Investigation of the [URE3] Phenomenon in S. cerevisiae

by

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ABSTRACT

This project's first goal was to express and purify Ure2p in both its [ure3-0] and [URE3] conformations. To this end, a S. cerevisiae strain and galactose-inducible URE2 expression vectors were made, and by all tests they seemed suitable for this purpose.

Ure2p which is C-terminally tagged with a 6His-FLAG epitope, can be easily purified out of yeast, but it is likely that this Ure2p can not be converted into its [URE3] form. To purify Ure2p in its [URE3] form, one may have to avoid tags.

To detect untagged Ure2p during purification, one must use polyclonal α-URE2 antibodies. Our antibodies cross-reacted with many other proteins, so we wanted to purify them. This necessitated the production of tagged Ure2p in E. coli, which was attempted but not accomplished, apparently because Ure2p was proteolyzed.

Both the chemical synthesis and the purification of the N-terminal 65 amino acids of Ure2p were very difficult. Finally, attempts to electroporate protein into yeast failed because conditions could not be found which permitted both the detectable uptake of protein and the survival of an appreciable number of cells.
The prion model of disease states that PrP\textsuperscript{Sc} proteins, whether spontaneously formed or introduced from the outside, cause neurodegenerative disorders, including scrapie, bovine spongiform encephalopathy, and Creutzfeld-Jacob disease (reviewed in Brown et al., 1991; Prusiner, 1991). The prion model also maintains that the mammalian PrP protein can exist in two forms: a normal form (PrP\textsuperscript{c}) and a prion form (PrP\textsuperscript{Sc}, see Figure 1). The PrP\textsuperscript{Sc} proteins are infectious because they convert the PrP\textsuperscript{c} proteins into more PrP\textsuperscript{Sc}s.

The existence of prions has been controversial for decades. Most evidence supports the model, but a few critical experiments have not been successfully performed, presumably because working with the PrP system is difficult.

In 1994, Wickner showed that prions might also exist in \textit{S. cerevisiae}. The yeast genetic phenomena [PSI\textsuperscript{+}] and [URE\textsubscript{3}] could be explained if [PSI\textsuperscript{+}] and [URE\textsubscript{3}] were prion forms of the proteins Sup35 and Ure2 (Wickner, 1994). If yeast do have prions, then working with them might be easier than working with PrP prions. Through [PSI\textsuperscript{+}] or [URE\textsubscript{3}], it might be possible to establish beyond reasonable doubt that prions exist.

Mammalian prion research, to date, has several weak points. For instance, critics worry that the infectivity associated with purified PrP\textsuperscript{Sc} might be due to a contaminating nucleic acid. The only way to satisfy these critics is to make the PrP synthetically (or in an unrelated organism like \textit{E. coli}), to purify it, convert it to its prion form, and then demonstrate its infectivity. For PrP, every step in this process has proved difficult.

The PrP protein is too large to make synthetically. For unknown reasons, expressing it at reasonable levels has been hard (Mehlhorn et al., 1996), and has only recently been achieved (Riek et al., 1996). Converting the normal form of the protein
to its prion form has also been challenging, and the best effort to date has produced so little putatively infectious protein that researchers can not detect a difference in infectivity after the conversion (Kocisko et al., 1994).

In my work, I hoped to use *S. cerevisiae*, both to learn about yeast prions and to address some of the issues that remained unresolved with PrP. First of all, I wanted to purify the hypothetical yeast prion protein, Ure2p, in its prion and non-prion forms. In mammals, infectivity is assayed by injecting infectious material into an animal and waiting for it to get sick. I wanted to find a way of "injecting" possibly infectious Ure2 protein into yeast cells, and assay whether they were converted into prion strains. I also hoped to convert normal Ure2 protein to its prion form *in vitro*. Using either *in vitro*--converted Ure2p or synthetic Ure2-N terminus, I hoped to "infect" normal yeast cells, thus showing that pure protein is sufficient to convert the cells to their prion form.

**About URE2.** Roughly twenty-five years ago, Francois Lacroute was investigating *S. cerevisiae*'s uridine biosynthesis pathway (see Figure 2). Surprisingly, his *ura2* URA3+ cells failed to grow on ureidosuccinate (USA). Consequently, he looked for mutations which would allow the cells to grow, and he discovered several. One type, he called *ure2*. These cells had a single, recessive, nuclear mutation which permitted growth on USA. A second "mutation" was *URE3*, a dominant, non-nuclear trait (Lacroute, 1971). Thus, *URE3* and *ure2* cells have the same phenotype with regard to USA use, but the traits are genetically distinct.

Later work by Coschigano and Magasanik (1991) resulted in the identification and cloning of *URE2*. By sequence, the Ure2 protein (Ure2p) is a 40 kD protein with C-terminal homology to glutathione-S-transferases. The protein's N-terminal 64 amino acids consist of 40% asparagine, and 20% serine and threonine.
URE2 is involved in the regulation of S. cerevisiae's nitrogen metabolism; specifically, it is responsible for repression of Gln3p in response to a good nitrogen source, such as glutamine or ammonium (Coschigano and Magasanik, 1991, see Figure 3). Gln3p activates the transcription of a variety of genes, including DAL5, the allantoate permease (reviewed in Magasanik, 1992). This permease is USA's means of entry into the yeast cell (Magasanik, 1992). Therefore, non-functional URE2 permits growth on USA because its absence permits Gln3p to activate transcription of the USA permease.

The prion connection. Wickner's groundbreaking paper (Wickner, 1994) proposed that the \([\text{PSI}^+]\) is a prion form of Sup35p, and that \([\text{URE3}]\) is a prion form of Ure2p. To support this hypothesis, he used cytoduction to confirm the dominant, cytoplasmically-inherited nature of \([\text{URE3}]\), and he demonstrated that the URE2 gene was necessary for \([\text{URE3}]\) maintenance. He also showed that growing \([\text{URE3}]\) cells on 5 mM guanidine hydrochloride effectively and reversibly cured cells of their \([\text{URE3}]\) phenotype.

In a second paper, Masison and Wickner (1995) showed that URE2's N-terminus is necessary for prion formation, while its C-terminus is necessary and sufficient for complementation. Consistent with the hypothesis that prion formation is a stochastic event whose probability depends on the amount of Ure2p, overexpression of URE2 increased the frequency at which cells "went prion" by 20-200 fold. Overexpression of the N-terminus increased the frequency to 3000 times the background level.

Recent research on \([\text{PSI}^+]\) has, by analogy, yielded some exciting clues on a possible mechanism of \([\text{URE3}]\) formation. Work by Chernoff et al., (1995), Paushkin et al. (1996), and Patino et al., (1996) strongly suggests that the prion form of Sup35p is an insoluble aggregate, with the N-terminal domain mediating aggregation. Certainly the prion form of PrP is an insoluble aggregate (Meyer et al., 1986, Gabizon
et al., 1988), and aggregation could explain the increases in protease resistance for 
PrPSc and for Ure2p in [URE3] strains (Masison and Wickner, 1995). Come et al. 
(1993) suggested that prion formation might involve a nucleation-dependent, self-
assembly mechanism. Consistent with this model is Glover and coworkers' (1997) 
finding that purified Sup35p precipitates into fibers with nucleation-propagation-
like kinetics.

MATERIALS and METHODS

Vector construction.

(a) Galactose-inducible expression vectors. I PCR-amplified URE2 with 
primers which gave it a 5' BamHI site, eliminated its stop codon, and gave it a 3' 
BspEI site followed by a SacI site. I inserted this fragment into the vector pBl-UASG 
(courtesy of Dr. Thomas Oehler, then in Dr. Leonard Guarente's lab, MIT), between 
its BamHI and SacI sites. This placed URE2 behind the galactose-inducible Gal1-
10 promoter, producing pUASG-URE2. Just upstream of the UASG was a XhoI site 
from pBl-UASG's polylinker.

I synthesized the oligo 5'-
GGTCCGGAGGTGGTGACTACAAGGACGACGATGA 
CAAGCACCATCATCACCATCACCATGTTGAGTGACTGAGAGCTCGG-3' and its 
complement. This oligo encodes the FLAG epitope (Eastman Kodak Co., Rochester, 
NY), six histidines, and a C-terminal cysteine. I annealed and digested the oligos 
with BspEI and SacI. I ligated this fragment to the XmnI-BspEI portion of pUASG-
URE2 and the XmnI-Sacl portion of pUASG-URE2. This triple ligation produced 
pUASG-URE2e.
I inserted the Xhol-Sacl fragment from pUASG-URE2e into pRS424 (Sikorski and Hieter, 1989), producing ET-URE2e. To make ET-URE2, I excised the tagged URE2 gene with BamHI and Sacl, and replaced it with a PCR-amplified, untagged URE2 gene.

(b) Other expression vectors. I synthesized the oligo 5'-CGCCTCGAGGTCCCCGGGTAGCTGCAGGAAGGATCCCGC-3' and its complement, and I annealed these to make a multiple cloning site. I digested this fragment with Xhol and BamHI, and inserted it into ET-URE2 and ET-URE2e, replacing their UASG regions. These vectors were CS-URE2 and CS-URE2e.

I digested pDB20 (Dr. Leonard Guarente, MIT) with Smal and Pstl to obtain a fragment containing the ADH1 promoter. I inserted this into similarly-digested CS-URE2 and CS-URE2e to produce pADH-URE2 and pADH-URE2e.

I PCR-amplified the copper-inducible promoter of YIp5-Sc3451 (Klein and Struhl, 1994), using primers which put at Smal site on its 5' end, and a Pstl site on its 3' end. I inserted this fragment into CS-URE2 and CS-URE2e, digested with Smal and Pstl, to make Sc3451-URE2 and Sc3451-URE2e.

To make tagged Ure2p in E. coli, I digested ET-URE2e with BamHI and Sacl, and inserted the URE2-containing fragment into similarly digested pAED4 (Dr. Paul Matsudaira, Whitehead Institute, Cambridge, MA). This produced pAED4-URE2e, a construct which is IPTG-inducible in E. coli strains containing the DE3 element (IPTG-inducible T7 RNA polymerase).

(c) URE2 deletion construct. I digested pJLZ113 (Dr. Leonard Guarente, MIT) with EcoRI and isolated the smaller fragment which contained URE2's ORF and some surrounding genomic DNA. I inserted this fragment into pUC18 (Boehringer Mannheim Co., Indianapolis, IN), producing pUC-URE2. I PCR-amplified LEU2 with primers which added a 5' Xhol site and a 3' SacII site. I inserted this fragment into similarly digested pUC-URE2, producing pΔU. All of the coding
region of URE2, except for several hundred base pairs at the C-terminus, were deleted. A partial EcoRI digest of this construct produced a fragment containing Aure2::LEU2, which I inserted into pBl-SK- (Stratagene, La Jolla, CA) a fragment usable to transform yeast and knock out URE2.

Strain Construction.

See Table 1 for a complete list of strains. Mating and sporulating STX21-4A and PSY316 Δtrp produced strains AvG1 and AvG3. I disrupted these strains' PEP4 genes with a pep4::HIS3 disruption construct (BJ3287, Dr. Elizabeth Jones, Carnegie Mellon University), producing AvG4 and AvG5. I then knocked out these strains' URE2 genes with pAU2 to make AvG8 and AvG9. Deleting the URE2 gene in strain 4445-27D produced AvG10. All gene disruptions were confirmed both by growth assays and by Southern blots.

Strain 3383 [URE3] #19 was isolated by plating 3383 onto a USA plate.

Strains 3385 c8Y and 3385 c5U are [URE3] variants of strain 3385, which became [URE3] by cytoduction.

Growth and media. Standard growth conditions (YPD or SD-based media, 30°C growth) were used unless otherwise indicated. All [URE3] strain plates were stored at room temperature, after they were grown up. The ureidosuccinate, or USA, (Sigma Chemical Company, St. Louis, MO) stock solution was 1% (w/v), pH 6.2-6.4. It was kept dark and at 4°C. I used USA at a final concentration of 100 μg/ml. To avoid possible heat degradation, I added USA to plate media after allowing the agar to cool somewhat. [URE3] strains were cured by growing them on SD plus minimal amino and nucleic acid supplements, and 5 mM guanidine hydrochloride.

Plating for [URE3]s. Overnight cultures were grown in appropriate media. 1 ml of these cultures was spun down, washed, and resuspended in sterile water. 100 μl of 1:10^4 dilutions were plated onto YPD to calculate the concentration of viable cells, and 100 μl of undiluted, washed culture was plated onto USA-plates to
calculate the concentration of [URE3] cells. When testing the effect of overexpressing URE2, all media contained 2% galactose and 1% raffinose instead of the usual 2% glucose.

Expression and purification of tagged Ure2p.

I inoculated AvG8, containing ET-URE2e, into 5 mL SD media supplemented with casamino acids, uridine, adenine, and 2% galactose. This culture was used as an inoculum for a 50 ml culture, which was in turn used to inoculate a 1 L culture, which was used to inoculate a 10 L fermenter run (Microferm Fermenter, New Brunswick Scientific Co., Inc., Edison, NJ). At each point, the cultures were checked under a microscope for contaminants.

I harvested the culture just before stationary phase, after about 26 hours. I resuspended the cell pellets in 0.5 ml of 3x lysis buffer per gram of wet cells (450 mM HEPES, pH 7.8, 3 mM EDTA, 30% glycerol, plus 3 mM DTT and 3x protease inhibitors added directly before use). I added the following (1x) protease inhibitors to all buffers, except dialysis buffers, directly before use: 100 mM PMSF, 200 mM benzamidine, 100 μg/ml pepstatin A, and 100 μg/ml bacitracin. Dialysis buffers contained only PMSF and benzamidine.

I lysed the cells on ice with 0.5 mm glass beads, beating them about 12 times, for 30 seconds each, with 2 minute rest-periods in between. The cell lysate was then centrifuged for 5 minutes at 5,000 rpm in a Sorvall RC-5B centrifuge (SA-600 rotor, Sorvall, Inc. Newton, CT). The supernatant was centrifuged in a SWTi rotor, in a Beckman ultracentrifuge, at 41,000 rpm, for 30 minutes. The lipid layer and the pellet were discarded.

I precipitated the majority of the Ure2p by adding ammonium sulfate to 40% of saturation, mixing the lysate for 5 minutes at 4 °C. Centrifugation in the Sorvall SA-600 rotor for 15 minutes at 15,000 rpm pelleted the protein.
I resuspended the ammonium sulfate pellet into 1x nickel loading buffer (50 mM HEPES, pH 7.8, 300 mM NaCl, 10% glycerol, 10 mM imidazole, 10 mM β-mercaptoethanol), plus 1x protease mix, and dialyzed this sample into nickel loading buffer. Any precipitate was removed by centrifugation.

I used 5 mL of Qiagen's Ni-NTA agarose resin (Qiagen, Inc. Santa Clarita, CA) per liter of original cell culture. I washed the resin with 10 column-volumes of nickel loading buffer, and added the dialyzed lysate. The lysate and the resin were mixed, in batch form, for 1 hour at 4 °C. I washed the resin once with a 3x-column volume of nickel column loading buffer, and twice with the same amount of a similar buffer which had 50 mM imidazole. I eluted the tagged Ure2p by mixing the resin twice, each time with one column volume of elution buffer (50 mM HEPES, pH 7.8, 300 mM NaCl, 10% glycerol, 500 mM imidazole, 10 mM β-mercaptoethanol, 1x protease mix), for 30 minutes at 4 °C.

The eluate was dialyzed into Buffer A (50 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA) and applied to the anion exchange column, Mono Q HR 5/5 (Pharmacia Biotech Inc., Piscataway, NJ). An increasing concentration of Buffer B (50 mM Tris, pH 7.5, 1 M NaCl, 1 mM EDTA), 29 mM/ml gradient, was used to elute Ure2p.

**Electroporation.** A single colony was inoculated into 100, 300, or 500 mL of YPD media and grown overnight. The culture with an A600 of 0.3-1.0 was used. During all the pre-electroporation steps, the cells were kept on ice.

I spun down a sufficient amount of the overnight culture, washed it twice in 1 ml of cold 1 M sorbitol, and resuspended it in enough 2 M cold sorbitol to yield a "2x" concentration of 1.5 x 10^8 cells/ml. I mixed these cells with an equal volume of cold sterile water, containing, if appropriate, 100 μM fluorescein-lactalbumin (final concentration, 50 μM). Stock solutions of fluorescein-lactalbumin were 1.25 mM, were kept at 4 °C, and protected from light. I used these stocks for 2-3 weeks.
I used 40 μL of cells per electroporation, in pre-chilled 2 mm cuvettes. Standard electroporation conditions were: 2.5 kV, 25 μF, and 800 Ω. Immediately after electroporating, I added about 1 ml of a 0.5x YPD, 1 M sorbitol solution. The cells were rocked gently at 30° for 30-60 minutes. After this point, they were again kept cold. I combined identical samples, and gently washed them twice with a cold, 1M sorbitol/saline (7.3 g NaCl/L) solution; the microfuge was set at 7,000 rpm. Samples were then resuspended in 300-400 ul of cold sorbitol/saline per electroporation. 5 μl of propidium iodide (10 μg/ml) were added, per electroporation.

Samples were analyzed with a FACScan analyzer (Becton Dickinson, San Jose, CA) or a FACS Vantage cell-sorter (Becton Dickinson). If the cells were sorted, they were gently centrifuged and resuspended into cold sorbitol/saline before being appropriately diluted and plated.

RESULTs and DISCUSSION

Working with [URE3] strains.

In the course of these experiments, I have learned several important lessons about the intricacies of handling [URE3] strains. Before describing my own experiments, I will summarize what I learned here, in hopes that it may be useful to others.

First of all, not all [URE3] strains are stable on YPD. Wickner reported on one strain which remained [URE3] after many generations of growth on YPD (Wickner, 1994), and in my experience, some [URE3] strains are stable on YPD for one or two streaks (maybe more). However, the [URE3] strain 3687, provided to me by Dr.
Wickner (National Institute of Health, Bethesda, MD), was very unstable on YPD. Streaking it out once onto YPD plates was sufficient for it to largely, if not entirely, lose its $[\text{URE3}]$ character. Without thorough testing, I would not recommend growing $[\text{URE3}]$ strains on YPD.

$[\text{URE3}]$ strains should not be repeatedly restruck onto USA plates. If they are, the strains tend to become $\text{ure2}^-$. As these are distinguishable from $[\text{URE3}]$s only by laborious cytoduction or curing experiments, it's best to reduce the probability of getting $\text{ure2}^-s$ in the first place. Instead, researchers should have several frozen stocks of their $[\text{URE3}]$ strains, and streak out from these stocks onto a USA plate. When the plate gets old, they should re-streak from the frozen stock.

I discovered that curing cells on guanidium chloride works best with minimal media, supplemented as needed. Curing candidate $[\text{URE3}]$ strains is less than 100% efficient on rich media, but it is 100% efficient on minimal. I do not know why this is the case.

From my own experience, and according to Dr. Reed Wickner (personal communication), yeast do not grow on USA in the presence of a complete complement of amino acids, e.g. casamino acids. I have not investigated which amino acids are responsible for this, nor do I know why the yeast fail to grow.

Twice I tested whether keeping $[\text{URE3}]$ strains at 4°C, rather than at room temperature, had any effect on $[\text{URE3}]$ 's stability. The first time it seemed that keeping the plate at 4°C completely cured the candidate $[\text{URE3}]$s, while an identical plate kept at room temperature was not cured. However, the second time, I did this experiment using known $[\text{URE3}]$ strains, and I found no difference. Dr. Wickner routinely keeps all his plates at room temperature and has not investigated possible effects of storing them at 4°C (personal communication). I advise researchers to keep their $[\text{URE3}]$ plates on their bench unless they first test whether their strains are stable at other temperatures.
Lastly, researchers should beware of cross-feeding. In this system, it seems to happen more efficiently than with simpler auxotrophies. Researchers should make sure that their colonies or streaks are really growing and not simply feeding off their neighbors.

**Developing Reagents.**

My first goal was to purify Ure2p from isogenic wildtype (\(\text{[ure3-o]}\)) and prion (\(\text{[URE3]}\)) strains. To do this, I needed appropriate yeast strains and a URE2-expression vector.

First of all, the appropriate strain had to be \(\text{ura2}\), \(\text{URA3}^+\). I wanted it to be \(\text{pep4}^-\) to facilitate protein purification. To avoid purifying genomic Ure2p along with the tagged Ure2p, the strain also had to be \(\text{ure2}^-\). It had to have a marker for the expression vector, and it had to be capable of becoming \(\text{[URE3]}\). I eventually made three candidate strains which met these criteria. The first two were AvG8 and AvG9, which seemed suitable for expression and purification of Ure2p.

I made the 2\(\mu\), galactose-inducible expression vectors ET-URE2 and ET-URE2e. In ET-URE2e, the \(\text{URE2}\) gene has a C-terminal tag, with the sequence Ser-Gly-Gly-Gly, the FLAG epitope, six histidines, and an additional C-terminal cysteine. The untagged construct was a control, in case the tag interfered with the \(\text{[URE3]}\) phenomenon. Sequencing the URE2-regions of ET-URE2 and ET-URE2e revealed no mutations. Both vectors also complement the \(\Delta\text{ure2}\) in AvG8 and AvG9, and complementation depended both on the presence of the \(\text{URE2}\) gene and on its induction.

Unfortunately, data from another set of experiments made me doubt whether AvG8 and AvG9 could produce \(\text{[URE3]}\) variants. After making strains AvG4 and AvG5, I repeatedly tried to identify \(\text{[URE3]}\) variants by plating them onto USA plates. I even tried overexpressing \(\text{URE2}\), using ET-URE2 and ET-URE2e, expecting an increase in the frequency of \(\text{[URE3]}\)s, as reported by Masison and Wickner (1995).
I found *ure2−* strains and some *URA*+ strains, but I found no candidate *URE3* strains. I began to suspect that something about my strains made it impossible for them to become *URE3*. If this was true for AvG4 and AvG5, it would also be true for AvG8 and AvG9. This would make them unsuitable for purification of Ure2p; I would have to find another strain.

I asked Wickner for his strain, 4445-27D, which he had published was capable of becoming *URE3* (Masison and Wickner, 1995). He provided it, and I deleted its *URE2* gene, producing AvG10, a third strain which seemed suitable for production of URE2-protein. However, a problem arose when I tested ET-URE2 and ET-URE2e's abilities to complement AvG10's *Δure2*.

The vector-only control failed to grow on inducing plates containing USA. AvG10 with ET-URE2, ET-URE2e, or pRS424 grew on plates containing glucose plus USA, and they all grew on plates containing galactose plus uridine. In other words, strain AvG10 grows very poorly on galactose. In the presence of uridine, it can grow sufficiently, but if it is only offered USA, it can not grow at all (see Table 2). I could not, therefore, determine if my vectors complemented AvG10's *Δure2*. More importantly, I realized it would be impossible to identify *URE3* variants of AvG10 with a galactose-inducible *URE2* expression vector. Identifying these variants would require the strain to grow on galactose plus USA.

To overcome this obstacle, I decided to try using other promoters in my expression vectors. I replaced the *Gal1-10* promoter with the strong, constitutive *ADH1* promoter (from pDM20, courtesy of Dr. Leonard Guarente, MIT) and with a copper-inducible promoter (from Ylp5-Sc3451, courtesy of Dr. Kevin Struhl, Harvard Medical School). I tested these constructs for their ability to complement in strains AvG9 and AvG10, and I compared their expression levels to those of ET-URE2 and ET-URE2e.
The *ADH1*-promoter constructs did not complement a deletion in *URE2*, and by Western, they produced very little protein. These data are consistent, but they do not explain why the *ADH1* promoter performed so poorly. The copper-inducible constructs produced large quantities of Ure2p, but they failed to complement the Δ*ure2* in both AvG10 and AvG9. There were no mutations in the *URE2* genes in these constructs, so the reason for their non-complementation is unclear. The failures of these promoters meant that I could not use AvG10 as a strain for purification of Ure2p.

At this point, there was only way that I could obtain a suitable strain. I had to hope that AvG8 or AvG9 could, despite my fears, become [URE3]. Although I had not been able to isolate spontaneous [URE3] variants in these strains, perhaps I could induce them to become [URE3] by cytoducing them with a known [URE3] strain. This I did, as explained below.

Production of [URE3] Strains.

I asked Dr. Wickner for several strains to use in my search for [URE3] versions of AvG4, AvG5, AvG8 and AvG9. He gave me 3383, a strain from which he had obtained [URE3]s, the [URE3] strain 3687, and a supposedly [URE3] variant of 4445-27D. I tested these strains to see whether they behaved as expected.

By the standard tests, Wickner's strain 3687 was [URE3], but his[URE3] variant of 4445-27D was suspect. It grew on USA plates, and this growth was curable by exposure to 5 mM guanidine hydrochloride, but it also grew on "-ura" plates. Oddly, I demonstrated that this "-ura" growth was also curable by exposure to 5 mM guanidine hydrochloride. I have no explanation for this effect, nor did Dr. Wickner, when I told him about it. I later found that similar variants could arise in AvG5 as well, so this phenomenon is not unique to one yeast strain. I did not explore these odd strains any further.
I attempted to find my own \[URE3\] variants of strains 3383 and 4445-27D. I thought that if I was successful, at least I could be sure that the plating technique worked, and that the problems I’d been having with AvG4 and AvG5 were particular to those strains. Amongst the usual background of \(ure2^-s\) and \(URA^+s\), I did obtain some candidate \[URE3\] strains. Because of problems handling \[URE3\] strains which I had not then resolved, I lost most of these candidates. However, I did not lose strain 3383 \[URE3\] #19. This strain grows on USA, does not grow on "-ura" plates, and is curable by growth on 5 mM guanidine hydrochloride.

Now that I had strains which I was reasonably sure were \[URE3\], I decided to see whether they could cytoduce other strains. However, before trying to cytoduce my own strains, I decided to work out the protocol with strains which I was sure could become \[URE3\].

Initially, I used the classic Rose protocol for cytoduction (Rose et al., 1990). I used this method several times, but I never obtained any cytoductee candidates. Next I tried Wickner's protocol (Ridley et al., 1984). This protocol has more steps, and because it uses auxotrophic markers to identify cytoductees, it restricts the possible pairings of donor and recipient more than the Rose protocol does. However, it had the advantage of working. I found that the frequency of \[URE3\] transfer ranges from 30\%-80\%.

Once I knew how to cytoduce strains, I tried cytoducing AvG5 with strains 3383 \[URE3\] #19 and 3687. In both cases, I obtained cytoductee AvG5 strains which behaved as \[URE3\]s: they grew on USA, were curable by growth on 5 mM guanidine hydrochloride, and they introduced \[URE3\] to other strains by cytoduction. It seemed likely I would be able to cytoduce AvG5's derivative strain, AvG9, while expressing Ure2p from an expression vector.

I used \[URE3\] strains 3687, 3385 c8Y, and 3385 c5U to cytoduce AvG9 containing ET-URE2 or ET-URE2e, while inducing. I only obtained 11 cytoductee
candidates from the ET-URE2e crosses, while the ET-URE2 crosses produced more than 30. Replica-plating patches of these candidates suggested that 6 of the ET-URE2e recipients and 27 of the ET-URE2 recipients were ura- but USA+. I restreaked six candidates from each recipient type onto inducing media containing USA, and onto inducing media containing guanidium hydrochloride. Four of the ET-URE2 recipients grew well on USA, but all of the ET-URE2e recipients grew minimally--similarly to the negative controls.

The four ET-URE2 recipients lost their ability to grow on USA after being cured. Uncured ET-URE2 recipients grew well. Curing the ET-URE2e \[URE3\] candidates reduced their growth on USA from marginal to zero; the uncured controls grew very slightly.

This result suggests that the untagged, inducible Ure2p can be converted into a \[URE3\] form, but that the tagged Ure2 protein can not. The tagged protein may be partly converted, as evidenced by the marginal growth on USA. However, it is likely either that not all of it is converted, or that its converted form is slightly different from the untagged protein’s form, such that the tagged protein is still partly functional. This result, if confirmed by future experiments, would make purification of Ure2p in its \[URE3\] form substantially more difficult, as one would not be able to use the 6His-M2 tag.

**Overexpression of URE2.**

I wanted to reproduce Masison and Wickner’s (1995) result that overexpression of Ure2p increased the frequency at which a strain generated \[URE3\] variants. Initially, I used ET-URE2 and ET-URE2e in strains AvG4, AvG5, and 4445-27D. I compared the frequency of \[URE3\] variants for these overexpressors with the frequencies for these strains containing the empty vector, pRS424. I did not see a reproducible change in the frequency of \[URE3\]s.
I obtained the materials with which the original observation had been made: strain 3469, and the vectors p554, p642, or p680. These vectors contained, respectively, no URE2, full-length URE2, and the N-terminus of URE2, all galactose-inducible. In strain 3469, I did observe an increase in the frequency of \([URE3]\) variants when the full-length gene was expressed (see Table 3), and I saw a larger increase when the N-terminus was expressed. When I put these vectors in AvG4, AvG5, and 4445-27D I did not see an increase for the full-length URE2 clone, but I did observe a large increase when I used the N-terminal fragment. From these results, I suspect that some strains may be more sensitive to levels of URE2-protein than others. In this case, only 3469 responded to full-length Ure2p, a less potent inducer of \([URE3]\)s, but all strains responded to overexpression of the truncated protein. This may be due to some difference in the strains' genetic backgrounds.

**Expression and Purification of Tagged Ure2p from S. cerevisiae and E. coli.**

I purified Ure2p, expressed from ET-URE2e, in AvG8 \([ure3-o]\) cells. I grew the cells in media containing casamino acids; other researchers should use minimally supplemented media if they want to purify Ure2p from identically-grown \([URE3]\) cells.

After growing and lysing the cells, I ultracentrifuged the extract to remove cell debris and lipids. A 40% ammonium sulfate cut precipitated almost all of the Ure2p. After resuspending and dialyzing the protein pellet, I purified it using nickel resin. This step of the purification, as described in materials and methods, may not have been optimal in several ways. I used a batch format, but a column format might have been better. Also, two of the resin washes used 50 mM imidazole, which resulted in significant elution of Ure2p. It might be better to use 10 mM imidazole in all three washes, although perhaps the higher imidazole was effective in removing additional contaminants. Third, I eluted the Ure2p in two washes of
one column-volume each; some Ure2p might still have remained on the resin. Further elutions or larger volumes might have released it.

I dialyzed the nickel column eluate and applied it to a MonoQ (anion-exchange) column. Ure2p eluted from this column at approximately 700 mM NaCl, in a peak with a substantial shoulder. Combining and concentrating these fractions tenfold produced a Ure2p solution that showed a doublet band on a Coomassie-stained gel. A Western blot using α-FLAG-M2 antibodies revealed the same doublet, plus a number of degradation products. Because I did not N-terminal sequence the bands, I am not sure whether one or both were N-terminally degraded.

I did not attempt to perfect this procedure because it became evident that the C-terminal tag was interfering with the protein's ability to become [URE3], and any further purification should be done with untagged Ure2p.

Without the M2 epitope tag, for purposes of detection, I would have to use some polyclonal αURE2 antibodies, kindly given me by Boris Magasanik of MIT. As these antibodies were not very clean, I decided to produce a small amount of Ure2p in E. coli to purify them. To this end, I tried to express tagged Ure2p using pAED4-URE2e in strain BB101 (Robert Sauer, MIT), but I did not see any induction on a Coomassie-stained gel. By Western, I saw many degradation products, but no full-length protein. I tried expressing the protein in the protease-deficient strain, BL21-DE3 (Novagen, Inc., Madison, WI), using induction times of 15 minutes to 2 1/2 hours, inducing in minimal media, and inducing at 30°C or room temperature, but by Coomassie staining, I never saw any induction. It is possible that the odd amino-acid composition of Ure2p gives it an ill-defined structure in E. coli, which makes it prone to proteolysis.

N-terminal Peptide.

Possessing a quantity of pure, N-terminal Ure2p might be useful in several ways. For example, one could use this peptide to make antibodies specific to the N-
terminus of URE2. These antibodies might help determine whether the N-terminus was involved in various protein-protein interactions. Also, if obtaining Ure2p in its [URE3] form proved difficult, the N-terminus might substitute for it in certain experiments. For instance, in establishing an assay for the infectivity of a protein, the N-terminus might serve as a positive control.

I asked David King of the University of California at Berkeley to synthesize and purify this 65-residue peptide. Unfortunately, he found that purification was extremely difficult. He did provide me with about 1 mg of pure peptide, in lyophilized form. This was only enough to do a few pilot experiments, experiments which failed for other reasons. However, I did make some potentially interesting observations about this peptide in solution. I had divided up the lyophilized peptide into three parts, only dissolving one part at a time. These solutions’ concentrations ranged up to 0.7 mM, as determined by amino acid analysis.

In one case out of three, the entire sample dissolved easily into water, but in the other two cases, there was a small amount of viscous residue which did not dissolve. I stored the peptide solutions at 4 °C, and over the course of several weeks, I noticed that in all cases, the amount of residue increased. In light of Glover and coworkers’ (1997) results, I would not be surprised if this precipitate were fibrous, or if its structure was similar to that of Ure2p from [URE3] cells.

Electroporation.

Electroporation is a highly efficient method of introducing molecules like DNA or FITC-dextran into yeast (Becker and Guarente, 1991; Bartoletti et al., 1989). It might also possible to introduce proteins into yeast, allowing electroporation to be used as a way to detect [URE3] protein. If a sample contained [URE3] protein, and if I efficiently introduced it into a collection of cells, some percentage of the cells should be converted from [ure3-o] to [URE3]. This percentage should be higher than the percentage obtained using normal Ure2p.
Other methods of introducing protein into yeast might include polyethylene glycol transformation, or spheroplast fusion. I decided against PEG transformation because I expected it would require much more protein than electroporation, which uses smaller volumes (40 uL compared to 1 mL or more). To my knowledge, spheroplast fusion has never been tried with yeast.

Before electroporating cells with Ure2p, I wanted to identify optimal electroporation conditions. Ideal conditions would maximize the number of cells which took up protein while killing as few as possible. I needed to assay both uptake of protein and cell death. To assay for the uptake of protein, I chose to electroporate fluorescein-tagged α-lactalbumin. This protein's size is 14 kD. If it was able to enter the cell, monomeric or dimeric N-terminal peptide should also be able to enter, as its molecular weight is under 7 kD. The fluorescent tag would allow cells which took up the lactalbumin to be distinguished from the rest, using flow cytometry.

Propidium iodide is a small molecule, often used in electroporation studies. It enters cells whose plasma membranes have been compromised, binds to a cell's DNA and fluoresces red (Krishan, 1975; Weaver et al, 1988). Thus, a flow cytometer can use a cell's red and green fluorescence, compared to controls, to estimate the fraction of cells which has both survived electroporation and which have taken up protein. In every experiment, I included two negative controls: non-electroporated cells which were exposed to the fluorescent protein, and electroporated cells which were not exposed to it.

First, I had to find optimal electroporation conditions. I tried altering a number of parameters, including the voltage, the time constant, and the number of pulses. I chose to use the diploid strain 3687 for the electroporation experiments because it seemed more sensitive to Ure2p levels. After electroporation, I was careful to treat the cells as gently as possible.
At voltages lower than 1 kV, I found that the yeast did not detectably take up protein. At voltages higher than 1 kV, the number of viable cells fell to under 10% (see Figure 4), as measured both by propidium iodide staining and by plating. Similarly, for time constants below 5 msec, the cells did not take up protein. For time constants above 5 msec, an increasing percent of cells took up protein, but only about 5% took it up and survived (data not shown). Using a lower time constant (2.4 msec) with various voltages, did not help, neither did using up to seven low-voltage, low-time constant pulses (2.4 msec, 0.5 kV). The cells did not take up any protein (data not shown). Overall, the best conditions seemed to use a high theoretical time constant (20 msec) and high voltage (2.5 kV). With these conditions, 85-95% of cells took up protein; about 5-10% of the cells lived, and about 5% of the total lived and took up protein.

Given the speed of flow cytometry and fluorescence-assisted cell sorting, the 5% result ought to have been enough. Therefore, I tried a co-electroporation experiment. I attempted to co-electroporate fluorescently-labeled lactalbumin with N-terminal peptide. Using a cell-sorter, I collected cells which fluoresced green but not red. These cells should be alive and should have taken up protein. I also collected cells which were alive but which had not taken up protein, as one negative control. Another negative control consisted of living, green cells that had been electroporated with the lactalbumin only. These two controls should have a background levels of \([\text{URE3}]\)'s. Those cells which received N-terminal peptide might have a higher level, if the peptide induces some fraction of them to become \([\text{URE3}]\).

Unfortunately, I found that virtually all of the cells that had taken up protein, and were supposedly alive, were actually dead. They failed to produce colonies on YPD plates. I repeated this experiment without the N-terminal peptide, fearing that it was killing the cells, but the results did not change. The supposedly alive, green cells were actually dead. I conclude that propidium iodide is less than 100% efficient
at labeling dead cells. My attempts to establish an assay for [URE3] protein failed because I could not electroporate protein into cells without killing them.

It is possible that the fluorescein-lactalbumin was responsible for the cells' death, but I think it unlikely. The overall percentage of viable cells was as low in the negative controls, when the cells were electroporated in the absence of the protein (data not shown).

CONCLUSIONS

Investigating prions by examining the [URE3] phenomenon in S. cerevisiae may produce results of interest to the prion community. It is, however, not a completely straightforward system, and it has several unexplained aspects. In particular, the meaning of curably ura+ strains is unclear, as is the reason why yeast strains can not grow on USA in the presence of a full complement of amino acids. It is also unclear why some [URE3] yeast strains seem stable on YPD, while at least one [URE3] strain is not. It may be that there is more than one type of [URE3] particle.

Difficulties in expressing Ure2p in E. coli suggest that it might be easiest to purify tagged Ure2p from yeast, and use that protein to purify polyclonal α-URE2 antibodies. Alternately, one might try the expression system which was used to express PrP to high levels (Riek et al., 1996; Melhorn et al., 1996).

Working with purified N-terminal peptide is also likely to be challenging as the peptide is difficult to produce synthetically and is very hard to purify away from synthesis by-products. One might attempt to make the peptide recombinantly, but given the difficulties in expressing full-length recombinant Ure2p, it might not be easy to express this peptide, either.
Once pure, the peptide is soluble in pure water, but it seems to precipitate from solution. This insolubility over time makes the peptide difficult to work with, but on the other hand, the precipitation reaction and the form of the precipitate might be interesting, as Sup35p was (Glover et al., 1997). Perhaps the aggregated N-terminus has a similar structure to Ure2p from [URE3] cells.

It seems likely that C-terminally tagged Ure2p is immune to becoming [URE3]. This is a somewhat surprising result, as it was the N-terminus which was necessary for conversion to [URE3] (Masison and Wickner, 1995). However, if a conversion system in vitro is ever developed, tagged Ure2p may be a useful reagent as a negative control.

Electroporation of protein into yeast, without killing the yeast, may be impossible. It could be that another yeast strain would be hardier, but failing that, researchers interested in introducing protein into yeast might want to explore other methods of doing so.

ACKNOWLEDGMENTS

I thank my advisors, Dr. Robert Sauer and Dr. Leonard Guarente, for their guidance during this work. Dr. Reed Wickner, Dr. Boris Magasanik, Dr. Elizabeth Jones, and Dr. Kevin Struhl kindly provided me with strains, vectors, and other materials, as mentioned above. I also thank all my labmates for their direction and advice, with especial gratitude to Alok Srivastava, and Dr. Wali Karzai.
Figure 1. Basic prion model. $X_A$ is the normally-folded form of protein $X$, while $X_B$ is the prion form. Wildtype $X_A$ can, at some low frequency, spontaneously convert into $X_B$. $X_B$ can induce more $X_A$ to become $X_B$ and is therefore "infectious".

Figure 2. The uridine biosynthesis pathway in *S. cerevisiae*. The names of the genes whose products catalyze particular steps appear below the arrow representing the relevant step. Only certain steps in the pathway are shown; the figure is derived from Jones and Fink, 1992).
Figure 3. In the presence of a good nitrogen source, such as NH$_4^+$, Ure2p is active. It represses the transcriptional activator, Gln3p, preventing the expression of a handful of nitrogen-regulated genes, including $DAL5$. In the absence of Dal5p, cells can not take up USA.

Figure 4. (a) Percentage of cells taking up fluorescein-labeled $\alpha$-lactalbumin as a function of electroporation voltage. (b) Percentage of cells surviving the electroporation, as assayed by plating, as a function of electroporation voltage.
<table>
<thead>
<tr>
<th>STRAIN</th>
<th>GENOTYPE</th>
<th>SOURCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>3383</td>
<td>MATa, kar1, ura2, leu2, his3</td>
<td>Wickner, 1994.</td>
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<td>3383 [URE3] #19</td>
<td>MATa, kar1, ura2, leu2, his3, [URE3]</td>
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<td>3385</td>
<td>MATa, kar1, ura2, leu2, his-</td>
<td>Wickner, 1994.</td>
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<td>3385 c8Y</td>
<td>MATa, kar1, ura2, leu2, his-, [URE3]</td>
<td>This paper.</td>
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<tr>
<td>3385 c5U</td>
<td>MATa, kar1, ura2, leu2, his-, [URE3]</td>
<td>This paper.</td>
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<td>3469</td>
<td>Mata/Mata, kar1, ura2, leu2, trp1/TRP1, his- /HIS+</td>
<td>Wickner, 1994.</td>
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<td>3687</td>
<td>MATa, kar1, ura2, leu2, his- [URE3]</td>
<td>R. Wickner, isogenic to 3385.</td>
</tr>
<tr>
<td>4445-27D</td>
<td>MATa, leu2, ura2, trp1, pep4</td>
<td>Masison and Wickner, 1995.</td>
</tr>
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<td>AvG1</td>
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<tr>
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<td>This paper.</td>
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<td>PSY316 Δtrp</td>
<td>MATa, ura3, leu2, lys2-801, his3, ade2-101</td>
<td>L. Guarente (MIT).</td>
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<td>STX21-4A</td>
<td>MATa, ura2, his6, arg4, thr1, met1, gal2</td>
<td>Yeast Genetic Stock Center, University of California at Berkeley.</td>
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Table 2: Difficulty in assaying complementation of the \( \Delta ure2 \) in strain AvG10

<table>
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<tr>
<th>Vector</th>
<th>Expected Result</th>
<th>Actual Result</th>
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<tr>
<td></td>
<td>Glu</td>
<td>Gal</td>
</tr>
<tr>
<td>pRS424</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ET-URE2 or ET-URE2e</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ET-URE2 or ET-URE2e in a[URE3] AvG10 strain</td>
<td>+</td>
<td>+</td>
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Table 3: Overexpression of \( URE2 \) \( ^a \)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Vector</th>
<th>p642</th>
<th>p680</th>
<th>ET-URE2(e)( ^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>3469</td>
<td>20-200x( ^c )</td>
<td>not reported</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3720</td>
<td>~7x( ^d )</td>
<td>~800x( ^d )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3469</td>
<td>~38x</td>
<td>&gt;100x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4445-27D</td>
<td>N.C.( ^e )</td>
<td>substantial increase( ^f )</td>
<td>N.C.</td>
<td></td>
</tr>
<tr>
<td>AvG4, AvG5</td>
<td>~2-6x</td>
<td>~40-100x</td>
<td>N.C.</td>
<td></td>
</tr>
</tbody>
</table>

(a) Numbers refer to fold-increases in the frequency of \( [URE3] \)s, when Ure2p is overexpressed, compared to the frequency of \( [URE3] \)s when Ure2p is normally expressed.

(b) As the results were identical for ET-URE2 and ET-URE2e, they were combined into one column.

(c) Wickner, 1994.

(d) Masison and Wickner, 1995. This number was derived by comparing the frequency of \( [URE3] \) colonies in this strain, when \( URE2 \) is overexpressed, to the frequency of \( [URE3] \) colonies in strain 3469, when not \( URE2 \) is not overexpressed. The frequency of \( [URE3] \)s in strain 3720, when Ure2p is not overexpressed, was not reported.

(e) N.C. means no change.

(f) There were too many cells on the plates to count and give a more accurate estimate.
REFERENCES


