Novel Non-Mendelian Determinant Involved in the Control of Translation Accuracy in *Saccharomyces cerevisiae*

Kirill V. Volkov,*,[1] Anna Yu. Aksenova,*,[1] Malle J. Soom,* Kirill V. Osipov,* Anton V. Svitin,* Cornelia Kurischko,[†] Irina S. Shkundina,[‡] Michael D. Ter-Avanesyan,[‡] Sergey G. Inge-Vechtomov* and Ludmila N. Mironova*,[2]

**Department of Genetics, St. Petersburg State University, St. Petersburg 199034, Russia, [†]Hans-Knöll Institut für Natürstoff-Forschung e. V., Jena D-07745, Germany and [‡]Institute of Experimental Cardiology, Cardiology Research Center, Moscow 121552, Russia*

Manuscript received May 25, 2001
Accepted for publication October 8, 2001

ABSTRACT

Two cytoplasmically inherited determinants related by their manifestation to the control of translation accuracy were previously described in yeast. Cells carrying one of them, [*PSI*$^+$], display a nonsense suppressor phenotype and contain a prion form of the Sup35 protein. Another element, [*PIN*$^+$], determines the probability of *de novo* generation of [*PSI*$^+$] and results from a prion form of several proteins, which can be functionally unrelated to Sup35p. Here we describe a novel nonchromosomal determinant related to the *SUP35* gene. This determinant, designated [*ISP*$^+$], was identified as an antisuppressor of certain *sup35* mutations. We observed its loss upon growth on guanidine hydrochloride and subsequent spontaneous reappearance with high frequency. The reversible curability of [*ISP*$^+$] resembles the behavior of yeast prions. However, in contrast to known prions, [*ISP*$^+$] does not depend on the chaperone protein Hsp104. Though manifestation of both [*ISP*$^+$] and [*PSI*$^+$] is related to the *SUP35* gene, the maintenance of [*ISP*$^+$] does not depend on the prionogenic N-terminal domain of Sup35p and Sup35p is not aggregated in [*ISP*$^+$] cells, thus ruling out the possibility that [*ISP*$^+$] is a specific form of [*PSI*$^+$]. We hypothesize that [*ISP*$^+$] is a novel prion involved in the control of translation accuracy in yeast.

RECENTLY, interest in the cytoplasmically inherited genetic determinants of the yeast *Saccharomyces cerevisiae* has been greatly increased. This is undoubtedly related to the fact that besides the well-studied DNA- or RNA-based nonchromosomal determinants, such as mtDNA, killer viruses, and 20S and 23S ssRNA replicons (reviewed in Wickner 1992; Chen and Clark-Walker 2000), several non-Mendelian genetic elements of yeast are similar in their fundamental properties to mammalian prions.

The prion concept originates from the study of several transmissible spongioform encephalopathies in mammals. Numerous lines of evidence indicate that the infectious agent causing these diseases is a self-perpetuating form of the cellular protein, PrP. This infectious protein was called a prion (for reviews, see Horwich and Weissman 1997; Prusiner 1998). Later it was shown that proteins capable of taking on self-propagating prion-like conformations also underlie certain phenotypes showing a non-Mendelian inheritance in Ascomycetes fungi. Among them there are two *S. cerevisiae* proteins, Sup35p and Ure2p, and one *Podospora anserina* protein, Het-s (for review, see Wickner *et al.* 1999). Thus, in lower eukaryotes, proteins may serve as genetic determinants. The [*Kil-d*] factor of yeast is also proposed to be a prion, although the corresponding protein is not known yet (Talloczy *et al.* 1998, 2000).

It is evident that the list of yeast prions can be extended. Runs of N or Q residues essential for the prion properties of Sup35p and Ure2p were revealed in almost 2% of yeast proteins (Michelitsch and Weissman 2000) and some of these proteins were recently identified as prions (Derkatch *et al.* 2001; Osherovich and Weissman 2001).

Among prion-based genetic determinants listed, the yeast [*PSI*$^+$] factor is the best studied one (for reviews, see Liebman and Derkatch 1999; Serio and Lindquist 2000). [*PSI*$^+$] is related to the functionally inactive self-maintained isomer of the Sup35 protein, which represents a homologue of the translation termination factor eRF3 of higher eukaryotes (Stansfield *et al.* 1995; Zhouravleva *et al.* 1995). In [*PSI*$^+$] cells this protein accumulates in aggregates, similar to prion protein in mammalian brains (Patino *et al.* 1996; Paushkin *et al.* 1996). [*PSI*$^+$] may be eliminated upon growth at low concentrations of guanidine hydrochloride (GuHCl), which is a protein-denaturing agent when used at ~1000-fold higher concentrations (Tuite *et al.* 1981) and can reappear with high frequency upon overproduction of Sup35p (Chernoff *et al.* 1993; Derkatch *et al.* 1996). Sup35p has the multidomain structure in which the conserved

[1]These authors contributed equally to this work.

[2] *Corresponding author:* Department of Genetics, St. Petersburg State University, Universitetskaya nab., 7/9, 199034 St. Petersburg, Russia. E-mail: mila@lm2016.spb.edu

C-terminal (C) domain of amino acids 254–685 is essential for translation termination and cell viability. The nonconservative N-terminal part of Sup35p is inessential for viability and may be subdivided into the middle (M) domain of unknown function and the N-terminal (N) domain of 123 amino acids necessary for the induction and propagation of [*PSI*$^+$] (Ter-Avanesyan *et al.* 1993, 1994). One of the important properties of [*PSI*$^+$] is the dependence of its propagation on the appropriate level of the Hsp104 chaperone protein: Both overexpression and inactivation of the gene encoding this chaperone cause elimination of [*PSI*$^+$] (Chernoff *et al.* 1995).

Partial inactivation of Sup35p either by switch into the prion form or by mutations has the same effect—appearance of the nonsense suppressor phenotype. However, the combination of [*PSI*$^+$] and *sup35* mutations usually is lethal (Cox 1977; Liebman and All-Robin 1984; Zhou *et al.* 1999), although certain *sup35* suppressor mutations may be selected in the [*PSI*$^+$] background (Ono *et al.* 1986; Inge-Vechtomov *et al.* 1988; Tikhodeev *et al.* 1990). Earlier we found that the suppressor phenotype of some *sup35* mutants selected in a [*psi*$^-$] strain can be changed upon growth in the presence of GuHCl (Volkov *et al.* 1997; Inge-Vechtomov *et al.* 1998). Interestingly, treatment with GuHCl decreased the suppressor efficiency in some *sup35* mutants, but increased it in other mutants. This allowed us to suggest that these mutants contain GuHCl-sensitive determinants that modify the suppressor phenotype.

In this work we studied the antisuppressor determinant, which we designated as [*ISP*$^+$] (*I*nversion of *S*uppressor *P*henotype). We showed that by some traits [*ISP*$^+$] resembles yeast prion determinants. Since [*ISP*$^+$] is related by its manifestation to the *SUP35* gene, it was reasonable to suggest that similarly to [*PSI*$^+$], [*ISP*$^+$] is based on the prion properties of Sup35p. This possibility seems to be intriguing, because its effect is opposite to that of [*PSI*$^+$] and therefore it should, contrary to [*PSI*$^+$], improve translation termination. However, by some essential properties, first of all by the independence of its induction and propagation on the prionogenic domain of Sup35p, [*ISP*$^+$] differs from [*PSI*$^+$]. Thus, [*ISP*$^+$] is more likely a prion form of some protein interacting with Sup35p.

MATERIALS AND METHODS

Yeast strains: Yeast strains used in this work are listed in Table 1. The [*psi*$^-$] derivative of the strain 2V-P3982 (the full name of this strain is du8-132-L28-2V-P3982) was used as the original strain for selection of *sup35* mutations suppressing the *ade1-14* (UGA), *lys2-87* (UGA), and *his7-1* (UAA) mutations. Strain 16A-D1608 with the chromosomal *SUP35* gene disrupted by the *TRP1* insertion contained plasmid pRSU2 carrying the wild-type *SUP35* (description of plasmids presented below).

Strain 25C-2V-P3982 contained the *sup35-25* mutant allele deleted from its 5′ terminus and encoding the C-domain of Sup35p (*sup35-25C*). It was obtained by the integration/excision method of Rose *et al.* (1990). The *Xho*I-*Bam*HI fragment of the plasmid pRSU1-25C was cloned into the same sites of the integrative plasmid pRS306. The plasmid obtained, designated as pRSU6-25C, was linearized with *Xba*I and integrated into the chromosomal *sup35-25* allele of the strain 25-2V-P3982. Selection of transformants was performed on SC-Ura medium. These transformants contained both *sup35-25* and *sup35-25C* in the chromosome divided by the fragment of plasmid DNA. Medium containing 5-fluoroorotic acid (5-FOA), described below, was used for the selection of clones with plasmid excision. Identification of the strain containing the chromosomal *sup35-25C* allele was performed by PCR and by Southern hybridization of chromosomal DNA with the labeled *SUP35* probe. The strain ΔHSP104-25-2V-P3982 contained the chromosomal *HSP104* gene disrupted by the *URA3* insertion. This strain was obtained by transformation of 25-2V-P3982 with the *Bam*HI-*Sal*I restriction fragment of pBC-HSP104::URA3. The disruption of *HSP104* was proved by Western blotting with anti-Hsp104p polyclonal antibody.

Plasmids: pYS-GAL104 is a *CEN-URA3* vector containing the *HSP104* gene under the control of the *GAL1* promoter (Lindquist and Kim 1996). The pBC-HSP104::URA3 plasmid is the integrative vector containing *HSP104* inactivated by the *URA3* insertion (the *Eco*RI-*Eco*RV fragment of the *HSP104* open reading frame was replaced with the *URA3* gene (V. Kushnirov, unpublished results). pSTR7 is the multicopy *SUP35*-containing plasmid, carrying *LEU2* as a selectable marker (Telckov *et al.* 1986). The multicopy *URA3* plasmid pFL44s-Δ3ATG contains *SUP35C* (Kushnirov *et al.* 1990).

The single-copy plasmids *CEN-LEU2* pRS315 and *CEN-URA3* pRS316, as well as *URA3* integrative pRS306 and multicopy pRS426 plasmids (Sikorski and Hieter 1989), were used for the construction of other vectors used in this study. To obtain centromeric and multicopy plasmids containing the *sup35-10* and *sup35-25* alleles, the *Xho*I-*Bam*HI fragment of pSTR7, carrying the wild-type *SUP35*, was ligated with pRS315 digested with the same restriction enzymes. The centromeric plasmid obtained was designated as pRSU1. The *Nsi*I-*Mlu*I fragment of this plasmid does not contain *SUP35* but retains sequences of the plasmid DNA flanking this gene. This fragment was used for transformation of the strains 10-2V-P3982 and 25-2V-P3982. Plasmids isolated from transformants contained the mutant *sup35* alleles (structure of the cloned fragments was examined by sequencing). The plasmids obtained were designated as pRSU1-10 and pRSU1-25. Cloning of the *Xho*I-*Bam*HI fragments of pRSU1, pRSU1-10, and pRSU1-25 into pRS316 allowed us to obtain the *CEN-URA3* plasmids pRSU2, pRSU2-10, and pRSU2-25, respectively. To obtain multicopy plasmids, which contain these alleles, the same fragments were cloned into pRS426. The plasmids obtained were designated as pRSU4, pRSU4-10, and pRSU4-25.

To obtain plasmids containing the 5′-deletion versions of the *sup35-10* and *sup35-25* alleles, the plasmid pRSU1-C, carrying *SUP35C*, was constructed first. For this purpose the *Mlu*I-*Nco*I fragment of pRSU1 was replaced with the same fragment of pFL44s-Δ3ATG. Strains 10-2V-P3982 and 25-2V-P3982 were transformed with the *Nsi*I-*Stu*I fragment of pRSU1-C. This fragment contained the regions corresponding to the C-domain of Sup35p at its ends, flanking the region of interest in mutant alleles. Recombination of this fragment with the chromosomal *sup35* alleles led to the replacement of *SUP35C* in pRSU1-C with *sup35-10C* and *sup35-25C*. The replacement was checked by sequencing of cloned *SUP35* fragments. The plasmids obtained were designated as pRSU1-10C and pRSU1-25C.

Cultivation procedures and genetic methods: The standard rich (YPD) and synthetic (SC) media were used (Sherman *et al.* 1986). The nonfermentable medium YPGly contained glyc-

erol (24 ml/liter) instead of glucose. Cycloheximide (Sigma, St. Louis) was added to YPD or YPGly at a concentration of 1 mg/ml. The standard procedures of yeast genetic analysis were used (Sherman *et al.* 1986).

The elimination of mitochondrial DNA was reached by ethidium bromide (Sigma) treatment (Goldring *et al.* 1970). The SC medium containing 1 mg/ml of 5-FOA (Boeke *et al.* 1984) purchased from Angus was used for the elimination of *URA3*-based plasmids. The induction of *GAL1* promoter was performed on SC-Ura medium containing 2% galactose (Sigma) as inducer and 2% raffinose (ICN Biomedical) as the carbon source, since strains used do not utilize galactose. Strains transformed with pYS-GAL104 were replicated five consecutive times on this media. After colony purification on YPD, single clones were used for further analysis.

For curing of yeast strains of the prion-like determinants, YPD containing 5 mm guanidine hydrochloride (Sigma) was used (GuHCl-test, see Tuite *et al.* 1981). The treatment procedure and subsequent examination of clones obtained were performed as described by Derkatch *et al.* (1997). It is noteworthy that the change of phenotype due to [*ISP*$^+$] loss was opposite to that observed after elimination of [*PSI*$^+$] and was manifested by restoration of the suppressor effect of *sup35-10* and *sup35-25* toward the *his7-1* and *lys2-87* mutations. Reappearance of [*ISP*$^+$] was followed by disappearance of the suppressor phenotype.

In cytoduction experiments, the *cyh2-1* [*rho*^{0}] strains were used as recipients. The strains of interest were mixed together on the surface of the YPD plate, incubated for 6–8 hr, and replica plated to YPGly medium containing cycloheximide. In 5–6 days the respiratory competent cycloheximide-resistant clones were isolated and their phenotypes were examined. Only those clones that corresponded in their chromosomal markers to the recipient strain were used for subsequent analysis.

Two different modifications of the fluctuation test were used for determination of the rates of spontaneous and GuHCl-induced [*ISP*$^+$] elimination and its reappearance after curing by GuHCl.

The rates of [*ISP*$^+$] loss were determined by means of the replicator test as described by Von Borstel (1978). Suspension of cells of the [*ISP*$^+$] strain 25-2V-P3982 (5×10^5 cells/ml) was plated by a 151-pintle replicator on two plates with SC media partially supplied with lysine (5 mg/liter). One of these plates also contained 5 mm GuHCl. In this test, the replica of each pintle, containing initially ~500 cells (N_0), may be considered as an independently growing culture. The loss of [*ISP*$^+$], which could occur either spontaneously or due to GuHCl action during the slow growth of the culture, should lead to the appearance of Lys$^+$ colonies. In 8 days the amount of replicas containing the different number of Lys$^+$ colonies (r) was counted on both plates. The distribution obtained was used to determine the average number (m) of Lys$^+$ colonies in each culture by the method of Ma-Sandri-Sarcar (MSS) maximum likelihood (Rosche and Foster 2000). The rate of [*ISP*$^+$] loss (μ) was determined as $m/1.44N_t$, where N_t is the terminal number of cells in a culture. To determine the average mean of N_t, five "zero spots," *i.e.*, replicas without Lys$^+$ colonies, were carefully cut out from the medium. Cells were washed with a measured amount of water and counted.

To estimate the rate of spontaneous [*ISP*$^+$] appearance (*i.e.*, reversions from suppressor to nonsuppressor phenotype), 10 colonies of the [*isp*$^-$] strain 25-2V-P3982, approximately equal in size and containing ~2.6×10^6 cells, were resuspended in water. An aliquot of cell suspension of each clone was spread on four YPD plates at a dilution to get ~350 colonies per plate. After 4 days of incubation plates were replica plated on SC medium lacking histidine and lysine, and after 5 days of incubation the number of [*ISP*$^+$] clones was determined in every culture. These numbers were used to obtain the maximum-likelihood estimation of the number of [*ISP*$^+$] clones per culture (m_{obs}) by the method of MSS maximum likelihood (Rosche and Foster 2000). The actual number was calculated as $m_{act} = m_{obs}\,[(z-1)/z\ln(z)]$, where z is the dilution factor. The rate of spontaneous [*ISP*$^+$] reappearance was estimated as $m_{act}/1.44N_t$, where N_t is the average number of cells in the colony.

To quantify the suppressor efficiency of *sup35* mutations and the antisuppressor effect of [*ISP*$^+$], [*isp*$^-$] and [*ISP*$^+$] strains were transformed with the nonsense codon readthrough assay plasmids pUKC815/817/818/819 (Stansfield *et al.* 1995) and subsequent examination of β-galactosidase expression level was performed as described. The *URA3*-carrying plasmid pUKC815 encodes a *PGK1-lacZ* gene fusion, while the pUKC817, pUKC818, and pUKC819 plasmids are identical to pUKC815 except that one of the three termination codons, UAA, UAG, and UGA, respectively, is present in-frame at the junction of the *PGK1* and *lacZ* genes (Stansfield *et al.* 1995). Suppression of the in-frame premature stop codons will result in β-galactosidase activity and the levels of β-galactosidase activity can therefore be used to quantify the readthrough of nonsense codons. The nonsense suppression levels were determined as a ratio of β-galactosidase activities in cells transformed with plasmids pUKC817, pUKC818, and pUKC819 to that of transformants with pUKC815. Individual transformants were grown selectively in SC supplemented with the required amino acids and bases to the midexponential phase.

DNA manipulations: Standard methods of DNA manipulations were used (Sambrook *et al.* 1989). Enzymes used were purchased from Fermentas (Vilnius, Lithuania). Transformation of *Escherichia coli* and yeast was performed as described (Inoue *et al.* 1990; Gietz *et al.* 1992). Amplification of chromosomal DNA containing the *SUP35* gene was performed by PCR with the use of primers M1280, GTCGGATCCTTGAAAGAC TCC ATTGTA and M1281, GACGAATTCAATGCTTTATGAT CGGTA. Two independent products of PCR were used for sequencing. Sequencing of the *SUP35* wild-type and mutant alleles was performed as described (Sanger and Coulson 1975) with the use of L1-COR 420 (MWG-BIOTECH). For sequencing, primers F1, GTACTGTAACAAAA AGCGGT; F2, CGACTTTCAAAAGCAACAAA; F3, AGTTGGTAAGGCCTAC TTG; F4, CTAACAAA ACCGCTGTGGA; R1, GCTTTATGAT CGGTATTAT; R2, TTCCACAGCGGTTTTGTTAG; R3, CAA AGTAGGCCTTACCAACT; and R4, TTTGTTGCTTTTGAAA GTCG were used.

Southern blot was performed with the use of a DIG DNA labeling and detection kit (Boehringer Mannheim, Mannheim, Germany) and the PCR product of *SUP35* was obtained with the use of M1280 and M1281 primers (see above).

Preparation, fractionation, and analysis of yeast cell lysates: Cell lysates were obtained and fractionated as described (Paushkin *et al.* 1996). The resulting fractions were analyzed for Sup35p distribution by Western blotting with polyclonal rabbit antibody against Sup35p.

RESULTS

Growth on GuHCl-containing medium increases nonsense suppression efficiency in two *sup35* mutants: The [*ISP*$^+$] determinant has been found in two strains, 10-2V-P3982 and 25-2V-P3982, bearing *sup35* mutations, *sup35-10* and *sup35-25*, respectively (Table 1). These mutants were selected in a [*psi*$^-$] derivative of the strain 2V-P3982 (Volkov *et al.* 1997) by simultaneous suppression of the *ade1-14*, *his7-1*, and *lys2-87* nonsense mutations, but subsequent examination of their phenotypes

TABLE 1

Strains of *S. cerevisiae* used in this work

Strain	Genotype
2V-P3982	*MAT*α *ade1-14 his7-1 lys2-87 ura3-*Δ *thr4-B15 leu2-1*
10-, 25-, 110-, 112-2V-P3982	Derivatives of 2V-P3982, bearing the *sup35-10*, *sup35-25*, *sup35-110*, *sup35-112* mutations
16A-P5154	*MAT***a** *his7-1 lys2-87 met13-A1 thr4-B15 leu2-1*
15B-P4422	*MAT***a** *ade1-14 his7-1 lys2-87 met13-A1 thr4-B15 leu2-1 sup35-10*
21V-P4424	*MAT***a** *ade1-14 his7-1 lys2-87 met13-A1 thr4-B15 leu2-1 sup35-25*
6A-P4475	*MAT***a** *his7-1 lys2-87 met13-A1 ura3-*Δ *thr4-B15 leu2-1 cyh2-1 sup35-25*
12G-P4468	*MAT*α *his7-1 lys2-87 leu2-1 met13-A1 ura3-*Δ *sup35-25*
20-13A-P4439	*MAT***a** *ade1-14 his7-1 lys2-87 met13-A1 thr4-B15 leu2-1 cyh2-1*
16A-D1608	*MAT*α *ade1-14 his7-1 lys2-87 ura3-*Δ *thr4-B15 leu2-3,112 SUP35*::*TRP1* [pRSU2]
25C-2V-P3982	Derivative of 25-2V-P3982 with *sup35-25* replaced for *sup35-25C*
ΔHSP104-25-2V-P3982	Derivative of 25-2V-P3982 containing the *hsp104*::*URA3* disruption allele

revealed that only *ade1-14* was slightly suppressed (Ade$^{\pm}$ phenotype). Notably, after growth in the presence of 5 mM GuHCl these mutants changed their phenotypes from Ade$^{\pm}$ His^{-} Lys^{-} to Ade^{+} His^{+} Lys^{+}. Since the change in the efficiency of *ade1-14* suppression was less obvious, we followed further phenotypic changes only by suppression of the *his7-1* and *lys2-87* mutations (Figure 1). Because GuHCl is known as an agent that eliminates yeast prions, the GuHCl-induced change of phenotype from nonsuppressor to suppressor could indicate that the mutants contained a prion-like antisuppressor determinant. Quantification of the antisuppressor effect

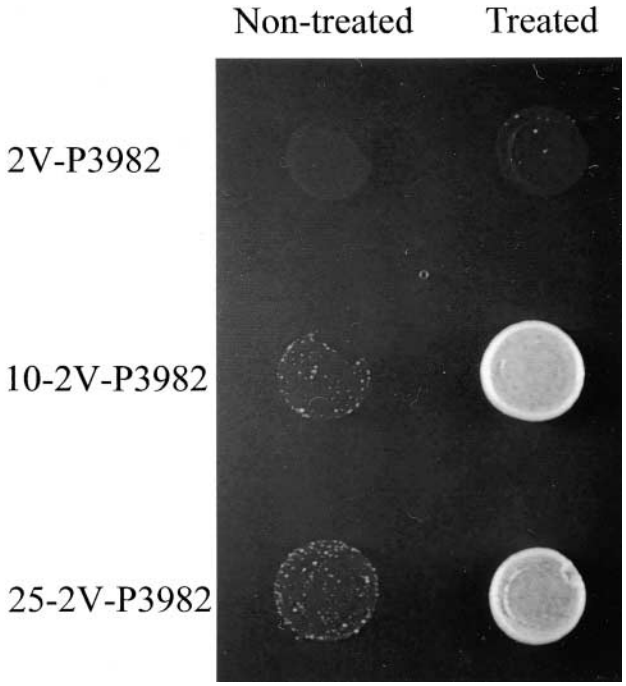

FIGURE 1.—GuHCl treatment changes phenotypes of the strains bearing *sup35-10* (10-2V-P3982) and *sup35-25* (25-2V-P3982) mutations, but does not alter the phenotype of the original strain 2V-P3982. Growth on SC-His, Lys medium after 3 days of incubation is shown.

of [*ISP*$^{+}$] performed with the use of stop codon readthrough assay plasmids demonstrated that [*ISP*$^{+}$] significantly decreased the efficiency of readthrough of all three types of nonsense codons, thus confirming omnipotence of its action (Table 2).

It is known that [*PSI*$^{+}$] can be cured with 100% efficiency by GuHCl treatment (TUITE *et al.* 1981). The efficiency of [*ISP*$^{+}$] loss upon growth on CuHCl-containing medium was substantially lower. Concentrations of GuHCl <5 mM were ineffective. Even after five consecutive incubations on YPD supplemented with 5 mM GuHCl a significant portion of clones in the mitotic progeny (up to 30%) retains the [*ISP*$^{+}$] phenotype (not shown).

Since GuHCl-induced loss is one of the diagnostic characteristics of yeast prions, we studied this trait in detail. It was found that in contrast to [*PSI*$^{+}$], which does not influence the sensitivity of yeast to GuHCl, [*ISP*$^{+}$] noticeably inhibited growth of the studied yeast strains on YPD with 5 mM GuHCl. Importantly, this inhibition was observed not only in the strains 10- and 25-2V-P3982, carrying *sup35* mutations in combination with [*ISP*$^{+}$]. It was even more evident in transformants of these mutants with the pRSU2 plasmid containing the wild-type *SUP35* (Figure 2). Since *sup35* mutations are recessive, this indicates that GuHCl sensitivity was caused by the [*ISP*$^{+}$] determinant itself. Interestingly, the inhibition of growth of [*ISP*$^{+}$] strains by GuHCl was noticeable only when strains grew on a solid medium. When [*ISP*$^{+}$] and [*isp*$^{-}$] strains grew in liquid YPD supplemented with GuHCl, the difference in their growth rates was not so pronounced (not shown), most probably due to enrichment of the [*ISP*$^{+}$] culture by [*isp*$^{-}$] cells, appearing *de novo* (see below).

Recently it was shown that GuHCl inhibits [*PSI*$^{+}$] propagation and the efficiency of curing correlates with the growth rate of the yeast strain (EAGLESTONE *et al.* 2000). The sensitivity of [*ISP*$^{+}$] strains to GuHCl significantly hampered understanding the reasons for [*ISP*$^{+}$] loss. Actually, it was not clear whether GuHCl caused [*ISP*$^{+}$] curing or selection for its loss. To distinguish be-

TABLE 2

Efficiency of stop codon readthrough in [*ISP*$^+$] and [*isp*$^-$] variants of the strain 25-2V-P3982

Strain	Plasmid		
	pUKC817 (UAA)	pUKC818 (UAG)	pUKC819 (UGA)
25-2V-P3982 [*ISP*$^+$]	0.1 ± 0.02	0.5 ± 0.12	0.5 ± 0.14
25-2V-P3982 [*isp*$^-$]	15.3 ± 1.53	21.8 ± 3.21	20.9 ± 1.81

The numbers correspond to the percentage of the level of β-galactosidase activity observed in transformants of the same strains with the control plasmid pUKC815. The average from six independent transformants is represented. The standard error of the mean is indicated.

tween these possibilities it was necessary to compare the rates of [*ISP*$^+$] loss when GuHCl was present or absent in the medium. These rates were determined by the fluctuation test as described in MATERIALS AND METHODS. The distribution of cultures by the number of Lys$^+$ colonies arising due to [*ISP*$^+$] loss on the media partially supplied with lysine is presented in Table 3. The rate of [*ISP*$^+$] loss on GuHCl-containing medium is ~40 times higher than on the medium lacking this agent. Thus, the data obtained indicated that GuHCl cured cells of [*ISP*$^+$].

[*ISP*$^+$] has dominant manifestation and shows nonchromosomal inheritance: Crossing of the [*ISP*$^+$] strain 10-2V-P3982 carrying *sup35-10* with the strain 15B-P4422 carrying the same suppressor mutation and manifesting the suppressor phenotype produced diploids with a nonsuppressor phenotype (Figure 3). The same was shown for diploids homozygous for *sup35-25* obtained in the cross of the [*ISP*$^+$] strain 25-2V-P3982 with the strain 21V-P4424 (not shown). Thus, the manifestation of [*ISP*$^+$] is dominant.

[*ISP*$^+$] behaves as a nonchromosomal genetic determinant. This was shown by studying its meiotic segregation in diploids obtained from the cross of [*ISP*$^+$] and [*isp*$^-$] strains. These diploids were homozygous for the suppressible *his7-1* and *lys2-87* mutations and were either heterozygous or homozygous for *sup35-10* and *sup35-25*. The His$^+$ Lys$^+$ segregants in their progeny contained *sup35* mutations and did not contain [*ISP*$^+$] (Sup$^+$ phenotype); the His$^-$ Lys$^-$ segregants might be either *SUP35* or *sup35* [*ISP*$^+$] (Sup$^-$ phenotype). Diploids heterozygous for *sup35-10* and *sup35-25* yielded mostly 4Sup$^-$:0Sup$^+$ tetrads whereas diploids homozygous for *sup35* mutations yielded only 4Sup$^-$:0Sup$^+$ tetrads (Table 4), indicating the non-Mendelian inheritance of [*ISP*$^+$]. It is noteworthy that treatment of segregants with GuHCl converts segregation in tetrads of diploids heterozygous for *sup35* mutations from 4Sup$^-$:0Sup$^+$ or 3Sup$^-$:1Sup$^+$ to 2Sup$^-$:2Sup$^+$ (Figure 4A) and from 4Sup$^-$:0Sup$^+$ to 0Sup$^-$:4Sup$^+$ in tetrads of diploids homozygous for *sup35* (Figure 4B).

The nonchromosomal nature of [*ISP*$^+$] was further confirmed by its transfer from cell to cell by cytoduction (a form of mating without fusion of the parental nuclei). In this experiment, the Sup$^-$ strain 25-2V-P3982 (*sup35-25* [*ISP*$^+$]) was used as a donor of cytoplasm. The recipi-

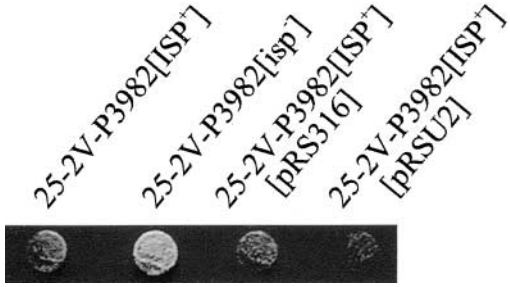

FIGURE 2.—Growth of the [*ISP*$^+$] strain 25-2V-P3982 on YPD supplemented with 5 mM GuHCl is inhibited comparatively to growth of its [*isp*$^-$] derivative. Transformation of [*ISP*$^+$] strain with the pRSU2 plasmid carrying wild-type *SUP35* does not restore its growth on GuHCl. The pRS316 plasmid, which does not contain *SUP35*, was used as a control. The same concentrations of cells were used in all cases.

TABLE 3

Data from fluctuation analysis of the [*ISP*$^+$] loss

No. of Lys$^+$ colonies observed (r)	No. of cultures with r Lys$^+$ colonies on the medium without GuHCl	No. of cultures with r Lys$^+$ colonies on the medium with GuHCl
0	52	10
1	47	16
2	25	19
3	14	22
4	8	32
5	4	23
6	1	14
7	0	6
8	0	4
9	0	3
10	0	1
11	0	0
12	0	1

The means of m were 0.84 for cultures growing without GuHCl and 1.99 for cultures growing on the medium with GuHCl. The means of N_t were 4.8×10^6 and 3.5×10^5, respectively. The means of μ were 1.2×10^{-7} on the medium without GuHCl and 4.0×10^{-6} on the GuHCl-containing medium. The rates difference was statistically significant ($t \gg t_{st;\ \alpha=0.05;\ \nu1+\nu2-2=300}$).

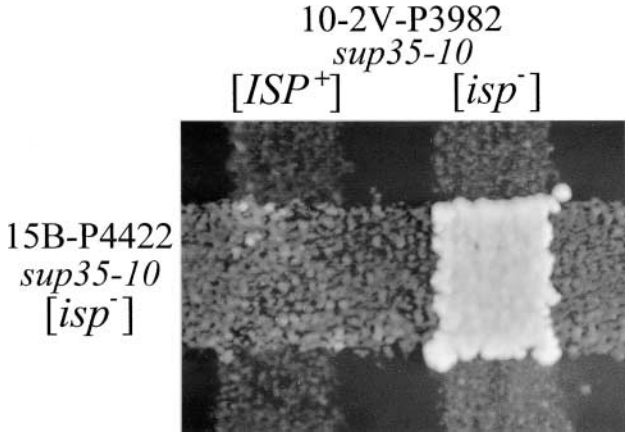

FIGURE 3.—Dominant manifestation of [*ISP*$^+$] in a diploid homozygous for the *sup35-10* allele (left). The [*isp*$^-$] derivative of 10-2V-P3982 was used in the control cross (right). The SC-His, Lys medium was used.

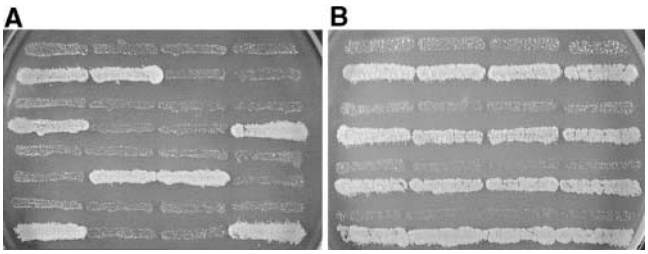

FIGURE 4.—GuHCl treatment changes phenotypes of segregants in the tetrads of diploids heterozygous (A) and homozygous (B) for the *sup35-25* mutation. Odd lines, growth of nontreated segregants; even lines, growth of GuHCl-treated segregants on SC-His, Lys medium after 3 days of incubation.

ent strain 6A-P4475 [rho^0] also contained *sup35-25* but had the Sup$^+$ phenotype because it was cured from [*ISP*$^+$] by GuHCl treatment. The [*isp*$^-$] derivative of the strain 25-2V-P3982 was used as donor of cytoplasm in the control cytoduction experiment. Most of the clones selected as cytoductants (*i.e.*, respiratory-competent and cycloheximide-resistant clones) had the Sup$^+$ phenotype. However, a portion of them were Sup$^-$. Importantly, the number of Sup$^-$ cytoductants was significantly higher when the [*ISP*$^+$] strain was used as a donor of cytoplasm (Table 5). Treatment with GuHCl changed the Sup$^-$ phenotype of all selected cytoductants to Sup$^+$. Sup$^-$ clones in the control could appear due to generation of [*ISP*$^+$] *de novo*, since frequency of its spontaneous appearance was relatively high (see below).

[*ISP*$^+$] can reappear after curing: The properties of [*ISP*$^+$] demonstrated above (curability by GuHCl, nonchromosomal mode of inheritance, dominance over [*isp*$^-$]) are similar to those of the yeast prion-based genetic determinants [*PSI*$^+$] and [*URE3*] (see LIEBMAN and DERKATCH 1999). An important feature of [*PSI*$^+$] and [*URE3*] is their ability to reappear after curing, because according to the prion model, the loss of a prion should be reversible as long as the gene encoding the prion protein is expressed (WICKNER 1994). Thus, if [*ISP*$^+$] is a prion, it should spontaneously reappear after curing. Indeed, we observed that a significant portion (up to 30%) of clones in the mitotic progeny of [*isp*$^-$] strains may be [*ISP*$^+$]. It is necessary to stress that since [*ISP*$^+$] manifests as an antisuppressor, the Sup$^+$[*isp*$^-$] strain may contain a fraction of Sup$^-$[*ISP*$^+$] cells, which can be revealed only by streaking culture to single colonies.

The rate of [*ISP*$^+$] reappearance was estimated on the basis of results of fluctuation tests (see MATERIALS AND METHODS). The distribution of cultures by the number of [*ISP*$^+$] (Lys$^-$ His$^-$) clones (r) in their mitotic progeny was as follows: 0-2, 1-2, 2-4, 3-1, 4-1 (the first number in every pair is r, and the second one is the number of corresponding cultures). m_{obs} determined by the method of MSS maximum likelihood (ROSCHE and FOSTER 2000) was 1.14 and m_{act} was 354.8. The rate of [*ISP*$^+$] appearance was $\sim 1.0 \times 10^{-4}$/cell/generation.

A high frequency of [*ISP*$^+$] clones in the mitotic progeny of [*isp*$^-$] strains may be caused not only by the high

TABLE 4

Nonchromosomal inheritance of [*ISP*$^+$]

Diploid	Total no. of tetrads	No. of tetrads with Sup$^-$:Sup$^+$ segregation		
		4:0	3:1	2:2
sup35-10/SUP35	32	27	1	4
sup35-25/SUP35	29	26	3	0
sup35-10/sup35-10	22	22	0	0
sup35-25/sup35-25	17	17	0	0

Diploids heterozygous for *sup35-10* and *sup35-25* were obtained from the cross of 10-2V-P3982 and 25-2V-P3982 with the strain 16A-P5154 and diploids homozygous for *sup35-10* and *sup35-25* were obtained from the cross of 10-2V-P3982 and 25-2V-P3982 with 15B-P4422 and 21V-P4424, respectively.

TABLE 5

The occurrence of [*ISP*$^+$] clones among cytoductants of the strain 6A-P4475

Donor	No. of cytoductants		% of [*ISP*$^+$] clones
	Total	[*ISP*$^+$]	
25-2V-P3982 [*ISP*$^+$]	71	12	16.9 ± 4.45
25-2V-P3982 [*isp*$^-$]	106	1	0.9 ± 0.91

Genotype of the donor strain 25-2V-P3982 is *MAT*α *ade1-14 his7-1 lys2-87 ura3-*Δ *thr4-B15 leu2-1 sup35-25*; genotype of the recipient strain 6A-P4475 is *MAT***a** *his7-1 lys2-87 met13-A1 ura3-*Δ *thr4-B15 leu2-1 cyh2-1 sup35-25* [rho^0]. The standard error (±SE) is indicated.

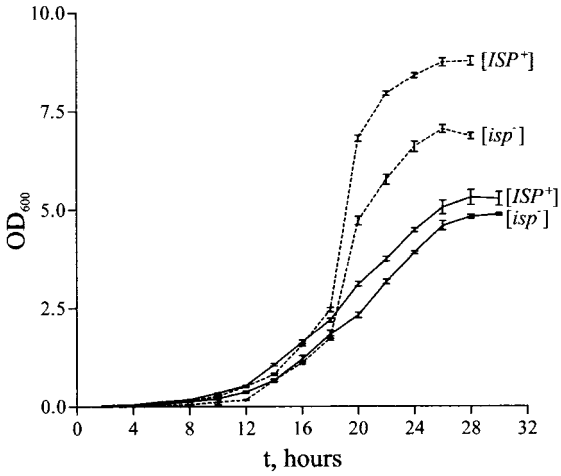

FIGURE 5.—[*ISP*$^+$] strains grow better in YPD than their [*isp*$^-$] derivatives. Dotted lines, 10-2V-P3982; solid lines, 25-2V-P3982.

rate of [*ISP*$^+$] appearance, but also by an advantage in propagation of [*ISP*$^+$] cells comparatively to [*isp*$^-$]. Indeed, the comparison of growth rates of [*isp*$^-$] and [*ISP*$^+$] variants of the strains 10-2V-P3982 and 25-2V-P3982 has shown that [*ISP*$^+$] noticeably improved their growth, probably interfering with the deleterious effect of *sup35* mutations (Figure 5).

Maintenance of [*ISP*$^+$] does not depend on the Hsp104 chaperone: The Hsp104 chaperone protein is critical for the propagation of yeast prions. For example, the disruption of the *HSP104* gene eliminates [*PSI*$^+$], while overexpression of the Hsp104p in [*PSI*$^+$] cells results in an antisuppressor phenotype and causes gradual [*PSI*$^+$] loss over successive cell generations (CHERNOFF *et al.* 1995; PAUSHKIN *et al.* 1996). Here we tested the effects of both the lack and overproduction of Hsp104p on the manifestation and maintenance of [*ISP*$^+$]. The effect of *HSP104* overexpression was studied with the use of pYS-GAL104 plasmid expressing *HSP104* under the control of *GAL1* promoter (see MATERIALS AND METHODS). The frequency of [*ISP*$^+$] loss was compared in transformants of the strains 10-2V-P3982 and 25-2V-P3982 incubated on galactose- and glucose-containing media. Data presented in Table 6 show that transient increase of the Hsp104p levels did not cause [*ISP*$^+$] elimination. The overexpression of *HSP104* from the same plasmid in the [*PSI*$^+$] variant of the original strain 2V-P3982 caused efficient (~60%) elimination of [*PSI*$^+$] (not shown).

To study the maintenance of [*ISP*$^+$] in the absence of Hsp104p, the chromosomal *HSP104* gene of the strain 25-2V-P3982 [*ISP*$^+$] was disrupted by the insertion of *URA3*. The inactivation of *HSP104* in three independently obtained clones did not alter the antisuppressor phenotype of the [*ISP*$^+$] strain (not shown). However, disruptants changed their phenotype from antisuppressor to suppressor upon growth on GuHCl. This means that they did not differ by their [*ISP*$^+$] status from the cells expressing Hsp104p. Thus, both lack and overexpression of Hsp104p did not affect [*ISP*$^+$] manifestation and propagation.

Manifestation but not propagation of [*ISP*$^+$] depends on the defined *SUP35* alleles: Determinant [*ISP*$^+$] was identified by an antisuppressor effect toward two *sup35* mutations. This does not necessarily mean that propagation of [*ISP*$^+$] is possible only in the background of these *sup35* alleles. To study this, [*ISP*$^+$] was transferred by cytoduction from the strain 25-2V-P3982 to the strain 20-13A-P4439 [*isp*$^-$] [rho^0], containing the wild-type *SUP35* gene. The [*ISP*$^+$] status of cytoductants could not be directly monitored in this strain, because the most clear manifestation of [*ISP*$^+$] is its ability to interfere with the suppressor effect of either *sup35-10* or *sup35-25* mutations. Therefore, to determine the [*ISP*$^+$] status of cytoductants obtained, 11 of them were crossed with the [*isp*$^-$] variant of the strain 12G-P4468. Six of the obtained diploids produced tetrads with an excess of Sup$^-$ segregants (4Sup$^-$:0Sup$^+$ and 3Sup$^-$:1Sup$^+$; Table 7). After GuHCl treatment 2Sup$^-$:2Sup$^+$ segregation was observed in all tetrads. The other five diploids yielded only 2Sup$^-$:2Sup$^+$ tetrads. The corresponding cytoductants probably did not get [*ISP*$^+$] from the donor strain. It is important that 2Sup$^-$:2Sup$^+$ segregation was also observed in 25 tetrads isolated in the control diploid (20-13A-P4439 × 12G-P4468), confirming the [*isp*$^-$] status of the strain 20-13A-P4439.

Thus, although the presence of *sup35* mutant alleles is necessary for [*ISP*$^+$] detection, they are not required for its propagation. To study the specificity of interac-

TABLE 6

The Hsp104 overproduction does not cure [*ISP*$^+$]

[*ISP*$^+$] strain	Media containing	No. of transformants studied: Total	No. of transformants studied: [*isp*$^-$]	Frequency of [*ISP*$^+$] loss (%)
10-2V-P3982	Glucose	3321	42	1.3 ± 0.03
	Galactose	3054	56	1.8 ± 0.03
25-2V-P3982	Glucose	1925	4	0.2 ± 0.05
	Galactose	758	2	0.3 ± 0.13

The pYS-GAL104 plasmid was used for transformation. ±SE is indicated.

TABLE 7

Genetic analysis of cytoductants bearing the wild-type *SUP35* allele

Strain	Total no. of tetrads	No. of tetrads with Sup$^-$:Sup$^+$ segregation		
		4:0	3:1	2:2
cyt1-20-13A-P4439	7	0	3	4
cyt2-20-13A-P4439	8	1	2	5
cyt3-20-13A-P4439	10	1	2	7
cyt13-20-13A-P4439	7	2	3	2
cyt26-20-13A-P4439	5	2	3	0
cyt27-20-13A-P4439	8	3	4	1

Hybrids analyzed were obtained from the crosses of cytoductants with 12G-P4468 (*MAT*α *his7-1 lys2-87 leu2-1 met13-A1 ura3-*Δ *sup35-25*).

tion of [*ISP*$^+$] with *sup35* mutations, two additional mutations, *sup35-110* and *sup35-112*, selected in the same original strain, were taken for the analysis. The suppressor phenotype of the strains 110-2V-P3982 (*sup35-110*) and 112-2V-P3982 (*sup35-112*) was not changed after treatment with GuHCl. These strains were crossed with the [*ISP*$^+$] strain cyt1-20-13A-P4439, containing the wild-type *SUP35* gene (see Table 7). Both diploids produced 2Sup$^+$:2Sup$^-$ tetrads (eight tetrads were analyzed in each case). This indicated that the *sup35-110* and *sup35-112* alleles are insensitive to the antisuppressor effect of [*ISP*$^+$]. This conclusion was confirmed by the examination of the phenotypes of diploids heteroallelic for the *sup35* mutations: *sup35-110/sup35-25* and *sup35-112/sup35-25*. These diploids were obtained from the cross of the [*ISP*$^+$] strain 21V-P4424 with the strains 110-2V-P3982 and 112-2V-P3982. In contrast to the [*ISP*$^+$] diploids homozygous for the *sup35-10* and *sup35-25* mutations, these diploids manifested the Sup$^+$ phenotype (Figure 6).

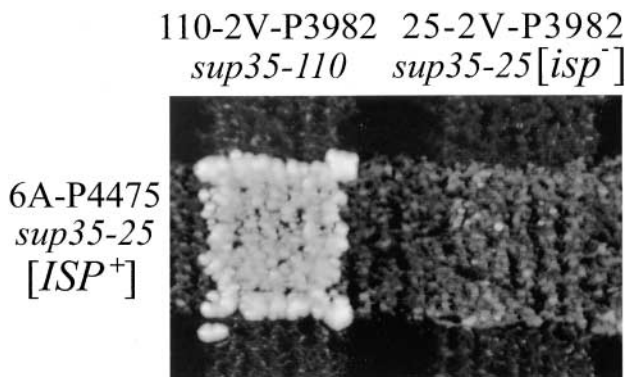

FIGURE 6.—[*ISP*$^+$] does not manifest an antisuppressor effect in diploids heteroallelic for the *sup35* mutations. Left, growth on SC-His, Lys medium of the *sup35-110/sup35-25* diploid is shown. Right, the diploid homoallelic for *sup35-25* was used as a control.

TABLE 8

Mutational alterations of the *SUP35* gene and encoded protein

Allele	Nucleotide replacement	Amino acid replacement
sup35-10	G1087A	Asp363Asn
sup35-25	C1133T	Thr378Ile
sup35-110	C1724A	Pro575His
sup35-112	G1237A	Val413Leu

All mutant *sup35* alleles used in the work were sequenced. The nucleotide sequences obtained were compared with the sequence of wild-type *SUP35* from the original strain 2V-P3982 because earlier it was found (VOLKOV *et al.* 2000) that the sequence of the *SUP35* gene from the *S. cerevisiae* strains of Peterhoff breeding stocks differs from that presented in GenBank (accession no. M21129) by 10 nucleotides. All studied *sup35* mutations caused amino acid replacements within the C-domain of Sup35p (Table 8).

Induction and propagation of [*ISP*$^+$] does not depend on the Sup35 protein: [*ISP*$^+$] is a genetic determinant interfering with the suppressor effect of certain *sup35* mutations. Manifestation and propagation of the [*PSI*$^+$] determinant also depends on the *SUP35* gene. We therefore asked whether [*ISP*$^+$] is somehow related to the prion properties of Sup35p. To address this question, we studied if the Sup35p N-terminal domain is required for [*ISP*$^+$] maintenance, since it was previously shown that this domain is indispensable for [*PSI*$^+$] (DOEL *et al.* 1994; TER-AVANESYAN *et al.* 1994). To this end the chromosomal *sup35-25* allele of the 25-2V-P3982 [*ISP*$^+$] strain was replaced with the *sup35-25C* 5′-deletion allele (see MATERIALS AND METHODS). The strain obtained was designated as 25C-2V-P3982. If [*ISP*$^+$] is a form of [*PSI*$^+$], such replacement should cause the loss of [*ISP*$^+$] and therefore the strain 25C-2V-P3982 should manifest the Sup$^+$ phenotype. Conversely, retention of the Sup$^-$ phenotype should indicate the independence of [*ISP*$^+$] propagation of the N-domain of Sup35p. We found that 25C-2V-P3982 did not differ phenotypically from 25-2V-P3982 (not shown). This could indicate the retention of [*ISP*$^+$] in the strain with the chromosomal *sup35-25C* allele and, thus, the independence of [*ISP*$^+$] maintenance of the Sup35p prion domain. However, this conclusion needed additional examination, since it was found that treatment of 25C-2V-P3982 with GuHCl did not restore the Sup$^+$ phenotype. This could be due either to an increased resistance of [*ISP*$^+$] to GuHCl in this strain or to a decreased suppressor effect of the 5′-deletion *sup35-25* allele. In the latter case, the strain bearing the *sup35-25C* deletion allele should manifest a Sup$^-$ phenotype independently of its [*ISP*$^+$] status.

In a separate experiment, which will be described elsewhere, we found that deletion of the 5′-region of

TABLE 9

Multicopy *sup35-10* and *sup35-25* alleles do not influence the frequency of [*ISP*$^+$] appearance

Strain	Plasmid	No. of transformants		
		Total	[*ISP*$^+$]	% of [*ISP*$^+$]
10-2V-P3982	pRS426	123	14	11.4 ± 2.86
	pRSU4-10	69	5	7.2 ± 3.12
25-2V-P3982	pRS426	123	11	8.9 ± 2.57
	pRSU4-25	116	4	3.4 ± 1.69

The [*ISP*$^+$] transformants were identified by inability to grow on the SC-His, Lys medium. ±SE is indicated.

sup35-10 and *sup35-25* alleles decreased their suppressor effect. For this reason we could not monitor directly the effect of GuHCl treatment of the strain 25C-2V-P3982: Both [*ISP*$^+$] and [*isp*$^-$] derivatives of this strain should have the Sup$^-$ phenotype.

[*ISP*$^+$] status of the strain 25C-2V-P3982 was verified by its cross with the [*isp*$^-$] strain 6A-P4475, containing the full-length *sup35-25* allele. The phenotype of the diploid obtained was Sup$^-$. This could be reasoned either by dominance of the antisuppressor effect of *sup35-25C* or by presence of [*ISP*$^+$] in 25C-2V-P3982. Notably, if this strain was treated with GuHCl before crossing, the diploid had a Sup$^+$ phenotype (not shown). This means that the nontreated strain, bearing *sup35-25C*, contained [*ISP*$^+$]. Taken together, these results strongly suggest the independence of [*ISP*$^+$] from the prion-determining N-terminal Sup35p domain.

The generation of [*ISP*$^+$] was not induced by overexpression of the *sup35-10* and *sup35-25* alleles used for its detection. The [*isp*$^-$] strains 10-2V-P3982 and 25-2V-P3982 were transformed with multicopy plasmids pRSU4-10 and pRSU4-25 bearing the *sup35-10* and *sup35-25* alleles. The plasmid pRS426, which does not contain *SUP35*, was used as a control. The frequency of [*ISP*$^+$] clones among transformants containing pRSU4-10 and pRSU4-25 was not higher than among transformants containing the control plasmid (Table 9).

Additional evidence for the lack of a relationship between [*PSI*$^+$] and [*ISP*$^+$] came from the study of Sup35p aggregation. Since it is known that solubility of the Sup35 protein differs in [*PSI*$^+$] and [*psi*$^-$] strains (Patino *et al.* 1996; Paushkin *et al.* 1996), we analyzed lysates of the 10-2V-P3982 [*ISP*$^+$] and 25-2V-P3982 [*ISP*$^+$] strains and their [*isp*$^-$] derivatives by centrifugation. This analysis did not reveal any influence of [*ISP*$^+$] on Sup35p aggregation (not shown).

DISCUSSION

We have discovered a novel non-Mendelian genetic determinant that decreases the suppressor effect of certain mutations in the *SUP35* gene. Two lines of evidence suggest that this determinant, designated as [*ISP*$^+$], appeared as a compensatory genetic change, neutralizing the deleterious effects of some *sup35* mutations: (i) Although these suppressor mutants were selected as Ade$^+$, His$^+$, Lys$^+$ prototrophs, their subsequent examination revealed only weak suppression of the *ade1-14* mutation; and (ii) [*ISP*$^+$] significantly improved growth of these *sup35* mutants in nonselective conditions. Remarkably, [*ISP*$^+$] is not the only nonchromosomal determinant affecting nonsense codon readthrough in yeast and related in manifestation to the *SUP35* gene. There is a well-known suppressor determinant [*PSI*$^+$], whose appearance and maintenance depend on *SUP35* and which was shown to be a prion-like form of the Sup35 protein (reviewed by Liebman and Derkatch 1999; Serio and Lindquist 2000). Another nonchromosomal determinant, [*PIN*$^+$], influences the probability of [*PSI*$^+$] induction after curing (Derkatch *et al.* 1997, 2000). In this work we have obtained evidence indicating a possible prion nature of [*ISP*$^+$], but we failed to reveal any dependence of its induction and propagation on the Sup35 protein.

We have shown that, similarly to known yeast prions, [*ISP*$^+$] can be cured upon growth in the presence of 5 mM GuHCl, albeit less efficiently than [*PSI*$^+$]. In addition, while known prion determinants do not cause sensitivity to GuHCl, the growth of [*ISP*$^+$] strains on solid media was inhibited by 5 mM GuHCl. This indicates that GuHCl causes not only curing of [*ISP*$^+$], but also probably some selection for the loss of this mitotically unstable genetic element.

An important trait shared by [*ISP*$^+$] and yeast prions is their ability to reappear after curing. Indeed, the high rate of reappearance is unlike any known nucleic acid replicon but is well explained by the prion model, which assumes that the loss of a prion should be reversible as long as the gene encoding the prion protein is expressed (Wickner 1994). On the other hand, [*ISP*$^+$] is distinct from the known yeast prions by its independence of the Hsp104 protein: Both lack and overproduction of this chaperone did not influence [*ISP*$^+$] manifestation and propagation. This does not obviously argue against the prion hypothesis for [*ISP*$^+$], since known yeast prions do not react to Hsp104p uniformly. For example, in contrast to [*PSI*$^+$], overproduction of Hsp104p does not cure [*PIN*$^+$] and [*URE3*] (Derkatch *et al.* 1997; Moriyama *et al.* 2000). Another presumable prion, [*KIL-d*], is even more similar to [*ISP*$^+$], since it is resistant to both absence and overproduction of Hsp104p (Talloczy *et al.* 2000). Finally, artificial [*PSI*$^+$], on the basis of the prion domain of Sup35p from *Pichia methanolica*, depends on the presence of Hsp104p, but is insensitive to its overproduction (Kushnirov *et al.* 2000a). The study of this artificial [*PSI*$^+$] also demonstrated that chaperones other than Hsp104p participate in prion propagation and that their involvement has a prion strain-spe-

cific character, *i.e.*, may not be revealed for every prion strain (Kushnirov *et al.* 2000b).

Thus, considerations presented above suggest that [*ISP*$^+$] is a nonchromosomally inherited element with genetic properties resembling those of yeast prions. In the frame of the prion model there are two hypotheses explaining its nature. The first one presumes that [*ISP*$^+$] is inducible and a self-propagating conformer of Sup35p, which differs from that of [*PSI*$^+$]. The second hypothesis suggests that there is another protein, whose prion form is manifested as [*ISP*$^+$]. The data obtained support the second hypothesis.

It is known that [*PSI*$^+$] depends on the N-terminal Sup35p domain and Sup35p is found in [*PSI*$^+$] cells in a form of heavy molecular weight aggregates (Patino *et al.* 1996; Paushkin *et al.* 1996). We failed to observe aggregated Sup35p in [*ISP*$^+$] cells. This is reasonable, since it is hard to imagine how aggregation of the mutant Sup35 protein could increase its activity in translation termination. We also have shown that maintenance of [*ISP*$^+$] did not depend on the first 253 amino acids of Sup35p. This leaves the possibility that [*ISP*$^+$] is related to the C-terminal portion of Sup35p. In favor of this could be the fact that [*ISP*$^+$] appeared in the strains carrying *sup35* mutations, which cause amino acid replacements in the C-domain of Sup35p. However, this does not mean that only these Sup35p mutant variants can adopt the [*ISP*$^+$]-specific conformation, since [*ISP*$^+$] can propagate in the *SUP35* wild-type background.

One of the key features indicating that the yeast nonchromosomal determinant is related to the prion state of a certain protein is high frequency of its appearance upon overproduction of that protein. For example, overexpression of Sup35p or Ure2p greatly increased the frequency of appearance of [*PSI*$^+$] or [*URE3*] determinants, respectively (Chernoff *et al.* 1993; Masison and Wickner 1995; Derkatch *et al.* 1996). In contrast to this, generation of [*ISP*$^+$] was not induced by overexpression of the *sup35-10* and *sup35-25* alleles used for its detection. Additional traits distinguishing [*ISP*$^+$] from [*PSI*$^+$] are their different behaviors in cytoduction experiments. While [*PSI*$^+$] usually showed 100% coincidence of transfer with mitochondria (Cox *et al.* 1988), this value was ~17% for [*ISP*$^+$]. It is noteworthy that both [*PSI*$^+$] and [*ISP*$^+$] behaved similarly in meiosis, showing typically nonchromosomal segregation. To explain this discrepancy one can suggest that unlike [*PSI*$^+$], [*ISP*$^+$] is localized in nuclei.

Taken together, the data obtained do not support the idea that [*ISP*$^+$] is a specific prion form of Sup35p. Therefore, though at present we cannot completely rule out this possibility, we favor the hypothesis that [*ISP*$^+$] is a prion form of unknown protein interacting with some Sup35 mutant proteins. At present two such *sup35* mutations have been identified. Interestingly, both caused amino acid changes in proximity to each other. Thus, this Sup35p region is probably involved in interaction with protein underlying [*ISP*$^+$]. Many proteins were shown to interact with Sup35p (Paushkin *et al.* 1997; Czaplinski *et al.* 1998, 2000; Bailleul *et al.* 1999; Wang *et al.* 2001) and some of them are able to enhance or inhibit its activity. However, prion properties are still not ascribed to any of them. It is possible to suggest that [*ISP*$^+$] is related to one of such modulators of Sup35p activity. It remains unclear how such interaction can take place if [*ISP*$^+$]-related protein is localized in the nucleus. One possibility is that this protein recycles between the cytoplasm and nucleus. If so, conversion into the prion form should hamper its export from the nucleus. Though at present there are no data confirming this suggestion, it is possible to mention that Sup35p interacts with Mtt1p (Czaplinski *et al.* 2000) and Itt2p (Urakov *et al.* 2001) for which nuclear localization was predicted.

To conclude, we stress that the determinant described in this article shows some properties that make it similar to yeast prions. If the hypothesis that it is a prion is correct, this should mean that at least two prion determinants, [*PSI*$^+$] and [*ISP*$^+$], are involved in the control of translation accuracy in yeast.

We thank Yu. Pavlov and M. Agaphonov for helpful discussion and critical reading of the manuscript, M. Tuite for the gift of nonsense codon readthrough assay plasmids, and V. Kushnirov for the pBC-HSP104::URA3 plasmid. This work was supported by grants from INTAS (99-00491, L.N.M. and M.D.T-A.), the Russian Foundation of Basic Research (99-04-49107, L.N.M., and 99-04-48601, M.D.T-A.), and Howard Hughes Medical Institute (55000337, M.D.T-A.).

LITERATURE CITED

Bailleul, P. A., G. P. Newnam, J. N. Steenbergen and Y. O. Chernoff, 1999 Genetic study of interactions between the cytoskeletal assembly protein Sla1 and prion-forming domain of the release factor Sup35 (eRF3) in *Saccharomyces cerevisiae*. Genetics **153:** 81–94.

Boeke, J. D., F. Lacroute and G. R. Fink, 1984 A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. **197:** 345–346.

Chen, X. J., and G. D. Clark-Walker, 2000 The petite mutation in yeasts: 50 years on. Int. Rev. Cytol. **194:** 197–238.

Chernoff, Y. O., I. L. Derkatch and S. G. Inge-Vechtomov, 1993 Multicopy *SUP35* gene induces *de-novo* appearance of psi-like factors in the yeast *Saccharomyces cerevisiae*. Curr. Genet. **24:** 268–270.

Chernoff, Y. O., S. L. Lindquist, B. Ono, S. G. Inge-Vechtomov and S. W. Liebman, 1995 Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [*PSI*$^+$]. Science **268:** 880–884.

Cox, B. S., 1977 Allosuppressors in yeast. Genet. Res. **30:** 187–205.

Cox, B. S., M. F. Tuite and C. S. McLaughlin, 1988 The psi factor of yeast: a problem in inheritance. Yeast **4:** 159–178.

Czaplinski, K., M. J. Ruiz-Echevarria, S. V. Paushkin, X. Han, Y. Weng *et al.*, 1998 The surveillance complex interacts with the translation release factors to enhance termination and degrade aberrant mRNAs. Genes Dev. **12:** 1665–1677.

Czaplinski, K., N. Majlesi, T. Banerjee and S. W. Peltz, 2000 Mtt1 is a Upf1-like helicase that interacts with the translation termination factors and whose overexpression can modulate termination efficiency. RNA **6:** 730–743.

Derkatch, I. L., Y. O. Chernoff, V. V. Kushnirov, S. G. Inge-Vechtomov and S. W. Liebman, 1996 Genesis and variability of [*PSI*] prion factors in *Saccharomyces cerevisiae*. Genetics **144:** 1375–1386.

Derkatch, I. L., M. E. Bradley, P. Zhou, Y. O. Chernoff and S. W. Liebman, 1997 Genetic and environmental factors affecting the *de novo* appearance of the [*PSI*$^+$] prion in *Saccharomyces cerevisiae*. Genetics **147:** 507–519.

Derkatch, I. L., M. E. Bradley, S. V. Masse, S. P. Zadorsky, G. V. Polozkov *et al.*, 2000 Dependence and independence of [*PSI*$^+$] and [*PIN*$^+$]: a two-prion system in yeast? EMBO J. **19:** 1942–1952.

Derkatch, I. L., M. E. Bradley, J. Y. Hong and S. W. Liebman, 2001 Prions affect the appearance of other prions: the story of [*PIN*$^+$]. Cell **106:** 171–182.

Doel, S. M., S. J. McCready, C. R. Nierras and B. S. Cox, 1994 The dominant *PNM2*$^-$ mutation which eliminates the ψ factor of *Saccharomyces cerevisiae* is the result of a missence mutation in the *SUP35* gene. Genetics **137:** 659–670.

Eaglestone, S. S., L. W. Ruddock, B. S. Cox and M. F. Tuite, 2000 Guanidine hydrochloride blocks a critical step in the propagation of the prion-like determinant [*PSI*$^+$] of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **97:** 240–244.

Gietz, D., A. St. Jean, R. A. Woods and R. H. Schiestl, 1992 Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20:** 1425–1431.

Goldring, E. S., L. I. Grossman, D. Krupnick, D. R. Cryer and J. Marmur, 1970 The petite mutation in yeast. Loss of mitochondrial deoxyribonucleic acid during induction of petites with ethidium bromide. J. Mol. Biol. **52:** 323–335.

Horwich, A. L., and J. S. Weissman, 1997 Deadly conformations-protein misfolding in prion disease. Cell **89:** 499–510.

Inge-Vechtomov, S. G., O. N. Tikhodeev and T. S. Karpova, 1988 Selective systems for obtaining recessive ribosomal suppressors in *Saccharomyces* yeast (in Russian). Genetika **24:** 1159–1165.

Inge-Vechtomov, S. G., S. P. Zadorsky, E. A. Ilmov, L. N. Mironova, V. L. Tikchomirova *et al.*, 1998 Yeast approach to protein "prionization": *SUP35*–[*PSI*$^+$] system, pp. 99–109 in *NATO ASI Series Volume: Prions and Brain Diseases in Animals and Humans*, edited by D. R. O. Morrison and J. Collings. Plenum Publishing, New York.

Inoue, H., H. Nojima and H. Okayama, 1990 High efficiency transformation of *Escherichia coli* with plasmids. Gene **96:** 23–28.

Kushnirov, V. V., M. D. Ter-Avanesyan, A. R. Dagkesamanskaya, Y. Chernoff, S. G. Inge-Vechtomov *et al.*, 1990 Deletion analysis of the *SUP2* gene in *Saccharomyces cerevisiae* (in Russian). Mol. Biol. **24:** 1037–1041.

Kushnirov, V. V., N. V. Kochneva-Pervukhova, M. B. Chechenova, N. S. Frolova and M. D. Ter-Avanesyan, 2000a Prion properties of the Sup35 protein of yeast *Pichia methanolica*. EMBO J. **19:** 324–331.

Kushnirov, V. V., D. S. Kryndushkin, M. Boguta, V. N. Smirnov and M. D. Ter-Avanesyan, 2000b Chaperones that cure yeast artificial [*PSI*$^+$] and their prion-specific effects. Curr. Biol. **10:** 1443–1446.

Liebman, S. W., and J. A. All-Robyn, 1984 A non-Mendelian factor, [eta$^+$], causes lethality of yeast omnipotent-suppressor strains. Curr. Genet. **8:** 567–573.

Liebman, S. W., and I. L. Derkatch, 1999 The yeast [*PSI*$^+$] prion: making sense of nonsense. J. Biol. Chem. **274:** 1181–1184.

Lindquist, S., and G. Kim, 1996 Heat-shock protein 104 expression is sufficient for thermotolerance in yeast. Proc. Natl. Acad. Sci. USA **93:** 5301–5306.

Masison, D. C., and R. B. Wickner, 1995 Prion-inducing domain of yeast Ure2p and protease resistance of Ure2p in prion-containing cells. Science **270:** 93–95.

Michelitsch, M. D., and J. S. Weissman, 2000 A census of glutamine/asparagine-rich regions: implications for their conserved function and the prediction of novel prions. Proc. Natl. Acad. Sci. USA **97:** 11910–11915.

Moriyama, H., H. K. Edskes and R. B. Wickner, 2000 [*URE3*] prion propagation in *Saccharomyces cerevisiae*: requirement for chaperone Hsp104 and curing by overexpressed chaperone Ydj1p. Mol. Cell. Biol. **20:** 8916–8922.

Ono, B., Y. Ishino-Arao, M. Tanaka, I. Awano and S. Shinoda, 1986 Recessive nonsense suppressors in *Saccharomyces cerevisiae*: action spectra, complementation groups and map positions. Genetics **114:** 363–374.

Osherovich, L. Z., and J. S. Weissman, 2001 Multiple Gln/Asn-rich prion domains confer susceptibility to induction of the yeast [*PSI*$^+$] prion. Cell **106:** 183–194.

Patino, M. M., J. J. Liu, J. R. Glover and S. Lindquist, 1996 Support for the prion hypothesis for inheritance of a phenotypic trait in yeast. Science **273:** 622–626.

Paushkin, S. V., V. V. Kushnirov, V. N. Smirnov and M. D. Ter-Avanesyan, 1996 Propagation of the yeast prion-like [*PSI*$^+$] determinant is mediated by oligomerization of the *SUP35*-encoded polypeptide chain release factor. EMBO J. **15:** 3127–3134.

Paushkin, S. V., V. V. Kushnirov, V. N. Smirnov and M. D. Ter-Avanesyan, 1997 Interaction between yeast Sup45p (eRF1) and Sup35p (eRF3) polypeptide chain release factors: implication for prion-dependent regulation. Mol. Cell. Biol. **17:** 2798–2805.

Prusiner, S. B., 1998 Prions. Proc. Natl. Acad. Sci. USA **95:** 13363–13383.

Rosche, W. A., and P. L. Foster, 2000 Determining mutation rates in bacterial populations. Methods **20:** 4–17.

Rose, M. D., F. Winston and P. Hieter, 1990 *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sambrook, J., E. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., and A. R. Coulson, 1975 A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J. Mol. Biol. **94:** 441–448.

Serio, T. R., and S. L. Lindquist, 2000 Protein-only inheritance in yeast: something to get [*PSI*$^+$]-ched about. Trends Cell Biol. **10:** 98–105.

Sherman, F., G. R. Fink and J. B. Hicks, 1986 *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.

Stansfield, I., K. M. Jones, V. V. Kushnirov, A. R. Dagkesamanskaya, A. I. Poznyakovski *et al.*, 1995 The products of the *SUP45* (eRF1) and *SUP35* genes interact to mediate translation termination in *Saccharomyces cerevisiae*. EMBO J. **14:** 4365–4373.

Talloczy, Z., S. Menon, L. Neigeborn and M. J. Leibowitz, 1998 The [*KIL-d*] cytoplasmic genetic element of yeast results in epigenetic regulation of viral M double-stranded RNA gene expression. Genetics **150:** 21–30.

Talloczy, Z., R. Mazar, D. E. Georgopoulos, F. Ramos and M. J. Leibowitz, 2000 The [*KIL-d*] element specifically regulates viral gene expression in yeast. Genetics **155:** 601–609.

Telckov, M. V., A. P. Surguchev, A. R. Dagkesamanskaya and M. D. Ter-Avanesyan, 1986 Isolation of a chromosomal DNA fragment containing *SUP2* gene of the yeast *Saccharomyces cerevisiae* (in Russian). Genetika **22:** 17–25.

Ter-Avanesyan, M. D., V. V. Kushnirov, A. R. Dagkesamanskaya, S. A. Didichenko, Y. O. Chernoff *et al.*, 1993 Deletion analysis of the *SUP35* gene of the yeast *Saccharomyces cerevisiae* reveals two non-overlapping functional regions in the encoded protein. Mol. Microbiol. **7:** 683–692.

Ter-Avanesyan, M. D., A. R. Dagkesamanskaya, V. V. Kushnirov and V. N. Smirnov, 1994 The *SUP35* omnipotent suppressor gene is involved in the maintenance of the non-Mendelian determinant [*PSI*$^+$] in the yeast *Saccharomyces cerevisiae*. Genetics **137:** 671–676.

Tikhodeev, O. N., E. V. Getmanova, V. L. Tikhomirova and S. G. Inge-Vechtomov, 1990 Ambiguity of translation in yeast: genetic control and modifications (in Russian), pp. 218–228 in *Molecular Mechanisms of Genetic Processes*. USSR Academy of Sciences, Nauka, Moscow.

Tuite, M. F., C. R. Mundy and B. S. Cox, 1981 Agents that cause a high frequency of genetic change from [psi$^+$] to [psi$^-$] in *Saccharomyces cerevisiae*. Genetics **98:** 691–711.

Urakov, V. N., I. A. Valouev, E. I. Lewitin, S. V. Paushkin, V. S. Kosorukov *et al.*, 2001 Mtt2, a novel protein modulating translation termination in *Saccharomyces cerevisiae*. BMC Mol. Biol. **2:** 9.

Volkov, K. V., E. A. Ilmov, L. N. Mironova and S. G. Inge-Vechtomov, 1997 The characteristics of the prion-like element of yeast-mutant factor [*PSI*$^+$] (in Russian). Dokl. Akad. Nauk **357:** 123–125.

Volkov, K. V., C. Kurischko, S. G. Inge-Vechtomov and L. N. Mironova, 2000 Polymorphism of the *SUP35* gene and its product in the yeast *Saccharomyces cerevisiae*. Russ. J. Genet. **36:** 155–158.

Von Borstel, R. C., 1978 Measuring spontaneous mutation rates in yeast. Methods Cell Biol. **20:** 1–24.

Wang, W., K. Czaplinski, Y. Rao and S. W. Peltz, 2001 The role of Upf proteins in modulating the translation read-through of nonsense-containing transcripts. EMBO J. **20:** 880–890.

Wickner, R. B., 1992 Double-stranded and single-stranded RNA viruses of *Saccharomyces cerevisiae*. Annu. Rev. Microbiol. **46:** 347–375.

Wickner, R. B., 1994 [*URE3*] as an altered URE2 protein: evidence for a prion analog in *Saccharomyces cerevisiae*. Science **264:** 566–569.

Wickner, R. B., K. L. Taylor, H. K. Edskes, M. L. Maddelein, H. Moriyama *et al.*, 1999 Prions in *Saccharomyces* and *Podospora* spp.: protein-based inheritance. Microbiol. Mol. Biol. Rev. **63:** 844–861.

Zhou, P., I. L. Derkatch, S. M. Uptain, M. M. Patino, S. Lindquist *et al.*, 1999 The yeast non-Mendelian factor [*ETA*$^+$] is a variant of [*PSI*$^+$], a prion-like form of release factor eRF3. EMBO J. **18:** 1182–1191.

Zhouravleva, G., L. Frolova, X. Le Goff, R. Le Guellec, S. Inge-Vechtomov *et al.*, 1995 Termination of translation in eukaryotes is governed by two interacting polypeptide chain release factors, eRF1 and eRF3. EMBO J. **14:** 4065–4072.

Communicating editor: J. Rine