Genetic and Environmental Factors Affecting the *de novo* Appearance of the *[PSI$^+$]* Prion in *Saccharomyces cerevisiae*

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ABSTRACT

It has previously been shown that yeast prion *[PSI$^+$]* is cured by GuHCl, although reports on reversibility of curing were contradictory. Here we show that GuHCl treatment of both *[PSI$^+$]* and *[psi$^-$]* yeast strains results in two classes of *[psi$^-$]* derivatives: Pin$^+$, in which *[PSI$^+$]* can be reinduced by Sup35p overproduction, and Pin$^-$, in which overexpression of the complete *SUP35* gene does not lead to the *[PSI$^+$]* appearance. However, in both Pin$^+$ and Pin$^-$ derivatives *[PSI$^+$]* is reinduced by overproduction of a short Sup35p N-terminal fragment, thus, in principle, *[PSI$^+$]* curing remains reversible in both cases. Neither suppression nor growth inhibition caused by *SUP35* overexpression in Pin$^+$ *[psi$^-$]* derivatives are observed in Pin$^-$ *[psi$^-$]* derivatives. Genetic analyses show that the Pin$^+$ phenotype is determined by a non-Mendelian factor, which, unlike the *[PSI$^+$]* prion, is independent of the Sup35p N-terminal domain. A Pin$^-$ *[psi$^-$]* derivative was also generated by transient inactivation of the heat shock protein, Hsp104, while *[PSI$^+$]* curing by Hsp104 overproduction resulted exclusively in Pin$^+$ *[psi$^-$]* derivatives. We hypothesize that in addition to the *[PSI$^+$]* prion-determining domain in the Sup35p N-terminus, there is another self-propagating conformational determinant in the C-proximal part of Sup35p and that this second prion is responsible for the Pin$^+$ phenotype.

CERTAIN neurodegenerative mammalian diseases, such as sheep scrapie, human Creutzfeldt-Jacob disease, and mad cow disease appear to be transmitted by a protein, without any nucleic acid. The infectious protein, called a prion, is proposed to be an altered form of a cellular protein that no longer functions normally and can convert the normal form of the protein (PrP^C) into its prion conformation (PrP^{Sc}). Infection by the prion is thought to trigger a chain reaction of conversion that results both in progressive accumulation of PrP^{Sc} and development of the disease (for reviews see PRUSINER 1994, 1996). One model for this chain reaction is that the prion and normal isoforms of the protein form a heterodimer, and that the interactions in this complex cause the normal isoform to take on the prion conformation. The newly created prion homodimer can then dissociate and dimerize with another molecule of normal protein. Prion protein may also form an aggregate as a secondary process. In an alternate model, a crystal seed composed of prion subunits induces normal protein subunits to join the prion crystal (for reviews see WICKNER *et al.* 1995; WEISSMANN 1996; CAUGHEY and CHESEBRO 1997).

WICKNER (1994) has recently noted that genetic data concerning the non-Mendelian yeast factors *[URE3]* and *[PSI]* can be explained by the prion hypothesis, and considerable additional data now support this model (TER-AVANESYAN *et al.* 1994; CHERNOFF *et al.* 1995; MASISON and WICKNER 1995; DERKATCH *et al.* 1996; PATINO *et al.* 1996; PAUSHKIN *et al.* 1996). It thus appears that prions may be a general phenomenon and the source of various inherited characteristics.

The non-Mendelian *[PSI$^+$]* factor was first described as a dominant element present in some strains of *Saccharomyces cerevisiae* that increases the efficiency of certain codon-specific nonsense suppressors (COX 1965, 1971). Later, *[PSI$^+$]* itself was shown to cause weak suppression of certain nonsense mutations even in the absence of suppressors (LIEBMAN and SHERMAN 1979; ONO *et al.* 1986; COX *et al.* 1988; TIKHODEEV *et al.* 1990). Despite extensive efforts, *[PSI$^+$]* was never linked to any extrachromosomal nucleic acid (see review, COX *et al.* 1988). The *[PSI$^+$]* factor can be eliminated from cells by a variety of agents (SINGH *et al.* 1979a; TUITE *et al.* 1981). Growth in 5 mM guanidine hydrochloride (GuHCl) causes 100% loss of *[PSI$^+$]*, while having no effect on Mendelian genes (TUITE *et al.* 1981).

Experimental data suggest that only cells carrying the *[PSI$^+$]* factor contain a prion form of Sup35p, which converts the normal, non-prion Sup35p isoform ($Sup35p^{Psi-}$) into the inactive (or less active) prion conformation ($Sup35p^{Psi+}$). Sup35p is the eukaryotic translational termination factor eRF3 (STANSFIELD *et al.* 1995; ZHOURAVLEVA *et al.* 1995; FROLOVA *et al.* 1996); its C-terminal portion (Sup35Cp) is essential for viability (TER-AVANESYAN *et al.* 1993). Cells that lack the nones-

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sential, N-terminal coding region of *SUP35* are viable (TER-AVANESYAN *et al.* 1993) but are unable to maintain *[PSI⁺]* (TER-AVANESYAN *et al.* 1994). This finding, that the N-terminus of Sup35p is required for *[PSI⁺]* maintenance, is analogous to the requirement for the mammalian *PrP* gene for susceptibility to prion infection (BUELER *et al.* 1993; PRUSINER *et al.* 1993) and fits the prion model since the host gene encoding PrP (or a prion susceptible form of Sup35p) must be present to provide a continuous supply of protein that can be converted to the prion form. Other genetic evidence that provides strong support for the prion model of *[PSI]* is our finding that an excess of Sup35p (or certain N-terminal regions of Sup35p, Sup35Np) induces the *de novo* appearance of *[PSI⁺]* (CHERNOFF *et al.* 1993; DERKATCH *et al.* 1996). While the precise mechanism of the *de novo* induction of *[PSI⁺]* caused by overproduction of Sup35p or its fragments is not known, either spontaneous folding of Sup35p (or Sup35Np) into the prion seed conformation or prion formation resulting from the interaction of two non-prion Sup35p (or Sup35Np) molecules would be more probable when the protein is present in excess.

The discovery that the propagation of *[PSI⁺]* depends upon the concentration of the chaperone Hsp104p (CHERNOFF *et al.* 1995) supports the prion model postulating that *[PSI⁺]* inheritance is based on maintenance of a self-propagating protein conformation, since the only known function of Hsp104p (PARSELL *et al.* 1994) is to facilitate protein conformational changes. Physical evidence for different structural states of Sup35p in *[PSI⁺]* and *[psi⁻]* strains has recently been reported by two groups (PATINO *et al.* 1996; PAUSHKIN *et al.* 1996). Sup35p accumulates in high molecular weight aggregates only in strains carrying *[PSI⁺]*.

The discovery of prion strains (DICKINSON and MEIKLE 1971; BRUCE and FRASER 1991) has challenged the prion hypothesis. Isogenic mice infected with different prion "strains" differ in the disease incubation period and the regions of the brain that are affected (HECKER *et al.* 1992). It has been suggested (BESSEN and MARSH 1992; BESSEN *et al.* 1995) that PrP proteins can have more than one self-propagating conformation and/or nucleation pattern that determines the strain-specific properties of prions. This hypothesis was supported by the discovery of strain-specific patterns of protease resistant PrP^{Sc} peptides (BESSEN and MARSH 1992) that are preserved during *in vitro* conversion of PrP^{C} into PrP^{Sc} (BESSEN *et al.* 1995), and by our recent finding that *[PSI⁺]* factors of different efficiencies can be induced by overproduction of plasmid-encoded Sup35p in the same yeast strain (DERKATCH *et al.* 1996).

In this article we examine *S. cerevisiae* strain derivatives obtained after growth in guanidine hydrochloride-containing media or after transient alteration of Hsp104p levels for their ability to be reinduced to become *[PSI⁺]* by an excess of Sup35p. Since curing of a prion does not imply the loss of its master gene, loss of the prion should not prevent it from arising again *de novo*. This is in contrast to a plasmid or virus that cannot arise *de novo* after curing. Surprisingly we found that cured *[psi⁻]* derivatives differ in their ability to support the *de novo* induction of *[PSI⁺]* as well as in the characteristic suppression and growth inhibition responses previously associated with excess Sup35p. The genetic relationship between the different types of *[psi⁻]* derivatives is explored and the hypothesis that there are different heritable $Sup35p^{Psi-}$ conformational variants is considered.

MATERIALS AND METHODS

Cultivation procedures: Standard yeast media and cultivation procedures were used (SHERMAN *et al.* 1986). Unless specifically mentioned yeast were grown in organic complete medium (YPD). The efficiency of suppression of the *[PSI⁺]* suppressible *ade1-14, lys9-A21, trp5-48* and *met8-1* nonsense alleles was estimated from the level of growth at 20° and/or 30° on media where suppression was required for propagation, *i.e.*, synthetic complete glucose media (SC) lacking adenine (SC −Ade), lysine (SC −Lys), tryptophan (SC −Trp) or methionine (SC −Met), respectively, as well as synthetic complete non-glucose media with 20 ml/liter ethanol (SEt) lacking adenine (SEt −Ade). Suppression of the *ade1-14* mutation was also scored by a color test on YPD since colonies of *ade1* mutants are red on YPD due to the accumulation of a red pigment. Suppression of these mutations prevents accumulation of this pigment resulting in lighter (pink or white) colonies. The intensity of the color change reflects the efficiency of suppression.

Transformants were grown in media selective for plasmid maintenance, *i.e.*, SC −Ura or SC −Leu. To eliminate plasmids bearing *URA3*, two subsequent replica platings of transformants on SC medium containing 1 mg/ml of 5-fluoroortic acid (+5-FOA; BOEKE *et al.* 1984) were used, and the loss of plasmids was confirmed by replica plating on SC −Ura. To obtain cells that lost a *LEU2*-containing plasmid but retained a *URA3*-containing plasmid, cotransformants were replica plated two times on YPD to allow for plasmid loss. Cultures were then streaked for single colonies on SC −Ura to select for colonies that retained the *URA3*-containing plasmid and replica plated on SC −Leu to identify colonies that lost the *LEU2*-containing plasmid.

YPD medium containing 1 mM guanidine hydrochloride (+1 mM GuHCl; purchased from Sigma) or 5 mM guanidine hydrochloride (+5 mM GuHCl or +GuHCl) was used to eliminate *[PSI⁺]* as well as to obtain strain derivatives not inducible to the *[PSI⁺]* state. Unless specifically mentioned the latter medium was used. To treat cells with GuHCl, they were patched and incubated on GuHCl-containing medium three consecutive times (~21 generations). No inhibition of growth was observed on GuHCl-containing media. Control experiments were performed analogously using YPD medium lacking GuHCl. Cells were then streaked for single colonies on YPD.

To induce the *GAL* promoter, organic or synthetic complete media containing 20 mg/ml galactose as a single carbon source was used (YPGal and SGal, respectively).

For phenotypic characterization of different *[psi⁻]* derivatives, organic complete media containing 20 ml/liter ethanol (YPEt) or 20 ml/liter glycerol (YPG) as a single carbon source and YPD media containing 0.2, 0.35, 0.5, 0.75, 1.0 or 1.5 mg/ml paromomycin (purchased from Sigma); 0.5, 1.0, 1.5, 2.0

or 2.5 mg/ml trichodermin (a gift of W. O. Godtfredsen, Leo Pharmaceutical Products, Denmark); 0.5, 1.0, 1.5, 2.0 or 3.5 M potassium chloride and 2.0 or 2.5 M ethylene glycol were used. Phenotypic suppression (SINGH *et al.* 1979b; PALMER *et al.* 1979) was estimated by comparing growth on SC −Ade and SC media both supplemented with 0.00, 0.01, 0.05, 0.1 and 0.2 mg/ml of paromomycin after 7 days of incubation at 30° and 20°.

Plasmids: The pGAL::SUP35 plasmid (also called pVK71; DERKATCH *et al.* 1996) is a YCp50 (ROSE *et al.* 1987)-based *CEN-URA3* vector that contains the promoterless *SUP35* gene under the control of the inducible *CYC1-GAL1 (GAL)* promoter.

A series of pEMBL-yex4 (CEZARINI and MURRAY 1987)-based plasmids containing either the complete *SUP35* gene (pEMBL-SUP35) or its deletion alleles controlled by the original *SUP35* promoter has been described earlier (TER-AVANESYAN *et al.* 1993). pEMBL-Δ3ATG contains a *SUP35* allele encoding the Sup35p C-terminus starting at amino acid 254. The maps of other *SUP35* deletion alleles used are presented in Figure 4. All these 2μ plasmids carry both the yeast *URA3* and *LEU2-d* selection markers. While these plasmids are normally maintained at 10–20 copies per cell (*e.g.*, on SC −Ura medium), they can be overamplified to ~100 copies per cell by selecting for expression of the defective *LEU2-d* allele on SC −Leu medium (see copy number determination in DERKATCH *et al.* 1996). The ability of the plasmids containing *SUP35* fragments to cause nonsense suppression, rescue *[PSI⁺]* maintenance, induce *[PSI⁺] de novo* at moderate copy number and inhibit growth of yeast strains at high copy number was studied previously (TER-AVANESYAN *et al.* 1993, 1994, DERKATCH *et al.* 1996).

pSTR7 (TELCKOV *et al.* 1986) is a YEp13 (BROACH and HICKS 1980)-based 2μ plasmid that contains the *LEU2* selectable marker and the complete *SUP35* gene. pYCH-U2 is a pFL38 (BONNEAUD *et al.* 1991)-based centromeric plasmid that contains the *URA3* selectable marker and the 4.2-kb *Bam*HI-*Xho*I insertion containing the complete *SUP35* gene from pSTR7.

pYS104 (SANCHEZ and LINDQUIST 1990) is a pRS316 (SIKORSKI and HEITER 1989)-based centromeric *URA3* plasmid containing the complete *HSP104* gene. The pYS-GAL104 plasmid (LINDQUIST and KIM 1996) is a centromeric vector containing the *URA3* marker as well as the *HSP104* gene under the control of the *GAL* promoter.

YRpHO (kindly provided by R. E. ESPOSITO) is a YIp5 (STRUHL *et al.* 1979)-based plasmid that contains an insert bearing the *HO* gene and an ARS sequence (JENSEN *et al.* 1983).

Strains: *S. cerevisiae* strains used in this study are listed in Table 1. Three independently obtained mutants of strain 74-D694, called 9ΔN-74-D694, 12ΔN-74-D694 and 19ΔN-74-D694, which contain the deletion *sup35-ΔN* allele in place of the wild-type *SUP35* allele, were obtained by the method of integration/excision (ROSE *et al.* 1990). The pEMBL-Δ3ATG plasmid bearing a functional *SUP35* fragment starting at codon 254 (TER-AVANESYAN *et al.* 1993, see also description below) was linearized with *Bal*I and integrated into the *SUP35* gene by selecting for transformants on SC −Ura and screening for those that are Leu⁻. +5-FOA medium was used to select for plasmid excision and PCR was used to identify mutants that contained only the deletion *sup35-ΔN* allele. The fourth such deletion strain (877ΔN-74-D694) was obtained by a one-step replacement procedure. The strain derivative [PSI⁺]7-74-D694, that is white on YPD due to suppression of the *ade1-14* nonsense mutation, was cotransformed with the *URA3*-containing plasmid pRS316 and the *Pst*I-*Xba*I fragment of pEMBL-Δ3ATG, which contains the upstream *SUP35* noncoding region fused with a complete *SUP35C* fragment starting at codon 254 (AUG). Ura⁺ transformants were screened for color on YPD plates. Red *([psi⁻])* transformants were cured of the pRS316 plasmid and subjected to Southern hybridization and PCR analysis. Among several hundred Ura⁺ transformants tested, one contained the *sup35-ΔN* allele. The 94ΔN-33G-D373 mutant containing the same *sup35-ΔN* allele was kindly provided by O. V. TARUNINA and S. G. INGE-VECHTOMOV (St. Petersburg State University, Russia).

Construction of the 74-D694 derivative bearing an *hsp104::URA3* disruption has been described previously (CHERNOFF *et al.* 1995). OT46 (also called Ura3-hsp104-del3U) is a Ura⁻ mutant of this strain selected as a spontaneous Ura⁻ clone on +5-FOA medium. The continuous presence of the disruption in this strain was confirmed by Southern analysis.

GF275-α6 is an isogenic *MATα* derivative of strain D1142-1A. To construct this strain, D1142-1A was transformed with YRpHO, which induces a mating-type switch. The plasmid was then eliminated and the resulting diploid was sporulated and dissected.

TABLE 1

Strains of the yeast *S. cerevisiae* used in this work

Name	Genotype	Source
74-D694	*MAT***a** *ade1-14*(UGA) *trp1-289*(UAG) *ura3-52 his3-Δ200 leu2-3,112*	CHERNOFF *et al.* (1995)
9ΔN-74-D694	*sup35-ΔN* mutant obtained in 74-D694	This study
12ΔN-74-D694	*sup35-ΔN* mutant obtained in 74-D694	This study
19ΔN-74-D694	*sup35-ΔN* mutant obtained in 74-D694	This study
877ΔN-74-D694	*sup35-ΔN* mutant obtained in [PSI⁺]7-74-D694 derivative of 74-D694	This study
OT46	*hsp104::ura3* disruption obtained in 74-D694	This study
64-D697	*MATα ade1-14*(UGA) *trp1-289*(UAG) *leu2-3,112 ura3-52 lys9-A21*(UAA)	This study
70-D697	*MATα ade1-14*(UGA) *trp1-289*(UAG) *leu2-3,112 ura3-52 lys9-A21*(UAA)	This study
33G-D373	*MATα pheA-10*(UAA) *his7-1*(UAA) *lys9-A21*(UAA) *trp1-289*(UAG) *ade2-144,717 ura3-52 leu2-3,112*	CHERNOFF *et al.* (1988, 1992)
94ΔN-33G-D373	*sup35-ΔN* mutant obtained in 33G-D373	O. V. TARUNINA and S. G. INGE-VECHTOMOV, unpublished data
D1142-1A	*MAT***a** *met8-1*(UAG) *trp5-48*(UAA) *ura3-1 cyc1-72*(UAA) *leu2-1*(UAA) *his4-166*(UGA) *aro7-1*(UAG) *lys2-187*(UGA)	EUSTICE *et al.* (1986)
GF275-α6	*MATα* derivtive of D1142-1A	This study

Analysis of phenotypes associated with *SUP35* overexpression: *Induction of the de novo appearance of the [PSI$^+$] factor:* Qualitative tests for the induction of the *de novo* appearance of *[PSI$^+$]* were performed as described previously (CHERNOFF *et al.* 1995; DERKATCH *et al.* 1996). Generally both transient induction of the *GAL::SUP35* construct and transient introduction of the multicopy pEMBL-SUP35 plasmid containing the wild-type *SUP35* gene with its normal promoter were used to test the inducibility of *[psi$^-$]* derivatives to the *[PSI$^+$]* state. In brief, pGAL::SUP35 and control YCp50 transformants were replica plated from SC −Ura to SGal −Ura where the *GAL* promoter was induced. SC −Ura-repressing medium was used as a control. Cultures were then replica plated to SC −Ade, SC −Trp or SC −Met where the *GAL* promoter was repressed and the appearance of *[PSI$^+$]* was detected by suppression of the *ade1-14, trp5-48* or *met8-1* nonsense mutations, respectively. Alternatively, transformants with pEMBL-based plasmids were replica plated from SC −Ura to media where suppression of a nonsense mutation was required for growth (*e.g.,* SC −Ade). If suppression suggestive of *[PSI$^+$] de novo* appearance was observed, plasmids were eliminated on +5-FOA (see above) to determine if suppression was independent of the presence of the plasmid. Suppression that remained after plasmid loss indicated the presence of *[PSI$^+$]*. Additional passes on SC −Ura medium, as well as overamplification of the plasmids on SC −Leu before transferring to adenineless media, were used to verify negative results. At least three transformants of each strain or GuHCl-treated derivative were analyzed. *[PSI$^+$]* induction was confirmed by demonstrating the stability of the suppressor phenotype on YPD and its instability on +GuHCl.

To test for *[PSI$^+$]* induction by transient overexpression of *SUP35* in *sup35-ΔN* mutants that cannot maintain *[PSI$^+$]*, the mutant strains as well as wild-type control strains were cotransformed with pYCH-U2 (CEN-URA3-SUP35) and pSTR7 (2μ-LEU2-SUP35) plasmids. The presence of pYCH-U2 allowed for *[PSI$^+$]* maintenance, while pSTR7 was used to induce *[PSI$^+$]*. Plasmids pFL38 and YEp13 were used as controls. Transformants were selected, patched and replica plated twice on SC −Ura −Leu medium. Colonies that lost pSTR7 (or YEp13) were obtained as described above, patched on SC −Ura and replica plated to SC −Ade to score for suppression indicative of *[PSI$^+$]* appearance.

Growth inhibition: Growth inhibition was examined as described previously (DERKATCH *et al.* 1996) by spotting transformants with pEMBL-based plasmids selected on SC −Ura to SC −Leu where a high plasmid copy number is required for growth (see above).

Nonsense suppression: Unlike induction of *[PSI$^+$] de novo* appearance, this phenotype is scored while Sup35p or its fragments remain overproduced (CHERNOFF *et al.* 1988, 1992; TER-AVANESYAN *et al.* 1993; DERKATCH *et al.* 1996). Transformants with centromeric or 2μ-based plasmids expressing *SUP35* alleles were grown on media selective for plasmid maintenance at low or moderate copy number, respectively. They were then replica plated onto media where suppression was required for propagation (*e.g.,* SC −Ade). Nonsense suppression caused by overexpression of the wild-type *SUP35* gene or its deletion alleles usually, but not always, correlates with *[PSI$^+$] de novo* appearance (DERKATCH *et al.* 1996).

DNA manipulations: Routine DNA manipulations were performed according to standard protocols (SAMBROOK *et al.* 1989). A radioactively labeled 3-kb *Kpn*I *HSP104*-containing fragment from pYS104 and a psoralene-biotin labeled 1.1-kb *Sal*I-*Hin*dIII *SUP35* fragment were used as probes for Southern (DNA) analyses. In the latter case labeling and detection were performed with a Schleicher and Schuell chemiluminescent kit according to company protocols. PCR, utilizing oligonucleotide primers 5′CAGTGTTCGAGTCTGTGTCA and 5′GGCATCAACATGACCCATGA homologous to the region upstream of the *SUP35* gene and to *SUP35C*, respectively, was used to identify a *sup35-ΔN* deletion in 877-ΔN-74-D694 by the size of the PCR product. Likewise in all other experiments, primers 5′CACTTCTTACCTTGCTCTTA and 5′TGAGAG GTGAAGTTTACTTG homologous to the regions upstream and downstream of the *SUP35* gene, respectively, were used to distinguish fragments of yeast chromosomal DNA containing wild-type *SUP35* and *sup35-ΔN* alleles by the size of the PCR products.

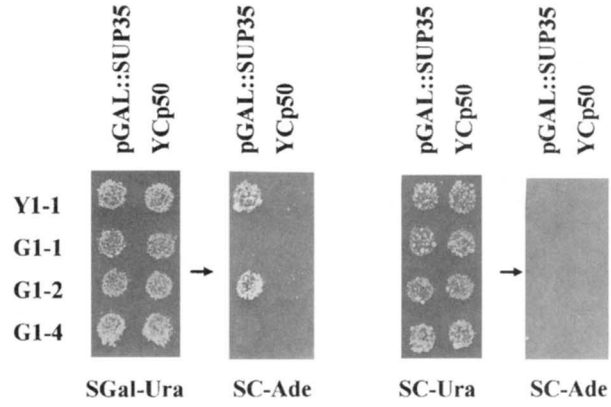

FIGURE 1.—GuHCl treatment affects the ability of derivatives of the Pin$^+$ *[psi$^-$]* 74-D694 strain to be induced to the *[PSI$^+$]* state by Sup35p overexpression. Three GuHCl-treated derivatives of 74-D694 (G1-1, G1-2 and G1-4) and a 74-D694 derivative (Y1-1) obtained in the control experiment where incubations were performed on YPD medium lacking GuHCl (see MATERIALS AND METHODS) are shown. Patches show growth of transformants bearing the *GAL::SUP35*-containing plasmid (pGAL::SUP35) or the control vector (YCp50) on media for *GAL* promoter induction (SGal −Ura) or repression (SC −Ura) and on repressing SC −Ade medium following *GAL* promoter induction or repression. Arrows indicate replica plating. Derivatives G1-1 and G1-4 are Pin$^-$ while G1-2 and Y1-1 remain Pin$^+$.

RESULTS

GuHCl treatment can cure Sup35p-mediated *[PSI$^+$]* inducibility: We have previously shown that the three *[psi$^-$]* strains, 74-D694, 64-D694 and 70-D697, give rise to *[PSI$^+$]* derivatives following overproduction of the whole Sup35p (DERKATCH *et al.* 1996). The *de novo* appearance of *[PSI$^+$]* was detected as growth on SC − Ade medium following transient induction of the *GAL::SUP35* construct or transient introduction of the multicopy pEMBL-SUP35 plasmid (see MATERIALS AND METHODS). Such strains are referred to as Pin$^+$ (inducible to *[PSI$^+$]*) in this article.

Surprisingly, when these *[psi$^-$]* strains were treated with 5 mM GuHCl and were colony purified on YPD, we observed that a significant proportion of the derivatives obtained could no longer be induced to become *[PSI$^+$]* following transformation with either pEMBL-SUP35 or pGAL::SUP35 (Figure 1 and Table 2). These strain derivatives that cannot be induced to the *[PSI$^+$]* state by Sup35p overproduction will be referred to as Pin$^-$. The

TABLE 2

GuHCl treatment affects the ability of yeast strains to be induced to *[PSI⁺]* by Sup35p overproduction

Strain	5 mM GuHCl treatment	No. of subclones analyzed		
		Total	Pin⁺	Pin⁻
74-D694	Yes	7	2	5
	No	3	3	0
64-D697	Yes	4	0	4
	No	2	2	0
70-D697	Yes	3	0	3
	No	2	2	0
Total	Yes	14	2	12
	No	7	7	0

efficiency of *[PSI⁺]* induction was not reduced in the GuHCl-treated derivatives that remain Pin⁺, and controls not treated with GuHCl all remained Pin⁺.

It was previously reported that moderate overexpression of the wild-type *SUP35* gene causes suppression of nonsense mutations in *[psi⁻]* strains (CHERNOFF *et al.* 1988, 1992), and that a high level of *SUP35* overexpression causes growth inhibition (DERKATCH *et al.* 1996). We now find that moderate *SUP35* overexpression does not cause suppression of nonsense mutations in Pin⁻ strains and their growth is only slightly inhibited when *SUP35* is amplified to high levels (Figure 2). In contrast, the level of nonsense suppression and growth inhibition is not reduced in the GuHCl-treated Pin⁺ derivatives compared to the original strains not treated with GuHCl (Figure 2). These data show that all known phenotypes associated with Sup35p overproduction, namely, *[PSI⁺]* induction, suppression and growth inhibition, are cured in Pin⁻ derivatives.

No other phenotypic differences were found between Pin⁺ and Pin⁻ *[psi⁻]* derivatives under various experimental conditions including those known to affect the growth of translational fidelity mutants (see HINNEBUSH and LIEBMAN 1991). Strains were spotted or serially diluted and pipetted on YPD, YPG and YPEt media as well as on YPD media containing different concentrations of paromomycin, trichodermin, potassium chloride, and ethylene glycol (see MATERIALS AND METHODS), and incubated at 30°, 20° and, in the case of YPD medium, at 37° (data not shown). In addition, Pin⁺ and Pin⁻ derivatives generally did not differ in the level of phenotypic suppression of the *ade1-14* mutation caused by low concentrations of paromomycin. Surprisingly however, the level of phenotypic suppression in one of the two Pin⁻ derivatives of 74-D694 examined was considerably higher than in other Pin⁺ and Pin⁻ derivatives analyzed, although there was no difference in sensitivity to these levels of paromomycin (data not shown). Possibly, the more easily suppressed Pin⁻ derivative contains an allosuppressor mutation or a *[pin⁻]* variant.

We next determined if *[psi⁻]* isolates obtained from

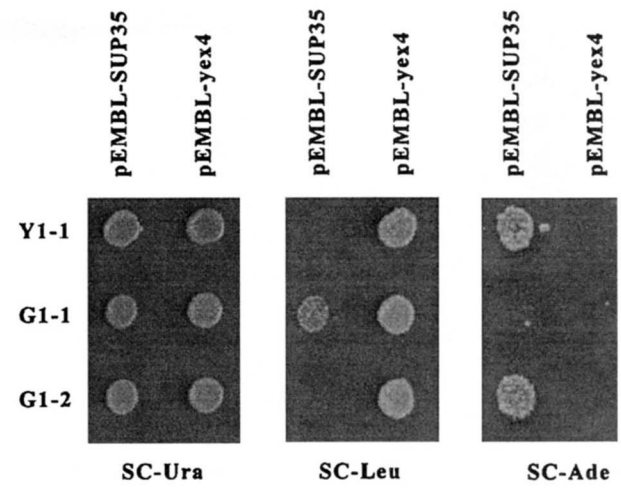

FIGURE 2.—Poor growth and suppression phenotypes associated with Sup35p overproduction are cured in Pin⁻ derivatives. G1-1 and G1-2 are, respectively, Pin⁻ and Pin⁺ GuHCl-treated derivatives of 74-D694. Y1-1 is a 74-D694 derivative obtained in the control experiment where incubations were performed on YPD medium (see MATERIALS AND METHODS). Spots show growth of transformants bearing the multicopy *SUP35*-containing plasmid (pEMBL-SUP35) or the control vector (pEMBL-yex4) on the indicated media. Plasmids are maintained at moderate copy number on SC −Ura and at high copy number on SC −Leu (growth inhibition test). Suppression of the *ade1-14* nonsense mutation is required for growth on SC −Ade medium.

[PSI⁺] can also be both Pin⁺ and Pin⁻. *[PSI⁺]* derivatives with different efficiencies of suppression obtained previously in 74-D694 (DERKATCH *et al.* 1996) were also treated with 1 or 5 mM GuHCl and colony purified on YPD. Such GuHCl treatment is known to efficiently cure *[PSI⁺]* (TUITE *et al.* 1981). Independent *[psi⁻]* derivatives were then transformed with pGAL::SUP35 to test for their ability to be induced to become *[PSI⁺]*. The results presented in Table 3 show that Pin⁻ subclones were found among the GuHCl-treated derivatives of all *[PSI⁺]* strains tested, regardless of the suppression efficiency associated with the original *[PSI⁺]* strain. A higher proportion of Pin⁻ derivatives was always obtained when the higher concentration of GuHCl was used. It is important to stress that each of the original *[PSI⁺]* derivatives also gave rise to some Pin⁺ GuHCl-cured *[psi⁻]* derivatives. Furthermore, these derivatives could be induced to become *[PSI⁺]* with the same frequency as the original strain, and the phenotypic characteristics of *[PSI]* factors preexisting in yeast strains did not affect the phenotypic characteristics of the *[PSI⁺]* factors reinduced (DERKATCH *et al.* 1996).

Effects of transient alterations in Hsp104 levels on *[PSI⁺]* inducibility: Similar experiments were performed in *[PSI⁺]* derivatives of 74-D694 and D1142-1A that were cured of *[PSI⁺]* by deletion of *HSP104* or transient overexpression of Hsp104p (CHERNOFF *et al.* 1995). To cure *[PSI⁺]* by Hsp104 overproduction, *[PSI⁺]* D1142-1A and two *[PSI⁺]* 74-D694 derivatives

TABLE 3

GuHCl effects on the ability to reinduce *[PSI⁺]* in 74-D694 derivatives cured of *[PSI⁺]*

[PSI⁺] derivative		No. of *[psi⁻]* subclones analyzed					
		After incubation on +1 mM GuHCl			After incubation on +5 mM GuHCl		
Name	Suppression efficiency	Total	Pin⁺	Pin⁻	Total	Pin⁺	Pin⁻
[PSI⁺]7-74-D694	Strong	2	1	1	8	0	8
[PSI⁺]8-74-D694	Strong	7	4	3	8	1	7
[PSI⁺]13-74-D694	Weak	8	5	3	7	1	6
[PSI⁺]14-74-D694	Weak	6	6	0	8	0	8
Total	Different	23	16	7	31	2	29

with different efficiencies of suppression were transformed with *GAL::HSP104* containing plasmid pYS-GAL104. *[PSI⁺]* elimination was induced on SGal −Ura medium as previously described (CHERNOFF *et al.* 1995). Resulting *[psi⁻]* derivatives were colony purified, cured of the pYS-GAL104 plasmid and transformed with pGAL::SUP35 to test for their ability to be induced to become *[PSI⁺]*. All seven independently obtained Hsp104-cured *[psi⁻]* derivatives of *[PSI⁺]* D1142-1A and all three independently obtained Hsp104-cured *[psi⁻]* derivatives of *[PSI⁺]* 74-D694 variants were Pin⁺. Two of the Hsp104-cured *[psi⁻]* Pin⁺ derivatives of *[PSI⁺]* 74-D694 variants were also tested for suppression and growth inhibition in the presence of pEMBL-SUP35 plasmid (see MATERIALS AND METHODS) and both phenotypes were detected in these derivatives. Note that two independently obtained 5 mM GuHCl-cured *[psi⁻]* D1142-1A derivatives and four independently obtained 5 mM GuHCl-cured *[psi⁻]* derivatives of 74-D694 *[PSI⁺]* variants were Pin⁻.

In another experiment we have used an *hsp104*-Δ strain OT46. OT46 was obtained from a *[PSI⁺]*-containing derivative, in which *[PSI⁺]* was induced by Sup35p overproduction in a Pin⁺ variant of strain 74-D694. *[psi⁻]* progeny of this *[PSI⁺]* derivative, induced by Hsp104 overproduction, remain Pin⁺ (see above). OT46 is *[psi⁻]* due to the presence of the *hsp104*-Δ allele. OT46 was cotransformed with the centromeric *HSP104*-containing plasmid, pYS104, and multicopy *SUP35*-containing plasmid, pSTR7. If this strain remained Pin⁺, we would expect pSTR7 to be able to cause suppression and to induce the *de novo* appearance of *[PSI⁺]* in the presence of pYS104, since the latter plasmid has previously been shown (SANCHEZ *et al.* 1992; CHERNOFF *et al.* 1995) to restore Hsp104 levels to approximately wild type. However, neither suppression nor an increase in the number of Ade⁺ revertants indicative of *[PSI⁺]* induction were detected in OT46 transformants bearing both pYS104 and pSTR7. This indicates that the *hsp104*-Δ disruption bearing OT46 strain has actually become Pin⁻.

Genetic analysis of the Pin⁺ phenotype: Five Pin⁻ and two Pin⁺ *[psi⁻]* derivatives of 74-D694 were crossed to Pin⁺ *[psi⁻]* isolates of 64-D697 and 70-D697. Introduction and transient expression of the *GAL::SUP35* construct in each of these diploids resulted in the *de novo* appearance of *[PSI⁺]* at similar frequencies regardless of the Pin status of the 74-D694 parent. This indicates that inducibility is a dominant trait. *[PSI⁺]* factors characterized by both weak and strong efficiencies of suppression of nonsense mutations were obtained in all these diploids (data not shown).

We have also demonstrated that the Pin⁺ phenotype is inherited in a non-Mendelian fashion, similarly to the prion elements *[URE3]* and *[PSI]*. In this experiment a Pin⁻ 74-694 derivative and a Pin⁺ 64-D697 isolate were mated and the resulting diploid was sporulated and dissected. All 28 segregants of the seven tetrads examined were Pin⁺. The efficiency of *[PSI⁺]* induction varied between the segregants, but the same degree of variability was observed in the analogous experiment with segregants from crosses between two Pin⁺ parents. We attribute variability to the meiotic segregation of multiple modifiers of translational suppression preexisting in the parental strains.

Analysis of diploids made by crossing Pin⁻ and Pin⁺ GuHCl-treated derivatives of 74-D694 to *[PSI⁺]* derivatives of both 64-D697 and 70-D697 revealed that the diploids were all *[PSI⁺]*. Furthermore, the efficiency of suppression of the homozygous *ade1-14* nonsense mutation was unaffected by the inducibility status of the 74-D694 parent (Figure 3). The frequency of *[PSI⁺]* loss, detected as red sectors on YPD medium, was likewise not affected by the inducibility status of the 74-D694 parent. Thus, the Pin⁻ phenotype is not dominant for causing the loss of *[PSI⁺]*.

One explanation for the Pin⁻ phenotype is that *[PSI⁺]* was induced but "hidden" in Pin⁻ derivatives. To check this possibility, two 74-D694 Pin⁻ derivatives were transformed with pGAL:SUP35 and, following transient induction on SGal −Ura medium, were crossed with a 64-D697 Pin⁺ *[psi⁻]* isolate on glucose. Suppression of the homozygous *ade1-14* mutation in the diploids would imply that *[PSI⁺]* was present in a hidden state in the pGAL:SUP35 transformants of the Pin⁻ parent after *GAL* promoter induction. However, all hybrids

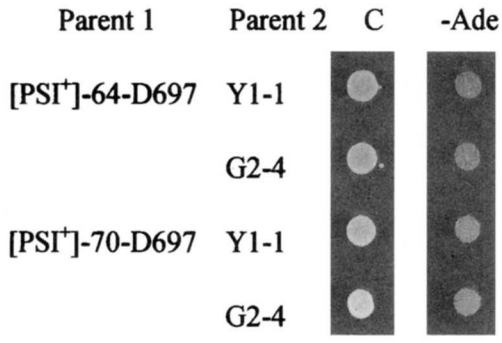

FIGURE 3.—*[PSI+]* can be maintained and is not antisuppressed in diploids from crosses of Pin$^-$ *[psi$^-$]* 74-D694 derivatives and *[PSI+]* derivatives of either 64-D697 or 70-D697. G2-4 is a Pin$^-$ GuHCl-treated derivative of 74-D694. Y1-1 is a Pin$^+$ 74-D694 derivative obtained in the control experiment where incubations were performed on YPD medium (see MATERIALS AND METHODS). Spots show growth of the diploids made by crossing the indicated parental strains. C indicates SC −His −Lys medium where only the hybrids but not the parental strains can grow; −Ade indicates SC −His −Lys −Ade medium. The level of growth on adenineless medium, determined by the efficiency of suppression of the homozygous *ade1-14* nonsense mutation by *[PSI+]*, is not affected by the inducibility status of the 74-D694 parental strain. Growth on SC −His −Lys −Ade medium was analyzed after 2, 3, 5 and 7 days of incubation at 30° and 20°, and the photograph was taken after 7 days of incubation.

remained Ade$^-$ after 20 days of incubation at 30° and 20°, proving that the absence of nonsense suppression after temporary Sup35p overproduction in the GuHCl-treated Pin$^-$ haploids is indeed due to the lack of *[PSI+] de novo* appearance and not due to the elimination of a factor required for *[PSI+]*-associated suppression. Control diploids made with GuHCl-treated 74-D694 Pin$^+$ derivatives that were previously transformed with pGAL:SUP35 and transiently induced on SGal −Ura medium before mating grew on adenineless media. A similar experiment was performed with a Pin$^-$ GuHCl-cured derivative of the strain GF275-α6 with the same result. In this experiment a *[psi$^-$]* Pin$^+$ derivative of an isogenic *MAT***a** D1142-1A strain was used as a mating partner, and the absence of *[PSI+]* in the diploid was confirmed on SC −Trp and SC −Met media.

The N-terminal domain of Sup35p does not determine the Pin$^+$ phenotype: We asked whether the N-terminal domain of Sup35p was responsible for the Pin$^+$ phenotype, since it was previously shown that the N-terminal region of Sup35p is required for *[PSI+]* maintenance (TER-AVANESYAN *et al.* 1994) and is sufficient for the *de novo* induction of *[PSI+]* when overproduced (DERKATCH *et al.* 1996; PATINO *et al.* 1996).

Phenotypes associated with *SUP35* overexpression were analyzed in strains containing independently obtained chromosomal deletions of the *SUP35* fragment encoding the N-terminal domain of Sup35p. Four such deletions were obtained in Pin$^+$ *[psi$^-$]* 74-D694 or 33G-D373 derivatives, while one was obtained in a *[PSI+]* 74-D694 isolate (see MATERIALS AND METHODS). When transformed with pEMBL-SUP35 plasmid, all these *sup35-*ΔN mutants showed clear growth inhibition and suppression,[1] the two phenotypes characteristic of Pin$^+$ strains.

The presence of the *sup35-*ΔN mutation makes it difficult to score for the induction of *[PSI+]* directly since strains lacking the N-terminal region of Sup35p cannot support *[PSI+]* maintenance and since *sup35-*ΔN is a dominant antisuppressor to *[PSI+]* (TER-AVANESYAN *et al.* 1993, 1994). However, when the three *sup35-*ΔN mutants obtained in a Pin$^+$ *[psi$^-$]* 74-D694 derivative were cotransformed with centromeric (pYCH-U2) and multicopy (pSTR7) plasmids containing wild-type *SUP35* (see MATERIALS AND METHODS), we found that they were indeed Pin$^+$. Plasmid pSTR7 causes suppression and induces *[PSI+]* in inducible strains, while pYCH-U2 is required for the maintenance of *[PSI+]* in *sup35-*ΔN strains but does not induce *[PSI+]* efficiently. Transformants were selected, patched and replica plated twice on SC −Ura −Leu. Colonies that lost pSTR7 (or YEp13) (see MATERIALS AND METHODS) were patched on SC −Ura and replica plated to SC −Ade. A fraction of the progeny of the pYCH-U2/pSTR7 cotransformants of all *sup35-*ΔN mutants was presumed to be *[PSI+]* because they showed weak suppression of the *ade1-14* nonsense mutation after pSTR7 was lost.[2]

To confirm the non-Mendelian nature of the determinant responsible for the Pin$^+$ phenotype in these three *sup35-*ΔN mutants, they were crossed with two GuHCl-treated Pin$^-$ derivatives of 64-D697, as well as with the original Pin$^+$ *[psi$^-$]* 64-D697 strain. In this experiment the diploids and meiotic progeny bearing the wild-type *SUP35* allele could be directly scored for inducibility to the *[PSI+]* state. All the diploids were Pin$^+$ although a low level of suppression efficiency was associated with *[PSI+]* in diploids because of the presence of the *sup35-*ΔN allele, which causes dominant antisuppression to *[PSI+]* (TER-AVANESYAN *et al.* 1993). However, the efficiency of suppression was the same whether

[1] The level of suppression caused by pEMBL-SUP35 and pSTR7 varied in the different *sup35-*ΔN isolates of 74-D694 as it did in wild-type *SUP35* isolates obtained following integration/excision. Suppression in 9ΔN-74-D694 was higher than in the original inducible 74-D694 strain and was not changed in 19ΔN-74-D694 and 877ΔN-74-D694. There was no observable suppression in 12ΔN-74-D694 transformants unless a centromeric *SUP35*-containing plasmid was also present.

[2] A quantitative estimate of the frequency of *[PSI+] de novo* appearance in 9ΔN-74-D694 and 19ΔN-74-D694 transformants was not possible since the dominant antisuppressor effect of the *sup35-*ΔN allele reduced the suppression efficiency of *[PSI+]*, sometimes to a level not detectable at 30°, while at 20° weak suppression caused by pYCH-U2 alone could be detected in some cells, probably due to an occasional uneven segregation of the centromeric plasmid. The frequency of *[PSI+] de novo* appearance in 12ΔN-74-D694 is also an underestimate due to the antisuppressor effect of the *sup35-*ΔN allele. However in this deletion derivative, pYCH-U2 did not cause any suppression, therefore all suppression of *ade1-14* at 30° and 20° was presumed to be due to *[PSI+]*, which allowed us to estimate that at least 45% of pYCH-U2/pSTR7 cotransformant progeny is *[PSI+]*.

[PSI⁺] was induced in diploids made with Pin⁺ or Pin⁻ 64-D697 derivatives. To analyze the meiotic progeny, one of the diploids (cross of the Pin⁺ 9ΔN-74-D694 deletion strain with a Pin⁻ 64-D697 derivative) was sporulated and dissected. PCR was used to distinguish between the wild-type and deletion *SUP35* alleles in the meiotic segregants. We showed that six Gal⁺ segregants from four tetrads carrying the wild-type *SUP35* allele were Pin⁺ since they could be induced to become *[PSI⁺]*. The results of nonsense suppression and growth inhibition tests in pEMBL-SUP35 transformants were in agreement with these data.

Since the determinant required for inducibility to the *[PSI⁺]* state was present in the *sup35-ΔN* mutants, we tested whether it could be eliminated by GuHCl treatment. All four independent *sup35-ΔN* mutants obtained in 74-D694 (see MATERIALS AND METHODS) were treated with 5 mM GuHCl and were then colony purified on YPD medium. Overamplification of pEMBL-SUP35 caused only weak growth inhibition, characteristic of Pin⁻ strains, in all GuHCl-treated subclones of each of the *sup35-ΔN* mutants. In addition, all suppressor activity caused by pEMBL-SUP35 in Pin⁺ *sup35-ΔN* mutants was missing in their GuHCl-treated derivatives, indicating that both the growth inhibition and suppression associated with the Pin⁺ phenotype can be cured by GuHCl in *sup35-ΔN* strains.

Overexpression of short but not long Sup35p N-terminal fragments can induce *[PSI⁺]* in Pin⁻ derivatives: We tested whether Pin⁻ derivatives that cannot be induced to become *[PSI⁺]* by overproduction of the complete Sup35p can be induced to become *[PSI⁺]* by overproduction of fragments of Sup35p. Two 74-D694 Pin⁻ derivatives were transformed with pEMBL-based plasmids containing *SUP35* fragments some of which were previously shown to be sufficient for *[PSI⁺]* induction in Pin⁺ isolates: pEMBL-ΔBal2, pEMBL-ΔBcl, pEMBL-ΔSal, pEMBL-ΔHpa, pEMBL-ΔBst (DERKATCH *et al.* 1996). Suppression of the *ade1-14* nonsense mutation was analyzed on SC −Ade and SEt −Ade media after incubation at 30° and 20°. The transformants were also spotted onto SC −Leu medium to score for the inhibition of growth by high copy number plasmids containing *SUP35* fragments (DERKATCH *et al.* 1996). The results are presented in Figure 4.

Transformation with the pEMBL-ΔBal2 plasmid containing the *SUP35* fragment encoding the first 154 amino acids of Sup35p caused suppression in the Pin⁻ derivatives that was nearly as efficient as in the original Pin⁺ strain. Furthermore, the plasmid caused induction of *[PSI⁺]*, since GuHCl-curable suppression remained after the plasmid was eliminated. When overamplified in Pin⁻ derivatives, pEMBL-ΔBal2 caused stronger growth inhibition than pEMBL-SUP35. However, growth inhibition caused by overamplification of pEMBL-ΔBal2 was weaker in Pin⁻ than in Pin⁺ derivatives. No suppression indicative of *[PSI⁺]* induction was observed in transformants of Pin⁻ derivatives with pEMBL-ΔBcl that contains a *SUP35* fragment 84 amino acids longer than in pEMBL-ΔBal2 or with pEMBL-ΔSal. The results for the pEMBL-ΔBal2 and pEMBL-ΔBcl plasmids were confirmed in two Pin⁻ GuHCl-treated derivatives of another strain, 64-D697.

Interestingly, neither pEMBL-ΔBal2 nor pEMBL-ΔSal caused suppression in *sup35-ΔN* strains, 877ΔN-74-D694 and 94ΔN-33G-D373, not treated with GuHCl after 20 days of incubation at 30°. In contrast, pEMBL-SUP35 causes nonsense suppression in these *sup35-ΔN* strains (see above) and both pEMBL-ΔBal2 and pEMBL-ΔSal cause nonsense suppression in the original Pin⁺ strains containing the complete *SUP35* gene (see Figure 4). These data confirm that an intact Sup35 protein is required for suppression.

DISCUSSION

According to the "protein only" model of prion phenomena, curing cells of $Sup35p^{Psi+}$ prion molecules should not prevent occasional $Sup35p^{Psi-}$ molecules from assuming a $Sup35p^{Psi+}$ prion shape in the future. In contrast, loss of a nucleic acid, such as a virus, required for the prion state would be predicted to preclude the reinduction of *[PSI⁺]* (WICKNER *et al.* 1995, 1996). Thus, the findings that cells cured of the *[PSI⁺]* or *[URE3]* prions can regain the lost prion upon overproduction of the corresponding wild-type protein (CHERNOFF *et al.* 1993; WICKNER 1994; DERKATCH *et al.* 1996) support the protein only model. While the results reported here do not question this postulate in general, they indicate that this simple model needs further elaboration, since isogenic "cured" derivatives differ in their ability to be induced to become *[PSI⁺]* by overproduction of the complete Sup35p protein.

The reversibility of GuHCl-induced curing of *[PSI⁺]* has been the subject of some uncertainty. While INGE-VECHTOMOV (TIKHODEEV *et al.* 1990; S. G. INGE-VECHTOMOV, personal communication) observed the spontaneous reappearance of *[PSI⁺]* after repeated GuHCl treatments, LUND and COX (1981) reported that *[psi⁻]* derivatives obtained by treatment with 5 mM GuHCl failed to revert to *[PSI⁺]*, although *[psi⁻]* derivatives induced by methanol, dimethylsulphoxide or KCl, as well as some *[psi⁻]* derivatives induced by 1 mM GuHCl (COX *et al.* 1988; COX 1994), did occasionally give rise to spontaneous *[PSI⁺]* revertants. The biggest difficulty with these experiments was their dependence on spontaneous reversion to *[PSI⁺]* that occurred at a very low frequency. Our finding that *[PSI⁺]* could be efficiently induced by overproduction of Sup35p even in derivatives cured of *[PSI⁺]* on 5 mM GuHCl (CHERNOFF *et al.* 1993; DERKATCH *et al.* 1996) made it appear as if curing with GuHCl was not irreversible and did not differ from curing with other agents. In the present article we explain the conflicting results obtained

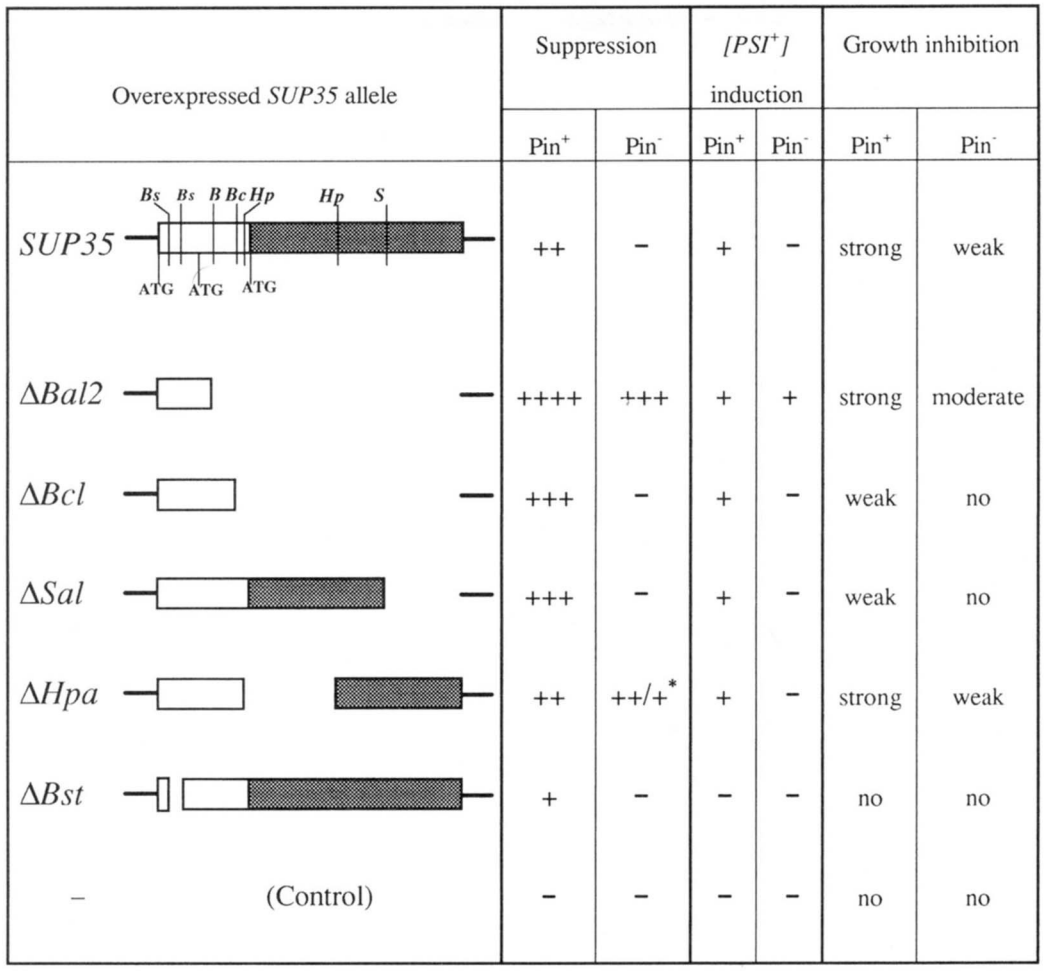

FIGURE 4.—Analysis of effects of overexpression of *SUP35* fragments in Pin⁻ 74-D694 derivatives. The *SUP35* open reading frame (ORF) is boxed; the open and filled portions correspond to the coding regions of Sup35p N-terminal and C-terminal domains, respectively. Restriction sites and their base pair positions relative to the beginning of the ORF are as follows: *Bs, Bst*EII (58, 202); *B, Bal*I (461, 1175); *Bc, Bcl*I (713); *Hp, Hpa*I (751, 1237); *S, Sal*I (1444). The positions of the first three ATG codons are marked. Two GuHCl-treated Pin⁻ and two Pin⁺ 74-D694 derivatives, one of which was also treated with GuHCl, were transformed with pEMBL-based plasmids pEMBL-SUP35, pEMBL-ΔBal2, pEMBL-ΔBcl, pEMBL-ΔSal, pEMBL-ΔHpa and pEMBL-ΔBst, respectively, containing the *SUP35* alleles indicated in the figure, or with control vector (see also MATERIALS AND METHODS). Suppression was analyzed by the level of growth of transformants containing the plasmids at moderate copy number on SC −Ade and SEt −Ade media at 30° and 20°. For example, very strong (++++), strong (+++), moderate (++) and weak (+) suppression correspond to growth after 2, 3–5, 5–7 and 7–14 days on SC −Ade medium at 30°, respectively. *[PSI⁺]* induction by the plasmids at moderate copy number was analyzed as described in MATERIALS AND METHODS. To analyze growth inhibition by plasmids at high copy number, transformants grown on SC −Ura medium were spotted on SC −Leu medium, where a high copy number of plasmids was required for growth (see MATERIALS AND METHODS). Strong and moderate inhibition of growth correspond to the absence of growth on SC −Leu media after 7 and 3 days of incubation, respectively, while weak inhibition of growth corresponds to a reduced level of growth on SC −Leu compared to the level of growth of transformants with control pEMBL-yex4 plasmid.

*pEMBL-ΔHpa-associated suppression that was eliminated together with the plasmid indicating the absence of *[PSI⁺]* induction was observed only at 20° after 2 weeks of incubation in one noninducible derivative of 74-D694 but was stronger and observed at 20° and 30° in the other derivative that showed higher phenotypic suppression (see first section of RESULTS).

with GuHCl-cured strains by clearly showing that there are two distinct types of derivatives that result from treatment with GuHCl: those that can be induced to revert to *[PSI⁺]* by overproduction of the complete Sup35p (Pin⁺) and those that cannot (Pin⁻). Furthermore, we have observed the spontaneous appearance of *[PSI⁺]* in Pin⁺, but not in Pin⁻ isolates (I. DERKATCH, M. BRADLEY and S. LIEBMAN, unpublished results). Note however, even Pin⁻ derivatives can be converted to *[PSI⁺]* when a short Sup35Np fragment is used as an inducer.

Growth inhibition was previously associated with moderate and high levels of Sup35p overproduction in *[PSI⁺]* and *[psi⁻]* strains, respectively (CHERNOFF *et al.* 1988, 1992; DAGKESAMANSKAYA and TER-AVANESYAN 1991; DERKATCH *et al.* 1996). We now find that in Pin⁻ derivatives overamplification of *SUP35* fragments unable to induce *[PSI⁺]* causes only weak or no growth

inhibition (see Figure 4). The pEMBL-ΔBal2 plasmid in Pin$^-$ derivatives induces *[PSI$^+$]* and causes a higher level of growth inhibition than any of the noninducing *SUP35* fragments, however growth inhibition is still reduced relative to that in Pin$^+$ strains. Possibly there is weaker growth inhibition in Pin$^-$ strains than in Pin$^+$ strains when *[PSI$^+$]* is induced.

Since all the meiotic progeny from a cross between a Pin$^+$ and a Pin$^-$ strain were Pin$^+$, a non-Mendelian factor must be required for inducibility to the *[PSI$^+$]* state. In addition, the fact that *HSP104* disruption or GuHCl treatment both result in the loss of the inducibility factor suggests that it could be a protein that is self-reproduced in a prion-like fashion, since the Hsp104p chaperone is known to be involved in prion maintenance and GuHCl is known to cure yeast prions (Tuite *et al.* 1981; M. Aigle, cited in Cox *et al.* 1988; Wickner 1994; Chernoff *et al.* 1995; for reviews see Cox *et al.* 1988; Cox 1994; Lindquist *et al.* 1995; Tuite and Lindquist 1996). We favor the hypothesis that this prion is an inducible conformation of the Sup35 protein. We also discuss the possibility that there is another prion protein whose proper conformation is necessary for the creation of *[PSI$^+$]* seeds.

The first possibility is that Pin$^+$ cells contain Sup35p$^{Psi-Pin+}$ molecules folded in a self-propagating prion conformation. Only Sup35p folded in this Sup35p$^{Psi-Pin+}$ conformation can permit intermolecular interactions of the Sup35p *[PSI$^+$]*-prion-determining domains (the N-termini), leading to the *de novo* formation of Sup35p^{Psi+} seeds. In contrast, the Sup35p$^{Psi-Pin-}$ molecules present in Pin$^-$ cells are incapable of *[PSI$^+$]* seed formation.

According to this hypothesis, Sup35p$^{Psi-Pin+}$ might be considered to be an intermediate state between Sup35p^{Psi+} and Sup35p$^{Psi-Pin-}$ conformations and therefore might cause weak Psi$^+$ phenotypes. However, no phenotypic differences between Pin$^+$ and Pin$^-$ derivatives were detected. In addition, no differences were detected in the levels of Sup35p aggregation determined as described previously (M. Patino *et al.* 1996) in protein extracts of Pin$^+$ and Pin$^-$ *[psi$^-$]* 74-D694 derivatives (M. Patino, I. Derkatch, S. Lindquist and S. Liebman, unpublished data).

Deletion analysis data clearly shows that the Pin$^+$ phenotype is not determined by the first 253 amino acids of Sup35p. This means that the inducibility domain responsible for the Pin phenotype is separate from the *[PSI$^+$]*-determining domain that is located within amino acids 1–154 of Sup35p (Derkatch *et al.* 1996). Thus, we consider the possibility that the Pin$^+$ determinant is in the C-proximal portion of Sup35p. Previous estimates of the frequency of the *de novo* appearance of *[URE3]* and *[PSI$^+$]* caused by amplified *URE2* and *SUP35* fragments, respectively, (Masison and Wickner 1995; Derkatch *et al.* 1996) and biochemical data reported by Patino *et al.* (1996) have already suggested that the

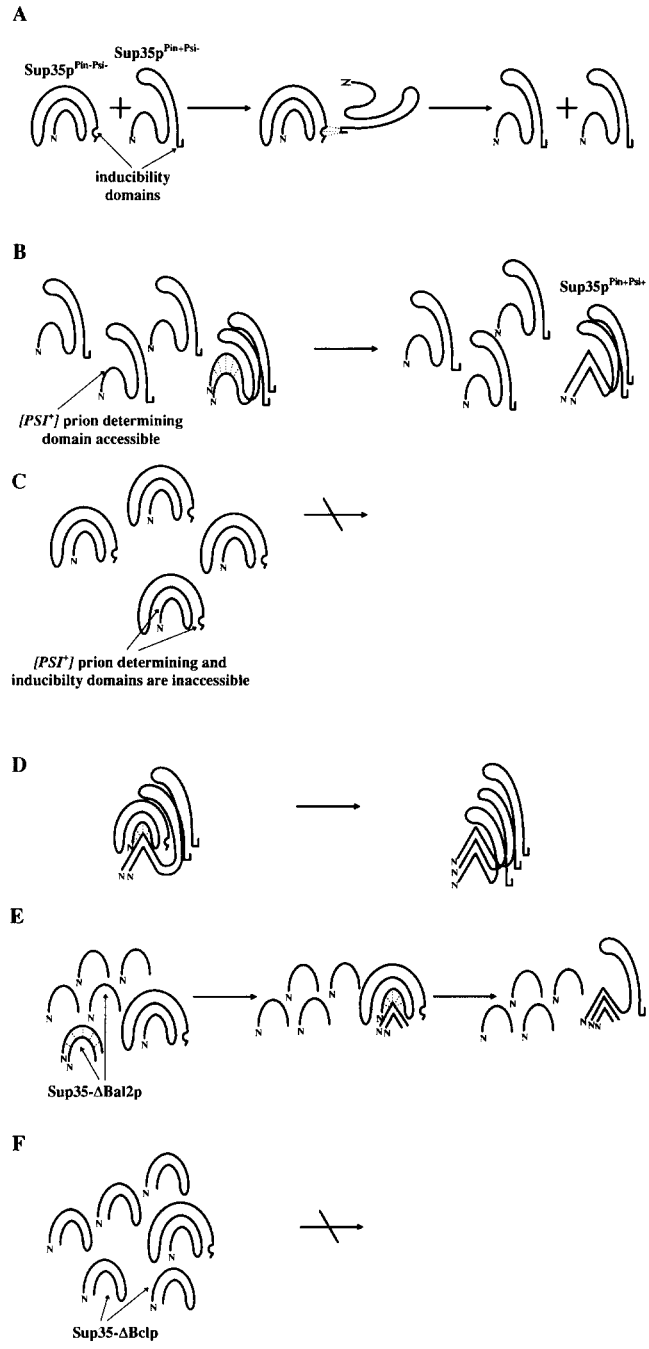

FIGURE 5.—A model suggesting that the Pin phenotype is determined by a self-propagating conformational determinant that is distinct from the *[PSI$^+$]* prion-determining domain and is located in the C-proximal part of Sup35p. See DISCUSSION for full description.

functionally distinct C-proximal domains may affect conformational stability and aggregational patterns of Ure2p and Sup35p N-terminal domains. We propose that newly made Sup35p is always in a Sup35$^{Psi-Pin-}$ conformation. However, newly synthesized Sup35p in a Pin$^+$ derivative takes on the Sup35p$^{Psi-Pin+}$ conformation by using the C-proximal region of the existing Sup35p$^{Psi-Pin+}$ as a template (Figure 5A). In contrast, Pin$^-$ derivatives lacking this template would contain

only $Sup35p^{Psi-Pin-}$ molecules. When the C-proximal region of Sup35p is folded in the inducible $Sup35p^{Pin+}$ conformation, the *[PSI⁺]*-prion-determining domain in the Sup35p N-terminus might be fully accessible permitting interactions between the *[PSI⁺]*-prion-determining domains in different molecules, leading to *[PSI⁺]* seed formation (Figure 5B). In contrast, when the C-proximal region is in the noninducible $Sup35p^{Pin-}$ conformation, a portion of the N-terminal *[PSI⁺]*-prion determinant may be hidden by the rest of the protein, thereby preventing the intermolecular interactions required for *[PSI⁺]* seed formation (Figure 5C). Likewise, the inducibility prion determinant in the C-proximal region is proposed to be inaccessible in $Sup35p^{Psi-Pin-}$ molecules so that an excess of these molecules would not permit intermolecular interactions between their C-proximal inducibility determining domains required for the formation of the $Sup35p^{Psi-Pin+}$ prion seeds (Figure 5C). However, partial exposure of the *[PSI⁺]*-prion determinant in the N-terminal region of the $Sup35p^{Psi-Pin-}$ protein might be sufficient for its interaction with the $Sup35p^{Psi+}$ prion form, directly triggering the conversion of $Sup35p^{Psi-Pin-}$ into $Sup35p^{Psi+}$ (Figure 5D).

Since, according to the above hypothesis, the Pin^- phenotype is caused by a conformation of a downstream region of Sup35p that blocks access to a part of the *[PSI⁺]*-inducing domain located within amino acids 1–154, one might suggest that the N-terminal Sup35p fragments lacking the troublesome downstream domain, but containing the N-terminal *[PSI⁺]*-prion-determining domain, should be able to form *[PSI⁺]* seeds even in Pin^- isolates. In agreement with this prediction we found that while *[PSI⁺]* is not induced when the complete Sup35p is overproduced in Pin^- derivatives, the overproduction of a short N-terminal Sup35p fragment (amino acids 1–154), encoded by the pEMBL-ΔBal2 plasmid, does cause *[PSI⁺]* induction. The formation of $Sup35Np^{Psi+}$ can occur either by the interaction of two $Sup35Np^{Psi-}$ molecules (shown in Figure 5E) or because the N-terminus of Sup35p spontaneously folds into the $Sup35Np^{Psi+}$ conformation in the absence of the Sup35p C-proximal region. In the longer Sup35p N-terminal fragment (amino acids 1–238), encoded by pEMBL-ΔBcl, the *[PSI⁺]*-prion-determining domain appears to be hidden since this fragment can only induce *[PSI⁺]* in Pin^+ isolates. Therefore this fragment is proposed to be able to take on inducible or noninducible conformations that are dependent upon the conformation of cellular Sup35p (Figure 5F). Note however, that a self-propagating determinant in Sup35p that causes this conformational alteration must be located downstream of amino acid 253, since *sup35-ΔN* mutants encoding protein lacking amino acids 1–253 remain inducible.

While it is not known if, after GuHCl treatment, isogenic Pin^+ and Pin^- derivatives can differ in the ability to support induction of another yeast prion, *[URE3]*, it is important to mention that transient deletion of its maintenance gene, *URE2*, did not eliminate inducibility to the *[URE3+]* state by overproduction of a complete Ure2p (Wickner 1994). This clearly indicates that the possibility of *[URE3]* induction is not determined by a self-propagating Ure2p conformation as hypothesized above for Sup35p and *[PSI]*.

The second hypothesis, that the Pin^+ phenotype is determined by another non-Mendelian factor, is also compatible with our data. This factor could be, for example, a chaperone that participates in Sup35p conformational changes and that is self-reproduced in a prion-like fashion. According to this hypothesis, the short Sup35p fragment encoded by pEMBL-ΔBal2 would not require the action of this factor to form *[PSI⁺]* seeds. However, the *[PSI⁺]* prion-determining domain in longer Sup35p fragments may be stabilized by the downstream regions making them unable to form *[PSI⁺]* seeds without the aid of the proposed prion-like chaperone.

Since overexpression of the genes encoding prion proteins could induce their prion state (Chernoff *et al.* 1993; Wickner 1994), we searched for a hypothetical prion determining the Pin^+ phenotype by overexpressing the two genes, *SUP45* and *HSP104*, whose products are known or presumed to interact with Sup35p (Chernoff *et al.* 1995; Stansfield *et al.* 1995). However, transient overexpression of Sup45p in cells with normal levels of Sup35p and transient overexpression of Hsp104p in cells with normal and increased levels of Sup35p failed to convert a Pin^- isolate into Pin^+ (I. Derkatch, M. Bradley and S. Liebman, unpublished results). Similarly, Hsp104 overproduction in strains containing an *HSP104* deletion failed to restore the Pin^+ phenotype (Y. Chernoff, unpublished data).

We have proposed previously that intermediate levels of Hsp104 are required to maintain partially unfolded $Sup35p^{Psi-}$ intermediate, which is a direct target for conversion to $Sup35^{Psi+}$ (Chernoff *et al.* 1995). Paushkin *et al.* (1996) have modified this hypothesis, making it compatible with the "seeded nucleation" model of prion formation (see Caughey and Chesebro 1997). They suggested that a conversion intermediate may be represented by a Sup35p oligomer, serving as an aggregation "seed." According to these authors, Hsp104 is needed to break the huge *[PSI⁺]* aggregates into several seeds thus assuring transmission of *[PSI⁺]* during cell division into daughter cells. Hsp104 overproduction further dissociates the seeds into monomers, which are not able to reproduce *[PSI⁺]*. In the absence of Hsp104 the huge *[PSI⁺]* aggregates are not broken down into small seeds thus reducing the possibility of *[PSI⁺]* transmission and leading to the loss of *[PSI⁺]*. Both models predict that *[psi⁻]* strains generated either by Hsp104 overproduction or by Hsp104 inactivation would be Pin^+, since each of them should contain the same unchanged monomeric form of the Sup35 protein. How-

ever, a *[psi⁻]* strain obtained by Hsp104 inactivation turned out to be Pin⁻. Thus while not rejecting the above models we propose that the absence of Hsp104 causes changes of Sup35p conformation, which are not easily reversible upon reintroduction of functional Hsp104, or causes the loss of an unidentified cofactor that takes part in $Sup35p^{Psi-}$ to $Sup35p^{Psi+}$ conversion.

Our results also show that *[PSI⁺]* curing by GuHCl cannot be explained exclusively by an increase in Hsp104 levels, as we proposed previously (CHERNOFF *et al.* 1995; LINDQUIST *et al.* 1995). Indeed, in contrast to the GuHCl treatment, Hsp104 overexpression in *[PSI⁺]* strains always resulted in Pin⁺ *[psi⁻]* derivatives. Apparently, GuHCl-induced curing includes an additional component, action of which results in "noninducibility" of some of the "cured" cells. Whether this effect should be attributed directly to the protein-denaturing activity of GuHCl itself or to the action of unknown protein(s), which are induced by GuHCl, is yet to be determined.

Whether the inducibility determinant is located within Sup35p or is encoded by a different element, the actuality of Pin⁺ and Pin⁻ derivatives suggests the existence of some non-Mendelian determinant that leads to changes in the conformational liability of the $Sup35p^{Psi-}$. Recent finding that certain sheep PrP alleles in Australia, New Zealand and Great Britain are identical, but that only the British sheep bearing these alleles contract sporadic scrapie, shows that the absence of scrapie in Australia and New Zealand is not due to the absence of susceptible PrP genotypes (HUNTER *et al.* 1997). While most of scrapie cases in the British flocks may be due to the presence of the PrP^{Sc} infectious agent, it is also possible that the Australian and New Zealand sheep cannot contract scrapie spontaneously because, unlike British sheep, they are analogous to the Pin⁻ yeast described here. Furthermore, the finding that the COOH-terminus of the PrP protein directing subcellular PrP trafficking is also required for PrP^{C} conversion into the scrapie isoform (KANEKO *et al.* 1997) suggests that conformational liability of proteins capable of undertaking prion conformations can be modulated. An interesting question arises: is the inducibility determinant described in this study a selfish element or does it provide a useful function in the cell, regulating activity of cellular Sup35p? In the latter case Sup35p molecules in different conformations or with different degrees of conformational liability might appear in the cell as a response to intracellular signaling or to variable environmental conditions, and may then be maintained through self-propagating elements.

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