Characterization of the interaction domains of Ure2p, a prion-like protein of yeast

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In the yeast *Saccharomyces cerevisiae*, the non-Mendelian inherited genetic element [*URE3*] behaves as a prion. A hypothesis has been put forward which states that [*URE3*] arises spontaneously from its cellular isoform Ure2p (the product of the *URE2* gene), and propagates through interactions of the N-terminal domain of the protein, thus leading to its aggregation and loss of function. In the present study, various N- and C-terminal deletion mutants of Ure2p were constructed and their cross-interactions were tested *in vitro* and *in vivo* using affinity binding and a two-hybrid analysis. We show that the self-interaction of the protein is mediated by at least two domains, corresponding to the first third of the protein (the so-called prion-forming domain) and the C-terminal catalytic domain.

Key words: binding assay, nitrogen starvation, non-Mendelian inheritance, *Saccharomyces cerevisiae*, two-hybrid system.

INTRODUCTION

It is well known that mutations in DNA can lead to phenotypes that are generally inherited according to Mendelian rules, but which are sometimes inherited differently. The study of non-Mendelian transmission has provided several lines of evidence for non-nuclear genetic determinants, such mitochondrial DNA, chloroplast DNA, viruses and plasmids. In yeast, the study of two non-Mendelian inherited phenotypes, [*URE3*] and [*PSI*$^+$], has shown that they were not explained by any of the known nucleic acids, either nuclear or non-nuclear. The yeast elements [*PSI*$^+$] and [*URE3*], discovered by Cox [1,2] and Lacroute and Aigle [3,4], correspond to two distinct phenotypes (the brackets indicate a phenotype in yeast nomenclature, and the capital letters indicate a dominant one). [*PSI*$^+$] enhances the suppression of nonsense codon [1,2,5] and [*URE3*] provokes a de-regulation of nitrogen catabolic repression [3,4]. Wickner and colleagues postulated that these two phenotypes ([*URE3*] and [*PSI*$^+$]) were inherited through a prion mechanism [6–8]. In mammals, the prion particle is correlated with an infectious agent causing transmissible spongiform encephalopathies [9]. This unconventional agent is presumed to be constituted mainly, if not entirely, by a particular protein, PrP (prion protein; [10,11]), encoded by the genome of the cell [12]. This protein is thought to become infectious by a conformational switch [13]. Once completed, this conformational switch is propagated by interactions between the normal PrP (PrP^{C}) and the misfolded one (PrP^{Sc}) [14]. The chain reaction which follows will thus cause the complete change of PrP from normal to pathogen form, which accumulates inside the neurone and finally kills it [15]. *Saccharomyces cerevisiae* is not subjected to an infectious process, but does present similar mechanisms with Sup35p (leading to [*PSI*$^+$]) and Ure2p (leading to [*URE3*]).

We present here the results of a two-hybrid analysis and affinity-binding experiments, using Ure2p as both bait and prey.

MATERIALS AND METHODS

Construction of two-hybrid vectors

The plasmids pAS2 and pACT3 were described previously [16]. pACT3-*URE2* was constructed by inserting a *Bam*HI/*Eco*RI fragment containing the open reading frame (ORF) of the *URE2* gene in frame with the activating domain of Gal4p. pAS2-*URE2* was constructed by digesting pACT3-*URE2* by *Xho*I and partially by *Nco*I. The fragment containing the ORF of the *URE2* gene was then ligated into pAS2 digested by *Nco*I and *Sal*I to generate pAS2-*URE2*. pEX plasmid was constructed as follows: pAS2 was digested by *Xba*I and *Eco*RV. The 4.5 kb fragment was then eluted, filled in with T4 DNA polymerase, and religated. The same strategy was used to generate pEX-*URE2* from pAS2-*URE2*.

N- and C-terminal deletions were constructed as follows. pEX-*URE2* was digested with appropriate restriction enzymes: ΔC (97–354), *Pml*I and *Bsu*36I; ΔC (152–354), *Apa*I and *Bsu*36I; ΔC (226–354), *Pfl*MI and *Bsu*36I; ΔN (1–63), *Not*I; ΔN (1–96), *Not*I and *Pml*I; and ΔN (1–152), *Not*I and *Apa*I. The linearized vector was treated with T4 DNA polymerase to form blunt ends and religated with T4 DNA ligase. pAS2-Δ*URE2* and pACT3-Δ*URE2* were constructed by gap-repair: the yeast strain Y190 was co-transformed with 1 μg of pAS2 or pACT3 linearized by *Eco*RI and with 1 μg of pEX-Δ*URE2* linearized by *Xmn*I. Yeast transformants were selected on medium lacking tryptophan. Finally, the plasmids were recovered in *Escherichia coli* XL1blue and verified by sequencing.

Construction of *in vitro* transcription vectors

ΔC (97–354)–*URE2*: the previously described [16a] N-terminal (6 × His)-influenza haemagglutinin (HA; YPYPVDYA) epitope–*URE2* fusion gene was inserted into the vector pBluescript II KS$^-$ and was thus under control of the T7 promoter. A C-terminal deletion of the *URE2* gene was constructed by digesting

Abbreviations used: PrP, prion protein; PFD, prion-forming domain; ORF, open reading frame; HA, influenza haemagglutinin.

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Table 1 Interaction between Ure2p and various Ure2p deletion mutants in the two-hybrid system

Quantitative β-galactosidase assays were done in two independent transformations. Activity was calculated in units of β-galactosidase activity per mg of protein in whole-cell extract. Values are expressed as a percentage of the activity measured in yeast transformed by pAS–*URE2* and pACT3–*URE2*. Standard error was less than 20%. ND, not determined.

		β-Galactosidase activity							
Prey	Bait ...	pAS2	*URE2*	ΔC (97–354)	ΔC (152–354)	ΔC (226–354)	ΔN (1–63)	ΔN (1–96)	ΔN (1–152)
pACT3		4.4	63	7.9	2	2.1	40	61	14
URE2		2.3	100	22	15	8.3	118	88	13
ΔN (1–152)		1.2	121	0.8	68	3	44	28	12
ΔC (152–354)		6	57	2	26	ND	17	28	75

pBluescript-*URE2* by *Pml*I and *Bsu*36I. The linearized vector was treated by T4 DNA polymerase to form blunt ends, and then religated with T4 DNA ligase.

To express the full-length protein Ure2p, the *URE2* coding sequence was inserted into the vector pBluescript II SK$^+$, under transcriptional control of the T7 promoter.

Yeast procedures

The Y190 strain (*MATa*; *GAL4*Δ; *GAL80*Δ; *ade2–101*; *his3*-Δ200; *leu2–3,112*; *trp1–901*; *ura3–52*; *URA3*::UAS-*GAL1-lacZ*; *LYS2*::*GAL1-HIS3*; CYH^R) was used to test the different interactions. Transformation of yeast cells was carried out as previously described [17]. The co-transformations were performed as indicated in Table 1.

The enzymic activities were determined as follows: yeast transformants were collected after a 4-day incubation directly from selective plates and harvested by centrifugation at 3000 ***g*** for 5 min at 4 °C. Cells were resuspended in 300 μl of ice-cold Z buffer (0.06 M $Na_2HPO_4 \cdot 7H_2O$, pH 7/0.04 M $NaH_2PO_4 \cdot H_2O$/0.01 M KCl/0.001 M $MgCl_2$/1 mM PMSF), and vortexed in presence of 150 μl of chilled glass beads (0.45 mm diameter), with two cycles of 1 min of blending and 1 min of cooling in ice. The extracts were then centrifuged at 12000 ***g*** for 10 min at 4 °C and supernatants (crude extract) were collected. Supernatant (5 μl) was used to quantify the proteins by a bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.).

β-Galactosidase assays were performed as follows: 200 μl of crude extract was added to 800 μl of buffer Z containing the substrate *O*-nitrophenyl-β-D-galactopyranoside, 4 mg/ml. After 0, 10, 30 and 120 min of incubation at 37 °C, 200 μl of each reaction was simultaneously stopped by adding 800 μl of 1 M Na_2CO_3. After measurement of the absorbance at 420 nm, β-galactosidase activity was calculated by linear regression. A β-galactosidase unit was defined as an A_{420} unit/min of reaction per μg of protein of total extract. The final results are represented as percentage of the activation given by the complete *URE2* ORF in the respective plasmids.

In vitro transcription/translation procedures

Full-length untagged Ure2p and ΔC (97–354)–(6 × His)-tagged Ure2p were synthesized *in vitro* using the Riboprobe® Transcription Systems and the wheat-germ extract (Promega, Madison, WI, U.S.A.). The final concentrations of Ure2p mRNA and ΔC (97–354)–Ure2p mRNA were about 160 μg/ml. The translation was carried out at 25 °C for 1 h in the presence of [^{35}S]methionine (15 mCi/ml; Amersham, Les Ulis, France), as described by the *Promega Technical Manual*. The final concentration of each protein was about 0.25–0.4 ng in 50 μl of translation mixture. Translation mixtures were put on ice for 10 min to stop the reaction.

Affinity-binding procedure

The ΔC (97–354)–(6 × His)-tagged Ure2p translation mixture (35 μl) was incubated with 20 μl of the full-length protein (equimolar amounts) and incubated for 1.5 h at 30 °C. In a control, 20 μl of the full-length Ure2p was incubated with 35 μl of the wheat germ extract.

After that, 55 μl of 2 × concentrated Na-phosphate buffer (100 mM NaH_2PO_4/600 mM NaCl/20mM imidazole) was added, and the mixture was laid on a Ni-NTA (nickel nitriloacetic acid) Spin Column (Qiagen, Courtaboeuf, France). The column was equilibrated with 400 μl of a 50 mM NaH_2PO_4 buffer, pH 4.8, containing 300 mM NaCl and 10 mM imidazole (Sigma, St. Louis, MO, U.S.A.). Centrifugations were performed for 3 min at 380 ***g***. Each sample was applied to a column. Then the column was washed with 200 μl of a 50 mM NaH_2PO_4 buffer, pH 4.8, containing 300 mM NaCl and 50 mM imidazole. Elution was performed in presence of 200 μl of a 50 mM NaH_2PO_4 buffer, pH 4.8, containing 300 mM NaCl and 250 mM imidazole. Sample aliquots were mixed with 50 mM Tris/HCl electrophoresis sample buffer, pH 6.8, containing 4% (w/v) SDS/2% (v/v) β-mercaptoethanol/12% (w/v) glycerol/0.01% Serva Blue G, and immediately incubated at 80 °C for 10 min. The samples were analysed by PAGE according to [18] in a 16.5% T, 6% C gel, overlaid with a 4% T, 3% C stacking gel. Rainbow ^{14}C-labelled methylated coloured proteins (Amersham) were used as molecular-mass markers. Gels were fixed, dried under vacuum and subjected to autoradiography using a Molecular Dynamics PhosphorImager and ImageQuant software.

RESULTS

Ure2p acts as an activator able to interact with itself

After a transformation of the yeast strain Y190 by pAS2-*URE2* alone (the bait), the transformants were collected. These yeast cells express a chimaeric protein that contains the binding domain of GAL4p and Ure2p. The possible effects of Ure2p on the transcription machinery can then be measured by the activation of a specially designed reporter gene that carrys the binding site for the DNA binding partner. For the strain used in our study, the reporter gene was β-galactosidase. A qualitative test showed a strong activation, since a deep blue colour due to the β-galactosidase appeared after 10 min. This result could be

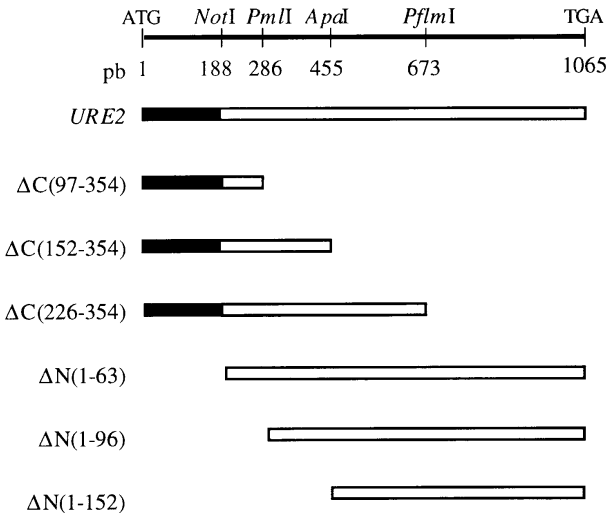

Figure 1 Deletion mutants fused to the galactose DNA-binding domain in the pAS2 plasmid or the galactose-activating domain in the pACT3 plasmid

The *URE2* sequence is shown with the PFD as a solid line. Restriction sites and the positions used to construct the deletion mutants are indicated.

due to a real transcriptional activation function for Ure2p and would make any screening procedure completely inefficient, due to the high background. When transformed with both pAS2-*URE2* and pACT3-*URE2* (the prey), the yeast cells produced a larger amount of β-galactosidase, as the same blue colour appeared after only 5 min. This result suggested that Ure2p interacting with itself could then increase the transcription of the reporter gene. To investigate this possibility we measured the level of β-galactosidase produced when the yeast cells were transformed with pAS2-*URE2* and pACT3 or with pAS2-*URE2* and pACT3-*URE2*. In this latter case, the β-galactosidase specific activity was two-fold higher than in the former case (results not shown). This higher amount of β-galactosidase in the cells transformed with both pAS2-*URE2* and pACT3-*URE2* compared with cells containing only Ure2p as bait indicated the existence of an interaction between Ure2p and itself.

A popular approach with the two-hybrid system uses the construction in pAS2 vector as bait in a screening procedure. Unfortunately, the basal level of transcription due to the original *URE2* construction made a screening procedure with a yeast genomic bank hazardous. Moreover, the previous construction did not permit the characterization of interacting domains. We thus decided to test the transcriptional activation of a collection of baits corresponding to different deletions of Ure2p.

The catalytic domain of Ure2p corresponds to an activating domain

Ure2p contains two domains (see Figure 1) [19]. The first one, corresponding to the first 25% of the protein, possesses the major 'prion' properties. This part of the sequence is strictly required for the appearance of [*URE3*] and by itself is sufficient to induce it. It has been shown recently that this domain alone allows the maintenance of [*URE3*] [20]. The rest of the protein corresponds to the catalytic domain, which represses Gln3p in the presence of a rich nitrogen source [21,22]. This domain retains all the properties of the full-length protein except the ability to present the so-called prion phenotype [*URE3*]. We made three N-terminal deletions of Ure2p that eliminate the first 63, 96 or 152 residues for the constructions ΔN (1–63), ΔN (1–96) and ΔN (1–152) respectively, and three C-terminal deletions that retain the first 96, 151 and 225 residues of Ure2p for the constructions ΔC (97–354), ΔC (152–354) and ΔC (226–354) respectively. These deletions, represented in Figure 1, are all in frame with the binding domain of Gal4p.

When expressed alone, all the ΔC–Ure2p–Gal4p constructions exhibited a very low level of β-galactosidase activity. However, yeast cells transformed by the hybrid Ure2p–Gal4p or ΔN–Ure2p–Gal4p (see Table 1, column 1) presented significant β-galactosidase activity. These results suggest a correlation between the catalytic domain of Ure2p and the transactivating activity detected in this study.

In order to determine if these deletions retained the ability to interact with Ure2p, we co-transformed yeast cells with each construction and with pACT3-*URE2*, and the level of β-galactosidase activity thus obtained was measured.

The prion domain interacts with Ure2p

Each deletion was tested for its ability to activate the transcription of the β-galactosidase reporter gene when expressed in yeast cells containing Ure2p in frame with the binding domain, and also in yeast cells containing only the binding domain. The strain Y190 was co-transformed by the different combinations of plasmids. After selection of the double-transformed yeast cells, they were broken and the amount of β-galactosidase was determined. The results obtained (Table 1, columns 1 and 2) are expressed as a percentage of the activity measured in yeast transformed by pAS2-*URE2* and pACT3-*URE2*.

In the same conditions, ΔN (1–152) and ΔN (1–96) displayed no significant differences in transactivating activities when expressed with Ure2p in frame with the activating domain or with the activating domain alone (88 versus 61, and 13 versus 14, respectively; these are arbitrary units, see Table 1).

On the other hand, the presence of Ure2p in frame with the binding domain dramatically increased the amount of β-galactosidase measured for the three ΔC deletions. The longest deletion, ΔC (97–354), which retained only the first 96 amino acids of Ure2p, exhibited a three-fold higher β-galactosidase activity in the presence of Ure2p in frame with the activating domain of Gal4p (22 versus 7.9). This increase clearly indicates an interaction between the first third of the protein that contains all the prion domain and Ure2p. Interestingly, ΔN (1–63) also exhibited a higher β-galactosidase activity when expressed with Ure2p fused in frame with the activating domain (118 versus 40).

In order to confirm the result observed with the two-hybrid system, we decided to test the putative interaction by affinity binding of *in vitro* translated proteins.

Interacting domains can be monitored by affinity binding

Full-length untagged Ure2p and truncated ΔC (97–354)–(6 × His)-tagged Ure2p were expressed using the wheat germ extract cell-free translation system. Equimolar amounts were mixed, and this mix was loaded on to a Ni-NTA column (see Figure 2). As a control, the full-length untagged protein was loaded on to a similar column in the same conditions. In both cases, the columns were washed with a 50 mM imidazole buffer and eluted with a 250 mM imidazole buffer. In the control, Ure2p was not detectable in the eluted fraction (Figure 2, Control, lane E). In contrast, a significant amount of the untagged Ure2p was co-eluted with the truncated ΔC (97–354)–(6 × His)-tagged Ure2p (Figure 2, Mix, lane E). The presence of the full-length Ure2p in this fraction is evidently due to an interaction between both polypeptides.

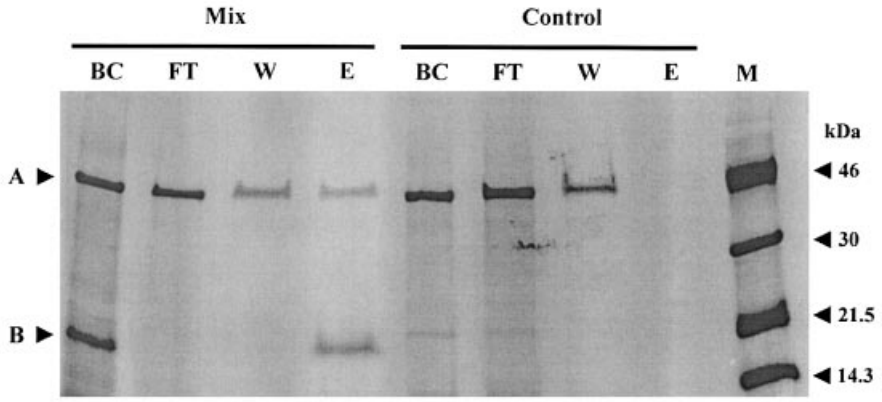

Figure 2 Autoradiogram of SDS gel electrophoresis of the full-length untagged Ure2p mRNA (A) and ΔC (97–354)–(6 × His)-tagged Ure2p mRNA (B) cell-free translation products

Mix: both full-length Ure2p and ΔC (97–354)–Ure2p were equimolarly mixed together. BC, mix before purification; FT, flow through the column; W, wash with 50 mM imidazole; E, elution with 250 mM imidazole. The molecular ratio A/B in lane E was 1/9. Control: the full-length Ure2p alone was used as control. Note: the ΔC (97–354)–Ure2p had an apparent molecular mass higher than that expected from its sequence (about 8 kDa), because of its unusual amino acid composition. Similar migration abnormalities have been described for another protein [30]. The autoradiogram was digitally processed using a Molecular Dynamics PhosphorImager and ImageQuant software.

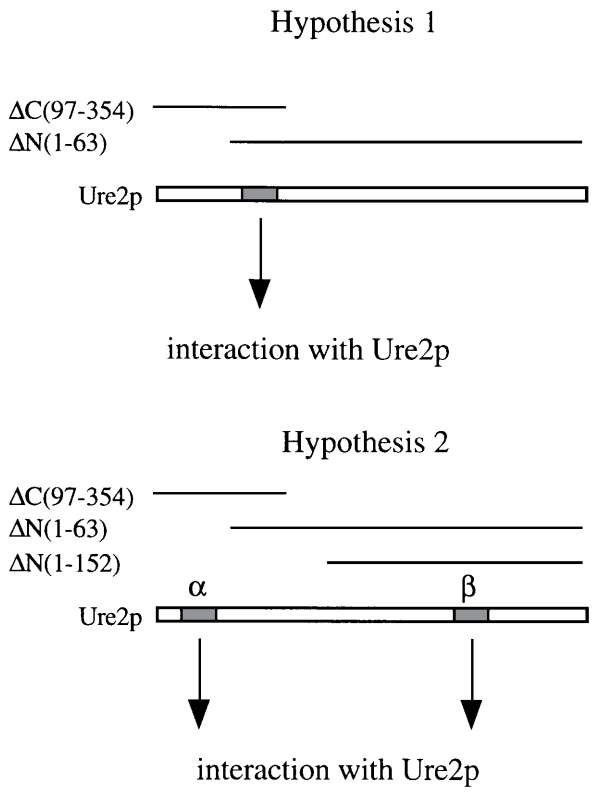

Figure 3 The two hypotheses of interacting domains of Ure2p

Hypothesis 1: there is one interacting domain (grey box), common to the ΔC (97–354) and ΔN (1–63) truncated proteins, as these constructions are both able to interact with Ure2p in the two-hybrid system. Hypothesis 2: Ure2p contains two interacting domains (grey boxes), α and β.

This result confirms the interaction previously detected between the prion-forming domain (PFD) of Ure2p and Ure2p. This interaction could be due either to a unique domain located in the PFD or alternatively to the presence of two different domains. To distinguish between these two hypotheses we decided to perform further two-hybrid experiments.

Ure2p contains at least two interacting domains

ΔN (1–63) and ΔC (97–354), which both interact with Ure2p, share a common part of *URE2* that could be considered as the sole interacting domain of Ure2p (see Figure 3, hypothesis 1). Alternatively, one can postulate two interacting domains (see Figure 3, hypothesis 2). The first one (α domain) would be located in the first third of Ure2p and would be present in ΔC (97–354), and the second one (β domain) located in the rest of the molecule, thus present in ΔN (1–63).

Both ΔN (1–152) and ΔN (1–96), which include the putative β domain but not the α domain, do not interact with Ure2p. This result argues against the second hypothesis but may also be due to a particular folding of chimaerical proteins coded by pAS2-ΔN (1–152) and pAS2-ΔN (1–96), which could mask this interacting domain, explaining the absence of significant interaction of Ure2p with these constructions. To address this question, we decided to test the truncated ΔN (1–152) construct not as bait, but as prey. Although this construction expressed the same primary sequence of *URE2*, the fusion with the activating domain of Gal4p rather than the binding domain could change the capacity of this truncated protein to interact with Ure2p.

The yeast cells were thus transformed with all the baits in combination with ΔN (1–152) as the prey. The β-galactosidase-specific activity was measured as previously described. The result is presented in Table 1, column 3. This deletion interacts with Ure2p when the full-length protein is used as the bait (121 versus 63). As ΔN (1–152) does not share the putative interacting domain common to ΔC (152–354) and ΔC (97–354), we therefore conclude that at least two different interacting domains exist (see Figure 3, hypothesis 2). This interaction demonstrates the presence of the β domain in the ΔN (1–152) construction.

In order to analyse the relationship between α and β domains, we used ΔC (152–354) as the prey (Table 1, column 4). This deletion mutant retains the α domain alone. The combination of ΔC (152–354) as bait with ΔN (1–152) as prey caused a 34-fold

increase in the β-galactosidase activity compared with the background level (68 versus 2). Such an interaction is also observed in the case of the reciprocal combination, ΔN (1–152) as bait and ΔC (152–354) as prey (75 versus 14). The interaction between ΔN and ΔC fragments of Ure2p demonstrates the ability of the α domain to interact with the β domain located in ΔN (1–152). In addition, we observed a strong interaction between the ΔC (152–354) constructions used both as bait and prey, as the β-galactosidase activity increased 13-fold compared with the control (26 versus 2). In conclusion, these experiments show that the α domain of the molecule, expressed in the ΔC (152–354) construction is able to interact with itself and with the β domain.

DISCUSSION

The two-hybrid approach is a popular way to increase our knowledge of a protein by identifying cellular partners. In some cases, it can help to assign a function to a gene [23]. This technique can also be used to further define previously reported interactions [24,25]. As the measurement of the interaction implies several factors, such as the number of copies of the plasmid per cell, efficiency of translation of the hybrid proteins, nuclear targeting of the baits and preys and finally the interaction of the two hybrids, it is not possible to strictly correlate the β-galactosidase activity with the affinity of the interaction *in vivo*. Moreover, the folding of the protein can be affected by its fusion to a part of Gal4p and therefore can lead to a tertiary structure completely different from the expected one. However, many studies have demonstrated the efficiency of this technique, either to characterize the interacting domains of a protein or to identify cellular partners. In this study, we demonstrate on the basis of this technique that Ure2p is able to interact with itself. This interaction involves two domains: the α domain, which is located between amino acids 1 and 63, and the β domain, which is located between amino acids 152 and 354. The α domain is able to interact with itself and with the β domain.

We have also demonstrated that the *in vitro*-expressed N-terminal part of Ure2p, comprising residues 1–96, is able to bind Ure2p. However, we observed in this case that the full-length Ure2p was not eluted in equimolar amounts with the tagged truncated Ure2p (see Figure 2). This fact suggests that this interaction is weak and/or unstable under the conditions of the experiment, which are far remote from the *in vivo* ones.

Ure2p and Sup35p form the family of yeast prions [7,26]. It has been shown that Sup35p can form fibres and this polymerization could explain the conversion of the cellular protein [27–29]. No such molecular data are yet available for Ure2p. This protein has not yet been purified either from yeast or from a heterologous system permitting a strong over-production, such as *E. coli*. The molecular mechanism explaining the formation and propagation of [*URE3*] thus remains largely unknown. Genetic data strongly suggest that the protein contains two domains acting independently in the yeast prion propagation [6,19,20]. The C-terminal domain of Ure2p (residues 66–354) fails to induce [*URE3*] formation and even decreases the rate of [*URE3*] appearance [19]. This domain can not propagate by itself the prion introduced by cytoduction [20]. In contrast, the N-terminal domain (called the PFD) is able to increase the rate of [*URE3*] appearance and may maintain the prion introduced by cytoduction, but less efficiently than the whole protein. Our data show that the first half of the protein (including the prion domain) can interact with itself but also with the second half (representing part of the catalytic domain). These interactions may reflect two kinds of events: one corresponding to the regular function of Ure2p and the other allowing the formation of an altered Ure2p, giving rise to the [*URE3*] phenotype. Our results are in agreement with the model of prion propagation described by Masison and co-authors [20], who proposed an interaction between the N-terminal prion-inducing domain and the C-terminal nitrogen-regulation domain of Ure2p. We now need precise biochemical data to achieve functional studies of this protein in regard to prion propagation but also to the cellular function of this nitrogen regulator. For this purpose, the hunting of cellular partners with our bait may be very rewarding.

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