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The Sup35 domains required for maintenance of weak, strong or undifferentiated yeast [*PSI*$^+$] prions

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Summary

The Sup35 protein can exist in a non-infectious form or in various infectious forms called [*PSI*$^+$] prion variants (or prion strains). Each of the different [*PSI*$^+$] prion variants converts non-infectious Sup35 molecules into that prion variant's infectious form. One definition of a 'prion domain' is the minimal fragment of a prion protein that is necessary and sufficient to maintain the prion form. We now demonstrate that the Sup35 N region (residues 1–123), which is frequently referred to as the 'prion domain', is insufficient to maintain the weak or strong [*PSI*$^+$] variants *per se*, but appears to maintain them in an 'undifferentiated' [*PSI*$^+$] state that can differentiate into weak or strong [*PSI*$^+$] variants when transferred to the full-length Sup35 protein. In contrast, Sup35 residues 1–137 are necessary and sufficient to faithfully maintain weak or strong [*PSI*$^+$] variants. This implicates Sup35 residues 124–137 in the variant-specific maintenance of the weak or strong [*PSI*$^+$] forms. Structure predictions indicate that the residues in the 124–137 region form an α-helix and that the 1–123 region may have β structure. In view of these findings, we discuss a plausible molecular basis for the [*PSI*$^+$] prion variants as well as the inherent difficulties in defining a 'prion domain'.

Introduction

It was suggested more than 30 years ago that a protein could act as an infectious agent if it existed in a self-propagating form that propagated by converting others into the infectious form (Griffith, 1967). The prion protein (PrP) is the original example of such a protein and is encoded by the mammalian gene *PRNP* (Oesch *et al.*, 1985). PrP's infectious form is a protein-based agent that causes several fatal mammalian diseases such as sheep scrapie, mad cow disease, Creuztfeldt–Jakob disease and many others, collectively known as the prion diseases (Prusiner, 1982; Prusiner *et al.*, 1998). One region of PrP has mostly α-helical structure in the protein's normal form but contains mostly β structure in the infectious form (Pan *et al.*, 1993). Intriguingly, multiple prion disease strains with distinct characteristics have been observed among inbred hosts with the same PrP genotype (Bruce, 1996), and it has been proposed that multiple infectious forms of PrP underlie these prion disease strains (Prusiner, 1991; Bessen and Marsh, 1992; Bessen *et al.*, 1995; Caughey *et al.*, 1997). Mammalian prion disease strains appear to be capable of crossing the 'species barrier' (Bruce *et al.*, 1994; Hill *et al.*, 1997; Scott *et al.*, 1999). For example, humans have acquired variant Creuztfeldt–Jakob by ingesting prion-infected beef.

The yeast *Saccharomyces cerevisiae* is host to at least three proteins that have normal forms and infectious prion forms: Sup35, Rnq1 and Ure2, which are unrelated to PrP (Wickner, 1994; Uptain and Lindquist, 2002). Sup35, the focus of this article, has a normal form referred to as [*psi*$^-$] that is essential for terminating protein synthesis when the ribosome encounters a stop codon (Stansfield *et al.*, 1995; Zhouravleva *et al.*, 1995). Cells harbouring the infectious prion form of Sup35, referred to as [*PSI*$^+$], contain aggregated Sup35 in their cytoplasm (Patino *et al.*, 1996; Paushkin *et al.*, 1996) and inefficiently terminate protein synthesis (Cox, 1965). Overproducing Sup35 induces the *de novo* appearance of [*PSI*$^+$] (Chernoff *et al.*, 1993), which is to be expected as the chance of the spontaneous appearance of a protein's prion form should increase with increasing concentrations of its normal form (Wickner, 1994). The yeast [*PSI*$^+$] prion has been adopted as a model system for studying the prion 'species barrier'. Indeed, a barrier to [*PSI*$^+$] transmission exists between Sup35 proteins from some yeast species but not others (Chernoff *et al.*, 2000; Kushnirov *et al.*, 2000; Santoso *et al.*, 2000; Nakayashiki *et al.*, 2001).

Sup35 has three distinct regions. The C region (residues 254–685) is essential for viability and is responsible for Sup35's termination factor activity (Ter-Avanesyan *et al.*, 1993). The middle region (M), which spans residues 124–253, is not required for viability or termination factor activity and may be important for propagating [*PSI*$^+$] because its absence reduces the mitotic stability of [*PSI*$^+$]

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(Liu *et al*., 2002). The M region displays a non-uniform composition with 42% of its residues carrying a charge. Under purified conditions *in vitro*, the presence of the M region slows the acquisition of the fibrous conformation formed by the N region. The N region spans residues 1–123, which are asparagine and glutamine rich (43%) and contain six imperfect oligopeptide repeats. The N region appears to be largely responsible for Sup35's prion activity. The ability to maintain [*PSI*$^+$] is abolished by a point mutation at residue 58 (Doel *et al*., 1994), by single or double mutations between residues 8 and 33 (DePace *et al*., 1998) and by multiresidue deletions in the N region (Ter-Avanesyan *et al*., 1994; Liu and Lindquist, 1999; Parham *et al*., 2001). In addition, transient overproduction of just the N region induces the *de novo* appearance of [*PSI*$^+$] (Derkatch *et al*., 1996; 2000; Patino *et al*., 1996). Interestingly, overproducing the N region efficiently induces [*PSI*$^+$] only in cells that harbour heterologous prions such as [*PIN*$^+$] (Derkatch *et al*., 1997; 2001). One exception to this rule is that transient overproduction of Sup35 fragments with a specific 17-residue C-terminal extension induces the *de novo* appearance of [*PSI*$^+$] even in cells that lack heterologous prions (Derkatch *et al*., 2000).

Overproducing Sup35 induces weak or strong [*PSI*$^+$] variants (or strains) that are analogous to the PrP disease strains and confer weak or strong loss of termination factor activity in the same genetic background (Derkatch *et al*., 1996; Zhou *et al*., 1999). Similarly, variants of the [*URE3*] (Schlumpberger *et al*., 2001) and [*PIN*$^+$] (Bradley *et al*., 2002) prions also exist. Distinct prion strains are faithfully transmitted to other cells by cytoduction, which is an incomplete form of yeast mating that transfers the cytoplasm, but not the nucleus (Conde and Fink, 1976; Kochneva-Pervukhova *et al*., 2001; Bradley *et al*., 2002). Also, when weak and strong [*PSI*$^+$] are combined in the same cell, the stronger [*PSI*$^+$] seems to take over (Zhou *et al*., 1999; Derkatch *et al*., 1999; Bradley *et al*., 2002).

Analyses of the properties and kinetics of Sup35 aggregates formed *in vitro* support the idea that [*PSI*$^+$] variants represent distinct heritable aggregations of Sup35 (Glover *et al*., 1997; Chien and Weissman, 2001; Kochneva-Pervukhova *et al*., 2001; Uptain *et al*., 2001; DePace and Weissman, 2002). Furthermore, [*PSI*$^+$] variants differ in levels of non-aggregated Sup35 (Zhou *et al*., 1999; Uptain *et al*., 2001) and prion aggregate size (Kryndushkin *et al*., 2003). The number of distinct [*PSI*$^+$] variants is unknown, but a continuum of weak to strong variants has been described (Kochneva-Pervukhova *et al*., 2001). In the absence of [*PIN*$^+$], strong [*PSI*$^+$] variants are lost at a very low rate during mitotic growth, whereas weak [*PSI*$^+$] variants are generally slightly less stable (Derkatch *et al*., 1996; Kochneva-Pervukhova *et al*., 2001). However, certain [*PIN*$^+$] variants destabilize weak but not strong [*PSI*$^+$] (Bradley and Liebman, 2003). Mutations in the *SUP35* gene favour the formation of certain variants over others (Chien *et al*., 2003) and have distinct effects on different [*PSI*$^+$] strains (Derkatch *et al*., 1999; King, 2001).

Although the N region is clearly important for Sup35's prion activity, the question of which sequences are essential to the maintenance of different [*PSI*$^+$] variants has not been systematically examined. In order to define such sequences, we tested the ability of different Sup35 fragments to maintain weak or strong [*PSI*$^+$] variants using a two-step cytoduction method similar to that pioneered by Ter-Avanesyan *et al*. (1994). By excluding heterologous prions required for the *de novo* appearance of [*PSI*$^+$] prions, we allow for an improved focus on the sequences important for maintaining [*PSI*$^+$]. Using this method, we find that the first 14 residues of the M region (124–137) are required to faithfully maintain weak and strong [*PSI*$^+$] variants, but are unimportant for propagating a general Sup35 prion aggregate that is undifferentiated with respect to the weak and strong variants.

Results

*Identification of Sup35 fragments that faithfully maintain weak or strong [*PSI$^+$*] variants*

The cytoplasms from [*psi*$^-$] [*pin*$^-$], weak [*PSI*$^+$] [*pin*$^-$] or strong [*PSI*$^+$] [*pin*$^-$] cells were donated to [*psi*$^-$] [*pin*$^-$] recipients that contained a chromosomal *SUP35* allele deleted for residues required to maintain [*PSI*$^+$] (*sup35*-Δ2-254) (Ter-Avanesyan *et al*., 1994) and that expressed plasmid-encoded Sup35 protein fragments (Table 1 and Fig. 1). The [*PSI*$^+$] phenotype maintained by the plasmid-encoded Sup35 fragments could not be seen directly in the primary cytoductants because the protein encoded by the recipient's chromosomal *sup35*-Δ2-254 allele can never enter the prion aggregate and thus terminates protein synthesis at normal levels whether the plasmid-encoded fragments are in a [*psi*$^-$] or [*PSI*$^+$] state (Ter-Avanesyan *et al*., 1993). Thus, to determine the prion states of the plasmid-encoded Sup35 protein fragments, the cytoplasms from the primary cytoductants were donated to a [*psi*$^-$] [*pin*$^-$] recipient (secondary cytoductant) that could easily be scored as [*psi*$^-$], weak [*PSI*$^+$] or strong [*PSI*$^+$] because its only *SUP35* allele was wild type, encoding Sup35 that was able to join prion aggregates. In addition, this recipient yeast strain contains the *ade1-14* nonsense allele (Chernoff *et al*., 1995) that is suppressed to different extents in [*psi*$^-$], weak [*PSI*$^+$] and strong [*PSI*$^+$] cells (Derkatch *et al*., 1996).

In the [*psi*$^-$] state, *ade1-14* cells do not grow on synthetic complete medium lacking adenine (SC-Ade) and turn red on YPD medium (Derkatch *et al*., 1996) because of the accumulation of an oxidized byproduct of adenine biosynthesis (Fisher, 1969). Weak [*PSI*$^+$] *ade1-14* cells

Table 1. Plasmids.

Plasmid	Marker(s)	Sup35	Source
pFL44	*URA3*	None	Bonneaud *et al.* (1991)
pFL-SUP35	*URA3*	1–685	Derkatch *et al.* (2000)
pFL-Bcl	*URA3*	1–238 + PGRVPSSN	Derkatch *et al.* (2000)
pFL-Bal2	*URA3*	1–154 + GVPSSN	Derkatch *et al.* (2000)
pFL-SUP35$_{1–147}$	*URA3*	1–147	This study
pFL-SUP35$_{1–137}$	*URA3*	1–137	This study
pFL-SUP35$_{1–123}$	*URA3*	1–123	This study
pFL-EcoRV	*URA3*	1–113 + WVPSSN	Derkatch *et al.* (2000)
pEM-EcoRV$^{No\ Ext}$	*URA3 leu2-d*	1–113 + W	Derkatch *et al.* (2000)
pEM-EcoRV^{Ext}	*URA3 leu2-d*	1–113 + SRVDLQ ACKLMIQYQRK	Ter-Avanesyan *et al.* (1993); Derkatch *et al.* (2000)

grow slowly on SC-Ade and become pink on YPD medium as they read through (suppress) the premature stop codon in *ade1-14* to produce some functional Ade1 protein. Strong [*PSI*$^+$] cells grow well on SC-Ade and become white on YPD medium (Derkatch *et al.*, 1996).

At least eight primary cytoductants were tested for every combination of a recipient expressing one Sup35 fragment with a [*psi*$^-$], weak [*PSI*$^+$] or strong [*PSI*$^+$] donor (Table 2). The Sup35 wild-type protein (residues 1–685) faithfully maintained the donated [*psi*$^-$], weak [*PSI*$^+$] or strong [*PSI*$^+$] states, except for occasional secondary cytoductants that were [*psi*$^-$] despite receiving donated weak [*PSI*$^+$] cytoplasm. The Sup35 1–238, 1–154, 1–147 and 1–137 fragments, which contain the entire N region and

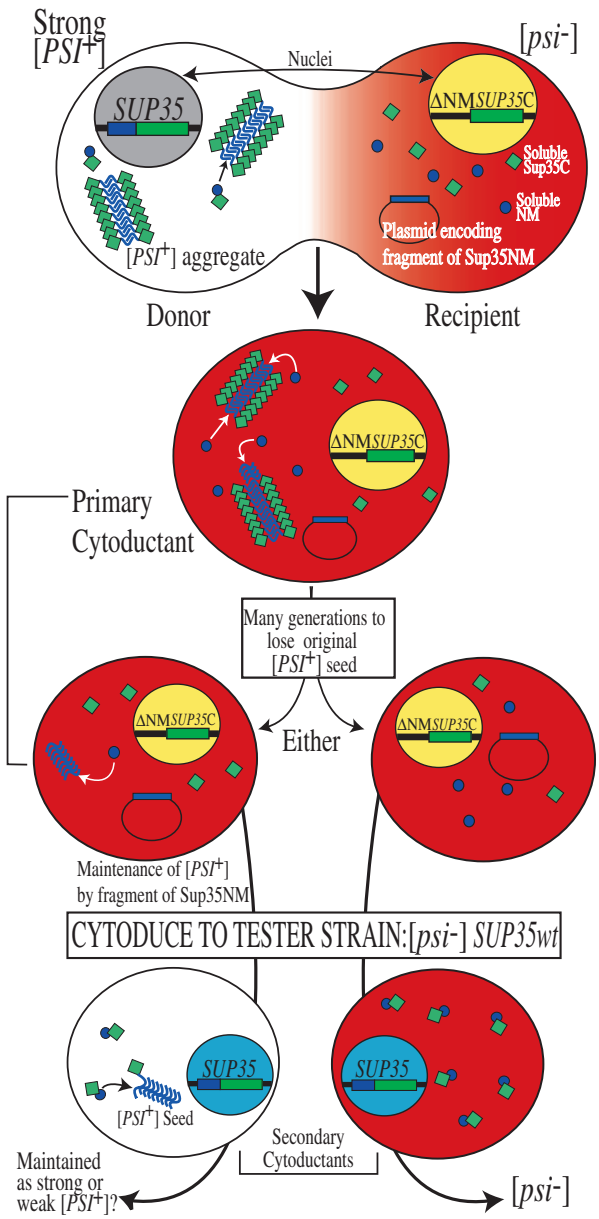

Fig. 1. Method for testing the ability of different Sup35 fragments to maintain [*PSI*$^+$] as well as the specificity of the original [*PSI*$^+$] variant. Shown above is the zygote of a donor and recipient cell. The donor, a derivative of yeast strain A3009 carrying a complete nuclear copy of *SUP35*, is shown bearing strong [*PSI*$^+$] aggregates. Here, soluble Sup35 (green diamond attached to blue circle) joins the [*PSI*$^+$] aggregates (ordered green diamonds and blue curves). Note that weak [*PSI*$^+$] or [*psi*$^-$] donor variants of A3009, although not depicted, were also used. The recipient is a [*psi*$^-$] derivative (L2275) of the yeast strain 74-D694, with a genomic deletion of the region of the *SUP35* gene encoding residues 2–254 (Δ*NMSUP35C*) and carrying a plasmid expressing one of the eight Sup35 N-terminal fragments tested. The primary cytoductants contain a mixture of the donor and recipient cytoplasms, but only the recipient's nucleus. The primary cytoductants are grown for at least 10 generations to ensure that the original Sup35 [*PSI*$^+$] seeds (ordered green diamonds and blue curves) are lost. To determine whether the soluble Sup35NM (blue circles) have been converted into prion aggregates (ordered blue lines), as shown on the left, or remain in the non-infectious form, as shown on the right, these primary cytoductants are used as donors in a second cytoduction. The recipient is a [*psi*$^-$] tester yeast strain, L2598, bearing the full-length wild-type *SUP35* gene that is required in order to score for the presence of [*PSI*$^+$]. If a Sup35NM seed is transmitted into the recipient, it will convert the wild-type soluble Sup35 into a Sup35 prion aggregate. Such cytoductants are then scored for the faithful retention of the original [*PSI*$^+$] variant from the A3009 donor. All yeast strains are [*pin*$^-$] to prevent the plasmid-encoded Sup35 fragments from inducing the *de novo* appearance of [*PSI*$^+$]. Cells are shown as red or white, when there is or is not, respectively, enough non-aggregated Sup35 or Sup35C to promote normal translational termination at the premature stop codon in the *ade1-14* allele present in all cells.

Table 2. Maintenance of [*PSI*$^+$] variants by Sup35 fragments.

Recipient		Donor					
		[*psi*$^-$]		Weak [*PSI*$^+$]		Strong [*PSI*$^+$]	
Sup35	NMD[a]	[*psi*$^-$]	[*PSI*$^+$]	[*psi*$^-$]	[*PSI*$^+$]	[*psi*$^-$]	[*PSI*$^+$]
None	Yes	12	0	15	0	15	0
1–685	Yes	16	0	0	19 w	0	37 s
	No	12	0	0	12 w	0	12 s
1–238	Yes	16	0	1	20 w	0	38 s
1–154	Yes	13	0	5	20 w	0	37 s
1–147	Yes	8	0	4	7 w	0	14 s
	No	13	0	2	21 w	0	16 s
1–137	Yes	8	0	4	5 w	1	14 s
	No	18	0	1	29 w	0	24 s
1–123	Yes	12	0	11	1 u	2	10 u
	No	17	0	27	11 u	6	28 u
1–113[b]	Yes	21	0	26	0	23	2 u
	No	22	0	42	0	22	18 u
$1–113_{Ura}$[c]	Yes	21	0	42	0	30	0
	No	15	0	25	0	19	4 u
$1–113_{Leu}$[c]	Yes	8	0	8	0	8	0
	No	24	0	59	1 u	42	14 u
$1–113^{Ext}$	Yes	0	9 w	0	18 w	0	16 w

a. NMD stands for nonsense-mediated decay. Yes indicates the presence of NMD in *UPF1* cells. No indicates the absence of NMD in *upf1* cells.
b. The pFL-EcoRV plasmid expresses 1–113 + WVPSSN.
c. The pEMBL-EcoRV$^{No\ Ext}$ plasmid expresses 1–113 + W and can be held at a normal copy number on SD-Ura ($1–113_{Ura}$) or at ≈ 100-fold increased copy number on SD-Leu ($1–113_{Leu}$).
Table entries indicate numbers of primary cytoductant colonies from each donor and recipient pairing scored as [*psi*$^-$] if only [*psi*$^-$] cells were in the colony, or as [*PSI*$^+$] (w for weak, s for strong, u for undifferentiated) if any [*PSI*$^+$] cells were in the colony. The Sup35 1–147, 1–137, 1–123 or 1–113 cytoductant colonies scored as [*PSI*$^+$] often contained few [*PSI*$^+$] cells (1–5%) and many [*psi*$^-$] cells.

variable amounts of the M region, behaved similarly to Sup35 wild type, except that there were many more [*psi*$^-$] cells among the cytoductants in experiments with the 1–147 and 1–137 fragments (see legend to Table 2). In all cases, however, donating strong [*PSI*$^+$] to recipients expressing any of these five proteins resulted in cytoductants that contained strong but never weak [*PSI*$^+$]. Conversely, donating weak [*PSI*$^+$] into these recipients resulted in cytoductants that contained weak but never strong [*PSI*$^+$]. The shortest fragment with these behaviours, Sup35 1–137, contained only the first 14 residues of the M region and represents the minimal amount of sequence information determined to be sufficient to faithfully maintain weak [*PSI*$^+$] or strong [*PSI*$^+$] variants.

*Identification of Sup35 fragments that propagate an 'undifferentiated' [*PSI$^+$*] rather than maintain the specific weak or strong variants*

The cytoplasms from weak [*PSI*$^+$] or strong [*PSI*$^+$] cells were donated to *sup35*-Δ2-254 recipients expressing Sup35 1–123 or Sup35 1–113, and the cytoplasms from these primary cytoductants were then donated to a *SUP35* wild-type, *ade1-14* [*psi*$^-$] recipient yeast strain that could be scored as [*psi*$^-$], weak [*PSI*$^+$] or strong [*PSI*$^+$]. As was true of the Sup35 1–147 and 1–137 fragments described above, the majority of these secondary cytoductants were red (indicative of [*psi*$^-$]). However, unlike the 1–147 and 1–137 cases, here both pink and white colonies (diagnostic for weak and strong [*PSI*$^+$] respectively) as well as unusual pink-white sectored colonies were always observed. This suggested to us that an 'undifferentiated' [*PSI*$^+$] variant, capable of turning into both strong and weak [*PSI*$^+$], had been maintained (Table 2). The 'undifferentiated' [*PSI*$^+$] variant never appeared after donating cytoplasm from [*psi*$^-$] cells or in experiments with larger (1–137 or longer) fragments.

We verified that the failures of Sup35 1–113 or 1–123 to faithfully maintain the weak or strong variants were not due to their having decreased protein levels compared with other Sup35 fragments such as Sup35 1–137 or 1–147. When the [*PSI*$^+$] maintenance experiment shown in Fig. 1 was repeated using an increased plasmid level to express higher levels of Sup35 1–113, the weak or strong variants were again not maintained (Table 2). It is important to note that the level of the fragments present in these cells was never as high as the normal wild-type level of Sup35, even when the highest plasmid copy number conditions were used (data not shown). As most of the plasmids lacked transcription termination signals and thus encoded 3′ extended transcripts that were targeted by the nonsense-mediated decay (NMD) pathway (Leeds *et al.*, 1992), the [*PSI*$^+$] maintenance experiment was repeated using a 74-D694 derivative deleted for *UPF1* and therefore defective in NMD. The inactivation of NMD increased the Sup35 fragment level (data not shown) and generally enabled them to maintain [*PSI*$^+$] more efficiently, but never changed the variant of [*PSI*$^+$] that was maintained (Table 2). Moreover, the amounts of two Sup35 protein fragments that faithfully maintain weak or strong [*PSI*$^+$] were identical to the amounts of two Sup35 protein fragments that do not maintain the weak or strong variants (Fig. 2).

We hypothesized that the large number of red ([*psi*$^-$]) colonies present among the secondary cytoductants (see legend to Table 2) arose because the primary cytoductant colonies were a mixture of [*psi*$^-$] and [*PSI*$^+$] cells. Indeed, when primary cytoductant colonies were restreaked, they gave rise to a minority of 'purified' colonies that could transmit undifferentiated [*PSI*$^+$] and a majority of colonies that did not transmit [*PSI*$^+$]. Furthermore, restreaking the purified colonies that could transmit undifferentiated [*PSI*$^+$] showed that most (≈ 85%) of these colonies were able to transmit undifferentiated [*PSI*$^+$]. The purified primary cytoductants capable of transmitting undifferentiated [*PSI*$^+$] contained aggregated Sup35 1–113. Similarly,

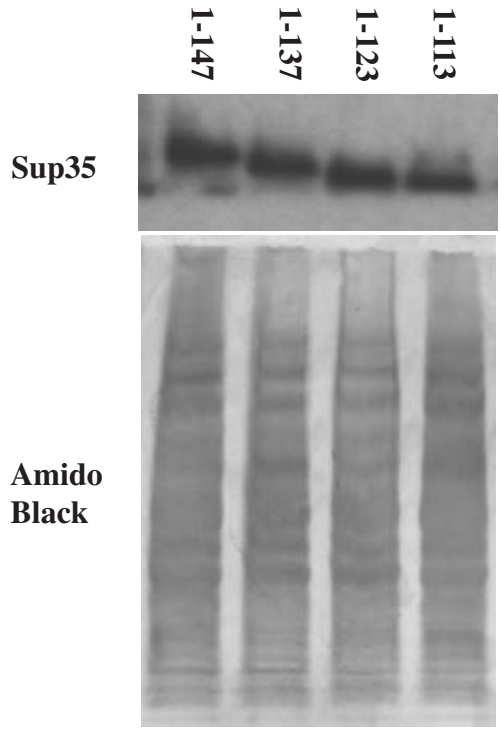

Fig. 2. Sup35 fragments that faithfully maintain (1–147 and 1–137) or fail to maintain (1–123 and 1–113) weak and strong [*PSI*$^+$] variants are expressed in similar amounts. A *sup35*-Δ2-254 *upf1* yeast strain, L2698, was transformed with plasmids pFL-SUP35$_{1–147}$, pFL-SUP35$_{1–137}$, pFL-SUP35$_{1–123}$ or pFL-EcoRV (1–113), expressing the indicated fragments of Sup35. Top, reactivity with a Sup35 N-specific antibody. Bottom, membrane stained with a non-specific protein stain, amido black.

purified strong or weak [*PSI*$^+$] primary cytoductants maintained with the larger Sup35 1–137 fragment, and composed of >95% strong or weak [*PSI*$^+$] cells, respectively, contained aggregated Sup35 1–137 (Fig. 3; data not shown).

When pink-white sectored colonies from undifferentiated [*PSI*$^+$] secondary cytoductants were restreaked, the new colonies were ≈47% strong [*PSI*$^+$] and ≈47% pink-white sectored (undifferentiated [*PSI*$^+$]), but ≈5% of the colonies were always weak [*PSI*$^+$]. Thus, it appears that the undifferentiated [*PSI*$^+$] variant, when donated into *SUP35* wild-type cells, can differentiate into strong and weak [*PSI*$^+$] variants. The pink-white sectored colonies were restreaked seven consecutive times, and the distribution of strong, undifferentiated and weak [*PSI*$^+$] colonies was unchanged after each restreaking. As is well known for weak and strong [*PSI*$^+$], the undifferentiated [*PSI*$^+$] was lost when cells were grown in the presence of guanidine hydrochloride.

Although these results show that Sup35 1–123 and 1–113 are not able to maintain the identity of weak [*PSI*$^+$] or strong [*PSI*$^+$] variants that were induced by overproducing Sup35 wild type, overproducing Sup35 1–123 and 1–113 does induce stable weak and strong [*PSI*$^+$] variants in [*PIN*$^+$] *SUP35* wild-type cells (data not shown; Derkatch *et al*., 2000). We hypothesized that the weak and strong [*PSI*$^+$] variants induced by overproducing Sup35 1–123 or 1–113 in [*PIN*$^+$] *SUP35* wild-type cells might somehow be different from the weak and strong [*PSI*$^+$] variants induced by overproducing Sup35 wild type and therefore might be faithfully maintained by the Sup35 1–123 or 1–113. However, we found that four weak [*PSI*$^+$] and five strong [*PSI*$^+$] variants induced by overproducing Sup35 1–113 (see *Experimental procedures*) were not faithfully maintained by Sup35 1–113, but were faithfully maintained by Sup35 wild type, 1–238 or 1–154 (data not shown). Thus, it appears that Sup35 1–113 (and by inference 1–123) lack the required sequence information for maintaining any weak or strong [*PSI*$^+$] variants.

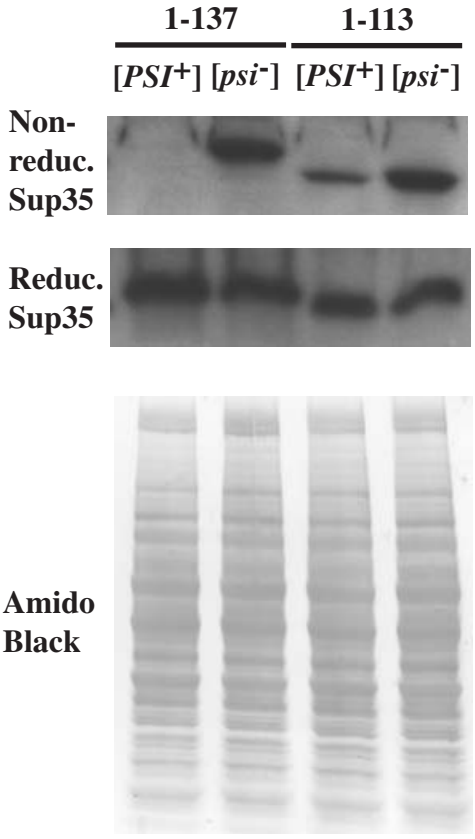

Fig. 3. Sup35 fragments are aggregated in [*PSI*$^+$] but not [*psi*$^-$] cells. A *sup35*-Δ2-254 *upf1* yeast strain, L2698, was transformed with plasmids pFL-SUP35$_{1–137}$ or pFL-EcoRV (1–113). Protein was from purified [*PSI*$^+$] or [*psi*$^-$] cytoductants of L2698 transformants that received cytoplasm from strong [*PSI*$^+$] or [*psi*$^-$] donors respectively. Top, reactivity with a Sup35 N-specific antibody after protein samples were treated with non-reducing or reducing conditions. Without reducing, the aggregated Sup35 fragments from [*PSI*$^+$] cells do not enter the gel. After reducing, equal amounts of soluble protein are present in [*PSI*$^+$] versus [*psi*$^-$] lanes. Bottom, membrane containing non-reduced protein stained with the non-specific protein stain, amido black.

Sup35 fragments that induce [PSI⁺] but do not maintain [PSI⁺]

In earlier work using a similar two-step cytoduction scheme, a Sup35 1–113 fragment with a 17-residue extension (1–113^{Ext}) seemed to maintain [*PSI*$^+$] (Ter-Avanesyan *et al*., 1994). We tested whether Sup35 1–113^{Ext} could maintain weak [*PSI*$^+$] or strong [*PSI*$^+$] (Table 2). Weak [*PSI*$^+$] was always recovered whether the donated cytoplasm was from [*psi*$^-$], weak [*PSI*$^+$] or strong [*PSI*$^+$] cells. These results indicate that the Sup35 1–113^{Ext} fragment is not amenable to the test of [*PSI*$^+$] maintenance. Sup35 1–113^{Ext} is extremely efficient at inducing [*PSI*$^+$] in [*pin*$^-$] cells such as these (Derkatch *et al*., 2000), and preferentially induces weak [*PSI*$^+$] variants (Kochneva-Pervukhova *et al*., 2001). Thus, it is highly probable that the recovered weak [*PSI*$^+$] were newly induced by Sup35 1–113^{Ext} in each cytoductant.

Discussion

We have shown that [*pin*$^-$] cells expressing Sup35 1–137 faithfully maintained the donated weak [*PSI*$^+$] or strong [*PSI*$^+$] variants, whereas [*pin*$^-$] cells expressing Sup35 1–123 (or 1–113) appear only to propagate the donated [*PSI*$^+$] as an undifferentiated variant. Thus, the 14 residues that span positions 124–137 enable the weak [*PSI*$^+$] or strong [*PSI*$^+$] variants to be faithfully maintained. The general method used here to identify the sequences needed to maintain specific prion variants can be applied to other prions that exhibit strain variation, such as the yeast [*URE3*] and [*PIN*$^+$] prions (Schlumpberger *et al*., 2001; Bradley *et al*., 2002) and the mammalian PrP prion (Bruce, 1996; Birkett *et al*., 2001).

The undifferentiated [PSI⁺] variant

We propose that residues 1–113 or 1–123 of the Sup35 N region are sufficient to propagate a new 'undifferentiated' [*PSI*$^+$] variant. When seeded by exposure to either weak or strong full-length Sup35 [*PSI*$^+$] aggregates, the [*PSI*$^+$] variants that appear in the primary cytoductants are undifferentiated, because no matter which [*PSI*$^+$] seed was used, cytoduction of the fragments back to a cell with full-length Sup35 causes the appearance of stable weak, stable strong and unstable strong/weak (undifferentiated) [*PSI*$^+$] variants. Another way of looking at these data is to say that the 1–113 or 1–123 fragments do not really maintain [*PSI*$^+$] at all, but rather cause the *de novo* induction of an undifferentiated variant of [*PSI*$^+$] in the primary cytoductants. According to this view, the donated [*PSI*$^+$] prions would serve the role normally ascribed to [*PIN*$^+$]. This would be possible as even the low level of these fragments present in cells with the high-copy plasmids encoding them (substantially lower than the normal level of the wild-type Sup35 protein; Kochneva-Pervukhova *et al*., 1998; data not shown) can induce the *de novo* appearance of [*PSI*$^+$] (Kochneva-Pervukhova *et al*., 1998; Derkatch *et al*., 2000).

The finding that [*PIN*$^+$] is a prion, and that its role in the induction of [*PSI*$^+$] can be supplied by another heterologous prion or overexpression of prion-like genes, led to the hypothesis that heterologous prions or prion-like aggregates template the *de novo* conversion of Sup35 to the [*PSI*$^+$] prion form (Derkatch *et al*., 2001). If this hypothesis is true, this blurs the difference between *de novo* induction and propagation. In propagation, homologous prion seeds lead to the faithful replication of the prion seed's variant or strain type. In *de novo* induction, a heterologous seed templates prion formation and results in the induction of a range of variant types. The transmission of [*PSI*$^+$] via the 1–113 or 1–123 fragments has elements of both propagation and induction. On the one hand, seeding is by a homologous [*PSI*$^+$] prion but, on the other hand, the prion seed's variant type is not faithfully transmitted. In our opinion, this phenomenon is best described as transmission of strong or weak [*PSI*$^+$] variants to an undifferentiated [*PSI*$^+$] state. Indeed, colonies containing the large pink/white sectors that characterize the undifferentiated [*PSI*$^+$] were never observed when [*PSI*$^+$] was induced *de novo* in the absence of [*PSI*$^+$] (in [*PIN*$^+$] cells) (Derkatch *et al*., 2000; Kochneva-Pervukhova *et al*., 2001; Bradley and Liebman, 2003). Although it is possible that only the [*PSI*$^+$] seed can promote the *de novo* appearance of the undifferentiated [*PSI*$^+$] variant, just as only the very high [*PIN*$^+$] was found to promote the *de novo* appearance of an unusual weak [*PSI*$^+$] that gives off stable strong [*PSI*$^+$] (Kochneva-Pervukhova *et al*., 2001; Bradley and Liebman, 2003), this seems unlikely. Furthermore, as the ability of the Sup35 fragments to induce [*PSI*$^+$] is known to increase with their decreasing lengths (Kochneva-Pervukhova *et al*., 1998), the same correlation would be expected to be observed in our experiments if the *de novo* induction hypothesis were true; however, the opposite correlation was seen (Table 2).

The undifferentiated [*PSI*$^+$] variant contains aggregated Sup35 1–113 (or 1–123) and ≈15% of purified primary cytoductant cells lose [*PSI*$^+$]. In contrast, the weak or strong [*PSI*$^+$] variants contain aggregated Sup35 1–137 (or longer), and <1% of purified primary cytoductant cells lose [*PSI*$^+$]. Surprisingly, undifferentiated Sup35 1–123 [*PSI*$^+$] aggregates initially seed Sup35 wild-type proteins as undifferentiated. Unlike [*PSI*$^+$] aggregates made only of Sup35 1–123, however, undifferentiated [*PSI*$^+$] aggregates made of the Sup35 wild-type proteins consistently differentiate into strong [*PSI*$^+$] and occasionally weak [*PSI*$^+$]. Thus, Sup35 wild-type protein stably maintains [*psi*$^-$], weak [*PSI*$^+$] or strong [*PSI*$^+$] forms, and can tempo-

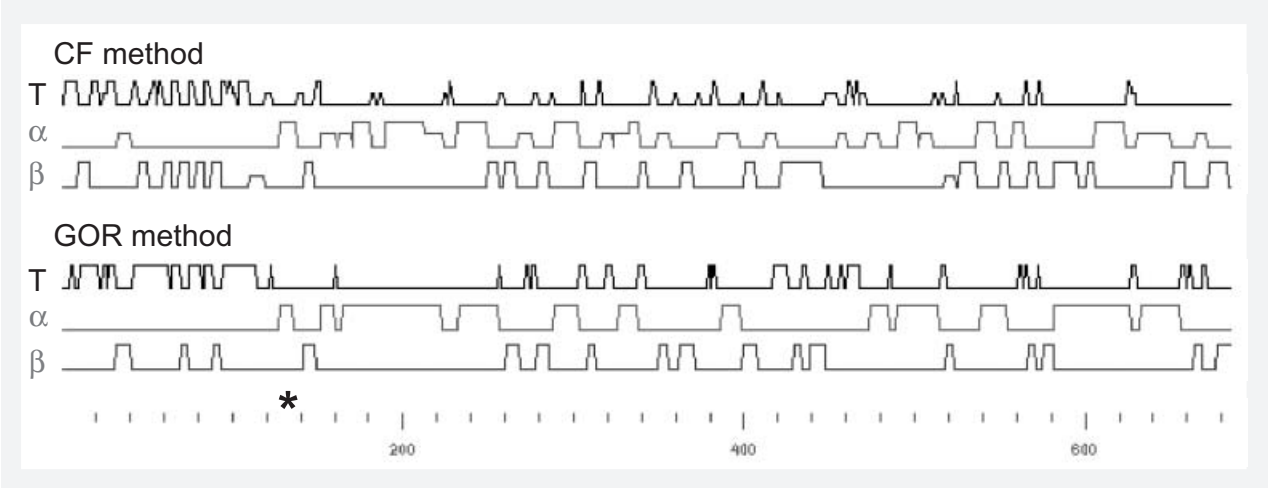

Fig. 4. Secondary structure predictions of Sup35. The helices (α) and strands (β) as predicted by two methods, Chou–Fasman (CF) (Chou and Fasman, 1978) and Garnier–Osguthorpe–Robson (GOR) (Garnier *et al*., 1996), are plotted along Sup35's 685 residues. Taller peaks indicate more reliable predictions than shorter peaks. The α-helix predicted for the residues MSLNDFQKQQKQAA of the 124–137 region is marked (*). Seven other independent methods (DPM, DSC, HNNC, MLR, PHD, Predator, SOPM) also predict α-helical structure for the 124–137 region. Four of the nine methods used (CF, DSC, GOR, SOPM) predict between three and 12 β-strands in the N region.

rarily propagate an undifferentiated [*PSI*$^+$] form if seeded by undifferentiated [*PSI*$^+$] aggregates.

*The molecular basis of [*PSI$^+$*] variants*

Numerous methods predict an α-helical secondary structure of the Sup35 124–137 region (Fig. 4). We present the following model of how an α-helix, if present in this region, could control the formation of weak versus strong [*PSI*$^+$]. As predicted by some methods, the Sup35 N region (residues 1–123) may contain several β-strands (Fig. 4). β-Strands containing polar residues, which predominate in the Sup35 N region, are prone to self-interactions through hydrogen bonding (Perutz *et al*., 1994), leading to the formation of various types of β structure. Peptides with only β structure often aggregate through intermolecular edge-to-edge hydrogen bonding (Mattice, 1989) and, in most natural proteins, the edge strands of β structures are covered by other secondary structural elements (helices, loops, etc.) to avoid edge-to-edge hydrogen bonding and aggregation (Richardson and Richardson, 2002). We propose that the α-helix predicted in the 124–137 region acts in this way to moderate the Sup35–Sup35 interaction driven by the Sup35 N region's putative β structure. If this is correct, the weak [*PSI*$^+$] and strong [*PSI*$^+$] variants could arise from Sup35's ability to take on two conformations that differ in their positioning of the 124–137 region relative to the 1–123 region. The undifferentiated [*PSI*$^+$] variant, which is faithfully maintained by Sup35 1–123 or 1–113 but differentiates frequently into strong or weak [*PSI*$^+$] when maintained by Sup35 wild type, could arise when the 124–137 α-helical region is completely absent, or when it is in a neutral position relative to the 1–123 region.

Although the actual 124–137 region of Sup35 is predicted to be α-helical, sequences artificially placed adjacent to the 1–123 region that form other structures such as a loop or bulge could also fulfil the role proposed in the above model (Richardson and Richardson, 2002) to maintain different positions relative to the 1–123 region that correspond to different heritable prion variations. Our data are also compatible with the possibility that a minimal length requirement of 137 residues enables the maintenance of [*PSI*$^+$] variants.

Defining the prion domain of Sup35

How can a prion domain be defined? Our work shows that separate domains need to be defined for prion induction and maintenance. The prion-inducing domain is the minimal sequence that can induce the appearance of the prion when overproduced. By this definition, the minimal prion-inducing domain of Sup35 would be as small as the first 61 residues, which is the smallest fragment reported to induce the appearance of [*PSI*$^+$] in Sup35 wild-type cells (King, 2001). The prion-maintaining domain would be defined as the minimal sequence that can maintain the prion state. Further complicating matters, the minimal prion-maintaining domain can be defined differently for different prion variants. The minimal prion-maintaining domain for undifferentiated [*PSI*$^+$] is Sup35 1–113 whereas for weak or strong [*PSI*$^+$], it is Sup35 1–137.

Certain protein fragments that are unable to maintain a prion can nevertheless induce the *de novo* appearance of

the prion. The confusion of prion induction with prion maintenance can be avoided using conditions that do not lead to the *de novo* appearance of that protein's prion form. For example, the protein fragments tested in our experiments did not induce the *de novo* appearance of [*PSI*$^+$] because they were only expressed in cells that lacked heterologous prions, such as [*PIN*$^+$], that normally allow these protein fragments to induce the *de novo* appearance of [*PSI*$^+$].

The minimal sequence that is sufficient for maintaining a prion in one context may not be capable of maintaining it in others. For example, Sup35 1–123 failed to maintain the identity of weak or strong [*PSI*$^+$] in our assay but, when it was fused to the 254–685 region of Sup35, the fusion was able to maintain weak and strong [*PSI*$^+$] (Liu *et al*., 2002). Interestingly, the fusion of Sup35 1–123 to Sup35 254–685 may recreate the situation in which a structural element covers the edge strand of the series of predicted β-strands in the N region.

Why is it useful to know the minimal sequence that can maintain a prion *in vivo*? The number of known prion proteins is small (≈ five), but it is suspected that many others may lie undiscovered. For example, database searches uncovered more than 100 potential new yeast prion proteins (and many more from other organisms) as those having sequence similarity to Sup35 or Ure2 prion domains (Michelitsch and Weissman, 2000; Sondheimer and Lindquist, 2000). A more precise definition of the functional prion domain sequences would refine such searches.

Some investigators have studied the *in vitro* aggregation properties of purified prion proteins as a more controlled way of studying prion replication, and it is unclear how similar these are to *in vivo* [*PSI*$^+$] aggregates. Purified Sup35 1–113 forms amyloid fibrils *in vitro* (Glover *et al*., 1997; King *et al*., 1997), but we find that Sup35 1–123 or 1–113 is not sufficient for maintaining the distinction between weak or strong [*PSI*$^+$] variants *in vivo*. This implies that the *in vitro* amyloid formation of Sup35 1–113 cannot correspond to *in vivo* prion variants.

As prion diseases are untreatable, controlling their transmission and spread is paramount. In some instances, prions are even thought to be transmitted between species with different prion protein sequences. Knowing the precise sequences required to maintain a prion should therefore lead to more predictability of its interspecies transmission range.

Experimental procedures

Cultivation procedures

Standard yeast media and cultivation were used (Sherman *et al*., 1986). Cells were grown at 30°C. Synthetic complete media contained dextrose (SC) or glycerol (SGly) and the required amino acids. Growth on yeast extract–peptone–dextrose medium (YPD) with 5 mM guanidine hydrochloride (+GuHCl) was used to eliminate [*PSI*$^+$] and [*PIN*$^+$] (Tuite *et al*., 1981; Derkatch *et al*., 1997). Growth on YPD medium with 40 mg l^{-1} ethidium bromide was used to convert [*RHO*$^+$] cells to [*rho*$^-$] (Goldring *et al*., 1970). Donating cytoplasm from one yeast strain to another (cytoduction) was performed between [*RHO*$^+$] donors and [*rho*$^-$] recipients, and at least one of the yeast strains involved had a non-functional *KAR1* allele in order to reduce the efficiency of nuclear fusion after mating (Conde and Fink, 1976).

*Testing Sup35 fragments for their ability to maintain [*PSI$^+$*] variants*

Plasmids (Table 1) were based on either the *URA3* 2μ vector, pFL44 (Bonneaud *et al*., 1991), or the *URA3 leu2-d* 2μ vector, pEMBL (Cesarini and Murray, 1987), that can be held at an ≈10-fold elevated copy number on SC media lacking uracil (SC-Ura) or an ≈100-fold increased copy number on SC media lacking leucine (SC-Leu) because of a defective *LEU2* promoter on this vector. New plasmids were constructed by inserting *Eco*RI-digested PCR amplicons, which were engineered to contain the 1099 bp upstream of the *SUP35* start codon and the 369 (amino acids 123), 411 (amino acids 137) or 441 (amino acids 147) bp downstream of the start codon followed by a stop codon, into *Eco*RI-digested pFL44. All plasmids were verified to have the correct structure by sequencing the inserts. The PCR target was always pFL-SUP35, and the primers were CCG GAA TTC AAG GAA TCG TCG AGA CCA CCA (#336) paired with CCG GAA TTC AAC CTT GAG ACT GTG GTT GGA A (#337), CCG GAA TTC AAG CGG CCT GCT TTT GTT GCT (#338) or CCG GAA TTC AGA CAA GCT TCA AAG TCT TCT T (#339).

A derivative of the yeast strain 74-D694 (*MAT*a *ade1-14 leu2-1 his3-Δ200 trp1-289 ura3-52*) (Chernoff *et al*., 1993) with the chromosomal *sup35-Δ2*-254 allele (L1936 [*psi*$^-$]) (Derkatch *et al*., 1997) was made [*pin*$^-$] and [*rho*$^-$] (L2275) as described above, and transformed with the plasmids listed in Table 1. The L2275 transformants were selected on SC-Ura and grown on SC-Ura or SC-Leu. Cytoplasms were donated to transformed L2275 recipients from [*psi*$^-$] (L2199), weak [*PSI*$^+$] (L2200) or strong [*PSI*$^+$] (L2201) [*RHO*$^+$] [*pin*$^-$] derivatives of the yeast strain A3099 (*MATα ade2-1 SUQ5 lys1-1 his3-11,15 leu1 ura3::kanMX4 kar1-1*) (Sondheimer and Lindquist, 2000). The L2275 cytoductants were selected by streaking mating mixtures on SGly-Ura or SGly-Leu media, and colonies were screened to identify those that had the recipient's mating type. The cytoplasm of each L2275 cytoductant was donated to a [*psi*$^-$] [*rho*$^-$] [*pin*$^-$] *cyh*^{R} and *kar1-d15* derivative (L2598) of the yeast strain L1845 (*MATα ade1-14 leu2-1 his3-Δ200 trp1-289 ura3-52*) (Bradley *et al*., 2002) by making patches from each cytoductant colony and crossing them to a lawn of L2598 on YPD medium. The L2598 cytoductants were selected by replica plating from YPD to SGly medium containing 3 mg l^{-1} cycloheximide (SGly + Cyh), in which the L2275 donors and any L2275/L2598 diploids are unable to grow. The secondary cytoductants were always unable to grow on –Ura medium, indicating that they did not acquire the plasmids from the donors. The [*PSI*$^+$]-mediated nonsense suppression of *ade1-14* in the L2598

cytoductants was determined by replica plating from SGly + Cyh to YPD and SC-Ade media. If the weak [*PSI*$^+$] versus strong [*PSI*$^+$] distinction was not clear because too few cells in the patch were [*PSI*$^+$], the L2598 cytoductants were instead streaked from SGly + Cyh to YPD medium, where individual [*PSI*$^+$] colonies were isolated and tested further on YPD and SC-Ade media.

Four weak [*PSI*$^+$] and five strong [*PSI*$^+$] isolates were induced in a [*psi*$^-$] [*PIN*$^+$] *SUP35* wild-type derivative (L1749) of the yeast strain 74-D694 by overproducing Sup35 1–113 from pFL-EcoRV. The resulting [*PSI*$^+$] [*PIN*$^+$] derivatives were grown briefly on YPD + GuHCl, which can eliminate the [*PSI*$^+$] and [*PIN*$^+$] prions independently (Tuite *et al*., 1981; Derkatch *et al*., 1997; 2000). Derivatives that had lost [*PIN*$^+$] but not [*PSI*$^+$] were identified as those lacking aggregated Rnq1, as determined by mating to a [*psi*$^-$] [*pin*$^-$] derivative (L1798) of the yeast strain SL1010-1A (*MAT*α *ade1-14 met8-1 leu2-1 his5-2 trp1-1 ura3-52*) (Zhou *et al*., 1999) already transformed with a *URA3*-based centromeric vector containing a fusion of *RNQ1* and GFP (Rnq1-GFP) (Sondheimer and Lindquist, 2000; Derkatch *et al*., 2001). The cytoplasms from the resulting weak [*PSI*$^+$] [*pin*$^-$] and strong [*PSI*$^+$] [*pin*$^-$] derivatives (L2528–L2536) were donated to a [*psi*$^-$] [*pin*$^-$] derivative (L1959) of the yeast strain A3099 to generate donors (L2550–L2558) suitable for the first cytoduction step shown in Fig. 1.

The yeast strain L2698 was generated by replacing wild-type *SUP35* with *sup35*-Δ2-254 (Derkatch *et al*., 1997) in a derivative of 74-D694 in which *UPF1* had previously been disrupted with *kanMX4* (Derkatch *et al*., 2000). The L2698 *sup35*-Δ2-254 *upf1* yeast strain was transformed with the plasmids listed in Table 1, and the [*PSI*$^+$] maintenance experiment shown in Fig. 1 was repeated.

Western detection of Sup35 fragments

L2698 transformants were grown in liquid SC-Ura, and harvested cells were resuspended in lysis buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 0.2% Triton X-100, 1.9 μg ml^{-1} aprotinin, 3.5 μg ml^{-1} E-64, 5 μg ml^{-1} leupeptin, 5 μg ml^{-1} pepstatin, 400 μg ml^{-1} 1,10 phenanthroline, 500 μg ml^{-1} phenylmethylsulphonyl fluoride (PMSF), 50 μg ml^{-1} *N*-(*p*-tosyl)lysine chloromethyl ketone (TLCK)] and mixed with 1000 μl of glass bead/lysis buffer slurries. Total protein lysates were obtained by vortexing tubes 10 separate times for 10 s with intermittent incubations on ice and removing cell debris at 10 000 *g* for 10 min. The protein concentrations of the cleared lysates were measured (Bio-Rad protein assay), and lysis buffer was added to normalize the samples. The reducing agent 2-mecaptoethanol was added at a final concentration of 5%, and the protein samples were placed in boiling water for 5 min. Proteins were separated by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Sup35 fragments were detected using a primary antibody directed against the Sup35 N region (a kind gift from S. Lindquist, Whitehead Institute) and a secondary antibody conjugated to alkaline phosphatase (Applied Biosystems).

In order to compare the aggregation state of Sup35 fragments, total protein was isolated in a modified lysis buffer (25 mM Tris, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 1.9 μg ml^{-1} aprotinin, 3.5 μg ml^{-1} E-64, 5 μg ml^{-1} leupeptin, 5 μg ml^{-1} pepstatin, 400 μg ml^{-1} 1,10 phenanthroline, 500 μg ml^{-1} PMSF, 50 μg ml^{-1} TLCK), and cell debris was removed at 3000 *g* for 5 min. The non-reduced samples were then normalized and loaded into the gel without any further treatment. Proteins were separated, transferred and detected as described above.

Sup35 secondary structure prediction

A total nine independent methods were used to predict the secondary structure of Sup35. The graphic in Fig. 4 displaying the predictions from two of the nine methods was generated at the Saccharomyces Genome Database (http://www.yeastgenome.org) with the Wisconsin Sequence Analysis Package® from the Genetics Computer Group. The other seven methods were accessed at the NPS@(Network Protein Sequence Analysis) website (http://npsa-pbil.ibcp.fr) (Combet *et al*., 2000).

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