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DOI: 10.1534/genetics.105.043489

The [URE3] Prion Is Not Conserved Among Saccharomyces Species

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Manuscript received March 17, 2005
Accepted for publication May 17, 2005

ABSTRACT

The [URE3] prion of *Saccharomyces cerevisiae* is a self-propagating inactive form of the nitrogen catabolism regulator Ure2p. To determine whether the [URE3] prion is conserved in *S. cerevisiae*-related yeast species, we have developed genetic tools allowing the detection of [URE3] in *Saccharomyces paradoxus* and *Saccharomyces uvarum*. We found that [URE3] is conserved in *S. uvarum*. In contrast, [URE3] was not detected in *S. paradoxus*. The inability of *S. paradoxus* Ure2p to switch to a prion isoform results from the primary sequence of the protein and not from the lack of cellular cofactors as heterologous Ure2p can propagate [URE3] in this species. Our data therefore demonstrate that [URE3] is conserved only in a subset of Saccharomyces species. Implications of our finding on the physiological and evolutionary meaning of the yeast [URE3] prion are discussed.

PRION is a commonly accepted term to describe the "infectious," conformationally altered form of an unusual class of proteins found in both mammals and fungi. They were originally implicated in a group of fatal neurodegenerative diseases in mammals, the transmissible spongiform encephalopathies, in which PrP^{Sc}, the prion form of the normal protein PrP^{C}, acts as an infectious agent (for reviews see Prusiner *et al.* 1998; Collinge 2001). In 1982, Prusiner proposed that PrP^{Sc} propagates by converting PrP^{C} into PrP^{Sc} by an autocatalytic process. Nevertheless, prions are not solely disease-causing agents. Indeed, more recently, it was shown that prions act as novel epigenetic determinants allowing adaptation of cells under certain conditions (True and Lindquist 2000; True *et al.* 2004). In the case of the [Het-s] prion of the fungus *Podospora anserina*, the prion form of the protein is the active form in a cell-cell recognition phenomenon (Coustou *et al.* 1997).

In *Saccharomyces cerevisiae*, two nonchromosomal elements, [URE3] and [PSI+], discovered a few decades ago (Cox 1965; Aigle and Lacroute 1975), were identified as the prion forms of Ure2p and Sup35p, respectively (Wickner 1994). Ure2p acts as a negative regulator in nitrogen catabolism repression (NCR). In the presence of a good nitrogen source, Ure2p binds the Gln3p transcriptional activator. In turn, this prevents the transcription of a number of genes involved in nitrogen catabolism, including the *DAL5* gene that encodes the allantoate permease. [URE3] proved to be an inactive form of Ure2p (Wickner 1994). Consequently, because of a lack of functional Ure2p, [URE3] and *ure2* cells can take up poor nitrogen sources even in the presence of good nitrogen sources in the medium (for review see Cooper 2002). Sup35p is involved in translation termination. In [PSI^+] cells, termination efficiency is strongly reduced, conferring suppression of nonsense mutations (for reviews see Uptain and Lindquist 2002; Tuite and Koloteva-Levin 2004). Both [URE3] and [PSI^+] are dominant in haploid crosses, display a non-Mendelian segregation in meiosis, and are efficiently eliminated (cured) on a medium containing 5 mM guanidine hydrochloride (GuHCl) (for review see Uptain and Lindquist 2002). Ure2p and Sup35p do not share any sequence similarities but in their N-terminal portion both contain stretches of asparagine and glutamine residues, termed the prion forming domain (PFD). The PFD is essential for prion appearance, maintenance, and propagation and is distinct from the functional domain of each protein (Ter-Avanesyan *et al.* 1994; Masison and Wickner 1995; Derkatch *et al.* 1996; Masison *et al.* 1997). *In vitro* studies showed that these PFDs are responsible for the formation of Ure2p and Sup35p amyloid aggregates, which are thought to be related to the prion-replicating species (Glover *et al.* 1997; King *et al.* 1997; Taylor *et al.* 1999; Thual *et al.* 1999; King and Diaz-Avalos 2004; Tanaka *et al.* 2004). Recently, the yeast prion world has become more populated. Rnq1p and New1p, two Asn/Gln-rich-domain-containing proteins, were identified as prions in *S. cerevisiae* (Santoso *et al.* 2000; Sondheimer and Lindquist 2000). *In silico* analyses identified 107 more polypeptides encoded by the *S. cerevisiae* genome that also contain a Asn/Gln-rich domain, indicating that additional prions might exist in this species (Michelitsch and Weissman 2000).

The physiological relevance of prions in yeast is still an open question. In the case of [PSI^+], genetic studies have

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shown that the prion may allow cells to thrive in certain fluctuating environments (Eaglestone et al. 1999; True and Lindquist 2000). In an attempt to analyze the potential adaptative role of [PSI$^+$], the conservation of [PSI$^+$] has been studied (Chernoff et al. 2000; Kushnirov et al. 2000; Santoso et al. 2000; Jensen et al. 2001; Nakayashiki et al. 2001; Resende et al. 2002, 2003). PFD of full-length genes of *SUP35* orthologs from distantly related yeast species have been cloned and their prion properties analyzed in *S. cerevisiae* (Chernoff et al. 2000; Kushnirov et al. 2000; Santoso et al. 2000; Zadorskii et al. 2000; Nakayashiki et al. 2001; Resende et al. 2002). These studies have shown that all the Sup35p orthologs tested can behave as [PSI$^+$] in *S. cerevisiae*. While the ability to form [PSI$^+$] seems to be well conserved throughout evolution, the same kind of studies on Ure2p indicate that [URE3] is less conserved. Indeed, several Ure2p orthologs from other yeast species do not behave as prions in *S. cerevisiae* (Edskes and Wickner 2002; Baudin-Baillieu et al. 2003). However, it is unclear whether the lack of prion behavior of the Ure2p orthologs results from the intrinsic inability of these proteins to adopt the prion isoform or from the lack in *S. cerevisiae* of species-specific cellular cofactors necessary for prion formation. With one exception (Nakayashiki et al. 2001), all previous studies on the evolutionary biology of yeast prions have been carried out through heterologous expression in *S. cerevisiae*. These studies thus do not address maintenance or loss of the prion properties of prion protein orthologs in their genuine cellular context. In our study, rather than analyzing heterologous expression of Sup35p or Ure2p orthologs in *S. cerevisiae*, we chose to directly determine whether [URE3] could exist in non-*cerevisiae* species.

To gain insight into the conservation of [URE3], we have developed genetic tools to monitor the appearance of [URE3] properties in *S. uvarum* and *S. paradoxus*, two yeast species closely related to *S. cerevisiae*. We first show that the nitrogen regulation function of the tested Ure2p orthologs has been conserved. Then we demonstrate that Ure2p of *S. uvarum* can behave as a prion in *S. uvarum*, whereas Ure2p of *S. paradoxus* cannot behave as a prion in *S. paradoxus*. Finally, we show that *S. cerevisiae* Ure2p can adopt a prion isoform in both *S. uvarum* and *S. paradoxus*. Our results clearly indicate that the lack of prion properties of Ure2p in *S. paradoxus* is an intrinsic property of the primary sequence of Ure2p and not due to the lack of species-specific cellular factors. This fact further reveals that [URE3] is not conserved throughout evolution in the Saccharomyces genus, in spite of *URE2* ortholog conservation.

MATERIALS AND METHODS

Nomenclature: To avoid confusion, we used Sc (*S. cerevisiae*), Sp (*S. paradoxus*), Su (*S. uvarum*), and Kl (*K. lactis*) in subscript to specify the gene origin. For prion nomenclature, [URE3] is used to name the prion status and [ure0] to name the wild-type status of Ure2p.

Plasmids construction: Table 1 presents the characteristics of all the plasmids used in this study. All the plasmids described below were obtained using the gap repair method (Orr-Weaver and Szostak 1983). Details of the constructions are available upon request. Cloning procedures of the *URE2* open reading frames (ORFs) or of the *URE2* PFD of the various yeast species into pYeHFn2L were described previously (Baudin-Baillieu et al. 2003). Monocopy plasmids bearing *URE2* ORFs were constructed from these plasmids by replacing the 2μ origin with an ARS-CEN origin obtained from pYeHFc1L (Cullin and Minvielle 1994). The *LEU2* cassette of pYe2L-*URE2*ΔC (Baudin-Baillieu et al. 2003) was replaced by the *URA3* cassette taken from pYeHFn2U (Cullin and Minvielle 1994) to obtain pYe2U-*URE2*ΔC.

To get the pYe2L-$DAL5_{Su}$ plasmid, the $DAL5_{Su}$ ORF, its promoter (319 bp upstream), and its terminator (317 bp downstream) were PCR amplified from the Su1a strain and cloned into pYeHFn2L between the *Bam*HI and *Bsu*36I sites. From this plasmid, to get the pYe2L-$pDAL5_{Su}$::$ADE2_{Sc}$ plasmid, the $DAL5_{Su}$ ORF was replaced by the one of $ADE2_{Sc}$, PCR amplified from the pYeHFn2A plasmid. To get the pYe2L-$URE2_{Su}$ plasmid, the $URE2_{Su}$ gene was PCR amplified from strain Su1a and cloned into pYeHFn2L between the *Bam*HI and *Bsu*36I sites. To get the pYe2L-$ure2_{Su}$::$URA3_{Sc}$, a part of $URE2_{Su}$ was replaced by the $URA3_{Sc}$ gene, PCR amplified from the pYeHFn2U plasmid. To get the pYe2L-HO_{Sp} plasmid, the HO_{Sp} gene was PCR amplified from strain Sp4707-22D and cloned into the pYeHFn2L plasmid between the *Bam*HI and *Bsu*36I sites. From this plasmid, to get the pYe2L-ho_{Sp}::*KanMX4* plasmid, the *HO* ORF was replaced by the *KanMX4* cassette, PCR amplified from the pFA-6A plasmid (Wach et al. 1994). To get the pYe2L-$DAL5_{Sp}$, the $DAL5_{Sp}$ ORF, its promoter (389 bp upstream), and its terminator (447 bp downstream) were PCR amplified from strain Sp4707-22D and cloned into pYeHFn2L between the *Bam*HI and *Bsu*36I sites. With this plasmid, the ORF of $DAL5_{Sp}$ was replaced by the ORF of $ADE2_{Sc}$, PCR amplified from the pYeHFn2A plasmid, resulting in the pYe2L-$pDAL5_{Sp}$::$ADE2_{Sc}$ plasmid. To create the pYe2L-$URE2_{Sp}$ plasmid, the $URE2_{Sp}$ gene was PCR amplified from strain Sp4707-22D and cloned into the pYeHFn2L between the *Bam*HI and *Bsu*36I sites. From this plasmid, to make the pYe2L-*ure2-Sp::LYS1Sc* plasmid, a part of $URE2_{Sp}$ was replaced by the *LYS1* gene, PCR amplified from genomic DNA of *S. cerevisiae*. For each plasmid, a test was performed in corresponding strains to check the functionality of the clones' ORFs. All sequences were obtained from the Génolevure project (Souciet et al. 2000), the genomic sequence projects (Cliften et al. 2003; Kellis et al. 2003), or from our data. The pH660 plasmid was kindly provided by Reed Wickner (Edskes and Wickner 2002).

Strain construction: All strains used in this study are listed in Table 2. Details of the constructions are available upon request. *S. uvarum* $Su_{[ure0]}$ was constructed from strain Su5-1A (Talarek et al. 2004) by transformation with a PCR product containing the $dal5_{Su}$::$ADE2_{Sc}$ cassette amplified from pYe2L-$pDAL5_{Su}$::$ADE2_{Sc}$ (the correct notation should be *dal5*::P_{DAL5} *ADE2*; for the sake of simplicity we noted the construction as *pDAL5::ADE2*). Integrative transformants were selected on minimal medium without adenine and with proline as poor nitrogen source. The *S. uvarum* strain $Su_{(\Delta ure2)}$ was constructed by transformation of the *S. uvarum* strain $Su_{[ure0]}$ with a PCR product containing the $ure2_{Su}$::$URA3_{Sc}$ cassette amplified from pYe2L-$ure2_{Su}$::$URA3_{Sc}$. Integrative transformants were selected on SD medium without adenine-containing ammonia as a good nitrogen source. Sp4707-22D, Sp4795-3B′/D, and Sp2B12D *S. paradoxus* original strains were used. From these

TABLE 1

Plasmids used in this study

Plasmid	Description	Reference
pYeHFn2L	2μ *LEU2*	Cullin and Minvielle (1994)
pYe2L-$URE2_{Sc}\Delta C$	2μ *LEU2* P_{GAL10} $URE2_{Sc}\Delta C$	Baudin-Baillieu *et al.* (2003)
pYe2L-$URE2_{Sp}\Delta C$	2μ *LEU2* P_{GAL10} $URE2_{Sp}\Delta C$	Baudin-Baillieu *et al.* (2003)
pYe2L-$URE2_{Su}\Delta C$	2μ *LEU2* P_{GAL10} $URE2_{Su}\Delta C$	Baudin-Baillieu *et al.* (2003)
pYe2L-$URE2_{Kl}\Delta C$	2μ *LEU2* P_{GAL10} $URE2_{Kl}\Delta C$	Baudin-Baillieu *et al.* (2003)
pYeHFn2U	2μ *URA3*	Cullin and Minvielle (1994)
pYe2U-$URE2_{Sc}\Delta C$	2μ *URA3* P_{GAL10} $URE2_{Sc}\Delta C$	This study
pYe2U-$URE2_{Sp}\Delta C$	2μ *URA3* P_{GAL10} $URE2_{Sp}\Delta C$	This study
pYe2U-$URE2_{Su}\Delta C$	2μ *URA3* P_{GAL10} $URE2_{Su}\Delta C$	This study
pYe2U-$URE2_{Kl}\Delta C$	2μ *URA3* P_{GAL10} $URE2_{Kl}\Delta C$	This study
pYe1L-$URE2_{Sc}$	*CEN LEU2* P_{GAL10} $URE2_{Sc}$	This study
pYe1L-$URE2_{Sp}$	*CEN LEU2* P_{GAL10} $URE2_{Sp}$	This study
pYe1L-$URE2_{Su}$	*CEN LEU2* P_{GAL10} $URE2_{Su}$	This study
pYe1L-$URE2_{Kl}$	*CEN LEU2* P_{GAL10} $URE2_{Kl}$	This study
pYe2L-$URE2_{Sp}$	2μ *LEU2* P_{GAL10} $URE2_{Sp}$	Baudin-Baillieu *et al.* (2003)
pH660	2μ *LEU2* P_{GAL1} $URE2_{Sp}$	Edskes and Wickner (2002)

strains, heterothallic strains were obtained by transformation with a PCR product containing the ho_{Sp}::*KanMX4* cassette from the plasmid pYe2L-*hoSp::KanMX4*. Integrative transformants were selected on rich medium containing 200 mg/liter G418 (Sigma, St. Louis). *S. paradoxus* strain $Sp_{[ure0]}$ was constructed from *S. paradoxus ade2* strain by transformation with a PCR product containing the $pDAL5_{Sp}$::$ADE2_{Sc}$ cassette, obtained from the plasmid pYe2L-$pDAL5_{Sp}$::$ADE2_{Sc}$. Integrative transformants were selected on minimal medium without adenine and with a poor nitrogen source. The *S. paradoxus* $Sp_{(\Delta ure2)}$ strain was constructed by transformation of *S. paradoxus* strain Sp12B with a PCR product containing the $ure2_{Sp}$::$LYS1_{Sc}$, obtained from pYe2L-$ure2_{Sp}$::$LYS1_{Sc}$. Integrative transformants were selected on SD medium without adenine containing a good nitrogen source. In each case, to confirm the disruption, transformants were analyzed by PCR. All transformants were also crossed with a strain of opposite mating type and the resulting diploids were sporulated and dissected to obtain strains with opposite mating type.

Medium and microbiological methods: Yeast cells were grown at 30° according to methods previously described for *S. cerevisiae* (Sherman 1991). YPDA was the YPD rich medium supplemented with 20 mg/liter of adenine. Synthetic dextrose (SD: 2% dextrose, 0.67% yeast nitrogen base with ammonia) was used as selective medium. The color phenotype was assayed on YPD4 medium [1% peptone, 1% yeast extract (Fisher), 4% dextrose]. Color phenotypes (white to dark red) were checked after 5–7 days at 30° and 2 days at 4°. The ureidosuccinic acid (USA) uptake phenotype in *ura2* strains was tested on SD medium to which the required amino acids and bases (except uracil) were added as well as 15 μg/ml USA (pH 6.7). Induction of the galactose promoter was performed on appropriate synthetic glucose-free medium containing 2% galactose (SG) and, if necessary, 2% raffinose. All mating, sporulation, and dissection procedures were carried out according to standard protocols (Sherman and Hicks 1991). For *S. uvarum* and *S. paradoxus* species, procedures were performed as previously described (Talarek *et al.* 2004). Asci dissections were performed by micromanipulation (Singer Instrument MSM). Curing [URE3] by GuHCl was performed in liquid or solid medium. In liquid medium, cells were grown for 20 generations in the presence of 2.5 mm or 5 mm GuHCl in YPD at 30°. The resulting population was then tested for [Ade] phenotype. In solid medium, a drop of [URE3] cells was put on YPD4 medium containing 2.5 mm or 5 mm GuHCl. The white/red phenotype was checked after 5–7 days at 30°.

Ure2p solubility assay by subcellular fractionation: Yeast [URE3] cells were grown in SD medium without adenine (in SD medium containing adenine for the [ure0] strain) to exponential phase and then diluted into YPDA medium and grown for 5 more hours. Total protein extract and fraction preparation were done as described previously (Ripaud *et al.* 2003). Urea was added to each fraction to a final concentration of 8 m. Prior to loading, the samples were boiled for 5 min. Equal quantities of each sample were analyzed by Western blot (12% SDS-PAGE, tricine buffer). Proteins were transferred onto nitrocellulose membranes, revealed with the ECL+ reagent (Pierce, Rockford, IL), and recorded with the VersaDoc Imaging System (Bio-Rad, Hercules, CA). Ure2p was quantified with Quantity One software (Bio-Rad). Polyclonal antibodies raised against Ure2p were affinity purified and diluted 1/3000 (Fernandez-Bellot *et al.* 2000).

RESULTS

Ure2p function is conserved among species: To analyze the conservation of [URE3] in the Saccharomyces genus, we searched for [URE3] cells in *S. paradoxus* and *S. uvarum*. For this purpose, we developed a reporter system allowing an easy detection of [URE3] in these species. We adapted the reporter system previously described by Schlumpberger *et al.* (2001) for *S. cerevisiae* and used this as a secondary screen for the identification of antiprion molecules (Bach *et al.* 2003). In this system the *ADE2* open reading frame is under the control of the *DAL5* promoter and inserted at the *DAL5* locus. *ADE2* transcription is thus under the control of the Gln3p transcriptional activator, which is inhibited by Ure2p in the presence of a good nitrogen source (Figure 1) (for review see Cooper 2002). Therefore, on rich and minimal media containing ammonia (a good nitrogen source), the [ure0] cells are red and [Ade^-], whereas the [URE3] cells are white and [Ade^+]. Thus,

TABLE 2

Strains used in this study

Strain	Species	Genotype	Reference
Sc$_{[ure0]}$	*S. cerevisiae*	*MAT***a** *his3-11,15 leu2-3,112 ade2-1 trp1-1 ura2::HIS3 pDAL5::ADE2*	Bach *et al.* (2003)
Sc$_{[URE3]}$	*S. cerevisiae*	*MAT***a** *his3-11,15 leu2-3,112 ade2-1 trp1-1 ura2::HIS3 pDAL5::ADE2* [URE3]	Bach *et al.* (2003)
Su5-1A	*S. uvarum*	*MAT***a**, *ura3-1 leu2::URA3 ade2::URA3*	Talarek *et al.* (2004)
Su$_{[ure0]}$	*S. uvarum*	*MAT*α *ura3-1 leu2::URA3 ade2::URA3 pDAL5::ADE2*	This study
Su$_{[URE3]S}$	*S. uvarum*	*MAT*α *ura3-1 leu2::URA3 ade2::URA3 pDAL5::ADE2* [URE3]S[a]	This study
Su$_{[URE3]I}$	*S. uvarum*	*MAT*α *ura3-1 leu2::URA3 ade2::URA3 pDAL5::ADE2* [URE3]I[b]	This study
Su$_{(\Delta ure2)}$	*S. uvarum*	*MAT***a**, *ura3-1, leu2::URA3, ade2::URA3, ure2::URA3, pDAL5::ADE2*	This study
Sp4707-22D	*S. paradoxus*	*MAT***aaa** *his4 ura3 ade1 leu2*	Hawthorne and Philippsen (1994)
Sp4795-3B′/D	*S. paradoxus*	*MAT*ααα *met1 ura1 ade2 aro7*	Hawthorne and Philippsen (1994)
Sp2B12D	*S. paradoxus*	**a**/α *trp5x/trp5y ade2/+ ade5,7/+ +/ade1 leu1x/leu1y his4/+ lys1/+ are4/+ met13x/met13y*	Herbert *et al.* (1988)
Sp12B	*S. paradoxus*	*MAT***a** *ade2 leu2 his4 lys1 pDAL5::ADE2*	This study
Sp$_{[ure0]}$	*S. paradoxus*	*MAT***a** *ho::KanMX4 his4 leu2 ade2 ura3 pDAL5::ADE2*	This study
Sp$_{(\Delta ure2)}$	*S. paradoxus*	*MAT***a** *ho::KanMX4 his4 leu2 ade2 ura3 lys1 ure2:: LYS1 pDAL5::ADE2*	This study
CC30	*S. cerevisiae*	*MAT***a** *his3-11,15 leu2-3,112 ade2-1 trp1-1 ura2::HIS3*	Fernandez-Bellot *et al.* (2000)

[a] [URE3]S, the S stands for spontaneous [URE3].
[b] [URE3]I, the I stands for induced [URE3].

the use of this reporter system allows discrimination between [URE3] and [ure0] cells simply by checking their color and their auxotrophy for adenine on rich and minimal media, respectively.

All *URE2* orthologs studied so far share much more similarity in their C-terminal functional domain than in their N-terminal PFD. They retain their Ure2p function when expressed in *S. cerevisiae* (Edskes and Wickner 2002; Baudin-Baillieu *et al.* 2003). Before testing the prion properties of Ure2p$_{(Sc,Sp,Su,Kl)}$ orthologs, we determined whether they could complement an *URE2* deletion in *S. uvarum* and *S. paradoxus*. To this purpose we transformed our reporter Δ*ure2* strains with plasmids expressing the Ure2p orthologs from a galactose-inducible promoter. The *S. uvarum* Su$_{(\Delta ure2)}$ strain was [Ade$^+$] on glucose minimal medium (Figure 2, SD). On galactose minimal medium, when Ure2p$_{Su}$ was expressed, the strains became [Ade$^-$]. The same result was observed with the other Ure2p orthologs (Figure 2, SG), indicating that all the orthologs complemented the *URE2* gene deletion in *S. uvarum*. We performed the same assay with the *S. paradoxus* Sp$_{(\Delta ure2)}$ strain and obtained the same results (data not shown). Thus the Ure2p function in nitrogen catabolism repression is conserved among the studied orthologs and this allowed us to study the conservation of their prion properties.

[URE3] appears spontaneously in *S. uvarum* but not in *S. paradoxus*: To determine whether or not [URE3] exists in *S. paradoxus* and *S. uvarum*, we searched for spontaneous [URE3] in *S. uvarum* and *S. paradoxus*. [ure0] *S. cerevisiae*, *S. paradoxus*, and *S. uvarum* strains (Sc$_{[ure0]}$, Sp$_{[ure0]}$ and Su$_{[ure0]}$, respectively) were grown on SD medium without adenine. Several [Ade$^+$] clones were obtained for each species (Table 3). Then the

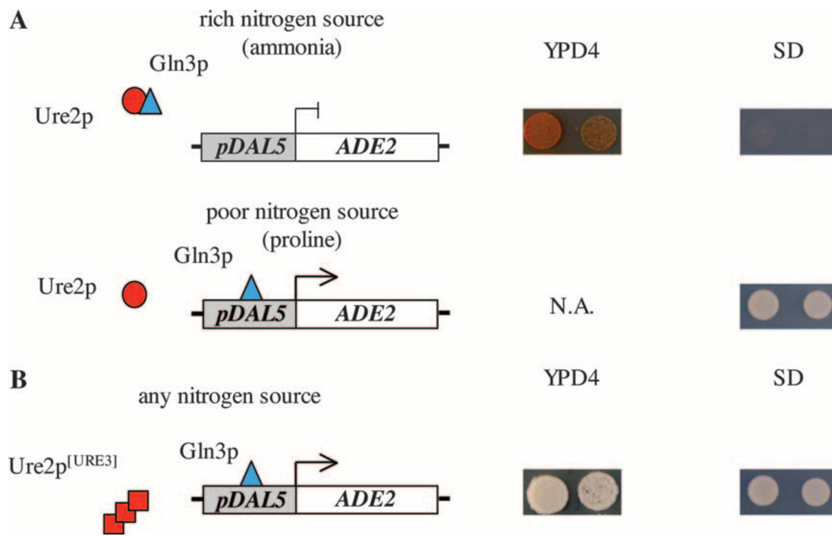


FIGURE 1.—Reporter system used to detect [URE3] in *S. cerevisiae*, *S. paradoxus*, and *S. uvarum*. (A) [ure0]: Ure2p in normal state. In an *ade2* strain on rich or minimal medium containing a good nitrogen source (such as ammonia), Ure2p binds Gln3p and prevents the transcription of *ADE2* from the *DAL5* promoter. On rich medium (YPD4) where adenine is limiting, colonies are red due to the lack of Ade2p activity. They cannot grow on glucose minimal medium without adenine (SD). On glucose minimal medium (SD) containing proline, a poor nitrogen source, Ure2p does not bind Gln3p. Gln3p can thus activate the transcription of *ADE2* from the *DAL5* promoter. Colonies are therefore [Ade$^+$] on proline medium. (B) [URE3]: Ure2p in prion state. In the [URE3] state, Ure2p is in an inactive form, and Gln3p can activate the transcription of *ADE2* from the *DAL5* promoter whatever the nitrogen source. Colonies are white on rich medium (YPD4) and [Ade$^+$] on SD medium. Due to the lack of Ure2p, an *ure2* mutant displays the same phenotype as that of an [URE3] strain. NA, not appropriate.

[URE3] status of 200 [Ade$^+$] clones was tested using three criteria. To be scored as [URE3], the [Ade$^+$] clones had to display the following characteristics. First, adenine prototrophy should be cured after GuHCl treatment; second it should be dominant in a cross with an *ade2* haploid; and third, it should display a non-Mendelian segregation in tetrads obtained after sporulation of the resulting diploid. As shown in Table 3, for *S. cerevisiae* the spontaneous frequency of appearance of [URE3] was 6.8×10^{-6}, a frequency comparable to those obtained in previous studies (Wickner 1994). For *S. uvarum* the spontaneous frequency of appearance of [URE3] was 4.2×10^{-5}, slightly higher than that observed for *S. cerevisiae*. This result indicates that Ure2p$_{Su}$ can spontaneously adopt a prion isoform in *S. uvarum*. To our knowledge, this is the first report of a spontaneous emergence of a yeast prion in a yeast species other than *S. cerevisiae*. For *S. paradoxus*, none of the tested [Ade$^+$] clones were [URE3]. Thus, in *S. paradoxus*, Ure2p$_{Sp}$ did not spontaneously adopt a prion state at a frequency detectable with our reporter system. This strongly suggests that [URE3] is not conserved in all Saccharomyces species.

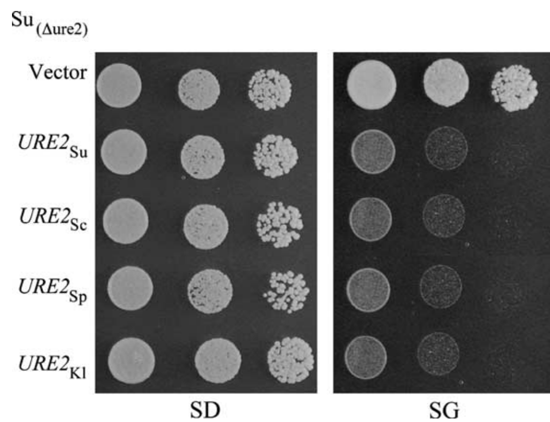


FIGURE 2.—Complementation of *ure2* by Ure2p orthologs. The *S. uvarum* Su $_{(\Delta ure2)}$ strain was transformed by plasmids carrying *URE2* orthologs under the control of a galactose-inducible promoter control (pYe2L-*URE2*). All the strains grew on dextrose minimal medium (SD) without adenine, because the absence of the *URE2* function allows the transcription of the reporter system. On galactose minimal medium without adenine (SG), strains did not grow, indicating that Ure2p was expressed and prevented the transcription of the *ADE2* gene.

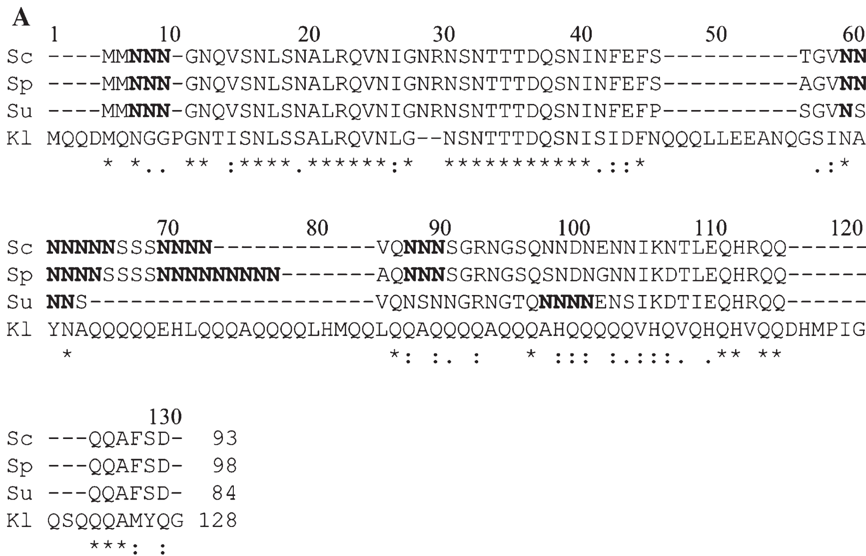
Ure2p PFDs can induce [URE3] in *S. uvarum* but not in *S. paradoxus*: Ure2p$_{Sc}$ consists of an N terminus PFD (residues 1–90) and a C-terminal domain (residues 91–354) that is required for activity and resembles glutathione *S*-transferases in sequence and structure and that has been proved to have glutathione peroxydase activity (Coschigano and Magasanik 1991; Bousset *et al.*

TABLE 3

Spontaneous appearance of [URE3] in *S. cerevisiae*, *S. paradoxus*, and *S. uvarum*

Species	[Ade$^+$] per 10^7 cells	[URE3] frequency
S. cerevisiae	710	6.8×10^{-6}
S. paradoxus	220	$<10^{-7}$
S. uvarum	830	4.2×10^{-5}

After overnight growth in YPD rich medium, 10^7 cells were plated onto SD medium without adenine. After 8 days of growth, the number of [Ade$^+$] were counted. The [URE3] status of 200 [Ade$^+$] clones was then monitored as described in the results (cure, dominance, segregation) except for *S. paradoxus* where all the [Ade$^+$] clones were analyzed. All the [Ade$^+$] clones that were cured by guanidine hydrochloride displayed a non-Mendelian segregation.



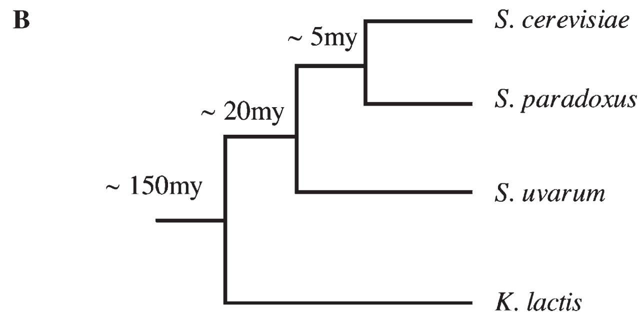


Figure 3.—Evolutionary analysis of Ure2p PFDs. (A) Multiple alignments of the Ure2p orthologs PFDs. The sequences are from a previous study (Baudin-Baillieu *et al.* 2003). Identities are indicated with an asterisk, strong and weak similarities with two and one point, respectively. Main Asn stretches are in boldface type. Sequences were aligned using the ClustalW algorithm (Higgins *et al.* 1996). (B) Phylogenetic relationships of yeast based on DNA sequences (Cliften *et al.* 2003; Kellis *et al.* 2003). The evolutionary distance was expressed in millions of years (my).

2001; Umland *et al.* 2001; Bai *et al.* 2004). In *S. cerevisiae*, overproduction of the Ure2p$_{Sc}$ PFD strongly increases the frequency of [URE3] appearance (Masison and Wickner 1995; Maddelein and Wickner 1999). We asked whether the overexpression of PFDs from Ure2p orthologs, as they were previously described (Figure 3A and Baudin-Baillieu *et al.* 2003), could increase [URE3] frequency in *S. uvarum* and promote [URE3] appearance in *S. paradoxus*. We transformed strains with plasmids expressing PFDs from a galactose-inducible promoter and analyzed the [URE3] phenotype of the resulting [Ade$^+$] clones obtained upon overexpression of PFD. In the *S. uvarum* Su$_{[ure0]}$ strain, overexpression of the PFD$_{Su}$ induced a threefold increase in the frequency of [URE3] appearance (Table 4A). Heterologous PFD$_{Sc}$ and PFD$_{Sp}$ were more effective than PFD$_{Su}$, inducing a 13-fold and a 25-fold increase in this frequency, respectively. By contrast, overexpression of the PFD$_{Kl}$ had no effect on the frequency of [URE3] appearance (Table 4A). These results parallel the one obtained in *S. cerevisiae* (Baudin-Baillieu *et al.* 2003). Thus, the PFDs capable of inducing [URE3] in *S. cerevisiae* were also able to induce [URE3] in *S. uvarum* and, conversely, the PFD (PFD$_{Kl}$) that failed to induce [URE3] in *S. cerevisiae* is also inactive in [URE3] in *S. uvarum* induction (Baudin-Baillieu *et al.* 2003). The different PFDs were also overexpressed in the *S. paradoxus* Sp[ure0] strain and [Ade$^+$] clones were recovered. The frequency of [Ade$^+$] clones obtained is seemingly the same with an empty vector or with any overexpressed PFD (Table 4B). Among 200 [Ade$^+$] clones tested, none were curable by GuHCl treatment and all displayed a Mendelian segregation (Table 4B). Thus we concluded that no [URE3] clones were obtained, indicating that Ure2p$_{Sp}$ could not adopt a prion isoform despite a strong overexpression of PFDs. It thus appears that Ure2p$_{Sp}$ cannot adopt a prion isoform in *S. paradoxus* either spontaneously or upon induction with PFDs.

Characterization of two distinct [URE3]$_{Su}$ strains in *S. uvarum*: Another aspect of prion biology is the existence of different prion strains that were first identified in mammals (Bruce *et al.* 1991). In *S. cerevisiae*, different [URE3] (Schlumpberger *et al.* 2001) and [PSI$^+$] strains (Uptain *et al.* 2001; Bradley *et al.* 2002; King and Diaz-Avalos 2004; Tanaka *et al.* 2004) have also been distinguished, depending on the strength of the phenotype. Genetic studies have shown that prion strains differ by their mitotic stability. In the case of Sup35p, the different [PSI$^+$] strains are characterized by differing amounts of Sup35p in the aggregated form and by the existence of different amyloid conformations (Uptain *et al.* 2001; Bradley *et al.* 2002; King and Diaz-Avalos 2004; Tanaka *et al.* 2004).

TABLE 4

[URE3] induction by overexpression of PFD in *S. uvarum* $Su_{[ure0]}$ and *S. paradoxus* $Sp_{[ure0]}$ strains

Plasmid	[URE3] frequency	Induction fold above the spontaneous frequency
	A. In *S. uvarum*	
Vector	2.1×10^{-6}	
PFD_{Sc}	2.7×10^{-5}	13
PFD_{Sp}	5.2×10^{-5}	25
PFD_{Su}	6.0×10^{-6}	3
PFD_{Kl}	1.9×10^{-6}	1
Plasmid	[Ade$^+$] frequency	[URE3] frequency
	B. In *S. paradoxus*	
Vector	3.11×10^{-5}	
PFD_{Sc}	2.40×10^{-5}	$<10^{-7}$
PFD_{Sp}	4.05×10^{-5}	$<10^{-7}$
PFD_{Su}	1.55×10^{-5}	$<10^{-7}$
PFD_{Kl}	1.44×10^{-5}	$<10^{-7}$

Strains were transformed with plasmids allowing the overexpression of the different $PFD_{(Sc,Sp,Su,Kl)}$ from a galactose-inducible promoter (pYe2L-*URE2*$_{(Sc,Sp,Su,Kl)}\Delta C$). Some clones of transformed strains were grown on raffinose/galactose minimal medium for 72 hr to induce overexpression. Then cells were plated onto glucose SD medium. After 5 days of growth, [ADE$^+$] clones were collected and their [URE3] state was then monitored as described in the RESULTS. The [URE3] state of 200 [Ade$^+$] clones was then monitored as described in the RESULTS. It should be noted that upon overexpression with PFD_{Sc}, PFD_{Sp}, and PFD_{Su}, in *S. uvarum* all the [Ade$^+$] clones obtained were [URE3].

In *S. uvarum*, $[URE3]_{Su}$ clones were obtained either spontaneously or upon overexpression of PFDs. All spontaneous *S. uvarum* [URE3] clones gave rise to light-red colonies on rich medium and grew poorly on SD medium (Figure 4; see [URE3]S; S for *s*pontaneous). Upon PFD overexpression, all [URE3] clones gave rise to white colonies on rich medium and grew as well on SD medium (Figure 4, [URE3]I, I signifying *i*nduced) as the wild type does (data not shown). To further differentiate between these two $[URE3]_{Su}$ strains, we

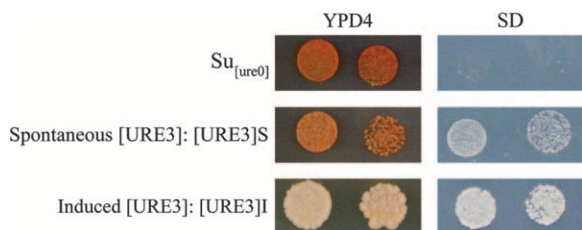


FIGURE 4.—Comparison of color phenotype and adenine prototrophy in *S. uvarum* $Su_{[ure0]}$ colonies and in two kinds of *S. uvarum* [URE3] strains, $Su_{[URE3]S}$ and $Su_{[URE3]I}$, obtained spontaneously and after PFD overexpression, respectively. Cells were grown on YPD4 and on SD medium at 30° for 5–7 days. The S and I indicate *s*pontaneous and *i*nduced [URE3], respectively.

TABLE 5

Mitotic and meiotic stability and dominance of $Su_{[URE3]S}$ and $Su_{[URE3]I}$

Strain	Mitotic stability (%)	[URE3] diploid (%)	Meiotic stability (%) 4::0	3::1	2::2	1::3	0::4
$Su_{[URE3]S}$	20	30	32	29	29	7	3
$Su_{[URE3]I}$	92	90	90	7	3	0	0

Mitotic stability was determined by counting the ratio of [Ade$^+$] clones after 20 generations in YPDA rich medium (2000 cells were counted for each strain). Dominance was determined by monitoring [Ade$^+$] diploids obtained after crosses between wild-type and [URE3] strains (30 diploids were counted for each strain). After sporulation and dissection of the resulting [Ade$^+$] diploids, meiotic stability was determined by analyzing the [URE3] segregation ([URE3]::[ure0]) (120 tetrads were dissected for each strain).

analyzed the mitotic and meiotic stability, dominance, and Ure2p solubility for each strain (Table 5).

Growth of both strains in YPDA during 20 generations showed that [URE3]I is more stable mitotically than [URE3]S. We then crossed the *S. uvarum* Su[ure0] strain with both [URE3] strains and analyzed the [URE3] status of the resulting diploids. This result indicated that [URE3]I is more invasive than [URE3]S. The phenotype of the progeny after sporulation of the previously obtained [URE3] diploids revealed that [URE3]I is meiotically more stable than [URE3]S. We then analyzed the solubility of Ure2p by subcellular fractionation (Figure 5). This indicated that more of the Ure2p was found in pellet fraction in the Su[URE3]I strain than in the Su[URE3]S strain. This suggests that overexpression of PFD leads to a strong [URE3] strain and a weak [URE3] strain was obtained spontaneously.

***S. paradoxus* can propagate $[URE3]_{Sc}$ and $[URE3]_{Su}$:** Unlike $Ure2p_{Su}$, $Ure2p_{Sp}$ cannot adopt a prion isoform in *S. cerevisiae* spontaneously, upon overexpression with PFDs, or in the presence of a preexisting $[URE3]_{Sc}$ (BAUDIN-BAILLIEU *et al.* 2003). We showed above that

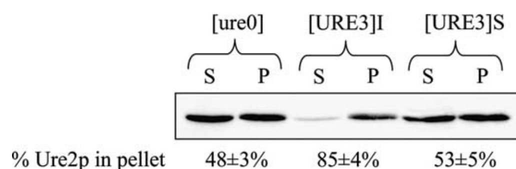


FIGURE 5.—Ure2p solubility in *S. uvarum* $Su_{[ure0]}$, $Su_{[URE3]S}$, and $Su_{[URE3]I}$ strains. $Su_{[ure0]}$ is the [ure0] *S. uvarum* strain, and the $Su_{[URE3]S}$ strain corresponds to the *S. uvarum* spontaneous [URE3] (Figure 4, Table 3). The $Su_{[URE3]I}$ strain corresponds to the *S. uvarum* [URE3] obtained after overexpression of PFD (Figure 4, Table 4). We determined the subcellular distribution of Ure2p and the percentage of Ure2p in the 100,000 × *g* pellet fraction (% Ure2p in the pellet) as described in MATERIALS AND METHODS. S, supernatant fraction; P, pellet fraction. The values were obtained from three independent experiments.

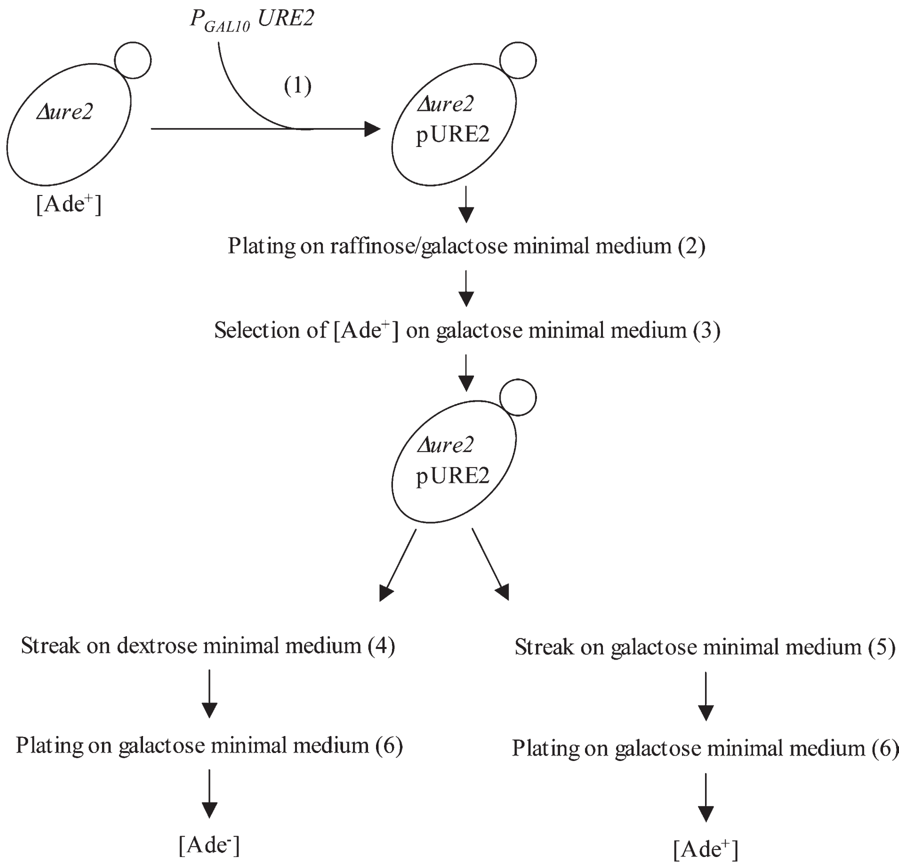


Figure 6.—Induction of $[URE3]_{Sc}$ in *S. uvarum* $Su_{(\Delta ure2)}$ and in *S. paradoxus* $Sp_{(\Delta ure2)}$ strains. (1) Cells were transformed with a plasmid, allowing the overexpression of Ure2p (pYe1L-*URE2*) from a galactose-inducible promoter. (2) Transformed strains were plated onto raffinose/galactose minimal medium supplemented with adenine. After 3 days, these strains were transferred onto galactose minimal medium without adenine to select $[Ade^+]$ clones (3). To test the [URE3] status of the $[Ade^+]$ clones, clones were replica plated on dextrose minimal medium to switch off the overexpression of Ure2p (4). Indeed, [URE3] cannot be maintained without continuous *URE2* expression. Clones remained on galactose (5) and one replica plated on dextrose minimal medium (4) were tested again on galactose minimal medium without adenine (6). Clones were [URE3] when cells remaining on galactose were $[Ade^+]$ and cells replica plated on glucose became $[Ade^-]$. The [URE3] status was confirmed by GuHCl treatments.

$Ure2p_{Sp}$ cannot adopt a prion isoform in *S. paradoxus* either spontaneously or upon overexpression of PFD. The lack of [URE3] in *S. paradoxus* could result from either an intrinsic inability of $Ure2p_{Sp}$ to switch to the prion state or the lack of cellular cofactors necessary for prion propagation (for review see Osherovich and Weissman 2002; Uptain and Lindquist 2002). To check if *S. paradoxus* displays all the necessary cofactors required for [URE3] propagation, we determined whether $Ure2p_{Sc}$ and $Ure2p_{Su}$ are able to give rise to [URE3] in this species. To avoid any cross-reaction, the *S. paradoxus* $Sp_{(\Delta ure2)}$ strain was used. This strain was transformed with plasmids overexpressing either $Ure2p_{Sc}$ or $Ure2p_{Su}$ from a galactose-inducible promoter. The method used to determine the [URE3] status is summarized in Figure 6. As the initial Δure2 strain has the same phenotype as potential [URE3] clones, this method is rather complex and is based on the fact that the prion phenotype cannot be maintained in the transient absence of Ure2p expression. [URE3] was obtained when $Ure2p_{Sc}$ or $Ure2p_{Su}$ was overexpressed (Table 6). These results indicated that *S. paradoxus* can harbor a [URE3] phenotype and that all the cellular cofactors required to induce, maintain, and propagate [URE3] are present in this species.

To further document prion conversion in a heterologous cellular context, we also tested, in the same way, whether $Ure2p_{(Sc,Sp,Kl)}$ can adopt a prion isoform in *S. uvarum*. To avoid any cross-reaction, the *S. uvarum* $Su_{(\Delta ure2)}$ strain was used. This strain was transformed with plasmids overexpressing Ure2p orthologs from a galactose-inducible promoter. In the *S. uvarum* $Su_{(\Delta ure2)}$ strain, [URE3] cells were obtained by overexpression of $Ure2p_{Sc}$ or $Ure2p_{Su}$ (Table 7). This result confirmed that in *S. uvarum* cellular cofactors required to induce, maintain, and propagate [URE3] were present. No [URE3] clones were obtained when $Ure2p_{Sp}$ or $Ure2p_{Kl}$ was overexpressed (Table 7), implying that these proteins themselves do not contain sequences necessary to allow prion formation and/or propagation.

$Ure2p_{Sp}$ cannot adopt a prion isoform in *S. paradoxus* even in the presence of a preexisting heterologous [URE3]: We could hypothesize that $Ure2p_{Sp}$, which failed to spontaneously adopt a prion isoform in

TABLE 6

Ure2p of *S. cerevisiae* and *S. uvarum* adopt a prion isoform in *S. paradoxus*

Ortholog	[URE3] frequency
$URE2_{Sc}$	5×10^{-6}
$URE2_{Sp}$	0
$URE2_{Su}$	1×10^{-6}
$URE2_{Kl}$	0

The $Sp_{(\Delta ure2)}$ strain was transformed with plasmids expressing Ure2p orthologs. The [URE3] phenotype was tested as described in Figure 6. The [URE3] state of 200 $[Ade^+]$ clones was monitored.

TABLE 7

Prion properties of Ure2p orthologs in *S. uvarum*

Ortholog	[URE3] frequency
$URE2_{Sc}$	1×10^{-5}
$URE2_{Sp}$	0
$URE2_{Su}$	2×10^{-6}
$URE2_{Kl}$	0

The $Su_{(\Delta ure2)}$ strain was transformed with plasmids expressing Ure2p orthologs. The [URE3] phenotype was tested as described in the legend of Figure 6. The [URE3] state of 200 [Ade^+] clones was monitored.

S. paradoxus, might be converted into a prion form in the presence of preexisting [URE3]. Therefore we tested whether the $Ure2p_{Sp}$ protein could be converted into a prion isoform in the presence of $[URE3]_{Sc}$ in *S. paradoxus*. We crossed a $[URE3]_{Sc}$ Δ*ure2 S. paradoxus* haploid strain (see above) with a *S. paradoxus* $Sp_{[ure0]}$ strain on galactose minimal medium to maintain $Ure2p_{Sc}$ expression. In the diploid strain both $Ure2p_{Sc}$ and $Ure2p_{Sp}$ are expressed. If $Ure2p_{Sp}$ is not converted into [URE3], the diploid should remain wild type for $Ure2p_{Sp}$ function. Among the 30 tested diploids, none was [Ade^+], showing that $Ure2_{Sp}$ remained in its functional isoform (data not shown). This result indicated that $Ure2p_{Sp}$ cannot adopt a prion isoform even in the presence of a preexisting [URE3].

DISCUSSION

One of the most intriguing questions surrounding fungal prions concerns their potential biological role. Prion properties of many orthologs of Ure2p and Sup35p have been studied in *S. cerevisiae* (Chernoff *et al.* 2000; Kushnirov *et al.* 2000; Santoso *et al.* 2000; Nakayashiki *et al.* 2001; Edskes and Wickner 2002; Resende *et al.* 2002; Baudin-Baillieu *et al.* 2003), but so far only one study has addressed the aggregation of Sup35p in a yeast species other than *S. cerevisiae* (Nakayashiki *et al.* 2001). Here we demonstrate that [URE3] can be obtained either spontaneously or upon overexpression of PFD in *S. uvarum*. Concerning *S. paradoxus*, two contradictory results have been obtained with $Ure2p_{Sp}$ when it has been expressed in *S. cerevisiae* (Edskes and Wickner 2002; Baudin-Baillieu *et al.* 2003). More precise analyses of these results (Edskes and Wickner 2002) tend to show that a readthrough phenomenon explains these contradictory results (supplemental data at http://www.genetics.org/supplemental/). Although we cannot formally rule out that [URE3] might exist in an atypical form in *S. paradoxus* with an extremely low probability of appearance, we have never obtained $[URE3]_{Sp}$ spontaneously, upon overexpression of PFD, or in the presence of preexisting [URE3]. We conclude that typical [URE3] cannot be formed in *S. paradoxus*. This suggests that [URE3] is not conserved throughout the Saccharomyces genus. Thus the presence of [URE3] is not correlated with the phylogenetic tree since *S. paradoxus* (which lacks [URE3]) is a more closely related species to *S. cerevisiae* than is *S. uvarum* (which harbors [URE3]) (Figure 3B).

Conservation of prion properties is mediated by primary sequence rather than by cellular factors: Among the Ure2p and Sup35p orthologs tested in *S. cerevisiae*, some exhibit prion properties whereas others do not. Two hypotheses may be proposed to explain this observation. First, species-specific cellular factors could allow or prevent prion apparition. Second, prion properties could be associated with an intrinsic behavior of each protein. We found that both $Ure2_{Sc}$ and $Ure2_{Su}$, two orthologs that can behave as prions in their own cellular context, can adopt a prion isoform in all three tested species. Consequently, all specie*s* possess the cellular factors necessary for [URE3] propagation. However, in *S. paradoxus*, $Ure2_{Sp}$ cannot adopt a prion isoform spontaneously, upon overexpression of PFDs, or even in the presence of a preexisting $[URE3]_{Sc}$. Thus $Ure2p_{Sp}$ does not behave as a prion in species that possess a cellular context permissive to prion emergence (this study and Baudin-Baillieu *et al.* 2003). These data suggest that the conservation of prion properties is mediated by the protein itself rather than by cellular cofactors. Further, the main cellular cofactors allowing prion emergence and propagation appear to be functionally conserved throughout the Saccharomyces genus.

An Asn/Gln-rich domain is not sufficient to confer prion properties: Several lines of evidence have indicated that the PFD of yeast prion proteins is necessary and sufficient for prion formation (Tuite 2000). In the case of Ure2p orthologs, PFDs have been defined from multiple alignments (Baudin-Baillieu *et al.* 2003 and Figure 3A). All these PFDs share a high content of Asn/Gln residues. Although the three PFDs that share a high degree of identity are able to induce [URE3] in both *S. cerevisiae* and *S. uvarum*, the PFD_{Kl} that contains a stretch of glutamines does not retain the prion-inducing properties in these two species. The abundance of Asn/Gln residues in a protein sequence has been used as a criterion to identify new potential prions in *S. cerevisiae* (Michelitsch and Weissman 2000). In addition, recent data indicate that [URE3] prion formation is driven primarily by the amino acid composition of the PFD, largely independent of its primary sequence (Ross *et al.* 2004). However, our results indicate that a high content of Asn/Gln does not systematically confer prion-inducing properties when it is attached to its globular domain. Indeed, expressed alone, the PFD_{Sp} is able to induce [URE3] in *S. cerevisiae* and *S. uvarum*. However, when it is attached to the globular domain, its prion-inducing properties are lost. These data confirm that an Asn/Gln-rich domain is not sufficient to

determine whether a protein can behave as a prion and that the prion-inducing properties of an Asn/Gln-rich domain can be very different when the Asn/Gln-rich domain is embedded in the full-length protein. It should be noted that *S. cerevisiae* and *S. paradoxus* Ure2p's share the exact same sequence in their globular domains only and that the differences are contained in the PFDs. Several results suggest that the PFD and the C-terminal domain of $Ure2p_{Sc}$ interact functionally with each other, leading to an inhibitory effect on the acquisition of the prion state (Fernandez-Bellot *et al.* 1999, 2000; Maddelein and Wickner 1999). However, it has been suggested that there are no physical interactions between the two domains of $Ure2p_{Sc}$ (Pierce *et al.* 2005). In the case of the [Het-s] prion, it has been shown that the potential prion-inducing properties of the PFD can be modulated by the PFD's interaction with its related globular domain (Balguerie *et al.* 2003). To explain the loss of prion-inducing properties of the PFD_{Sp} when fused to the C-terminal domain, it is tempting to speculate that this is due to the N79D change because asparagine-to-aspartate mutations were shown to have a drastic effect on [*PSI*$^+$] induction (Osherovich and Weissman 2001). However, this mutation is also present in the PFD_{Su} sequence with no dramatic consequences. Another possibility to explain the loss of prion properties in $Ure2p_{Sp}$ could be that the longer asparagine stretch and/or the change of a few amino acids couples the PFD to the C-terminal domain in a manner that would prevent [URE3] apparition. In the same way, overexpression of PFDs that usually induce [URE3] in *S. cerevisiae* and *S. uvarum* species (this study and Baudin-Baillieu *et al.* 2003) does not lead to such an induction in *S. paradoxus* probably because the PFD of Ure2p embedded in the whole protein is unable to interact with overexpressed PFD and allow prion propagation. In conclusion, the prion property is linked to the whole protein and is not restricted to its sole PFD.

[URE3] as an evolutionary significant epigenetic metabolic switch?: While prions are lethal pathogens in mammals (for review see Dobson 1999), their physiological meaning is different in other organisms. For instance, in the case of the [Het-s] prion of the fungus *P. anserina,* the prion form of the protein is the active form in the cell-cell recognition phenomenon that might be beneficial for that species (Coustou *et al.* 1997). Also, several studies support the idea that [PSI$^+$] confers some advantage to the cells harboring it (Eaglestone *et al.* 1999; True and Lindquist 2000; Namy *et al.* 2002). It has also been proposed that the metastable [PSI$^+$] state offers the opportunity for emergence of new traits (True *et al.* 2004). Conservation of the prion properties throughout evolution among the various Sup35p orthologs supports this hypothesis (Chernoff *et al.* 2000; Kushnirov *et al.* 2000; Santoso *et al.* 2000; Jensen *et al.* 2001; Nakayashiki *et al.* 2001; Resende *et al.* 2002, 2003). Concerning [URE3], the situation described here reveals a more complex relationship between genes and phenotypes. Sequences of the PFDs evolved more quickly than those of the globular domain in Ure2p (Baudin-Baillieu *et al.* 2003). Moreover, prion property is lost or retained without correlation with the importance of these variations. Surprisingly, *S. uvarum* retains the prion function whereas *S. paradoxus* does not, although as compared to *S. cerevisiae*, PFD_{Su} shows a larger difference in sequence than PFD_{Sp} (Figure 3A). To explain this paradox, it can be hypothesized that a selective pressure acts on the prion property in *S. cerevisiae* and *S. uvarum*, but not in *S. paradoxus.* Two facts are in agreement with this idea:

i. *S. cerevisiae* and *S. uvarum* are found in the same natural biotope, often in composite populations (Naumov *et al.* 2000). Conversely, *S. paradoxus* has never been found in these biotopes (I. Masneuf, personnal communication). It is thus reasonable to propose that the selective pressure, whatever it is, could act on *S. cerevisiae* and *S. uvarum* but not on *S. paradoxus.*
ii. Δ*ure2* strains of *S. cerevisiae* have been reported to be more selectively competitive on natural substrate compared to wild-type strains (Salmon and Barre 1998). The same kind of behavior would be expected for [URE3] strains since they have the same phenotype regarding the NCR.

Altogether, this suggests that: (1) there is a loose relation between amino acid sequence and prion property (Ross *et al.* 2004) and (2) a high rate of variations of those sequences can lead to this original evolution story.

We thank S. Saupe, M. Blondel, B. Daignan-Fornier, I. Sagot, and C. Schwimmer for helpful discussions and critical reading of the manuscript. We thank Mohan Gupta for looking over the English. We are grateful to R. B. Wickner and H. Edskes for generously providing pH660 plasmid. This work was supported by a grant from the Groupement d'Intérêt Scientifique "Infections à Prion" and the Fondation pour la Recherche Médicale. N.T. was supported by grants from the French Ministère de la Recherche and from the Centre National de la Recherche Scientifique.

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Communicating editor: A. Nicolas