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The yeast non-Mendelian factor [*ETA*$^+$] is a variant of [*PSI*$^+$], a prion-like form of release factor eRF3

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The yeast non-Mendelian factor [*ETA*$^+$] is lethal in the presence of certain mutations in the *SUP35* and *SUP45* genes, which code for the translational release factors eRF3 and eRF1, respectively. One such mutation, *sup35-2*, is now shown to contain a UAG stop codon prior to the essential region of the gene. The non-Mendelian inheritance of [*ETA*$^+$] is reminiscent of the yeast [*PSI*$^+$] element, which is due to a self-propagating conformation of Sup35p. Here we show that [*ETA*$^+$] and [*PSI*$^+$] share many characteristics. Indeed, like [*PSI*$^+$], the maintenance of [*ETA*$^+$] requires the N-terminal region of Sup35p and depends on an appropriate level of the chaperone protein Hsp104. Moreover, [*ETA*$^+$] can be induced *de novo* by excess Sup35p, and [*ETA*$^+$] cells have a weak nonsense suppressor phenotype characteristic of weak [*PSI*$^+$]. We conclude that [*ETA*$^+$] is actually a weak, unstable variant of [*PSI*$^+$]. We find that although some Sup35p aggregates in [*ETA*$^+$] cells, more Sup35p remains soluble in [*ETA*$^+$] cells than in isogenic strong [*PSI*$^+$] cells. Our data suggest that the amount of soluble Sup35p determines the strength of translational nonsense suppression associated with different [*PSI*$^+$] variants.

Keywords: [*ETA*]/prion/[*PSI*]/release factor/*SUP35*

Introduction

The prion concept was originally used to describe an infectious protein, PrP, hypothesized to cause several transmissible spongiform encephalopathies, including human Kuru, Creutzfeldt–Jakob disease, mad cow disease and sheep scrapie (Griffith, 1967; Prusiner, 1982). The infectivity of prions is proposed to be due to a self-propagating protein conformation, capable of converting the normal form of the protein (PrP^C) into its prion (PrP^{Sc}) conformation (for reviews see Prusiner, 1996; Caughey and Chesebro, 1997; Weissmann, 1999).

[*PSI*$^+$] and [*URE3*] are heritable yeast factors that have been proposed to be prion-like forms of Sup35p and Ure2p, respectively (Wickner, 1994). [*PSI*$^+$] causes increased read-through of stop codons with or without other nonsense suppressors (for review see Cox *et al*., 1988). [*URE3*] derepresses genes coding for nitrogen catabolic enzymes that would normally be repressed by a good nitrogen source (Aigle and Lacroute, 1975). Both [*PSI*$^+$] and [*URE3*] exhibit a non-Mendelian pattern of inheritance and can be eliminated by stress-inducing compounds and the protein-denaturing agent guanidine hydrochloride (GuHCl) (Singh *et al*., 1979; Tuite *et al*., 1981; Aigle and Lacroute, 1975, cited by Wickner, 1994).

Genetic and biochemical evidence strongly supports the hypothesis that [*PSI*$^+$] is a self-propagating conformation of Sup35p. Sup35p is the yeast homologue of the eukaryotic release factor eRF3 (a GTPase) which, together with the other release factor Sup45p/eRF1, recognizes stop codons and facilitates the release of nascent proteins from ribosomes (Frolova *et al*., 1994; Stansfield *et al*., 1995; Zhouravleva *et al*., 1995). Sup35p can be divided roughly into two domains. The C-terminal region (432 amino acids) is homologous to EF-1α, contains GTP-binding domains, is essential for viability (Kushnirov *et al*., 1988; Wilson and Culbertson, 1988; Ter-Avanesyan *et al*., 1993) and is probably responsible for release-factor activity. The N-terminal region (253 amino acids) is not essential for viability, but is required for [*PSI*$^+$] maintenance (Doel *et al*., 1994; Ter-Avanesyan *et al*., 1994). Moreover, overexpression of this N-terminal region, as well as of the complete *SUP35* gene, induces the appearance of [*PSI*$^+$] *de novo* (Chernoff *et al*., 1993; Derkatch *et al*., 1996; Patino *et al*., 1996). The fact that an appropriate level of the chaperone protein Hsp104 is required for the propagation of [*PSI*$^+$] suggests that [*PSI*$^+$] heredity is based on a change in the conformation of Sup35p (Chernoff *et al*., 1995). Indeed, in [*PSI*$^+$] cells Sup35p forms insoluble aggregates ($Sup35^{PSI+}$), which are partially resistant to proteinase K, whereas in [*psi*$^-$] cells Sup35p is soluble ($Sup35^{psi-}$) (Patino *et al*., 1996; Paushkin *et al*., 1996). The aggregation of Sup35p in [*PSI*$^+$] cells apparently reduces the level of functional Sup35p, providing a simple explanation for the nonsense-suppressor phenotype of [*PSI*$^+$]. Sup35p or its N-terminal region, purified from *Escherichia coli*, forms amyloid fibers *in vitro* and these fibers form much more rapidly when they are 'seeded' by previously formed fibers (Glover *et al*., 1997; King *et al*., 1997). Moreover, in cell-free lysates, Sup35p can be converted from a soluble to an aggregated form characteristic of [*PSI*$^+$] cells upon incubation with $Sup35^{PSI+}$ (Paushkin *et al*., 1997a). These data support the hypothesis that the conversion of newly synthesized soluble Sup35p into aggregated $Sup35^{PSI+}$ by pre-existing aggregated $Sup35^{PSI+}$ is responsible for the propagation of the [*PSI*$^+$] factor.

In addition to [*PSI*$^+$] and [*URE3*] there may be other prions in yeast. Indeed, [*PIN*$^+$] and [*ETA*$^+$], two other GuHCl-curable non-Mendelian elements of yeast, have been proposed to be yeast prions (Wickner *et al*., 1996;

Derkatch *et al*., 1997; Liebman and Derkatch, 1999), although the molecular basis of these factors is unknown. [*PIN*$^+$] determines the possibility that [*PSI*$^+$] can appear *de novo* in yeast strains (Derkatch *et al*., 1997). [*ETA*$^+$] causes a lethal interaction with certain recessive mutant alleles of the release factors, e.g. *sup35-2* and *sup45-2*, which themselves have a nonsense-suppressor phenotype (Liebman and All-Robyn, 1984). When an [*ETA*$^+$] strain is crossed to a strain bearing either *sup35-2* or *sup45-2*, almost all the *sup35-2* or *sup45-2* meiotic segregants are dead, whereas the wild-type *SUP35* or *SUP45* segregants are alive. This behavior of [*ETA*$^+$] is reminiscent of the lethal interaction of [*PSI*$^+$] with the allosuppressor mutations *sal3* and *sal4* (Cox, 1977), which are now known to be alleles of *SUP35* (Crouzet and Tuite, 1987) and *SUP45* (Crouzet *et al*., 1988), respectively. Unlike *sup35-2* and *sup45-2*, the *sal3* and *sal4* alleles do not cause suppression by themselves but rather enhance the efficiency of the tRNA suppressor *SUQ5*. It was therefore suggested that both [*ETA*$^+$] and [*PSI*$^+$] may be related to translation termination and that [*ETA*$^+$] might be a variant of [*PSI*$^+$] (Liebman and All-Robyn, 1984; Wickner *et al*., 1996). Indeed, the existence of [*PSI*$^+$] variants with various efficiencies of suppression and mitotic stability was observed in our recent study (Derkatch *et al*., 1996). However, earlier studies uncovered apparent differences between [*PSI*$^+$] and [*ETA*$^+$] (Liebman and All-Robyn, 1984): (i) [*ETA*$^+$] had not been shown to cause nonsense suppression; (ii) [*PSI*$^+$] was inherited by all of the meiotic progeny, but [*ETA*$^+$] passed to only 70–85% of the meiotic progeny; and (iii) [*ETA*$^+$] did not cause lethality in the presence of the *SUP3-o* and *SUP11-o* tRNA suppressors (Liebman and All-Robyn, 1984), but [*PSI*$^+$] did (Cox, 1971). Thus, [*ETA*$^+$] might be a non-Mendelian factor that is independent of [*PSI*$^+$] and *SUP35*.

In this study, we directly tested the relationship between [*ETA*$^+$] and [*PSI*$^+$]. As is true for [*PSI*$^+$], we found that the maintenance of [*ETA*$^+$] requires the N-terminal region of Sup35p, and that the appearance of [*ETA*$^+$] can be induced *de novo* by Sup35p overproduction. Furthermore, the level of Hsp104 is critical for [*ETA*$^+$] propagation. Moreover, we observed that Sup35p aggregates in [*ETA*$^+$] but not in [*eta*$^-$] strain derivatives. In addition, growth inhibition caused by overexpression of *SUP35* and weak nonsense suppression can be detected in [*ETA*$^+$] but not in [*eta*$^-$] derivatives. We conclude that [*ETA*$^+$] is actually a weak, unstable [*PSI*$^+$] factor.

Results

[ETA$^+$] causes weak nonsense suppression

Previously, strong nonsense suppression was not detected in [ETA$^+$] strains (Liebman and All-Robyn, 1984). Because the [ETA$^+$] strains used previously did not have markers suitable for detecting weak nonsense suppression, we re-scored [ETA$^+$] strains for suppression of nonsense mutations using the *ade1-14* (UGA) marker. Two [ETA$^+$] strains with desirable markers were constructed by a cross of the originally described [ETA$^+$] strain SL611-17A (Liebman and All-Robyn, 1984) and [*psi*$^-$] [*eta*$^-$] 74-D694 using classical tetrad analysis. Two of the segregants from this cross, SL1010-1A and SL1010-6B, when crossed to the *sup35-2* mutant, SL429-10B, gave rise to inviable

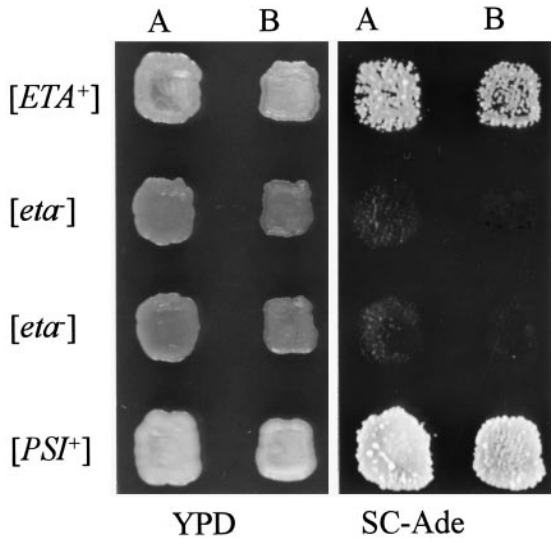

Fig. 1. Comparison of suppression of the *ade1-14* nonsense mutation in isogenic [*ETA*$^+$], [*eta*$^-$] and strong [*PSI*$^+$] derivatives of SL1010-1A and SL1010-6B. Yeast cells were grown on YPD at 30°C for 4 days and on SC-Ade at 20°C for 14 days (note that [*ETA*$^+$] strains grew more poorly on SC-Ade at 30°C than at 20°C). The growth on SC-Ade and the lack of red pigment on YPD indicate suppression of the *ade1-14* nonsense mutation. The [*eta*$^-$] derivatives were obtained from the [*ETA*$^+$] strains, SL1010-1A (**A**) and SL1010-6B (**B**), either by growth on GuHCl medium (upper) or by selection for spontaneous loss of [*ETA*$^+$] (lower). The strong [*PSI*$^+$] derivatives were obtained in spontaneous [*eta*$^-$] derivatives of SL1010-1A and SL1010-6B by transient overexpression of *SUP35*.

sup35-2 meiotic progeny (0:24 and 1:13, *sup35-2*:*SUP35* viable segregants, respectively), indicating the presence of [*ETA*$^+$]. Throughout this paper, only these strains and the [*ETA*$^+$] strains originally described by Liebman and All-Robyn (1984) will be referred to as [*ETA*$^+$]. The level of *ade1-14* nonsense suppression in both SL1010-1A and SL1010-6B was weak but unambiguous (Figure 1): they were pink (instead of red) on YPD medium after 3–4 days of incubation at 30°C and grew on SC-Ade medium after 10–14 days of incubation at 20°C.

Next, we tested the ability of GuHCl to 'cure' the suppressor phenotype in these strains. Passage on plates containing GuHCl was previously shown to eliminate both [*ETA*$^+$] and [*PSI*$^+$] (Tuite *et al*., 1981; Liebman and All-Robyn, 1984). After SL1010-1A and SL1010-6B strains were grown for ~21 cell generations on YPD containing 5 mM GuHCl, their color changed from pink to red and they no longer grew on SC-Ade (Figure 1). The absence of [*ETA*$^+$] in GuHCl-treated derivatives was confirmed by crossing them to a *sup35-2* mutant: the *sup35-2* progeny were viable. Suppression was also occasionally lost spontaneously (Figure 1), and this was correlated with the loss of [*ETA*$^+$]: a cross of a spontaneous non-suppressor (red) colony to a *sup35-2* mutant gave rise to viable *sup35-2* progeny. These results demonstrate that both SL1010-1A and SL1010-6B contain [*ETA*$^+$] and that [*ETA*$^+$] is associated with weak nonsense suppression.

The N-terminal region of SUP35 is required for the maintenance of [ETA$^+$]

The C-terminal region of Sup35p (amino acids 254–685) is essential for cell growth, whereas deletion of the N-terminal region (amino acids 1–253) is viable but cannot

Table I. Deletion of the N-terminal region of Sup35p eliminates [*ETA*$^+$]

Gene replacements of *SUP35* in [*ETA*$^+$] SL1010-1A	No. of tetrads with viable:non-viable spores from crosses to SL429-10B (*sup35-2*)				*sup35-2*:*SUP35* viable spores
	4:0	3:1	2:2	1:3	
SUP35[a]	0	1	23	0	1:48
SUP35-ΔN[b]	11	0	1	0	23:23
SUP35-ΔN[b]	10	0	2	0	21:23
SUP35-ΔN[b]	10	2	0	0	22:24

[a]The data from two independent wild-type controls are combined.
[b]Independently obtained disruptions.

Table II. Disruption of *HSP104* abolishes [*ETA*$^+$]

Gene replacements of *HSP104* in [*ETA*$^+$] SL611-17A	No. of tetrads with viable:non-viable spores from crosses to SL429-10B (*sup35-2*)				*sup35-2*:*SUP35 viable spores*
	4:0	3:1	2:2	1:3	
HSP104	0	1	10	5	1:27
hsp104-Δ::LEU2[a]	9	4	0	0	26:22
hsp104-Δ::LEU2[a]	9	1	1	0	21:20
hsp104-Δ::LEU2[a]	7	3	3	0	21:22
hsp104-Δ::LEU2[a]	8	2	1	1	20:21

[a]Independently obtained disruptions of the *HSP104* gene.

maintain [*PSI*$^+$] (Ter-Avanesyan *et al.*, 1994). If [*ETA*$^+$], like [*PSI*$^+$], is a prion form of Sup35p, then deletion of the N-terminal region of *SUP35* should eliminate [*ETA*$^+$].

The N-terminal region of *SUP35* was deleted by the method of integration and excision in the [*ETA*$^+$] strain, SL1010-1A (see Materials and methods). Following the excision step, derivatives that retained the intact *SUP35* gene as opposed to the *SUP35-ΔN* allele, were used as controls. These *SUP35* wild-type control derivatives maintained pink color on YPD and caused lethality when combined with *sup35-2* (Table I), indicative of [*ETA*$^+$]. In contrast, the three independent *SUP35-ΔN* derivatives were all red on YPD. Moreover, when crossed to a *sup35-2* mutant, they produced viable meiotic progeny containing *sup35-2* (Table I). This clearly shows that the *SUP35-ΔN* derivatives became [*eta*$^-$]. Thus, the N-terminal region of the *SUP35* gene is required for [*ETA*$^+$] propagation.

The level of Hsp104 is crucial for [ETA$^+$] maintenance

Another characteristic of [*PSI*$^+$] is that it is cured by either deletion or overexpression of the chaperone protein Hsp104 (Chernoff *et al.*, 1995). To test whether Hsp104 affects the propagation of [*ETA*$^+$], four independent disruptions of the *HSP104* gene were made in the [*ETA*$^+$] strain, SL611-17A (see Materials and methods). Analyses of crosses to a *sup35-2* mutant established that all four *hsp104-Δ::LEU2* disruption derivatives lost the [*ETA*$^+$] phenotype, whereas the wild-type *HSP104* control strain remained [*ETA*$^+$] (Table II). This demonstrates that Hsp104 is required for [*ETA*$^+$] maintenance.

To investigate the effects of overexpression of the *HSP104* gene on [*ETA*$^+$], plasmid pYS-Gal104, containing the *HSP104* gene under the control of the galactose-inducible promoter, *GAL1*, was introduced into two *ade1-14* strains containing [*ETA*$^+$], SL1010-1A and SL1010-6B. Transformants were pink on YPD following growth on glucose medium without uracil, where the plasmid was under selection but the *GAL* promoter was repressed. In contrast, almost all cells were red on YPD following transient growth on galactose medium without uracil, where overexpression of *HSP104* was induced. Following these treatments and colony purification on YPD, colonies from SL1010-1A and SL1010-6B were crossed to the tester strain SL429-10B containing *sup35-2* and their meiotic progeny were analyzed (Table III). Clearly, [*ETA*$^+$] was eliminated by transient growth on galactose but not glucose. When the pRS416 control plasmid lacking the *HSP104* gene was used, [*ETA*$^+$] was not eliminated either by growth in glucose or galactose media (data not shown). Thus, either deletion or overexpression of the *HSP104* gene causes the loss of [*ETA*$^+$].

Overexpression of SUP35 can induce [ETA$^+$]-like factors

It has been shown that [*PSI*$^+$] can be induced *de novo* by Sup35p overproduction in certain strains (Chernoff *et al.*, 1993; Derkatch *et al.*, 1996). We tested whether overexpression of *SUP35* can also induce the appearance of [*ETA*$^+$]-like factors. A spontaneous [*eta*$^-$] derivative of SL1010-1A was transformed with a *SUP35*-bearing plasmid, pEMBL-SUP35, or the control plasmid, pEMBL-yex4. Transformants were first selected on SC-Ura and subsequently plated on YPD to allow for plasmid loss. White and pink colonies with increased efficiency of *ade1-14* nonsense suppression (Ade$^+$) were observed in plasmidless derivatives of pEMBL-SUP35 transformants but not pEMBL-yex4 transformants. Nonsense suppression in both white and pink colonies (indicative of strong [*PSI*$^+$] and weak [*PSI*$^+$] or [*ETA*$^+$], respectively) was curable by growth on 5 mM GuHCl medium. Two pink Ade$^+$ derivatives of pEMBL-SUP35 transformants, as well as two Ade$^-$ derivatives of pEMBL-yex4 transformants, were crossed to a *sup35-2* strain, SL429-10B, to score for [*ETA*]. Analysis of their progeny shows that the two Ade$^+$ colonies contained a factor that was lethal in

Table III. Overexpression of *HSP104* eliminates [*ETA*$^+$]

Strains transformed with pYS-Gal104	Transient growth on	Color on YPD	No. of tetrads from cross to SL429-10B (*sup35-2*) with viable:nonviable spores[a]				*sup35-2*:*SUP35* viable spores[a]
			4:0	3:1	2:2	1:3	
[*ETA*$^+$] SL1010-1A[b]	SC-Ura	pink	0	0	18	2	0:38
	SGal-Ura	red	14	5	1	0	37:36
[*ETA*$^+$] SL1010-6B	SC-Ura	pink	1	0	5	2	2:14
	SGal-Ura	red	3	4	0	0	12:12

[a]These results were all obtained following transient growth on the synthetic media lacking uracil and containing glucose (SC-Ura) or galactose (SGal-Ura) as the single carbon source as listed.
[b]The data from two independent transformants are combined.

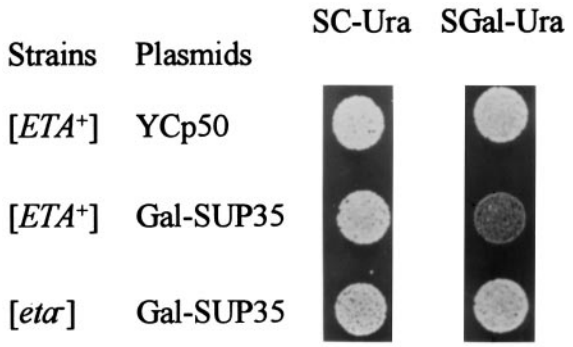

Fig. 2. Growth of an [*ETA*$^+$] strain is inhibited by overexpression of the *SUP35* gene. [*ETA*$^+$] SL1010-1A and an [*eta*$^-$] derivative obtained on GuHCl were transformed with the indicated plasmids. Spots show growth of representative transformants on SC-Ura medium where the *GAL::SUP35* is repressed, and on SGal-Ura medium where the *GAL::SUP35* is induced. Incubation was at 30°C for 5 days.

combination with *sup35-2* (0:17 and 0:16 *sup35-2*:*SUP35* viable segregants, respectively), whereas the two Ade$^-$ control clones did not contain such a factor (15:16 and 16:15, *sup35-2*:*SUP35* viable segregants, respectively). Therefore, like [*PSI*$^+$], [*ETA*$^+$] factors can be obtained *de novo* by transient overexpression of *SUP35*.

Overexpression of the SUP35 gene causes weak growth inhibition in [ETA$^+$] strains

Overexpression of the *SUP35* gene severely inhibits the growth of [*PSI*$^+$] strains, and plasmids carrying *SUP35* are very unstable in [*PSI*$^+$] strains (Chernoff *et al*., 1988, 1992; Dagkesamnskaya and Ter-Avanesyan, 1991; Derkatch *et al.*, 1996). Similarly, an instability of *SUP35*-containing plasmids in [*ETA*$^+$] strains was noticed previously, although *SUP35* overexpression did not cause any visible inhibition of growth (Dagkesamnskaya and Ter-Avanesyan, 1991). Here we re-examined the effects of overexpression of *SUP35* on the growth of [*ETA*$^+$] strains.

A centromeric plasmid, pGal-SUP35, or a control plasmid, YCp50, was introduced into [*ETA*$^+$] SL1010-1A and into [*eta*$^-$] derivatives obtained on GuHCl, in which [*PSI*$^+$] cannot be induced. All transformants grew well on glucose medium lacking uracil, where the plasmid was under selection but the *GAL* promoter was repressed. However, on galactose medium lacking uracil, where overexpression of *SUP35* was induced, growth inhibition was observed in the [*ETA*$^+$], but not in any of the [*eta*$^-$] derivatives (Figure 2). When the experiment was repeated with the [*ETA*$^+$] strain SL1010-6B, a slight inhibition of growth by *SUP35* overexpression was observed but only within 3 days at 30°C.

Interestingly, when both SL1010-1A and SL1010-6B [*ETA*$^+$] cells transformed with pGal-SUP35 were replica plated from galactose medium lacking uracil to glucose medium lacking adenine and colony purified, some colonies showed stronger suppression than the untransformed strains. Furthermore, this increase in suppression was sometimes maintained even after the pGal-SUP35 plasmid was lost. Since this suppression was cured in both strains by growth on 5 mM GuHCl medium, it suggests that strong [*PSI*$^+$] factors appeared.

[ETA$^+$] strains contain less soluble Sup35p than [eta$^-$] strains but more than strong [PSI$^+$] strains

The physical state of Sup35p is strikingly different in [*PSI*$^+$] and [*psi*$^-$] cells. Sup35p is soluble in [*psi*$^-$] cells and functions normally in translation termination; however, in [*PSI*$^+$] cells, the majority of Sup35p is in large aggregates where it is presumably unable to participate in translation termination (Patino *et al.*, 1996; Paushkin *et al.*, 1996). To examine the physical state of Sup35p in [*ETA*$^+$] cells, we compared the solubility of Sup35p in lysates from isogenic SL1010-1A derivatives containing [*ETA*$^+$], strong [*PSI*$^+$] or lacking both elements using differential centrifugation (see Materials and methods; Figure 3A). In each case, the sedimentation of ribosomal protein L3 was independent of the yeast strain used. In addition, no difference in protein amount or composition between corresponding sucrose-cushion fractions from the different derivatives was detected by Coomassie Blue staining of the transfer membrane with the exception of lane 15 which appears to be slightly underloaded (Figure 3A). Unlike the ribosomal proteins, the distribution of Sup35 differed between the individual isogenic cell lysates, particularly in the top and pellet fractions. As expected, the majority of Sup35p from [*eta*$^-$] cells was soluble and was found in the top sucrose-cushion fraction (Figure 3A, lane 24). Notably, less Sup35p was soluble in [*ETA*$^+$] cells than in [*eta*$^-$] cells (Figure 3A, compare lane 16 with 24), and more Sup35p was found in the pellet fraction of lysates from [*ETA*$^+$] than from [*eta*$^-$] cells (Figure 3A, compare lane 10 with 18). Thus, Sup35p forms aggregates in [*ETA*$^+$] cells. However, the extent or type of aggregation in [*ETA*$^+$] cells differs from that in [*PSI*$^+$] cells since we observed more Sup35p in the pellet fraction (Figure 3A, lanes 2 and 26) and less in the top fraction from [*PSI*$^+$] cells (Figure 3A, lanes 8 and 32).

To determine more quantitatively the relative amounts

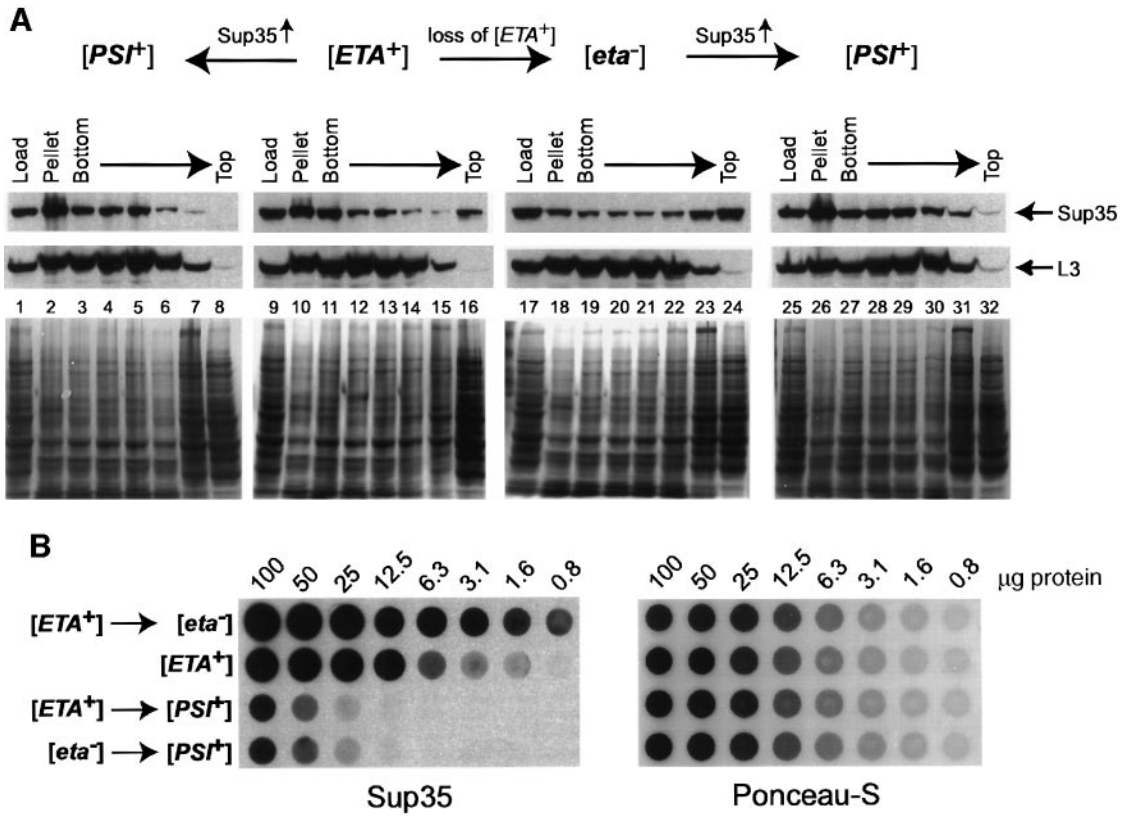

Fig. 3. An [*ETA*$^+$] strain contains more soluble Sup35 protein than isogenic [*PSI*$^+$] strains, but less than an [*eta*$^-$] strain. (**A**) Analysis of Sup35p solubility in SL1010-1A derivatives by differential centrifugation: the fractionation of Sup35 from four isogenic [*ETA*] and [*PSI*] strains was analyzed by differential centrifugation. Upper panel: immunoblots of sucrose-cushion fractions probed with either Sup35p or L3 antisera. Lanes 1, 9, 17 and 25 (load) represent the composition of total proteins layered on the sucrose cushion. Lanes 2, 10, 18 and 26 contain proteins found in the pellet fraction. Lanes 8, 16, 24 and 32 contain proteins from the top fraction. The other lanes represent intermediate (left) to upper (right) sucrose-cushion fractions, from bottom to top. Lower panel: Coomassie Blue-stained PVDF membranes. The phenotype and origin of the [*ETA*] and [*PSI*] strains are indicated above the immunoblots. The [*eta*$^-$] derivative arose by spontaneous loss of [*ETA*$^+$] in SL1010-1A. The two [*PSI*$^+$] strains were obtained by transient overexpression of *SUP35* either in [*ETA*$^+$] SL1010-1A (leftmost) or in [*eta*$^-$] SL1010-1A (right-most). (**B**) Semi-quantitative Western dot-blot analysis: the total protein from top sucrose-cushion fraction of each strain (lanes 8, 16, 24 and 32 of Figure 3A) was adjusted to 0.66 mg/ml, serially diluted in 2-fold increments and applied to a PVDF membrane. Left, reactivity with Sup35p antisera. Right, the same membrane stained by Ponceau S, a non-specific protein stain. The phenotype and origin of the strains are indicated on the left.

of soluble Sup35p in these isogenic strains, we performed semi-quantitative Western dot-blot analysis using the top fraction from each sucrose cushion (see Materials and methods). The fractions were adjusted to contain equivalent amounts of protein, serially diluted and then spotted on a membrane. Ponceau S staining of the membrane demonstrated that equivalent amounts of protein were applied for each of the dilution series (Figure 3B). The amount of Sup35p, however, was not equivalent. We estimate that there was ~50-fold more soluble Sup35p in [*ETA*$^+$] cells than in [*PSI*$^+$] cells and ~200-fold more soluble Sup35p in [*eta*$^-$] cells than in [*PSI*$^+$] cells. The intermediate level of soluble Sup35p in [*ETA*$^+$] cells compared with either strong [*PSI*$^+$] or [*eta*$^-$] correlates well with the intermediate degree of nonsense suppression exhibited by [*ETA*$^+$] cells.

To visualize the aggregates of Sup35p in living yeast cells, plasmid pCUPSUP35GFP containing a *SUP35–GFP* fusion was transformed into isogenic [*ETA*$^+$], strong [*PSI*$^+$] and [*eta*$^-$] derivatives of both SL1010-1A and SL1010-6B. In [*eta*$^-$] transformants of both strains, when *SUP35-GFP* transcription was induced with 5 μM $CuSO_4$ for 4 h, fluorescence was evenly distributed in >94% of the cells. The remaining cells contained intense fluorescent foci, probably due to the induction of [*PSI*$^+$] *de novo* (Patino *et al.*, 1996). In contrast, 60–90% of the fluorescent cells of isogenic [*ETA*$^+$] derivatives transformed with *SUP35–GFP* showed intense fluorescent foci after 4 h of induction (Figure 4). As expected, intense fluorescent foci were also observed in the [*PSI*$^+$] cells (Figure 4). When *GFP* not fused to *SUP35* was induced for 4 h, fluorescence was diffusely distributed in [*ETA*$^+$], [*PSI*$^+$] and [*eta*$^-$] transformants.

These results suggest that pre-existing Sup35p aggregates promote the aggregation of newly synthesized Sup35p in [*ETA*$^+$] strains. However, since *SUP35* overexpression in [*ETA*$^+$] strains might induce the appearance of [*PSI*$^+$], it was necessary to rule out the possibility that the Sup35–GFP aggregation observed in the [*ETA*$^+$] strains was caused by newly appearing [*PSI*$^+$]. [*ETA*$^+$] cells were plated on YPD following 4 h of induction by 5 μM $CuSO_4$ and were analyzed for the appearance of strong [*PSI*$^+$], scored as more efficient nonsense suppression than that in the original [*ETA*$^+$] strain (see Materials and methods). Strong [*PSI*$^+$] appeared in 0.8–2% of these [*ETA*$^+$] pCUPSUP35GFP transformants. When [*eta*$^-$] pCUPSUP35GFP transformants were analyzed, the appearance of [*PSI*$^+$] was <3%. Thus the majority of aggregates seen in the

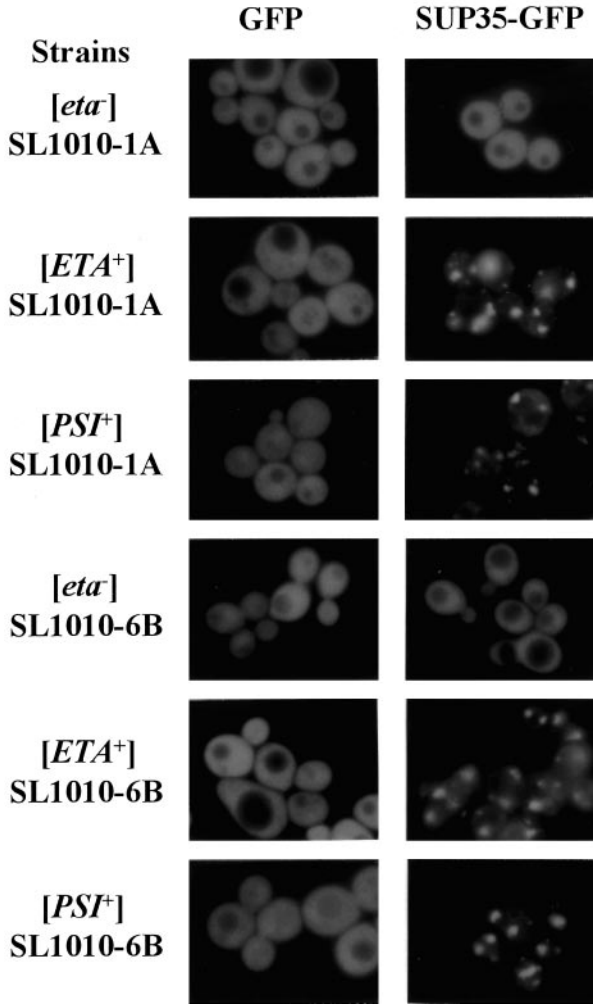

Fig. 4. The SUP35–GFP fusion protein forms aggregates in [*ETA*$^+$] and [*PSI*$^+$] strains. The fluorescent images of the cells of the indicated strains transformed with pCUPGFP (GFP) or pCUPSUP35GFP (SUP35GFP) were taken after 5 μM $CuSO_4$ was added to early log-phase cultures and incubation continued for 4 h.

[*ETA*$^+$] derivatives were caused not by newly induced [*PSI*$^+$] but by pre-existing [*ETA*$^+$].

The lethality caused by the combination of [ETA$^+$] and the sup35-2 mutation cannot be rescued by excess Sup45p

The above experiments establish that [*ETA*$^+$] is a variant of [*PSI*$^+$]. To investigate the mechanism by which the [*ETA*$^+$] and *sup35-2* combination causes lethality, we tested if this lethality could be overcome by overexpression of *SUP45*. This seemed possible since some data suggest that eRF1 (Sup45p) is the only release factor that is indispensable for translation termination while eRF3 (a Sup35p homologue) only stimulates the eRF1 activity (Frolova *et al*., 1994; Zhouravleva *et al*., 1995; Drugeon *et al.*, 1997; Le Goff *et al*., 1997).

Meiotic progeny from crosses of the *sup35-2* strain SL1020-3C and transformants of [*ETA*$^+$] strain SL1010-1A, carrying *SUP45* plasmids with moderate (YEp13-SUP45) or high (pJDB-SUP45) copy number, showed the typical [*ETA*$^+$] phenotype (0:17 and 0:33, *sup35-2*:*SUP35* viable segregants, respectively). Since the YEp13-SUP45 and pJDB-SUP45 plasmids were retained in 50–60% and nearly 100% of the viable meiotic progeny, respectively, it is clear that neither moderate nor high-level *SUP45* overexpression can prevent the lethal interaction between [*ETA*$^+$] and *sup35-2*. We also showed that Sup45p overproduction at high or moderate levels is unable to rescue a deletion of *SUP35* in a [*psi*$^-$] [*eta*$^-$] background (J.Kuna, I.L.Derkatch and S.W.Liebman, unpublished data).

The sup35-2 suppressor contains a UAG mutation

To understand the basis for the lethality of [*ETA*$^+$] and [*PSI*$^+$] with the *sup35-2* allele, we compared the sequences of the *SUP35* and *sup35-2* open reading frames (ORFs). There was a single nucleotide change (T→A) between the wild-type *SUP35* and the suppressor *sup35-2* ORFs. This mutation substitutes a UAG stop codon for the leucine residue at amino acid position 110. Because amino acids 254–685 of Sup35p are essential for viability (Ter-Avanesyan *et al.*, 1993), the *sup35-2* mutant strain should only be viable if the UAG codon was efficiently read through and/or if translation were initiated downstream of the UAG. To clarify this, Western blot analyses were performed on crude yeast lysates prepared from the wild-type (L475) and *sup35-2* (SL429-10B) strains using antibodies specific for either amino acids 137–151 (M) or for amino acids 87–102 (N) (data not shown). In both lysates, both antibodies reacted with a protein corresponding to full-length Sup35p. Notably, Sup35p was expressed at a lower level in the *sup35-2* strain than in the wild-type strain. Another polypeptide, found only in the *sup35-2* lysate, migrated at ~12 kDa and reacted with N antibody, but not with M antibody. This protein is presumably the prematurely terminated, 109 amino-acid Sup35p fragment. The signal from the ~12 kDa protein was much weaker than that of the full-length protein, indicating that the truncated protein was present at a much lower steady-state concentration than Sup35p.

Discussion

We have linked the inheritance of the [*ETA*$^+$] factor, a non-Mendelian, heritable element in yeast, to an altered self-propagating prion-like conformation of Sup35p. First, deletion of the N-terminal region of *SUP35* caused the loss of [*ETA*$^+$]. Secondly, [*ETA*$^+$] caused nonsense suppression. Thirdly, Sup35p formed aggregates in [*ETA*$^+$] but not in [*eta*$^-$] cells. Fourthly, transient *SUP35* overexpression induced the appearance of [*ETA*$^+$] *de novo*. Fifthly, moderate *SUP35* overexpression inhibited growth of [*ETA*$^+$] cells but not [*eta*$^-$] strain derivatives. Sixthly, an appropriate level of the chaperone protein Hsp104 was required for the propagation of [*ETA*$^+$] but not for the normal function of Sup35p. Earlier observations that [*ETA*$^+$] is characterized by a non-Mendelian pattern of inheritance and can be cured by low concentrations of GuHCl (Liebman and All-Robyn, 1984) also support this argument. Thus, [*ETA*$^+$] shares all the notable features of [*PSI*$^+$] and therefore should be considered a variant of [*PSI*$^+$].

Several aspects of [*ETA*$^+$] distinguish it from previously described strong [*PSI*$^+$] variants. For example, nonsense suppression was weaker in [*ETA*$^+$] than in [*PSI*$^+$] cells. [*ETA*$^+$] was less stable in mitosis and meiosis than [*PSI*$^+$],

and the growth of [*ETA*$^+$] cells was only moderately inhibited in the presence of excess Sup35p. Recently, it was reported that weak variants of [*PSI*$^+$] exhibiting weak nonsense suppression efficiencies and reduced mitotic stabilities could be induced *de novo* in isogenic cells by Sup35p overproduction (Derkatch *et al*., 1996). Thus, we conclude that [*ETA*$^+$] is also a weak variant [*PSI*$^+$].

The intermediate level of soluble Sup35p in [*ETA*$^+$] cells compared with either strong [*PSI*$^+$] or [*eta*$^-$] correlated well with the intermediate degree of nonsense suppression exhibited by [*ETA*$^+$] cells. This result suggests a possible biochemical explanation for the phenotypic difference between strong and weak [*PSI*$^+$] variants: the larger the fraction of the soluble Sup35p, the more efficient the translational termination and the weaker the nonsense suppression phenotype.

Currently, there are two major models for how prions are propagated, and our data can be interpreted within the framework of either. According to the seeded-nucleation model, the propagation of prions involves the joining of non-prion protein with a pre-existing prion aggregate (Jarret and Lansbury, 1993). The dimerization model postulates that the non-prion molecule acquires the prion conformation during transient interaction with another protein in the prion conformation and that aggregation, when it occurs, is a secondary process (Cohen *et al*., 1994). If the seeded-nucleation model, favored by some investigators of [*PSI*$^+$] (Paushkin *et al*., 1997a), is correct, then [*PSI*$^+$] variants may differ in the efficiency with which the normal form of Sup35p joins the [*PSI*$^+$] aggregates or in the stability of [*PSI*$^+$] aggregates themselves. Indeed, 50-fold more Sup35p remained soluble in [*ETA*$^+$] cells than in isogenic [*PSI*$^+$] cells (see Figure 3). In the context of the dimerization model, different efficiencies of translation termination could be due to different levels of inactivation of soluble Sup35p upon conformational change before it goes into an aggregate. This might reflect the existence of different soluble Sup35^{PSI+} conformations with different aggregational properties in weak and strong [*PSI*$^+$] variants. Note that other models could also be devised that would also accommodate our data.

Although most models for [*PSI*$^+$] focus on the changes in the Sup35p protein, it is important to consider the effect of other proteins. [*ETA*$^+$] and [*PSI*$^+$] may differ because the type or amount of other proteins associated with the Sup35^{PSI+} aggregates are different. Indeed, it has been reported that Upf1p, which functions in nonsense-mediated mRNA decay, and Sup45p, the translational release factor eRF1, are found in [*PSI*$^+$] aggregates (Paushkin *et al*., 1997b; Czaplinski *et al*., 1998) and that excess Sup45p reduces the appearance of [*PSI*$^+$] *de novo* (Derkatch *et al*., 1998). Such co-aggregated proteins could change the stability of the aggregates, and thereby alter the levels of soluble Sup35p.

Our previous findings that UAG tRNA suppressors antisuppress *sup35-2* suggested that the *sup35-2* allele might contain a UAG nonsense mutation (Song and Liebman, 1985). Indeed, we now show that *sup35-2* encodes a UAG mutation at codon 110, near the end of the [*PSI*$^+$] prion domain. Since the C-terminal region of *SUP35* is essential for viability (Ter-Avanesyan *et al*., 1993), one might expect a nonsense mutation near the beginning of the gene to be lethal unless it is frequently read through or unless ribosomes reinitiate translation further downstream. Indeed, Western blot analyses support the former explanation since there was considerable expression of the full-length protein in *sup35-2* strains. One reason for the readthrough of the stop codon is that it is followed by a cytidine residue. UAGC has been shown to be misread as sense three- to 10-times more frequently than other tetranucleotide stop codons in *Saccharomyces cerevisiae* (Bonetti *et al*., 1995). Likewise, the non-acidic Asn codon at the –2 position relative to the UAG places this stop codon in a context that is inefficient for termination (Mottagui-Tabar *et al*., 1998). In addition, although some full-length Sup35p was present in the *sup35-2* strain, there was less of it in the mutant strain than in the wild-type strain. This also contributes to the nonsense suppression phenotype of the *sup35-2* strain: reduced levels of eRF3 result in increased suppression of all stop codons, including the UAG mutation in *sup35-2*.

We have also investigated the relationship between the *sup35-2* allele and [*PSI*$^+$] variants. In crosses between the *sup35-2* strain and [*ETA*$^+$] strains or other [*PSI*$^+$] variants in SL1010-1A and other genetic backgrounds, almost all of the *sup35-2* meiotic progeny die (Liebman and All-Robyn, 1984; this study; our unpublished observations). Such a lethal interaction may be explained if the low level of Sup35p in *sup35-2* segregants is sequestered into prion-induced aggregates. This would further reduce the amount of eRF3, causing it to fall below the level required to sustain viability in the *sup35-2* spores. This hypothesis of the lack of soluble Sup35p causing lethality is consistent with our finding that excess Sup45p fails to rescue [*ETA*$^+$] *sup35-2* spores.

The presence of the truncated Sup35p$_{1–109}$ polypeptides in *sup35-2* strains may explain an interesting paradox. [*ETA*$^+$] is not very stable in meiosis and 15–30% of *SUP35* segregants are [*eta*$^-$] (Liebman and All-Robyn, 1984). Thus, one would expect 15–30% of the *sup35-2* segregants of an [*ETA*$^+$] *SUP35/sup35-2* heterozygous diploid to be viable. Instead, almost all the *sup35-2* segregants die. This is likely because Sup35p$_{1–109}$ adopts the [*ETA*$^+$] conformation more easily than the complete Sup35p (Derkatch *et al*., 1996; Kochneva-Pervukhova *et al*., 1998). Thus, all the *sup35-2* progeny would retain [*ETA*$^+$] and would die. However, the *SUP35* progeny would lose [*ETA*$^+$] at an appreciable frequency because they do not synthesize Sup35p$_{1–109}$.

Finally, we note that a strong [*PSI*$^+$] variant appeared in [*ETA*$^+$] strains following transient overexpression of *SUP35*. Although excess Sup35p causes growth inhibition in [*ETA*$^+$] strains, the cells still grow. The strong [*PSI*$^+$] induced in [*ETA*$^+$] strains could be formed (i) *de novo* from newly synthesized Sup35p$^{psi–}$ and coexist with [*ETA*$^+$], (ii) *de novo* after [*ETA*$^+$] was lost because [*ETA*$^+$] is not stable, or (iii) by converting the pre-existing Sup35^{ETA+} conformation into a strong Sup35^{PSI+} conformation. We also noticed that strong [*PSI*$^+$] is dominant over [*ETA*$^+$] in diploids (unpublished data; Derkatch *et al*., 1999). These results raise the interesting questions of whether one prion conformation can be converted into another prion conformation, and whether two prion conformations can coexist.

Table IV. Plasmids used in this study

Plasmids	Constructs	References
pEMBL-yex4	*URA3 leu2-d* 2μ	Cesarini and Murray (1987)
pEMBL-Δ3ATG	*SUP35-ΔN* in pEMBL-yex4	Ter-Avanesyan *et al.* (1993)
pEMBL-SUP35	*SUP35* in pEMBL-yex4	Ter-Avanesyan *et al.* (1993)
YCp50	*URA3* CEN	Rose *et al*. (1987)
pGal-SUP35	*GAL1::SUP35* in YCp50	Derkatch *et al*. (1996)
pYS-Gal104	*URA3 GAL1::HSP104* CEN	Chernoff *et al.* (1995)
pYABL5	*hsp104-Δ::LEU2* 2μ	Chernoff *et al*. (1995)
YEp13	*LEU2* 2μ	Broach *et al*. (1979)
YEp13-SUP45	*SUP45* in YEp13	Chernoff *et al.* (1992)
pJDB207	*leu2-d* 2μ	Beggs (1981)
pJDB-SUP45	*SUP45* in pJDB207	Chernoff *et al.* (1992)
pCUPGFP	*URA3 CUP1::GFP* CEN	J.J.Liu and S.Lindquist, unpublished
pCUPSUP35GFP	*URA3 CUP1::SUP35GFP* CEN	J.J.Liu and S.Lindquist, unpublished

Materials and methods

Strains and plasmids

SL1010-1A ([*ETA*$^+$] *MAT*α *ade1-14 met8-1 leu2-1 his5-2 trp1-1 ura3-52*) and SL1010-6B ([*ETA*$^+$] *MAT*α *ade1-14 met8-1 leu2-1 his5-2 trp1-1 ura3-52 lys2-1*) were derived from a cross between the previously described [*ETA*$^+$] strain, SL611-17A ([*ETA*$^+$] *MAT*α *met8-1 leu2-1 his5-2 trp1-1 lys2-1*; Liebman and All-Robyn, 1984) and a [*psi*$^-$] [*eta*$^-$] derivative of strain 74-D694 (*MAT***a** *ade1-14 leu2-3,112 his3-Δ200 trp1-289 ura3-52*) (Chernoff *et al.*, 1995). The [*eta*$^-$] SL1010-1A and [*eta*$^-$] SL1010-6B derivatives were obtained either by 5 mM GuHCl treatment or by selecting for spontaneous loss of [*ETA*$^+$] during subcloning. The former were shown to be [*pin*$^-$] (not inducible to [*PSI*$^+$] by overexpression of *SUP35*), while the latter were shown to be [*PIN*$^+$] (inducible to [*PSI*$^+$] by overexpression of *SUP35*) (Derkatch *et al.*, 1997). Using the suppression phenotype to score for [ETA$^+$], we estimated that the frequency of spontaneous loss of [*ETA*$^+$] during subcloning was <3% in SL1010-1A and 2–8% in SL1010-6B. [*PSI*$^+$] derivatives of SL1010-1A and SL1010-6B, which exhibited strong nonsense suppression, were obtained by transient overexpression of *SUP35* in either [*ETA*$^+$] or [*eta*$^-$] derivatives.

Deletions of the region of *SUP35* encoding amino acid residues 1–253 of Sup35p in the [*ETA*$^+$] derivatives of SL1010-1A and SL1010-6B were constructed using plasmid pEMBL-Δ3ATG (Table IV) by the method of integration and excision (Rose *et al*., 1990). Following the excision step, either the *SUP35-ΔN* deletion or the wild-type *SUP35* allele was retained and these were distinguished by PCR analysis using primers CACTTCTTACCTTGCTCTTA and TGAGAGGTGAAGTTTACTTG. To obtain *hsp104-Δ::LEU2* mutants, a *Pvu*I–*Hin*dIII fragment containing an *hsp104-Δ::LEU2* disruption from plasmid pYABL5 (Chernoff *et al*., 1995) was used to replace the *HSP104* allele in SL611-17A by the one-step gene replacement method (Rothstein, 1983). Disruptions were verified by PCR analysis using two sets of primers: (i) CCTTCAAGACGCTGCTAAGA and GAGTCGGCATCTTCATCTCT, homologous to the *HSP104* gene; (ii) GCGCAGATCTAACTGTGGGAATACTCAGG, homologous to *LEU2* and GAGTCGGCATCTTCATCTCT, homologous to *HSP104*.

Other strains used in this study are SL1020-3C (*MAT***a** *ade1-14 met8-1 leu2-3, 112 trp1-1 lys2-1 ilv1-1 ura3-52 sup35-2*, [*psi*$^-$] [*eta*$^-$]), SL429-10B (*MAT***a** *ade3-26 met8-1 leu2-1 his5-2 trp1-1 lys1-1 aro7-1can1-132 cyc1-176 ilv1-1 sup35-2*, [*psi*$^-$] [*eta*$^-$]) and L475 (Liebman and Cavenagh, 1980) which contains the wild-type *SUP35* allele from which the *sup35-2* mutation was induced.

Plasmids used in this study are listed in Table IV. Yeast transformation was according to Ito *et al.* (1983).

Media and growth conditions

Standard yeast media were used (Sherman *et al*., 1986). Yeast strains were grown at 30°C unless specified. Transformants were grown in synthetic media selective for plasmid maintenance. To eliminate [*ETA*$^+$] or [*PSI*$^+$], cells were replica plated three times on YPD medium supplemented with 5 mM GuHCl and then colony purified on YPD. The *GAL1* promoter was induced by growth on appropriate synthetic media where 20 mg/ml galactose was the single carbon source (e.g. SGal-Ura). The *CUP1* promoter was induced by growth on appropriate synthetic media supplemented with 5 μM $CuSO_4$.

Genetic methods

Standard yeast genetic procedures of crossing, sporulation and tetrad analysis were used (Sherman *et al*., 1986). The *sup35-2* mutation was scored by suppression of the *met8-1* (UAG) or *leu2-1* (UAA) nonsense mutations on synthetic complete medium lacking methionine (SC-Met) or leucine (SC-Leu), respectively. Strains were scored for [*ETA*$^+$] by crossing to a *sup35-2* containing tester strain. If most of the tetrads had only two viable spores and none or only a few of the *sup35-2* segregants were viable in comparison with the wild-type *SUP35* segregants, the strains were scored as [*ETA*$^+$] (Liebman and All-Robyn, 1984). Suppression of *ade1-14* (UGA) was used to score for [*PSI*$^+$] as previously described (Derkatch *et al*., 1996) and was also used to score for [*ETA*$^+$] in this study. The better the growth on SC-Ade medium and the less red the color on YPD medium, the higher the suppression of the *ade1-14* mutation.

PCR amplification and sequencing of sup35-2 and Western analysis of Sup35-2p

Yeast genomic DNA (Hoffman and Winston, 1987) was PCR amplified using Vent DNA polymerase (New England BioLabs, Beverly, MA). DNA used for coding-strand sequence was generated using oligonucleotides (Research Genetics, Huntsville, AL) Bal2-1, CTCACTAGTCATATGTCGGATTCAAACCAAGG and Sup35C-3BamH1, CGCGGATCCTTACTCGGCAATTTTAAC. DNA used for non-coding strand sequence was generated using oligonucleotides SMU3, CGGAGCTCCAAAGCTCCCATTGCTTCTG and SMU4, CGGGATCCGAAAACGTGATTGAAGGAGTTG. To minimize the possibility of PCR-generated sequencing errors, eight independent amplification reactions were pooled for both the coding and non-coding strand sequencing reactions. Sequencing (Sanger *et al.*, 1977), which was performed by the University of Chicago Cancer Research Center DNA Sequencing Facility, was obtained for both strands of the *SUP35* or *sup35-2* alleles from L475 and SL429-10B, respectively. Western analyses of total protein extracts were as described by Patino *et al.* (1996) and rabbit Sup35p affinity-purified antipeptide polyclonal antibodies to amino acids 137–151 (M) (Patino *et al.*, 1996) and amino acids 87–102 (N) (Quality Controlled Biochemicals, Hopkinton, MA) were used.

Analysis of Sup35p aggregates

Differential sedimentation and Western blot analyses were performed as described by Paushkin *et al.* (1996) with modifications. [*PSI*$^+$], [*ETA*$^+$] and [*eta*$^-$] [*psi*$^-$] derivatives of SL1010-1A were grown in YPD at 25°C to a density of 4–5×10^7 cells/ml. Harvested cells were suspended in buffer A [25 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DDT), 4% v/v glycerol, 4 mM Pefabloc, 5 μg/ml aprotinin and 5 μg/ml leupeptin]. Cells were lysed using acid-washed glass beads and a BioSpec bead beater at 6°C. The crude lysates were partially clarified by differential centrifugation at 4000 *g* for 20 min at 4°C. Total protein (2 mg) of each supernatant fraction was layered upon 30% w/v sucrose in buffer A and centrifuged at 200 000 *g* for 34 min in a Beckman SW60 rotor. Fractions of 200 μl were then drawn from the top of the sucrose cushion. The pelleted proteins were resuspended in 200 μl of 30% sucrose in buffer A. To follow the fractionation of Sup35p, 20 μg total protein from each fraction was resolved by 7.5% SDS–PAGE, transferred to PVDF membranes, immunoblotted with rabbit Sup35p antipeptide polyclonal serum to amino acids 137–151 (Patino

et al., 1996) and a mouse monoclonal antibody raised against ribosomal protein L3 (gift from J.Warner, Albert Einstein College of Medicine, NY), and visualized by enhanced chemiluminescence (ECL).

To estimate more precisely the relative amount of Sup35p in the uppermost [*PSI*] and [*ETA*] sucrose-cushion fractions, semi-quantitative Western dot-blot analysis was performed as follows. The total protein from the top fraction was normalized to 0.66 mg/ml and then serially diluted in 2-fold increments. Protein concentration was determined by Bradford protein assay using BSA as standard. The diluted protein, which ranged from 100 to 0.78 μg total protein, was applied to PVDF membranes using a Dot Blot apparatus (V&P Scientific, Inc.). The membrane was immunoblotted with the Sup35p antisera and visualized by ECL. The amount of total protein per dot was assessed by staining the PVDF membrane with Ponceau S.

To visualize Sup35p aggregates *in vivo*, isogenic [ETA^+] and [eta^-] SL1010-1A and SL1010-6B were transformed with the pCUPSUP35GFP or the control pCUPGFP plasmid. Transformants were grown to early log phase (OD_{600} = 0.2–0.4) in SC-Ura medium selective for plasmid maintenance. $CuSO_4$ was added to a final concentration of 5 μM. After 4 h of growth, samples were taken for observation under a fluorescence microscope, Axioskop (Carl Zeiss, Inc).

Induction of the appearance of [PSI$^+$] de novo

The induction of [PSI^+] by *SUP35*-carrying plasmids pGal-SUP35 and pEMBL-SUP35 in strains SL1010-1A and SL1010-6B was tested essentially as described previously (Derkatch et al., 1996). The appearance of Ade^+ derivatives with GuHCl-curable nonsense suppression following transient overexpression of *SUP35* indicated the appearance of [PSI^+] in [eta^-] strains. When the analysis was carried out in [ETA^+] strains, the appearance of derivatives that, following plasmid loss, had a higher level of GuHCl-curable nonsense suppression than in the original [ETA^+] strains, indicated the appearance of strong [PSI^+].

To determine the frequency of the *de novo* appearance of [PSI^+] caused by plasmid pCUPSUP35GFP bearing the *SUP35–GFP* fusion after Cu^{2+} induction, transformants grown in liquid SC-Ura medium containing 5 μM $CuSO_4$ for 4 h were plated on YPD (80–200 cells/plate). Colonies with a higher level of *ade1-14* suppression (whiter color) than the control pCUPGFP transformants treated the same way, were scored as [PSI^+]. By growing several of these colonies on YPD + 5 mM GuHCl medium we confirmed that the acquired suppression was curable. The rare colonies that showed a few white sectors were not counted as [PSI^+] because the [PSI^+] induction probably occurred after plating on YPD.

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