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Induction of Distinct [*URE3*] Yeast Prion Strains

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[*URE3*] is a non-Mendelian genetic element in *Saccharomyces cerevisiae*, which is caused by a prion-like, autocatalytic conversion of the Ure2 protein (Ure2p) into an inactive form. The presence of [*URE3*] allows yeast cells to take up ureidosuccinic acid in the presence of ammonia. This phenotype can be used to select for the prion state. We have developed a novel reporter, in which the *ADE2* gene is controlled by the *DAL5* regulatory region, which allows monitoring of Ure2p function by a colony color phenotype. Using this reporter, we observed induction of different [*URE3*] prion variants ("strains") following overexpression of the N-terminal Ure2p prion domain (UPD) or full-length Ure2p. Full-length Ure2p induced two types of [*URE3*]: type A corresponds to conventional [*URE3*], whereas the novel type B variant is characterized by relatively high residual Ure2p activity and efficient curing by coexpression of low amounts of a UPD-green fluorescent protein fusion protein. Overexpression of UPD induced type B [*URE3*] but not type A. Both type A and B [*URE3*] strains, as well as weak and strong isolates of type A, were shown to stably maintain different prion strain characteristics. We suggest that these strain variants result from different modes of aggregation of similar Ure2p monomers. We also demonstrate a procedure to counterselect against the [*URE3*] state.

The [*URE3*] state was first described in 1971 (29) as a new phenotype inherited in a non-Mendelian manner, but the underlying genetic basis for its unusual mode of inheritance remained enigmatic for more than 20 years. In 1994, Wickner suggested protein conformation-based inheritance of [*URE3*] by a prion-like conversion of Ure2 protein (Ure2p) into an inactive form (54). This hypothesis was based on three observations. First, cells can be cured of the [*URE3*] state by growth in the presence of millimolar concentrations of guanidine, but after curing the phenotype can reappear spontaneously, with a frequency similar to that of untreated wild-type cells. Second, [*URE3*] appears at higher frequencies when Ure2p is overexpressed. Third, the phenotype caused by [*URE3*] is identical to that of a *ure2* deletion mutant, and yet [*URE3*] appearance and maintenance depend on the expression of Ure2p. Wickner also proposed that a second non-Mendelian state in *Saccharomyces cerevisiae*, termed [*PSI*], was caused by a similar autocatalytic inactivation of the Sup35 protein (Sup35p), which is involved in translation termination in yeast.

Since then, numerous studies have provided further support for this model. The prion isoforms of Ure2p and Sup35p acquire partial resistance to proteinase K (33, 37, 39). Using fusion proteins with green fluorescent protein (GFP), it has been shown that both proteins form aggregates in vivo in cells displaying the respective prion phenotype but not in wild-type cells (19, 37). Both proteins also form amyloid-like aggregates in vitro (22, 27, 43, 47, 51), a process that can be seeded by preformed aggregates and by extracts from cells in the prion state (22, 38). All prion-like characteristics of Ure2p and Sup35p depend upon the presence of an N-terminal prion domain that is dispensable for the normal function of either protein (33, 49). In both cases, the prion domain contains a high level of polar uncharged amino acids, in particular, asparagine and glutamine. In vitro, the Ure2p prion domain (UPD) promotes not only its own aggregation but also the refolding of other sequences attached to it (43). Similar polar uncharged domains of other yeast proteins have been shown to support a prion state, either in their native contexts or when they replace the prion domain of Sup35p (52, 55), indicating that prion-like phenomena might be more widespread in nature than previously assumed. Other features characteristic of mammalian prion diseases have been reproduced in the [*URE3*] and [*PSI*] systems, such as mutations that can favor or disfavor prion formation and propagation (13, 18, 21, 30) and, in the case of [*PSI*], the existence of a species barrier (6, 28, 42). Furthermore, Hsp104p (7, 34) and a number of other chaperone proteins in yeast (8, 35) have been shown to influence the induction and maintenance of [*PSI*] and [*URE3*]. A recent study demonstrated induction of [*PSI*] by transfection with in vitro aggregated Sup35 prion domain (45). These experiments provide direct evidence for the central point of the protein-only hypothesis, that a purely proteinaceous particle can show infectious behavior due to an autocatalytically propagating abnormal configuration.

For mammalian prions, different strains have been described that cause disease forms that differ in incubation time as well as in clinical symptoms and brain pathology (17). The occurrence of such strains, which requires invoking the existence of multiple stable conformations of a protein, has long been used as an argument against the protein-only hypothesis (4, 9). Considerable evidence supports the argument that strain characteristics are enciphered in the disease-causing isoform of the prion protein in mammals (2, 36, 40, 41, 44, 48). Strain variants of [*PSI*] which differ in mitotic stability and the efficiency of nonsense suppression have been demonstrated previously (16). However, the molecular basis for this strain difference is not known. Demonstration of clearly distinguishable and stably

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maintained variants of yeast prion states would provide an opportunity to determine how prion strain differences can be enciphered by protein conformation.

Ure2p in yeast is a negative regulator of the utilization of poor nitrogen sources in the presence of preferred nutrients such as ammonia or glutamine (12, 32). As a consequence of regulation by Ure2p, cells are unable to import ureidosuccinic acid (USA) in the presence of ammonia. In *ure2* mutant cells, USA can substitute for uracil to allow *ura2* mutants to grow on minimal medium in the presence of ammonia. This phenotype can therefore be used to select for the loss of Ure2p function in a *ura2* mutant background. The target for Ure2p activity is the transcriptional activator Gln3p, a GATA-type zinc finger protein essential for the expression of a wide range of genes involved in nitrogen uptake and metabolism (32). Current data indicate that Ure2p acts by binding Gln3p in the cytoplasm, thereby sequestering it away from the nucleus (1). Here, we demonstrate a new colony color reporter for Ure2p function, which allows the observation of at least two distinct types of [*URE3*] strains. Transient overexpression of full-length Ure2p induced both types of [*URE3*], whereas overexpression of UPD induced only one type of [*URE3*]. These observations allow us to speculate about how prion strains can be enciphered and how the initial conversion of Ure2p to the prion state occurs in yeast cells.

MATERIALS AND METHODS

Reagents and media. Enhanced chemiluminescence reagents and horseradish peroxidase-conjugated donkey anti-rabbit antiserum were obtained from Amersham Pharmacia Biotech (Piscataway, N.J.). The antiserum to Ure2p was described previously (43). Other reagents were purchased from Sigma-Aldrich (St. Louis, Mo.).

Standard yeast media, cultivation conditions, and methods were employed (24). Yeast cells were grown at 30°C. USA^+ strains were maintained in minimal medium, consisting of 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, the required amino acids, and 100 mg of USA/liter. The color phenotype was assayed on standard yeast extract-peptone-dextrose (YPD) plates made from 1% Bacto yeast extract, 2% Bacto peptone, 2% glucose, and 2% Bacto agar. To induce [*URE3*] by overexpression, strains carrying plasmids pMS46, pMS57, or pRW680 were grown in glucose-free media containing 2% galactose for 24 to 48 h prior to plating on USA medium containing glucose. All USA^+ isolates were cured of the overexpressing plasmids prior to detailed phenotypical characterization. Cytoduction experiments were performed as described previously (24). In each case, strain YRW3383 carrying the *kar1-1* mutation was used as either the donor or acceptor strain. As acceptor strains, [rho^0] derivatives of YRW3383 and YMS23 were isolated after ethidium bromide treatment (24). For maximum cytoduction efficiency, donor strains were grown on USA medium prior to mating. Cytoductants were identified as showing the acceptor genotype, showing the corresponding mating type, and being restored to [RHO^+].

Counterselection against the [*URE3*] state. Any drug whose uptake is regulated by the Ure2p-Gln3p system could be used potentially for assaying Ure2p function. Substances that are essential for growth, such as USA in *ura2* mutants, allow positive selection for the prion state on plates containing ammonia as the primary nitrogen source. Conversely, a toxic substance can be used to select against [*URE3*], potentially allowing the identification of conditions or gene products that cure cells of the prion state. High levels of α-aminoadipate (α-AA) are toxic for yeast cells carrying wild-type alleles of both the *LYS2* and *LYS5* genes but only in the absence of ammonia (24, 56). Therefore, we tested whether uptake of α-AA is regulated by Ure2p. Indeed, a strain carrying a chromosomal deletion of *URE2* was unable to grow on α-AA plates containing ammonia, whereas a wild-type strain was unaffected by the drug. When a variety of [*URE3*] strains was tested for growth on α-AA in the presence of ammonia, in all cases, colonies still formed at an efficiency up to 1,000-fold lower than that of the wild type. When colonies from these plates were tested on USA, they were found to be cured of the prion state. Therefore, colony formation by [*URE3*] strains on α-AA is due to instability of the prion phenotype. Although this spontaneous loss of the prion state limits the usefulness of counterselection for screening purposes, it was found to be a valuable tool for estimating the relative strength and stability of [*URE3*] isolates (see Fig. 3). To select for strains with functional Ure2p, minimal medium containing 2 g of α-AA/liter and standard ammonia concentrations was used.

TABLE 1. Plasmids used in this study

Plasmid	Description[a]	Source or reference
pMS46	2μm *LEU2* P_{GAL1} *UPD*	This study
pMS57	2μm *LEU2* P_{GAL1} *URE2*	This study
pMS64	*CEN LEU2* P_{URE2} *URE2*	This study
pMS68	2μm *LEU2* P_{URE2} *UPD*	This study
pMS82	2μm *LEU2* P_{URE2} *URE2*	This study
pMS86	2μm *LEU2* P_{DAL5} *ADE2*	This study
pMS87	Int. *LEU2* P_{DAL5} *ADE2*	This study
pMS90	*CEN LEU2* P_{DAL5} *ADE2*	This study
pRW680	2μm *LEU2* P_{GAL1} *URE2N*	33
pVTG12	*CEN LEU2* P_{URE2} *URE2N-GFP*	19
pH327	*CEN LEU2* P_{URE2} *URE2-GFP*	19

[a] Int., integrating plasmid.

Plasmid construction. To construct plasmids pMS40 and pMS41, the UPD fragment of *URE2* was amplified by PCR using primers UPD1 (5′-GGATCCT CTAGACATGATGAATAACAACGGCAACC-3′) and UPD2 (5′-CAGTG CCAAGCTTTGGCTTTGGCTACCATTGCGGC-3′). The product was cut with *Hin*dIII/*Xba*I and inserted into plasmids YEp351 (26) and YEp351G (pRW554) (54), respectively. The oligonucleotide Ssp-Stop (5′-AGCTGATAA TATTATC-3′) was inserted into the *Hin*dIII site of pMS40 and pMS41 to yield plasmids pMS59 and pMS46, respectively. The PCR product of primers UPD1 and URE2-T (5′-CTTTATTGAAAGCTTCAGATCTACAGTGACAA CACCC-3′) was cut with *Not*I/*Hin*dIII and inserted into pMS41 to obtain pMS57. The promoter and open reading frame of *URE2* were amplified using the overlapping primer pairs P-URE+ (5′-TGGTCTGAGCTCGCGAAAAAGA AAAAGGGC-3′) and ATG-URE− (5′-GCCGTTGTTATTCATCATGTCT AGAACAACTTAATTTGCAGC-3′) and ATG-URE+ (5′-GCTGCAAATT AAGTTGTTCTAGACATGATGAATAACAACGGC-3′) and URE2-T2 (5′-TGAAAGCTGCAGATCTACAGTGACAACACCC-3′). These were recombined in a second PCR step, cut with *Sac*I/*Pst*I, and inserted into pFL36 (3) to yield plasmid pMS64. A 0.7-kb *Sac*I/*Xba*I fragment from pMS64 was inserted into pMS59 to obtain pMS68, and a 2-kb *Sac*I/*Bgl*II fragment from pMS64 was inserted into YEp351 to produce pMS82. To construct the P_{DAL5}*ADE2* reporter, a segment comprising 561 bp upstream of the *DAL5* gene and the *ADE2* open reading frame was amplified using the overlapping primer pairs PDAL+ (5′-CTTTTACATCAGCACAATATCC-3′) and DAL-ADE− (5′-CCAACT GTTCTAGAATCCATCTGCAGTTTTTTTTTTACACTATTTG-3′) and DAL-ADE+ (5′-CAAATAGTGTAAAAAAAAAACTGCAGATGGATTCTAGAA CAGTTGG-3′) and T-ADE− (5′-CTGCATGTCGACGCCTTATATGAACTGT ATCG-3′). These were recombined in a second PCR step, cut with *Bam*HI/*Sal*I, and introduced into YEp351 to obtain pMS86. A 2.6-kb *Bam*HI/*Sal*I fragment from pMS86 was then inserted into YIp5 and pRS313 to obtain plasmids pMS87 and pMS90, respectively. All constructs were checked by sequencing. Plasmids pRW680, pVTG12, and pH327 were generously provided by R. B. Wickner. The plasmids used in this study are listed in Table 1.

Strain construction. The *URA2* gene in strain W303 was replaced by a *loxP-kan*^{r}*-loxP* cassette generated by PCR using primers URA2Δlox-1 (5′-TAAACCTTACCTAATAGAATATAACAATCATAATATGGCCGCAT AGGCCACTAGTGGATCTG-3′) and URA2Δlox-2 (5′-TATAAATTTAAAA TACGGATAGGTCTCTTATCATTCACATCAGCTGAAGCTTCGTACGC-3′) and pUG6 as templates (23), generating strain YMS11. After removal of the *kan*^{r} marker by Cre recombinase action using plasmid pSH47 (23) to produce YMS12, the same system was used to replace the *URE2* gene by a *loxP-kan*^{r}*-loxP* cassette generated using primers URE2Δlox-1 (5′-ATTGTTTTAAGCTGCA AATTAAGTTGTACACCAAATGATGGCATAGGCCACTAGTGGATCTG-3′) and URE2Δlox-2 (5′-CCTTCTTCTTTCTTTCTTGTTTTTAAAGCAGCC TTCATTCCAGCTGAAGCTTCGTACGC-3′) to obtain strain YMS13. Correct replacement of the target sequence in all three strains was confirmed by Southern blotting and PCR. Plasmid YIp5 was linearized using *Stu*I and integrated into YMS12 at the *URA3* locus to yield strain YMS15. Plasmid pMS87 was also linearized using *Stu*I and integrated into YMS12 and YMS13 to obtain strains YMS23 and YMS24, respectively. Transformants from this step were selected on

TABLE 2. Yeast strains used in this study

Strain	Genotype	Reference or source
W303	*MAT*α *trp1-1 ade2-1 leu2-3,112 his3-11,15 ura3*	50
YMS12[a]	*ura2*Δ::*loxP*	This study
YMS13[a]	*ura2*Δ::*loxP ure2*Δ::*loxP-kan*^{r}*-loxP*	This study
YMS15[a]	*ura2*Δ::*loxP ura3*::YIp5	This study
YMS23[a]	*ura2*Δ::*loxP ura3*::pMS87	This study
YMS24[a]	*ura2*Δ::*loxP ure2*Δ::*loxP-kan*^{r}*-loxP ura3*::pMS87	This study
YCC34[a]	*MAT***a** *URA3 ura2*Δ::*HIS3* [*URE3*]	21
YRW3560	*MAT***a** *URA3 ura2 leu2 his*$^-$ *kar1-1* [*URE3*]	33
YRW3383	*MAT***a** *URA3 ura2 leu2 his*$^-$ *kar1-1*	54

[a] Genotype is otherwise identical to that of strain W303.

minimal medium containing USA in the absence of ammonia. Strains YRW3560 (33) and YRW3383 (54) were generously provided by R. B. Wickner. Strain YCC34 (21) was generously provided by C. Cullin. The strains used in this study are listed in Table 2.

Proteinase K digestion. Crude extracts were prepared from cells grown in USA medium (supplemented with uracil for wild-type controls) to an optical density at 600 nm (OD_{600} of 1 to 1.5. Briefly, an amount of cells corresponding to 20 ml of culture at an OD_{600} of 1 was washed, resuspended in 400 μl of TNT buffer (25 mM Tris-HCl [pH 7.4], 100 mM NaCl, 0.2% Triton X-100), and vortexed with 200 μl of acid-washed glass beads (425- to 600-μm-diameter; Sigma) for 20 min at 4°C. No protease inhibitors were added. Extracts were cleared by spinning at 6,000 × *g* for 10 min at 4°C, and then they were immediately frozen on dry ice. Protein concentration was determined using the BCA assay system (Pierce). For ultracentrifugation, 250 μg of total protein in 100 μl of TNT buffer was spun at 100,000 × *g* for 1 h, the supernatant was transferred to a fresh tube, and the pellet fraction was resuspended in 100 μl of TNT buffer. Twenty-five microliters of 5× sample buffer was added, and samples were boiled for 5 min. For proteinase K digestion, 150 μg of total protein from the same extracts was diluted to 54 μl in TNT buffer, mixed with 6 μl of appropriate serial dilutions of the proteinase, and incubated at 37°C for 30 min. Digestion was stopped by the addition of phenylmethylsulfonyl fluoride to a concentration of 1 mM, then 15 μl of 5× sample buffer was added, and the mixture was boiled for 5 min. Urea was added to each sample to a final concentration of at least 7 M. Prior to loading, the samples were again boiled for 5 min. For electrophoresis, 25 μl (50 μg) of samples was used per lane. Electrophoresis, Western blotting, and immunodetection were performed as previously described (43). All blots were treated with 0.2 M NaOH for 30 min to ensure detection of all Ure2p fragments.

RESULTS

Reporter system for [*URE3*]. So far, the only commonly used way to detect the [*URE3*] prion state has been selection for growth on USA media. Because the assay is based on selection, it does not allow easy visualization of spontaneous loss or curing of the prion state. Cells without active Ure2p also secrete uracil into the medium (29, 34), and consumption of the available ammonia can diminish Ure2p activity. Both of these contribute to considerable background growth on USA medium. In addition, the selection assay cannot distinguish different levels of residual Ure2p activity, since growth rates on USA might be negatively affected by the accumulation of abnormally folded protein as well as by the rate of USA uptake.

To facilitate the study of [*URE3*], an *ADE2*-based reporter for Ure2p activity was designed (Fig. 1). Strains deficient in *ADE2* are auxotrophic for adenine and accumulate a red pigment when grown on media where adenine is limiting (such as YPD complete medium), whereas wild-type strains are ade-

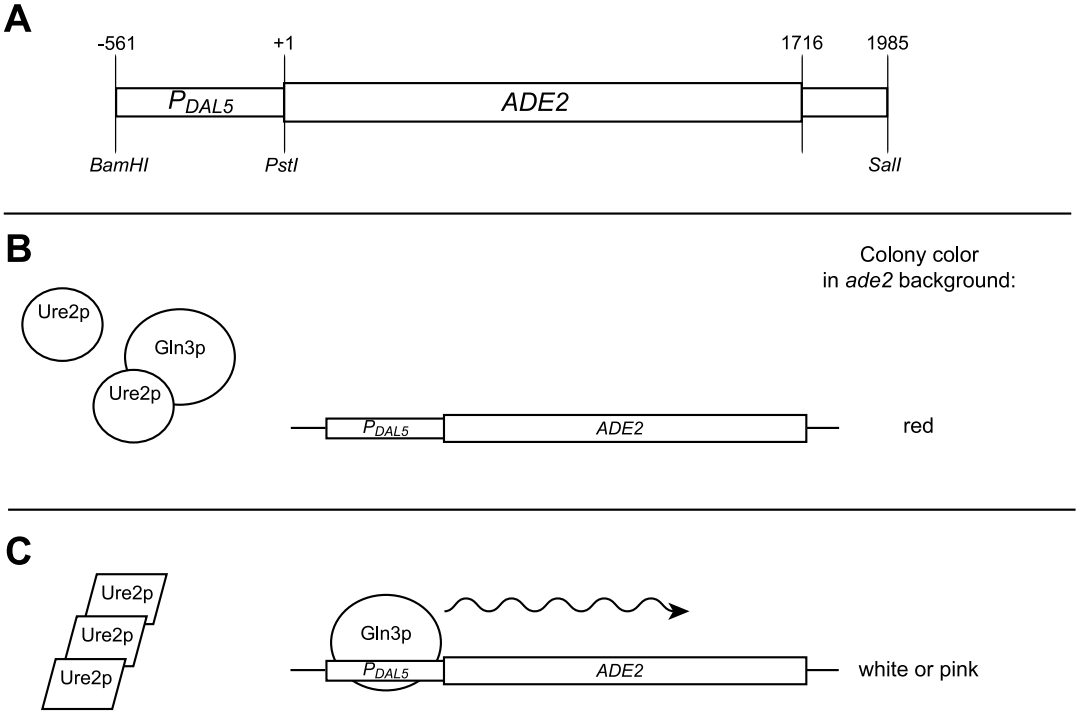

FIG. 1. *ADE2* reporter for Ure2p function. (A) Structure of the reporter construct. Base numbering starts with the start codon of the *ADE2* opening reading frame as +1. The 561-bp segment amplified from the genomic region upstream of the *DAL5* start codon contains the binding sites for Gln3p. Sequences between +1 and +1985 were amplified from the genomic *ADE2* sequence. The *ADE2* open reading frame ends at position 1716. (B) Regulation of the reporter. In the presence of ammonia, Ure2p binds Gln3p and prevents transcription from the *DAL5* regulatory region. Colonies are red due to the lack of Ade2p. (C) In the [*URE3*] state, Ure2p is aggregated and inactive. Gln3p can now activate transcription of *ADE2*, resulting in lighter colony color and adenine prototrophy.

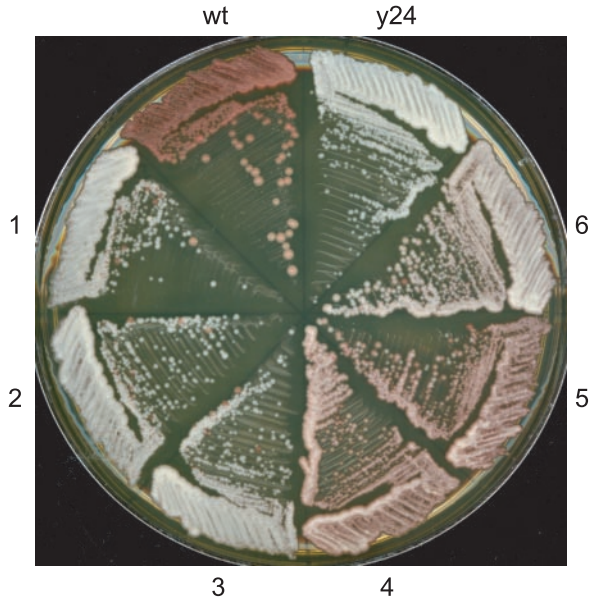

FIG. 2. Cytoduction of [*URE3*] into reporter strain YMS23. The [*URE3*] element was introduced into strain YMS23 from strains YCC34 and YRW3560 by cytoduction. Representative cytoductants displaying the [*URE3*] phenotype were streaked on YPD. Sectors: wt, wild-type; 1 to 3, cytoductants from YCC34 (UFL); 4 to 6, cytoductants from YRW3560 (URW); y24, *ure2* deletion strain YMS24.

nine prototrophic and form white colonies. The reporter consists of *ADE2* under the control of the Ure2p-regulated *DAL5* promoter. The *DAL5* promoter was chosen for two reasons. First, unlike other genes regulated by nitrogen sources, *DAL5* expression appears to be unaffected by specific substrates other than the presence or absence of good nitrogen sources (11, 32). Second, the *DAL5* gene product is the transporter responsible for uptake of USA (53) and whose regulation allows selection for [*URE3*] strains. The color phenotype generated by such a P_{DAL5}*ADE2* reporter can therefore be expected to report the prion state with similar accuracy.

When an integrated version of the reporter pMS87, based on the yeast plasmid YIp5, was introduced into the *ade2* yeast strain YMS15, the resulting strain YMS23 formed red colonies. On medium without adenine, the cells were able to form microcolonies (data not shown), indicating some leakiness of the repression by Ure2p. In contrast, YMS24, which contains pMS87 integrated into the *ure2* deletion mutant YMS13, was able to grow in the absence of adenine with an efficiency similar to that of the wild type (data not shown) and formed colonies on YPD medium that were almost completely white (Fig. 2). Strain YMS24 transformed with plasmid pMS64, which expresses Ure2p from a single-copy plasmid under the control of its own promoter, formed red colonies on YPD with occasional white sectors, indicating a loss of the *URE2* plasmid. These results demonstrate that the chromosomally integrated version of the *ADE2* reporter is efficiently regulated by Ure2p activity.

In contrast, when this P_{DAL5}*ADE2* reporter was placed on a 2μm-based, multicopy plasmid (pMS86) and transformed into the yeast strain YMS15, the resulting strain became Ade^+ and white, indicating that the construct is not downregulated efficiently by Ure2p. Even colonies of cells carrying the reporter construct on a *CEN*-based, single-copy vector (pMS90), were completely white (data not shown).

Previously described [*URE3*] isolates. To test the usefulness of the new reporter system in monitoring [*URE3*], the prion elements from two strains, YRW3560 (33) and YCC34 (21) were transferred by cytoduction into YMS23. Cytoduction allows cytoplasmic mixing of two yeast strains without the exchange of chromosomal DNA. [*URE3*] in YCC34 represents the isolate originally described by Lacroute (29). Cytoductant clones obtained from this donor strain are hereafter referred to as UFL; those from YRW3560, an isolate originally described by Masison and Wickner (33) are hereafter referred to as URW. Cytoductants carrying the prion element from both sources were able to grow on USA and formed pink colonies on YPD medium. The obtained clones differed somewhat in color from each other, but both were at least slightly darker than the *ure2* mutant strain, indicating that inactivation of Ure2p was not complete (Fig. 2). Despite some variation in colony color (Fig. 2, compare sectors 5 and 6), all UFL cytoductants were lighter in color than URW cytoductants, indicating that some difference between the two prion isolates is stably inherited, similar to strain variants observed for the [*PSI*] yeast prion (16). UFL thus appears to confer a more severe reduction of Ure2p function than does URW.

When [*URE3*] cells were plated on nonselective YPD plates, spontaneous loss of the prion state could easily be observed by the formation of red colonies or red sectors in pink colonies. Cells from such red sectors were tested on USA and found to be unable to grow, as was expected. Sectored colonies were particularly abundant when cells were plated directly from USA medium onto YPD. Up to 50% of all colonies showed sectoring, confirming that the stability of [*URE3*] is considerably lower than that observed for [*PSI*] using a similar colony color phenotype (see Fig. 3B in reference 16 for comparison). In contrast, when cells were grown in YPD prior to plating on YPD, most of the colonies were either homogeneously pink or red, indicating that the change from USA to YPD medium causes some loss of [*URE3*]. Spontaneous loss of [*URE3*] is also apparent in sectors 1 to 6 in Fig. 2, which contain the cytoductant clones.

Induction of new [*URE3*] by overexpression of Ure2p or UPD. Overexpression of either Ure2p or UPD in YMS23 resulted in greatly elevated frequencies of [*URE3*] appearance, as reported previously (33). Clones growing on USA were isolated from strains carrying a multicopy plasmid expressing UPD or Ure2p under control of the strong, inducible *GAL1* promoter (pMS46 and pMS57, respectively). When transferred to YPD plates, USA^+ clones isolated from the strain overexpressing full-length Ure2p displayed various shades of pink, from almost white to dark pink or red (Fig. 3). In striking contrast, USA^+ clones isolated from the UPD overexpressing strain were all red and indistinguishable from the parental wild-type strain. Apparently, in these red isolates, Ure2p activity is low enough to allow uptake of USA but not low enough to produce a sufficient level of Ade2p to prevent accumulation of the red pigment. The difference in the spectrum of induced USA^+ phenotypes was reproducible in several experiments using different overexpressing plasmids. Specifically, plasmids pMS68 (expressing UPD under the control of the *URE2* pro-

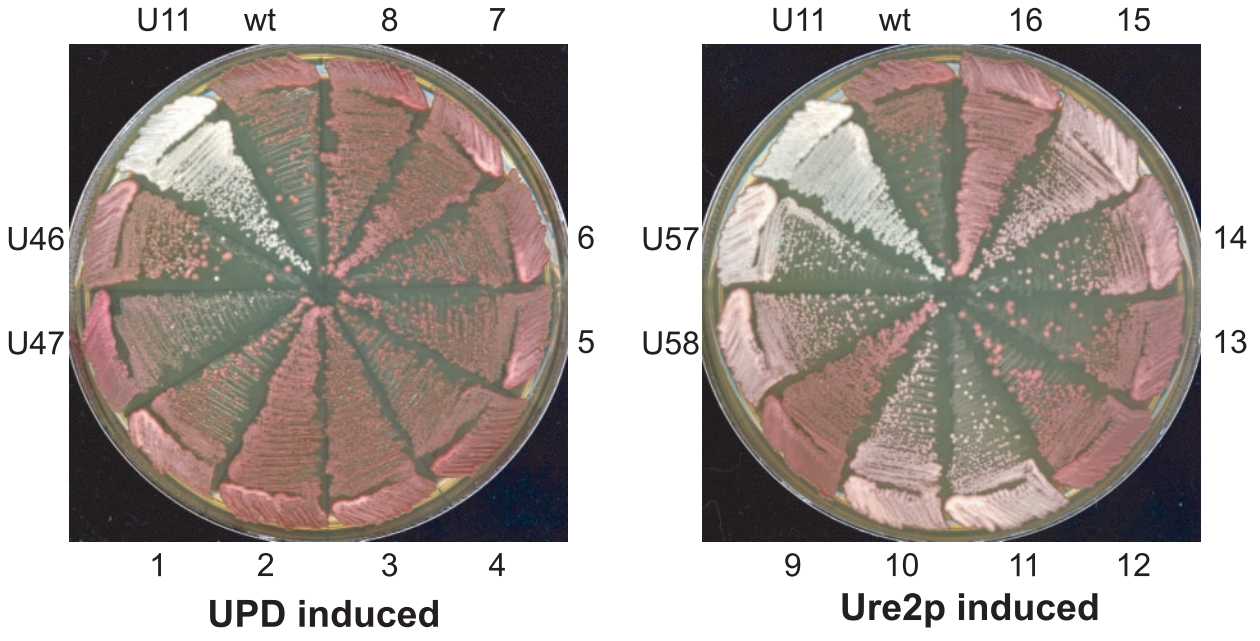

FIG. 3. Color phenotypes of new [*URE3*] elements induced by overexpression. Clones isolated following overexpression of UPD are on the left, and clones isolated following overexpression of full-length Ure2p are on the right. Wild-type (wt) YMS23 and *ure2* mutant (U11) cells are included as color controls. U46, U47, U57, and U58 are the clones used for phenotypic characterization (see text). Clones 1 to 8 and 9 to 16 were randomly selected from a second, independent overexpression experiment using the same plasmids. Colonies were picked from USA plates, purified, and streaked on YPD.

moter from a 2μm plasmid) and pRW680 (equivalent to pMS46, which expresses a slightly shorter UPD fragment) also produced red USA^+ isolates, which were indistinguishable by color from wild-type colonies.

Characterization of USA^+ clones. In order to further elucidate the differences between these prion strain variants, four representative clones were initially chosen for further characterization: U46 and U47 were induced by UPD overexpression from pMS46 and formed red colonies; and U57 and U58 were induced by Ure2p expressed from pMS57 and formed light pink and pink colonies, respectively (Fig. 3). The UFL and URW isolates obtained by cytoduction into YMS23 (Fig. 2) were used as controls.

In addition to colony color, USA^+ clones were characterized by the efficiency of curing by guanidine, aggregation state of Ure2p, resistance of Ure2p to proteinase K digestion, fluorescence pattern in strains expressing a UPD-GFP fusion protein, and cytoduction efficiency.

In order to assess guanidine curing, USA^+ isolates were grown in YPD medium with or without 5 mM guanidine hydrochloride, and serial dilutions were spotted on USA and α-AA plates, respectively (Fig. 4). The ability to grow on USA was found to be directly related to the color phenotype. Pink clones, such as U57 and U58, grew almost as efficiently as *ure2* mutants, whereas the red variants U46 and U47 grew markedly slower. In contrast, U46 and U47 showed considerable growth on α-AA, whereas the stronger isolates did not (Fig. 4). With the exception of U46 and the mutant strains, all USA^+ clones were 90% or more cured of the prion state after about 10 generations in the presence of 5 mM guanidine hydrochloride. Curing was evident from diminished growth on USA as well as improved growth on α-AA.

Similar to mammalian prion protein, Ure2p from [*URE3*] cells has been reported to be partially resistant to proteinase K digestion (33). The four representative clones, U46, U47, U57, and U58, as well as URW and UFL were tested in the same way in order to determine whether the observed strain variability can be associated with a biochemical difference between Ure2p from those isolates. Ure2p was found mostly in the soluble fraction of extracts from the wild-type strain, whereas it was completely insoluble in all USA^+ isolates except U46 after centrifugation at 100,000 × *g* (shown for the wild type, UFL, U47, and U58 in Fig. 5A). Ure2p was completely digested after treatment with 0.6 μg of proteinase K/ml for 30 min in both the wild-type and the U46 strains. In contrast, in the five remaining strains, full-length Ure2p could still be observed following digestion with up to 4 μg of proteinase K/ml. Furthermore, a characteristic pattern of partial degradation products was visible, with three bands in the range of 25 to 30 kDa. A fourth band at ≈13 kDa was already present in the untreated extracts but not in extracts prepared using proteinase inhibitors. This band is therefore most likely due to partial degradation of Ure2p by lysosomal proteinases after disruption of the cells. The same band is also the end product of the proteinase K digestion, since it intensifies with increasing concentration of the proteinase used. A band with similar electrophoretic mobility and proteinase resistance is observed when UPD is expressed in yeast (data not shown) as well as in the case of UPD purified from *Escherichia coli* (43). Accordingly, it can be assumed that the fragment generated in extracts from the [*URE3*] strains also corresponds to UPD.

The analysis was repeated in strains overexpressing Ure2p from the multicopy plasmid pMS82 (Fig. 5B). Even under these conditions, no difference in Ure2p could be detected

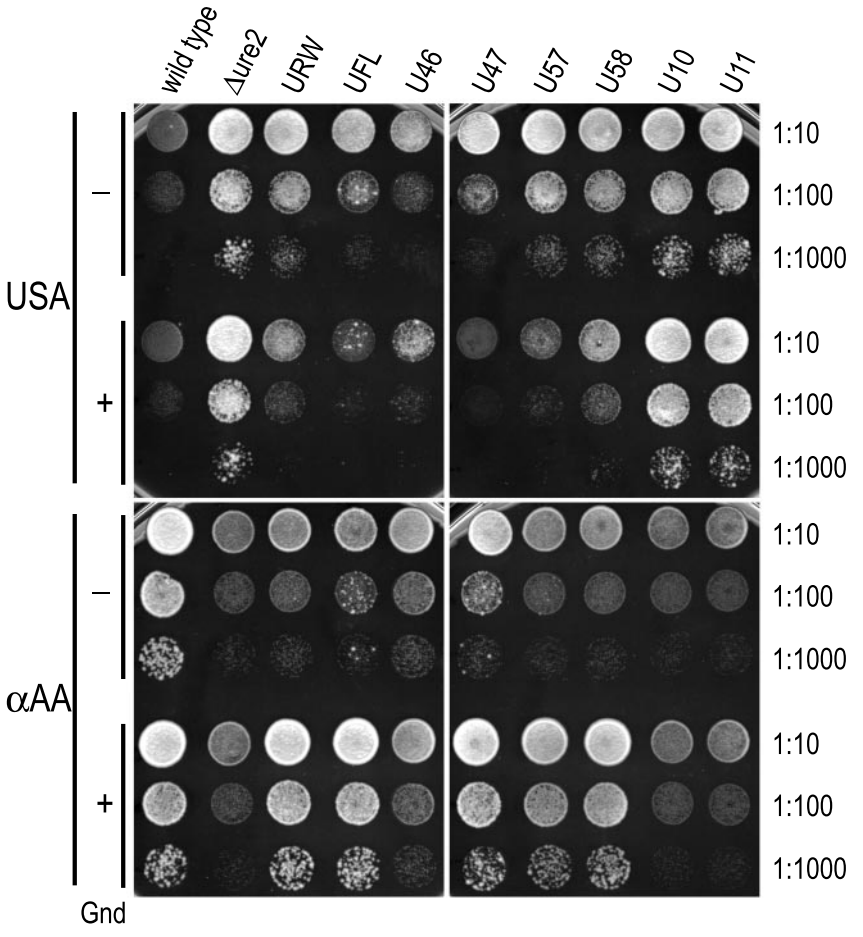

FIG. 4. Guanidine curing of USA$^+$ isolates. [*URE3*] cells were grown in nonselective YPD medium with or without 5 mM guanidine for about 10 generations, and serial dilutions were spotted onto USA and α-AA plates to select for cells with inactive or active Ure2p, respectively. Wild-type (YMS23) and Δ*ure2* (YMS24) strains that are not affected by guanidine were used as controls. Also shown are cytoductants carrying the [*URE3*] isolate from strains YRW3560 (URW) and YCC34 (UFL), USA$^+$ isolates U46 and U47 (induced by transient UPD overexpression), U57 and U58 (induced by Ure2p overexpression), and U10 and U11 (two *ure2* mutant strains).

between wild-type and U46 cells. The overexpressing strains showed more clearly the accumulation of a fragment with an apparent molecular mass of 13 kDa, as well as two slightly smaller fragments, as the final product of the digestion reaction. There was little degradation of this species, even at concentrations of proteinase K up to 40 μg/ml (Fig. 5B).

The experiments did not show any significant differences in the digestion patterns of U47, U57, U58, URW, or UFL, indicating that the conformation of Ure2p is similar in all these isolates. The molecular basis for the strain variability is therefore unclear. A closer look at the data presented in Fig. 5, however, shows a small amount of aggregated Ure2p that did not migrate into the sodium dodecyl sulfate gel, but instead it was stuck in the gel-loading pockets in the cases of U57, U58, URW, and UFL but not U47. Although this appears to be a small difference, it was consistently observed in several independent experiments. Even in U47 cells expressing high levels of Ure2p from pMS82, no Ure2p signal was observed in the gel pocket (Fig. 5B), despite the overexpression. It seems therefore plausible that the strain variability is caused by a difference in the aggregation pattern of Ure2p rather than the conformation of individual protein monomers.

Recent studies (5, 25) suggest that Ure2p is phosphorylated in its active state and that dephosphorylation leads to inactivation of the protein. Phosphorylation leads to a slight shift in electrophoretic mobility of the protein and the appearance of a double band. A similar double band is visible in extracts of wild-type and U46 strains (Fig. 5), whereas all type A and B [*URE3*] strains show only a single band. However, phosphatase treatment, as described in reference 5, of the cell extracts used for the experiments whose results are shown in Fig. 5 did not change the Ure2p banding pattern in our study (data not shown).

Studies by Edskes et al. (19) show that, in cells coexpressing UPD-GFP or Ure2-GFP fusion proteins, the aggregation state of the fusion protein depends on the [*URE3*] phenotype. In wild-type cells expressing either GFP fusion protein, the entire cytoplasm shows weak, homogeneous fluorescence. In contrast, in [*URE3*] strains, the fluorescence coalesces into one or several bright spots (foci) in each cell. Clones U46, U47, U57, and U58 as well as UFL and URW were transformed with pVTG12 and pH327, which express UPD-GFP and Ure2-GFP fusion proteins, respectively. Transformants were tested for growth on USA and the pattern of intracellular fluorescence.

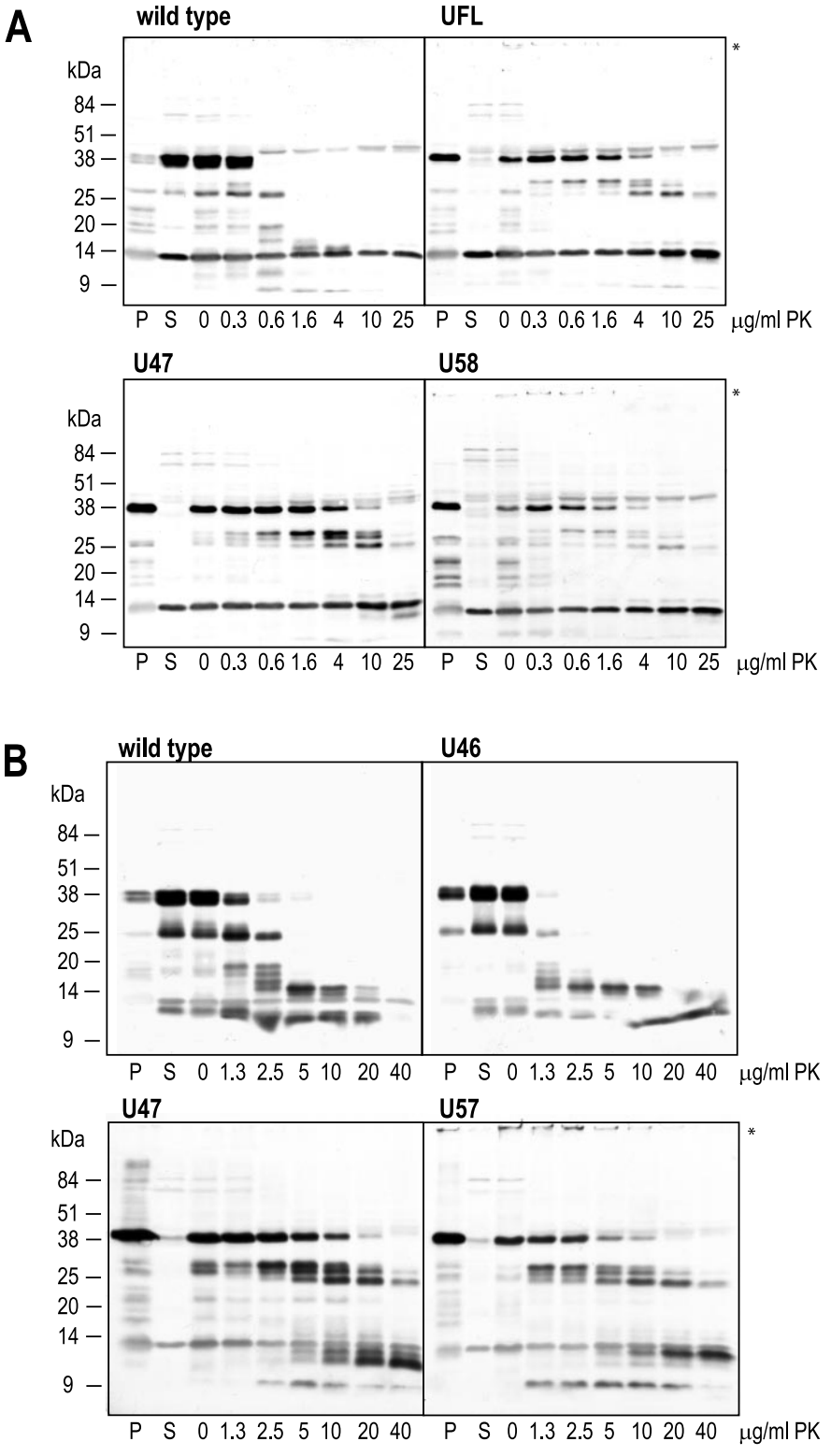

FIG. 5. Insolubility and proteinase K resistance of Ure2p in USA$^+$ isolates. Cells were grown in USA medium (supplemented with uracil for wild-type controls) at 30°C. Extracts were prepared in the absence of proteinase inhibitors and subjected to ultracentrifugation or digestion with proteinase K. Western blots were analyzed with anti-GST-Ure2 antiserum. (A) Wild-type and [*URE3*] (isolates UFL, U47, and U58) strains without any plasmid; (B) wild-type and USA$^+$ (isolates U46, U47, and U57) strains overexpressing Ure2p from pMS82. In each gel, lane P represents the pellet and lane S represents the supernatant fraction after centrifugation at 100,000 × *g*. For the remaining lanes, samples of whole-cell lysates were treated with increasing amounts of proteinase K (PK) as indicated. The asterisk indicates the position of the gel-loading pocket on the blot membrane.

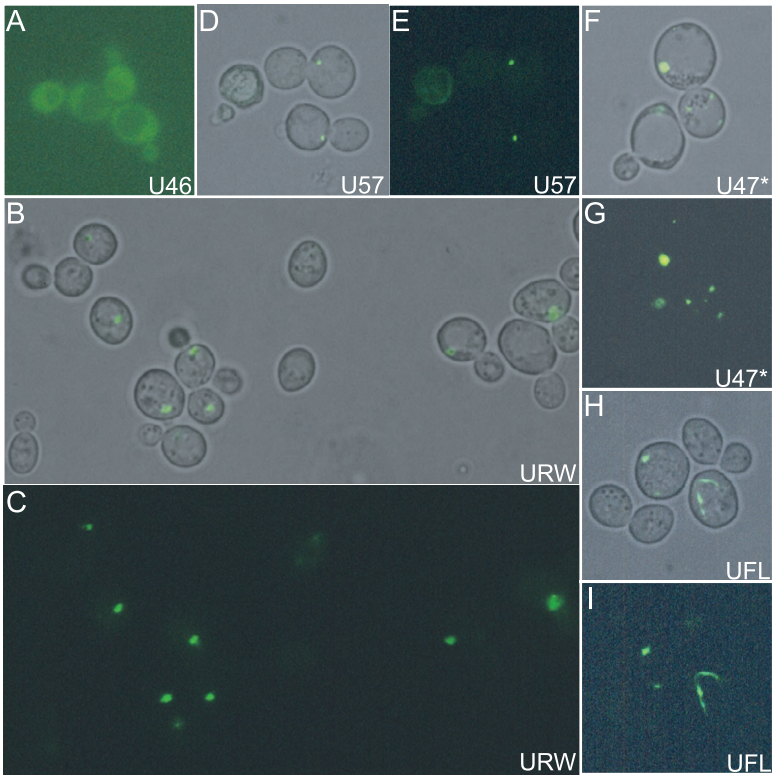

FIG. 6. Autofluorescence of USA$^+$ strains expressing a UPD-GFP fusion protein. USA$^+$ isolates of strain YMS23 (Fig. 2 and 3) were transformed with pVTG12 (expressing Ure2N-GFP) and grown in USA medium without leucine prior to microscopic observation. (A) Clone U46, showing homogeneous distribution of UPD-GFP, identical to wild-type cells (data not shown); (B and C) clone URW; (D and E) clone U57; (F and G) cells of clone U47 that remained USA$^+$ after transformation with pVTG12; (H and I) cells of clone UFL, showing fiber-like aggregates of UPD-GFP. Panels A, C, E, G, and I show autofluorescence; in panels B, D, F, and H, autofluorescence was overlaid on the corresponding bright light image to visualize cell shapes. Exposure time for the image in panel A was approximately two to three times longer than for the other autofluorescence images.

In clones U57, U58, UFL, and URW, most cells showed one or two fluorescent foci per cell (Fig. 6B to E). Occasionally, elongated structures were observed, reminiscent of fibers formed by purified UPD and Ure2p in vitro (Fig. 6H and I). Sometimes these structures spanned the entire length of the cell. In contrast, U46, like wild-type cells, showed homogeneous staining (Fig. 6A). Surprisingly, U47 transformed with either construct was efficiently cured of the prion state, even though the GFP fusion protein was expressed at relatively low levels. After loss of the GFP fusion plasmid, the cells did not regain the ability to grow on USA, indicating that the [*URE3*] state had not been suppressed but was actually cured. Curing by expression of GFP fusion proteins or UPD alone has been demonstrated previously (19), but in those cases GFP-UPD expression levels had to be in the order of 20 to 50 times higher than those produced by pVTG12. No curing was observed with either pVTG12 or pH327 for any of the other five USA$^+$ isolates tested in this experiment. While curing of U47 by pH327 was always complete, in some transformants with pVTG12 a small percentage of cells in the original colony retained the USA$^+$ phenotype. Under the microscope, such cells exhibited numerous fluorescent foci (Fig. 6F and G).

Classification of additional USA$^+$ isolates. Following this initial characterization, a larger number of USA$^+$ clones was examined using the same methods. All clones induced by transient UPD overexpression showed the characteristics of either U46 or U47, whereas virtually all clones obtained by Ure2p overexpression behaved like either U57 or U47. Specifically, of 16 red clones that were cured by guanidine and isolated after either UPD or Ure2p overexpression, 15 were also cured by expression of UPD-GFP (Table 3). Based on these observations, three types of USA$^+$ isolates can be distinguished following transient overexpression of UPD or Ure2p (Table 3). Clones similar to U57, classified as type A isolates, and clones similar to U47, classified as type B isolates, were cured by guanidine. However, type A clones were generally pink and showed aggregation of UPD-GFP into fluorescent foci under the microscope, whereas type B clones were red and lost the prion phenotype after transformation with the UPD-GFP-expressing plasmid. Type B clones also tended to grow more readily on α-AA. Cytoductants containing [*URE3-RW*] from YRW3560 or [*URE3-FL*] from YCC34 had all the characteristics of type A.

The third type of USA$^+$ isolate, designated type III clones (similar to U46), often showed very weak growth on USA, tended to grow better on α-AA, and was not affected by guanidine. While type A and B isolates showed all of the characteristics of [*URE3*], no sign of abnormal Ure2p could be found in type III isolates, meaning they do not appear to be [*URE3*]. Although we cannot exclude the possibility that a mutation causes this phenotype, we consider this possibility unlikely because of the following observations. Type III USA$^+$ isolates

TABLE 3. Properties and classification of various USA^+ isolates

Test or characteristic	Result for USA^+ type (representative clone)				
	Wild type (none)	[URE3] A (U57, U58)	[URE3] B (U47)	III (U46)	Mutant (U10, U11)
Color	Red	Pink	Red	Red	White
Growth on USA[a]	−	++	+	+	+++
Growth on α-AA[a]	++	−	+/−	+/−	−
Cured by guanidine	NA[b]	Yes	Yes	No	No
Proteinase K-resistant Ure2p	No	Yes	Yes	No	No
GFP-UPD appearance and/or curing	Homogeneous	Foci	Cured	Homogeneous	Homogeneous
Cytoduction	NA	>95%	>95%	No	No
Generated by UPD overexpression[c]	NA	0	7/12	5/12	0
Generated by Ure2 overexpression[c]	NA	9/19[b]	8/19[b]	0	0

[a] −, no growth; +/−, mostly microcolonies formed; +, single colonies formed in 4 to 5 days; ++, single colonies formed in 3 to 4 days; +++, single colonies formed in 3 days.
[b] NA, not applicable.
[c] Number of strains in this group/total number of USA^+ strains.
[d] One isolate in this group was pink but was otherwise type B; one isolate was cured by guanidine but was otherwise type III.

are clearly induced by UPD overexpression. These clones are also only partially complemented by expression of Ure2p from the single-copy plasmid pMS64 or the multicopy plasmid pMS82, and they lose the USA^+ phenotype upon continued propagation on nonselective media. The molecular basis of the defect in these isolates is therefore unclear at present.

Stable inheritance of [*URE3*] yeast prion strains. To determine whether the observed variants of [*URE3*] are faithfully inherited, cytoduction experiments were performed. The prion state in U47, U57, and U58 was first transferred from YMS23 to YRW3383 and subsequently from YRW3383 back into YMS23. In each case, at least three clones obtained from the first round of cytoduction were used as donors for the second round. After these two transfers, clones containing [*URE3*] from U47 still showed all type B characteristics, including curing by low expression levels of UPD-GFP, while clones carrying [*URE3*] from either U57 or U58 were still type A. Moreover, U57-derived isolates all maintained a lighter colony color than those derived from U58, indicating that even the presumably more subtle differences within type A isolates are stably inherited (Fig. 7). The cytoduction efficiencies of [*URE3*] for all three isolates were above 95%, provided that the donor cells were grown on USA medium prior to mating. We were unable, however, to obtain any cytoductants that retained the USA^+ phenotype from the U46 isolate, although a substantial portion of the occasional diploids that were formed in these experiments were still weakly USA^+.

Spontaneous USA^+ isolates. Colonies with the ability to grow on USA appeared with a low frequency (about 2×10^{-6} to 5×10^{-6}) in wild-type strains without overexpression of Ure2p or UPD. When such clones were isolated from YMS23, about 40% were completely white with no apparent sectoring, indicating considerably lower Ure2p activity and greater mitotic stability than in any [*URE3*] clone isolated following transient overexpression of Ure2p. Most of the remaining clones were red; some were pink. In four white clones, the *URE2* gene was sequenced. Two of these clones contained nonsense mutations (U9, S210Stop; U11, Q32Stop), leading to the expression of truncated, inactive versions of Ure2p. In the other two clones, missense mutations were identified (U10, G182R; U13, D310H) which could also explain the USA^+ phenotype. Thirty clones (13 white, 1 pink, 16 red) were tested for guanidine curing of the USA^+ phenotype, and all were found to be fully resistant, indicating that they either contained a *ure2* mutation or exhibited the type III USA^+ phenotype. In all white and pink clones tested, the USA^+ phenotype was complemented by the plasmid pMS64, indicating a mutation in *URE2*. In contrast, several of the red clones retained the ability to grow on USA medium after transformation, as observed for type III isolates. Apparently, in the absence of overexpression, [*URE3*] is generated spontaneously only at very low frequencies in the W303 strain background used for our studies.

DISCUSSION

Novel reporter system for [*URE3*]. Previously, Ure2p function has been monitored almost exclusively by the growth of Ure2p-deficient *ura2 URA3* strains on media containing USA instead of uracil in the presence of ammonia. Studies of the [*URE3*] state have been hampered by background growth due to secretion of uracil by cells deficient in Ure2p function (29); by the frequent isolation of weak, unstable, or atypical USA^+ isolates; and by the lack of an additional, nonselective phenotype. By putting an *ADE2* reporter gene under the control of the *DAL5* promoter, adenine biosynthesis and colony color become regulated by the Ure2p-Gln3 system, which allows facile quantitative observation of this regulation by colony color intensity. We have used this reporter system to visualize the stability of the [*URE3*] prion state and to identify different strain variants of the [*URE3*] prion state.

Different strains of [*URE3*]. For many years, the existence of strains of mammalian prions, which are responsible for different disease phenotypes, was used as an argument against prions consisting only of protein (4, 9). Considerable evidence argues that the biological properties of mammalian prion strains are enciphered in the secondary and tertiary structures of PrP^{Sc} (2, 36, 40, 41, 44, 48). The question remains, however, how a single protein can exist in multiple self-propagating conformations.

Our studies demonstrate that different strains of [*URE3*] can be generated reproducibly in fully isogenic cells, excluding the potential involvement of *URE2* sequence variation in the for-

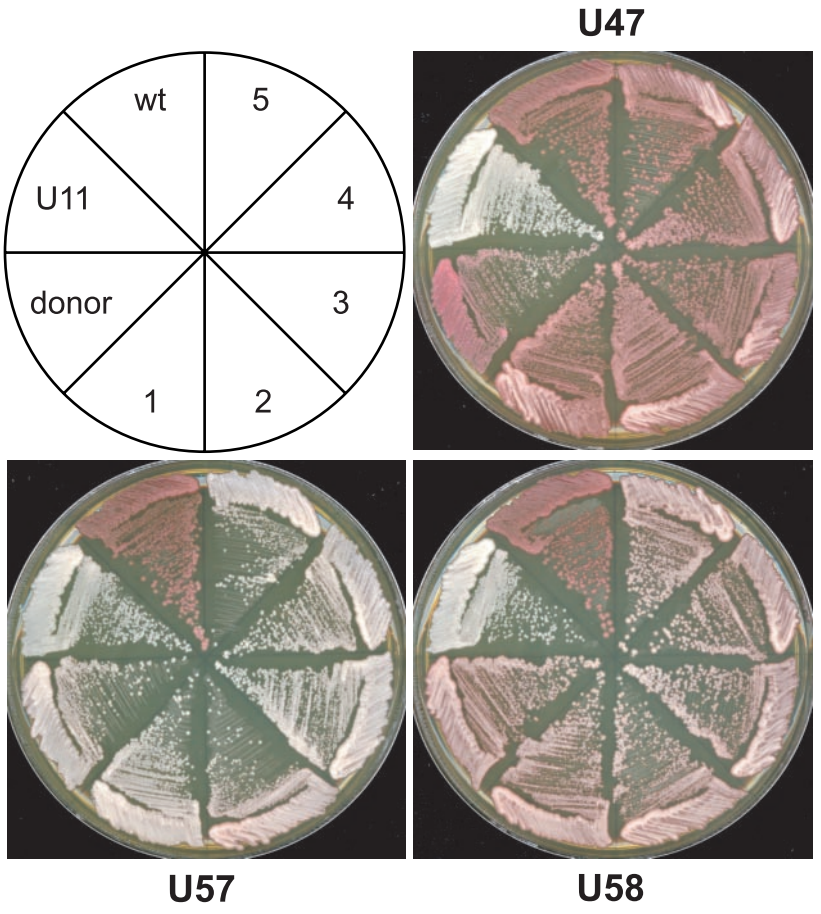

FIG. 7. Stable inheritance of different [*URE3*] strain variants. [*URE3*] clones U47, U57, and U58 (Fig. 3) were cytoduced from YMS23 into strain YRW3383 and then back into YMS23. Each plate shows a wild-type (wt) and *ure2* mutant (U11) control, the original [*URE3*] clone (donor), and five representative cytoductants (1 to 5) streaked on YPD plates.

mation or maintenance of the [*URE3*] strains. Posttranslational modifications of Ure2p, which is a cytosolic protein, appear to be limited to a phosphorylation event that has been implicated in the regulation of Ure2p function (5, 25). Therefore, any strain information has to be enciphered in the Ure2 protein structure itself.

Based on colony color and the response to expression of a GFP-UPD fusion protein, we defined two types of [*URE3*] isolates, A and B. Since proteinase digestion experiments did not detect significant differences in the conformation of Ure2p from these different isolates, we presume that they do not exhibit major variations in secondary or tertiary structure. Alternatively, [*URE3*] prion strain variations could be due to differences in the aggregation state or aggregation pattern (for example differences in the monomer surfaces that mediate aggregation) of Ure2p. Our observation that some of the Ure2p in extracts from type A but not from type B [*URE3*] strains is more likely to become stuck in the loading pocket of a sodium dodecyl sulfate-polyacrylamide gel, despite the harsh denaturation conditions used in the experiment, is consistent with different aggregation patterns: similarly folded monomers could form different oligomers and higher polymers, depending on the kind of seed that initiated the conversion to [*URE3*].

Response to GFP fusion proteins. We observed that a UPD-GFP fusion protein is surprisingly potent in curing type B [*URE3*] isolates even at low expression levels. In a previous report, efficient curing by the same fusion protein or UPD alone required massive overexpression, whereas Ure2-GFP was more effective (19). In our experiments, all 15 red USA^+ isolates that were curable by guanidine (i.e., type B) were also cured by small amounts of UPD-GFP, while 8 of the 9 pink isolates (type A) were not affected by the presence of the fusion protein. Thus, curing by UPD-GFP offers a definitive way to distinguish the two types of [*URE3*], at least in our strain background.

We propose that the GFP fusion protein binds to Ure2p in type B [*URE3*] in a manner that does not allow further propagation of the prion isoform. The observation that expression of the GFP fusion protein cures type B but not type A [*URE3*] indicates that the interaction surface between the normal and the prion forms of the protein, where binding and conversion are assumed to occur, are different in these [*URE3*] strain variants. A small number of cells in type B isolates remains stably USA^+ even with the expression of UPD-GFP. These cells still form red colonies. Under the microscope, they display slightly different fluorescent foci, which appear larger and more numerous than those found in type A [*URE3*] (Fig. 6F and G). Remarkably, when such cells were cured of the plasmid expressing the GFP fusion protein, all of them were USA^- (unpublished observations), indicating that in these cells, the

UPD-GFP fusion protein was required to maintain the prion state.

A recent study by Speransky et al. (46) showed that Ure2p forms globular structures consisting of filamentous aggregates in [*URE3*] but not in wild-type yeast cells that overexpress Ure2p. Our observation of fibrous, fluorescent aggregates in a small percentage of [*URE3*] cells expressing low amounts of GFP-UPD (Fig. 6H and I) complements this work, and provides further evidence for the hypothesis that amyloid fiber formation might be the underlying mechanism of [*URE3*] formation and inheritance.

Comparison to [*PSI*] yeast prion strains. Different strains of yeast prions have also been observed for [*PSI*], using an *ADE1* reporter to distinguish between stronger and weaker isolates of [*PSI*] by colony color (16). Weak [*PSI*] isolates are less efficient in nonsense suppression and are spontaneously lost at a much higher rate than are strong isolates. Each prion strain was shown to breed true in that it does not convert from weak to strong or vice versa. One study demonstrated that overexpression of Sup35p can destabilize some weak [*PSI*] isolates but not others (15). Weak and strong isolates of [*PSI*] seem to correspond most closely to weak and strong isolates of type A [*URE3*]. The characteristics of type B [*URE3*] distinguish it further from type A because of its specific induction by UPD overexpression and by the strikingly different effects of the GFP fusion proteins.

A recent study by Chien and Weissman (10) provides another example of [*PSI*] prion strains. A strain background was used which expressed Sup35p with a part of the prion domain replaced by a corresponding fragment from *Candida albicans* Sup35p. Overexpression of GFP fusion proteins with the Sup35 prion domain from either *S. cerevisiae* or *C. albicans* induced [*PSI*] in this chimeric background, indicating the absence of a species barrier. However, the two fragments used to induce [*PSI*] generated different prion strain variants. Furthermore, fibers of this chimeric prion domain generated in vitro also showed distinct patterns of proteinase-resistant fragments, depending on the protein with which they were seeded. This observation provides direct evidence for differences in the secondary or tertiary structure of the protein that could determine prion strain characteristics.

Initiation of new [*URE3*] elements. It has been speculated previously (31) that [*URE3*] elements are initiated by N-terminal fragments of Ure2p generated by intracellular proteolytic cleavage. Since no such cleavage products are detected in standard Western blots, they cannot be very abundant. However, like UPD overexpressed from plasmids, N-terminal fragments should have a much higher propensity than full-length Ure2p to induce [*URE3*], which could compensate for low abundance. Fragments corresponding to the prion domain in size and proteinase K resistance do appear in cell extracts that have been prepared without the use of proteinase inhibitors (Fig. 5), indicating that yeast contains proteinases that could generate this kind of fragment. Since we demonstrate that induction by full-length Ure2p overexpression leads to a prion phenotype that is distinct from that induced by UPD, this hypothesis can be tested. UPD seeds do not induce the pink (type A) variant of [*URE3*] in our strain background. Therefore, such type A strains are most likely initiated by full-length Ure2p itself. Conversely, type B [*URE3*] can be induced by UPD overexpression and is also found following Ure2p overexpression. It is possible, therefore, that type B [*URE3*] is seeded by N-terminal proteolytic fragments in the case of Ure2p overexpression as well.

Spontaneous appearance of the prion state. Surprisingly, in contrast to earlier studies in which [*URE3*] isolates were obtained spontaneously at a frequency of approximately 10^{-5} (54), we were unable to isolate any [*URE3*] strains without overexpressing Ure2p or UPD. It is noteworthy that the original work describing [*URE3*] was based on a single isolate and also mentioned difficulties in obtaining additional clones (29). The difference is likely due to variations in the strain background used in these studies. The [*PIN*] factor is another prion-like state in yeast, which plays a role in the induction of [*PSI*] (14). It is possible that [*PIN*] or another prion-like state is also necessary for the spontaneous appearance of [*URE3*]. Since chaperone proteins have been shown to influence prion formation and maintenance in yeast, another explanation for the failure to generate spontaneous [*URE3*] could be differences in the expression of such proteins in different strain backgrounds. Finally, the Mks1 protein has been reported to inhibit Ure2p function and to be necessary for initial conversion of wild-type yeast cells to [*URE3*] (20), indicating that regulation of Ure2p function could also influence the generation of new [*URE3*] elements. The question of how [*URE3*] is generated spontaneously therefore cannot be answered at this point. Most likely, one would expect a distribution similar to that observed when full-length Ure2p is overexpressed, although lower protein levels might affect seed formation by the full-length protein more strongly than by UPD. [*URE3-FL*], the only spontaneous [*URE3*] clone used in this study, clearly falls into the type A prion strain classification.

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