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

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**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 spongiform 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, Hets (for review, see Wickner et al. 1999). Thus, in lower eukaryotes, proteins may serve as genetic determinants. The [Kild] 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 Lieberman 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
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+].

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 (Coix 1977; Lieberman 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; Tikhoodev 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+] (Inversion of Suppressor Phenotype). 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::L-28-2V-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 TRPI 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 Xhol-BamHI 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 Xhol 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 BamHI-SaFl restriction fragment of pBC·HISP104·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·HISP104·URA3 plasmid is the integrative vector containing HSP104 inactivated by the URA3 insertion (the EcoRl-EcoRV 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 (Telegov et al. 1986). The multicopy URA3 plasmid pFL44·ΔATG contains SUP35C (Kushnirov et al. 1990).

The single-copy plasmids CEN·LEU2/pRS315 and CEN·URA3/pRS316, as well as URA3 integrative pRS06 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 Xhol·BamHI 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·MluI 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 sup35·10 alleles (structure of the cloned fragments was examined by sequencing). The plasmids obtained were designated as pRSU1-10 and pRSU1·25. Cloning of the Xhol·BamHI 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·NcoI fragment of pRSU1 was replaced with the same fragment of pFL44·ΔATG. Strains 10·2V-P3982 and 25·2V-P3982 were transformed with the Nsi·MluI 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 YPGl 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 p18S-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 Tuttle 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 his-t and lys-2-87 mutations. Reappearance of [ISP+] was followed by disappearance of the suppressor phenotype.

In cytoduction experiments, the cyh2-1 [rho0] 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 replicates plated to YPGl 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 252-VP-P3982 (5 × 10^6 cells/ml) was plated by a 151-pin 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 pin, containing initially ~500 cells (N0), 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 replicates 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, where N is the terminus number of cells in a culture. To determine the average mean of N, five “zero spots,” i.e., replicates 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 252-VP-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 ~550 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 (mobs) by the method of MSS maximum likelihood (Rosche and Foster 2000). The actual number was calculated as mobs = m0(1 − 1/z ln(z)), where z is the dilution factor. The rate of spontaneous [ISP+] reappearance was estimated as mobs/1.44N, where N 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 read-through assay plasmids pUKC815/817/818/819 (Stansfeld 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 (Stansfeld 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 read-through 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 (Isou 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, GTCCGATTCCTGAAAAGAC TCCATTGTA and M1281, GACGAATCTAATGTTATGAT CCGTA. Two independent products of PCR were sequencing. Sequencing of the SUP35 wild-type and mutant alleles was performed as described (Sanger and Coulson 1975) with the use of Li-COR 420 (MWG-BIOTECH). For sequencing, primers F1, GTACGTGTAACAAAAGCCGT; F2, CGACCTTCAAAGCAGAAA; F3, AGTGGTGAAGGCTTAC TTG; F4, CTAAAGAAAACCCGGCTGGA; R1, GCTTTATAT CGGGATTAT; R2, TTCCACAGGCGTTTTGTAG; R3, CAA ATGGCGCTTACA; and R4, TTTGGTGCTTGTGAAAA 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 [psr+] 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
revealed that only ade1-14 was slightly suppressed (Ade± phenotype). Notably, after growth in the presence of 5 mM GuHCl these mutants changed their phenotypes from Ade± 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 of [ISP+] performed with the use of stop codon read-through 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 GuHCl-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.
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 non-chromosomal 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 non-suppressor 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-

### TABLE 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>pUKC817 (UAA)</th>
<th>pUKC818 (UAG)</th>
<th>pUKC819 (UGA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-2V-P3982 [ISP+]</td>
<td>0.1 ± 0.02</td>
<td>0.5 ± 0.12</td>
<td>0.5 ± 0.14</td>
</tr>
<tr>
<td>25-2V-P3982 [isp−]</td>
<td>15.3 ± 1.53</td>
<td>21.8 ± 3.21</td>
<td>20.9 ± 1.81</td>
</tr>
</tbody>
</table>

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 indicated.

![Figure 2](image2.png)

**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 pRS516 plasmid, which does not contain SUP35, was used as a control. The same concentrations of cells were used in all cases.

### TABLE 3

<table>
<thead>
<tr>
<th>No. of Lys+ colonies observed (r)</th>
<th>No. of cultures with r Lys+ colonies on the medium without GuHCl</th>
<th>No. of cultures with r Lys+ colonies on the medium with GuHCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>52</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>47</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
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<td>7</td>
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<td>6</td>
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<td>0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>1</td>
</tr>
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</table>

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 were 4.8 × 10^5 and 3.5 × 10^6, 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 > t_0.05; v1+v2−z=36).
ent strain 6A-P4475 [rho0] 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, non-chromosomal mode of inheritance, dominance over [isp\(^-\)]) are similar to those of the yeast prion-based genetic determinants [PSI\(^+\)] and [URE3] (see Liebman and Derratches 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 Importantly, the number of Sup\(^-\) cytoductants was significantly higher when the [ISP\(^+\)] strain was used as 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).

**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.

**TABLE 4**

Nonchromosomal inheritance of [ISP\(^+\)]

<table>
<thead>
<tr>
<th>Diploid</th>
<th>Total no. of tetrads</th>
<th>4:0</th>
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<th>2:2</th>
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<tr>
<td>sup35-10/ISP(^+)</td>
<td>32</td>
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<td>sup35-10/ISP35</td>
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<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>sup35-25/ISP35</td>
<td>17</td>
<td>17</td>
<td>0</td>
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</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Donor</th>
<th>No. of cytoductants</th>
<th>% of [ISP(^+)] clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-2V-P3982 [ISP(^+)]</td>
<td>71</td>
<td>16.9 ± 4.45</td>
</tr>
<tr>
<td>25-2V-P3982 [isp(^-)]</td>
<td>106</td>
<td>0.9 ± 0.91</td>
</tr>
</tbody>
</table>

Genotype of the donor strain 25-2V-P3982 is MAT\(a\) ade1-14 his7-1 lys2-87 ura3-Δ trh4-B15 leu2-1 sup35-25; genotype of the recipient strain 6A-P4475 is MAT\(a\) his7-1 lys2-87 met3-1Δ ura3-Δ trh4-B15 leu2-1 cyh2-1 sup35-25 [rho0]. The standard error (±SE) is indicated.
The frequency of disruption of the HSP104 for the propagation of yeast prions. For example, the status of cytoductants obtained, 11 of them were crossed. Indeed, the comparison of growth rates of the trol of GAL1 and an antisuppressor phenotype and causes gradual 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⁻] [rho0], 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**

<table>
<thead>
<tr>
<th>[ISP⁺] strain</th>
<th>Media containing</th>
<th>No. of transformants studied</th>
<th>Frequency of [ISP⁺] loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>[isp⁻]</td>
</tr>
<tr>
<td>10-2V-P3982</td>
<td>Glucose</td>
<td>3321</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Galactose</td>
<td>3054</td>
<td>56</td>
</tr>
<tr>
<td>25-2V-P3982</td>
<td>Glucose</td>
<td>1925</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Galactose</td>
<td>758</td>
<td>2</td>
</tr>
</tbody>
</table>

The pYS-GAL104 plasmid was used for transformation. ±SE is indicated.
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 \(110\text{-}2\text{V-P3982}\) (\(sup35-110\)) and \(112\text{-}2\text{V-P3982}\) (\(sup35-112\)) was not changed after treatment with GuHCl. These strains were crossed with the \([ISP^+]\) strain \(c yt1\text{-}2\text{A-P4439}\), containing the wild-type \(SUP35\) gene (see Table 7). Both diploids produced \(2\text{Sup}^{+}\):\(2\text{Sup}^-\) tetrad (eight tetrad 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 \([ISP^+]\) (Figure 6). These diploids were obtained from the cross of the \([ISP^+]\) strain \(2\text{V-P4424}\) with the strains \(110\text{-}2\text{V-P3982}\) and \(112\text{-}2\text{V-P3982}\). In contrast to the \([ISP^+]\) diploids homozygous for the \(sup35-10\) and \(sup35-25\) mutations, these diploids manifested the \(Sup^-\) phenotype (Figure 6).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total no. of tetrad</th>
<th>No. of tetrad with Sup^+:Sup^- segregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>(c yt1\text{-}2\text{A-P4439})</td>
<td>7</td>
<td>4:0</td>
</tr>
<tr>
<td>(c yt2\text{-}2\text{A-P4439})</td>
<td>8</td>
<td>1:2</td>
</tr>
<tr>
<td>(c yt3\text{-}2\text{A-P4439})</td>
<td>10</td>
<td>1:2</td>
</tr>
<tr>
<td>(c yt13\text{-}2\text{A-P4439})</td>
<td>7</td>
<td>1:2</td>
</tr>
<tr>
<td>(c yt2\text{-}2\text{A-P4439})</td>
<td>5</td>
<td>2:3</td>
</tr>
<tr>
<td>(c yt2\text{-}2\text{A-P4439})</td>
<td>8</td>
<td>3:1</td>
</tr>
</tbody>
</table>
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).

**TABLE 9**

Multicopy sup35-10 and sup35-25 alleles do not influence the frequency of [ISP\(^+\)] appearance

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>No. of transformants</th>
<th>% of [ISP(^+)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-2V-P3982</td>
<td>pRS426</td>
<td>123</td>
<td>11.4 ± 2.86</td>
</tr>
<tr>
<td></td>
<td>pRSU4-10</td>
<td>69</td>
<td>7.2 ± 3.12</td>
</tr>
<tr>
<td>25-2V-P3982</td>
<td>pRS426</td>
<td>123</td>
<td>8.9 ± 2.57</td>
</tr>
<tr>
<td></td>
<td>pRSU4-25</td>
<td>116</td>
<td>3.4 ± 1.69</td>
</tr>
</tbody>
</table>

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

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 Lieberman 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 (Talagavy 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 (Kushnir 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 non-chromosomal 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; Maxisson 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; Czapinski 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 Mtl1p (Czapinski 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.

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