFP inducers. [PSI$^+$ chi$^-$] yeast containing HA epitope-tagged *C. albicans*, *P. methanolica*, or *K. lactis* maintainer plasmid indicated by CA-EF, PM-EF, and KL-EF, respectively, and the indicated inducer plasmid were grown in selective medium to early-log phase. Following 40 hours of copper induction, the number of [PSI$^+$ CHI$^+$] colonies (CFUs) was determined by plating onto no ADE medium.

(C) In vivo observation of species specific aggregation. Shown are fluorescence images of [PSI$^+$] yeast co-expressing fusion proteins between the indicated species of PrDM and a yellow (YFP) and cyan (CFP) variant of GFP driven by an inducible copper promoter. The left, middle and right panels display the Cyan, Yellow and Combined fluorescence, respectively. Schematics on the right indicate the identity of the PrDM-GFP variant fusion proteins. Dotted lines denote the cell outlines.

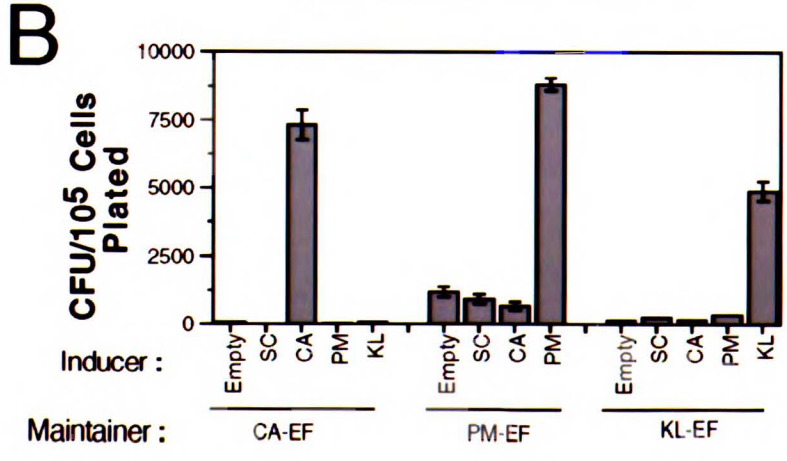
(D) Stability of propagation of a [CHI^+] prion. A robust [PSI^+ CHI_{CA}^+] isolate was patched serially onto complete medium. Following the indicated number of passages, an aliquot was patched on either low or no ADE medium, as indicated, to test for the presence of the suppression phenotype caused by [CHI^+]. For comparison, [psi^-] and [PSI^+] yeast are shown.

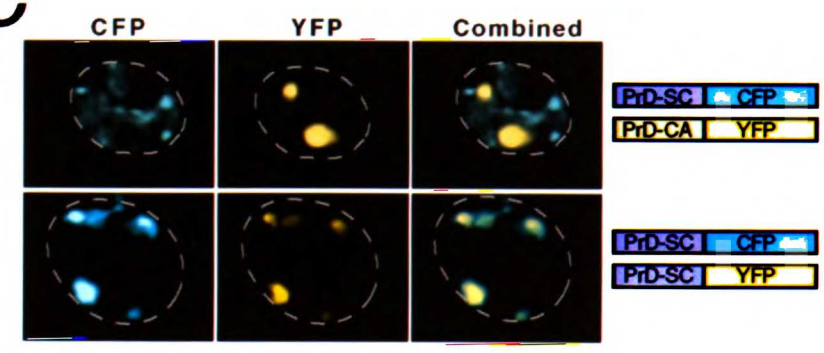
(E) Centrifugation assay to examine the solubility of foreign PrDM-EF. Extracts from the indicated yeast strains were centrifuged at 100,000g, and soluble (S) or pelleted (P) fractions were assayed by immunoblots with antibodies (α-HA) specific to the epitope-tagged $PrDM_{CA}$-EF or with antibodies (α $PrDM_{SC}$) specific to the *S. cerevisiae* Sup35p.

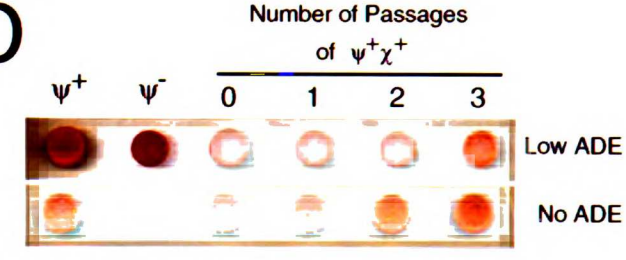
(F) Formation and propagation of [CHI^+] in the absence of *S. cerevisiae* Sup35p. A yeast strain was constructed in which the chromosomal *SUP35* gene was deleted and replaced with an episomal copy $PrDM_{CA}$-EF. Shown are examples of this strain either prior to or after induction of the [CHI^+] prion, denoted [Ψ° χ^-] and [Ψ° χ^+], respectively, plated on either low or no ADE medium. For comparison [PSI^+] and [psi^-] yeast are also shown.

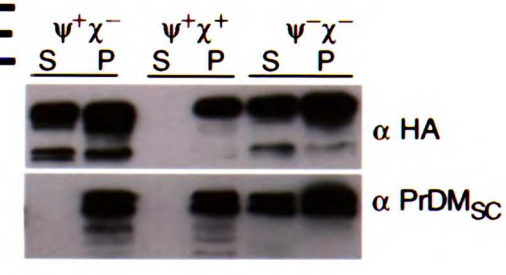


	Homotypic Inducer	Heterotypic Inducer
Not Prion	No Conversion	No Conversion
Species Barrier	Conversion	No Conversion
No Barrier	Conversion	Conversion









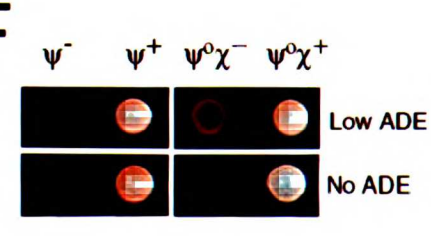


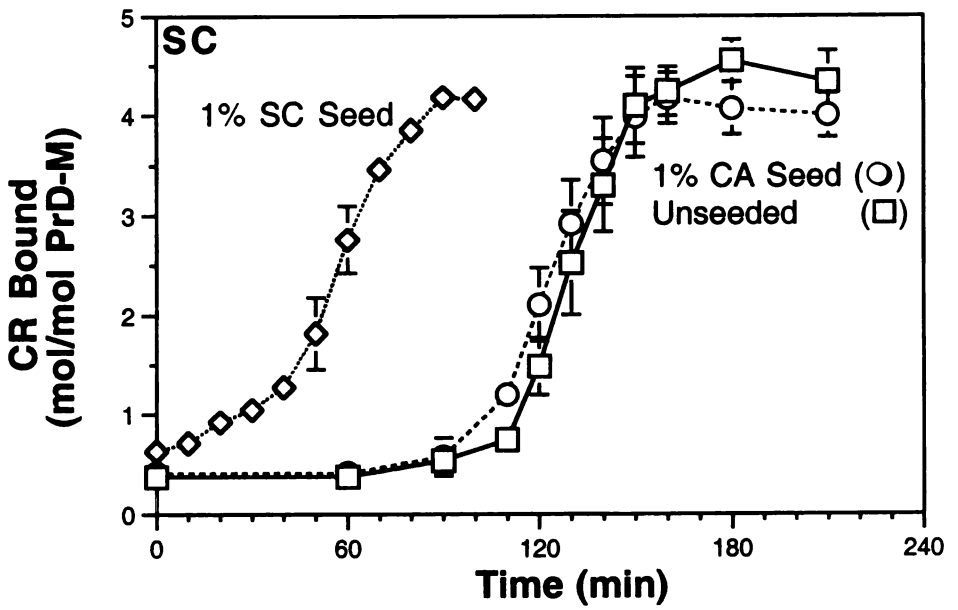
Figure 4. In Vitro Amyloid Fibril Formation of $PrDM_{SC}$ and $PrDM_{CA}$

To initiate conversion, concentrated pure PrDM protein in urea was diluted into conversion buffer and subjected to continuous slow rotation. At indicated times, the extent of fibril formation was assayed by Congo red binding. Each curve was conducted in triplicate. Bars indicate errors larger than the symbol size.

(A) Conversion kinetics of 2.5 μM *S. cerevisiae* PrDM in the absence (square), and presence of 1% (wt/wt) pre-formed $PrDM_{SC}$ (diamond) and $PrDM_{CA}$ (circle) fibrils.

(B) Conversion kinetics of 2.5 μM *C. albicans* PrDM in the absence (square), and presence of 1% (wt/wt) pre-formed $PrDM_{CA}$ (diamond) and $PrDM_{SC}$ (circle) fibrils.

A


SC
1% SC Seed
1% CA Seed (○)
Unseeded (□)
CR Bound
(mol/mol PrD-M)
Time (min)
0
1
2
3
4
5
0
60
120
180
240

B

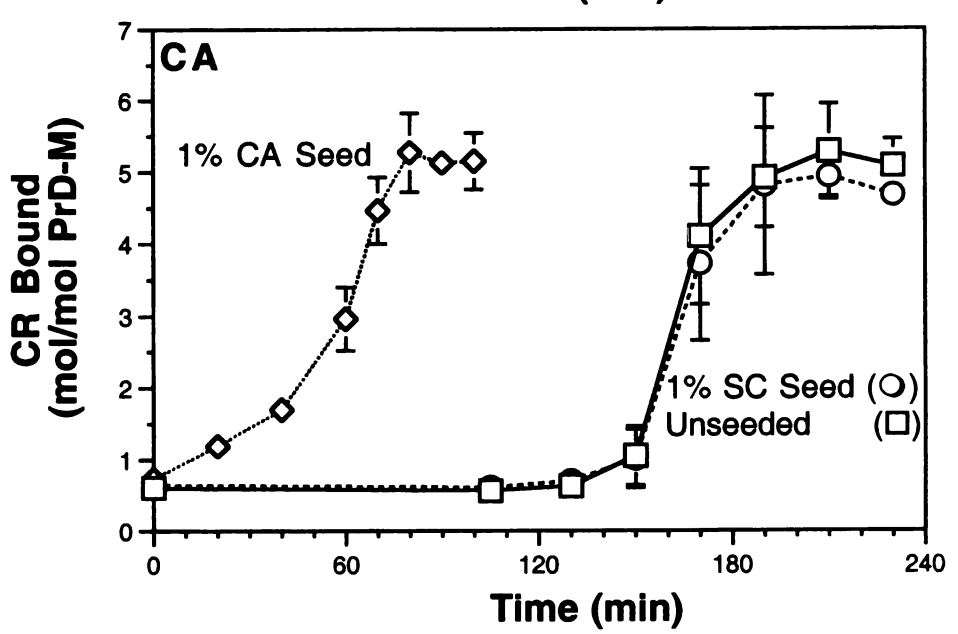

CA
1% CA Seed
1% SC Seed (○)
Unseeded (□)
CR Bound
(mol/mol PrD-M)
Time (min)
0
1
2
3
4
5
6
7
0
60
120
180
240

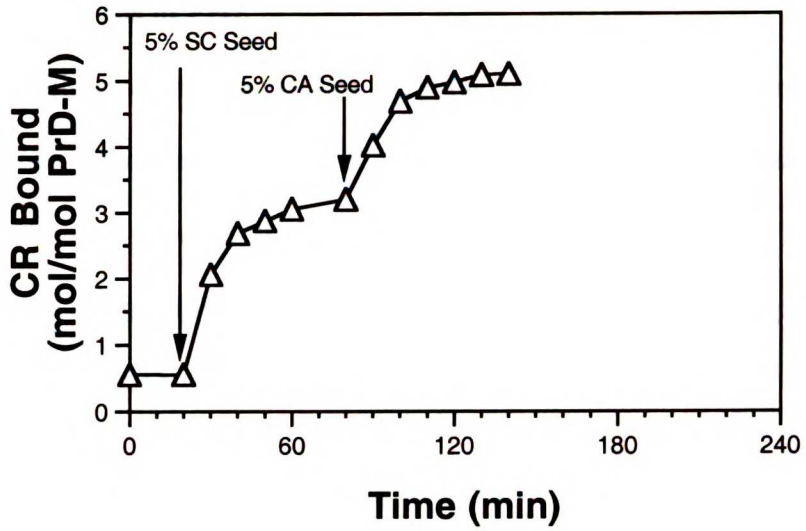
Figure 5. In vitro Amyloid Fibril Formation of a $PrDM_{SC}$ / $PrDM_{CA}$ Mixture

(A) Conversion kinetics of equimolar mixture (2.5μM) of $PrDM_{SC}$ and $PrDM_{CA}$ seeded with 5% (wt/wt) $PrDM_{SC}$ fibril and subsequently with $PrDM_{CA}$ fibril at indicated timepoints.

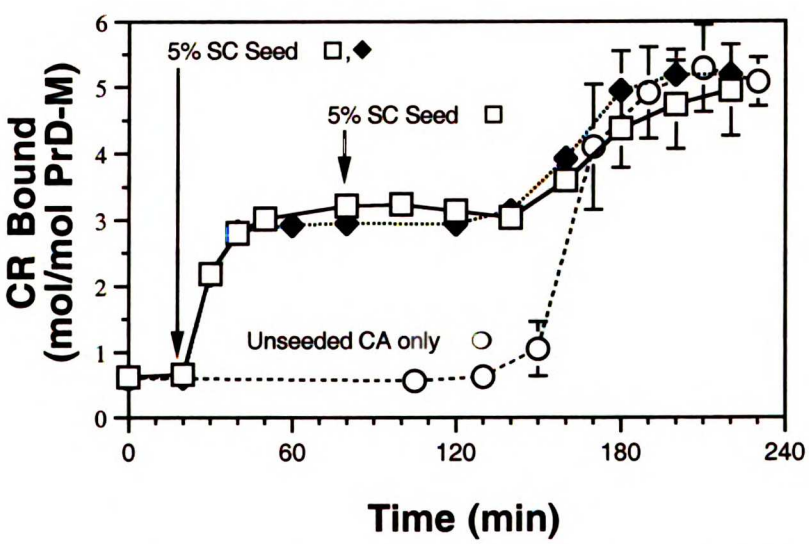
(B) Conversion kinetics of an equimolar mixture (2.5μM) of $PrDM_{SC}$ and $PrDM_{CA}$ seeded with 5% (wt/wt) $PrDM_{SC}$ fibril only (filled diamond) twice with $PrDM_{SC}$ fibril (square) compared to unseeded kinetics of $PrDM_{CA}$ (circle).

(C) Electron micrographs of the converted $PrDM_{SC}$ and $PrDM_{CA}$ mixture. $PrDM_{SC}$ and $PrDM_{CA}$ are labeled by 5nm and 15nm gold particles, respectively, using species specific antibodies. Examples of fibers decorated exclusively with 5nm (left) or 15nm (right) gold particles from the same electron micrograph are shown. Although some fibrils were poorly labeled, no fibers were decorated by both antibodies. Scale bar corresponds to 100nm.

A


5% SC Seed
5% CA Seed
CR Bound
(mol/mol PrD-M)
Time (min)
0
60
120
180
240
1
2
3
4
5
6

B


5% SC Seed
5% SC Seed
Unseeded CA only
CR Bound
(mol/mol PrD-M)
Time (min)
0
60
120
180
240
1
2
3
4
5
6

C

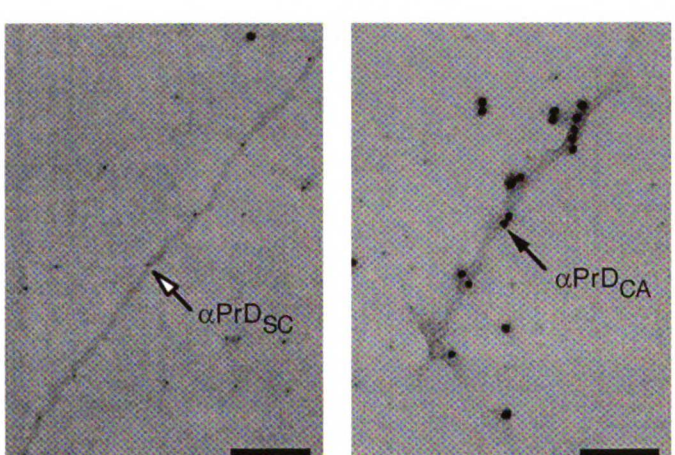

αPrD$_{SC}$
αPrD$_{CA}$

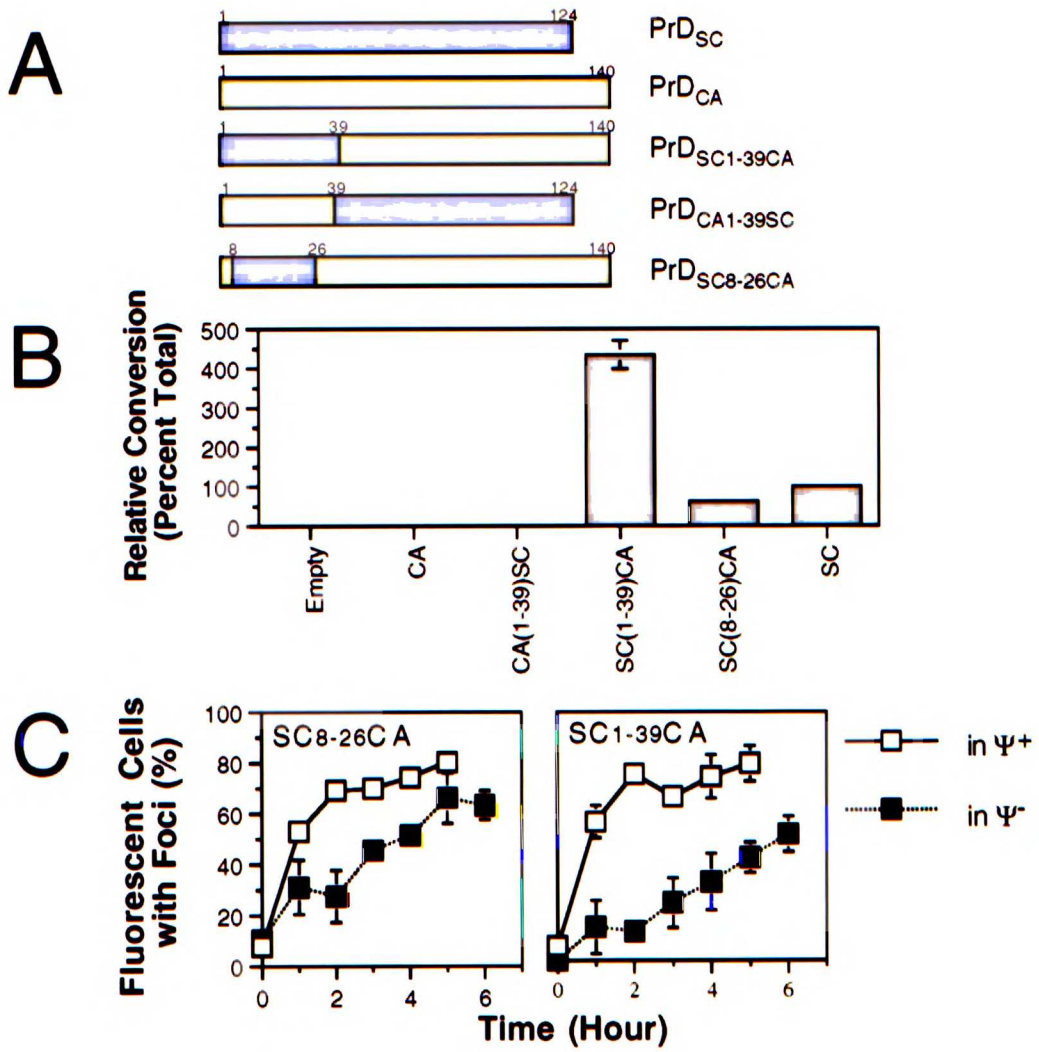
Figure 6. Chimeric Analysis of PrD

(A) Schematics of chimeric PrD sequences. Residue numbers are indicated on top. Chimeric region residue numbers are that of *S. cerevisiae* sequence. *S cerevisiae* regions are denoted by purple and *C. albicans* by light yellow.

(B) Efficiency, relative to $PrDM_{SC}$, of conversion of [psi$^-$] to [PSI$^+$] by overexpression of the indicated PrDM-GFP fusion.

(C) Quantitative foci formation kinetics. [PSI$^+$] and [psi$^-$] yeast, as indicated, containing the indicated chimeric PrDM-GFP fusion were grown in selective medium and induced at early-log phase. Shown is a plot of the percentage of fluorescent cells with visible foci as a function of induction time.

(D) Hypothetical model to explain chimera conversion data. The N-terminal region of PrD is envisioned as having many of the critical species-specific interactions involved in recruitment of new monomers to the growing amyloid, whereas the C-terminal region may be primarily involved in intramolecular interactions that stabilize the prion form.


A
1
124
PrD$_{SC}$
1
140
PrD$_{CA}$
1
39
140
PrD$_{SC1-39CA}$
1
39
124
PrD$_{CA1-39SC}$
8
26
140
PrD$_{SC8-26CA}$
B
Relative Conversion (Percent Total)
500
400
300
200
100
0
Empty
CA
CA(1-39)SC
SC(1-39)CA
SC(8-26)CA
SC
C
Fluorescent Cells with Foci (%)
100
80
60
40
20
0
SC8-26CA
SC1-39CA
0
2
4
6
Time (Hour)
in Ψ+
in Ψ-
D
N-
N-
S. cerevisiae
C. albicans

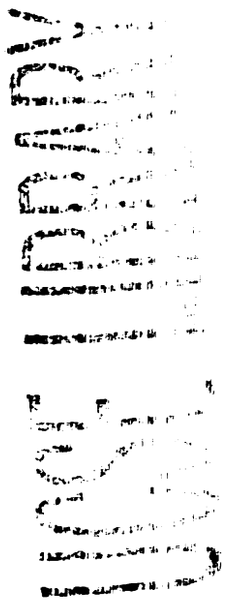
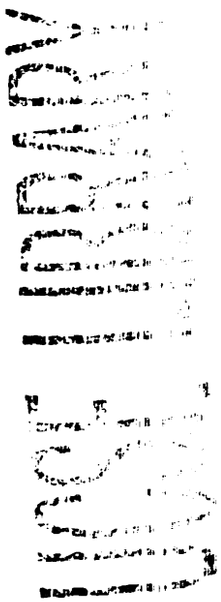


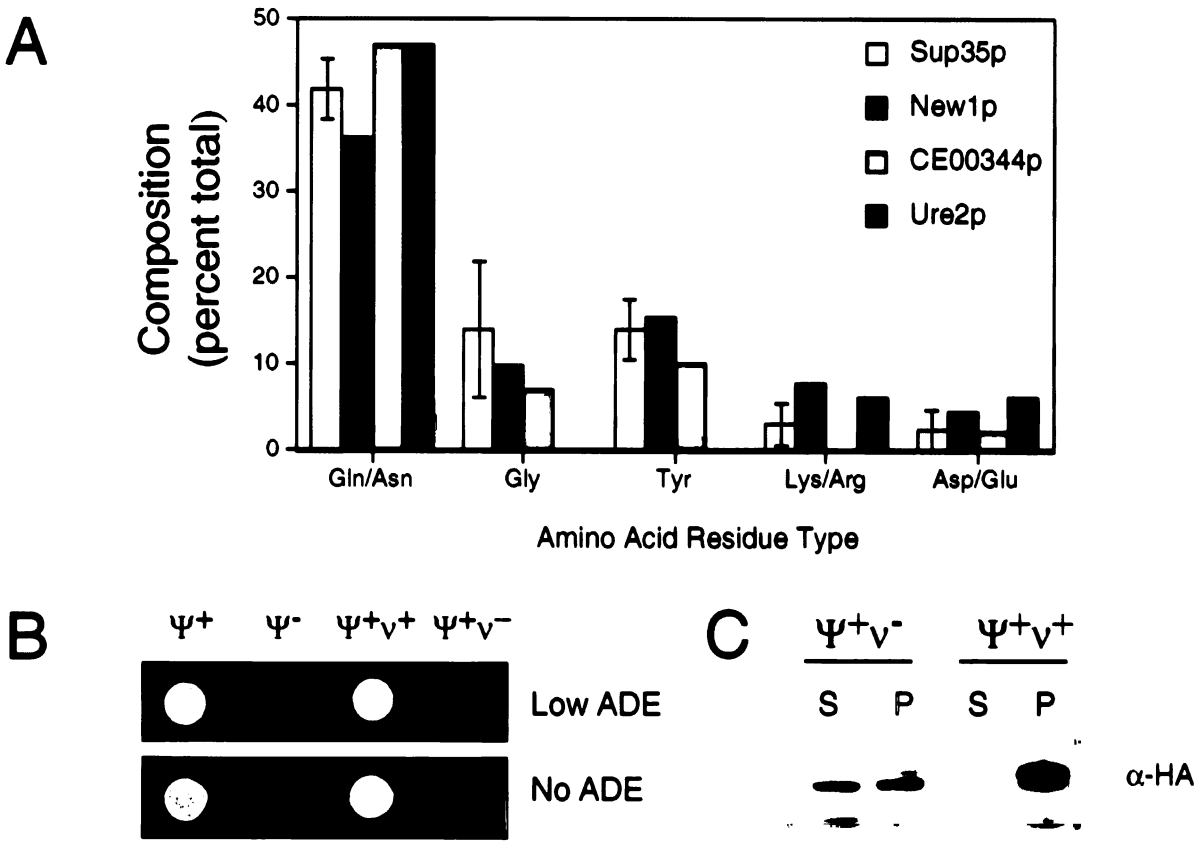
Figure 7. The N-terminus of New1p Can Act as a Prion.

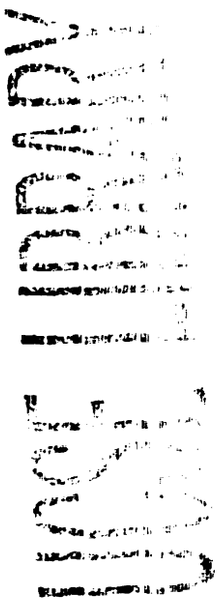
(A) The amino acids content of Sup35p PrD, residues 10-100 of New1p, residues 230 to 319 from the *C. elegans* ORF CE00344, and the first 90 amino acids of Ure2p are displayed. For the PrD plot, the average value among the various yeast species is used with error bars indicating the maximum variation.

(B) Phenotypic consequences of prion formation by the New1p$_{1-153}$-HA$_3$-EF fusion protein. Shown are examples of [PSI$^+$ nu$^-$] and [PSI$^+$ NU$^+$] isolates grown on medium with low amounts of adenine (above) and no adenine (below), illustrating the conversion from an antisuppressed to a suppressed state following [Nu$^+$] induction. For comparison, [PSI$^+$] and [psi$^-$] strains are shown.

(C) Centrifugation assay to follow the solubility of the New1p$_{1-153}$-HA$_3$-EF fusion, performed as in Figure 3E.




A
50
40
30
20
10
0
Composition (percent total)
Sup35p
New1p
CE00344p
Ure2p
Gln/Asn
Gly
Tyr
Lys/Arg
Asp/Glu
Amino Acid Residue Type
B
Ψ+
Ψ-
Ψ+ν+
Ψ+ν−
Low ADE
No ADE
C
Ψ+ν-
Ψ+ν+
S
P
S
P
α-HA



Chapter 4

Prion-Based Switch of Biological Functions

This chapter describes the construction of and unpublished data regarding the fusion of Sup35 PrD to the cytoplasmic enzyme His3p and nuclear transcriptional activator LexAB42. Also discussed briefly is the use of these reagents in screens for factors that modulate the formation or propagation of the [PSI^+] factor as well as the broader biological implication of the modularity of this domain.

Summary

The prion conformation of yeast [PSI$^+$] factor is determined by the amino-terminal PrD of the translational termination factor Sup35p. The ability of this domain to confer prion-based regulation of protein activity is determined to be modular and transferable. Here, we have extended this prion-based control of the activities to a cytoplasmic enzyme His3p and a nuclear transcriptional activator fusion of LexA and B42. Fusions of the Sup35 PrD to these two proteins result in normal and inactivated functions in [psi$^-$] and [PSI$^+$] cells, respectively. Furthermore, the inactivated prion state of these fusion proteins is propagated faithfully through cellular divisions, until it is reversed by exposure to agents that cure the [PSI$^+$] prion. The extension of the prion-based modulation of protein activities to these two cellular proteins provides novel tools for genetics screen or manipulations of the [PSI$^+$] system and also suggests an evolutionary role of prions as molecular switches in the regulation of protein functions.

Introduction

Prion proteins could exist in two stable structural conformations, the normal and the self-propagating prion states. First identified in context of human neurodegenerative diseases, prions were originally thought of only as a novel class of protein-based pathogens (for review, see Prusiner, 1998). Here, an abnormal β-sheet rich prion conformation of the mammalian PrP, termed PrP^{SC}, was postulated to be the infectious pathogen responsible for the disease transmissions and pathogeneses. Upon infection of a new host, PrP^{SC} particles were capable of converting the normal α-helical form of PrP, termed PrP^{C}, into the prion isoform. These converted prion molecules then served as further catalyst for the conformational changes of newly synthesized PrP^{C} molecules. This self-catalyzed conversion was thought to be the basis of prion infectivities, and to underlie the different modes of transmissions of prion diseases.

The recent identification of yeast prions by Reed Wickner as the mechanisms for the genetic inheritances of the [PSI^{+}] and [URE3] determinants had expanded the original prion hypothesis to include protein conformations as novel genetic elements (for review, see Wickner, 1994, 1999). The [PSI^{+}] determinant was first identified as a non-Mendelian factor that mediated suppression of nonsense stop codons. This element was later shown to be caused by a self-propagating prion-like conformation of the translational termination factor Sup35p. In a [psi^{-}] cell, Sup35p was soluble and active, thus able to mediate proper translational termination. In contrast, in a [PSI^{+}] yeast, Sup35p was in an insoluble and inactive prion aggregate, thus unable to mediate faithful

translation termination. Similar to the self-catalyzed conversion of mammalian prion, the [PSI$^+$] factor propagated in vivo by a prion-like mechanism. Here, the aggregated form of Sup35p promoted a self-propagating conversion of the soluble protein into the prion form.

The inactive Sup35 prion conformation in [PSI$^+$] yeast led to increased readthrough or suppression of nonsense stop codons in these cells. In the original strain from which this factor was identified, this translational suppression led to readthrough of the nonsense stop codon of a marker *ade1-14*, thus producing full length and active Ade1p. Conversely, faithful translation termination in [psi$^-$] cells carrying this marker led to the production of truncated and inactive Ade1p (Cox, 1965; also see Serio and Lindquist, 1999 for review). Therefore, the use of this convenient marker allowed researchers to distinguish [PSI$^+$] from [psi$^-$] cells by virtue of adenine auxotrophy: [PSI$^+$] cells were able to grow on media lacking adenine and were white on media containing low amount of the amino acid, whereas [psi$^-$] cells were not able to grow on media lacking adenine and were red on low adenine media.

Sup35p is divided into three distinct functional domains. The last or most carboxyl-terminal of these domains is responsible for the protein's biological function in polypeptide translation (Hoshino et al., 1989; Kushnirov et al., 1990a, b; Samsonova et al., 1991; Ter-Avanesyan, 1993). This domain, composed of residues 254 to 685 is homologous to the elongation factor EF-1α and is responsible for the translational termination activities of Sup35p. Thus, as could be expected, this domain is essential for

cell viability. In contrast, the first two amino-terminal domains of Sup35p are dispensable for normal cell growth. The first, termed the PrD (for Prion determining Domain, see below) is comprised of residues 1 to 123 and has an unusually high glutamine/asparagine and low charged residues content. Additionally, it also has five imperfect oligopeptide repeats reminiscent to, but not homologous to that found in the PrP sequence (Liu and Lindquist, 1999). The second or middle domain, appropriately termed the M domain, is composed of residues 124 to 253 and is rich in charged residues. No function has yet been assigned to this domain, although it is postulated to be a binding site for a co-factor necessary for the translational termination activity of Sup35p (Paushkin et al., 1997).

Although the Sup35 PrD was not important for cell viability as yeast carrying deletion of this domains grow well, it was required for the formation and propagation of the [PSI^+] prion. The role of the PrD in the de novo formation of [PSI^+] prion aggregates was shown when it was discovered that transient overexpression of PrD in [psi^-] cells resulted in an increased rate of the appearance of [PSI^+] colonies (Chernoff et al., 1993). The role of the PrD in the maintenance of the [PSI^+] state was demonstrated as when the wildtype chromosomal copy of *SUP35* gene in a haploid [PSI^+] cell was replaced by a series of amino-terminal truncation mutants, the yeast were found to revert to the [psi^-] state (Ter-Avanesyan, 1994). Furthermore, certain fortuitous or engineered mutations in this domain were found to interfere with the [PSI^+] prion propagation, or resulted in suppression of its translational readthrough phenotypes (DePace et al., 1998; Doel et al., 1994, Liu and Lindquist, 1999).

The formation of Sup35p aggregates, thought to be the basis of the propagation of the [PSI$^+$] prion, was shown when lysates of [psi$^-$] and [PSI$^+$] cells were subjected to ultracentrifugation (Paushkin, 1996). The insoluble Sup35p aggregates from [PSI$^+$] cells partitioned to the pellet fraction, whereas the soluble monomeric proteins from [psi$^-$] cells were largely found in the supernatant. This aggregation of the full length Sup35p was driven by intermolecular protein interactions mediated by the PrD. Indeed this explained the role of this domain in the formation and maintenance of the [PSI$^+$] prion aggregates: overexpression of the PrD in [psi$^-$] cells first resulted in the de novo induction of the PrD fragment aggregates, which then recruited full length Sup35p. The further maintenance of the prion element could then be performed solely by the recruitment of endogenous wildtype Sup35p into the prion aggregates, as the PrD expression plasmid could subsequently be lost (Chernoff et al., 1993; Derkatch et al., 1996; Ter-Avanesyan et al., 1994). Correspondingly, the [PSI$^+$] state could not be maintained in a yeast expressing Sup35p lacking the PrD, as the absence of this domain resulted in the absence of soluble monomers that could be recruited into the prion aggregates. This abolished the faithful propagation of the prion aggregates, presumably by dilution of the Sup35 aggregates through several rounds of cellular divisions.

The ability of the Sup35 PrD to mediate a [PSI$^+$]-dependent protein aggregation in vivo was demonstrated by its fusion to green fluorescent protein (GFP). In [psi$^-$] cells, expression of the PrDM-GFP fusion protein resulted in an even cytoplasmic fluorescence, consistent with a state of soluble PrD. In contrast, expression of this protein in [PSI$^+$]

cells resulted in fluorescent foci, suggesting that the PrD fusion was in a large inclusions or in an aggregated state (Patino et al., 1996). Furthermore, consistent with the ability of the PrD to induce the appearance of the [PSI$^+$] state when overexpressed, overexpression of the PrDM-GFP fusion protein in [psi$^-$] cells first resulted in diffuse cytoplasmic fluorescence, with slow formation of foci over a period of more than 10 hours. The appearance of these foci were also accompanied with increased conversion to the prion state, as assayed by plating of these cells onto media selective for [PSI$^+$] yeast, and thus seemed to represent prion conversion events.

In addition to the demonstration that Sup35p was capable of existing in two different conformational states in vivo, the PrDM-GFP fusion protein also suggested that the [PSI$^+$]-dependent aggregation of the PrD was modular and transferable. This modularity allowed the design of a prion-based regulation of protein activity, achieved by an amino-terminal fusion of the PrD. In effect, this allowed the creation of a novel class of protein-based genetic element: in [psi$^-$] cells, biological functions of these proteins would be normal, whereas in [PSI$^+$] cells, they would be curtailed.

To this effect, Lindquist and coworkers had recently described the creation of such element by fusing the Sup35 PrDM to either wildtype or constitutively active glucocorticoid steroid receptor (GR) (Li and Lindquist, 2000). When this fusion protein was expressed in a [psi$^-$] cell carrying a β-galactosidase reporter gene under control of GR, a characteristic blue color was observed upon addition of steroids and X-gal substrate. This suggested that in these cells, GR was soluble and active. In contrast, no

β-galactosidase activity was observed in [PSI$^+$] cells, which remained white in the color assay, suggesting that the PrDM-GR fusion protein was inactive. Protein immunoblots showed that this effect was not due to lowered expression of the GR fusion protein. Instead, the [PSI$^+$]-dependent inactivation of GR seemed to be due to its insolubility by PrD-mediated aggregation, although this remained to be shown directly by lysates ultracentrifugation experiments.

Consistent with the hypothesis that the PrDM-GR fusion proteins being in a prion-like aggregated conformations, it was determined that the white [PSI$^+$] haploid cells were dominant when mated to blue [psi$^-$] cells of the opposite mating type. Furthermore, chemical agents or biological factors that were shown to cure [PSI$^+$] prion, such as exposure to low amount of guanidine hydrochloride or overexpression of the molecular chaperone HSP104p, also eliminated the PrDM-GR fusion protein aggregates (Chernoff et al., 1995; Tuite et al., 1981). White cells containing aggregated fusion protein turned blue upon growth on guanidine or when HSP104p was overexpressed in these cells. Consistent with the reversible curing of [PSI$^+$] prion, a low level of spontaneous generation of white colonies from the cured blue colonies was observed.

Metastable conformational states in a prion-based molecular switch of protein activity are paramount to their use as a regulatory mechanism. A prion switch must remain in its current conformation until a signal is given for it to change to the other conformation. The [PSI$^+$] system fulfills this requirement well, as the rate of spontaneous conversion of [psi$^-$] yeast to the [PSI$^+$] state, as well as the rate of prion curing, is very

low (approximately 0.0001%) (Y.O. Chernoff, personal communication; Santoso and Weissman, unpublished observations). Indeed, a high level of spontaneous conversion to the prion or reversion to the normal states would result in high background if this system were to be used in genetic screens or selections. In contrast to the low level of spontaneous interconversion between the conformational states in the [PSI^+] system, an interconversion rate as much as 10,000 times higher was observed for the PrDM-GR fusion proteins (Li and Lindquist, 2000). Moreover, high level of expression of the GR fusion protein was required to assay the aggregation state of the construct, which provided a possible explanation for the high level of spontaneous conversion to the prion-like aggregated state. This could be due to the intrinsic propensity of steroid receptors to aggregate when expressed in yeast (Brian Freeman, personal communication).

Result and Discussion

In order to construct prion-based molecular switches that did not involve modification of translational termination activities, we fused the Sup35 PrDM to a cytoplasmic enzyme His3p (Struhl and Davis, 1977) and to a nuclear transcriptional activator protein LexAB42 (Gyuris et al., 1993). Consistent with the findings of Lindquist and coworkers, we discovered that activities of these two diverse proteins could be regulated by a prion-like aggregation mechanism similar to that found in the [PSI^+] system. The expression of a fusion protein composed of the PrDM domains and His3p allowed a *his3*$^-$ yeast to grow on media lacking histidine in a [PSI^+]-dependent manner (Figure 1A). Here, in a [psi^-] yeast, the PrDM-His3p fusion protein was soluble and active. This rendered the cell prototrophic for histidine, as shown by its ability to grow on media devoid of this amino acid (Figure 1B). In contrast, the fusion protein was aggregated and inactive in [PSI^+] cells, rendering the cell unable to grow on media lacking histidine. In practice, residual activity of the aggregated His3p enzyme was enough to allow [PSI^+] cells to grow on selective media. The addition of a competitive inhibitor 3-aminotriazole (3-AT) to the media (Durrin et al., 1992), however, was sufficient to inhibit the residual activity of the enzyme in a [PSI^+] cell. The addition of 20mM 3-AT to the media allowed ready distinction between [PSI^+] and [psi^-] cells: [PSI^+] cells were unable to grow on the selective media, whereas the growth of [psi^-] cells was unaffected.

The use of histidine prototrophy of [psi$^-$] cells allowed for a genetic selection that is opposite to that of the canonical selection of [PSI$^+$] cells by use of the nonsense mutant allele *ade1-14* (Cox, 1965). Translational suppression of nonsense stop codon in the *ade1* marker resulted in the synthesis of full length and active Ade1p, and thus adenine prototrophy or the ability to grow on media lacking adenine in [PSI$^+$], but not in [psi$^-$] yeast. Thus, with this marker, one would be able to perform a genetic selection for [PSI$^+$] cells. In contrast, as PrDM-His3p was active and soluble in [psi$^-$] but not in [PSI$^+$] yeast, one would be able to perform the *opposite* genetic selection, that is to select for [psi$^-$] cells by plating on media lacking histidine.

In principle, the fusion of Sup35 PrDM to a nuclear transcriptional activator or repressor would allow the greatest flexibility in the variety of reporter signals. For example, by putting reporter genes encoding for GFP or β-galactosidase under transcriptional control of these factors, one would be able to use either a fluorescence or a color-based assay to determine the prion state of the yeast (Figure 2A). Similarly, by using selectable markers as reporters, one would easily be able to use this system to perform genetic selections. To this effect, we had fused the Sup35 PrDM to a transcriptional activator protein composed of a fusion of the DNA-binding domain of LexA, which normally functioned as a repressor of the bacterial SOS genes (Ebina et al., 1983), and the acidic prokaryotic B42 transactivation peptide (Ma and Ptashne, 1987). The combination of these two domains in trans was successfully used in a yeast two hybrid system, using a reporter of a *LacZ* gene encoding for β-galactosidase under control of multiple LexA operators (Gyuris et al., 1993; Golemis et al., 1996).

Similar to the case of His3p fusion, fusion of LexA-B42 to Sup35p PrDM resulted in a [PSI]-dependent regulation of the transcriptional activation of the fusion protein (Figure 2B). In [psi$^-$] yeast carrying a β-galactosidase gene, soluble PrDM-LexAB42p resulted in transcription and synthesis of this reporter protein. Upon addition of X-gal substrate to these colonies, the characteristic blue color was produced. In contrast, the fusion protein was aggregated and inactive in [PSI$^+$] cell, and thus unable to activate reporter gene transcription. This resulted in no production of the blue color upon the addition of the X-gal substrate to the cells. The high transcriptional activation activity of the LexAB42 fusion protein, undoubtedly the reason for its use in the yeast two hybrid screens for protein-protein interactions, allowed us to use much lower protein expression level compared to the GR fusion described by Lindquist (Li and Lindquist, 2000). Unlike the high level of interconversion in the GR fusion system, our LexAB42 fusion system showed no appreciable spontaneous conversion to or curing of the [PSI$^+$] state. Furthermore, exposure to guanidine hydrochloride, which was shown to cure the [PSI$^+$] prion, also cured these cells of the PrDM-LexAB42 aggregate, as shown by their blue color upon addition of the color assay substrate (Figure 2B).

The ability to confer a heritable aggregation state mediated by the PrD to proteins unrelated to the Sup35 EF allowed novel genetic screens or selections that did not depend on suppression of translational nonsense stop codons. Although the conventional [PSI$^+$] system had been used successfully in genetic screens for factors involved in prion formation or propagation (e.g. Bailleul et al., 1999; Chernoff et al., 1995; DePace et al., 1998; Newnam et al., 1999), these screens were often complicated by the reliance on

translational suppression phenotypes. By use of an orthogonal signal as an additional secondary screen, such as the PrDM-LexAB42p, one would be able to eliminate candidate genetic factors such, as ribosomal mutants that passed the initial screen by modifying translational suppression (L.Z. Osherovich and J.S. Weissman, unpublished data).

In addition to providing a useful reagent for genetic screens or selections, the demonstrated modularity of the PrD and the transferability of its aggregation domain suggested a hypothesis that this domain could have evolved as a regulatory mechanism for modulating protein activity. This hypothesis was further strengthened by the discovery that the sequence and the ability of homologous Sup35 PrDs to aggregate in a prion-like fashion across were conserved across a broad range of budding yeast species (Santoso et al., 2000 or see Chapter 3). As a mechanism of inheritance of genetic information, the prion or protein based system allowed the cell to inhibit or modulate the activity of a specific protein and propagate this change indefinitely while retaining the ability to restore fully this protein activity (Lindquist, 1997). As it was shown that certain environmental conditions favored the survival of yeast in [PSI^+] state, whereas others favored [psi^-] yeast, prion-based genetic inheritance provides a yeast cell with a mechanism of adapting to these environmental changes and of propagating these changes to its mitotic progenies (Eaglestone et al., 1999; Tuite et al., 1981).

The identification of novel protein domains in the *Saccharomyces cerevisiae* genome that had the unusually high glutamine/asparagine and low charged residue

content similar to that of Sup35 PrD suggested that these domains might also have prion-like properties (M. Michelitch and J.S. Weissman, manuscript in preparation; Santoso et al., 2000; Sondheimer and Lindquist, 2000). For example, genetics and ultracentrifugation data recently showed that the amino-terminal domain of New1p (Santoso et al., 2000) and the carboxyl-terminal domain of Rnq1p (Sondheimer and Lindquist, 2000) could exist stably in two conformational states similar to that found in the [PSI] system. Overexpression of these domains induced the conversion of these cells to the aggregated state, which were then propagated stably through mitoses and meioses. Furthermore, passage of these cells carrying either the New1p or Rnq1p aggregates in media containing small amounts of guanidine hydrochloride, which was shown to cure the [PSI^+] prion, also resulted in the reversible curing of these novel prion-like aggregates.

The identification of the novel prion domains above suggested that yeast prions might not be limited to the two known prions [PSI^+] and [URE3]. Indeed, the prion phenomena might not be limited to mammalian and yeast systems, as a genetic element in *Podospora anserina* that determined vegetative incompatibility was recently postulated to be based on a prion-mechanism (Coustou et al., 1997). Taken together, these discoveries lead to an exciting possibility that prions may constitute a novel class of regulation of protein activities, as well as a novel genetic mechanism of propagating this information. Therefore, we are looking forward to further research to determine the biological functions of the proteins containing the novel prion domains, and the roles of prion-based mechanisms in the regulation of the activities of these proteins.

Experimental Procedures

General Procedures and Reagents

Isogenic *S. cerevisiae* [psi$^-$] and [PSI$^+$] strains 74-D694 [*Mat* **a**, *ade1-14(UGA)*, *his3*, *leu2*, *trp1*, *ura3*] were used in this study (Chernoff et al., 1995). Nucleic acid and yeast manipulations were performed according to standard protocols (Ausubel, 1987). Plasmid sequences were confirmed by dye termination sequencing (Perkin-Elmer). Curing of [PSI$^+$] prion was performed by streaking [PSI$^+$] cells onto plates containing 5mM guanidine hydrochloride (Tuite et al., 1981). Oligonucleotide primers were as follows:

P1 aattccatcaccatcatcaccatcaccactaagtcgacgagct

P2 cgtcgacttagtggtgatggtgatgatggtgatgg

P3 gggggtcgacggatccggggaattcccgcggggaatgaaagcgttaacggccaggcaac

P4 ccccccactagtcagccagtcgccgttgcgaataacc

P5 ggactagtatgcataccggtggaggggggggaggacctccaaaaaagaagagaaaggt

P6 ccccccgcggccgcttacgtacgcccctgcagagaggcataatctggcacatcataa

P7 gggggctcgagcctaggatatccttttgttgtttccgg

P8 ccccccgtcgacgacgtctccgcccggaattaattcaagctt

Construction of PrDM-His3p and PrDM-LexAB42 Expression Plasmids

The *HIS3* gene was PCR-amplified (primers P1, P2) and cloned into the EcoRI - SacI sites of p316SpPrDMEF vector described in DePace et al., 1998. The *LexA* and *B42* encoding genes were amplified from pLexA vector (primers P3, P4) and pB42AD vector (primers P5, P6) obtained from Clontech. The *ADH1* promoter was amplified from

pLexA vector (primers P7, P8) and was cloned into the XhoI - SalI sites of the TRP1 marked CEN/ARS plasmid pRS314 (Christianson et al., 1992). PrDM from p316SpPrDMEF (SalI - BamHI), LexA (BamHI - SpeI) and B42 (SpeI- SacI) were then subcloned into this vector to get p314ADH1pPrDM-LexAB42.

Assays for His3p and LexAB42 Functions

To assay for the His3p fusion protein activity, [PSI^+] and [psi^-] yeast were transformed with p316SpPrDMEF. Transformants were then patched onto SD-URA-HIS plates supplemented with 0, 10, 20, 30, or 40 mM 3-aminotriazole (Sigma). Growth of these colonies were scored and documented after four days of incubation at 30°C. To assay for the LexAB42 transcriptional activity, [PSI^+] and [psi^-] yeast were transformed with p314ADH1pPrDM-LexAB42 and the reporter p8OplacZ (Clontech). Transformants were patched onto SD-TRP-URA for four days. A 10ml agarose overlay of 0.5% melted agarose, 0.5M Sodium Phosphate pH 7.0, 0.1% SDS and 0.05% X-Gal was poured onto the yeast plates. Color was scored and documented after approximately 30 minutes of incubation at room temperature.

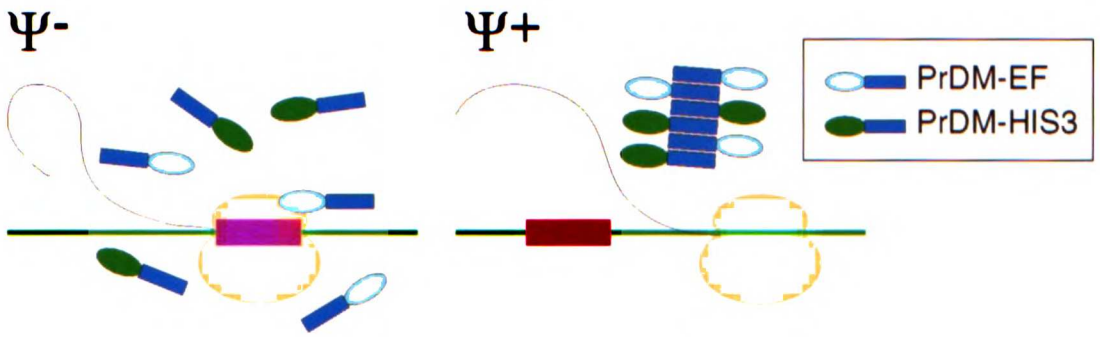
Figure Legends

Figure 1. Examination of the Effect of PrDM Fusion to His3p on Histidine Auxotrophy

(A) Schematic model of PrDM-His3p interaction with wildtype Sup35p (PrDM-EF) in [psi$^-$] and [PSI$^+$] yeast. In [psi$^-$] cell, soluble Sup35p mediates translational termination at the *ade1* nonsense stop codon (red bar) and soluble PrDM-His3p is able function in the synthesis of histidine. In contrast, in the suppressed [PSI$^+$] cell, the absence of soluble Sup35p and active PrDM-His3p results in translational readthrough and histidine auxotrophy, respectively. Below, effects of the [PSI] state of the yeast on PrDM aggregation, translational termination and histidine synthesis are shown.

(B) Effect of [PSI] state on growth of the cell in media lacking histidine. Shown are aliquots of [PSI$^+$] and [psi$^-$] yeast episomally expressing PrDM-His3p from a URA3 marked plasmid on SD-HIS-URA supplemented with increasing amount of 3-aminotriazole (3-AT), a competitive inhibitor for His3p activity.

A



	Ψ-	Ψ+
PrDM Aggregated?	No	Yes
Non-sense Stop Suppression/ Adenine Synthesis	Unsuppressed/ Truncated Ade1p	Suppressed/ Full Length Ade1p
HIS3 Activity/ Histidine Synthesis	Normal/ Histidine Produced	Abrogated/ No Histidine Produced

B

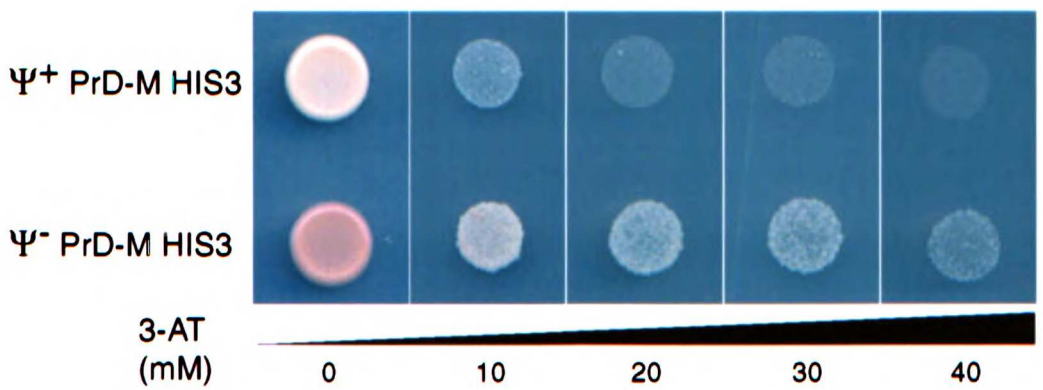
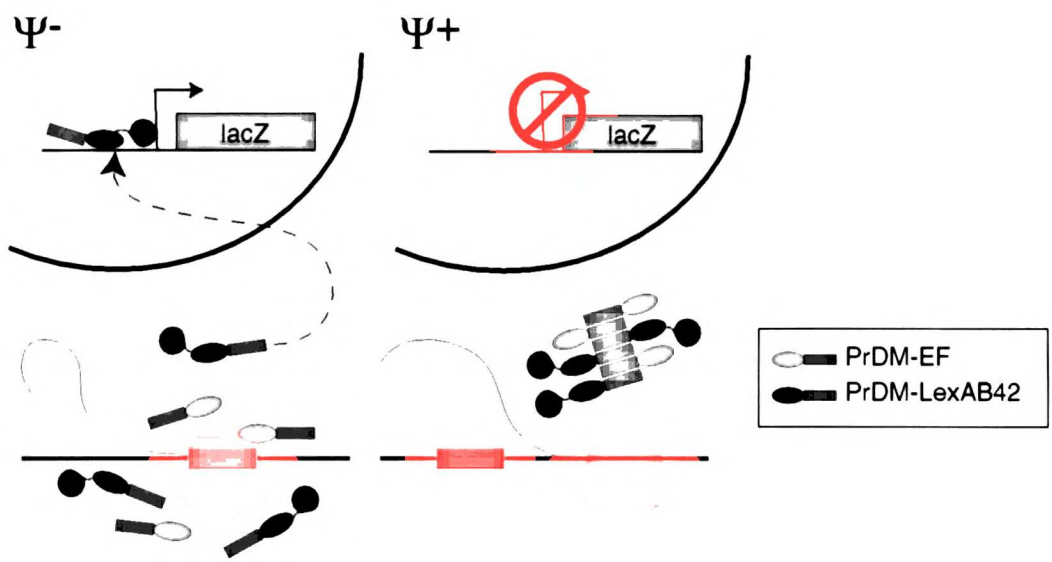


Figure 2. Examination of the Effect of PrDM Fusion on the Transcriptional Activity of LexAB42 Protein.

(A) Model of PrDM-LexAB42 interaction with wildtype Sup35p (PrDM-EF) in [psi$^-$] and [PSI$^+$] cells. In [psi$^-$] cells, soluble Sup35p is able to mediate translational termination at the *ade1* nonsense mutation (red bar) and soluble PrDM-LexAB42p is transported to the nucleus to activate the transcription of the *LacZ* reporter gene, which encodes β-galactosidase. In contrast, in [PSI$^+$] cell aggregated Sup35p results in the absence of translational termination activity, which leads to readthrough of stop codon. Similarly, aggregated PrDM-LexAB42p results in the absence of soluble transcription activator and no synthesis of the *LacZ* reporter gene. Below, effects of the [PSI] state of the yeast on PrDM aggregation, translational termination and transcription of the β-galactosidase reporter are shown.

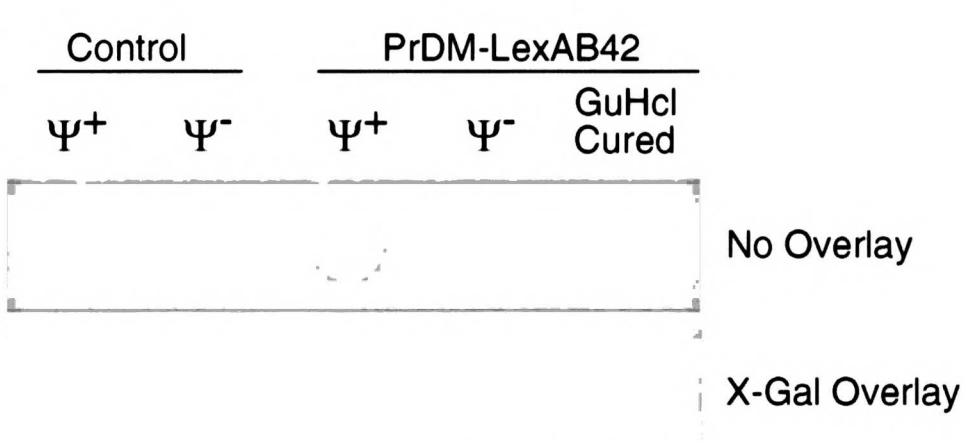
(B) Effect of [PSI] state on the color of yeast on a β-galactosidase color assay. Shown are aliquots of [PSI$^+$] and [psi$^-$] yeast episomally expressing PrDM-LexAB42p from a TRP1 marked plasmid and a URA3 marked reporter plasmid encoding for β-galactosidase on SD-TRP-URA. Also shown is an aliquot of the [PSI$^+$] yeast expressing PrDM-LexAB42p after passage in media containing guanidine hydrochloride. For comparison, [psi$^-$] and [PSI$^+$] cells carrying empty plasmids are shown.

A



	Ψ-	Ψ+
PrDM Aggregated?	No	Yes
Non-sense Stop Suppression/ Adenine Synthesis	Unsuppressed/ Truncated Ade1p	Suppressed/ Full Length Ade1p
LexAB42 Transcriptional Activity	Normal/ β-galactosidase Transcribed	Abrogated/ No β-galactosidase Produced

B



Chapter 5

Conclusion

The discovery of the [PSI$^+$] factor more than thirty years ago marked the beginning of a long period filled with complex, baffling and sometimes contradictory series of genetic analyses and observations (for review, see Liebman and Derkatch, 1999; Serio and Lindquist, 1999; Wickner, 1999). This factor was first identified in a genetic screen for nonsense suppressors in *Saccharomyces cerevisiae*, specifically as a non-Mendelian enhancer of a weak tRNA mutant (Cox, 1965). Almost immediately, efforts to characterize the [PSI$^+$] factor genetically resulted in data that were confusing and even paradoxical. Therefore, determination and efforts of Cox and coworkers in pursuing the characterization of this factor was to be commended.

Cox had proposed, correctly, that the [PSI$^+$] factor was a cytoplasmic element, which was dominant in a genetic cross with a haploid cell without the element, termed [psi$^-$] yeast. This cytoplasmic genetic inheritance was reminiscent to that of the known cytoplasmic factors such as plasmids, mitochondrial DNAs and viruses. Efforts to link [PSI$^+$] to these factors, however, proved to be unfruitful (Leibowitz and Wickner, 1978; Tuite et al., 1982; Young and Cox, 1972). Furthermore, experiments with mutagens suggested that at least one chromosomal gene was required for the propagation of the cytoplasmic [PSI$^+$] factor (Cox et al., 1980; Tuite and Cox, 1980).

To add further complexity, it was discovered that the [PSI$^+$] and [psi$^-$] states were metastable. More specifically, although they were inherited well mitotically and meiotically, interconversions between these two states (~0.1-1 in 10^6) occurred at rates

higher than expected for genetic mutations. This rate of interconversion could be increased greatly (up to 100% of the yeast cells) by addition of chemical agents (Singh et al., 1979; Tuite et al., 1981). For example, growth in presence of small concentration of guanidine hydrochloride was sufficient to cause very efficient conversion of [PSI$^+$] yeast to the [psi$^-$] state. Moreover, this conversion was found to be reversible, as [PSI$^+$] yeast could be re-isolated from the cured [psi$^-$] cells at some low rate ($\sim 1 \times 10^7$)

The slow but steady progress in understanding the molecular basis of [PSI$^+$] phenotypes and propagation became a torrent once the correct framework of viewing the baffling and contradictory data was supplied by Reed Wickner in 1994. Wickner proposed that [PSI$^+$], along with another non-Mendelian element [URE3], were yeast prions (Wickner, 1994). Unlike the canonical mammalian prions, yeast prions were not involved in disease production, nor were they spread laterally from cell to cell. Rather, they caused changes in phenotypes and were inherited through cell division or obtained by mating. Wickner noted that these yeast prions fulfilled properties expected of a prion. These included the non-Mendelian inheritance of these prion factors and their ability to be transferred through mating or cytoduction, the requirement of the chromosomal gene encoding for the prion protein, the induction of the prion state by overexpression of the prion protein, and the reversible curing of the prion element (Wickner, 1994; see also Serio and Lindquist, 1999).

Since the seminal proposal by Wickner that [PSI$^+$] factor was a yeast prion, recent research by Lindquist, Ter-Avanesyan and coworkers demonstrated that soluble and

active Sup35p resulted in the [psi$^-$] state, whereas insoluble and inactive Sup35p, which was propagated in a prion-like fashion, resulted in the [PSI$^+$] state (Patino et al., 1996; Paushkin et al., 1996, 1997). It was discovered that the prion state could be regulated by a molecular chaperone Hsp104p (Chernoff et al., 1995), as both overexpression and absence of this protein caused efficient curing of the [PSI$^+$] element. Other molecular chaperones were also implicated as factors that regulate prion formation or propagation. For example, Chernoff and co-workers demonstrated that Ssa1p, a member of the Hsp70 family could interfere with Hsp104p mediated [PSI$^+$] curing (Newnam et al., 1994).

The discovery that purified amino-terminal Sup35p fragment, termed PrDM, formed amyloid fibrils that polymerized in a nucleation dependent manner provided biochemical support of the yeast prion hypothesis (DePace et al., 1998; Glover et al., 1997; King et al., 1997). Sup35 PrDM purified under denaturing conditions readily formed amyloid fibrils upon dilution to aqueous buffer, with a cooperative kinetics preceeded by a significant lag phase, which could be eliminated by the addition of a small amount of pre-formed fibrils (DePace et al., 1998; Glover et al., 1997) or lysates of [PSI$^+$] yeast (Paushkin et al., 1997). Furthermore, Sup35p mutants that caused poor incorporation or curing of wildtype amyloid in vivo also showed decreased propensities to form fibrils in vitro (DePace et al., 1998 or Chapter 2). These mutations mapped exclusively to a short amino-terminal region in the PrD that was unusually rich in glutamines and asparagines.

The analogies between mammalian and yeast prions were further strengthened by the discovery that prion induction was limited by a species barrier (Chernoff et al., 2000; Kushnirov et al., 2000; Santoso et al., 2000 or Chapter 3). Here, it was discovered that the sequence and the ability of PrDM of Sup35p homologs from diverse spectrum of budding yeast species to aggregate in a prion-like fashion were conserved. Prion induction by Sup35p, however, was restricted to protein molecules of one species: a barrier prevented the prion particles from one species to convert monomers of another species. This species barrier was remarkably reproduced in vitro, where ongoing polymerization of one PrDM species did not interfere with conversion of another protein species. Chimeric analysis isolated a region that played important role in conferring this species specificity. Surprisingly, this region was identical to that one previously discovered to play an important role in prion formation and propagation (DePace et al., 1998).

Unlike the context of pathologies in which mammalian prions were first discovered, yeast prions were postulated to offer a unique mechanism of regulation of protein activities. Tuite and coworkers noticed that [psi$^-$] and [PSI$^+$] yeast had preferential survival rates in different environmental conditions (Eaglestone et al., 1999; see also Chernoff et al., 1998; Lindquist, 1997). For example, although there was no difference in the growth rates of [psi$^-$] and [PSI$^+$] yeast in rich media, [PSI$^+$] cells exhibited enhanced tolerance to ethanol or heat than [psi$^-$]. Similarly, [psi$^-$] cells exhibited cold tolerance (Y.O. Chernoff, personal communication). Intriguingly, conditions that selected for one of the prion states also promoted conversion to the

opposite state, perhaps as mechanism to ensure that both prion states were populated to ensure colony survival in the event of drastic change in growing conditions. For example, ethanol, which favored survival of [PSI+] cells also, promoted their conversion to [psi$^-$] (Eaglestone et al., 1999).

Taken together, these genetics, cell biological and biochemical data have contributed much to our understanding of the yeast prion phenomena, including intragenic sequences or extragenic factors that modulate prion formation and propagation, or the possible role of [PSI$^+$] as an evolutionary mechanism of regulation of protein activities. However, many more questions regarding the mechanistic details of prion conversion or curing, analogies to the strain phenomenon found in the mammalian prions, and specific roles of the [PSI$^+$] prion in yeast biology, remain unanswered. It is therefore with forward acknowledgement of the risk that these questions may not remain unanswered for long in this fast moving field, several of noteworthy areas of future research are highlighted below.

Although it is generally accepted that overproduction of Sup35 PrDM fragments in vivo caused de novo formation of aggregation of these fragments, which then can recruit full length Sup35p molecules, detailed mechanism by which this process occurred are not yet known (Chernoff et al., 1993; Derkatch et al., 1996; Ter-Avanesyan et al., 1994). For example, can well folded Sup35p be incorporated into the growing amyloid? Alternatively, is this process restricted only to nascent or partially unfolded proteins? The discovery of a fascinating non-Mendelian factor termed [PIN$^+$], whose presence is

required for efficient prion induction, added another layer of complexity to this question (Derkatch et al., 1997). What is the nature of the [PIN$^+$] factor? Does this factor control induction of yeast prions other than [PSI$^+$]? The identification of molecular chaperones as regulators of prion formation or propagation provided an important first glimpse into factors that involved in these processes (Chernoff et al., 1995; Newnam et al., 1999). For example, either overexpression or absence of Hsp104p interfere with the propagation of the [PSI$^+$], but not of the [URE3] prions. In contrast, exposure to guanidine hydrochloride causes efficient curing of both prions. What are the mechanistic details of the interaction of Hsp104p with the [PSI$^+$] prion particles? How does guanidine cause curing of both [PSI$^+$] and [URE3] prions? Surely, however, these identified proteins do not constitute an exhaustive list. Are there other proteins that contribute to prion formation and propagation? Are there homologous molecular chaperones that play analogous roles in mammalian prion formations or pathogeneses?

A fascinating aspect of mammalian prion involves the existence of prion "strains". In this context, prion strains refer to different pathological symptoms, susceptibility of neural tissues to degeneration, or even patterns of protease resistance of the prion particles (reviewed in Prusiner, 1998). In context of yeast prion [PSI$^+$], strains refer to the various strength of nonsense codon suppression and stabilities of prion propagation (Cox, 1965; Derkatch et al., 1996; Zhou et al., 1999). What distinguishes prion strains? Preliminary data suggest that different the yeast prion strains can be recapitulated in vitro using pure proteins, suggesting that the strain identities are encoded in protein conformations (P. Chien and J.S. Weissman, unpublished data).

Lastly, as described briefly above, the discovery that [PSI$^+$] and [psi$^-$] states offer survival advantages in different growing conditions (Eaglestone et al., 1999) hints at a possible role of prions as molecular switches or regulators of protein activities. This assertion is further strengthened by the discovery of the conservation of the ability of PrDs of Sup35p homologs from a wide spectrum of budding yeast species, and the ability of the PrDs to confer control of protein functions in a modular and transferable manner (Li and Lindquist, 2000; see also Chapter 4). The exact mechanism by which the [PSI$^+$] confer survival advantage to yeast in certain growing conditions, however, is unknown. Is this achieved by translational readthrough in specific chromosomal loci, which produced proteins with novel domains and thus altered activities? Moreover, if prions are indeed evolutionarily beneficial, such as by acting as regulators of protein activities, how many prions are there? What are their functions? Indeed, these questions are partially answered by the discovery of novel protein domains in yeast that can form prion-like aggregates (Santoso et al., 2000; Sondheimer and Lindquist, 2000). Lastly, can prions also serve as molecular switches of protein functions in higher eukaryotes? Genomic analyses of the nematode *Caenorhabditis elegans* for the proteins with similar amino acid compositions as the Sup35 PrD provides tantalizing clues that the answer to that question may be yes (M. Michelitch and J.S. Weissman, manuscript in publication).

Undoubtedly these fast advances in the field of yeast prions were partially due to the published work on mammalian prions, which provided a general framework for experimental designs. Even so, the availability of established yeast genetics tools, fast generational time of yeast and facile biochemical assays provided unique advantages of

using the [PSI$^+$] system to study properties that were general to prions or specific only to the yeast prions. Therefore, it would not be surprising or unexpected that our understanding of the biological properties of the yeast and mammalian prions, as well as other amyloidogenic proteins, would soon be enriched.

Appendix A

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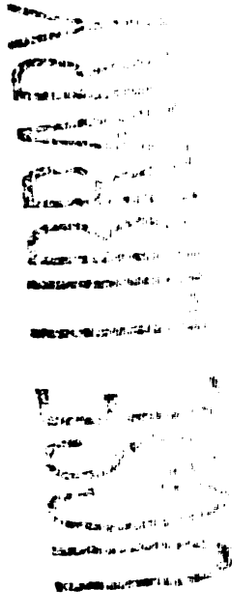

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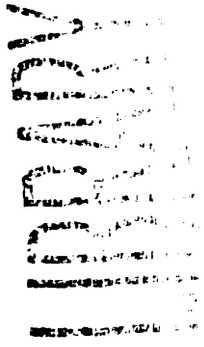

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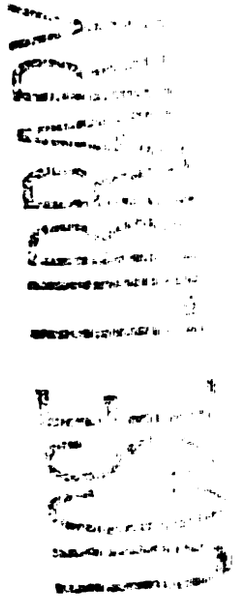

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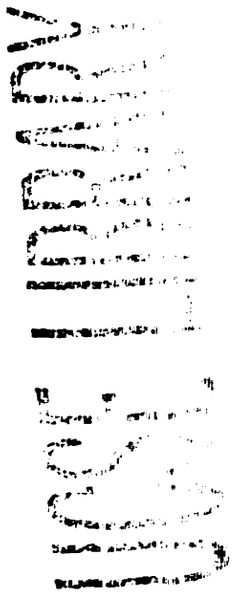

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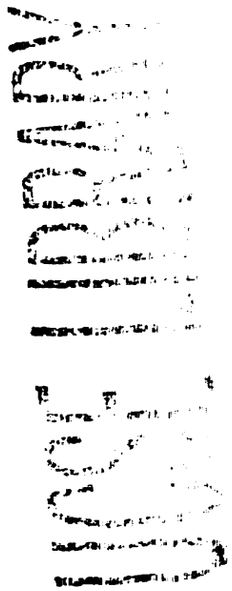

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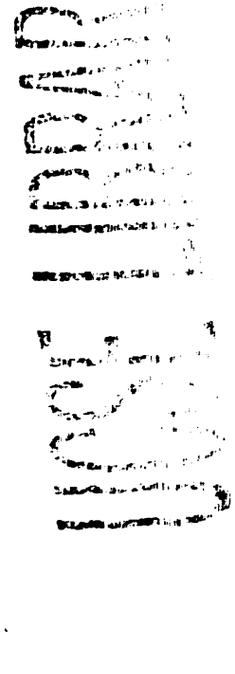

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