The Cellular Concentration of the Yeast Ure2p Prion Protein Affects Its Propagation as a Prion

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The [URE3] yeast prion is a self-propagating inactive form of the Ure2p protein. We show here that Ure2p from the species *Saccharomyces paradoxus* (Ure2p_{sp}) can be efficiently converted into a prion form and propagate [URE3] when expressed in *Saccharomyces cerevisiae* at physiological level. We found however that Ure2p_{sp} overexpression prevents efficient prion propagation. We have compared the aggregation rate and propagon numbers of Ure2p_{sp} and of *S. cerevisiae* Ure2p (Ure2p_{sc}) in [URE3] cells both at different expression levels. Overexpression of both Ure2p orthologues accelerates formation of large aggregates but Ure2p_{sp} aggregates faster than Ure2p_{sc}. Although the yeast cells that contain these large Ure2p aggregates do not transmit [URE3] to daughter cells, the corresponding crude extract retains the ability to induce [URE3] in wild-type [ure3-0] cells. At low expression level, propagon numbers are higher with Ure2p_{sc} than with Ure2p_{sp}. Overexpression of Ure2p decreases the number of [URE3] propagons with Ure2p_{sc}. Together, our results demonstrate that the concentration of a prion protein is a key factor for prion propagation. We propose a model to explain how prion protein overexpression can produce a detrimental effect on prion propagation and why Ure2p_{sp} might be more sensitive to such effects than Ure2p_{sc}.

INTRODUCTION

Prions were originally defined as a unique class of infectious agents composed solely of protein. In mammals, prions cause fatal neurodegenerative diseases, such as Creutzfeldt–Jacob disease in human, sheep scrapie, and bovine spongiform encephalopathy (Prusiner *et al.*, 1982; Aguzzi *et al.*, 2007; Collinge and Clarke, 2007). All these diseases are linked to abnormal self-propagating conformations of a cellular protein termed PrP. The yeast *Saccharomyces cerevisiae* possesses at least four different prion proteins (Sup35, Ure2p, Rnq1p, and Swi1p) that can generate phenotypes ([PSI⁺], [URE3], [PIN⁺] and [SWI⁺], respectively) as a direct consequence of an inherited conformational change (Wickner, 1994; Derkatch *et al.*, 2001; Du *et al.*, 2008). Yeast prions are denoted within brackets and by capital letters to specify that they correspond to cytoplasmically transmitted inheritable elements that are dominant in genetic crosses. The yeast prion proteins exhibit clear mechanistic similarities with PrP. In particular, mammalian and yeast prions have the ability to polymerize into amyloid aggregates (for reviews, see Baskakov, 2007; Wickner *et al.*, 2007). Interestingly, biochemical pathways controlling prion formation and/or maintenance seem conserved from yeast to humans because antiprion drugs, isolated in yeast-based screening assays,

inhibit mammalian prion propagation and vice versa (Bach *et al.*, 2003).

Numerous results highlight the clear connection between the yeast prion phenotype and the aggregation of the protein involved in this process (Patino *et al.*, 1996; Edskes *et al.*, 1999; Paushkin *et al.*, 1997; Zhou *et al.*, 2001). Reconstitution of in vivo infectivity from amyloid fibers formed in vitro from purified protein has definitively proven the protein-only hypothesis for prion formation and established indubitably the link between amyloid fibers and the prion form propagated in cells (Tanaka *et al.*, 2004, 2006; Brachmann *et al.*, 2005; King and Diaz-Avalos, 2004; Patel and Liebman, 2007). Other proteins form amyloids in an autocatalytic manner, but only prion proteins are infectious. The exact nature of the self-propagating species still remains unclear. Indeed, the Ure2p aggregates isolated from [URE3] cells differ from amyloid filaments formed in vitro (Ripaud *et al.*, 2004). Genetics studies on yeast prions have revealed the existence of cellular factors required for prion aggregation and propagation. Notably, the de novo appearance and subsequent propagation of yeast prions is highly dependent on the expression levels of a number of molecular chaperones (reviewed in Perrett and Jones, 2008). Biochemical analysis of [PSI⁺] propagating yeast extracts showed that Ssa1/Ssa2 chaperones were the major components interacting with infectious Sup35 aggregates (Bagriantsev *et al.*, 2008).

To ensure an efficient transmission of prion in actively dividing cultures, prion aggregates must be continually subdivided to yield additional replication templates known as prion seeds or propagons. The disaggregating Hsp104 chaperone has the most critical and universal role in yeast prion maintenance, and it is essential to remodel existing prion complexes to generate or maintain propagons (Chernoff *et al.*, 1995; Satpute-Krishnan *et al.*, 2007). Then, propagons are

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partitioned to daughter cells, where they continue to direct the conversion of neosynthesized protein to the prion form and ensure inheritance of the associated phenotype.

The Ure2p prion protein controls catabolism of nitrogen sources. In the presence of a good nitrogen source, Ure2p blocks the uptake of poor nitrogen sources by sequestering the transcription factor Gln3p in the cytoplasm (Beck and Hall, 1999; Cox *et al.*, 2000). One of these genes activated by Gln3p is the allantoin permease gene, *DAL5* (Turoscy and Cooper, 1987). Because of the structural similarity between allantoin and ureidosuccinate (USA), an essential intermediate of uracil biosynthesis, Dal5p can take up USA (Aigle and Lacroute, 1975). Prion conversion of Ure2p leads to its loss of function and consequently, enables the uptake of ureidosuccinate (USA) and permits growth on an ad hoc selective medium. Ure2p is a two-domain protein. The asparagine and glutamine rich N-terminal domain of Ure2p, termed prion-forming domain, is unstructured in the soluble form of the protein and is crucial for prion properties, whereas its C-terminal domain is compactly folded and sufficient to perform the cellular function in nitrogen regulation (Masison and Wickner, 1995). Although the catalytic function of Ure2p in nitrogen regulation was found to be conserved among all analyzed *Saccharomyces* species, controversial results were obtained concerning the conservation of prion-forming properties (Edskes and Wickner, 2002; Baudin-Baillieu *et al.*, 2003). In particular, we did not detect [URE3] prion capacity with Ure2p from *Saccharomyces paradoxus* (Ure2p_{Sp}), whereas H. Edskes and coworkers reported that this protein could form a prion. The conflicting studies differed in two experimental aspects. One difference was the expression level of Ure2p in yeast cells, the other was the existence of a silent mutation in the construct used by Edskes *et al.* that could lead to an artifactual translational read-through of *URE2* (Talarek *et al.*, 2005).

De novo prion generation is a stochastic process. Transient overproduction of the prion protein increases the frequency of prion formation (Wickner, 1994; Derkatch *et al.*, 1996). This property was proposed by R. Wickner as one of the indicators that a heritable non-Mendelian state is due to a prion-based determinant and is now regarded as a fundamental property of such systems (for review, see Wickner *et al.*, 2006). The rationale behind this argument is simply that a higher concentration of the protein enhances the probability of prion nucleation. However, high amounts of prion protein can also interfere with propagation (Edskes *et al.*, 1999; Ripaud *et al.*, 2003; Allen *et al.*, 2005). For example, high overexpression of Ure2p leads to curing of the [URE3] prion.

A complex relationship apparently exists between prion properties and the amount of the prion protein. In the present work, we wondered whether the concentration of Ure2p could interfere with its prion properties. We report here that, when expressed at physiological level, Ure2p_{Sp} can convert into the prion form either spontaneously or after contact with preexisting [URE3]. But, we show that the Ure2p concentration controls the ability to induce and propagate [URE3]. We found that the number of [URE3] propagons is lower when propagated with Ure2p_{Sp} than with Ure2p_{Sc} and diminishes with increase of the cellular concentration of Ure2p. Together, our results suggest that the aggregation dynamics of Ure2p and [URE3] propagation depends both on the expression level of Ure2p and on the sequence of the prion-forming domain. This work shows that concentration of a prion protein contributes to determine its prion properties.

MATERIALS AND METHODS

Nomenclature

To avoid confusion, we used *S. cerevisiae* (Sc) or *Saccharomyces paradoxus* (Sp) in subscript to specify the gene origin. For prion nomenclature, [ure3-0] is used to name the soluble or nonprion status of Ure2p and [URE3] is used to name the prion status.

Plasmids and DNA Manipulations

All plasmids used in this study are listed in Table 1. The full-length *URE2* open reading frames (ORFs) of the *S. cerevisiae* and *S. paradoxus* are placed under control of the inducible *GAL10-CYC1* promoter and are carried either by the monocopy plasmid (with an ARS-CEN origin of replication) pG1URE2_{Sc} and pG1URE2_{Sp} or by the multicopy plasmid (with a 2 μ origin of replication) pG2URE2_{Sc} and pG2URE2_{Sp}. The multicopy plasmids pG2URE2_{Sc}-green fluorescent protein (GFP) and pG2URE2_{Sp}-GFP were originally described by Ripaud *et al.* (2003) and Immel *et al.* (2007), respectively. pFL39-URE2_{Sp}, containing the *URE2*_{Sp} ORF flanked by promoter and terminator sequence of *URE2*_{Sc}, was constructed as follows. The *URE2*_{Sp} ORF was amplified using oligonucleotide 37 (5'-GTCATATTGTTTAAAGCTGCAAAT-TAAGTTGTACACCAATGATGAATAACAACGGTAAC-3'), which introduces a sequence homologous to the *URE2*_{Sc} promoter at the 5' of the gene and oligonucleotide 41 (5'-TTTCCTCTCTCTCTCTCTCTGTTTTTAAGCAGCCTTTATTACACGCAATGCCTT-3'), which introduces a sequence homologous to the *URE2*_{Sc} terminator at the 3' of the gene. This fragment was cloned into the pFL39URE2_{Sc}, carrying a genomic fragment of *URE2*_{Sc}, by using the gap repair method (Orr-Weaver and Szostak, 1983).

Strains, Media, and Genetic Methods

All strains used in this study are disrupted for the *URA2* gene. *S. cerevisiae* strain CC30 (*MAT α* , *trp1-1*, *ade2-1*, *leu2-3*, 112, *his3-11*, 15, Δ *ura2::HIS3*) was used as the [ure3-0] parent and is termed URE2_{Sc} in this publication (Fernandez-Bellot *et al.*, 2000). The strain AB34, termed here URE2_{Sc} [URE3], is isogenic to URE2_{Sc} and carries the [URE3] strain originally described by Aigle and Lacroute (1975) that was transmitted by cytoduction. To integrate the

Table 1. Plasmids used in this study

Plasmid	Description	Reference/source
pFL39URE2 _{Sc}	<i>URE2</i> _{Sc} genomic fragment cloned in pFL39 vector (CEN-ARS TRP1)	Gift of Ozier-Kalogeropoulos O
pFL39URE2 _{Sp}	<i>URE2</i> _{Sp} ORF with <i>URE2</i> _{Sc} promoter in pFL39 vector (CEN-ARS TRP1)	This study
pG	2 μ <i>LEU2</i>	pYeHFn2L in Cullin and Minvielle-Sebastia (1994)
pG1URE2 _{Sc}	CEN-ARS <i>LEU2</i> P _{GAL10} - <i>URE2</i> _{Sc}	pYe1L-URE2 _{Sc} in Talarék <i>et al.</i> (2005)
pG2URE2 _{Sc}	2 μ <i>LEU2</i> P _{GAL10} - <i>URE2</i> _{Sc}	pYe2L-URE2 _{Sc} in Baudin-Baillieu <i>et al.</i> (2003)
pG1URE2 _{Sp}	CEN-ARS <i>LEU2</i> P _{GAL10} - <i>URE2</i> _{Sp}	pYe1L-URE2 _{Sp} in Talarék <i>et al.</i> (2005)
pG2URE2 _{Sp}	2 μ <i>LEU2</i> P _{GAL10} - <i>URE2</i> _{Sp}	pYe2L-URE2 _{Sp} in Baudin-Baillieu <i>et al.</i> (2003)
pG2URE2 _{Sc} -GFP	2 μ <i>TRP1</i> P _{GAL10} - <i>URE2</i> _{Sc} -GFP	pYe2T-scURE2GFP in Ripaud <i>et al.</i> (2003)
pG2URE2 _{Sp} -GFP	2 μ <i>TRP1</i> P _{GAL10} - <i>URE2</i> _{Sp} -GFP	pYe2T-spURE2GFP in Immel <i>et al.</i> (2007)
pWG2URE2 _{Sc}	2 μ <i>TRP1</i> P _{GAL10} - <i>URE2</i> _{Sc}	pYe2T-URE2 _{Sc} in Baudin-Baillieu <i>et al.</i> (2003)
pH327	CEN-ARS <i>LEU2</i> P _{URE2} - <i>URE2</i> _{Sc} -GFP	Edskes <i>et al.</i> (1999)

URE2_{sp} ORF into the *URE2* locus of *S. cerevisiae*, the AF36 strain (*MAT α* , *trp1-1*, *ade2-1*, *leu2-3*, 112, *his3-11*, 15, Δ *ura2::HIS3*, *cyh2r*, Δ *ure2::CYH2*) (Baudin-Baillieu *et al.*, 2003) was transformed with a *SacI*-*PstI* fragment from pFL39-*URE2_{sp}*. The substitution of the *CYH2* allele from the *URE2_{sc}* locus in the AF36 strain leads to cycloheximide resistance. For this reason, integrative transformants were selected on YPD medium supplemented with 1 μ g/ml cycloheximide. The resulting strain was named AF30 and backcrossed successively three times with *URE2_{sc}* to create strain *URE2_{sp}* (*MAT α* , *trp1-1*, *ade2-1*, *leu2-3*, 112, *his3-11*, 15, Δ *ura2::HIS3*, *P_{URE2sc}::URE2_{sp}*). The strain *URE2_{sp}* [URE3] is isogenic to *URE2_{sc}*, and it propagates the [URE3] strain introduced by crossing from *URE2_{sc}* [URE3]. *URE2_{sc}* and *URE2_{sp}* ORFs were discriminated by *NotI* restriction of the polymerase chain reaction (PCR) fragment generated using oligonucleotides 214 5'-GATGAATAACAACG-GCAACC-3' and 220 5'-CCGTAAACTCTTCTAACCTC-3'.

Growth and handling of *S. cerevisiae* involved standard techniques. Standard yeast media have been described previously (Baudin-Baillieu *et al.*, 2003). Induction of protein expression under control of Gal10 promoter was performed by growth in medium containing either 4% raffinose or 2% galactose + 4% raffinose as the carbon source. The [URE3]/USA⁺ phenotype of *ura2* strains was tested on a minimal medium containing ammonium as the nitrogen source and supplemented with appropriate amino acids and 15 mg/ml USA. Strains were cured of the [URE3] prior by growth on YPDA medium supplemented with 3 mM guanidine hydrochloride (GuHCl).

Quantifying the [URE3] Prion Formation

To evaluate spontaneous prion apparition, serial 10-fold dilution of cells were plated on medium supplemented with USA or with uracil. Colonies were counted after incubation for 3 d at 30°C for plates containing uracil and after incubation for 5 d for plates supplemented with USA. Frequency of USA⁺ colony formation was calculated as the ratio of the number of colonies on USA to number of colonies on uracil. To determine the induction rate of transient overexpression of *Ure2p* on prion formation, cells were grown for 3 d in medium containing 2% galactose and 2% raffinose dropout medium lacking leucine. Frequency of USA⁺ colony formation was determined as described above on SD medium lacking leucine supplemented with USA or with uracil. All frequencies indicated in this work were determined as the average of at least five independent experiments. To rule out a potential masking of the [URE3] phenotype on dextrose medium supplemented with USA, because of the presence of residual *Ure2p* after induction on galactose medium, cells were grown on dextrose medium supplemented with uracil for 2 d before selection of USA⁺ colonies. This supplementary step allows growth of the cells and dilution of any remaining *Ure2p*. By this approach, the obtained results led to the same conclusions as when selection of USA⁺ colonies was performed immediately after the transient overexpression of *Ure2p*.

[URE3] Curing by Guanidine Hydrochloride and Counting Propagons

After overnight growth on YPD (except for *URE2_{sc}* pG1*URE2_{sc}* which were grown on 2% YPGalactose), cells were grown in YPD with 3 mM GuHCl. Cultures were maintained in exponential growth (between 0.1 and 1 OD₆₀₀) by successive dilutions into liquid YPD medium with 3 mM GuHCl. At regular time points, aliquots of cultures were withdrawn and plated on SD medium supplemented either with uracil or with USA. The frequency of USA⁺ colonies was determined as described above. The number of propagons was derived from the kinetics of GuHCl-induced prion curing according to Cox *et al.* (2003). The data are plotted as the percentage of [URE3] cells as a function of generations of growth in YPD + GuHCl. The number of propagons can be calculated from the value of 2^g, where g is the number of generations elapsed at the time point when 36.8% of cells had lost the prion, assuming that the propagon number halves at each generation.

Protein Extraction, Sedimentation Analysis, and Western Blotting

Total yeast extracts (glass beads disruption with Fast-prep24 [MP Biomedicals, Irvine CA] in TNT buffer: 25 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.2% Triton X), sedimentation analyses, Western blotting and treatment with antibodies were performed as described previously (Ripaudo *et al.*, 2003; Immel *et al.*, 2007). Before heating at 95°C, urea was added to a 8 M final concentration to the loading buffer of all extracts from yeast cells propagating the prion. To measure the amount of *Ure2p* in various strains, yeast extracts were prepared by alkaline lysis. Briefly, 5 OD₆₀₀ units of yeast cells in exponential phase were permeabilized with 1 ml of 0.185 M NaOH, 0.2% mercaptoethanol for 10 min on ice. Trichloroacetic acid was added to a final concentration of 5%, and the samples were incubated for an additional 10 min on ice. Precipitates were collected by centrifugation at 14,000 $\times$ g for 5 min. The pellets were neutralized and dissolved in 30 μ l of dissociation buffer (4% SDS, 0.1 M Tris-HCl, pH 6.8, 4 mM EDTA, 20% glycerol, 2% 2-mercaptoethanol, and 0.02% bromophenol blue) and 15 μ l of 1 M Tris base, and heated at 95°C for 5 min. One to 5 μ l was loaded onto a 12% SDS-polyacrylamide gel. Western blotting and immunoblotting with *Ure2p* and *Adel3p* antibodies

were performed as described previously (Ripaudo *et al.*, 2003). Signal intensity was quantified using the Amount One software (Bio-Rad, Hercules, CA).

Microscopy Techniques

URE2_{sc} and *URE2_{sp}* cells propagating [URE3] were transformed with the pG2*URE2_{sc}*-GFP and pG2*URE2_{sp}*-GFP plasmids, respectively. Cells were grown on YNB medium (0.67%) supplemented with 2% dextrose, appropriate amino acids, and 15 mg/l USA to 0.8–1 OD₆₀₀. Cells were washed and suspended in YNB medium (0.67%) supplemented with 4% raffinose or 2% galactose. Cells were then pelleted at different times and resuspended in DABCO maintaining solution (218 mM diazabicyclo 2-2-2 octane [Sigma-Aldrich], 25%, vol/vol phosphate-buffered saline, and 75%, vol/vol glycerol). Cells were observed and photographed with a DMRB microscope (Leica, Wetzlar, Germany) with a 100 $\times$ HCX PL fluotar objective.

Preparation of Yeast Extract and Test of [URE3] Infectivity

Yeast extract were prepared as described in the supplemental experimental procedures of Brachmann *et al.* (2005). *URE2_{sc}* pG2*URE2_{sc}*-GFP cells propagating [URE3] were grown as described above for microscopy analyzes. Approximately 150 OD of cells were resuspended in 250 μ l of ST buffer (1.2 M sorbitol and 10 mM Tris-HCl, pH 7.5) and disrupted with 0.5-mm glass beads in ice for 20 s using the Fast-prep24 (MP Biomedicals). Whole cells extract were briefly clarified at 500 $\times$ g for 30 s.

Yeast extract were transformed into [ure3-0] cells as described in the supplemental experimental procedures of Shorter and Lindquist (2006). Briefly, spheroplasts were recovered from *URE2_{sc}* yeast cells after digestion with lyticase (L-5263; Sigma-Aldrich). Spheroplasts were resuspended in STC buffer (1.2 M sorbitol, 10 mM CaCl₂, and 10 mM Tris-HCl, pH 7.5) and mixed with yeast extracts (15 μ l of whole cells extracts for 100 μ l of spheroplasts suspension), a *LEU2* plasmid (pRS426; 0.02 mg/ml), and salmon sperm DNA (0.1 mg/ml). Fusion was induced by addition of 9 volumes of polyethylene glycol (PEG) buffer (20%, wt/vol PEG8000, 10 mM CaCl₂, and 10 mM Tris-HCl, pH 7.5). Then, 330 Leu⁺ transformants were spotted onto SD supplemented with USA. To ensure USA⁺ colonies corresponded to [URE3], test for curing of the USA⁺ phenotype were carried out by streaking cells for single colonies onto YPD containing 3 mM guanidine HCl and then checked for the loss of USA⁺ phenotype. The numbers of [URE3] colonies relative to total transformants was then determined. A negative control without extracts was performed, which only rarely gave rise to spontaneous [URE3] clones. To make sure that potential surviving cells from yeast extract did not yield false positives in transformation experiments, we plated the cells extracts (incubated without pRS426 by using the transformation procedure described above) and have verified that it did not yield Leu⁺ colonies.

RESULTS

Ure2p_{sp} Can Adopt a Prion Form When Expressed at Physiological Level

To determine whether *Ure2p_{sp}* can sustain [URE3] propagation when it is expressed at physiological level, we constructed an *S. cerevisiae* strain in which the *URE2_{sp}* ORF was integrated at the *URE2* locus under control of the endogenous *URE2_{sc}* promoter (this strain is termed *URE2_{sp}*). The expression of *Ure2p_{sp}* was similar to that of *Ure2p_{sc}*, as determined by Western blotting (Figure 1A). As reported previously, *Ure2p_{sp}* can perform the nitrogen catabolism repression function of *Ure2p_{sc}*, and as a consequence, the *URE2_{sp}* strain shows a USA⁻ phenotype (Figure 1B) (Baudin-Baillieu *et al.*, 2003).

We wondered whether the *Ure2p_{sp}* protein could be converted into a prion form in the presence of preexisting [URE3]. For that purpose, we crossed the *URE2_{sp}* strain with a *URE2_{sc}* strain propagating [URE3]. The resulting *URE2_{sp}* $\times$ *URE2_{sc}* [URE3] diploids displayed the USA⁺ phenotype, whereas the *URE2_{sp}* $\times$ *URE2_{sc}* [ure3-0] diploid remain wild type for *Ure2p* function (Figure 1B). To determine whether [URE3] could be transmitted to cells expressing only *Ure2p_{sp}*, we analyzed the meiotic progeny of the [URE3] diploid. We observed a non-Mendelian inheritance of the USA⁺ phenotype (Figure 1C). This USA⁺ phenotype segregated independently of the *URE2_{sc}* or *URE2_{sp}* alleles (determined by PCR analysis; data not shown). USA⁺ phenotypes were cured after growth on YPD medium containing 3 mM

Figure 1. Ure2p_{Sp} can form and propagate the [URE3] prion. The $URE2_{Sp}$ open reading frame was inserted in place of the $URE2_{Sc}$ open reading frame at the resident $URE2$ locus to create a $URE2_{Sp}$ strain. (A) Comparative analysis of expression levels of Ure2p_{Sp} and Ure2p_{Sc}. Quantification of Ure2p was performed by Western blot analysis with an anti-Ure2p antibody. An antibody against Ade13p was used as loading control. Molecular weights are indicated on the right side. (B) Comparative growth on SD + uracil and SD + USA medium. Growth of cells was observed after 4 d at 30°C. The presence of functional Ure2p in the cells makes the strain unable to grow on SD + USA. Orthologous $URE2$ alleles expressed in each strain and [ure3-0] or [URE3] prion states are indicated. (C) Non-Mendelian segregation of [URE3] in tetrads from $URE2_{Sc}$ /URE2_{Sp} [URE3] diploids. Meiotic progeny (spores A, B, C, and D) of five different tetrads (T1 to T5) were spotted on SD supplemented with uracil or USA. To test whether the USA⁺ phenotype in the spore was curable, cells were streaked for single colonies on YPD medium containing 3 mM GuHCl. Single colonies were then tested for their ability to use USA (USA post-GuHCl). The segregations of the prion phenotype in the meiotic progeny (36 tetrads) are shown in the table. (D) Spontaneous apparition of USA⁺ colonies in strains with integrated $URE2_{Sc}$ or $URE2_{Sp}$ alleles. Frequencies of USA⁺ colonies were determined as described in *Materials and Methods*. SEs are indicated.

GuHCl. We conclude from these results that Ure2p_{Sp} can propagate the [URE3] prion.

The aggregation and resulting insolubility of Ure2p are hallmarks of the [URE3] prion state (Edskes *et al.*, 1999; Ripaud *et al.*, 2004). To characterize biochemically the prion form of Ure2p_{Sp}, we analyzed the solubility of Ure2p_{Sp} in USA⁺ cells by cellular fractionation after ultracentrifugation of yeast extracts. We found that Ure2p_{Sp} is specifically aggregated in [URE3] cells (Figure 2A). Furthermore, the cellular distribution of Ure2p_{Sp} was monitored by GFP fluo-

Figure 2. Biochemical and cellular characterization of the [URE3] prion propagated by Ure2p_{Sp}. (A) Sedimentation analysis of Ure2p_{Sp} in a $URE2_{Sp}$ strain with or without [URE3]. S, supernatant fraction, P, pellet fraction. Fractions were analyzed by Western blot with an anti-Ure2p antibody. Molecular weight markers are given on the right side. (B) Fluorescence microscopy assay of Ure2p_{Sp} aggregation status. Ure2p_{Sp}-GFP in [ure3-0] and [URE3] $URE2_{Sp}$ cells were observed after 2 h of growth in galactose medium.

rescence. For that purpose, $URE2_{Sp}$ [ure3-0] and [URE3] strains were transformed with a plasmid expressing an inducible fusion of Ure2p_{Sp} and the green fluorescent protein (Ure2p_{Sp}-GFP). Induction of Ure2p_{Sp}-GFP expression in cells containing [URE3] led primarily to a punctate fluorescence pattern, whereas [ure3-0] cells displayed diffuse fluorescence (Figure 2B). These data indicate that the [URE3] prion of Ure2p_{Sp} corresponds to a self-propagating aggregate.

Together, these results show that Ure2p_{Sp} can propagate the [URE3] prion. We next wondered whether Ure2p_{Sp} can also promote spontaneously [URE3] formation, because apparition and propagation of yeast prion could be mediated by distinct mechanisms (Derkatch *et al.*, 2000; Zhou *et al.*, 2001; Bradley and Liebman, 2004). Frequency of USA⁺ formation was determined for a $URE2_{Sp}$ strain. Spontaneous USA⁺ formation occurred at about the same rate as in $URE2_{Sc}$ cells (~2 in 10⁵ cells) (Figure 1D). The majority of the USA⁺ cells were cured in presence of 3 mM GuHCl. As expected, when reselected on USA-containing medium, USA⁺ clones occurred in such cured clones at a similar frequency as in the original cells (data not shown). We conclude from this set of experiments that when expressed at physiological level, Ure2p_{Sp} can spontaneously form [URE3] and stably propagate it.

High Overexpression of Ure2p_{Sp} Abolishes Its Prion Properties

In apparent contradiction with the results reported in the previous section, we had reported previously that Ure2p_{Sp} is not able to convert into the [URE3] prion form (Baudin-Baillieu *et al.*, 2003; Talarek *et al.*, 2005). Because the only difference between the present experiment and these previous studies was the use of different promoters, we wondered whether the capacity to support prion propagation could be dependent on Ure2p cellular concentration. So, we expressed different amounts of Ure2p_{Sc} and Ure2p_{Sp} in $URE2_{Sc}$ and $URE2_{Sp}$ [URE3] cells, respectively. We used monocopy or multicopy plasmids carrying the $URE2_{Sc}$ or $URE2_{Sp}$ ORF under control of the *GAL10* promoter. After growth in galactose medium, the amount of Ure2p was quantified by Western blotting experiments (Figure 3A). Monocopy and

Figure 3. Overproduction of Ure2p_{sp} suppresses [URE3] prion properties. (A) Estimation of Ure2p_{sc} and Ure2p_{sp} quantities. URE2_{sc} and URE2_{sp} alleles were placed under control of *GAL10*-inducible promoter and carried by a monocopy (pG1) or a multicopy (pG2) plasmids. Ure2p production was quantified by Western blot analysis. An antibody against Ade13p was used to normalize the quantification. The Ure2p level was expressed as a ratio of the amount expressed from the plasmid to the one expressed in a URE2_{sc} strain. Molecular weights markers are given on the right side. (B) High amounts of Ure2p_{sp} interfere with propagation of [URE3]. URE2_{sc} and URE2_{sp} strains propagating [URE3] prion were transformed with the control plasmid (pG), monocopy (pG1URE2_{sc}, pG1URE2_{sp}) or multicopy (pG2URE2_{sc}, pG2URE2_{sp}) plasmids. All strains were grown on galactose medium supplemented with uracil for 48 h and tested for their phenotype on dextrose and galactose medium containing USA (SD + USA, SG + USA). (C) Induction rate of USA⁺ colonies after transient overproduction of the URE2 orthologues. URE2_{sc} strain was transformed with the indicated plasmid carrying URE2_{sc} or URE2_{sp} alleles under control of the *GAL10* promoter. Individual transformants were streaked on galactose-raffinose dropout medium lacking leucine and grown for 3 d at 30°C. USA⁺ frequency was calculated as described in *Materials and Methods*. SEs are indicated.

multicopy plasmids led to approximately a 50- and a 500-fold overexpression, respectively, compared with Ure2p expressed from URE2 at the chromosomal locus. It was noted that the amount of Ure2p_{sp} is slightly higher than Ure2p_{sc} regardless of the promoter. *S. cerevisiae* and *S. paradoxus* Ure2p share the exact same sequence in their globular domains, and the differences are restricted to the prion-forming domain (Supplemental Figure S1) (Edsikes and Wickner, 2002; Baudin-Baillieu *et al.*, 2003). Because this domain is essential to stabilize the catalytic domain of Ure2p (Shewmaker *et al.*, 2007), the sequence differences in the prion-forming domain sequence between two orthologues might result in distinct stability of Ure2p.

We then tested the ability of the yeast cells to propagate [URE3] by testing their capacity to grow on galactose medium containing USA. As a control, we confirmed that the transformation per se did not impair the prion phenotype (Figure 3B, SD+USA). As published previously, a

strong overexpression of Ure2p_{sc} is compatible with growth on USA medium, whereas a very strong overexpression of Ure2p_{sp} is not (Figure 3B, SG+USA) (Baudin-Baillieu *et al.*, 2003). Intermediate overexpression of Ure2p_{sc} or Ure2p_{sp} did not affect [URE3] propagation (Figure 3B, SG+USA). The observation that Ure2p_{sp} overexpression led to severe inhibition of prion propagation contrasts with the fact that transient overexpression of Ure2p increases the frequency of prion formation (Wickner, 1994; Baudin-Baillieu *et al.*, 2003). So, we have also determined the effect of massive Ure2p_{sp} transient overexpression on [URE3] formation. We transiently expressed various amounts of Ure2p_{sc} or Ure2p_{sp}. Prion apparition was assayed as formation of USA⁺ colonies on a dextrose medium (Figure 3C). As reported previously, an increase of Ure2p_{sc} promoted the appearance of [URE3] at a higher frequency (Wickner, 1994). The difference in the induction rate was not statistically significant when Ure2p_{sc} was overexpressed either from a monocopy or multicopy plasmid. A mild overproduction of Ure2p_{sp} also led to a high induction of [URE3] formation. In contrast, such an induction rate was not observed after a high transient overexpression of Ure2p_{sp}. We got the same results when transient overexpressions were performed in a URE2_{sp} strain (data not shown).

Overall, our results indicate that prion formation rate is not strictly correlated with the expression level of Ure2p_{sc} or Ure2p_{sp} and in particular that high overexpression of Ure2p_{sp} dramatically lowers [URE3] formation rate.

Ure2p_{sp} and Ure2p_{sc} Aggregate in [URE3] Cells in an Ordered Pathway

Given that there are clear links between the yeast prion phenotype and the aggregation of the protein involved in this process, we decided to compare the aggregation dynamics of Ure2p_{sp} and Ure2p_{sc} in vivo. To that purpose, we used the Ure2p-GFP protein that when induced in [URE3] cells, decorates the preexisting prion aggregates and allows their visualization (Fernandez-Bellot *et al.*, 2002). The expression system is based on high copy number plasmids, the coding sequence of Ure2p_{sc}-GFP or Ure2p_{sp}-GFP being under the control of the inducible galactose promoter. Different patterns of aggregation, which were never detected in [ure3-0] cells, were observed. We grouped these patterns in four classes (Figure 4). We distinguish cells with small numerous foci quoted as the small aggregates class. We also observed cells with one large dot among smaller foci (medium aggregates class). The two other classes of aggregation are branching structures in which elongations are short (large aggregates) or long (very large aggregates). Formation of these distinct aggregates was not due to the overexpression of the Ure2p_{sc}-GFP construct itself because the same results were obtained when a low expression level of the Ure2p_{sc}-GFP fusion was used to decorate the Ure2p aggregates (Supplemental Figure S2).

We quantified the proportion of each of the four defined classes at different times after the induction of the expression of the fluorescent protein in a cell population (Figure 5A). The switch from dextrose to galactose medium was realized during late exponential phase. Because of the metabolic transition in this condition, less than one generation occurs during the first 24 h. We thus consider that we are dealing with a nondividing cell population. For each observation, at least two classes coexisted, but their ratio changed during the kinetics. Immediately after the induction of Ure2p_{sc}-GFP or Ure2p_{sp}-GFP, most cells belong to the small aggregates class. Few hours later, the proportion of this class has a clear tendency to decrease, and more cells with medium aggre-

Figure 4. Classes of Ure2p-GFP aggregates in [URE3] cells. [URE3] cells were transformed with multicopy pG2URE2_{sc}-GFP and pG2URE2_{sp}-GFP plasmids. The cells were grown overnight on dextrose medium supplemented with USA. In exponential phase ($OD_{600} = 1$), the cells were placed in galactose medium supplemented with uracil, and different times after the induction, the fluorescent proteins were observed in the cells. Typically observed patterns are shown.

gates class were observed. Finally, after a longer induction time, these two classes have completely disappeared and the branching structures were observed (large and very large aggregates class). In individual cells we never observed large aggregates and small aggregates together. The number of visible aggregates in each cell decreased in the course of

expression of Ure2p-Gfp, suggesting a coalescence of Ure2p aggregates. The simplest interpretation of these observations is that Ure2p_{sp} and Ure2p_{sc} aggregate in the same ordered pathway in [URE3] cells and that the observed aggregates types derive from each other leading to aggregates of increasing size and in decreasing number.

Figure 5. Aggregation dynamics of Ure2p-GFP in [URE3] cells. [URE3] cells were transformed with multicopy pG2URE2_{sc}-GFP and pG2URE2_{sp}-GFP plasmids. The cells were grown overnight on dextrose medium supplemented with USA. In exponential phase ($OD_{600} = 1$), the cells were placed in galactose (A) or raffinose (B) supplemented with uracil. Different times after the induction, the proportion of cells (among at least 100 fluorescent cells) containing the different class of aggregates were evaluated.

High Overexpression of Ure2p Accelerates the Aggregation Dynamics of Ure2p

We have demonstrated that the [URE3] prion properties depend on the amount of Ure2p. We therefore studied the aggregation kinetics of Ure2p_{sc} and Ure2p_{sp} at two distinct cellular concentrations of Ure2p_{sc}-GFP and Ure2p_{sp}-GFP. To this purpose, we have induced the expression either with raffinose or raffinose + galactose as the carbon source. Raffinose, contrary to dextrose, does not repress the *GAL10* promoter, allowing a weak expression of the protein. In this condition, the proportion of cells containing small aggregates decreased slowly with both orthologues (Figure 5B). Indeed, ~40% of the cells still correspond to the small aggregates class 24 h after the onset of induction. In contrast, when fluorescent proteins were overexpressed by inducing with galactose, this cell population had totally disappeared 9 h after the induction of overexpression. Moreover, in overexpression conditions, we observed emergence of cells with large aggregates as soon as 6 h after the onset of induction, whereas they were never formed when Ure2p_{sc}-GFP or Ure2p_{sp}-GFP was expressed at low levels. These results show that the concentration of cellular Ure2p influences its aggregation dynamics in [URE3] cells.

We also observed that, in galactose + raffinose medium, there is a significant difference in the aggregation dynamic of Ure2p_{sc}-GFP and Ure2p_{sp}-GFP (Figure 5A). The proportion of cells exhibiting small aggregates decreases more rapidly with Ure2p_{sp}-GFP. In fact, 6 h after galactose induction, 58% of cells expressing Ure2p_{sc}-GFP contained small aggregates, whereas the proportion of this population was only 12% with Ure2p_{sp}-GFP. Moreover, Ure2p_{sp}-GFP formed large aggregates more efficiently than Ure2p_{sc}-GFP because after 24 h, 92% of the cells expressing Ure2p_{sp}-GFP contained these particular aggregates compared with 35% for cells expressing Ure2p_{sc}-GFP. In fact, at all time points, aggregates were larger in Ure2p_{sp}-GFP than in Ure2p_{sc}-GFP-expressing cells. These results clearly demonstrated that the aggregation dynamics rely not only on the cellular concentration of the prion protein but also on its primary sequence.

The Aggregation Dynamics of Ure2p Defines the Number of [URE3] Propagons

We wondered whether the differences in aggregation dynamics of Ure2p in [URE3] cells (*URE2_{sc}*, *URE2_{sc}* overexpressing Ure2p and *URE2_{sp}* strains) could be correlated with variations in the number of [URE3] propagons per cell. We have estimated the number of [URE3] propagons by the method described by Eaglestone *et al.* (2000). This analysis is based on chemical inactivation of Hsp104 by low concentrations of GuHCl that inhibits prion replication and thus leads to rapid prion loss in dividing cells by progressive dilution of the propagons (Eaglestone *et al.*, 2000; Ferreira *et al.*, 2001). [URE3] strains (*URE2_{sc}*, *URE2_{sc}* overexpressing Ure2p and *URE2_{sp}* strains) were transferred during log phase into YPD medium supplemented with 3 mM GuHCl; at regular time points, cells density was determined and cultures were spread on plates to determine the percentage of [URE3] loss. In the strain overexpressing Ure2p_{sc}, the overexpression was stopped when cells were transferred into YPD supplemented with 3 mM GuHCl.

As expected, the kinetics of [URE3] curing displayed two phases; there was a lag phase during which no curing occurred followed by a linear increase in the number of cured cells over time (Figure 6) (Eaglestone *et al.*, 2000; Ripaud *et al.*, 2003). The curing profile for the [URE3] *URE2_{sc}* strain

Figure 6. Kinetics of [URE3] elimination following guanidine hydrochloride treatment. [URE3] cells were grown in YPD supplemented with 3 mM guanidine hydrochloride as described in *Materials and Methods*. At regular time points, cultured aliquots were removed, and [URE3] loss was estimated by calculating the frequency of USA⁺ colonies (see *Materials and Methods*). The percentages of [URE3] cells have been plotted as a function of generations, which have been estimated from OD₆₀₀. The kinetics of [URE3] curing reported in this figure were determined as the average of at least four independent experiments. The number of propagons can be calculated from the value of 2^g , where g is the number of generations elapsed at the time point when 36.8% of cells had lost the prion (63.2% [URE3]), assuming that the propagon number halves at each generation.

was much slower than that observed in either the [URE3] *URE2_{sc}* strain overexpressing Ure2p_{sc} or the [URE3] *URE2_{sp}* strain. For the two latter strains, the elimination of [URE3] was so fast that the lag phase was not always apparent in the four performed independent curing kinetics (Figure 6). The number of propagons in [URE3] cells can be inferred from these kinetics of elimination. From the method developed by B. Cox (Cox *et al.*, 2003), we estimate the apparent number of propagons to be 52 for [URE3] *URE2_{sc}* strain. This number is much larger than that found for either [URE3] *URE2_{sp}* or [URE3] *URE2_{sc}* overexpressing Ure2p (15 and 10 propagons, respectively). These results show that the number of propagons is influenced by the primary sequence of the Ure2p prion-forming domain, and remarkably, that an increase of cellular concentration of Ure2p decreases the number of propagons. In other terms, we have correlated a variation of aggregation dynamic of Ure2p with variations in the numbers of [URE3] propagons.

Infectivity of Ure2p Aggregates and Their Ability to Propagate as a Prion Can Be Dissociated

In a previous study, we showed that the formation of large aggregates of Ure2p correlates with [URE3] elimination (Ripaud *et al.*, 2003). We confirm that in these experimental conditions, when only the large and very large aggregates were observed, the majority of cells lost the [URE3] prion. Indeed, when the overexpression of Ure2p_{sc}-GFP in [URE3] cells was stopped after 24 h of induction, only 5×10^{-4} cells still maintained the USA⁺ phenotype on glucose medium (data not shown). This strong and fast prion elimination led us to propose that curing of [URE3] occurred by inactivation of propagons through the massive aggregation of Ure2p

Figure 7. Functional characterization of Ure2p-GFP large aggregates. (A) Infectivity of sonicated yeast crude extract from *URE2_{sc}* [URE3] + pG2URE2_{sc}-GFP containing large aggregates was performed as described in Materials and Methods. A control transformation was carried out with buffer without yeast extract. The number of [URE3] colonies relative to total transformants was determined from 330 colonies. (B) *URE2_{sc}* [URE3] strains, transformed with pG2URE2_{sc}-GFP, were grown as described in Figure 4. Twenty-four hours after galactose induction, an aliquot of this culture was briefly sonicated and plated as isolated cells on solid dextrose medium. Microcolonies were observed after 12 h. The mother cells containing large aggregate are indicated with an arrowhead.

(Ripaudo *et al.*, 2003). This inactivation could result from the formation of structurally distinct off-pathway nonprion Ure2p aggregates due to overexpression. Alternatively, inactivation could simply be because of the sequestration of the propagons in the mother cell through their assembly into bigger aggregates. In the latter hypothesis, the Ure2p large aggregates should be structurally related to the bona fide prion aggregates and should therefore conserve their ability to induce [URE3] apparition once introduced in [ure3-0] yeast cells. To test this hypothesis, we transformed [ure3-0] yeast cells with a crude extract of a strain containing large Ure2p_{sc}-GFP aggregates together with a plasmid bearing the *LEU2* marker. Then, Leu⁺ transformants were tested for the presence of [URE3] by their capacity to grow in the presence of USA. The prion phenotype was further confirmed by the phenotypic reversibility of the growth on USA medium after having treated the cells with 3 mM GuHCl. Approximately 22% of the Leu⁺ transformants were [URE3]. The results clearly indicate that the cells with Ure2p_{sc}-GFP large aggregates contain Ure2p aggregates that are still infectious (Figure 7A) and that are thus structurally related with the propagating prion aggregates.

The above-mentioned finding prompted us to investigate whether the cells with large aggregates of Ure2p are able to generate new transmissible aggregates. To that purpose, the progeny of cells with the large Ure2p-GFP aggregates were analyzed for their fluorescent aggregates content. After one generation, the proportion of cells with large aggregate clearly decreased, suggesting that large aggregates are nontransmissible (Supplemental Table S1). In accordance with the observed fast prion elimination, in microcolonies, daughter cells of mothers containing large aggregates Ure2p-GFP consistently contained no aggregates (Figure 7B). In a control experiment, the daughter cells of mothers propagating [URE3] prion exhibited the same ag-

gregation pattern (small aggregates, visualized by the expression of a low level of Ure2p_{sc}-GFP from the pH327 plasmid) (Supplemental Figure S3).

Together, these results suggest that the infectivity of Ure2p aggregates and their ability to propagate as a prion can be dissociated.

DISCUSSION

In this study, we showed that Ure2p of *S. paradoxus* possesses the capacity to switch into a [URE3] prion conformation. Our analyses demonstrated that the expression level of Ure2p_{sp} strongly modulates this capacity. The ability to induce the prion form and/or to propagate this self-inactivation is observed only when Ure2p_{sp} was not expressed at high level. In contrast, Ure2p_{sc} and Ure2p of *S. uvarum* orthologue exhibited these prion properties regardless of the amount of Ure2p that is expressed (Baudin-Baillieu *et al.*, 2003).

Ure2p_{sp} prion propagation is possible in *S. cerevisiae* (Edskes and Wickner, 2002; this study). In a previous study, however, we failed to isolate [URE3] clones when Ure2p_{sp} was expressed in its genuine cellular context, namely, in *S. paradoxus* (Talarek *et al.*, 2005). Expression level of Ure2p_{sp} in *S. paradoxus* was similar to the ones allowing prion propagation in *S. cerevisiae*. Why [URE3] propagation from Ure2p_{sp} could not be detected in *S. paradoxus* is unclear at present. It might be that Ure2p_{sp} prion propagation cannot occur in that host or else that prion formation occurs at a very low frequency.

The native yeast prion proteins change from the functional form to the prion form more frequently if the parent genes are overexpressed. As a rule, it is widely accepted that "the more you express, the more prion you get." The observed frequency of prion formation after a transitory overexpression of Ure2p_{sc} or Ure2p_{sp} from a monocopy plasmid is 100-fold higher than the rate of spontaneous acquisition of the prion at normal expression level. When the transient overexpression is performed from a multicopy plasmid, the cellular level of Ure2p is increased ~10-fold compared with Ure2p level produced from a monocopy plasmid. If the induction of [URE3] apparition was strictly proportional to the amount of Ure2p, the prion formation should also increase in the same extent. Surprisingly, strong overexpression of Ure2p_{sc} led to the same induction rate as mild overexpression. The effect was even more dramatic with Ure2p_{sp} because increasing its protein concentration (from mild to strong overexpression) decreases (rather than increases) the frequency with which [URE3] arises. So, increasing the pool of Ure2p does not continually augment the occurrence of the event that initiates prion formation.

Yeast prion determinants are related to amyloid polymerization. In vitro, purified Ure2p spontaneously forms amyloid fibers, and the introduction of such fibers into [ure3-0] yeast cells leads to the [URE3] prion phenotype (Brachmann *et al.*, 2005; Immel *et al.*, 2007). But not all amyloid-forming proteins are prions. Tanaka *et al.* (2006) developed an elegant model, experimentally validated, that accounts for the difference between a noninfectious and a prion (infectious) amyloid. They proposed that the infectious nature of an amyloid is defined by two fundamental parameters: the rate of polymer elongation and its susceptibility to shearing. An amyloid aggregate can be propagated as a prion in a population of dividing cells only if, in combination, these parameters surpass a threshold to allow sustained propagation. Possible explanations for the impairment of prion properties when Ure2p is strongly overexpressed could therefore be

related to a change either in the rate of amyloid growth or in the brittleness of the aggregates. The fragmentation frequency of an amyloid fiber is defined by the intrinsic physical properties of this particular amyloid conformation. It is unlikely that an increased level of Ure2p results in a change of conformational structure of its amyloid fibers. Conversely, *in vitro*, the speed of Ure2p fiber growth is linearly proportional to the concentration of soluble Ure2p (Fay *et al.*, 2003). Similarly, our *in vivo* analysis highlighted that the aggregation dynamics of Ure2p is faster when cellular concentration of Ure2p increases. We showed that the Ure2p aggregates isolated from cells which no longer propagate [URE3] still retain their ability to induce [URE3] once artificially introduced in wild-type [ure3-0] cells. This result suggests that the large aggregates are structurally related to the amyloid [URE3] prion aggregates and do not correspond to a distinct off-pathway aggregate. The high levels of Ure2p increase the speed of polymerization of fibers. Consequently, it is possible that above a threshold Ure2p amount, the formed aggregates are not self-propagating because their size increase and coalescence is too rapid. In this model, rapidly growing and coalescing Ure2p aggregates would fail to be segregated properly to daughter cells during cell divisions, as shown by our microscopy observations (Figure 7B). In agreement with this model, is the fact that increasing the cellular concentration of Ure2p results in a reduction of [URE3] propagon number (Figure 6).

Here, we showed that the aggregation pathway of the two Ure2p orthologues share the same intermediates states. However, a major difference lies in the aggregation dynamics that is faster for Ure2p_{sp} than for Ure2p_{sc}. Consequently, we observed many more cells containing very large aggregates when Ure2p_{sp} is overexpressed. Similarly, our *in vitro* analyses showed that Ure2p_{sp} exhibits a higher propensity to aggregate than Ure2p_{sc} (Immel *et al.*, 2007). We have also demonstrated that Ure2p_{sp} is able to assemble in an infectious amyloid and that this one was significantly more resistant than the Ure2p_{sc} amyloid to mechanical shearing (Immel *et al.*, 2007). Interestingly, our propagon counting assay indicated that the apparent number of propagons per cell in the [URE3] Ure2p_{sp} strain was much smaller than that found in [URE3] Ure2p_{sc} (Figure 7). Therefore, we can speculate that Ure2p_{sp} would tend to form aggregates, which could resist more efficiently to fragmentation and would be unable to be transmitted to daughter cells. The most likely possibility to explain this higher stability could be the longer asparagine stretch present in the prion-forming domain (Supplemental Figure S1). Indeed, the prion aggregation properties of the described yeast proteins rely on their content in glutamine and asparagine (Osherovich *et al.*, 2004). These stretches could stabilize the polymer by specific polar zipper structures and asparagine-ladders (Perutz *et al.*, 2002; Nelson *et al.*, 2005). Thus, independently of its rate of polymerization, the Ure2p_{sp} prion aggregates would be less prone to fragmentation than prion aggregates of Ure2p_{sc}.

In Figure 8, we have summarized all these observations and integrated them in the analytical model proposed by Tanaka *et al.* (2006). Our results suggest that there would be another threshold above which stable prion propagation would not be possible. In this area on the diagram (top left area), the prion particles would be too large and/or to few to allow efficient propagation at cell division. Thus, although in the Tanaka model increasing fiber growth rate favors prion stability, it might be that above a given value, the polymerization rate can become detrimental to prion maintenance.

Together, our results suggest that the concentration of a prion protein modifies a fundamental parameter (the amy-

Figure 8. A diagram of the parameters determining the ability of an amyloid to be prion. In this diagram adapted from Figure 1B in Tanaka *et al.* (2006), we suggest, without direct experimental evidence, relative positions for the [URE3] prion propagated by Ure2p_{sc} and Ure2p_{sp} with different amount levels. Based on previous *in vitro* evidence and the results reported here, Ure2p_{sp} is represented at a position in the diagram reflecting a higher aggregation rate and a lower fragmentation ability as Ure2p_{sc}. An increase in the Ure2p level leads to an increase in fiber growth rate and the size of prion aggregates, the arrows represent this variation. Our addition to the model defines another threshold (white line) in addition to the threshold defined by Tanaka *et al.* (2006) (black line). Above of this limit, stable prion propagation is not possible. So, a change of Ure2p_{sp} level would transform a prion amyloid into a nonprion amyloid.

loid polymerization rate), which contributes to determine its transmission ability. It is possible that variation in prion protein expression could also interfere with prion propagation in mammals. Prion accumulation occurs in specific neuronal target areas. The reasons that define this cellular tropism are still largely unknown. Interestingly, it was reported that the level of prion protein differs markedly between different neuronal types (Ford *et al.*, 2002). It would be interesting to study whether the concentration of cellular PrP could contribute to the determination of the variable cellular tropism of prion accumulation.

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