Conservation of a portion of the *S. cerevisiae* Ure2p prion domain that interacts with the full-length protein

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The [URE3] prion of *Saccharomyces cerevisiae* is a self-propagating inactive amyloid form of the Ure2 protein. Ure2p residues 1–65 constitute the prion domain, and the remaining C-terminal portion regulates nitrogen catabolism. We have examined the *URE2* genes of wild-type isolates of *S. cerevisiae* and those of several pathogenic yeasts and a filamentous fungus. We find that the normal function of the *S. cerevisiae* Ure2p in nitrogen regulation is fully complemented by the Ure2p of *Candida albicans*, *Candida glabrata*, *Candida kefyr*, *Candida maltosa*, *Saccharomyces bayanus*, and *Saccharomyces paradoxus*, all of which have high homology in the C-terminal nitrogen regulation domain. However, there is considerable divergence of their N-terminal domains from that of Ure2p of *S. cerevisiae*. [URE3^{Sc}] showed efficient transmission into *S. cerevisiae* ure2Δ cells if expressing a Ure2p of species within *Saccharomyces*. However, [URE3^{Sc}] did not seed self-propagating inactivation of the Ure2p's from the other yeasts. When overexpressed as a fusion with green fluorescent protein, residues 5–47 of the *S. cerevisiae* prion domain are necessary for curing the [URE3] prion. Residues 11–39 are necessary for an inactivating interaction with the full-length Ure2p. A nearly identical region is highly conserved among many of the yeasts examined in this study, despite the wide divergence of sequences found in other parts of the N-terminal domains.

The word "prion" means "infectious protein." Considerable evidence supports a prion basis for the transmissible spongiform encephalopathies (TSEs) of mammals, with an amyloid form of the PrP protein as the culprit (1). The gene for PrP controls the clinical and pathological features of the TSEs (2–7). The scrapie agent is far more radiation resistant than even small genome viruses (8), and purification of the infectious agent purifies an amyloid form of PrP (9). PrP is clearly necessary for and central to infectivity, but showing that it is sufficient has been difficult.

[URE3] (10) and [PSI+] (11) are nonchromosomal genes of *S. cerevisiae* whose molecular basis was long obscure. Genetic evidence first identified [URE3] and [PSI+] as prions of the yeast Ure2p and Sup35p, respectively (12). Three criteria distinguishing prions from viruses and plasmids are (*i*) after curing a prion, it can arise again *de novo* in the cured strain, (*ii*) overexpression of the protein increases the frequency with which the prion arises *de novo*, and (*iii*) the prion's propagation depends on the chromosomal gene encoding the protein, but the presence of the prion has a similar phenotype to recessive mutation of the chromosomal gene (12). Both [URE3] and [PSI+] satisfy all three criteria as prions of Ure2p and Sup35p, respectively (reviewed in refs. 13–16)

Ure2p is a regulator of nitrogen catabolism, acting by binding to the Gln-3 GATA transcription factor and thereby keeping the latter in the cytoplasm when the medium contains a rich nitrogen source such as NH_3 or glutamine (17–22). This prevents the transcription of genes, such as *DAL5*, encoding enzymes or transporters needed to use poor nitrogen sources (23–25). The N-terminal 65–90 residues of Ure2p largely determine the prion properties of the 354 residue protein (26, 27), whereas the C-terminal 261 residues are sufficient for nitrogen regulation (18, 26). Overexpression of the prion domain is sufficient to induce the *de novo* appearance of [URE3] at rates far higher than even the elevated rates observed on overexpression of the full-length Ure2p protein (26, 27). A similar phenomenon has been observed for the Sup35p prion domain and induction of [PSI+] appearance (28). Moreover, expression of just the prion domain is sufficient to maintain [URE3] (29).

The first biochemical evidence for the yeast prions and hint of their molecular basis was the observation that Ure2p is protease-resistant in extracts of [URE3] cells, but not in extracts of normal strains (26). The similarity of this finding to the protease resistance of PrP in scrapie brains (9) was, of course, highly suggestive. Moreover, it is the prion domain of Ure2p that forms the protease-resistant core of the prion form (26, 30, 31). Ure2p is aggregated *in vivo* specifically in [URE3] strains, and this aggregation requires the prion domain (32).

Ure2p purified from yeast is a stably soluble dimer (30, 33), but the synthetic prion domain, Ure2p1–65, spontaneously and rapidly forms amyloid fibers *in vitro* (30). Moreover, in the same way that expression of the prion domain induces the *de novo* appearance of the [URE3] prion *in vivo*, its presence *in vitro* induces the full-length Ure2p to form amyloid filaments containing both the prion domain fragment and the full-length molecule (30). The self-propagation of this reaction, the specificity for the Ure2p prion domain, and the similarity of the protease-resistance patterns of this *in vitro* amyloid to that of Ure2p in [URE3] cells strongly support the concept that [URE3] is a self-propagating inactive amyloid form of Ure2p.

Ure2p filaments have been directly observed *in vivo* specifically in [URE3] cells (31). These filaments were observed in large networks, localized to limited areas of the cytoplasm, with generally only a single network observed in a single cell section. Other areas of the cytoplasm were depleted of Ure2p (31).

Several lines of evidence suggest a structure for the Ure2p amyloid in which the prion domain forms a central β-sheet-rich core surrounded by the appended functional domain. Protease digestion of the 400 nm wide amyloid filaments formed by full-length Ure2p leaves narrow filaments morphologically similar to those formed by the prion domain alone and composed of the prion domain (30). Aggregated Ure2p from [URE3] cells shows little reaction with

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Abbreviations: TSE, transmissible bovine encephalopathy; GST, glutathione *S*-transferase; GFP, green fluorescent protein; USA, ureidosuccinate; YPAD, yeast extract peptone adenine dextrose.

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antibody to the prion domain, but reacts well with anti-Ure2C (31). The same is true of Ure2p filaments seen by electron microscopy specifically in [URE3] cells (31). This observation suggests that, in this structure, the prion domain is inside and the C-terminal domain is outside.

The Ure2p prion domain was fused to the N terminus of several enzymes with small substrates (34), including glutatione *S*-transferase (GST), which is similar to the C-terminal domain of Ure2p (18, 35, 36). Amyloid formed in each case, and the enzymatic activity of the fusion proteins were essentially the same in the amyloid form as in the soluble form, when suitable correction was made for diffusion effects (34). These results suggested that amyloid formation does not inactivate Ure2p function by changing the conformation of the C-terminal domain, but rather by either sterically blocking interaction of Ure2p with Gln3p or by Ure2p being diffusion-limited in its filament form (34). Several of the fusion proteins formed monofilaments with a helical form. The helical repeat length was consistent within a given filament, but, remarkably, varied dramatically from one filament to another, even though the filaments were composed of the same fusion protein (34). This finding indicates that the geometry of Ure2p filament formation is determined by some stochastic process that occurs during filament initiation.

The epidemic of bovine spongiform encephalopathy in the United Kingdom, followed by over 100 human TSE cases caused by consumption of infected bovine material has highlighted the importance of cross-species transmission of the mammalian TSEs. A similar phenomenon has been demonstrated in variants of [PSI+] in which the region of Sup35 of various yeasts corresponding to the prion domain of Sup35Sc was fused to the *S. cerevisiae* C-terminal domain of Sup35 (37–39). These studies demonstrated that the N termini of the Sup35 proteins of *Pichia methanolica* and *Candida albicans* can act as prion domains. Moreover, the divergence of these prion domains correlated with a "species barrier," much like that long documented for the mammalian TSEs.

We previously showed that overexpression of fragments of Ure2p or fusions of such fragments with green fluorescent protein (GFP) could efficiently cure the [URE3] prion (32). Here we have defined the portions of Ure2N and Ure2C required for this curing activity. As one approach to the functional significance of the Ure2N curing region, we examined homologs of Ure2p and found strong conservation of this part of the otherwise rapidly evolving N-terminal domain.

Materials and Methods

Yeast Strains and Media. Media were as described by Sherman (40). The ureidosuccinate (USA) uptake phenotype of *ura2* strains was tested on synthetic dextrose plates to which was added the required amino acids and bases (except uracil) and 100 μg/ml of USA.

S. cerevisiae "wild-type" cultures were obtained from three sources. Some were locally purchased; these include cultures sold for making bread (SAF Perfect Rise yeast, Lesaffre, Bruxelles, Belgium; Fleischmann Active Dry yeast, www.breadworld.com; Peter McPhie's Sour Dough strain, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda) as well as cultures sold for home beer and wine making (Red Star Dry Wine Yeast, Premier cuvee; Wyeast catalog no. 1007, www.wyeastlab.com, German Ale; Wyeast catalog no. 2112xL, California Lager; White Labs catalog no. WLP002, www.whitelabs.com, English Ale yeast; Boots home beer making genuine brewers yeast). Yeast cultures from Centraalbureau voor Schimmelcultures (CBS, www.cbs.knaw.nl) in the Netherlands (CBS400, palm wine from *Elaies guineensis*, Ivory Coast; CBS405, bili wine from *Osbeckia grandiflora*, West Africa; CBS429, fermenting must of champagne grapes; CBS2087 flower of lychee, Tonkin, China; CBS2247, grape must, Cape Province, South Africa; CBS3093, alpechin, Spain; CBS4734, from juice of sugar cane; CBS5112, grape must, Spain; CBS5287, grapes, Russia; CBS6216, tap water, Rotterdam, The Netherlands; CBS7957, cassava flour, Sao Paulo, Brazil). Clinical isolates of *S. cerevisiae* (41) were kindly provided by J. McCusker (Duke University Medical Center, Durham, NC). YJM145 is a segregant from YJM128 which was cultured from the lung of a man with immune deficiency syndrome. YJM413 is a segregant from clinical isolate YJM454. YJM280 is a segregant from YJM273, which was cultured from peritoneal fluid of a patient. YJM320 is a segregant from YJM309, which was cultured from the blood of a patient. YJM326 is a segregant from clinical isolate YJM310. YJM339 is a segregant from YJM311, which was cultured from the bile tube of a patient.

Saccharomyces bayanus (YJM562) and *Saccharomyces paradoxus* (YJM498) were kindly provided by J. McCusker. *Candida glabrata* (37A; ref. 42), *Candida kefyr* (telemorph is *Kluyveromyces marxianus*) (B4425) (43), *Candida maltosa* (B4430) (44), *C. albicans* (Darlington strain) (45), and *Candida lipolytica* (telemorph is *Yarrowia lipolytica*) (B3163) (46) were kindly provided by K. J. Kwon-Chung (National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda). *Ashbya gossypii* (47) was purchased from American Type Culture Collection (catalog no. 8717).

Plasmid Constructions. PCR used *Pfu* Turbo polymerase (Stratagene) unless otherwise stated.

Construction of yeast expression plasmids. pH7 (2μ *LEU2* P_{ADH1}) and pH317 (2μ *LEU2* P_{GAL1}) have been described (32, 48). pH199, a 2μ *LEU2* plasmid containing GFP under control of the *ADH1* promoter, has also been described (32).

To create pH722 (*LEU2 CEN* P_{URE2}), first the *Nhe*I–*Bam*HI bordered *ADH1* promoter of pH124 (32) was replaced by the similarly bordered *GAL1* promoter from pH250 (48), creating pH316 (*LEU2 CEN* P_{GAL1}). Then, a 413-bp *URE2* promoter fragment bordered by *Nhe*I and *Bam*HI sites was amplified by PCR from *S. cerevisisae* strain S288C using oligos HE194 (5′-AGAGCTAGCTTAGTAGAGCTGTGTAGAG-3′) and HE195b (5′-TTGGGATCCAACTTAATTTGCAGCTTAAAAC-3′) and cloned into the *Eco*RV site of pBC KS+ creating pH497. Replacing the *Nhe*I–*Bam*HI bordered *GAL1* promoter of pH316 with the similarly bordered *URE2* promoter from pH497 resulted in pH722. Likewise, replacing the *Nhe*I–*Bam*HI bordered *GAL1* promoter of pH317 with the *URE2* promoter from pH497 resulted in pH723 (*LEU2* 2μ P_{URE2}).

The *Hin*dIII and *Xba*I sites were removed from the *TRP1* gene in the 2μ *TRP1* vector pRS424 (49) by site-directed mutagenesis using oligos HE128 (5′-AAGAGAGCCCCGAAAGTTTA-CATTTTATGTTAGCTG-3′) and HE129 (5′-GGCCGCA-GAATGTGCTCTTGATTCCGATGCTGACTTG-3′), respectively, resulting in plasmid pH342. The *ADH1* promoter was amplified from pH7 by using oligos HE66 (5′-AGAGCTAGCAT-TACGCCAGCAACTTCT-3′) and HE67 (5′-ACAAGATCTTA-ATGCAGCCGGTAGAG-3′) and ligated into *Pvu*II digested pH342 creating pH401 (*TRP1* 2μ P_{ADH1}). The *TRP1* and *ADH1* promoters are facing each other in this construct.

Truncations of the Ure2p C terminus fused to GFP. pH328 contains the C-terminal part of *URE2* starting at Asp-66 (32). Further N-terminal deletions were made by amplifying *URE2*–GFP fusions from pH326 (32). PCR products were cloned into the *Eco*RV site of pBC KS+ (Stratagene), sequenced, and inserted as *Bam*HI–*Xho*I fragments into the *Bam*HI–*Xho*I window of pH7.

Deletions in the C-terminal fragment of the *URE2*–GFP fusion proteins were created through amplification of *URE2* fragments from pH13 (32), cloned into the *Eco*RV site of pBC KS+ and sequenced. The truncated fragments bordered by *Bam*HI and *Not*I sites were fused to GFP through exchange with the *Bam*HI–*Not*I fragment from pVTG4 (32).

Plasmids expressing truncations of the Ure2p N terminus fused to GFP. pVTG4 containing a part of *URE2* terminating at Arg-65 has been described (32). Further N-terminal deletions were made by ampli-

Table 1. Curing and complementation by Ure2C-GFP fusion proteins

Plasmid	*URE2* part*	GFP signal	Curing,† USA−/Total	Complementation of *ure2*Δ
pH328	1–2, 66–354–GFP	+	40/40	+
pH409	1, 86–354–GFP	+	40/40	+
pH410	1, 106–354–GFP	+	40/40	−
pH550	1, 111–354–GFP	+	40/100	−
pH495	1, 116–354–GFP	+/−	0/100	−
pH411	1, 126–354–GFP	−	0/40	−
pH445	1–2, 66–293–GFP	+	0/40	−
pH444	1–2, 66–313–GFP	+	0/40	−
pH443	1–2, 66–333–GFP	+	0/40	−
pH494	1–2, 66–344–GFP	−	0/100	−
pH760	1–2, 66–347–GFP	+	98/100	−
pH549	1–2, 66–349–GFP	+	100/100	−
pH199	–GFP	+		−

*The portions of Ure2p are shown as residue numbers. After the Ure2p portion is the sequence GGR followed by GFP.

†Curing was assayed in strain YHE64 (*MAT*α *ura2 leu2 trp1* [URE3]) as USA− transformants/total. USA complementation was assayed in strain YHE887 (*MAT*α *ura2 leu2 ure2*).

fying *URE2*–GFP fusions from pVTG4. PCR products were cloned into the *Eco*RV site of pBC KS+, sequenced, and transferred as *Bam*HI–*Xho*I fragments into the *Bam*HI–*Xho*I window of pH7.

C-terminal truncations were made by amplifying the *ADH1* promoter and parts of *URE2* from pVTG4. PCR products were cloned into the *Eco*RV site of pBC KS+ and sequenced. The truncated *URE2* fragments bordered by *Bam*HI and *Not*I sites were fused to GFP through exchange with the *Bam*HI–*Not*I fragment from pVTG4. In pH767, S33 is encoded by AGC instead of AGT, and in pH548, T41 is encoded by ACT instead of ACA.

The *URE2N*$^{1\text{-}45\text{-}SGR}$-GFP fragment from pH547 was transferred as a *Bam*HI–*Xho*I fragment into the *Bam*HI–*Xho*I window of pH401 creating pH792.

Cloning *URE2* from Different *S. cerevisiae* Strains. Yeast strains were grown on yeast extract peptone adenine dextrose (YPAD) to single colonies. Colonies were resuspended in 50 μl H_2O containing 3 mg/ml zymolyase and incubated at 37°C for 30 min. One microliter of this suspension was used to amplify the *URE2* gene with oligos HE252 (5′-CTGCAAATTAAGTTGTACACC-3′) and HE253 (5′-TTCCTCCTTCTTCTTTCTTTC-3′). PCR products were cloned into *Eco*RV digested pBC KS+ and sequenced.

Cloning *URE2* Homologs from Different Fungi. All yeasts were grown in liquid YPAD, harvested, and genomic DNA was extracted as described (50). *A. gossypii* was grown on solid YPAD, and genomic DNA was extracted as described (51). Degenerate PCR primers were designed based on the alignment of *S. cerevisiae URE2* and *C. albicans URE2* (strain SC5314 of *C. albicans*; http://www-sequence.stanford.edu/group/candida/). Two degenerate sense strand primers, HE207 (5′-CCIAAYGGITTYAARGTIGCIATH-3′; Y = C + T; R = A + G; H = A + C + T) and HE208 (5′-GGICAYGCICCIATGATHGGICAR-3′), and three degenerate antisense strand primers, HE209 (5′-RTAIGCIGCIGCGTTYTCIGTRTC-3′), HE210 (5′-RTCIACIACRTTRTTCCAIGGIAC-3′), and HE211 (5′-CATCATRTGYTTIGTCCAYTTRTA-3′), were used to amplify *URE2* homologs by using PCR supermix (GIBCO/BRL). PCR products were cloned into pCR2.1/TOPO (Invitrogen) and sequenced. Based on these sequences, nested organism-specific inverse primer sets were designed. Genomic DNA was digested with one of a number of restriction endonucleases and the generated fragments were self ligated. The 5′ and 3′ regions of the *URE2* homologs were amplified by using PCR supermix and cloned into pCR2.1/TOPO. To ensure that full-length *URE2* ORFs were identified, PCR primers were designed that hybridized to the 5′ and 3′ untranslated regions. After amplification of *URE2*, the PCR products were cloned into *Eco*RV-digested pBC KS+ and sequenced. No PCR product could be obtained that contained the 5′ untranslated region of the *URE2* gene of *S. paradoxus*. A PCR product could be obtained when genomic DNA of *S. paradoxus* was used in a PCR reaction with the *S. bayanus*-specific 5′ oligo. Finally, the *URE2* homologs were amplified by PCR using oligos that created a *Bam*HI site followed by the nucleotides CAA upstream of the start AUG and a *Xho*I site immediately downstream of the stop codon. *C. lipolytica* was amplified by using a PCR primer that created a *Hin*dIII site immediately downstream of the stop codon of *URE2* as it contains an internal *Xho*I. PCR products were cloned into the *Eco*RV site of pBC KS+ (Stratagene) and sequenced. The different *URE2* ORFs were cloned as *Bam*HI–*Xho*I fragments into the different expression vectors. Only *C. lipolytica URE2* was cloned as a *Bam*HI–*Hin*dIII fragment into the different expression vectors. If *URE2* was expressed under the control of the *S. cerevisiae URE2* promoter, the ORFs were first cloned into expression vectors containing the *GAL1* promoter. Subsequently, the *Nhe*I–*Bam*HI bordered *GAL1* promoter was replaced by the similarly bordered *URE2* promoter.

Results

Interaction Domains of Ure2p Based on Curing. We previously showed that overexpression of parts of Ure2p or of their fusions with GFP led to efficient curing of the [URE3] prion (32). Ure2C (residues 66–354) fused to GFP could both complement the nitrogen regulation function of Ure2p and cure. We now find that the N terminus of the curing region of Ure2C (fused to GFP) is between residues 111 and 116, whereas its C terminus is between residues 333 and 349 (Table 1). The amounts of fusion protein expressed from the various constructs was checked by the level of green fluorescence (Table 1). Comparable amounts were expressed from most constructs, but some constructs showed decreased expression, making the N terminus of the region needed for curing ambiguous. The minimal portion of the fusion proteins for complementing a *ure2*Δ mutation is residues 86–354.

Although overexpression of the N-terminal domain of Ure2p induces [URE3] prion formation, this same domain, when overexpressed in a [URE3] strain, cures cells of the prion (32). Curing also takes place when the Ure2p N terminus fused to GFP is overexpressed in a [URE3] strain (32). By making N- and C-terminal deletions in the Ure2 domain of these GFP fusion proteins (Table

Table 2. N-terminal domain interactions with Ure2p

Plasmid	*URE2* part	GFP signal*	Curing,† USA−/total	Interference,‡ USA+/10^6
pH199	–GFP	+ cytopl.	15/718	13
pVTG4	M1, **M2**–R65–GFP	+	439/440	68,000
pH545	M1, **N3**–R65–GFP	+	200/200	93,000
pH486	M1, **N4**–R65–GFP	+	100/100	73,000
pH762	M1, **N5**–R65–GFP	+	100/100	22,000
pH487	M1, **G6**–R65–GFP	+	9/300	11,000
pH408	M1, **N7**–R65–GFP	+	229/340	109,000
pH763	M1, **V9**–R65–GFP	+	197/200	35,000
pH764	M1, **N11**–R65–GFP	+	6/180	79,000
pH349	M1, **S13**–R65–GFP	+	0/118	71
pH350	M1, **R24**–R65–GFP	+ cytopl.	1/118	3
pH351	M1, **S34**–R65–GFP	+	0/118	8
pH352	M1, **N45**–R65–GFP	+ cytopl.	0/118	3
pH769	M1–**S33**, S63–R65–GFP	+ cytopl.	5/100	4
pH768	M1–**I35**, S63–R65–GFP	+ cytopl.	4/100	1
pH767	M1–**F37**, S63–R65–GFP	+ mainly cytopl.	4/100	4,600
pH442	M1–**F39**, S63–R65–GFP	+	0/240	130,000
pH484	M1–**V43**, S63–R65–GFP	−	0/200	66
pH548	M1–**N44**, S63–R65–GFP	+	129/300	60,000
pH547	M1–**N45**, S63–R65–GFP	+	101/200	57,000
pH546	M1–**N46**, S63–R65–GFP	+	80/200	61,000
pH485	M1–**N47**, S63–R65–GFP	+	295/300	39,000
pH766	M1–**N49**, S63–R65–GFP	+	86/100	70,000
pH441	M1–**N50**, S63–R65–GFP	+	139/140	63,000
pH765	M1–**S53**, S63–R65–GFP	+	95/100	53,000

*The GFP signal of most fusion constructs transformed into the [URE3] strain was aggregated. Others showed an even cytoplasmic distribution ('cytopl.').

†Curing was tested as in Table 1.

‡Interference was measured as USA+ cells per 10^6 cells. All USA+ clones tested became USA− on loss of the plasmid, indicating that this is not [URE3] induction, but simply interference with Ure2p action.

2), we more accurately defined this [URE3] curing region. We find that residues 5–47 are needed for this curing activity. Comparable levels of protein were expressed for nearly all of the constructs as judged by the level of GFP fluorescence.

Unstable Inactivation of Ure2p by Overexpressed Ure2N–GFP. Overexpression of Ure2N–GFP from pVTG4 (*CEN* P_{ADH1} *URE2N*) results in the frequent appearance of USA+ colonies in either strain 3686 (*MATα trp1 ura2 leu2*) (Table 2) or YHE142 [3686 × 3385 (*MATa kar1 ura2 leu2 his*$^-$)]. However, loss of the plasmid from 18 of these colonies uniformly (324 colonies tested) resulted in loss of the USA+ phenotype, whereas subclones that retained the plasmid remained USA+ (314 colonies tested). Moreover, this USA+ phenotype was not well transferred by cytoplasmic mixing, again indicating that it is not a stably self-propagating change. Cytoduction into 3385ρ^o + pVTG12 (*CEN LEU2* P_{URE2} *URE2N*–GFP) gave 23 cytoductants, of which 21 were USA− and two were weakly USA+. Cytoduction into 3385ρ^o + pH312 (vector) produced only 25 USA− cytoductants. The failure of cytoduction and the instability of the USA+ phenotype shows that this is not caused by generation of the [URE3] prion.

Deletion mutants of the prion domain were tested for ability to inactivate Ure2p as above (Table 2). Most of the fusion proteins inactivating Ure2p appeared to be aggregated *in vivo* (Table 2), suggesting that Ure2p may have been recruited into aggregates, even though these would not stably propagate. In contrast to the aggregation of overproduced Ure2p–GFP fusion proteins, their production at normal levels generally leads to aggregation only in [URE3] cells (32). The part of Ure2N needed for this high frequency conversion to USA+ extends from residue 11 to residue 39 (Table 2). We will see below that this region corresponds roughly to a conserved sequence in a variety of yeasts.

Natural Variants of Ure2. The N-terminal region of Ure2p contains a number of asparagine runs and is generally asparagine/serine rich. This finding could suggest a high degree of plasticity in this region. In searching for natural variation of Ure2p, we first examined various strains of *S. cerevisiae* including pathogenic isolates from immunocompromised patients, and strains used for brewing and baking from geographically widely scattered sources (Table 3). These strains contained 10 silent codon changes, all in the C-terminal domain. All but one were clustered in the region between A224 and A264. Only three changes that altered the amino acid sequence were found, but each, N23S, N70Y, and insertion of an N (AAT codon) between V43 and N44, was in the prion domain. Half of the *URE2* sequences (13 of 26) of the strains examined were identical to that of strain S288C. Thus, although amino acid changes are found in the N terminus of Ure2p, the sequence of this region is completely conserved in many of the strains tested, so that [URE3] could occur in wild-type strains of *S. cerevisiae*.

Conservation of Ure2 N Terminus Among Different Fungi. *URE2* homologs were isolated by using degenerate PCR from *S. bayanus*, *S. paradoxus*, *C. glabrata*, *C. kefyr*, (telomorph is *K. marxianus*), *A. gossypii, C. maltosa*, *C. albicans*, and *C. lipolytica* (telomorph is *Y. lipolytica*). Two *URE2* alleles were identified in the analyzed strain of *C. kefyr*. All *URE2* homologs have a highly conserved C-terminal domain that starts at Ser-100 of the *S. cerevisiae* sequence (see Table

Table 3. Amino acid changes in natural isolates of *S. cerevisiae*

Strain	Changes
Σ1278b; YJM145; YJM413; YJM280; YJM326; SAF; Fleischmann; Boots home; McPhie sourdough; CBS2087 Lychee, China; CBS4734 Sugar cane; CBS7957 Cassava flour Brazil; German Ale	None
YJM320	A224
CBS400 Palm wine–Ivory Coast; CBS405 Bili wine, West Africa	A264
CBS3093 Alpechin, Spain; CBS5112 Grape must Spain	L231
CBS5287 Grapes Russia	V245
English Ale; California lager; CBS6216 tap water Roterdam	N23S
CBS429 grapes, France; Red Star Wine; CBS2247 Grape must, S. Africa	N70Y V258
YJM339	N43b S10, E260, R344

N43b means insertion of an asparagine residue after amino acid 43. A224 means a change in codon 224 without changing the amino acid encoded.

4). The N-terminal regions show substantial divergence, although they remain asparagine/glutamine rich (Fig. 1). Interestingly, the N-terminal region identified by deletion analysis as needed for curing of [URE3] is conserved among *S. cerevisiae*, *S bayanus*, *S. paradoxus*, *C. glabrata*, *C. kefyr*, and *A. gossypii*. This region is missing from *C. maltosa, C. albicans*, and *C. lipolytica*. As *C. albicans* is an asexual organism, it was possible that this domain is missing in the particular strain we examined. However, a *C. stellatoidae* strain we examined (synonymous with *C. albicans*), and two additional *C. albicans* strains (present in the database) all had N-terminal domains identical to the first *C. albicans* strain, and lacked the conserved N-terminal region. The *URE2* sequences of *S. bayanus* and *S. paradoxus* are nearly identical, but differ in the asparagine-rich domain between Ser-40 and Leu-81 of the *S. cerevisiae* sequence, indicating a relative plasticity of this region.

Complementation of a *S. cerevisiae ure2* Deletion by *URE2* Homologs. The *URE2* ORFs of *S. cerevisiae*, *S. bayanus*, *S. paradoxus*, *C. glabrata*, *C. kefyr*, *A. gossypii, C. maltosa*, *C. albicans*, and *C. lipolytica* were placed under control of the *S. cerevisiae* URE2 promoter in pH722 (*LEU2 CEN* P_{URE2}). Complementation was assayed as the inability to grow on USA plates. *C. lipolytica URE2* did not complement, and *A. gossypii URE2* complemented weakly. For both the complementation was not improved significantly when expressed from the 2μ plasmid pH723 (also under control of the *S. cerevisiae* URE2 promoter). All of the other homologs complemented the *ure2* deletion in strain YHE888 (*MAT*α *ura2 leu2*::*hisG trp1*::*hisG ure2*::*G418*), preventing USA uptake with ammonia, while allowing it with proline as a nitrogen source.

Table 4. Conservation of *URE2* C-terminal domains

Species	Identity with *S. cerevisiae*, %		
	Amino acid	DNA	Complementation
S. Paradoxus	100	94	+
S. Bayanus	99	85	+
C. Glabrata	92	78	+
C. Kefyr-1	91	79	+
C. Kefyr-2	91	79	+
K. Lactis	91	77	+
A. gossypii	89	71	−/+*
C. Maltosa	82	73	+
C. Albicans	80	73	+
C. Lipolytica	78	66	−*

Comparison starts at Ser-100 of *S. cerevisiae* and equivalent positions in other organisms. The sequence from *K. lactis* was obtained from the GenBank database (gi: 14009513). Complementation is based on expression of the whole sequence with the *S. cerevisiae URE2* promoter on a CEN plasmid in YHE888.

*Not improved if the *URE2* homolog is on a multicopy plasmid.

Induction of [URE3] by Overproduced *URE2* Homologs. The *URE2* homologs were placed under control of the *GAL1* promoter and overexpressed in a strain with an intact chromosomal *S. cerevisiae* URE2 gene. Induction of [URE3] was assayed as appearance of USA+ colonies (Table 5). Only *S. paradoxus* and, to a slight degree, *S. bayanus* were able to induce the appearance of [URE3] at higher than background rates. The lower than background rates for most of the other homologs probably reflects their masking the spontaneous [URE3] events by complementing the functional deficit of *S. cerevisiae* Ure2p and not being themselves inactivated by the *S. cerevisiae* [URE3] (see below).

Curing of [URE3] by Overexpression of Ure2p Homologs. Each of the Ure2p homologs was expressed from the *GAL1* promoter on a *LEU2 CEN* plasmid in strain YHE64 (*MAT*α *ura2 leu2 trp1* [URE3]). Transformants were confirmed to still have [URE3] as judged by the USA+ phenotype (20 USA+ of 20 tested in each case). This finding shows that homolog production is efficiently repressed on glucose medium, a critical point for this experiment. Transformants were grown to single colonies on YPAGal2%Raf1% to overexpress the Ure2p homolog, and colonies were then replicaplated to leucine dropout plates containing dextrose. Leu+ colonies were spotted on a grid on dextrose media lacking leucine, and replicaplated twice to allow growth and dilution of any remaining heterologous Ure2p. Patches were then replicaplated to USA plates to score retention or loss of [URE3]. There was no curing by any of the *Saccharomyces* Ure2 or by the nonfunctional *C. lipolytica* Ure2p. However, each of the other Ure2s completely cured [URE3]. That this is curing, and not masking of the USA+ phenotype, is shown by the fact that the glucose-repressed transformants are USA+, and the cells are again repressed by glucose after the expression of homolog.

Can Overexpressed Ure2N$^{1\text{-}45}$-GFP Inactivate Ure2 Homologs? Strain YHE888 (*ure2*) containing plasmids expressing *URE2* homologs

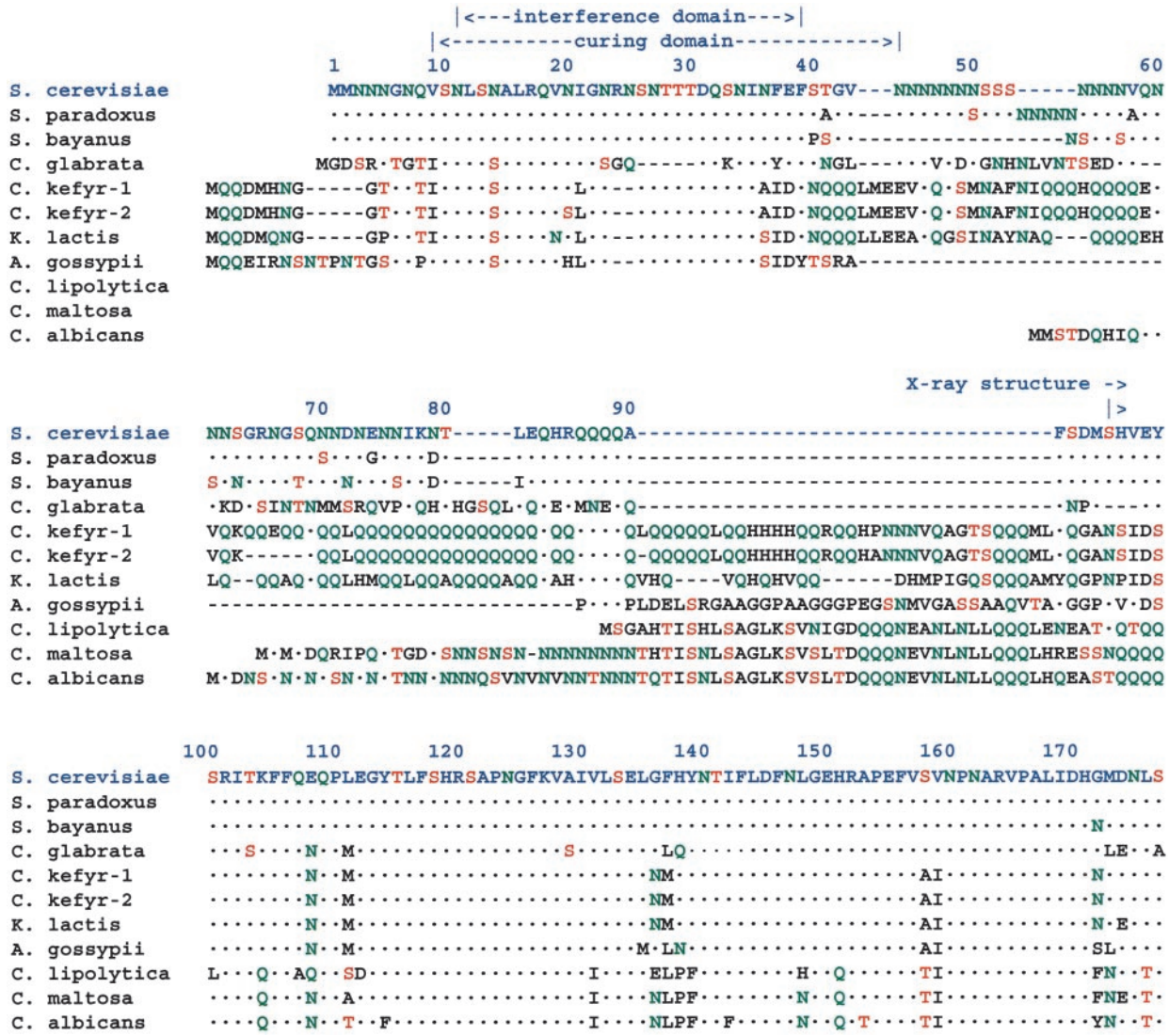

Fig. 1. Alignment of Ure2p homologs from yeasts and a filamentous fungus by using GCG pileup. The "." indicates identity, whereas "–" indicates a gap. S and T residues are red, and Q and N residues are green. The C-terminal (nitrogen regulation) domain begins at residue 100 in the *S. cerevisiae* sequence and continues to residue 354, though the figure only shows through residue 174. The "interference domain" is the portion of Ure2N which when overexpressed as a GFP fusion interferes with Ure2p activity. The "curing domain" is the portion that, fused to GFP, is needed for curing [URE3].

(*LEU2 CEN* P_{URE2}*URE2*^{homolog}) was transformed with either pH401 (*TRP1* 2μ P_{AHD1}) or pH792 (*TRP1* 2μ P_{AHD1}*URE2N*$^{1-45}$-GFP), and transformants were plated at serial dilutions on USA plates. Only the *S. cerevisiae* Ure2p was inactivated through overexpression of *URE2*N$^{1-45}$-GFP.

Transformants containing the *S. cerevisiae*, *C. glabrata*, or *C. kefyr URE2* were also plated quantitatively giving the same result as above. It is surprising that the nearly identical *S. bayanus* and *S. paradoxus* Ure2s were not inactivated. The fact that the source of Ure2p determined whether interference was observed indicates that Ure2N$^{1-45}$ was not interfering with the action of another component of the nitrogen control pathway.

Ability of Ure2 Homologs to Propagate [URE3]. *URE2* homologs were expressed in strain YHE888 (*ure2*) under control of the *S. cerevisiae* URE2 promoter from a centromeric *LEU2* plasmid. [URE3] was introduced into these strains by cytoduction from strain 4833–3B (*MAT***a** *ura2 arg1 kar1-1* [URE3-1]), and the cytoductants were examined for the USA phenotype. Only the *URE2* homologs from *S. bayanus* (11 USA+ of 11 cytoductants) and *S. paradoxus* (6 USA+ of 11 cytoductants) were able to propagate [URE3]. These are the same two Ure2s that can induce *S. cerevisiae* [URE3] appearance when overexpressed. The Ure2s of *C. kefyr* (both genes), *C. albicans*, *C. maltosa*, and *C. glabrata* gave only USA– cytoductants. The *Ashbya* Ure2p could not be tested because of its incomplete complementation ability.

Discussion

Curing of [URE3]. Because propagation of [PSI+] (52) and [URE3] (53) require Hsp104, interference with its production can cure either prion. Guanidine at millimolar concentrations can also cure either [PSI+] (54) or [URE3] (12, 55) by a mechanism that appears to involve inactivation of Hsp-104 (56, 57). [URE3] is also cured by overexpression of parts of Ure2p, particularly when they are fused to GFP (32). This curing by the "hair of the dog" method has a potentially broad application, and is known to be effective in curing tissue culture cells of PrP (58). We proposed that the Ure2p fragments or fusion proteins join the growing filaments, but do not themselves provide a growing point, thus poisoning the linear amyloid crystal, but there are other possibilities.

Table 5. Ability of URE2 homologs to induce or cure [URE3] in *S. cerevisiae*

URE2 gene	Plasmid	Induction, USA+/10^6 cells	Plasmid	Curing, USA+/USA−
Vector	pH317	22	pH316	39/1
S. cerevisiae	pH739	7,700	pH740	40/0
S. bayanus	pH661	52	pH679	40/0
S. paradoxus	pH660	6,300	pH678	40/0
C. glabrata	pH659	<1	pH677	0/40
A. gossypii	pH656	1	pH674	0/40
C. kefyr-1	pH713	2	pH711	0/40
C. kefyr-2	pH714	2	pH712	0/40
C. albicans	pH563	6	pH672	0/40
C. maltosa	pH657	<0.1	pH675	0/40
C. lipolytica	pH658	22	pH676	40/0

Strain YHE711 (*MATα ura2 leu2::hisG*) was transformed with 2μ plasmids carrying *URE2* homologs under control of the *GAL1* promoter. Individual transformants were grown to saturation in leucine dropout medium containing 2% galactose and 1% rafinose and plated in 10-fold dilutions onto USA plates to assay [URE3] induction. For curing, centromeric plasmids were transformed into YHE64. Transformants were confirmed to still carry [URE3], then grown to single colonies on YPAGal2%Raf2% to overexpress the Ure2p homolog. Leu+ colonies were grown as patches three times on dextrose, then tested for USA phenotype.

Here, we defined the parts of the C-terminal and N-terminal domains of Ure2p necessary for their curing activities. The borders of the part of Ure2C needed for curing [URE3], are approximately residues 111 and 347. The domain needed for complementation is larger, extending from approximately residue 86 to residue 354. Interaction of Ure2p with Gln3p requires at least Ure2p residues 151–330 (22), and dimer formation has been demonstrated for Ure2p residues 90–354, but not for Ure2p residues 111–354 (33). The C-terminal domain of Ure2p may cure [URE3] by forming heterodimers with the full-length Ure2p. This interaction might compete with its incorporation into the filaments. [URE3] prion stability is not affected by the nitrogen source (29), so it is unlikely that interactions with Gln3p or other factors involved in nitrogen regulation explain this curing phenomenon. Mks1p is necessary for [URE3] prion generation, not for propagation, indicating that possible interactions with this protein are not likely to be involved.

By using the yeast two hybrid method, evidence for an interaction of Ure2p residues 1–96 with Ure2p residues 152–354 has been obtained (59). However, our results show that a larger segment of Ure2C is needed for the curing, arguing against explaining the curing by this interaction of N terminus and C terminus competing for N terminus–N terminus interactions.

We find that the part of Ure2N (as a fusion with GFP) needed for curing of [URE3] is N5 to N47, a relatively short region. In this case, the crystal poisoning mechanism, in which "impurities" prevent crystal growth, is particularly attractive. A slightly smaller region, amino acid residues N11 to N44, is necessary for interfering with Ure2p activity on overexpression. All of the constructs able to interfere with Ure2p appear to be aggregated as judged by the nonhomogeneous distribution of GFP fluorescence. It is possible that the overexpressed Ure2–GFP fusion protein forms aggregates that sequester the full-length Ure2p, but that these aggregates are not self propagating, or at least do not initiate a self-propagating aggregation of the full-length Ure2p. Negative complementation of Sup35p by a fragment of its prion domain has also been observed, but whether or not this is associated with aggregation is not yet known (60).

Whatever the mechanism of inhibition, the N11 to N44 region probably interacts with full-length Ure2p, though we do not yet have direct evidence for this interaction. This region corresponds quite closely with the part of the N-terminal domain that is conserved among a group of Ure2p homologs (Fig. 1). The conservation of this region, despite wide divergence of sequence in the N-terminal part, suggests that this region is important for some function. In addition to the two-hybrid data (59), functional data suggests that Ure2N and Ure2C interact. Deletion of Ure2 residues 1–65 weakens the ability of Ure2C to carry out its function in nitrogen regulation (29). Likewise, deletions of all or parts of Ure2C dramatically increase the frequency with which Ure2p changes to the prion form (26), suggesting that the C-terminal domain stabilizes the N-terminal prion domain, perhaps by an interaction.

Ure2 Homologs. In surveying clinical isolates, brewing strains, and baking strains of *S. cerevisiae* from a variety of sources, we find that the *URE2* sequence is well conserved, and the few amino acid changes observed are in the N-terminal prion domain. Similar results were obtained by Jensen *et al.* (61) studying the Sup35 protein. Examination of *URE2* genes from a series of yeasts and the fungus *A. gossypii* shows that the C-terminal part of Ure2p is highly conserved. Although Ure2p is homologous to the θ group of GSTs (18, 62), that similarity is only about 30%, whereas these proteins are 80–90% identical to each other in their C-terminal domains. For example, the "cap region" constitutes a loop with an α helix (36) of the *S. cerevisiae* Ure2p that is missing in most GSTs, but is present in all of the Ure2p homologs studied here as well as in that of *Kluyveromyces lactis* (GenBank accession no. AAK51642). Moreover, most of the yeast and fungal homologs fully complement the *S. cerevisiae* ure2Δ mutant, indicating that the nitrogen regulation function is conserved.

Unlike other θ class GSTs, the *S. cerevisiae* Ure2p has Ala-122 and His-187 instead of the consensus Ser and Tyr, respectively. In the sequences obtained here, the Ala for Ser substitution is maintained, but the *C. lipolitica*, *C. maltosa*, and *C. albicans* Ure2 proteins contain the consensus Tyr-187, and the consensus Gly-136 is replaced with Asp in *C. kefyr*, *K. lactis*, *C. maltosa*, and *C. albicans*, and with Glu in *C. lipolitica*. Thus, all of the Ure2p homologs diverge at critical residues from the GST consensus sequence.

All of the Ure2 homologs studied here have an asparagine/glutamine-rich N-terminal extension not found in the enzymatically active GSTs or in homologs from *Schizosaccharomyces pombe* or *Neurospora crassa* (ref. 63 and www-genome.wi.mit.edu). Interestingly, precisely those homologs with an N-terminal extension have the "cap region" insert in the C-terminal domain. Are they functionally related? The functional significance of the N-terminal extension of Ure2p remains a mystery. It is unlikely that prion formation helps cells regulate nitrogen catabolism, because it differs from the normal regulation mainly in lacking flexibility. Although Ure2C can regulate nitrogen catabolism without the prion domain, this regulation is less efficient than that carried out by the full-length Ure2p (26, 29). This helper activity of the N terminus may be sufficient to explain its retention in evolution.

A domain that is only present in yeasts closely related to *S.*

cerevisiae and the filamentous fungus *A. gossypii* (also closely related to *S. cerevisiae*) is the region between S10 and I35. The conservation of this region, despite the wide sequence divergence of the remainder of the N termini, suggests the presence of some functional constraint. There is no similarly conserved portion of the Sup35p prion domain (37–39).

The curing of [URE3] by homologs of Ure2p is striking in that all complementing Ure2s can cure except for the Ure2s of *Saccharomyces* species, which can participate in the [URE3] process. The results suggest that the C-terminal domain is doing the curing in these cases. However, it is not the complementation *per se* masking the [URE3] phenotype, because cells are assayed for [URE3] when the expression of the homolog is repressed on glucose.

Species Barrier for [URE3]. Our efforts to transmit [URE3] from *S. cerevisiae* Ure2p to Ure2p of other fungi was only successful for the Ure2s from other *Saccharomyces* species. These were the most closely related in N-terminal sequence, and would thus be expected to have the lowest barrier to transmission. The fact that transmission of [URE3] was not observed to the *C. glabrata* or *C. kefyr* Ure2 proteins from the *S. cerevisiae* Ure2p, despite all having the conserved sequence in the residue 10–40 region, indicates that this region is not sufficient to allow prion transmission.

Similar results were obtained on testing the Ure2p homologs for ability to induce the *de novo* appearance of *S. cerevisiae* [URE3]. Only *S. paradoxus*, and to a slight extent *S. bayanus*, were able to induce [URE3] appearance. Others behaved like Ure2C (26) in showing an apparent decrease in frequency of [URE3], indicative of efficient complementation and inability to be affected by [URE3]. *C. lipolytica* did not induce, but does not complement *ure2*Δ either, and showed a normal background frequency of [URE3].

Further work will be needed to determine whether the non-*Saccharomyces* Ure2ps can form prions on their own. It will also be important to determine whether the conserved S10 to I35 peptide participates in amyloid formation, and with what, if any, part of Ure2C it interacts.

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