

Yeast prion protein derivative defective in aggregate shearing and production of new ‘seeds’

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According to the nucleated polymerization model, *in vivo* prion proliferation occurs via dissociation (shearing) of the huge prion polymers into smaller oligomeric ‘seeds’, initiating new rounds of prion replication. Here, we identify the deletion derivative of yeast prion protein Sup35 (Sup35- Δ 22/69) that is specifically defective in aggregate shearing and ‘seed’ production. This derivative, [PSI⁺], previously thought to be unable to turn into a prion state, in fact retains the ability to form a prion ([PSI⁺] Δ 22/69) that can be maintained in selective conditions and transmitted by cytoplasmic infection (cytoduction), but which is mitotically unstable in non-selective conditions. Moreover, the full-size Sup35 prion ‘seeded’ by [PSI⁺] Δ 22/69 retains its mitotic stability defect. The [PSI⁺] Δ 22/69 cells contain more Sup35 protein in the insoluble fraction and form larger Sup35 aggregates compared with the conventional [PSI⁺] cells. Moderate excess of Hsp104 disaggregase increases transmission of the [PSI⁺] Δ 22/69 prion, while excess Hsp70-Ssa chaperone antagonizes it, opposite to their effects on conventional [PSI⁺]. Our results shed light on the mechanisms determining the differences between transmissible prions and non-transmissible protein aggregates.

Keywords: chaperone/oligopeptide repeats/prion/protein aggregation/*Saccharomyces cerevisiae*

Introduction

Prions are ‘infectious’ isoforms of normal proteins that are capable of reproducing themselves by converting a normal protein, encoded by the same gene, into a prion (Prusiner, 1982). First discovered as infectious agents causing neurodegenerative diseases in mammals and humans (for review see Prusiner, 1998), prions were then implicated in certain cytoplasmically inherited traits in yeast (Wickner, 1994). In this way, prions provide a new mechanism for genetic transmission of information, coded in structural rather than sequence templates (for review see Chernoff, 2001).

It has been proposed that prions are aggregated proteins propagated via nucleated polymerization, so that

pre-existing multimeric prion ‘seeds’ initiate polymerization of the soluble protein (for reviews see Lansbury and Caughey, 1995; Kushnirov and Ter-Avanesyan, 1998). Indeed, the prion proteins characterized thus far form insoluble aggregates *in vivo*, when present in prion state, and *in vitro* (for reviews see Serio *et al.*, 1999; Serio and Lindquist, 2000; Chernoff, 2001). In some cases, these aggregates possess characteristic features of amyloids (ordered fiber-like structures with high β -sheet content). This relates prion phenomena to amyloidoses and neural inclusion disorders, such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, amyotrophic lateral sclerosis and others (for review see Koo *et al.*, 1999).

Yeast prion [PSI⁺], a polymerized isoform of the translation termination factor Sup35 (eRF3), has been successfully used as a model to study cellular control of prion transmission (for reviews see Serio and Lindquist, 2000; Chernoff, 2001). The [PSI⁺] strains are defective in translation termination, which provides a convenient experimental assay for [PSI⁺]. Prion formation can be induced *de novo* in the [psi⁻] background by transient overproduction of the Sup35 protein (Chernoff *et al.*, 1993; Derkatch *et al.*, 1996), possibly due to increased aggregation at higher protein concentrations. Successful transmission of [PSI⁺] in cell generations requires an intermediate level of the chaperone protein Hsp104 (Chernoff *et al.*, 1995), a disaggregase previously implicated in solubilizing temperature-induced protein aggregates (Parsell *et al.*, 1994). Excess Hsp104 causes frequent loss of [PSI⁺] (Chernoff *et al.*, 1995), apparently due to increased solubilization of prion aggregates (Patino *et al.*, 1996; Paushkin *et al.*, 1996). However, inactivation or loss of Hsp104 also results in the loss of [PSI⁺] (Chernoff *et al.*, 1995) via blockage of aggregate partitioning or shearing (Wegrzyn *et al.*, 2001). This leads to accumulation of the large agglomerates lacking prion replicating activity. It appears that the ability of prion polymers to dissociate into the small oligomeric ‘seeds’ (shearing) is crucial for prion transmission by infection or in cell generations, and provides the major distinction between prion aggregates and non-infectious protein inclusions. Yeast prions other than [PSI⁺], such as [PIN⁺] (Derkatch *et al.*, 1997) and [URE3] (Moriyama *et al.*, 2000), also appear to require Hsp104, implicating it as a universal *trans*-catalyst of prion transmission. However, the *cis*-factors that control aggregate shearing remain unknown.

Yeast Sup35 consists of three regions: Sup35C, essential and sufficient for the translation termination function; middle Sup35M; and the N-terminal prion-forming domain (Sup35N), responsible for prion formation and propagation (for reviews see Serio and Lindquist, 2000; Chernoff, 2001). Sup35N includes an N-terminal Gln- and Asn-rich stretch (QN-stretch) resembling huntingtin and

Table I. Genotypes and origins of the yeast strains

Strain	Genotype/description	Source
GT81	<i>MATa/MATα ade1-14/lade1-14 his3/his3 leu2/leu2 lys2/lys2 ura3/ura3 trp1/trp1 [PSI⁺ PIN⁺]</i> (self-diploid)	Chernoff <i>et al.</i> (2000)
GT159	the haploid <i>MATa [psi⁻ PIN⁺]</i> derivative of GT81	Chernoff <i>et al.</i> (1999)
GT109	the <i>SUP35/sup35::HIS3</i> derivative of GT81	Chernoff <i>et al.</i> (2000)
GT111	the <i>[psi⁻ PIN⁺]</i> derivative of GT109	Chernoff <i>et al.</i> (2000)
GT113	the <i>[psi⁻ pin⁻]</i> derivative of GT109	Chernoff <i>et al.</i> (2000)
GT247-1C	<i>MATa ade1-14 his3 leu2 lys2 ura3 trp1 sup35::HIS3 [LEU2 sup35-Δ22/69] [psi⁻ PIN⁺]</i>	this study
GT247-1D	the same as GT247-1C but <i>MATα</i>	this study
GT249-21A	the same as GT247-1D	this study
GT255-2D	<i>MATa ade1-14 his3 lys2 ura3 leu2 trp1 sup35::HIS3 [LEU2 SUP35] [psi⁻ PIN⁺]</i>	this study
c10B-H49 [p ⁻] (OT102)	<i>MATα lys1-1 his3 leu1 ade2-1 SUQ5 kar1-1 cyh^R [psi⁻ rho⁻]</i>	Kochneva-Pervukhova <i>et al.</i> (1998)
GT388	<i>MATα lys1-1 his3 leu1 ura3 ade2-1 SUQ5 kar1-1 cyh^R [psi⁻ rho⁻]</i>	this study

other proteins associated with poly-Gln diseases (De Pace *et al.*, 1998), and oligopeptide repeats (ORs) between amino acid (aa) positions 40 and 90, resembling those observed in the mammalian prion protein PrP (Cox, 1994). Deletion of *SUP35N* makes yeast cells unable to propagate [*PSI⁺*] (Ter-Avanesyan *et al.*, 1994), while overproduction of Sup35N alone is sufficient to induce [*PSI⁺*] formation in the cells bearing an intact chromosomal copy of *SUP35* (Derkatch *et al.*, 1996). A number of *sup35N* mutations were identified that negatively affect prion propagation, leading to the ‘[*PSI⁺*] no more’ (*PNM*) phenotype. These include aa substitutions within the QN-stretch (De Pace *et al.*, 1998; King, 2001) and substitutions or deletions within the OR region (Ter-Avanesyan *et al.*, 1994; Liu and Lindquist, 1999; Parham *et al.*, 2001). In several cases, it was observed that the mutant Sup35 derivatives are poorly recruited into the Sup35^{PSI⁺} aggregates and/or show markedly decreased rates of amyloid formation *in vitro* (De Pace *et al.*, 1998; Kochneva-Pervukhova *et al.*, 1998; King, 2001; Parham *et al.*, 2001). The molecular effects of the other mutations remain unclear.

Among *PNM* mutations, the *sup35-Δ22/69* (formerly called *sup35-ΔBstEII*) deletion, lacking a piece of Sup35N between aa positions 22 and 69, is of specific interest. It abolishes the interaction between Sup35N and some of its partners *in vivo* (Bailleul *et al.*, 1999) and slightly decreases the rates of spontaneous Sup35 aggregation *in vitro* (Glover *et al.*, 1997). This led to the suggestion that the defect in prion propagation is due to inability of the mutant protein to enter the Sup35^{PSI⁺} aggregates (Kochneva-Pervukhova *et al.*, 1998; Serio and Lindquist, 2000). However, overproduction of Sup35-Δ22/69, and especially of Sup35N-Δ22/69 in cells bearing full-size Sup35 protein resulted in partial loss of the Sup35 termination function, detected as nonsense suppression, a phenotype similar to that of [*PSI⁺*] (Ter-Avanesyan *et al.*, 1993; Derkatch *et al.*, 1996). In contrast to [*PSI⁺*], suppression was co-retained and co-lost with the Sup35-Δ22/69 (or Sup35N-Δ22/69) encoding plasmid.

Here, we perform a detailed analysis of the Sup35-Δ22/69 prion-forming potential *in vivo*. Our data show that the Sup35-Δ22/69 protein can form prion aggregates. However, these aggregates are unusually large and are rapidly lost in non-selective conditions, apparently due to the inability to produce small ‘seeds’. These features and

patterns of interaction with Hsp104 implicate *sup35-Δ22/69* as the first prion protein mutation that is specifically defective in aggregate shearing and transmission.

Results

Construction of yeast strains bearing the *sup35-Δ22/69* allele

Isogenic haploid strains bearing the complete *sup35::HIS3* deletion (Chernoff *et al.*, 2000) on the chromosome and either *sup35-Δ22/69* or the wild-type (*SUP35⁺*) allele on the single-copy (centromeric) plasmid were constructed as described in Materials and methods. We have confirmed that the *sup35-Δ22/69* spore clones that originate from [*PSI⁺*] diploids have lost [*PSI⁺*], while [*PIN⁺*] prion, needed for [*PSI⁺*] induction by overproduced Sup35 (Derkatch *et al.*, 1997, 2001), was not affected (data not shown). Most of the experiments described below were performed using strains GT247-1C, GT247-1D, GT249-21A (all *sup35-Δ22/69*), GT255-2D (*SUP35⁺*) and their derivatives (see Table I).

The Sup35-Δ22/69 protein can both induce the prion state and be turned into a prion

In contrast to the previously reported results (Derkatch *et al.*, 1996, 1997), we have observed that the transient galactose-induced overexpression of the *sup35N-Δ22/69* construct under the *GAL* promoter in the strains containing the *UGA* allele *ade1-14* slightly but reproducibly increased the frequency of Ade⁺ colonies on glucose medium (i.e. after the *GAL* promoter was turned off; Figure 1A). This effect was detected in both GT255-2D bearing the *SUP35⁺* allele on the *CEN* plasmid and the isogenic [*psi⁻ PIN⁺*] strain GT159 bearing the *SUP35⁺* allele on the chromosome. Apparently, such a low level of Ade⁺ induction (~4-fold over background) was not detected in the previous experiments employing the multicopy (rather than galactose-inducible) *sup35-Δ22/69* constructs (Derkatch *et al.*, 1996, 1997) due to lower sensitivity of that experimental system.

Moreover, transient overexpression of *SUP35*, *SUP35N* or *sup35N-Δ22/69* under the *GAL* promoter induced intense formation of Ade⁺ colonies in the *sup35-Δ22/69* [*psi⁻ PIN⁺*] strains (Figure 1A). Although *GAL-sup35N-Δ22/69* remained the least efficient inducer, it induced

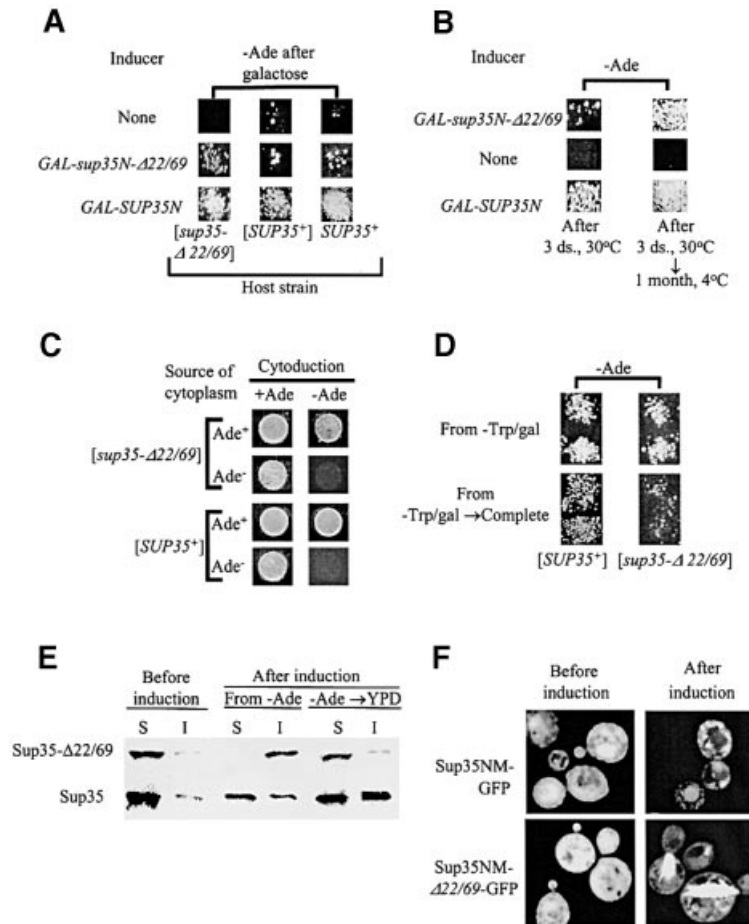


Fig. 1. The Sup35-Δ22/69 protein can both induce the prion state and be turned into a prion. (A) Transient overproduction of Sup35N or Sup35N-Δ22/69 induces the appearance of Ade⁺ colonies in the *[psi⁻ PIN⁺]* strains bearing *sup35-Δ22/69* or *SUP35⁺*. Yeast strains: GT247-1C (*[sup35-Δ22/69]*, left column); GT255-2D (*[SUP35⁺]*, central column) and GT159 (*SUP35⁺*, right column). Plasmids: left and central columns, pRS316GAL control (none), pRS316GAL-SUP35N (*GAL-SUP35N*) and pFL39GAL-SUP35NΔBst (*GAL-sup35N-Δ22/69*); right column, pFL39GAL control (none), pFL39GAL-SUP35N (*GAL-SUP35N*) and pFL39GAL-SUP35NΔBst (*GAL-sup35N-Δ22/69*). The -Ade/glucose plates were photographed after 10 days at 30°C. Similar results were obtained at 25°C (not shown). The strains GT247-1D and GT249-1A (not shown) produced the same result as GT247-1C. The plasmid pGAL-SUP35, bearing a complete *SUP35* gene under the *GAL* promoter, induced Ade⁺ formation at about the same efficiency as pRS316GAL-SUP35N (not shown). (B) Induction of prion derivatives of Sup35-Δ22/69 is increased at low temperature. Yeast strain: GT249-21A (*[sup35-Δ22/69]*). Plasmid designations are as above (A, left and central columns). After induction on -Ura/galactose medium for 3 days at 30°C (left) or for 3 days at 30°C followed by 1 month at 4°C (right), cultures were velveteen replica plated onto -Ade/glucose medium. Plates were photographed after 10 days at 30°C. Strains GT247-1C and GT247-1D (not shown) produced the same result as GT249-21A. (C) Transmission of the newly induced prion derivatives of Sup35-Δ22/69 by cytoduction. Ade⁺ derivatives, induced in GT247-1C (*[sup35-Δ22/69]*, line 1) or in GT255-2D (*[SUP35⁺]*, line 3) by pFL39GAL-SUP35N, were mated to the lawn of the recipient strain c10B-H49 on YPD, and following 1 day of incubation, transferred to medium selective for cytoductants (+Ade; see Materials and methods) and the same medium lacking adenine (-Ade) to identify cytoductants able to suppress the *ade2-1* mutation. The original uninduced strains (lines 2 and 4) were used as cytoplasm donors in the control experiment. Plates were photographed after 3 (left column) or 7 (right column) days of incubation. The same results were observed with the recipient strain GT388 (not shown). (D) Instability of the newly induced prion derivatives of Sup35-Δ22/69. Yeast strains are as in (A), left and central columns. Plasmid: pFL39GAL-SUP35N. After induction on -Trp/galactose medium for 3 days at 30°C, cultures were replica plated either directly onto -Ade/glucose medium (plate on the top) or first onto complete synthetic glucose-containing medium and then (after 1 day of incubation) onto -Ade/glucose medium. Plates were photographed after 10 days at 30°C. Post-induction incubation on complete medium decreases the frequency of Ade⁺ colonies in *sup35-Δ22/69* but not in *SUP35⁺*. The same result was obtained with the inducer plasmid pGAL-SUP35 and also in the case when YPD was used instead of the synthetic complete medium (not shown). (E) Centrifugation analysis of aggregation of the Sup35 and Sup35-Δ22/69 prions. Yeast strains GT247-1C (Sup35-Δ22/69) and GT255-2D (Sup35), bearing the plasmid pRS316GAL-SUP35N, were incubated on -Ura/galactose medium for 3 days and velveteen replica plated onto -Ade/glucose medium. The Ade⁺ colonies, induced in each strain, were collected after 2 weeks of growth at 30°C, pooled together and either used directly for protein isolation (from -Ade) or incubated in liquid YPD on the shaker at 30°C and 200 r.p.m. (-Ade → YPD) for 24 h (Sup35-Δ22/69) or up to 72 h (Sup35) prior to protein isolation. Uninduced cultures of the respective strains (before induction) were used as *[psi⁻]* controls. Proteins were isolated and fractionated into the soluble (S) and insoluble (I) fractions by centrifugation at 12 000 g as described (Newnam *et al.*, 1999), run on the SDS-PAGE gel, transferred onto the nitrocellulose membrane and reacted with the Sup35NM specific antibodies. The larger fraction of the Sup35-reacting material was insoluble in the Ade⁺ cultures bearing Sup35-Δ22/69, compared with those bearing full-size Sup35. However, insoluble Sup35-Δ22/69 disappeared after 24 h in YPD, while full-size Sup35 aggregates remained even after 72 h in YPD. (F) Fluorescence microscopy of the Sup35 and Sup35-Δ22/69 prion aggregates. Derivatives of the yeast strains GT255-2D (*[SUP35⁺]*) and GT247-1C (*[sup35-Δ22/69]*), bearing the plasmids pRS316-NMGFP (Sup35NM-GFP) and pRS316-NMΔBstGFP (Sup35NM-Δ22/69-GFP), respectively, were transformed with the plasmid pFL39GAL-SUP35N, incubated on -Ura/galactose medium for 3 days and velveteen replica plated onto -Ade/glucose medium. Cells from Ade⁺ colonies, grown after 2 weeks of incubation at 30°C, were analyzed by fluorescence microscopy as described in Materials and methods. Uninduced cultures of the respective strains (before induction) were used as *[psi⁻]* controls. Prion aggregates are seen as fluorescent clumps.

Table II. Individual analysis of Ade⁺ derivatives, obtained in the *SUP35*⁺ and *sup35-Δ22/69* backgrounds

Inducee	Inducer	Cytoplasm recipient	Ade ⁺ derivatives		
			Stable Ade ⁺	Unstable Ade ⁺	Total Ade ⁺ analyzed
Originally induced Ade ⁺ colonies					
<i>SUP35</i> ⁺	Sup35N	N/A	24	0	24
<i>SUP35</i> ⁺	Sup35N-Δ22/69	N/A	10	14	24
<i>sup35-Δ22/69</i>	Sup35N	N/A	0	58	58
<i>sup35-Δ22/69</i>	Sup35N-Δ22/69	N/A	8 ^a	52	60
Ade ⁺ cytoductants					
<i>SUP35</i> ⁺	Sup35N	<i>SUP35</i> ⁺	16	0	16
<i>sup35-Δ22/69</i>	Sup35N	<i>SUP35</i> ⁺	0	40	40

^aProven to be suppressor mutants.

more Ade⁺ colonies in the *sup35-Δ22/69* background than in the *SUP35*⁺ background (Figure 1A). Like [*PSI*⁺] induction in the *SUP35*⁺ strains (for review see Chernoff, 2001), Ade⁺ induction in the *sup35-Δ22/69* strains was strictly dependent on the presence of [*PIN*⁺] (data not shown), was higher at 25°C than at 30°C (data not shown) and was significantly increased by prolonged incubation of induced cultures at 4°C (Figure 1B).

As Ade⁺ phenotype is indicative of *ade1-14*_{UGA} suppression due to a translation termination defect, one possible explanation of our results is that Sup35-Δ22/69 protein can be turned into a partially defective state in response to overproduction, as has been shown previously for the complete Sup35 protein. Alternatively, one could suggest that transient suppression of *ade1-14* by the overproduced Sup35 derivative in the [*PIN*⁺] environment leads to accumulation of the Ade1 read-through product, which is retained in cell generations at a level sufficient for growth on –Ade medium. To distinguish between these possibilities, we studied a transfer of the suppressor state to the [*psi*⁻] strains c10B-H49[*p*⁻] and GT388 (Table I) bearing a different nonsense reporter (*ade2-1*_{UAA}) by cytoplasmic infection (cytoduction). Ade⁺ derivatives induced in the *sup35-Δ22/69* or *SUP35*⁺ strains were used as cytoplasm donors. In both cases, Ade⁺ cytoductants were obtained, although the efficiency of transfer was apparently lower with the *sup35-Δ22/69* donor, as could be judged from heterogeneous growth on the selective medium (Figure 1C). As read-through Ade1 protein would be incapable of compensating for the *ade2* defect, this result shows that nonsense suppression in the *sup35-Δ22/69* background was caused by the transmissible isoform of the Sup35-Δ22/69 protein. As this isoform is maintained and reproduced for the number of cell generations sufficient to form a visible Ade⁺ colony, it certainly bears the major characteristics of the [*PSI*⁺] prion.

Mitotic instability of the Sup35-Δ22/69 prion isoform

To check whether prion derivatives of Sup35-Δ22/69 are capable of stable propagation in non-selective conditions, the galactose-induced cultures were incubated on complete glucose medium, non-selective for suppression, for 1–2 days before replica plating onto –Ade medium. While this step did not have any detectable effect on Ade⁺ induction by complete Sup35 or Sup35N in the *SUP35*⁺ strain, it greatly decreased the frequency of Ade⁺ colonies

induced by Sup35, Sup35N or Sup35N-Δ22/69 in the *sup35-Δ22/69* strain (Figure 1D), suggesting that most of the newly induced Sup35-Δ22/69 prion derivatives are mitotically unstable and can easily be lost in non-selective conditions. To confirm this, we picked the individual Ade⁺ colonies induced by Sup35N in the *SUP35*⁺ background and by Sup35N or Sup35N-Δ22/69 in the *sup35-Δ22/69* background, streaked them out on non-selective (YPD) medium and analyzed four subclones of each colony. While the vast majority of the *SUP35*⁺ Ade⁺ colonies produced Ade⁺ subclones, most of the *sup35-Δ22/69* Ade⁺ colonies produced Ade⁻ subclones (Table II). Eight exceptional stable Ade⁺ derivatives of the *sup35-Δ22/69* strain were found to contain nuclear suppressor mutations, apparently acquired during growth of the initially unstable Ade⁺ culture under the selective pressure (data not shown). These data confirm mitotic instability of the Sup35-Δ22/69 prion derivatives in non-selective conditions.

Quite remarkably, a large fraction of the Ade⁺ colonies induced by the transient overproduction of Sup35N-Δ22/69 in the *SUP35*⁺ strain (Figure 1A) were also mitotically unstable (Table II). As the frequency of Ade⁺ induction in this experiment was low (see above), it is possible that most, if not all, of the remaining stable Ade⁺ colonies originated from spontaneous [*PSI*⁺] formation or from *ade1-14* reversions, rather than from Sup35N-Δ22/69-mediated induction. Likewise, the Ade⁺ cytoductants that received cytoplasm from the *sup35-Δ22/69* Ade⁺ donor (Figure 1D) were mitotically unstable, in contrast to cytoductants that received cytoplasm from the *SUP35*⁺ Ade⁺ donor (Table II), despite the fact that the recipient strain contained the wild-type *SUP35*⁺ allele in both cases. Cytoduction experiments with the *ura3* recipient strain GT388 confirmed that the transfer of the Ade⁺ phenotype is not associated with the transfer of the *URA3* plasmid bearing the *sup35-Δ22/69* allele (data not shown). This indicates that the mitotic stability defect of the initial Sup35-Δ22/69 prion derivative can not be corrected by the addition of or substitution for the full-size Sup35 protein. A similar phenomenon was described recently for chimeric *Candida-Saccharomyces* Sup35 constructs (Chien and Weissman, 2001).

Differences in cytoductant stability also confirm that cytoplasmic transmission of the Ade⁺ state is not due to the presence of residual inducer protein in the donor cytoplasm, as both *sup35-Δ22/69* and *SUP35*⁺ Ade⁺ derivatives used for cytoduction were induced by one

and the same inducer, Sup35N. Indeed, the control experiment using the donor cultures taken directly from galactose medium and containing high levels of the Sup35N inducer generated stable Ade⁺ cytoductants independently of whether the donor strain was SUP35⁺ or *sup35-Δ22/69* (data not shown).

Aggregation of the Sup35-Δ22/69 protein in the cells containing mitotically unstable prions

Sup35 aggregation is associated with the [PSI⁺] state (Patino *et al.*, 1996; Paushkin *et al.*, 1996). To check whether the Sup35-Δ22/69 prion isoform is also aggregated, we isolated and analyzed proteins from the Ade⁺ colonies, freshly induced by GAL-SUP35N in the *sup35-Δ22/69* and SUP35⁺ backgrounds. In contrast to the control (before induction) cultures containing soluble Sup35-reacting material, the Ade⁺ derivatives contained a significant fraction of insoluble Sup35. Moreover, the *sup35-Δ22/69* Ade⁺ derivatives grown in the selective conditions contained almost all of the Sup35-reacting material in the low-speed precipitated fraction corresponding to large-size aggregates, while the SUP35⁺-based Ade⁺ derivatives grown in the same conditions showed less than half of the total Sup35 protein in this fraction (Figure 1E). Further growth of the *sup35-Δ22/69* Ade⁺ derivatives in non-selective (YPD) medium resulted in the shift of all Sup35-Δ22/69 protein back to the soluble fraction, while the SUP35⁺ Ade⁺ derivatives grown in non-selective medium retained insoluble Sup35 (Figure 1E). This correlated with the rapid loss of the Sup35-Δ22/69 prion in non-selective conditions (Figure 1D; Table II).

To visualize prion aggregates, we transformed the isogenic [*psi*⁻ PIN⁺] *sup35-Δ22/69* and [*psi*⁻ PIN⁺] SUP35⁺ yeast strains with centromeric plasmid bearing the SUP35NM-Δ22/69 or SUP35NM fragment, respectively, fused in-frame to green fluorescent protein (GFP) and expressed under the control of the endogenous SUP35 promoter. Prion induction by GAL-SUP35N was performed as described previously. Cells from the Ade⁺ colonies of both strains contained fluorescent clumps, which were not present in the 'before induction' samples and appear to have resulted from aggregation of the prion isoform of Sup35NM (or Sup35NM-Δ22/69)-GFP (Figure 1F). In agreement with differential centrifugation results, 10–30% of cells in the *sup35-Δ22/69* Ade⁺ colonies contained huge rod-like structures, which were not observed in the cells from the SUP35⁺ Ade⁺ colonies (Figure 1F). Further incubation in non-selective conditions resulted in a sharp decrease in frequency of the aggregate-containing cells in the *sup35-Δ22/69* but not in the SUP35⁺ population (data not shown).

Effects of the chaperone proteins on the Sup35-Δ22/69 prion

To check whether low stability of the Sup35-Δ22/69 prion is related to the large aggregate size, we performed the Ade⁺ induction experiments in the SUP35⁺ and *sup35-Δ22/69* strains bearing an extra copy of the HSP104 gene on the centromeric plasmid. Normal levels of the Hsp104 disaggregase promote shearing and propagation of the [PSI⁺] aggregates, while excess Hsp104 leads to aggregate solubilization that results in prion loss (Chernoff *et al.*, 1995; Patino *et al.*, 1996; Paushkin *et al.*, 1996; Wegrzyn

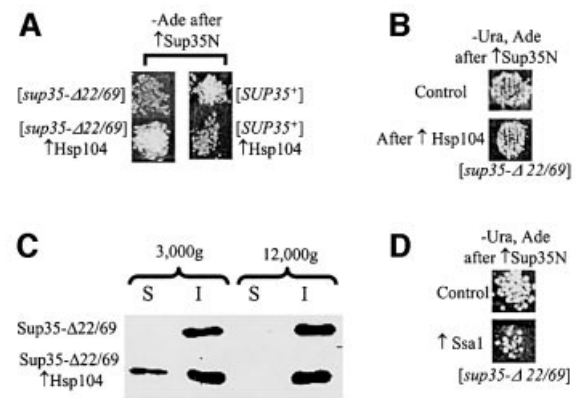


Fig. 2. Effects of the chaperone proteins on the Sup35-Δ22/69 prion. (A) The continuous presence of an extra copy of HSP104 increases the frequency of the detectable Ade⁺ derivatives in the *sup35-Δ22/69* strain but decreases it in the SUP35⁺ strain. The yeast strains GT247-1C ([*sup35-Δ22/69*]) and GT255-2D ([SUP35⁺]) were co-transformed with the TRP1-based plasmid pFL39GAL-SUP35N and either URA3-based plasmid pYS104, containing the HSP104 gene (↑Hsp104), or matching control plasmid pRS316GAL. Co-transformants were incubated on -Trp, Ura/galactose medium for 3 days and velveteen replica plated onto -Ade/glucose medium. Plates were photographed after 10 days at 30°C. (B) Transient overproduction of Hsp104 does not influence Ade⁺ induction in the *sup35-Δ22/69* background. The yeast strain GT247-1C ([*sup35-Δ22/69*]) was co-transformed with the TRP1-based plasmid pFL39GAL-SUP35N and either URA3-based plasmid pGAL104-URA3, containing the HSP104 gene under the GAL promoter (↑Hsp104), or matching control plasmid pRS316GAL. Co-transformants were incubated on -Trp, Ura/galactose medium for 3 days and velveteen replica plated onto -Ade, Ura/glucose medium. Plates were photographed after 10 days at 30°C. (C) Moderate excess of Hsp104 promotes partial dissociation of the Sup35-Δ22/69 aggregates. Ade⁺ colonies induced in GT247-1C (Sup35-Δ22/69) by pFL39GAL-SUP35N in the presence of plasmid pYS104, containing the HSP104 gene (↑Hsp104), or matching control plasmid pRS316GAL (control), were collected after 2 weeks of growth on -Ade, Ura at 30°C, pooled together and used for protein isolation and centrifugation analysis of the Sup35-Δ22/69 aggregation, as described above (see the legend to Figure 1E). Centrifugation was performed either at 12 000 or 3000 g. The presence of excess Hsp104 increases the relative amount of the Sup35-Δ22/69 protein remaining in the soluble fraction at 3000 g, which appears to correspond to the smaller aggregates. (D) The presence of an extra copy of SSA1 decreases the frequency of Ade⁺ colonies in the *sup35-Δ22/69* strain. The yeast strain GT247-1C ([*sup35-Δ22/69*]) was co-transformed with the TRP1-based plasmid pFL39GAL-SUP35N and either URA3-based plasmid pRS316K-SSA1-K10, containing the SSA1 gene (↑Ssa1), or matching control plasmid pRS316GAL. Co-transformants were incubated on -Trp, Ura/galactose medium for 3 days and velveteen replica plated onto -Ade, Ura/glucose medium. Plates were photographed after 14 days at 30°C.

et al., 2001). As expected, an extra copy of HSP104, expressed from its own promoter, decreased the frequency of Ade⁺ colonies induced in the SUP35⁺ background. However, it increased the frequency of Ade⁺ colonies induced in the *sup35-Δ22/69* background (Figure 2A). This stimulatory effect was observed only at moderate levels of excess Hsp104, as high-level Hsp104 overexpression from the copper-induced CUP1 promoter antagonized formation of detectable Ade⁺ colonies in both strains (data not shown). Transient overexpression of HSP104 (simultaneously with the SUP35N inducer) from the GAL promoter did not influence the frequency of Ade⁺ colonies in the *sup35-Δ22/69* background (Figure 2B), confirming that the continuous presence of excess Hsp104 is needed for its effect on the Sup35-Δ22/69 prion.

Table III. Analysis of colonies produced by Ade⁺ derivatives of the *sup35-Δ22/69 [URA3 HSP104]* strain in non-selective conditions

Class	Phenotype		Total number
	Ade ⁺	Ade ⁻	
Retained [<i>URA3 HSP104</i>]	13	32	45
Lost [<i>URA3 HSP104</i>]	3	36	39
Total	16	68	84

Individual analysis of the Ade⁺ colonies that were induced in the *sup35-Δ22/69* background at moderately increased Hsp104 levels confirmed that excess Hsp104 increases longevity of the Sup35-Δ22/69 prion derivatives. Among 11 independent Ade⁺ derivatives analyzed, seven produced Ade⁺ colonies in non-selective conditions. Moreover, the Ade⁺ phenotype was retained more frequently in the progeny retaining the *HSP104* plasmid (Ura⁺) than in those losing the *HSP104* plasmid (Ura⁻; Table III). However, excess Hsp104 was not able to compensate completely for the mitotic transmission defect. All the colonies, even those retaining the *HSP104* plasmid, had lost the Ade⁺ phenotype upon the next round of incubation in non-selective conditions (data not shown).

To check whether a moderate excess of Hsp104 influences Sup35-Δ22/69 prion aggregation, we isolated proteins from the fresh *sup35-Δ22/69* Ade⁺ colonies, induced in the presence or absence of the *HSP104 CEN* plasmid, and analyzed them by differential centrifugation. In both cases, most Sup35-Δ22/69 was precipitated at 12 000 g. However, the presence of excess Hsp104 led to the appearance of a Sup35-Δ22/69 fraction remaining in solution at lower centrifugation speed, 3000 g (Figure 2C). This shows that an increase in the mitotic stability of the Sup35-Δ22/69 prion correlates with the appearance of the smaller aggregates, apparently generated by the disaggregating activity of Hsp104.

The Ssa protein of the Hsp70 family was previously shown to counteract the [PSI⁺]-antagonizing effect of excess Hsp104 (Newnam *et al.*, 1999), to increase [PSI⁺]-mediated suppression (Newnam *et al.*, 1999) and to stimulate *de novo* [PSI⁺] induction by excess Sup35 or Sup35N (K.Allen and Y.Chernoff, unpublished observations). In contrast, we have observed that excess Ssa protein decreases the frequency of detectable Ade⁺ colonies induced in the *sup35-Δ22/69* background (Figure 2D). As we have previously proposed that Ssa is a factor promoting aggregate growth (Chernoff, 2001; Wegrzyn *et al.*, 2001), its antagonizing effect on the Sup35-Δ22/69 prion agrees with the notion that the low mitotic stability of this prion derivative is related to its abnormally large aggregate size.

Discussion

Our data show that the deletion derivative of the Sup35 protein, Sup35-Δ22/69, formerly described as '[PSI⁺] no more' (Ter-Avanesyan *et al.*, 1994), retains both aggregate-forming and -inducing activities and is able to enter protein aggregates *in vivo* (Figure 1A, E and F). The overproduction-induced aggregation-prone isoform of

Sup35-Δ22/69 possesses the major characteristic features of the [PSI⁺] prion. When selection for the partial loss of the translation termination function is applied (i.e. in the *ade1-14_{UGA}* strain incubated on -Ade medium), the Sup35-Δ22/69 aggregation-prone isoform can be maintained and propagated in cell divisions long enough to form Ade⁺ colonies (Figure 1A). No low-molecular-weight protein fragments corresponding to the Sup35N inducer protein were detected in the Ade⁺ cultures (data not shown), confirming that inducer is not retained by the time the Ade⁺ colony is formed. Moreover, the suppressor state could be transmitted by cytoduction to a recipient strain bearing a different nonsense reporter, *ade2-1_{UAA}* (Figure 1C), confirming that suppression is not caused by the residual read-through Ade1 protein accumulated during the inducer's overproduction. Thus, it is clear that partial loss of termination activity becomes an inherent property of the Sup35-Δ22/69 protein itself. Therefore, we recognize the Sup35-Δ22/69 aggregation-prone isoform as a prion and designate it [PSI⁺]^{Δ22/69}.

However, in contrast to the conventional [PSI⁺] prions formed by the full-size Sup35 protein, the [PSI⁺]^{Δ22/69} prion is rapidly lost once cells resume growth in non-selective conditions. This loss was detected both by recovery of Sup35-Δ22/69 translation termination function (Figure 1D; Table II) and shift of the Sup35-Δ22/69 protein from the insoluble to soluble fraction (Figure 1E). Moreover, this mitotic instability pattern is retained by the [PSI⁺]^{Δ22/69}-derived prion that was transmitted by cytoduction to the strain containing full-size Sup35 protein (Table II). This shows that Sup35 lacking the 22/69 region forms a prion that is inherently defective in mitotic transmission in the absence of selective pressure. This defect appears to be determined by the structural patterns of the initial prion 'nucleus', as it can not be corrected by the following addition of the full-size Sup35 molecules.

The physical properties of the Sup35-Δ22/69 aggregates also differ from the conventional Sup35^{PSI+} prions by a larger proportion of protein in the low speed precipitating fraction, and a larger size and unusual morphology of GFP-tagged clumps (Figure 1E and F). Our previous data show that [PSI⁺] propagation *in vivo* probably occurs via small oligomers, while large cytologically visible aggregates represent the 'dead ends' of the prion replication cycle (Wegrzyn *et al.*, 2001). This agrees with the recent *in vitro* results suggesting that the process of Sup35NM amyloid formation proceeds via oligomeric intermediates (Serio *et al.*, 2000). Thus, accumulation of the huge aggregates in the [PSI⁺]^{Δ22/69}-containing cells suggests a defect in aggregate shearing and production of the small oligomeric 'seeds'.

In strong support of this model, we have observed that a moderate excess of Hsp104 disaggregase exhibits opposite effects on the newly formed [PSI⁺] and [PSI⁺]^{Δ22/69} prions: it is antagonizing conventional [PSI⁺] but increases the frequency of detectable [PSI⁺]^{Δ22/69} derivatives by improving their mitotic transmission (Figure 2A). As Hsp104 is responsible for aggregate shearing and required for production of new oligomeric 'seeds' (Wegrzyn *et al.*, 2001), a moderate excess of Hsp104 most likely assists [PSI⁺]^{Δ22/69} by promoting partial dissociation of large aggregates, so that a larger number of 'seeds' is generated. This is confirmed by differential centrifugation results

(Figure 2C). However, a further increase in Hsp104 levels becomes counterproductive as it leads to aggregate loss via solubilization. Completely stable $[PSI^+]^{\Delta 22/69}$ derivatives were not recovered even in the extra-*HSP104*-containing strain, indicating that excess Hsp104 is not able either to fully compensate for the transmission defect caused by the $\Delta 22/69$ deletion or irreversibly alter the inherent structural parameters of aggregates that are responsible for the shearing defect.

Another chaperone (Ssa of the Hsp70 family) that assists in propagation of the conventional $[PSI^+]$ prion in normal conditions (Newnam *et al.*, 1999) exhibits an antagonistic effect on $[PSI^+]^{\Delta 22/69}$ (Figure 2D). This agrees with the previously observed inhibitory effect of excess Ssa on transmission of the large $[PSI^+]$ aggregates generated at low levels of Hsp104 activity (Wegrzyn *et al.*, 2001) and is most likely explained by the previously hypothesized aggregation-promoting role of Ssa leading to a further increase of aggregate size (Chernoff, 2001; Wegrzyn *et al.*, 2001).

Our data explain the previously observed nonsense-suppressor effect of overproduced Sup35- (or Sup35N-) $\Delta 22/69$ in the cells containing full-size Sup35 (Ter-Avanesyan *et al.*, 1993; Derkatch *et al.*, 1996). Apparently, overproduction induces unstable Sup35- $\Delta 22/69$ prions, which convert full-size Sup35 into a prion. However, the resulting prion remains unstable and is rapidly lost, as demonstrated in the cytoduction experiments. In support of this model, we have observed low-efficient induction of mitotically unstable Ade⁺ derivatives by the transient Sup35N- $\Delta 22/69$ overproduction in the strain bearing full-size Sup35 (Figure 1A; Table II). A low frequency of such ‘heterologous’ induction could be due to the previously reported anti-suppressor effect of the *sup35- $\Delta 22/69$* allele on conventional $[PSI^+]$ (Ter-Avanesyan *et al.*, 1994). It is possible that inclusion of the Sup35- $\Delta 22/69$ protein into wild-type $[PSI^+]$ aggregates inhibits shearing and reproduction. Indeed, continuous overproduction of Sup35N- $\Delta 22/69$ in the weak conventional $[PSI^+]$ strain results in an increased loss of the prion state (Y.O.Chernoff, unpublished observations).

Previous observations suggested that newly emerged isolates of $[PSI^+]$ and $[PIN^+]$ frequently display mitotic instability, but can be stabilized upon further propagation (Derkatch *et al.*, 2000; I.Derkatch, Y.O.Chernoff and S.G.Inge-Vechtomov, unpublished observations). It is possible that prion generation occurs via a two-step process including initial formation of the unstable aggregate and its conversion into the ‘shearing-prone’ state that ensures stable transmission. Interestingly, the heterologous $[PSI^+]$ derivatives, formed by a chimeric Sup35 protein bearing the highly divergent Sup35N (or NM) region of the distantly related yeast *Pichia methanolica*, are characterized by lower mitotic stability (Chernoff *et al.*, 2000; Kushnirov *et al.*, 2000; Santoso *et al.*, 2000), a larger proportion of protein in the insoluble fraction, lower sensitivity to excess Hsp104 (Kushnirov *et al.*, 2000) and larger aggregate size (Y.O.Chernoff and E.Lewitin, unpublished observations), compared with the conventional endogenous *Saccharomyces cerevisiae* $[PSI^+]$ prions. This indicates that the Sup35 sequence divergence between *Saccharomyces* and *Pichia* might affect the shearing step.

The *sup35- $\Delta 22/69$* deletion is the first documented *sup35* allele that specifically affects aggregate shearing and ‘seed’ production. The $\Delta 22/69$ deletion overlaps two subregions of Sup35N previously shown to influence prion propagation: it removes the last several aa residues of the N-terminal QN-stretch (De Pace *et al.*, 1998) and the first 2.5 ORs (Cox, 1994), as well as the region between the QN-stretch and ORs. It has yet to be determined which alteration contributes most to the shearing defect observed. Previous data indicate that point mutations (Kochneva-Pervukhova *et al.*, 1998) or deletions (Parham *et al.*, 2001) within ORs decrease the ability of Sup35 to enter prion aggregates. However, the shearing defect has not been reported for these mutants, nor was it reported for the aa substitutions within the QN-stretch (De Pace *et al.*, 1998). It is not yet known whether correct spacing between the QN and OR regions is important for aggregate shearing. It is also possible that interactions between Sup35 and other proteins (Hsp104 and/or its co-factors) involved in aggregate shearing are impaired in the Sup35- $\Delta 22/69$ aggregates. Indeed, $\Delta 22/69$ decreases or abolishes *in vivo* interaction between Sup35N and some other proteins, including Sla1, which influences $[PSI^+]$ formation and propagation by an unknown mechanism (Bailleul *et al.*, 1999). However, if the defect of prion shearing is indeed due to a lack of protein–protein interactions, this should mean that protein interaction patterns of prion aggregates are determined completely by the protein that initiated prion formation, as full-size Sup35 aggregates ‘seeded’ by Sup35- $\Delta 22/69$ appear to retain the same pattern (Table II).

Previously, it was reported that Sup35- $\Delta 22/69$ protein exhibits slow (relative to full-size Sup35) kinetics of aggregate formation *in vitro* (Glover *et al.*, 1997). Part of that could be due to the shearing defect, although the complex nature of the Sup35- $\Delta 22/69$ deletion makes it possible that initial aggregation of Sup35- $\Delta 22/69$ is also impaired. Indeed, overproduced Sup35- $\Delta 22/69$ induced its own prionization less efficiently than overproduced Sup35 or Sup35N (Figure 1A). Further experiments are needed to uncover the specific molecular mechanisms of the shearing defect.

Our data provide a framework for detailed analysis of the mechanisms differentiating between the transmissible prions and non-transmissible protein aggregates. Our results also agree with the recent observations made in mammalian systems, indicating that huge deposits of aggregated proteins may represent ‘dead ends’ of amyloid propagation, while toxic effects are determined by the small proliferating intermediates (for review see Koo *et al.*, 1999). In this case, aggregate shearing rather than aggregate formation *per se* could become the most prospective target of anti-prion treatments.

Materials and methods

Yeast plasmids

All the yeast plasmids used were single-copy (centromeric) shuttle vectors. The plasmids pRS315-SUP35 Δ Bst (*LEU2*) and pRS316-SUP35 Δ Bst (*URA3*) were constructed by inserting the *XhoI-XbaI* 3.5 kb fragment of pEMBL-SUP35- Δ BstEII (Ter-Avanesyan *et al.*, 1993), which bears the *sup35- $\Delta 22/69$* allele under the endogenous *SUP35* promoter, into *SalI-XbaI*-cut pRS315 or *XhoI-XbaI*-cut pRS316 (Sikorski and Hieter, 1989), respectively. The plasmids bearing *SUP35*

and its derivatives under the galactose-inducible (*GAL*) promoter were constructed as follows. The *TRP1* plasmid pFL39GAL-SUP35N and the control matching plasmid pFL39GAL were obtained by inserting, respectively, the 1.5 kb *PvuII* fragment of pRS316GAL-SUP35N (Baillieu *et al.*, 1999) and the 1.2 kb *PvuII* fragment of pRS316GAL (Liu *et al.*, 1992) into *PvuII*-cut pFL39 (Bonneaud *et al.*, 1991). The *URA3* plasmid pGAL-SUP35ΔBst and *TRP1* plasmid pFL39GAL-SUP35NΔBst were constructed from pGAL-SUP35 (Derkatch *et al.*, 1996) and pFL39GAL-SUP35N (see above), respectively, by deleting the 144 bp *BstEII*–*BstEII* region, corresponding to the Sup35 aa positions 22–69. The *URA3* plasmid pGAL-SUP35NΔBst was constructed from pGAL-SUP35ΔBst by deleting the 1.8 kb region between *HindIII* sites, which removes *SUP35C* and part of *SUP35M*. The *URA3* plasmids pRS316-SpGFP and pRS316-NMGFP were constructed by substituting the *BamHI*–*SacI* fragment of pRS315-Sp-SUP35HA (De Pace *et al.*, 1998), bearing the *SUP35* ORF, with either the 0.7 kb *BamHI*–*SacI* fragment of p316CG (Serio *et al.*, 1999), containing the *GFP* ORF, or the 1.6 kb *BamHI*–*SacI* fragment of pmCNMG (Serio *et al.*, 1999), containing the *SUP35NM*–*GFP* ORF, respectively. The resulting constructs express either superglow GFP or chimeric Sup35NM–GFP protein under the control of the *SUP35* promoter. The plasmid pRS316-NMΔBstGFP has been obtained from pRS316-NMGFP by removing the 144 bp *BstEII* fragment of pRS316-NMGFP, coding for Sup35 aa positions 22–69. The *URA3* plasmid pRS316K-SSA1-K10, containing the *SSA1* gene under its own promoter, was kindly provided by E.Craig. The *URA3* plasmid pCUP-HSP104 bearing *HSP104* under *CUP1* promoter has been constructed by K.Bapat in Y.O.Chernoff's laboratory. The *URA3* plasmids pYS104, which bears the *HSP104* gene under its own promoter (Sanchez *et al.*, 1992), pGAL104-*URA3*, which bears *HSP104* under the *GAL* promoter (Chernoff *et al.*, 1995), and the *LEU2* plasmid pASB2, bearing the complete *SUP35* gene under its own promoter (Borchsenius *et al.*, 2000), were described earlier.

Yeast strains

Strains used in this study are listed in Table I. Isogenic diploid strains GT81, GT109, GT111 and GT113 were described previously (Chernoff *et al.*, 2000). The original [*PSI*⁺] self-diploid GT81 is heterozygous by *MAT* locus and homozygous by all other genes. GT109 is a derivative of GT81 that is heterozygous by the *sup35::HIS3* disruption. The strains GT111 and GT113 are [*psi*⁻ *PIN*⁺] and [*psi*⁻ *pin*⁻] derivatives of GT109, respectively. The haploid strain GT159 (Chernoff *et al.*, 1999) is a [*psi*⁻ *PIN*⁺] derivative of GT81-1C, a meiotic spore clone of GT81. Haploid strains bearing the *sup35::HIS3* allele on the chromosome and either the *sup35-Δ22/69* or *SUP35*⁺ allele on the single-copy (centromeric) plasmid were obtained by sporulating and dissecting GT111 (GT247-1C and GT247-1D) or GT109 (GT249-21A), transformed with the plasmid pRS315-sup35ΔBst. GT255-2D was obtained by sporulating and dissecting GT111 transformed with pASB2. If necessary, the plasmid shuffle was performed to substitute the *LEU2* plasmids for the *URA3* plasmids. The respiratory deficient *kar1* strain c10B-H49 [*p*⁻] (Kochneva-Pervukhova *et al.*, 1998) was used as a recipient in cytoduction experiments. This strain contains the tRNA suppressor *SUQ5*, able to suppress *ade2-1* only in the presence of [*PSI*⁺] (Cox, 1994). The spontaneous *ura3* derivative of this strain, GT388, was selected on the 5-FOA medium (see Kaiser *et al.*, 1994).

Genetic and microbiological techniques

Standard yeast genetic techniques, media and cultivation conditions were used (Kaiser *et al.*, 1994). Yeast cultures were incubated at 30°C, unless stated otherwise. The presence of [*PSI*⁺] was tested for by suppression of *ade2-1*_{UAA} mutation in the presence of *SUQ5* (strains c10B-H49 [*p*⁻] and GT388) or *ade1-14*_{UGA} mutation (all the other strains), resulting in growth on the medium lacking adenine (–Ade) as described previously (Cox, 1994; Chernoff *et al.*, 1995). Dissection was performed on the micromanipulator Ergaval Series 10 from Carl Zeiss (Jena, Germany). To induce [*PSI*⁺], yeast transformants were usually incubated on galactose medium, selective for the corresponding plasmid, for 3–4 days and velveteen replica plated onto –Ade/glucose medium, as described previously (Derkatch *et al.*, 1996). Growth on –Ade was indicative of prion induction, leading to suppression of the *ade1-14*_{UGA} allele. For cytoduction experiments, the donor strains GT247-1C, GT255-2D, GT159 or their freshly induced Ade⁺ derivatives, selected on –Ade medium, were mated to the karyogamy-defective *kar1* (Conde and Fink, 1976) recipient strains c10B-H49 ([*p*⁻]) or GT388 (Table I). The recipients lack functional mitochondrial DNA and bear the recessive *cyh*^r mutation. After 1 day mating on YPD medium, cell mixtures were velveteen replica plated to synthetic medium containing glycerol as the

sole carbon source and cycloheximide (5 mg/l) to select for cytoductants receiving the mitochondria from the donor strain. In parallel, cell mixtures were replica plated onto the same medium lacking adenine to identify cytoductants able to suppress the *ade2-1* mutation. At least eight independent repeats of each experiment demonstrated in Figures 1A–D, 2A, B and D were performed per strain/plasmid combination, with the same result.

DNA and protein analysis

Standard procedures were used for DNA isolation and plasmid constructions. Yeast proteins were isolated and fractionated by centrifugation according to Patino *et al.* (1996) with some modifications (Newnam *et al.*, 1999; see the legend to Figure 2C). Proteins were run on SDS–PAGE gels and reacted with the Sup35NM-specific antibodies described earlier (Wegrzyn *et al.*, 2001). Reaction to the secondary anti-rabbit antibodies and chemiluminescent detection were performed by using the ECL detection kit from Amersham.

Fluorescence microscopy

GFP images shown in Figure 1F were scanned using a Zeiss LSM510 UV confocal laser scanning microscope (Carl Zeiss Inc., New York, NY) at wavelength 488 nm and image analysis was conducted using a Zeiss LSM image browser (Carl Zeiss). Alternatively, the samples were analyzed using the microscope Lumam-12 (LOMO, St Petersburg, Russia) and the video–computer interface Video-test FISH (ISTA, St Petersburg, Russia) at wavelength 400–460 nm (data not shown).

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