The EMBO Journal Vol. 19 No. 13 pp. 3215–3222, 2000

The yeast prion [*URE3*] can be greatly induced by a functional mutated *URE2* allele

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The non-Mendelian element [*URE3*] of yeast is considered to be a prion form of the Ure2 protein. The [*URE3*] phenotype occurs at a frequency of 10^{-5} in haploid yeast strains, is reversible, and its frequency is increased by overexpressing the *URE2* gene. We created a new mutant of the Ure2 protein, called H2p, which results in a 1000-fold increase in the rate of [*URE3*] occurrence. To date, only the overexpression of various C-terminal truncated mutants of Ure2p gives rise to a comparable level. The *h2* allele is, thus, the first characterized *URE2* allele that induces prion formation when expressed at a low level. By shuffling mutated and wild-type domains of *URE2*, we also created the first mutant Ure2 protein that is functional and induces prion formation. We demonstrate that the domains of *URE2* function synergistically *in cis* to induce [*URE3*] formation, which highlights the importance of intramolecular interactions in Ure2p folding. Additionally, we show using a green fluorescent protein (GFP) fusion protein that the *h2* allele exhibits numerous filiform structures that are not generated by the wild-type protein.

Keywords: aggregation/GFP/prion/*Saccharomyces cerevisiae*/[*URE3*]

Introduction

The genetic properties of some non-Mendelian determinants of yeast, such as [*URE3*] (Lacroute, 1971; Aigle and Lacroute, 1975) and [*PSI*] (Cox, 1965; Ter-Avanesyan *et al*., 1994), support the hypothesis that they are yeast prions (Wickner, 1994). This hypothesis is based on the genetic properties of these elements: they are dominant and display a non-Mendelian segregation; their phenotype is reversible; they depend on the expression of the regular form of the protein (Ure2p and Sup35p, respectively); and their frequency is increased by the overexpression of the normal protein (reviewed by Wickner *et al*., 1995).

The *URE2* gene codes for a 354-amino-acid protein that contains two domains. The N-terminal domain is responsible for inducing [*URE3*] formation *de novo*. This N-terminal domain was originally defined as the first 65 amino acids of Ure2p, the prion forming domain (PFD) (Masison and Wickner, 1995), but has now been more widely defined as the first 94 Ure2p amino acids that are able to induce [*URE3*] (Komar *et al*., 1999). The C-terminal domain of the Ure2 protein contains the catalytic domain (Coschigano and Magasanik, 1991), which inhibits the activity of the transcription factor Gln3p in the presence of a rich nitrogen source (Courchesne and Magasanik, 1988). Ure2p thus contributes to nitrogen catabolism repression by allowing cells to import poor nitrogen sources, like allantoin, when necessary, and by maintaining an optimal level of glutamine for growth. Recently, it was also demonstrated that Ure2p plays a role in the TOR kinases signaling pathway, which regulates a range of cellular functions including cell proliferation and response to nutrients (Cardenas *et al*., 1999).

The prion conversion process of the mammalian prion protein, PrP, remains poorly understood. The yeast [*PSI*] model, however, provides a clearer picture of prion formation: when the yeast Sup35 protein converts to the prion form, it becomes insoluble and pellets when centrifuged at 15 000 *g* (Paushkin *et al*., 1996). Autocatalytic conversion of the yeast Sup35 protein to the [*PSI*] prion has been demonstrated *in vitro*, and the [*PSI*] phenotype is associated with the aggregation state of the Sup35 protein (Paushkin *et al*., 1996, 1997).

Polymorphisms of the different prion proteins have been widely studied, especially polymorphisms of the mammal prion protein PrP. Several amino acid changes of PrP influence incubation time and/or susceptibility to transmissible spongiform encephalopathy (TSE) in animal models or human. The single 101L mutation in mouse PrP generated in transgenic mice alters the incubation time of a TSE infection (Manson *et al*., 1999). This mutation is equivalent to the P102L substitution in the human PrP that is associated with Gerstmann–Straussler–Scheinkel syndrome (GSS), a hereditary form of TSE in humans (Hsiao *et al*., 1992). Other amino acid substitutions alter susceptibility to TSE. In sheep, polymorphisms at amino acids 136, 154 and 171 in the PrP gene affect both the occurrence of the disease and the mean survival time of affected animals (Goldmann *et al*., 1994; Hunter *et al*., 1994). The presence of a valine or a methionine at position 129 of human PrP was described as influencing susceptibility to and the incubation period of two hereditary forms of TSE: fatal familial insomnia and a subtype of familial Creutzfeld–Jakob disease (Goldfarb *et al*., 1992). Also the incidence of iatrogenic transmission of Creutzfeld–Jakob disease by growth hormone treatment has been associated with polymorphisms of the PrP gene. Early in 1993, a homozygous genotype with a valine at position 129 of the PrP gene was described as playing a role in the disease incidence in French patients treated with extractive growth hormone (Labauge *et al*., 1993; Masson *et al*., 1994). The molecular mechanism by which these different forms of the PrP protein influence incubation times and

phenotypic expression remains unknown, although thermodynamic and kinetic models have been proposed to explain the role of these single mutations (Cohen, 1999).

In the yeast *Saccharomyces cerevisiae*, several mutants have been identified that enhance or inhibit [*URE3*] or [*PSI*] formation. The PNM2 mutation that eliminates the [*PSI*] element was identified as a single transition in the *SUP35* gene that generates a glycine to aspartic acid substitution in the PFD of the Sup35 protein (Doel *et al*., 1994). *Trans* acting factors that influence prion phenotypes in yeast have also been described. For example, the Hsp104 chaperone protein is required to maintain the [*PSI*] phenotype (Chernoff *et al*., 1995). Nonsense mutations in the *URE2* gene that lead to the expression of the N-terminal domain of Ure2p or missense mutations that result in the expression of an out-of-frame catalytic domain of Ure2p favor the formation of [*URE3*] (Wickner, 1994; Masison *et al*., 1997). Until now, however, alleles of *URE2* or *SUP35* that induce high levels of [*URE3*] and [*PSI*] prion phenotype, respectively, were all proteins that lost their functional acitivity.

Here we describe the creation of a mutated allele of *URE2*, called *h2*, which induces [*URE3*] formation at a high frequency when expressed on a monocopy plasmid. We characterized this mutant using genetic and cellular approaches, and analyzed the effects of mutated domains on Ure2 activity and prion formation by shuffling the different mutated domains. In this process, we obtained the first prion-inducing Ure2 protein that is still functional. Finally, by expressing H2–GFP (green fluorescent protein) fusion protein in yeast, we studied the aggregation patterns of protein encoded by the *h2* allele.

Results

Selection of inducing alleles

In order to select alleles of *URE2* that induce [URE3] at a high frequency, we transformed the CC30 yeast strain with a pFL39-*URE2* mutagenized library, and 10^5 cells were replicated on ureidosuccinate (USA) medium, which permits the selection of the [*URE3*] phenotype. Eight clones presenting three or more [USA$^+$] papillae, thus likely to present a high frequency of [*URE3*] appearance, were selected. Each clone was then replated on USA-containing medium and one was selected for further analysis as it presented a high level of [USA$^+$] clones. The transforming plasmid from this clone was re-introduced into the wild-type strain CC30. The transformed strain then exhibited a 1000-fold increase in [USA$^+$] frequency. The sequence of the *URE2* allele carried on the transforming plasmid and called *h2* revealed 14 mutations, of which 10 lead to amino acid substitutions in Ure2p (Figure 1). Two substitutions were in the PFD (defined as the domain spanning amino acids 1–94), and eight mutations were located in the catalytic domain of Ure2p. Complementation assays demonstrated that the *h2* allele was not able to complement the Δ*ure2* strain AF36.

To characterize the [USA$^+$] clones induced by the *h2* allele, we first plated these [USA$^+$] colonies on a non-selective medium in order to cure the clones of plasmids. In parallel, in the same way we analyzed [USA$^+$] clones induced by a multicopy plasmid carrying the PFD of *URE2*. Table I shows that 24 [USA$^+$]-independent clones

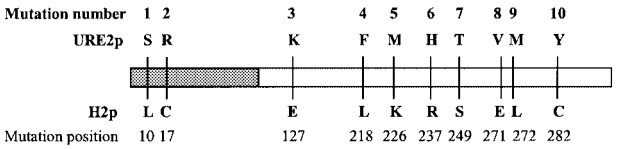

Fig. 1. Amino acid substitutions are indicated with solid lines and position; above is the wild-type *URE2* sequence and below is the *h2* allele. The shaded box indicates amino acids 1–94 including the PFD; the white box indicates the catalytic part of the protein.

induced by the overexpression of a plasmid carrying the PFD reverted to [USA$^-$] when the inducing plasmid was lost. In contrast, among 15 [USA$^+$] clones induced by the *h2* allele, five remained [USA$^+$] after the loss of the plasmid, suggesting the formation of [*URE3*], as the [USA$^+$] phenotype was maintained after the loss of the *h2* allele. We analyzed this phenotype further and demonstrated that it was dominant by crossing these clones with the CC30 strain. After sporulation, tetrads showed a characteristic non-Mendelian inheritance of the [USA$^+$] phenotype (data not shown).

N- and C-terminal mutations have a cis synergistic effect

By shuffling different regions of the *h2* and *URE2* genes, we generated new *URE2* alleles which encode proteins that combine either the mutated PFD of H2p and a wild-type Ure2p catalytic domain (*hM 1,2*), or a wild-type Ure2p PFD with the mutated catalytic domain of H2p (*hM 3-10*). These constructs were expressed on a monocopy plasmid and tested for their ability both to complement a Δ*ure2* strain and to induce the [*URE3*] phenotype. Results are shown in Table II, lines 3 and 4. The two alleles increased the frequency of [*URE3*] clones 10 and 6 times, respectively. The *hM 3-10* allele carrying the mutated catalytic domain was not able to complement a Δ*ure2* strain. However, the *hM 1,2*, carrying a wild-type catalytic domain of *URE2*, was still functional.

The [*URE3*]-inducing effect of the two hybrid alleles, *hM 1,2* and *hM 3-10*, was lower than that observed with the entire *h2* allele. To determine whether the two mutated domains could interact *in trans*, we expressed the *hM 1,2* and *hM 3-10* alleles in the same cell simultaneously. As the rate of [*URE3*] appearance partially depends on the concentration of Ure2p, we performed 10 separate co-transformations. The western blot performed with these strains indicated that the concentration of Ure2p was proportional to the copy number of the plasmids (Figure 2). Each transformant was then plated on a medium containing USA. The results shown in Figure 2 lead to several observations. Whereas the overexpression of Ure2p increases [*URE3*] appearance (Masison and Wickner, 1995), two copies of the *URE2* gene cause a decrease in the rate of [*URE3*] (Figure 2, blue arrow). This effect is even stronger with three copies. However, the concentration of Ure2p in cells is higher in this latter case (Figure 2, compare lane 1 with lanes 2 and 6 on the western blot). In the same way, co-expressing a *URE2* gene with *hM 1,2* or *hM 3-10* constructs partially inhibited their inducing effect (Figure 2, green arrow). However, the inducing effect of the *h2* allele is not significantly affected by the presence of the expression of a wild-type *URE2* gene.

Table I. Phenotype of cells after loss of plasmids inducing the [USA$^+$] phenotype

Inducing plasmid	Number of [USA$^+$] clones analyzed	Number of [USA$^+$] clones after loss of the plasmid
pY2L-Nter	24	0
pFL39-*h2*	15	5

When strain CC30 was co-transformed by vectors expressing *hM 1,2* and *hM 3-10* constructs, the level of [USA$^+$] clones was not greater than that observed for the cells transformed by *hM 1,2* alone. The low level of [USA$^+$] clones compared with the control (co-transformation of the CC30 strain by *h2* and *URE2* genes; Figure 2, red arrow) indicates that the mutated domains have a synergistic inducing effect when located on the same protein.

Effect of the mutations of the h2 allele

In order to assess the individual input of each mutation in the [*URE3*]-inducing effect, each mutation was re-introduced by directed mutagenesis of the *URE2* gene. We showed that no single mutation had a significant effect on the rise of [*URE3*]. We further investigated the effect of the mutations by combining different pairs of mutations, always selecting one mutation located in the PFD of the protein and a second mutation in the catalytic domain. We observed that several pairs of mutations had a significant effect on [*URE3*] induction, confirming that the two domains of the protein play a synergistic role in the inducing properties of Ure2p (Table II). Furthermore, we noticed that most of the inducing properties of the *h2* allele were determined by the first three mutations, which are located in the PFD and in the catalytic domain of Ure2p (Figure 1; mutant allele *hM 1,2,3*).

We also tested each construct for its ability to complement the Δ*ure2* strain AF36. One of the inducing mutation pairs (*hM 1,8*) retained the catalytic function of the *URE2* gene, but not the second (*hM 2,3*) (Table II). Thus, different mutations in the *URE2* gene produce either functional or non-functional prion-inducing proteins.

h2–GFP fusions present several aggregation patterns

It has been reported several times that the [*URE3*] phenotype may be related to an aggregation state of the Ure2p protein in the cytoplasm of the cells (Edskes *et al*., 1999). To study Ure2p aggregation, we constructed a gene that generates a protein fusion between GFP and the protein encoded by the *h2* allele. This construct was expressed under the control of the *URE2* promoter, on a monocopy or a multicopy plasmid (pYeFc1L and pYeFc2L expression vectors, respectively). As controls, we constructed the same GFP fusion with the wild-type *URE2* gene.

These plasmids were then used to transform the CC30 strain in order to measure the prion-inducing effect. The *h2*–GFP allele was still able to induce [*URE3*] at a high frequency, but its effect was somewhat reduced compared with that of the *h2* allele (Table III, line 2). The decrease in induction observed when expressing the *h2*–GFP fusion construct was also observed for the *URE2*–GFP fusion

Table II. Complementation test and [*URE3*] induction by different mutated alleles of *URE2*

Allele	USA growth	[URE3] induction
URE2	–	1
h2	+	1000
hM 1,2	–	10
hM 3-10	+	6
hM 1	–	1
hM 2	+/–	1
hM 3	+	1
hM 4	–	1
hM 5	+/–	1
hM 6	–	1
hM 7	+	1
hM 8	+/–	1
hM 9	–	1
hM 10	+	1
hM 1,3	+/–	1
hM 1,7	+	1
hM 1,8	–	10
hM 1,9	–	1
hM 2,3	+	12
hM 2,7	+/–	1
hM 2,8	+/–	1
hM 2,9	+	1
hM 1,2,3	+	500

Numbers written after *hM* indicate the position of mutations re-inserted, as indicated on Figure 1.
The absence of growth (–) indicates the functionality of the *URE2* allele. +/– indicates very slow growth.
[*URE3*] induction is given as 1 for the inducing effect of the wild-type *URE2* gene, corresponding to a frequency of 10^{-5}. Standard deviation is <10%.

protein expressed on the multicopy plasmid pYeFc 2L that was used as a control. By transforming the CC34 strain with the monocopy and multicopy plasmids bearing the H2–GFP fusion protein, we ensured that the *h2*–GFP fusion did not cure [*URE3*] in a strain initially carrying the [*URE3*] prion (Table III, line 3). The C-terminal GFP fusion is able to decrease slightly the inducing effect of *URE2*, but when overexpressed on the pYeFc 2L plasmid, it has a destabilizing effect on a pre-existing [*URE3*] phenotype, as previously described (Edskes *et al*., 1999).

The proteins encoded by these constructs exhibited different expression patterns that did not depend on the [*URE3*] phenotype but on the expression levels of each construct (Figure 3). When expressed on the monocopy plasmid pYeFc 1L, the *URE2*–GFP and *h2*–GFP fusion constructs generated, in 100% of cells, a diffuse green color in both strains CC30 and CC34 (Figure 3A, B and C).

However, when expressed on the multicopy plasmid pYeFc 2L, the GFP fusion proteins produced by the two constructs showed different patterns of expression (Figure 3D, E and F). When transformed by the control *URE2*–GFP, ~30% of the cells presented a diffuse expression of GFP and ~70% showed either one big spot or several small green spots, localized in the cytoplasm, either in the CC30 [USA$^-$] strain (Figure 3D, insets) or in the CC34 [*URE3*] strain (Figure 3E). However, the CC34 [*URE3*] strain transformed with the *h2*–GFP construct

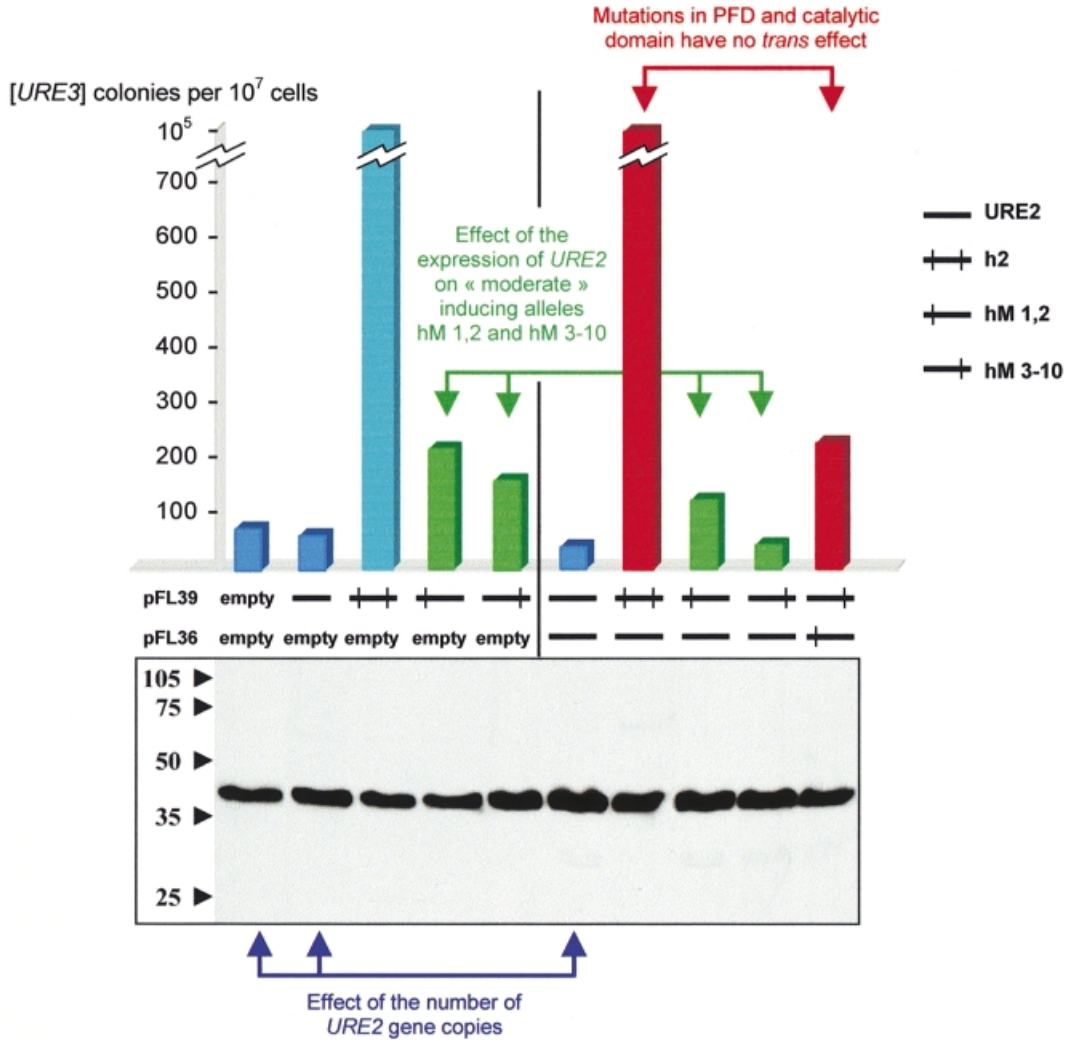

Fig. 2. *Cis* and *trans* effect of the co-expression of different mutated alleles of *URE2*. The CC30 strain was co-transformed with different plasmids based on pFL39 and pFL36 leading to the expression of *URE2*; *h2*; *hM 1,2*; *hM 3-10*. The 10 different combinations were analyzed for their inducing properties by plating 10^7 cells on USA-selective medium. Standard deviation is not >10%. Bottom, the expression levels of Ure2p in each co-transformed strain were compared by western blot analysis. Numbers indicate the molecular weights (in kilodaltons).

presented a diffuse color in ~10% of the cells and a green spot in 40% of the cells (Figure 3F). In addition, 50% of these cells presented a new coloration pattern, with some long cytoplasmic filiform structures that sometimes branched out (Figure 3F, insets). Furthermore, these aggregation patterns were observed regardless of the [*URE3*] phenotype in both the CC30 and CC34 strains.

Discussion

The molecular and biochemical ways in which the [*URE3*] phenotype is generated from the Ure2 protein remain unclear. The prion hypothesis postulates that [*URE3*] is the product of an autocatalytic inactivation of Ure2p. This inactivation implies a Ure2p–Ure2p interaction, leading to the inactivation of this protein. As a consequence, USA can be imported into the cell, leading to a [USA$^+$] phenotype. However, this [USA$^+$] phenotype can also be due to classical mutations affecting the *URE2* gene. In this case, the [USA$^+$] phenotype would segregate as expected for a nuclear mutation. Only the segregation of the [USA$^+$] phenotype or its curability allows the prion state to be distinguished from mutations that affect Ure2p expression. A third case may also exist that could lead to the same [USA$^+$] phenotype but with distinct genetic properties. This case may be found upon expression of a Ure2p 'inhibitor'. Such a molecule would inhibit Ure2p, thus permitting the entry of USA. The [USA$^+$] phenotype would only exist, however, while this inhibitor was expressed. After the loss of such an inhibitor, Ure2p should regain activity and give rise to the [USA$^-$] phenotype. This kind of inactivation would not correspond to an autocatalytic process and such a mechanism, even if it involves the PFD as the inhibitor molecule, could not correspond to [*URE3*]. Both [USA$^+$] clones obtained by overexpression of the PFD or the *h2* allele in our experiment were therefore analyzed after the loss of the inducing construct. Surprisingly, the PFD can inactivate Ure2p when expressed in these strains, but this inactivation proved to be transitory, given that no [USA$^+$] clone retained that phenotype after the loss of the plasmid. In contrast, at least in the genetic background of our strains,

Table III. Number of [*URE3*] clones per 10^6 cells on USA medium

GFP fusion construct	Control	pYeFc1L *URE2*–GFP	pYeFc1L *h2*–GFP	pYeFc2L *URE2*–GFP	pYeFc2L *h2*–GFP
CC30	39	60	129	50	~1000
CC34	[*URE3*]	[*URE3*]	[*URE3*]	cured	[*URE3*]

[*URE3*] indicates that all cells were able to grow on USA medium, showing that the [*URE3*] phenotype of the CC34 strain was not affected by the expression of the different constructs. Only the multicopy expression of the *URE2*–GFP fusion cured the CC34 strain, which is consistent with what was previously reported.

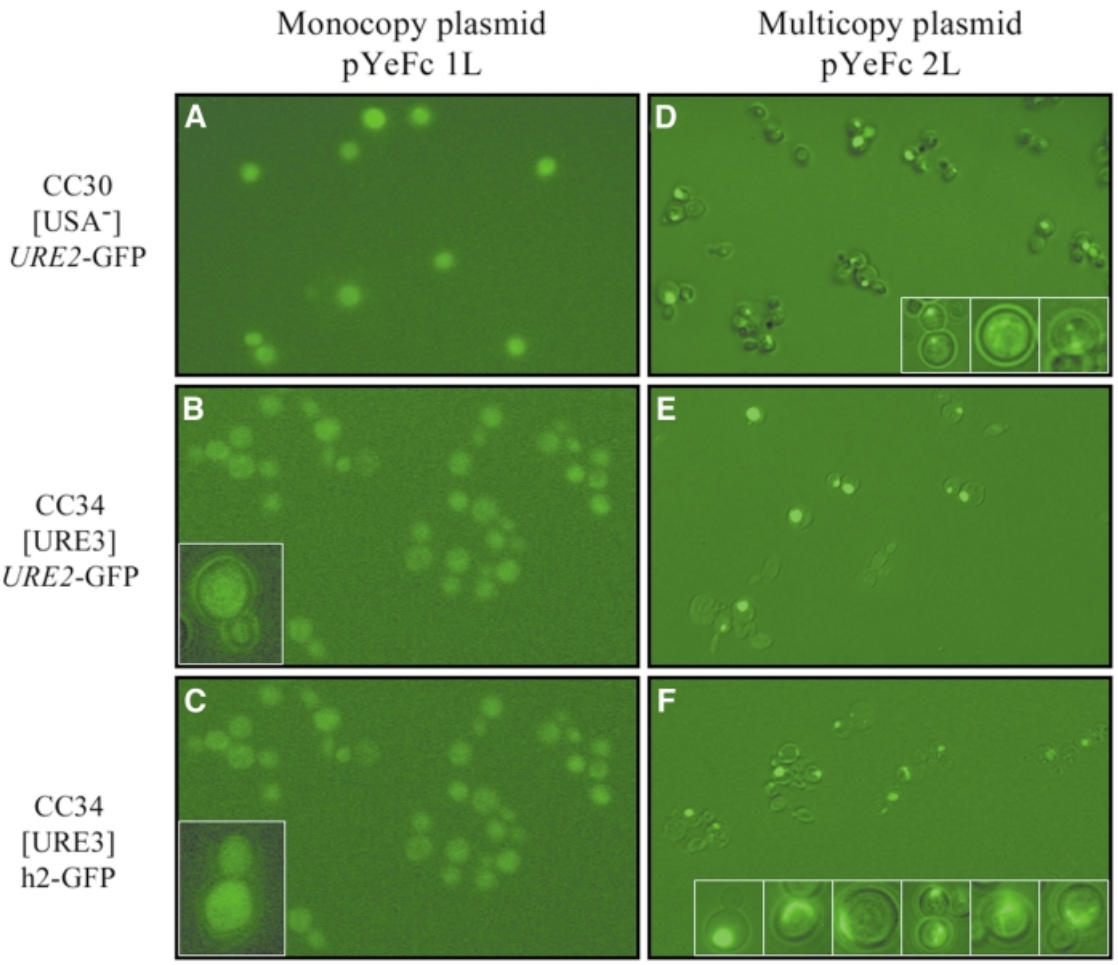

Fig. 3. Microscopy observation of CC30 and CC34 strains transformed by monocopy and multicopy plasmids expressing *URE2*–GFP and *h2*–GFP. (**A** and **D**) Cellular distribution of *URE2*–GFP in the CC30 [USA⁻] strain expressed on a monocopy and a multicopy plasmid, respectively. (**B** and **E**) Cellular distribution of *URE2*–GFP in the CC34 [URE3] strain expressed on a monocopy and a multicopy plasmid, respectively. (**C** and **F**) Cellular distribution of *h2*–GFP in the CC34 [URE3] strain expressed on a monocopy and a multicopy plasmid, respectively. Insets in (B) and (C) show the diffuse pattern exhibited by the expression on a monocopy plasmid of the wild-type and mutant *URE2* gene, whatever the [URE3] or [USA⁻] phenotype. Insets in (D) show the spots and clumps typically observed by expressing the wild-type *URE2* gene on a multicopy plasmid, and insets in (F) show the typical filiform and ramified structures observed by expressing the *h2* allele on a multicopy plasmid.

the *h2* element can promote an inactivation phenotype, [USA⁺], which can be maintained even after the loss of the inducing element. Indeed, *h2* efficiently promotes the conversion of Ure2p to its prion form $Ure2p^{[URE3]}$. Thus, several ways to inactivate the Ure2 protein exist; some are connected to the prion mechanisms, which are persistent, and others are transitory inactivation mechanisms.

The protein encoded by the *h2* allele of *URE2* differs from the wild-type gene by 10 mutations located both in the PFD and the catalytic domain of the protein. By shuffling sequences between wild-type and mutated genes, we generated several new *URE2* alleles including one that both encodes a functional Ure2p and induces [*URE3*]. This allele is particularly exciting as all the inducing proteins so far reported can not fulfill the functional criteria. These previous constructs may act by destabilizing Ure2p through an artificial mechanism that would never occur in the yeast cell. In contrast, the functional inducing allele that we generated mimics the mechanism behind [*URE3*] formation. The balance between functional $Ure2p^{C}$ and

Ure2p$^{[URE3]}$ in this case merely favors Ure2p$^{[URE3]}$. The existence of this allele emphasizes the 'protein only' model for [*URE3*] formation.

To dissect the role of the mutations in *h2*, the functional inducing allele (*hM 1,2*) and its counterpart (*hM 3-10*) obtained by the complementary shuffling were co-expressed. Co-expression of the *hM 1,2* and *hM 3-10* alleles induces the [*URE3*] phenotype at a level similar to that observed with the expression of each allele alone. Thus, there is no *trans* synergistic effect of the mutated domains of *URE2*, whereas this effect is maximal when the two mutated domains are carried by the same molecule. These data suggest that mutations of the H2 protein modify the intramolecular interactions necessary for its folding. Co-expression in yeast of various inducing constructs also generated additional data (Figure 2): when expressed on a monocopy plasmid, the *URE2* gene did not induce [*URE3*], as it does when overexpressed. On the contrary, it inhibited [URE3] appearance, and the inhibition effect was proportional to the number of *URE2* gene copies, at least from one to three. This result suggests that the catalytic domain of Ure2p, known for its inhibiting properties on the PFD, exceeds the effect of the PFD when the number of copies of the *URE2* gene is low. This inhibitory effect, however, appears to be overcome by overexpression of the PFD on a multicopy plasmid. This inhibitory effect of the catalytic domain is also observed on the weaker inducing alleles *hM 1,2* and *hM 3-10*.

To analyze further the role of each mutation in the *h2* allele, we re-introduced these 10 mutations into *URE2* as single mutations. Although some of them abolish the function of Ure2p, they do not behave as [*URE3*] inducers. The inducing effect of *h2*, therefore, does not appear to be the consequence of the loss of function of Ure2p. Genetic analysis of *URE2* alleles carrying two of the *h2* mutations showed that two pairs of mutations had an inducing effect on [*URE3*] formation. One of these pairs (*hM 2,3*) abolishes the catalytic function of *URE2*, whereas the other one (*hM 1,8*) preserves it. In both cases, the changes occurring in the catalytic domain are important since an acidic amino acid replaces a neutral or a basic amino acid. The more drastic change, E127K, by itself provokes the loss of *URE2* function. In the PFD (which is dispensable for the catalytic function of Ure2p), the changes (S10L and R17C) involve two basic amino acids. The synergistic effect could be due to intramolecular interactions involving electrostatic forces.

Wickner has proposed that the mechanism responsible for the formation of the prion form relies on a polymerization of the normal protein into filaments that lead to a conformational change of the protein and then its loss of function (Edskes *et al*., 1999). In the case of the [*PSI*] determinant, many arguments support this hypothesis: the crystal form of Sup35 protein is indeed able to convert the normal form of Sup35p into a prion form efficiently. Moreover, the formation of Sup35p fibrils is strictly related to the appearance of the prion phenotype. However, in the case of the mammal prion protein PrP, no data show any absolute connection between fibrils and the converted resistant form PrPSc. In the genetic background of our yeast strains, we observed a similar phenomenon.

We found, while expressing the fusion *URE2*–GFP in our strains, that there is no strict connection between the existence of aggregates and the [*URE3*] phenotype (E.Guillemet, E.Fernandez-Bellot, C.Thual and C.Cullin, submitted). We also observed that some factors that increase the appearance of [*URE3*] also stimulate aggregate formation. The results presented here indicate that aggregate formation is not related to phenotype but to *URE2* expression level; there is no correlation between the frequency of [*URE3*] induction and the frequency of cells presenting aggregates. When the inducing allele *h2* is expressed under the control of the *URE2* promoter, the number of cells presenting aggregates is not significantly greater than when the wild-type *URE2* gene is expressed. Moreover, in the [USA$^+$] clones, not all the cells present aggregates as would be expected.

The nature of the structures observed *in vivo* remains to be characterized. If the [*URE3*] phenotype corresponds to a certain aggregation level, the expression of GFP fusion proteins demonstrated that this level is not observable by optical microscopy. Moreover, the spots observed when Ure2–GFP or H2–GFP are expressed on a multicopy plasmid, if they are correlated with an increase in [*URE3*], are not directly dependent on [*URE3*] formation. It is possible that various levels of aggregation *in vivo*, based on what we previously observed *in vitro* (Thual *et al*., 1999), correspond to various partially or completely inactive states of protein, and that these states may or may not be autocatalytic.

Materials and methods

Construction of a mutant URE2 library and the selection of prion-inducing alleles

The *URE2* gene, carried on a pFL39 plasmid, was amplified by PCR in the presence of 200 μM each of dGTP, dTTP and dCTP, and several concentrations of dATP ranging from 1 to 50 μM, using *Taq* DNA Polymerase (Promega). When amplification efficiency was poor, the corresponding PCR product was cloned by gap-repair into the pFL39 plasmid.

Construction of hM 1,2 and hM 3-10 alleles

The 5′ sequences corresponding to the PFD of the *h2* allele were used to replace the wild-type sequences by digesting the pFL39-*h2* and pFL39-*URE2* plasmids with *Bsi*WI and *Not*I. The eluted pFL39 vector containing the wild-type 3′ sequences of the *URE2* gene was then ligated with a fragment containing the *h2* 5′ sequences to give the pFL39 *hM 1,2* vector, and reciprocally the eluted pFL39 vector containing the 3′ region of the *h2* gene was ligated with a fragment containing wild-type 5′ sequences to generate the pFL39 *hM 3-10* vector.

Construction of pFL39-hM 1,2,3

The pFL39-*hM 1,2,3* vector, containing the first three mutations of the *h2* allele, was constructed by digesting pFL39-*h2* with *Xho*I and *Apa*I. The 0.9 kb fragment containing the mutated 5′ region of the *h2* gene was ligated into pFL39-*URE2* digested with *Xho*I and *Apa*I.

Construction of pFL vectors with leucine as the selectable marker

The pFL39 vectors containing different constructs were cut by *Bgl*II to remove the selectable marker tryptophan. A *Bgl*II–*Bgl*II fragment containing the *LEU2* gene was then inserted to give the pFL36 vectors containing the different *URE2* alleles.

Expression levels of Ure2p in co-transformed strains

Yeast cultures were grown on a solid medium selective for plasmid markers. The cells were harvested, washed in water, and lysed by vortexing with glass beads in 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). Cell debris was removed by centrifugation at 15 000 *g* for 15 min. Total protein extract (50 μg) of each lysate was deposed on an SDS–polyacrylamide gel, according to standard procedures. After transfer from the gel to

nitrocellulose membrane (Schleicher and Schuell), the blot was probed with a polyclonal rabbit antibody against Ure2p. Coomassie Blue staining was also performed to confirm that an equal amount of protein was loaded in the lanes.

Directed mutagenesis of the URE2 gene

The different mutations of the *h2* allele were re-inserted into the *URE2* gene by PCR-directed mutagenesis, using Advantaq DNA polymerase (Clontech). The *URE2* gene was amplified with the ure2Xho primer (5′-GCTTCCTTACTCGAGGTTG-3′) and the hMxR primers (x corresponding to the number of the mutation; hM1R: 5′-GGAATTCAACACTTGGTTG-3′; hM2R: 5′-GTTTACTTGACAGAGCGCATTGG-3′; hM3R: 5′-CTATAGCAACTTCGAATCCATTAG-3′; hM4R: 5′-GACGTTTGGAGGAACAACCATG-3′; hM5R: 5′-CTTGTCCAATCTTTGGCGCATG-3′; hM6R: 5′-CTTTTGTGAACGGAAGTATCTG-3′; hM7R: 5′-AACCT-CATCCGAATATCTTTCTA-3′; hM8R: 5′-CTAATTCCATCTCCAGCGCTTC-3′; hM9R: 5′-GTCTAATTCCAACACCAGCGCT-3′; hM10R: 5′-ACCAGCTGAGCATGCAGCCGC-3′), and with the ure2Nco primer (5′-CATTATTCCATGGGACAAAGGCC-3′) and the hMxD primers (hM1D: 5′-CAACCAAGTGTTGAATCTCTC-C-3′; hM2D: 5′-CCAATGCGCTCTGTCAAGTAAAC-3′; hM3D: 5′-CTAATGGATTCGAAGTTGCTATAG-3′; hM4D: 5′-CATGGTTGTTCCTCCAAACGTC-3′; hM5D: 5′-CATGCGCCAAAGATTGGACAAG-3′; hM6D: 5′-CAGATACTTCCGTTCACAAAAG-3′; hM7D: 5′-TAGAAAGATATTCGGATGAGGTT-3′; hM8D: 5′-GAAGCGCTGGAGATGGAATTAG-3′; hM9D: 5′-AGCGCTGGTGTTGGAATTAGAC-3′; hM10D: 5′-GCGGCTGCATGCTCAGCTGGT-3′). The amplification products carrying the same mutation were mixed and re-amplified with the ure2Xho and ure2Nco primers to reconstitute a whole *URE2* gene carrying a single mutation. Alleles carrying two mutations were constructed in the same way.

Strains, media and complementation tests

CC30 strain: *MAT***a**, *trp1-1, ade2-1, leu2-3,112, his3-11,15,* Δ*URA2::HIS3*. CC34 strain: *MAT***a**, *trp1-1, ade2-1, leu2-3,112, his3-11,15,* Δ*URA2::HIS3*, [*URE3*]. The CC34 strain carries the [*URE3*] element described originally by Aigle and Lacroute (1975), which was transmitted to the CC30 strain by cytoduction. Functional complementation tests were carried out with the AF36 strain: *MAT***a**; *trp1-1*; *ade2-1*; *leu2-3,112*; *his3-15,15*; *URA2::HIS3*; *cyh2*^{r}; *URE2::CYH2*. Transformations were carried out as previously described (Gietz *et al*., 1995). Complete medium (YPGA) was prepared with 1% bacto-yeast extract, 2% bacto-peptone, 2% glucose, 2.5% bacto-agar, 20 mg/ml adenine. Minimal medium (W0) was prepared with 0.7% nitrogen base without amino acids (Difco), 2% glucose, 2.5% agar, supplemented with amino acids. [URE3] selection is based on the USA uptake in the *ura2*$^-$ strain, caused by the inactivation of Ure2p after its conversion to the prion form. [*URE3*] colonies may then grow on a minimal medium supplemented with appropriate amino acids, except uracil, and 15 mg/l USA, as previously described (Lacroute, 1971).

Construction of h2–GFP fusions

The pYeFc 1L-*URE2*–GFP and pYeFc 2L-*URE2*–GFP plasmids were constructed by digesting pYeFc 1L-V10-*URE2*–GFP and pYeFc 2L-V10-*URE2*–GFP vectors with *Bam*HI and *Pvu*II to remove the V10 (PGK) promoter and the first 850 nucleotides of the *URE2* gene. A fragment containing the *URE2* promoter and the first 850 nucleotides of the *URE2* gene was amplified by PCR with primers 208 (5′-CCGGCGCGGATCCCTACCGTCCTCTATGTCTCC-3′) and 209 (5′-GTGTTGTACCAGCTGAGTATGCAGCCGC-3′), with plasmid pFL39-*URE2* as the template. After digesting this fragment with *Bam*HI and *Pvu*II, it was re-ligated with the digested pYeFc *URE2*–GFP plasmids to give the pYeFc 1L-*URE2*–GFP and pYeFc 2L-*URE2*–GFP plasmids. Plasmids pYeFc 1L-*h2*–GFP and pYeFc 2L-*h2*–GFP were constructed in the same way by amplifying the *h2* allele under the control of the *URE2* promoter with pFL39-*h2* as the template.

Microscopy observations

Cells were grown overnight on selective medium and were suspended in a 50 µl Dabco solution [218 mM diazabicyclo-2-2-2-octane (Sigma), 25% phosphate-buffered saline, 75% glycerol]. Cells were photographed using a DMRB microscope (Leica, Germany) with a PL APO 63× objective.

Acknowledgements

We gratefully thank Professor François Lacroute for constructing the mutagenized library and his helpful suggestions, and Ranjiv Khush for critical reading of the manuscript. The work was supported by grants from Action Concertée Coordonnée Science du Vivant No. 10 (9510001) to C.C. and an EC Contract No. BI104-98-6045, Maintenance and transmission of yeast prions: a model system.

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Received March 24, 2000; revised and accepted May 12, 2000