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Research Article

Guanidine reduces stop codon read-through caused by missense mutations in *SUP35* or *SUP45*

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Abstract

Sup35 and Sup45 are essential protein components of the *Saccharomyces cerevisiae* translation termination factor. Yeast cells harbouring the [*PSI*$^+$] prion form of Sup35 have impaired stop codon recognition (nonsense suppression). It has long been known that the [*PSI*$^+$] prion is not stably transmitted to daughter cells when yeast are grown in the presence of mM concentrations of guanidine hydrochloride (GuHCl). In this paper, Mendelian suppressor mutations whose phenotypes are likewise hidden during growth in the presence of millimolar GuHCl are described. Such GuHCl-remedial Mendelian suppressors were selected under conditions where [*PSI*$^+$] appearance was limiting, and were caused by missense mutations in *SUP35* or *SUP45*. Clearly, antisuppression caused by growth in the presence of GuHCl is not sufficient to distinguish missense mutations in *SUP35* or *SUP45*, from [*PSI*$^+$]. However, the Mendelian and prion suppressors can be distinguished by subsequent growth in the absence of GuHCl, where only the nonsense suppression caused by the [*PSI*$^+$] prion remains cured. Recent reports indicate that GuHCl blocks the inheritance of [*PSI*$^+$] by directly inhibiting the activity of the protein remodelling factor Hsp104, which is required for the transmission of [*PSI*$^+$] from mother to daughter cells. However, the nonsense suppressor activity caused by the GuHCl-remedial *sup35* or *sup45* suppressors does not require Hsp104. Thus, GuHCl must antisuppress the *sup35* and *sup45* mutations via an *in vivo* target distinct from Hsp104. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: ***Saccharomyces cerevisiae;*** **GuHCl; translation termination**

Introduction

The genetic code is translated into polypeptides with the help of charged transfer-RNA molecules (tRNAs) that ferry amino acids to the ribosomal/mRNA decoding site. When the ribosome encounters stop codons — either UAA, UAG or UGA nonsense codons — nature's programming causes translation to terminate. These stop codons are recognized by release factor *proteins* instead of tRNAs. In *Saccharomyces cerevisiae*, two essential proteins, Sup45 (Breining *et al*., 1986; Himmelfarb *et al*., 1985) and Sup35 (Kushnirov *et al*., 1988; Wilson and Culbertson, 1988) correspond to the eukaryotic release factors eRF1 and eRF3, respectively (Frolova *et al*., 1994). Sup45 and Sup35 interact to form a translation termination factor (Stansfield *et al*., 1995b; Zhouravleva *et al*., 1995) and mutations in *SUP45* or *SUP35* can cause impaired stop codon recognition (nonsense suppression) (Inge-Vechtomov and Andrianova, 1970; Crouzet *et al*., 1988; Crouzet and Tuite, 1987).

Sup35 can take on at least two forms; normal and prion. The prion form is infectious and is thought to convert the normal proteins into the prion form (for reviews see Cox, 1994; Liebman and Derkatch, 1999; Wickner *et al*., 1999) The prion form of Sup35, known as [*PSI*$^+$], requires the N-terminal region for its maintenance, and [*PSI*$^+$] can be induced *de novo* by overproduction of the same region (Ter-Avanesyan *et al*.,

1994; Doel *et al.*, 1994; Derkatch *et al.*, 1996; Patino *et al.*, 1996). In addition, fusing this region to an unrelated protein was sufficient to transfer prion properties to the fusion protein (Li and Lindquist, 2000). [*PSI*$^+$] causes read-through of stop codons and it is common to monitor this phenotype using yeast auxotrophic for adenine, which have a premature stop codon that interrupts the *ADE1* gene (Inge-Vechtomov *et al.*, 1988). Yeast that are [*psi*$^-$] efficiently terminate translation at the premature stop codon. They do not, therefore, produce much adenine or grow on medium lacking adenine. Rather, on rich medium they accumulate an intermediate of adenine biosynthesis that gives the cells a red colour (Fisher, 1969). In contrast, yeast that are [*PSI*$^+$] occasionally read through the premature stop codon in *ADE1*, enabling them to grow on medium lacking adenine and to stay white or pink on rich medium.

Yeast prions (for review, see Wickner *et al.*, 2001) are stably transmitted from mother to daughter cells in most environments, yet each of the known yeast prions is cured by growth in medium containing 1–5 mM guanidine hydrochloride (GuHCl; Tuite *et al.*, 1981; Wickner, 1994; Derkatch *et al.*, 1997; Sondheimer and Lindquist, 2000). GuHCl is commonly used to distinguish phenotypes that are associated with prions, which are curable, from those associated with Mendelian traits, which are not curable. While all of the effects of low levels of guanidine on yeast metabolism are not known, it appears that GuHCl cures prions by inactivating Hsp104 (Ferreira *et al.*, 2001; Jung and Masison, 2001; Jung *et al.*, 2002; Ness *et al.*, 2002), a protein chaperone (Parsell *et al.*, 1994) required for the maintenance of various yeast prions (Chernoff *et al.*, 1995; Derkatch *et al.*, 1997; Moriyama *et al.*, 2000).

Here we show that missense mutations in the *SUP35* or *SUP45* genes cause nonsense suppression that is remedied during growth in the presence of mM GuHCl. This novel nonsense suppression phenotype is distinct from [*PSI*$^+$] in numerous ways, e.g. it exhibits Mendelian segregation and its maintenance does not require Hsp104. These findings illustrate that growth in the presence of 1–5 mM GuHCl affects the accuracy of translation, and does so independently of Hsp104.

Materials and methods

Cultivation procedures

Conventional yeast media and cultivation techniques were employed (Sherman *et al.*, 1986). Yeast extract/peptone/dextrose (YPD) medium and YPD with 5 mM guanidine hydrochloride (YPD + GuHCl) was used throughout unless indicated otherwise. Synthetic medium lacking adenine (SD–Ade), histidine (SD–His) or uracil (SD–Ura) was used to select for suppressors, diploids and transformants, respectively.

Isolating and characterizing suppressors of *ade1–14*

All suppressors of *ade1–14* are spontaneous derivatives of the yeast strain 74-D694 (*MAT***a** *ade1–14 trp1–289 his3-*Δ*200 leu2–3,112 ura3–52*) (Chernoff *et al.*, 1993). The version of 74-D694 (L1751) used to isolate suppressors of *ade1–14* had been cured of the [*PIN*$^+$] prion, thereby rendering it almost incapable of acquiring spontaneous [*PSI*$^+$] (Derkatch *et al.*, 1997). Adenine prototrophs were selected on SD–Ade medium for up to 14 days before being assayed for [*PSI*$^+$] using the GuHCl curability test. Each Ade$^+$ papilla was colony-purified on YPD and the resulting colonies were patched onto both YPD and YPD + GuHCl. The derivatives having a deeper red colour on the latter media were transferred from YPD + GuHCl back to YPD. Although this step of the GuHCl curability test is not always performed, it is essential for distinguishing between [*PSI*$^+$] and the Mendelian GuHCl-remedial mutants described in this paper, because only the nonsense suppression associated with [*PSI*$^+$] fails to return after growth in the presence of GuHCl. Sup35 sedimentation assays were performed with cells grown in YPD as described previously (Patino *et al.*, 1996).

Allelism and linkage assays

Allelism tests were performed by crossing suppressor containing derivatives of 74-D694 to wild-type, *sup35* or *sup45* derivatives of strain 8A-P3532 (*MAT*α *ade1–14 his7-1 met13-A1*) (kind gifts of S. Inge-Vechtomov). Diploids of 74-D694 derivatives and each of the three 8A-P3532 strains were selected on SD–His, where the appearance of a red

rather than a white or pink colour indicated that complementation of the suppressor mutation had occurred. L1845 (*MATα ade1–14 trp1–289 his3-Δ200 leu2–3,112 ura3–52*; Bradley *et al*., 2002) is a nearly isogenic opposite mating type derivative of 74-D694 that was used to demonstrate linkage between the nonsense suppression and the GuHCl-remedial traits.

Sequencing *sup35* and *sup45* alleles

The mutant *sup35* and *sup45* alleles were PCR amplified in at least five independent reactions using either the *SUP35* primers, p213 (CGGAGC TCCAAAGCTCCCATTGCTTCTG) and p214 (CGGCATCCGAAAACGTGATTGAAGGAG TTG), or the *SUP45* primers p241 (TGTTGGT-GTGGCCTTAACGAC) and p242 (CACGGTC-CTCTAAACCCACTA). All PCR reactions from a single strain were pooled and both strands were sequenced (University of Chicago CRC facility). Sequencing primers for *SUP35* were p131 (TCTTCATCGACTTGCTCGGA), p216 (GGTCA TGTTGATGCCGGTAA), p218 (TGGCTATGTG TGG TGAGCAAG), p214 (CGGCATCCGAAA ACGTGA TTGAAGGAGTT), p222 (CGACGTG-GTTCATTGTATCC) and p132 (TTACCGGCAT-CAACATGACC). Sequencing primers for *SUP45* were p241 (TGTTGGTGTGGCCTTAACGAC), p243 (GGTCGCCGAAGTTGCTGTTCA), p242 (CACGGTCCTCTAAACCCACTA) and p244 (CC TTACATGCTAGTCTTGGATCG).

HSP104 inactivation

HSP104 was inactivated in suppressor (L2327, L2328, L2330, L2331, L2503), [*psi*$^-$] (L1751), and [*PSI*$^+$] (L1762) derivatives of 74-D694 by transformation with a *PvuI–Hin*dIII fragment of pYABL5, which contains an *hsp104::LEU2* disruption cassette (Chernoff *et al*., 1995). Upon obtaining Leu$^+$ transformants, disruptions were verified by PCR using both a pair of primers that produce an amplicon only in *HSP104* strains (CCTTCAA-GACGCTGCTTAAGA and GAGTCGGCATCTT CATCTCT) and a pair of primers that produce an amplicon only in *hsp104::LEU2* strains (GCGCA-GACTTAACTGTGGGAATACTCAGG and GAG TCGGCATCTTCATCTCT).

LacZ read-through assay

Centromeric plasmids pUKC815, 816, 817 and 819 (Stansfield *et al*., 1995a) were transformed into suppressor (L2328 and L2336) and [*psi*$^-$] (L1751) 74-D694 derivatives. Transformants were grown in plasmid-selective medium and 3×10^6 or 6×10^6 cells were inoculated into 5 ml YPD or YPD + GuHCl cultures, respectively. Triplicate samples from cultures grown to $OD_{600} \approx 1.0$ were treated essentially as described (Coligan, 1995), with the following modifications. The cells were suspended in 500 µl Z buffer, 10 µl 0.1% SDS and 20 µl chloroform. The samples were then vortexed for 15 s and equilibrated for 15 min in a 30 °C water bath. After adding 100 µl 4 mg/ml *O*-nitrophenyl-*β*-D-galactosidase (ONPG), samples were vortexed for 5 s and the reaction was carried out for 3 h in a 30 °C waterbath before stopping the reaction by adding 500 µl 1 M sodium carbonate. After centrifugation at $5000 \times g$, the supernatants were measured at both 420 nm and 550 nm wavelengths and the Miller units of each sample was calculated as $(OD_{420}) - (OD_{550} \times 1.75)$. The read-through for each of the three stop codons was calculated as the percentage of LacZ expression relative to the construct lacking a stop codon grown in the same medium.

Results and discussion

After the [*pin*$^-$] [*psi*$^-$] *ade1-14* yeast strain L1751 had been maintained in stationary phase for a prolonged period, we detected the rare appearance of Ade$^+$ colonies that occasionally corresponded to the appearance of [*PIN*$^+$] [*PSI*$^+$] cells (Derkatch *et al*., 2000). While applying the GuHCl curability assay to screen these Ade$^+$ colonies for the presence of [*PSI*$^+$], we also found [*psi*$^-$] cells carrying suppressors of *ade1–14*. We now report that such suppressors represent three distinct groups (Figure 1A, Table 1). Group I consists of *dominant* (see Table 1) suppressors, which generally have the same colour on YPD as they do on YPD + GuHCl. Group II contains *recessive* (see Table 1) suppressors that in patch assays have a similar colour on YPD and YPD + GuHCl but are slow-growing on YPD + GuHCl in single colony assays. Group III is comprised of recessive (see Table 1) suppressors that have deeper red colour when grown on

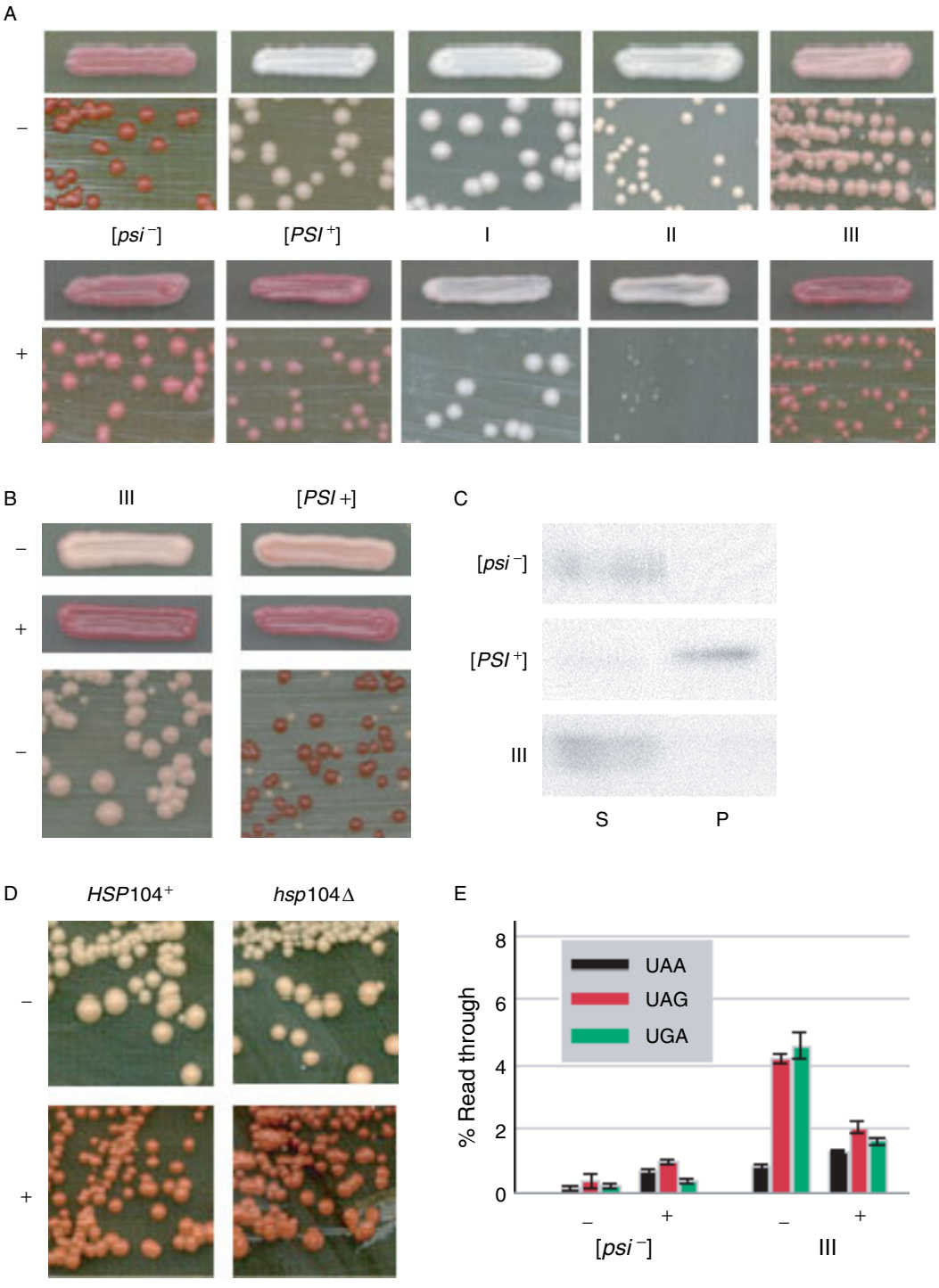

Table 1. Genetic characterization of *ade1-14* nonsense suppressors

		Allelism tests					Mutation	
Group	**No. Total**	**Dom.**	**Rec.**	***SUP35***	***SUP45***	**Other**	**Nonsense**	**Missense**
III	20	0	20	11	5	4	0	14
II	19	0	19	13	3	3	15	0
I	38	38	0	n.a.	n.a.	n.a.	n.d.	n.d.

In order to demonstrate the relative occurrences of each group, the numbers displayed here are from only one experiment in which all Ade$^+$ colonies were picked and tested thoroughly. We performed allelism tests by crossing Ade$^+$ derivatives of [*pin*$^-$] [*psi*$^-$] 74-D694 to three different 8A-P3532 strains. Crosses to wild-type 8A-P3532 revealed whether nonsense suppression was dominant (dom.) or recessive (rec.). Recessive suppressors were scored as being allelic to *SUP35* if the 8A-P3532 *sup35* strain failed to complement the nonsense suppression. Allelism to *SUP45* was determined similarly, using the 8A-P3532 *sup45* strain. No [*PSI*$^+$] colonies were found, and recessive nonsense suppressors that were complemented by both the 8A-P3532 *sup35* and *sup45* strains (other) were not studied further. Mutation results (nonsense or missense) were determined by sequencing the alleles indicated by the allelism tests. n.a., not applicable; n.d., not determined.

YPD + GuHCl than when grown on YPD. We refer to Group III as GuHCl-remedial nonsense suppressors. Unlike [*PSI*$^+$], which is cured of its suppression upon growth in the presence of GuHCl, the [*psi*$^-$] GuHCl-remedial suppressors regain their original nonsense suppression phenotype upon removal from GuHCl medium (Figure 1B). The non-curable GuHCl-remedial nonsense suppressors also lack the aggregated Sup35 characteristic of [*PSI*$^+$] (Figure 1C).

We established Mendelian segregation of the non-curable suppressors. Furthermore genetic linkage between the GuHCl-remedial and nonsense suppression traits indicated that the two phenotypes are caused by the same mutation. Meiotic progeny from crosses of two Group III members (L2327 and L2328) to a wild-type strain showed complete linkage of the two traits (all 13 progeny from the L2327 cross that inherited nonsense suppression out of 29 total progeny examined were also GuHCl-remedial; all 10 progeny with nonsense suppression out of 20 total progeny from the L2328 cross were GuHCl-remedial). The GuHCl-remedial and nonsense suppression phenotypes thus result from a single Mendelian mutation in each derivative. Recessive nonsense suppression (like that observed in Groups II and III) usually results from mutations in *SUP35* or *SUP45* (Inge-Vechtomov and Andrianova, 1970; Crouzet *et al*., 1988; Crouzet and Tuite, 1987). Most of the recessive nonsense suppressors were indeed found to be allelic with either *SUP35* or *SUP45* (Table 1).

We sequenced many of the *SUP35* or *SUP45* alleles implicated by the allelism tests and found a striking difference: Group II alleles contained only nonsense mutants, while Group III alleles

Figure 1. (A) Shown are [*psi*$^-$] (L1751), [*PSI*$^+$] (L1762) and three groups (I–III) of *ade1–14* suppressors (isolated in 74-D694), grown at 30 °C on YPD (–) or YPD + GuHCl (+) for 5 days as either patches (top panels) or single colonies (bottom panels). Representative members of Group I (L2604), Group II (L2495) and Group III (L2331) are shown. (B) GuHCl-remedial nonsense suppression is never permanently eliminated in Group III suppressors. Group III suppressors and [*PSI*$^+$] were grown on YPD(–) or YPD + GuHCl (+). After approximately 21 generations on YPD + GuHCl, the cells were subcloned to YPD (small white colonies are petites induced by GuHCl). A representative member of Group III (L2331) is shown. (C) Group III mutants do not have aggregated Sup35. Total protein lysates from [*psi*$^-$], [*PSI*$^+$] and six different Group III suppressors (L2327–L2332) were fractionated into supernatant (S) and pellet (P) portions. All of the Group III suppressors had a Sup35 S : P ratio like the [*psi*$^-$] strain. A representative member of Group III (L2331) is shown. (D) Group III GuHCl-remedial nonsense suppression does not require *HSP104*. Wild-type and *HSP104* deletion (*hsp104*Δ) derivatives of four Group III suppressors (L2327, L2328, L2330 and L2331) were grown on YPD (–) or YPD + GuHCl (+). A representative (L2331) is shown. (E) GuHCl influences translation termination. For each plasmid, two or three independent transformants of [*psi*$^-$] or Group III (L2336) strains were grown in YPD (–) or YPD + GuHCl (+) and three samples from each culture were assayed for LacZ expression. Shown are the averages and standard deviations from a single representative transformant of each strain grown in each medium. Similar results were also obtained for other transformants and for another Group III strain (L2328)

Table 2. Sequencing results

Strains		Mutations	
		DNA	**Protein**
Group II			
sup35	L2492	C 64 T	Q 22 STOP
sup35	L2502	C 147 G	Y 49 STOP
sup35	L2500	C 211 T	Q 71 STOP
sup35	L2493	C 214 T	Q 72 STOP
sup35	L2499	G 229 T	G 77 STOP
sup35	L2501	G 334 T	G 112 STOP
sup35	L2496	G 514 T	E 172 STOP
sup35	L2504	G 514 T	E 172 STOP
sup35	L2503	G 553 T	E 185 STOP
sup35	L2494	C 629 A	S 210 STOP
sup35	L2495	C 668 G	S 223 STOP
sup35	L2497	G 1609 T	E 537 STOP
sup45	L2507	G 366 A	E 366 STOP
sup45	L2506	G 1115 A	W 372 STOP
sup45	L2505	G 1243 T	E 414 STOP
Group III			
sup35	L2329	G 959 T	R 320 I
sup35	L2336	A 1052 G	Y 351 C
sup35	L2330	G 1070 A	G 357 D
sup35	L2333	G 1256 A	R 419 H
sup35	L2328	G 1429 T	D 477 Y
sup35	L2334	A 1607 C	N 536 T
sup35	L2332	T 1957 C	C 653 R
sup45	L2327	G 144 T	M 48 I
sup45	L2520	G 187 T	V 63 F
sup45	L2522	T 188 A	V 63 D
sup45	L2331	G 659 A	G 220 D
sup45	L2523	G 757 T	V 253 F
sup45	L2519	T 824 A	V 275 D
sup45	L2521	A 1229 C	Y 410 S

Strains are listed by Group II or III classification, *SUP35* or *SUP45* allelism, and the laboratory identification numbers given to each strain. DNA sequencing results of the coding strand are summarized as the nucleotide change (wild-type at left; mutant at right) at the base pair position indicated (where 1 corresponds to the first base pair of the first codon). The protein column shows the codon affected by the mutation with the wild-type amino acid on the left and the mutant amino acid on the right. The Group II and III suppressors are from various experiments, including the one documented in Table 1.

contained only missense mutants (Table 2). It is not surprising that nonsense mutations in *SUP35* and *SUP45* are not remedial because the anti-suppression caused by GuHCl should also reduce read-through of the *sup35* or *sup45* premature nonsense codons, thereby lowering production of the release factor and restoring suppression. Similar feedback hypotheses have been described previously to explain viability of nonsense mutations in the essential *SUP35* and *SUP45* genes (Stansfield *et al*., 1996; Zhou *et al*., 1999). According to this hypothesis, a nonsense mutation in *SUP35* or *SUP45* is not lethal because the premature stop codon is read through due to the lower level of termination factor. Since all of the many different missense mutations obtained throughout *SUP35* or *SUP45* (Table 2) result in GuHCl-remedial nonsense suppression, we hypothesize that GuHCl may be influencing termination through a target other than the altered *sup35* or *sup45* proteins.

The only documented target of GuHCl *in vivo* is Hsp104. If Hsp104 were the target responsible for the antisuppressor effect, deletion of *HSP104* from Group III strains should have the same effect as growth in the presence of GuHCl. However, deleting *HSP104* did not affect the phenotypes of the Group III suppressors in either the absence or presence of GuHCl (Figure 1D). As expected, deleting *HSP104* from a control [PSI^+] strain resulted in the complete loss of [PSI^+] (data not shown). This proves that growth in the presence of 1–5 mM GuHCl has an effect on translation's accuracy not involving Hsp104. Likewise, removal of *HSP104* from Group II strains did not change their slow-growth in the presence of GuHCl (data not shown).

To confirm that the deeper red colour observed for Group III suppressors in the presence of GuHCl is due to increased translation termination, we measured translation termination with a LacZ-based reporter assay (Stansfield *et al*., 1995a). In this system, stop codons placed in frame between the start of the *PGK1* gene and the *LacZ* gene allow for calculating the percentage of read-through by comparing LacZ expression relative to a control construct that lacks a stop codon between the two fused frames. As expected, the wild-type [psi^-] strain exhibited extremely low levels of read-through in both the absence and presence of GuHCl (Figure 1E). A Group III suppressor grown in the absence of GuHCl had elevated read-through compared to wild-type. The Group III suppressor grown in the presence of GuHCl had less read-through compared to the same transformants grown in the absence of GuHCl. Thus, GuHCl reduces the translational read-through associated with the Mendelian suppressor mutants.

Conclusions

These characterizations show that Mendelian GuHCl-remedial nonsense suppressors are not [*PSI*$^+$] variants, neither do they contain non-prion, aggregated Sup35 that could become soluble in the presence of GuHCl. The existence of nonsense suppressors such as these stresses that the GuHCl curing test to identify [*PSI*$^+$] must be done completely (as shown in Figure 1B), since the relief of nonsense suppression in the presence of GuHCl is also a property of yeast that are [*psi*$^-$] but have acquired GuHCl-remedial Mendelian nonsense suppressors. Some additional hypotheses regarding the influence of GuHCl on translation termination can be imagined. First, GuHCl may act as a molecular 'glue' to promote the formation of the termination factor (i.e. Sup45–Sup35 binding). Second, GuHCl may directly inactivate a protein such as Itt1, which acts as an inhibitor of translation termination (Urakov *et al*., 2001), or GuHCl may interfere with any protein, such as Upf1 or Mtt1 (Weng *et al*., 1996; Czaplinski *et al*., 1998, 2000), that modulates the efficiency of translation termination. Third, GuHCl might cause a stress that indirectly stimulates translation termination. Finally, GuHCl could be stimulating translation termination by directly influencing the peptidyl-transferase centre of the ribosome.

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