Mechanism of inhibition of Ψ^+ prion determinant propagation by a mutation of the N-terminus of the yeast Sup35 protein

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The *SUP35* gene of *Saccharomyces cerevisiae* encodes the polypeptide chain release factor eRF3. This protein (also called Sup35p) is thought to be able to undergo a heritable conformational switch, similarly to mammalian prions, giving rise to the cytoplasmically inherited Ψ^+ determinant. A dominant mutation (*PNM2* allele) in the *SUP35* gene causing a Gly58→Asp change in the Sup35p N-terminal domain eliminates Ψ^+ . Here we observed that the mutant Sup35p can be converted to the prion-like form *in vitro*, but such conversion proceeds slower than that of wild-type Sup35p. The overexpression of mutant Sup35p induced the *de novo* appearance of Ψ^+ cells containing the prion-like form of mutant Sup35p, which was able to transmit its properties to wild-type Sup35p both *in vitro* and *in vivo*. Our data indicate that this Ψ^+ -eliminating mutation does not alter the initial binding of Sup35p molecules to the Sup35p Ψ^+ -specific aggregates, but rather inhibits its subsequent prion-like rearrangement and/or binding of the next Sup35p molecule to the growing prion-like Sup35p aggregate.

Keywords: prion/release factor eRF3/*Saccharomyces cerevisiae*/translation termination

Introduction

In mammals, prions are postulated to produce spongiform encephalopathies, such as scrapie in sheep, kuru, Creutzfeldt–Jakob disease (CJD), Gerstmann–Straussler–Scheinker syndrome in man and similar fatal neurological diseases of animals, through the sole agency of an infectious protein (reviewed by Prusiner, 1994; Horwich and Weissman, 1997). This prion protein, PrP, a cell surface protein expressed in cells of mammalian brains, becomes infectious when it acquires a new conformation. Once in this conformation, it interacts with other PrP molecules and influences them to adopt the same conformation. The repeating cycles of this reaction generate new infectious material that spreads to adjacent cells and kills the affected neurons leading to a typical manifestation of these diseases. The infectious form of PrP (PrP^{Sc}) differs from its normal form (PrP^C) by poor solubility in detergents, high resist-

ance to proteolysis and a marked propensity for aggregation, being primarily β -sheet unlike α -helical PrP^C (Prusiner *et al.*, 1983; Oesch *et al.*, 1985; Meyer *et al.*, 1986). Two models have been proposed for the conversion process. In accordance with the heterodimer model, the conversion reaction occurs between monomeric PrP^C and PrP^{Sc} molecules (Cohen *et al.*, 1994), while the PrP^{Sc} aggregation is a secondary process. The seeded-polymerization model suggests that the properties of PrP^{Sc} are acquired within the framework of the polymer and the conformational rearrangement occurs during binding of PrP^C to the PrP^{Sc} polymer (Brown *et al.*, 1991; Jarrett and Lansbury, 1993).

Recently, the prion concept was used to explain the unusual genetic features of some genetic determinants in the yeast *Saccharomyces cerevisiae* and fungus *Podospora anserina* (Wickner, 1994; Coustou *et al.*, 1997). One such determinant in yeast is the cytoplasmically inherited factor Ψ^+ , which controls the efficiency of translation termination (reviewed in Cox *et al.*, 1988). Ψ^+ has been proposed to arise, like prions, from the ability of the Sup35 protein to switch to an alternative conformational and functional state (Wickner, 1994). Some properties typical of mammalian prions, such as aggregation and resistance to proteases, have been shown for Sup35p in the Ψ^+ state (Patino *et al.*, 1996; Paushkin *et al.*, 1996). The cell-free conversion of Sup35p from Ψ^- cells (Sup35p ^{Ψ^-}) to the prion-like Ψ^+ -specific form (Sup35p ^{Ψ^+}) was reproduced *in vitro* (Paushkin *et al.*, 1997a). This conversion reaction was repeated through several consecutive cycles, thus modeling *in vitro* continuous Ψ^+ propagation. Size fractionation of lysates of Ψ^+ cells demonstrated that the converting activity was associated solely with Sup35p ^{Ψ^+} aggregates, in agreement with the seeded-polymerization model for Ψ^+ propagation. Purification of Sup35p ^{Ψ^+} to apparent homogeneity showed that the converting activity copurified with Sup35p aggregates, thus confirming the basic assumption of the prion model for Ψ^+ , that the converting agent is an altered form of Sup35p (Paushkin *et al.*, 1997a). The prion hypothesis for Sup35p was further strengthened by the observations that purified bacterially expressed Sup35p can self-assemble *in vitro* into amyloid-like filaments (Glover *et al.*, 1997; King *et al.*, 1997).

Sup35p is a yeast homologue of the eRF3 translation termination factor of higher eukaryotes (Stansfield *et al.*, 1995; Zhouravleva *et al.*, 1995). It represents a multi-domain protein, in which the C-terminal (C) domain is essential for translation termination and cell viability. The Sup35p N-terminal region of 253 amino acids is not essential for viability and can be subdivided into the N-terminal (N) domain of 123 amino acids, required for Ψ^+ maintenance, and the middle domain, for which no function has yet been ascribed (Ter-Avanesyan *et al.*, 1993, 1994). The Sup35p N domain plays a key role in

Fig. 1. Schematic representation of the Sup35 protein and its derivatives. Designations of the *SUP35* deletion alleles and corresponding protein fragments are presented on the left. Multicopy plasmids carrying the *SUP35* gene and its deletion alleles were originally described by Ter-Avanesyan *et al.* (1993). Amino acid numbers are indicated. **SUP35-P2* and *sup35-P2ΔS* differ from the corresponding *SUP35* alleles by a single G→A transition at position +173 in the coding region for the N domain of the protein (Doel *et al.*, 1994).

the Ψ^+ phenomenon, since it is required for the Ψ^+ propagation *in vivo* and is solely responsible for the Sup35p prion conversion and oligomerization into amyloid-like fibrils *in vitro* (Glover *et al.*, 1997; King *et al.*, 1997; Paushkin *et al.*, 1997a). The *SUP35* deletion alleles encoding N-terminally truncated Sup35p cannot support Ψ^+ propagation, but in the heterozygote they do not interfere with Ψ^+ . In contrast, the *PNM2* mutation (designated hereafter as *SUP35-P2*), which defines a Gly58→Asp change in the Sup35p N domain (Doel *et al.*, 1994), is dominant for Ψ^+ elimination. This indicates that the mutant Sup35p protein (Sup35P2p) can actively interfere with the process of Sup35p prion-like conversion (McCready *et al.*, 1977).

In this study we observed that Sup35P2p can incorporate efficiently into Sup35p prion aggregates, while overexpressed Sup35P2p could generate and support the Ψ^+ state. The prion conversion of Sup35P2p proceeded at reduced rate and the conversion of Sup35p was also slowed down in the presence of Sup35P2p. This increases the time of Ψ^+ 'replication' with respect to a cell division, which may cause the Ψ^+ loss.

Results

The Sup35P2 protein can undergo a prion-like rearrangement *in vitro*

To test whether Sup35P2p can participate in the prion conversion, we tried to perform its conversion *in vitro*, as described previously for wild-type Sup35p (Paushkin *et al.*, 1997a). A lysate of P-5V-H19 Ψ^- strain carrying the chromosomal *SUP35-P2* mutation was mixed with Sup35ΔSp Ψ^+ seeds (see Figure 1 for designations of Sup35p variants), obtained as sedimented material of the lysate of strain 1-5V-H19 Ψ^+ overexpressing Sup35ΔSp (Paushkin *et al.*, 1997b). As a control, a lysate of the Ψ^- strain 5V-H19, expressing wild-type Sup35p, was mixed with the same seeds. Both full-length Sup35p and Sup35P2p proteins were convertible to an aggregated (Figure 2) and protease-resistant (data not shown) form, although the amount of converted material was at least 2-fold lower in the case of Sup35P2p. This indicates that the prion-like conversion of Sup35P2p can be seeded by Sup35p Ψ^+ , but proceeds more slowly than that of the wild-type Sup35p.

Fig. 2. Conversion of Sup35P2p Ψ^- and Sup35p Ψ^- to an aggregated form caused by Sup35ΔSp Ψ^+ . Immunoblot analysis of Sup35p. Experiment: lysates of Ψ^- cells were mixed with Ψ^+ sedimented material containing Sup35ΔSp, incubated for 20 min or 2 h and analyzed as described. Control: analysis of Sup35P2p in Ψ^- after 2 h of incubation. The distribution of Sup35p in Ψ^- lysate also did not change after 2 h of incubation (data not shown). Total, the mix of Ψ^- lysate and Ψ^+ sedimented material. Lysate, lysate of Ψ^- cells of P-5V-H19; cytosol, sucrose and pellet; supernatant, intermediate fraction and sedimented material obtained after centrifugation of lysates and mixes.

Ψ^+ induction by overexpression of the Sup35P2 protein

The overexpression of Sup35p or its N-terminal part can induce the *de novo* appearance of the Ψ^+ determinant (Chernoff *et al.*, 1993; Derkatch *et al.*, 1996). The ability of Sup35P2p to undergo the prion-like conversion suggested that its overexpression could also induce Ψ^+ appearance. To test this suggestion, we studied the ability of multicopy plasmids encoding the full-length Sup35P2 protein and its C-terminally truncated version to induce Ψ^+ in strain 1-5V-H19. It is noteworthy that since Sup35Cp encoded by strain 1-5V-H19 lacks the prionogenic N-terminal region and cannot convert into the prion-like form, Ψ^+ induced in transformants of this strain must be derived only from the prion-like rearrangement of the plasmid-encoded Sup35P2 protein. This strain carried the cytoplasmic [*PIN* $^+$] determinant, which is required for the efficient Ψ^+ induction (Derkatch *et al.*, 1997). The Ψ^+ phenotype could not be scored in this strain due to the antisuppressor effect of chromosomal *SUP35-C* allele. Therefore, to monitor the Ψ^+ generation, we transferred cytoplasm from the 1-5V-H19 transformants to a tester strain c10B-H49 Ψ^- , using a 'cytoduction' procedure (see Materials and methods).

The levels of plasmid-encoded Sup35P2p and Sup35P2ΔSp in 1-5V-H19 transformants did not differ noticeably from those of Sup35p and Sup35ΔSp, exceeding the levels of chromosomally encoded Sup35p ~15-fold (data not shown). Overexpression of all four proteins caused Ψ^+ induction with comparable frequency (Table I). The Ψ^+ cells are characterized by Sup35p accumulation in the form of high-molecular-weight aggregates, which allowed biochemical testing of the Ψ^+ state of the 1-5V-H19 strain. High-speed centrifugation of lysates showed the presence of such aggregates in all studied 1-5V-H19 transformants (data not shown). One of the centrifugation pellets, containing Sup35P2ΔSp, was used to seed the cell-free prion conversion of Sup35p (see below), which further confirms its Ψ^+ state.

Table I. Ψ^+ induction in transformants of the 1-5V-H19 strain

Inducing allele	Total number of cytoductants	Number of Ψ^+ cytoductants	% of Ψ^+ cytoductants
<i>sup35-P2ΔS</i>	6940	34	0.5 ± 0.2
<i>sup35-ΔS</i>	4750	53	1.1 ± 0.3
<i>SUP35-P2</i>	2060	13	0.6 ± 0.2
<i>SUP35</i>	2630	40	1.5 ± 0.4
Control	19 150	0	0

Control, the pEMBL-yex4 plasmid lacking the *SUP35* sequence. Cytoductants of the strain c10B-H49 were selected on cycloheximide-containing medium as described in Materials and methods. The Ψ^+ state of Ade⁺ cytoductants was confirmed by the GuHCl test. The Ψ^+ induction data represent an average from three independent transformants. The standard deviation is indicated.

Table II. Ψ^+ induction in transformants of the P-5V-H19 strain

Inducing allele	Total number of cytoductants	Number of Ψ^+ cytoductants	% of Ψ^+ cytoductants
<i>sup35-N1</i>	1150	226	19.7 ± 5.7
<i>sup35-N2</i>	1042	51	4.9 ± 1.6
<i>sup35-NM</i>	2940	16	0.5 ± 0.1
<i>sup35-ΔS</i>	3060	12	0.4 ± 0.2
<i>SUP35</i>	5600	14	0.3 ± 0.1
<i>sup35-P2ΔS</i>	2200	18	0.8 ± 0.3
<i>SUP35-P2</i>	2520	9	0.4 ± 0.2
Control	2810	0	0

For details, see Table I.

The chromosomal *SUP35-P2* mutation does not prevent Ψ^+ induction by extra copies of the *SUP35* gene

The use of *SUP35-P2* mutant strain P-5V-H19 Ψ^- instead of 1-5V-H19 also allowed the Ψ^+ induction by overexpressed Sup35P2p, Sup35p and their deletion variants, as was revealed in cytoduction experiments (Table II). Furthermore, in contrast to the 1-5V-H19 strain, the newly generated Ψ^+ could be observed directly in P-5V-H19. The overexpression of truncated Sup35 proteins caused suppression of the *ade2-1* ochre mutation (Figure 3A). The Ade⁺ phenotype of transformants was mitotically unstable and was lost simultaneously with the plasmid on a non-selective medium. The incubation of transformants in the presence of 1.5 mM GuHCl strongly inhibited their subsequent growth on adenine omission medium (Figure 3B) thus suggesting that the suppressor phenotype of transformants reflects their Ψ^+ status. The centrifugation revealed the presence of aggregated Sup35p in lysates of these transformants and thus confirmed their Ψ^+ status (data not shown).

The transformants with multicopy plasmids carrying the complete *SUP35* or *SUP35-P2* alleles did not grow on adenine omission medium, but nevertheless were capable of transmitting the Ψ^+ determinant to the strain c10B-H49 Ψ^- by cytoduction (Figure 3A). The different suppressor efficiency in the studied P-5V-H19 transformants is probably related to the different levels of functional Sup35p. These levels are decreased by Sup35p aggregation in all cases, but in the transformants overexpressing truncated Sup35p only a small portion of soluble Sup35p is functional, which results in inefficient translation termination and the suppressor phenotype.

Fig. 3. Ψ^+ induction in transformants of the strain P-5V-H19 with multicopy plasmids carrying either *SUP35* and *SUP35-P2* constructs. (A) The vertical streaks are c10B-H49 Ψ^- . The strains streaked horizontally are transformants with plasmids carrying indicated *SUP35* alleles. The cross of Ade⁻ transformants carrying *SUP35* and *SUP35-P2* plasmids with the c10B-H49 Ψ^- tester strain allowed the selective growth of cytoductants on adenine omission medium containing glycerol as a sole carbon source. All the rest of the transformants were able to grow on this adenine omission medium. The plates were incubated for 5 days at 30°C. (B) Growth of transformants carrying the *sup35-P2ΔS* multicopy plasmid on adenine (-Ade) and uracil (-Ura) omission media after incubation on medium selective for the plasmid with 1.5 mM GuHCl (+GuHCl) or without GuHCl (-GuHCl).

The overexpression of Sup35p in Ψ^+ strains strongly inhibits cell growth (Dagkesamanskaya and Ter-Avanesyan, 1991). It was suggested that this was due to excessive aggregation of Sup35p and another release factor, Sup45p (a yeast homologue of the eRF1 polypeptide chain release factor of higher eukaryotes; Stansfield *et al.*, 1995) which is efficiently absorbed by aggregated Sup35p in the strain 5V-H19 Ψ^+ (Paushkin *et al.*, 1997b). Since the N-terminally truncated variants of Sup35p are able to bind Sup45p, but are not incorporated into Ψ^+ -specific Sup35p aggregates (Paushkin *et al.*, 1996, 1997b), they restore translation termination and override the inhibitory effect. The Sup35P2 protein also alleviates the inhibitory effect of overexpressed Sup35p in the Ψ^+ state, but is likely to act in a somewhat different way. In the strains expressing Sup35P2p molecules the overall rate of Sup35p prion conversion is decreased and the levels of Sup35p variants and Sup45p in a soluble fraction should be higher, which explains the viability of the described P-5V-H19 Ψ^+ transformants.

Sup35P2p Ψ^+ can seed the prion-like conversion of Sup35p *in vitro*

The ability of prion aggregates of Sup35P2p to seed the conversion of Sup35p *in vitro* was tested. The aggregated fractions of the Ψ^+ transformants of 1-5V-H19, overexpressing either Sup35P2ΔSp or Sup35ΔSp, were used as seeds for the conversion reaction. The incubation of Sup35p Ψ^- with these seeds resulted in its conversion into an aggregated form (Figure 4). The extent of conversion reaction was ~2-fold lower in the case of Sup35P2ΔSp seeds. The aggregates resulting from these conversion reactions were recovered by centrifugation and used to initiate new rounds of conversion. Such a cycle was repeated consecutively four times (Figure 4). The Sup35P2ΔSp Ψ^+ and Sup35ΔSp Ψ^+ , which were used to

Fig. 4. Consecutive cycles of Sup35p prion-like conversion initiated by Sup35P2ΔSp^{Ψ+} or Sup35ΔSp^{Ψ+}. Immunoblot analysis of Sup35p. (Panel 1) The first cycle. Lysates of Ψ⁻ cells of the strain 5V-H19 were mixed with Ψ⁺ sedimented material containing Sup35P2ΔSp or Sup35ΔSp with the ratio of 1 Sup35ΔSp^{Ψ+} or Sup35P2ΔSp^{Ψ+} to 2 Sup35p^{Ψ-}, incubated for 3 h and analyzed as described. (Panels 2 and 3) In the second and third cycles the sedimented material obtained in the previous cycle was mixed with the 5V-H19 Ψ⁻ lysate (with the ratio of 1 molecule of Sup35p from the pellet to ~10 molecules of Sup35p^{Ψ-}). (Panel 4) Fourth cycle. The sedimented material obtained in the third cycle was mixed with the lysate of 5V-H19 Ψ⁻ transformed with Sup35NMP-encoding plasmid (at Sup35p ratio of 1 to 4). Sup35p^{Ψ-} from 5V-H19 lysate used in cycles 1 to 3 (Control 1) and Sup35NMP^{Ψ-} used in the cycle 4 (Control 2) were not aggregated after 3 h of incubation. Designation of fractions is given in the legend to Figure 2.

start the first conversion cycle, were only barely detectable among the products of the second reaction and could not be detected in the products of the third reaction. It is noteworthy that the disappearance of Sup35P2ΔSp^{Ψ+} seeds was accompanied by an increase in the efficiency of the conversion reaction up to the level of control reactions, started by Sup35ΔSp^{Ψ+} seeds.

Discussion

No treatment is currently available for the prion diseases of man and animals. In contrast, in yeast two mechanisms are known which can cure cells of the prion-like Ψ⁺ determinant. The first is associated with the chaperone protein Hsp104 (Hsp104p). Either the lack or overexpression of Hsp104p eliminates Ψ⁺, the prion state of Sup35p (Chernoff *et al.*, 1995). This suggests that a certain optimal level of Hsp104p is required for the maintenance of Ψ⁺. Here we attempted to elucidate the other mechanism, related to the PNM2 (*SUP35-P2*) mutation which defines the Gly58→Asp change in Sup35p (Doel *et al.*, 1994). The Ψ⁺ loss caused by this mutation is a dominant genetic trait, and a heterozygous diploid gradually loses the ability to produce Ψ⁺ spores over successive generations (McCready *et al.*, 1977). In contrast, the *SUP35* 5'-deletion alleles are genetically recessive for Ψ⁺ elimination (Ter-

Avanesyan *et al.*, 1994), a property evidently related to the inability of N-terminally truncated Sup35p to interact with the Ψ⁺-specific aggregates of full-length Sup35p and interfere with aggregate formation (Paushkin *et al.*, 1996). This suggests that Sup35P2p has the opposite properties, being able to incorporate into Sup35p^{Ψ+} aggregates and inhibit their formation. In support of this suggestion we observed that Sup35P2p is able to incorporate into Sup35p^{Ψ+} aggregates (Figure 2), and moreover, can form such aggregates on its own. The overexpression of Sup35P2p induced the Ψ⁺ determinant, which showed the basic features of the yeast prions, since Sup35P2p^{Ψ+} could transmit its prion properties to the normal Sup35p molecules both *in vitro* (Figure 4) and *in vivo* (Tables I and II). The prion properties of Sup35P2p differed from that of Sup35p quantitatively rather than qualitatively. The rate of the prion-like conversion was decreased when Sup35P2p was used either as convertible material or as prion-like seeds. This suggests that the *SUP35-P2* mutation simply decreases the efficiency of Sup35p prion-like conversion. At the same time, Sup35P2p incorporates into Sup35p^{Ψ+} aggregates with the same efficiency as Sup35p, as we observed in competition experiments (data not shown). Therefore, Sup35P2p can efficiently compete with wild-type Sup35p for binding to growing Sup35p^{Ψ+} aggregates, and, once bound, slows down the prion polymerization of Sup35p. The slowed down steps could be either the conformational rearrangement of Sup35P2p or joining of the next Sup35p/Sup35P2p molecule, or both.

Could the deceleration of Sup35p prion conversion be sufficient to cause the Ψ⁺ elimination? The Ψ⁺ propagation depends on a balance between the Sup35p polymerization and dissolution of the polymers by chaperone Hsp104p. The overexpression of Hsp104p causes Ψ⁺ elimination and in some cases as little as 2-fold excess of Hsp104p is sufficient for this (Chernoff *et al.*, 1995). Thus, an ~2-fold reduction of the rate of Sup35P2p prion conversion, which was observed *in vitro*, may be sufficient to allow Hsp104p to dissolve the Sup35p^{Ψ+} aggregates. The increase of Sup35P2p levels should greatly accelerate its prion conversion, since the rate of Sup35p conversion is proportional to the concentrations of both prion and normal forms of Sup35p. The overexpression of Sup35P2p compensates for its reduced conversion ability and allows the Ψ⁺ propagation. The cells with standard Sup35P2p levels should differ from the cells overexpressing this protein only by the decreased rate of Sup35P2p prion conversion, which appears to be the only reason for the Ψ⁺ elimination. The lower polymerization rate of Sup35P2p was also supported by the observation of compatibility of overexpressed Sup35P2p with the Ψ⁺ state. The overexpression of Sup35p in Ψ⁺ strains is semi-lethal, probably because the excessively efficient polymerization leaves virtually no Sup35p and an associated factor, Sup45p (homologous to the eRF1 release factor of higher eukaryotes; Frolova *et al.*, 1994) in a soluble functional state. The analogous strains with Sup35P2p are viable, which suggests a higher proportion of soluble Sup35p due to a lower polymerization rate.

It should be noted that, similarly to the data presented in this paper, several cases have been described in mammals where the simultaneous presence of different variants of PrP protein interfere with the conversion of PrP^C into the

PrP^{Sc} prion form. The expression of hamster PrP in cultured mouse neuroblastoma cells, persistently infected with mouse scrapie, blocked prion formation for both mouse and hamster PrP. A similar effect was observed during the expression of mouse PrP with three hamster PrP-specific methionines at positions 108, 111 and 138. Mouse PrP with only Met108 and Met111 reduced total PrP^{Sc} formation 6-fold and itself was converted to a protease-resistant form (Priola *et al.*, 1994; Priola and Chesebro, 1995). The effect of the expression of hamster PrP in mice was less prominent; the mouse PrP^{Sc} formation was not blocked, although some inhibition of it was observed (Prusiner *et al.*, 1990). The frequency of sporadic appearance of CJD is notably decreased in heterozygotes in comparison with homozygotes for Met129 or Val129 of PrP (Palmer *et al.*, 1991). These data demonstrate that prion formation may be reduced or blocked in the presence of different alleles of the same prionogenic protein. It should be noted that, in the above cases, the inhibitory PrP represented either heterologous proteins or allelic variants. One can assume that more efficient inhibitors of prion aggregate formation could be isolated among PrP mutants. However, a simple procedure to screen for such mutants in mammals is lacking. In this respect, the generation and characterization of new prion-eliminating mutations in the yeast *SUP35* gene could be helpful in elucidating the structural rules defining the inhibitory potential of altered prion proteins.

Materials and methods

Strains and media

The *S.cerevisiae* strains used were: 5V-H19 (MATa *ade2-1 SUP35 can1-100 leu2-3,112 ura3-52 Ψ^+*), 1A-H19 (MATa *ade2-1 SUP35 lys1-1 his3-11,15 leu2-3,112 Ψ^+*) and c10B-H49, which is a cycloheximide-resistant Ψ^- derivative of 10B-H49 (MATa *ade2-1 SUP35 lys1-1 his3-11,15 leu1 kar1-1*) (Ter-Avanesyan *et al.*, 1994). The *sup35-C* allele of the strain 1-5V-H19 encodes a truncated Sup35 protein lacking amino acids 1–253 and causes dominant antisuppression and recessive inability to propagate Ψ^+ (Ter-Avanesyan *et al.*, 1994). The strain P-5V-H19 was constructed by replacing the wild-type *SUP35* gene with its *SUP35-P2* allele, which is dominant for the Ψ^+ elimination (Doel *et al.*, 1994). This was performed by co-transformation of the Ψ^+ haploid strain 5V-H19 with the *LEU2*-carrying YEp13 plasmid and DNA digest of the plasmid pSM111 (Doel *et al.*, 1994) with *XhoI* and *XbaI*, containing the *SUP35-P2* gene. Approximately 2–3% of *Leu*⁺ transformants were pink and adenine-requiring independently of the presence of YEp13 plasmid. To confirm the presence of the *SUP35-P2* mutation in the pink transformants, one of them, P-5V-H19, was crossed with the Ψ^+ strain 1A-H19. Diploid cells obtained from this cross manifested the Ade⁻ phenotype and did not carry Ψ^+ because it resulted only in asci with Ade⁻ spores, thus indicating that the corresponding transformant carried the *SUP35-P2* allele instead of the wild-type *SUP35* gene.

We used standard rich (YEED) and synthetic (SC) media for yeast (Sherman *et al.*, 1986). Non-fermentable media contained glycerol (24 ml/l) as a sole carbon source. Yeast strains were grown at 30°C.

Plasmids, DNA manipulation and transformation

DNA manipulations were performed by standard protocols (Sambrook *et al.*, 1989). A series of multicopy pEMBLyex4-based plasmids (Cesareni and Murray, 1987) containing the *SUP35* gene or its 3'-deletion alleles (Figure 1; Ter-Avanesyan *et al.*, 1993), YEp13 (Broach *et al.*, 1979) and pSM111 (Doel *et al.*, 1994) plasmids were used. For obtaining multicopy *SUP35-P2* plasmids, the *XhoI*–*SacI* or *Sall* fragments of pSM111 carrying the complete *SUP35-P2* allele or its 3'-deleted version *sup35-P2ΔS*, were ligated with the pEMBL-yex4 vector digested by *XhoI* and *SacI* or *XhoI* and *Sall*, respectively. DNA transformation of yeast cells was performed as described (Gietz *et al.*, 1995). Transformants with all plasmids used (except YEp13) were selected and grown on uracil omission medium.

Genetic methods

Yeast strains were cured of the Ψ^+ determinant by growth on media supplemented with 1.5 mM GuHCl (Tuite *et al.*, 1981). The Ψ^- colonies of *ade2-1 SUP35*-carrying strains were identified by their pink color and adenine requirement since the weak serine-inserting tRNA suppressor *SUP35* cannot suppress the *ade2-1* ochre mutation in the absence of Ψ^+ determinant (Cox, 1965). Non-suppressive petites (ρ^-) of the strain c10B-H49 were obtained by ethidium bromide treatment (Goldring *et al.*, 1970).

For performing cytoduction experiments, strains of interest were mated with the strain c10B-H49 $\Psi^- \rho^-$ which carries the *kar1-1* mutation that blocks karyogamy (Conde and Fink, 1976). The strains were mixed together on the surface of a YEED plate, incubated for 1 day, and then replica-plated to adenine omission medium containing glycerol as a sole carbon source. Respiratory competent (ρ^+) colonies were scored as cytoductants. Mating of transformants of the strain 1-5V-H19 carrying multicopy *SUP35* plasmids or transformants of P-5V-H19 bearing *SUP35* and *SUP35-P2* plasmids, with the strain c10B-H49 $\Psi^- \rho^-$ allowed for the isolation of cytoductants selectively: only those cells of the recipient strain that received Ψ^+ determinant could grow on the glycerol-containing adenine omission medium, whereas transformants and diploid cells were Ade⁻. Only those transformants that could co-transfer ρ^+ and Ψ^+ by cytoduction to the $\Psi^- \rho^-$ tester strain, c10B-H49, were considered to possess the Ψ^+ determinant. In all cases, white Ade⁺ colonies were confirmed to be Ψ^+ by 'curing' with GuHCl (see above). To quantify the induction of Ψ^+ in strains 1-5V-H19 and P-5V-H19 by plasmids carrying different *SUP35* constructs, the procedure for the selection of cytoductants was modified. Transformants of 1-5V-H19 and P-5V-H19 were crossed with the strain c10B-H19 $\Psi^- \rho^-$ carrying the *cyh*^r mutation. This mutation is semidominant, so diploids are more sensitive to cycloheximide than the recipient haploid strain. Therefore, cytoductants were selected from the mating mixture of cells by transfer to medium containing cycloheximide (3 µg/ml) with glycerol as the sole carbon source. The cells of transformants and resulting diploids were sensitive to cycloheximide and did not grow on this medium. Respiratory competent and cycloheximide resistant colonies were scored as cytoductants and tested for their ability to grow on adenine omission medium. The frequency of Ψ^+ induction in transformants was taken as equal to the fraction of Ψ^+ cells among the ρ^+ cytoductants because Ψ^+ and ρ^+ show 100% coincidence of transfer by cytoduction (Cox *et al.*, 1988).

Preparation, fractionation and analysis of yeast cell lysates

Yeast cultures were grown in liquid YEED medium or in a defined medium selective for plasmid markers to an OD₆₀₀ of 1.5. The cells were harvested, washed in water and lysed by vortexing with glass beads in buffer A (25 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 2% glycerol) containing protease inhibitors, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamide, 0.1 mM sodium metabisulfite, 0.5 µg/ml TPCK, 0.5 µg/ml TLCK, 2.5 µg/ml antipain, 0.5 µg/ml leupeptine, 1.0 µg/ml pepstatin A, 2.0 µg/ml aprotinin. Cell debris was removed by centrifugation at 15 000 g for 10 min at 4°C. To obtain sedimented material containing Sup35p^{Ψ+}, the lysates of 1-5V-H19 Ψ^+ strain transformed with the multicopy plasmids encoding either Sup35ΔSp or Sup35P2ΔSp were loaded on a sucrose layer (1 ml, 30%) and centrifuged at 200 000 g for 30 min at 4°C. The sedimented material was resuspended in buffer A for further use in conversion reactions performed as described previously (Pauskhin *et al.*, 1997a).

Protein samples were separated on 10–15% SDS–polyacrylamide gels according to Laemmli (1970) and electrophoretically transferred to nitrocellulose sheets (Towbin *et al.*, 1979). Western blots were probed with polyclonal rabbit anti-Sup35p antibody and developed using the Amersham ECL system.

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