The PCR product was digested with Nco I and Xho I and cloned into pGEX-KG vector (*16*). GST-KAP C140S was constructed by a PCR method as described (*17*) with the oligonucleotide 5′-TTAATACACAGCTATGGAGG-3′ and its antisense. Phosphorylated Cdk2 was prepared as in (*15*) and incubated with 100 μg of GST-KAP or GST-KAP C140S in buffer containing 20 mM tris-Cl (pH 7.5), 50 mM NaCl, and 0.1 mM EDTA at 23°C for 45 min.

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22. Expression and purification of human GST-Cdk2 and bovine PA–cyclin A (staphylococcal PA-tagged) was as described (*4*). Phosphorylation of GST-Cdk2 or Cdk2 by anti-CAK immunoprecipitates (from mouse Swiss 3T3 cells) in the presence of [γ-^{32}P]ATP was as described (*13*); phosphorylation was quantitated with a phosphorimager (Molecular Dynamics). GST-Cdk2 was immobilized on GSH-agarose and cleaved with thrombin as described (*4*); the cleaved Cdk2 in the supernatant was incubated with PA–cyclin A (100 ng) at 4°C for 15 min. Phosphorylated Cdk2 (100 ng in 1 μl) was added to *Xenopus* cytostatic factor–arrested egg extract (10 μl) treated with 5 mM EDTA and incubated at 23°C. Anti-Cdk2 and immunoblotting were as described (*13*).
23. HeLa cells were labeled with [^{35}S]methionine for 6 hours as described (*14*). Cell extracts were prepared (*13*), and 500 μg was immunoprecipitated (*13*) with 1 μg of affinity-purified anti-KAP directed against the COOH-terminal 18 residues of KAP (C-18; Santa Cruz Biotechnology, Santa Cruz, CA).
24. Phosphorylated Cdk2 (100 ng) (*15*) was added to a HeLa cell extract (200 μg) that had been immunoprecipitated with anti-KAP as described (*15*) and treated with 5 mM EDTA. After incubation at 23°C for 30 min, the Cdk2 was recovered by immunoprecipitation with anti-Cdk2 (*13*).
25. PP2A was purified from bovine brain (provided by K. P. Lu) and was used at a concentration of 0.24 U/ml. The Cdk2 Thr^{160} dephosphorylation assay was as described (*18*).
26. Human *Wee1Hu* cDNA (*19*) cloned into pGEX-KG was provided by K. Yamashita. Mouse GST-Cdc25B was as described (*20*). Monoclonal antibody to cyclin A was provided by J. Gannon. Phosphorylation of GST-Cdk2 Tyr^{15} was done by incubating GST-Cdk2 (10 μg) with GST-Wee1 (1 μg), 15 mM $Mg(OAc)_2$, and 10 μCi of [γ-^{32}P]ATP in a volume of 10 μl at 23°C for 60 min. The GST fusion proteins were recovered with GSH-agarose, cleaved with thrombin, and treated with hirudin; the cleaved Cdk2 (100 ng) in the supernatant was incubated with 100 ng of GST-KAP or GST-Cdc25B as described (*18*).
27. Cyclin A–H6 is a histidine-tagged truncated bovine cyclin A that can bind Cdk2 (R. Y. C. Poon and T. Hunt, unpublished data) and was expressed in bacteria and purified as described (*21*). GST-Cdk2 was cleaved with thrombin and treated with hirudin as above. The cleaved Cdk2 (100 ng) was incubated with cyclin A–H6 (100 ng) and GST-KAP (1 μg) with bovine serum albumin (5 μg) as a carrier at 23°C for 30 min; the GST-KAP was then recovered with GSH-agarose.
28. GST-Cdk2 was phosphorylated with CAK immunoprecipitates in the presence of 1 mM ATP and 15 mM Mg^{2+}; the phosphorylated GST-Cdk2 was immobilized on GSH-agarose, cleaved with thrombin, and treated with hirudin as above. The cleaved Cdk2 (100 ng) was incubated with cyclin A–H6 (1 μg) and then with GST-KAP or C140S mutant (1 μg), followed by cyclin A–H6 (1 μg) as indicated; each incubation was at 23°C for 15 min. The histone H1 kinase assay was as described (*4*).
29. KAP was subcloned into pcDNA3 vector (Invitrogen) from the Bam HI–Xho I fragment of GST-KAP in pGEX-KG. KAP in pcDNA3 or pcDNA3 vector (20 μg) was transfected into human 293 embryonic kidney cells (5 × 10^5 cells per 10-cm plate) by a calcium phosphate precipitation method. Cell extracts were prepared and resolved by SDS–polyacrylamide gel electrophoresis (PAGE) (15%, 15 cm in length) followed by immunoblotting with anti-Cdk2, anti–cyclin A (*4*), and anti-Cdk7 (provided by E. Nigg).
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Prion-Inducing Domain of Yeast Ure2p and Protease Resistance of Ure2p in Prion-Containing Cells

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The genetic properties of the [URE3] non-Mendelian element of *Saccharomyces cerevisiae* suggest that it is a prion (infectious protein) form of Ure2p, a regulator of nitrogen catabolism. In extracts from [URE3] strains, Ure2p was partially resistant to proteinase K compared with Ure2p from wild-type extracts. Overexpression of Ure2p in wild-type strains induced a 20- to 200-fold increase in the frequency with which [URE3] arose. Overexpression of just the amino-terminal 65 residues of Ure2p increased the frequency of [URE3] induction 6000-fold. Without this "prion-inducing domain" the carboxyl-terminal domain performed the nitrogen regulation function of Ure2p, but could not be changed to the [URE3] prion state. Thus, this domain induced the prion state in trans, whereas in cis it conferred susceptibility of the adjoining nitrogen regulatory domain to prion infections.

A prion is an infectious protein, an altered form of a normal protein that may have lost its normal function but has acquired the ability to convert the normal form into the altered (prion) form. This concept originated in studies of scrapie of sheep, kuru and Creutzfeldt-Jakob diseases of humans, and bovine spongiform encephalopathy (mad cow disease) (*1*, *2*). These diseases are believed to be caused by a self-propagating conformational change of a highly conserved protein denoted PrP.

We previously suggested that [URE3] and [PSI], two non-Mendelian elements of yeast, are prion forms of the chromosomally encoded Ure2p and Sup35p, respectively (*3*). [URE3] (*4*, *5*) or mutations in the chromosomal *URE2* gene (*6*) each produce derepression of nitrogen catabolic enzymes that would normally be repressed by a good nitrogen source (*7*). [PSI] (*8*, *9*) or mutations in *SUP35* (*10*) both increase the efficiency of nonsense suppressor tRNAs in yeast. We proposed that [PSI] and [URE3] are prions on the basis of three lines of evidence.

1) Each is reversibly curable. Curing of [PSI] by high osmotic strength (*11*) or guanidine (*12*) produces colonies from which [PSI]-containing clones can again be derived, at some low frequency (*12*, *13*). Likewise, curing of [URE3] by 5 mM guanidine produces strains from which [URE3] clones can again be isolated (*3*). This is unlike nucleic acid replicons which, once cured, do not return unless introduced from other cells. Guanidine curing may be mediated by induction of Hsp104 (*14*).

2) [PSI] and [URE3] depend for their propagation on the chromosomal *SUP35* and *URE2* genes, respectively (*3*, *5*, *15*, *16*). The phenotypes of recessive *sup35* and *ure2* mutants closely resemble those produced by the presence of [PSI] and [URE3], respectively. In contrast, recessive mutants unable to propagate nucleic acid replicons have phenotypes opposite to those produced by the presence of these genomes.

3) Overproduction of Ure2p induces the generation of [URE3] (*3*) and overproduction of Sup35p induces the generation of [PSI] (*13*). The presence of more of the normal form increases the likelihood that the spontaneous prion change will occur.

The relative protease resistance of PrP from diseased animals compared with that from normal animals was an early indication that the mammalian scrapie agent was an altered form of this protein (*17*). It has also been critical in the recent demonstration of in vitro conversion of PrP^C to PrP^{Sc} (*2*). By immunoblotting with a polyclonal antibody to Ure2p (*3*), we detected equal amounts of similarly migrating Ure2p in extracts of strains with and without [URE3] (Fig. 1A, lanes marked X). Ure2p in extracts of normal strains was digested by proteinase K in less than 1 min to products that run off the gel, whereas Ure2p from isogenic [URE3] (prion-con-

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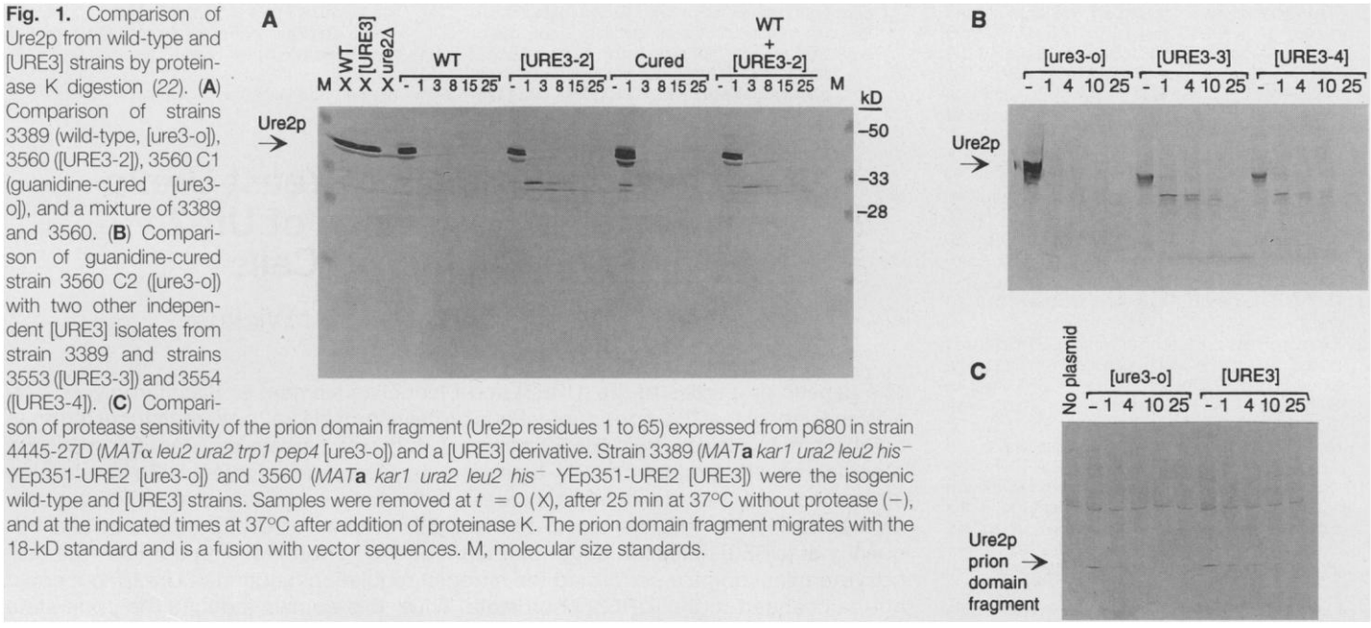

Fig. 1. Comparison of Ure2p from wild-type and [URE3] strains by proteinase K digestion (*22*). (**A**) Comparison of strains 3389 (wild-type, [ure3-o]), 3560 ([URE3-2]), 3560 C1 (guanidine-cured [ure3-o]), and a mixture of 3389 and 3560. (**B**) Comparison of guanidine-cured strain 3560 C2 ([ure3-o]) with two other independent [URE3] isolates from strain 3389 and strains 3553 ([URE3-3]) and 3554 ([URE3-4]). (**C**) Comparison of protease sensitivity of the prion domain fragment (Ure2p residues 1 to 65) expressed from p680 in strain 4445-27D (*MAT*α *leu2 ura2 trp1 pep4* [ure3-o]) and a [URE3] derivative. Strain 3389 (*MAT***a** *kar1 ura2 leu2 his*$^-$ YEp351-URE2 [ure3-o]) and 3560 (*MAT***a** *kar1 ura2 leu2 his*$^-$ YEp351-URE2 [URE3]) were the isogenic wild-type and [URE3] strains. Samples were removed at t = 0 (X), after 25 min at 37°C without protease (−), and at the indicated times at 37°C after addition of proteinase K. The prion domain fragment migrates with the 18-kD standard and is a fusion with vector sequences. M, molecular size standards.

taining) strains was digested more slowly, with fragments of about 32 and 30 kD persisting for over 15 min (Fig. 1, A and B). Two isogenic strains, cured of [URE3] by growth on rich medium containing 5 mM guanidine HCl (*3*), showed the same proteinase K sensitivity of Ure2p as the parental wild-type strain (Fig. 1, A and B). All three [URE3] isolates tested showed increased protease resistance of Ure2p (Fig. 1, A and B). Ure2p was stable in both wild-type and [URE3] extracts unless protease was added (Fig. 1A), and treatment of an equal mixture of extracts from wild-type and [URE3] strains showed an essentially additive result (Fig. 1A), indicating that the difference in degradation was not due to proteases in the wild-type extract or inhibitors in the [URE3] extract, but to a difference in the structure or associations of Ure2p.

[URE3] arises at a frequency of about 10^{-5} (*3*, *5*). Overexpression of Ure2p increased this frequency by 20- to 200-fold (*3*) (Fig. 2). Deletions from the COOH-terminus of Ure2p eliminated complementation of a chromosomal *ure2* deletion, but the truncated protein could induce [URE3] in a strain with a normal chromosomal *URE2* with an efficiency 100-fold greater than that of the intact protein, that is, 3000-fold above the spontaneous rate (Fig. 2). The NH_2-terminal 65 amino acids sufficed for this increased [URE3]-inducing activity, so we call this the "prion-inducing" domain. In-frame deletion of eight residues within the nitrogen regulatory domain around the Apa I site (Fig. 2) mimicked the COOH-terminal deletions. The prioninducing domain is 40% asparagine and 20% serine and threonine (Fig. 2) (*18*).

Deletion of part or all of the prion-inducing domain eliminated the ability of the overexpressed truncated Ure2p to induce [URE3] (Fig. 2), but the remaining COOH-terminal part could complement a chromosomal *ure2* deletion (Fig. 2) (*18*). We therefore refer to it as the nitrogen regulatory domain (Fig. 2); it contains the region related to glutathione-S-transferases (*18*).

Overexpression of the nitrogen regulatory domain alone failed to induce the [URE3] (prion) change, and prevented detection of the spontaneous [URE3] derivatives. Expression of deletion mutants lacking the prion domain consistently gave a >30-fold reduction in [URE3] clones (Fig. 2). Thus, inactivation of the nitrogen regulatory domain required a covalently attached prion-inducing domain. Deletion of residues 2 to 65 from *URE2* on a single-copy (CEN) plasmid under control of the normal *URE2* promoter resulted in reduced complementation of *ure2*Δ (Fig. 2) (*18*). Introduction of [URE3] by cytoduction into such strains (4444-3A is *MAT*α *his3 leu2ura2ure2::URA3*/pDM12) resulted in no improvement of growth on ureidosuccinate.

To determine whether the prion-inducing domain was portable, we prepared plasmids in which amino acid residues 1 to 65 of Ure2p were fused to β-galactosidase. Overexpression of this fusion protein did not induce [URE3], nor was β-galactosi-

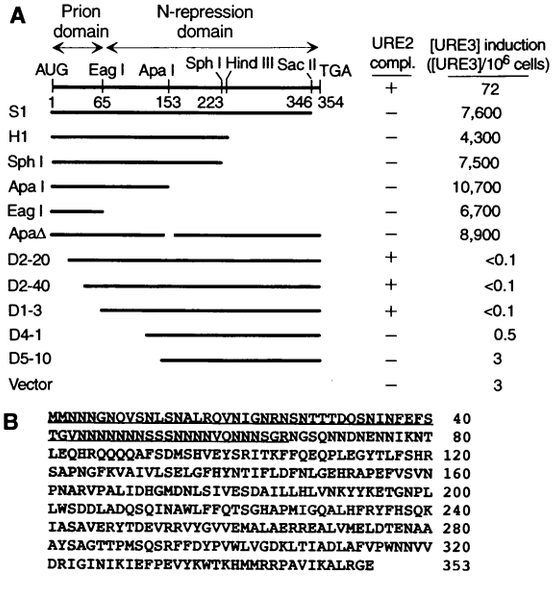

Fig. 2. Definition of prion-inducing and nitrogen regulatory domains of *URE2*. (**A**) Deletion mutants (*23*) were expressed from the *GAL1* promoter and tested for complementation of *ure2*Δ (*24*) or for the ability to induce the [URE3] state (*25*). (**B**) The Ure2p sequence (*18*) is shown with the prion-inducing domain underlined.

dase inactivated when cells spontaneously became [URE3]. Thus, the Ure2p prion domain can inactivate the Ure2p nitrogen regulatory domain, but not an arbitrary protein to which it is attached.

We compared the protease sensitivity of the overexpressed prion-inducing domain of Ure2p in wild-type and prion-containing ([URE3]) strains. This fragment was more rapidly digested in the wild-type than in the [URE3]$^-$ strain (Fig. 1C), although the difference was less marked than when the entire Ure2 protein was examined. Although only fragments of Ure2p were resistant to proteinase K, the apparent size of the prion-inducing domain did not change on digestion, suggesting that it is within the protease-resistant domain of the prion form of Ure2p.

The nonoverlapping of the NH_2-terminal [URE3] (prion)–inducing and COOH-terminal nitrogen regulation domains narrows the possible models of [URE3]. That the amount of Ure2p produced was similar in strains with and without [URE3] (Fig. 1A) likewise eliminates models based on regulation of Ure2p production.

Specific interactions of the NH_2-terminal prion-inducing domain and the COOH-terminal nitrogen catabolite repression domain are suggested by the following: (i) the NH_2-terminal part of Ure2p improves the activity in nitrogen catabolite repression of the COOH-terminal domain; (ii) the COOH-terminal domain appears to stabilize the NH_2-terminal domain, preventing its conversion to the prion form; (iii) the COOH-terminal nitrogen regulatory domain was not altered unless attached to the prion domain; and (iv) attachment of the prion domain to β-galactosidase did not make it subject to inactivation in [URE3] strains. We suggest that the [URE3] prion state is generated by specific interactions of the prion domains with each other and with the nitrogen regulatory domain.

The NH_2-terminal 114 residues of *SUP35* are required for the propagation of [PSI] (*16*). Whether the COOH-terminal region essential for cell growth is also needed for propagation of [PSI] could not be tested. The [PSI] phenotype of increased nonsense suppression was only expressed when the NH_2-terminal domain was expressed in cis with the essential COOH-terminal domain, that is, as one molecule (*16*). Although the authors propose that "the Sup35p serves as a trans acting factor required for the maintenance of [*psi*$^+$]" (*16*, p. 675), our prion model for [PSI] (*3*) is more consistent with the results. Our results likewise indicate that the COOH-terminal portion of Ure2p is not inactivated by the NH_2-terminal prion-inducing domain unless it is covalently attached. Whether overproduction of part of PrP could induce the prion change is not yet known.

The Ure2p prion domain resembles neither PrP nor the region of Sup35p needed to propagate [PSI]. Although both Sup35p and PrP have similar octapeptide repeats (*19*), these repeats seem to be dispensable for propagation of scrapie (*1*). Mutations resulting in increased prion formation (familial Creutzfeldt-Jakob disease) are distributed through most of the PrP gene (*1*). Similarly, we find that deletions in the large COOH-terminal domain result in a markedly increased frequency of [URE3] generation. The absence of apparent structural similarity among these prions, PrP, Sup35p, and Ure2p, suggests that prions can arise in various ways, producing analogous phenomena by substantially different detailed mechanisms.

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7. Nomenclature. For *URE2*, as for all chromosomal genes, the dominant allele is shown in uppercase italicized letters. The wild-type allele is dominant and the mutant, *ure2*, is recessive. Ure2p means the protein product of *URE2*. The brackets in [URE3] indicate that it is a non-Mendelian element and the uppercase letters that it is dominant, although [URE3] strains are isolated as "mutants." The wild-type (recessive) state is [ure3-o]. Aspartate transcarbamylase, whose product is ureidosuccinate, is encoded by *URA2*. Uptake of ureidosuccinate is normally repressed by Ure2p in the presence of ammonia, so the ability of *ura2* mutants to grow on ureidosuccinate (without uracil) in the presence of ammonia is used to assess *URE2* and [URE3].
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22. Strains were grown at 30°C in 200 ml of H-leu medium to an absorbance at 600 nm (A_{600}) of 1.0. Strain 4445-27D was transferred to the same medium with galactose in place of glucose for 12 hours before collecting cells. Cells were suspended in 1 pellet volume of 50 mM tris-HCl (pH 7.5), 100 mM NaCl and lysed with glass beads. Extracts were centrifuged for 15 min at 15,000*g* and the supernatant fraction retained. Each reaction included 150 μg of protein in 60 μl. Proteinase K (2 μg) was added to 44 μl of extract, 8-μl samples were removed periodically, added to sample buffer [63 mM tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.7 M 2-mercaptoethanol, 0.025% bromphenol blue, and 3 mM phenylmethylsulfonyl fluoride], and placed on dry ice. Samples were boiled for 2 min, analyzed by electrophoresis on 15% SDS-polyacrylamide gels (A) or 4 to 20% gradient gels (B and C), and probed with affinity-purified antibody to Ure2p (anti-Ure2p) (*3*). A cross-reacting band migrating slightly faster than Ure2p was present even in *ure2*Δ strains.
23. Plasmid constructions. YEp351G-URE2 is a high-copy *LEU2* plasmid with *URE2* under control of the *GAL1* promoter (*3*). Plasmid p644 has, in addition, the f1 ori inserted in the Sal I site just 3′ to the *URE2* insert such that the strand encoding *URE2* is produced. Deletions of p644 were made (*20*) with oligonucleotide D1 (5′-TCATTATTTTGGCTACCATT-CATCATTTGGGATCC-3′) deleting amino residues 3 to 65 (D1-3), D4 (5′-AGTACTATAGCAACTTT-GAA-CATCATTTGGGATCC-3′) deleting residues 3 to 125 (D4-1), D5 (5′-ATCTTCGCCAAGATT-GAAATC-CATCATTTGGGATCC-3′) deleting residues 3 to 144 (D5-10), ApaΔ (5′-GCATTAGGGT-TCACAG-CGCCAAGATTGAAA-3′) deleting amino acid residues 151 to 158, D2-20 (5′-CTGTTCCT-GTTTCCTAT-CATTTGGGATCCGGGG-3′) deleting residues 2 to 20), D2-40 (5′-GTTATTATTAT-TATTATTTACACCTGT-CATTTGGGATCCGGGG-3′) deleting residues 2 to 40, and Prter2 (5′-CATTATTTTGGCTACCATT-CTA-GCCGCTGT-TATTGTTTTG-3′), which inserts a UAG codon after amino acid 64. This construct has effects similar to those produced by p680 (Eag I, see below). Other deletion mutations of YEp351G-URE2 were made by digestion with restriction enzymes as follows. Hind III digestion and religation eliminated amino acid residue 231 to the COOH-terminus and the part of the insert 3′ of the *URE2* open reading frame, producing p665 (H1). Digestion and religation with Sac II removed residues 347 to the COOH-terminus and about 0.8 kb 3′ of the gene, producing p664 (S1). Sph I digestion and religation of YEp351G-URE2 removed amino acid residues 224 to the COOH-terminus, producing p676 (Sph I). Digestion of YEp351G-URE2 with Sph I and Eag I followed by polishing the ends with Klenow fragment and ligation removed all amino acids except residues 1 to 64, producing p680 (Eag I). Digestion of YEp351G-URE2 with Sph I and Apa I followed by polishing the ends with Klenow fragment and ligation removed all amino acids except residues 1 to 151, producing p679 (Apa I). A *HIS3*-CEN plasmid (pDM12), carrying the *URE2* gene with its normal promoter but deleted for the prion-inducing domain (amino acid residues 2 to 65), was constructed in two steps. The 1.2-kb Bam HI–Eco RI fragment of D1-3 (p682) was introduced into p576 [p532 mutagenized with oligonucleotide UB (*3*)] cut with the same enzymes, producing pDM12a. The Sal I–Eco RI fragment of pDM12a was inserted into pRS313 (*21*) cut with the same enzymes, producing pDM12.
24. Complementation of *ure2*Δ. Plasmids with various parts of *URE2* under control of the *GAL1* promoter were introduced into strain 4441-6B or 4441-8B (both *MAT*α *his3,x leu2 ura2 ure2::URA3* GAL$^+$) and grown on synthetic minimal medium containing galactose as carbon source, histidine, and either uracil or ureidosuccinate. Growth was scored after 4 days.
25. Induction of [URE3]. Plasmids with various parts of *URE2* under control of the *GAL1* promoter were introduced into strain 3720 (*MAT*α *leu2 ura2* GAL$^+$). After 5 days of growth on galactose medium with uracil, cells were plated on SD containing ureidosuccinate. Colonies appearing at 4 days were counted.

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