The PCr product was digested with Xho I and Xho I and cloned into pGEX-KG (16). GST-Cdk2/CAP140S was constructed by a PCR method as described (17) with the oligonucleotide 5'-TTATACAGCTATGGAGG-3' and its antisense. Phosphorylated Cdk2 was prepared as in (15) and incubated with 100 μg of GST-KAP or GST-CAP140S 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 Cdk2 from the Bacteriophage T7 poly-A signal was described as above. Phosphorylation of GST-Cdk2 or Cdk2 by anti-Cdk2 antibodies from mouse (3T3) cells was performed as described (19). Phosphorylation assay was quanititated with a phosphomager (Molecular Dynamics). GST-Cdk2 was immobilized on GSH-agarose and cleaved with thrombin as described (20); the cleaved Cdk2 in the supernatant was purified with a monoclonal antibody to cyclin A (R. Y. C. Poon and T. Hunt, unpublished data) and was expressed in bacteria and purified as described (21).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 heparin 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.

23. Cyclin A-H6 is a histidine-tagged truncated bovine cyclin A that can bind Cdk2 (R. Y. C. Poon and T. Hunt, unpublished data), and it was expressed in bacteria and purified as described (21).GST-Cdk2 was cleaved with thrombin and treated with heparin as above. The cleaved Cdk2 (100 ng) was incubated with cyclin A-H6 (100 ng) and GST-CAP (1 μg) with bovine serum albumin (5 μg) as a carrier at 23°C for 30 min; the GST-CAP was then recovered with GSH-agarose.

24. GST-Cdk2 was phosphorylated with Cdk2 and activated in the presence of 1 mM ATP and 15 mM MgCl2; the phosphorylated GST-Cdk2 was immobilized on GSH-agarose, cleaved with thrombin, and treated with heparin as above. The cleaved Cdk2 (100 ng) was incubated with cyclin A-H6 (1 μg) and then with GST-KAP or GST-CAP (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).

25. KAP was subcloned into pcDNA3 vector (Invitrogen) from the plasmid pP514 (18) fragment of GST-KAP cDNA pGEX-KG. KAP in pcDNA3 or pcDNA3 vector (20 ng) was transfigured into human 293 embryonic kidney cells (5 × 10⁵ 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 (1:1000), anti-cyclin A (4), and anti-Cdk7 (provided by E. Nog).

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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 Ure2p was detected at the amino-terminal 65 residues. The frequency of [URE3] induction 6000-fold. Without this “prion-inducing domain” the carboxy-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] have two non-Mendelian elements of yeast, are strain 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 Sup35 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 intrav conversion of PrP(63-230) to PrP(23-103). By immunovesting 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-
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 protease 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 NH2-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 ApaI site (Fig. 2) mimicked the COOH-terminal deletions. The prion-inducing 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 ure2Δ (Fig. 2) (18). Introduction of [URE3] by cytoduction into such strains (4444-3A is MATα his3 leu2ura2::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-
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 URE1, as for all chromosomes, the normal 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 [URE1] indicate that the mutation is a deletion and the uppercase letters that it is dominant, although [URE3] strains are isolated as "mutants." The wild-type (recessive) state is [ure2-3]. Asparagine 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 co2-5 mutants to grow on a nitrogen source (ureidosuccinate) is a useful test for 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 YEPD medium to an absorbance at 600 nm (A600) of 1.0.

23. Plasmid constructions. YEp351-URE1 is a high copy LEU2 plasmid with URE2 under control of the GAL1 promoter (5). Plasmid pD64 has, in addition, theori inserted in the SalI site just 3' to the URE2 insert such that the strand encoding URE2 is produced. Deletions of pD64 were made (20) with oligonucleotides D1 (5'-TCATTGCTACATT-GCATTTGGGATC-3') deletes amino residues 3 to 65 (D1-3), D4 (5'-AGATACAGAACATGG-GCATTTGGGATC-3') deletes residues 3 to 65 (D4-1), and YEp351-CACCAAGTT-GAAATC-CATTTGGGATC-3') deleting residues 3 to 144 (D6-10), ApaAl (5'-GATTAGGTC-TCCG-CACCAAGTT-GAAATC-CATTTGGGATC-3') deleting amino acid residues 151 to 158, D2-20 (5'-CTGCTCTATTTTCTTAT-CATTTGGGATC-3') deleting residues 2 to 20, D2-40 (5'-CTGCTCTATTTTCTTAT-CATTTGGGATC-3') deleting residues 2 to 40, and Prt62 (5'-CATTTTGGCTACATT-CATA-GCCCGTGT-TGTGT-3') which inserts a UAG codon after amino acid 64. This construct effects similar to those produced by pD68 (Eag 1, see below). Other deletion mutations of YEp351-URE2 were made by digestion with restriction enzymes as follows. HindIII digestion and restriction eliminated amino acid residue 251 to the COOH-terminal and the part of the insert 3' of the URE2 open reading frame, producing pD665 (HI). Digestion and religation with SacII removed residues 347 to the COOH-terminal and about 0.8 kb 3' of the gene, producing pD64 (S1). SpI digestion and religation of YEp351-URE2 removed amino acid residues 224 to the COOH-terminal, producing pD655 (S2). Digestion of YEp351-URE2 with SpI and EagI followed by polishing the ends with Klenow fragment and ligation removed all amino acids except residues 1 to 64, producing pD670 (EagI). Digestion of YEp351-URE2 with SpI and ApaI followed by polishing the ends with Klenow fragment and ligation removed all amino acids except residues 1 to 151, producing pD671 (ApaI). 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 Barn H-Eco RI fragment of D1-3 (pD68) was introduced into pS765 [p532 methylated with oligonucleotide UB (3)] cut with the same enzymes, producing pDM12A. The 0.6-kb EcoRI fragment of pDM12A was inserted into pRS132 (21) cut with the same enzymes, producing pDM12.
24. Complementation of ure2Δ. Plasmids with various parts of URE2 under control of the GAL1 promoter were introduced into strain 4441-6B or 4441-8B (both MA7α has,3x leu2 ura2:2 PRAG GAL*). and grown on synthetic minimal medium containing galactose as carbon source, histidine, and either uracil or uridesosuccinate. 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 (MA7α leu2 ura2:2 PRAG GAL*). After 5 days growth on galactose medium with uracil, cells were plated on SD containing uridesosuccinate. Colonies appearing at 4 days were counted.
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