

Discrimination Between Defects in Elongation Fidelity and Termination Efficiency Provides Mechanistic Insights into Translational Readthrough

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The suppression of stop codons (termed translational readthrough) can be caused by a decreased accuracy of translation elongation or a reduced efficiency of translation termination. In previous studies, the inability to determine the extent to which each of these distinct processes contributes to a readthrough phenotype has limited our ability to evaluate how defects in the translational machinery influence the overall termination process. Here, we describe the combined use of misincorporation and readthrough reporter systems to determine which of these mechanisms contributes to translational readthrough in *Saccharomyces cerevisiae*. The misincorporation reporter system was generated by introducing a series of near-cognate mutations into functionally important residues in the firefly luciferase gene. These constructs allowed us to monitor the incidence of elongation errors by monitoring the level of firefly luciferase activity from a mutant allele inactivated by a single missense mutation. In this system, an increase in luciferase activity should reflect an increased level of misincorporation of the wild-type amino acid that provides an estimate of the overall fidelity of translation elongation. Surprisingly, we found that growth in the presence of paromomycin stimulated luciferase activity for only a small subset of the mutant proteins examined. This suggests that the ability of this aminoglycoside to induce elongation errors is limited to a subset of near-cognate mismatches. We also found that a similar bias in near-cognate misreading could be induced by the expression of a mutant form of ribosomal protein (r-protein) S9B or by depletion of r-protein L12. We used this misincorporation reporter in conjunction with a readthrough reporter system to show that alterations at different regions of the ribosome influence elongation fidelity and termination efficiency to different extents.

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Introduction

The suppression of premature stop mutations that confer an auxotrophic phenotype has frequently been used to identify translation factors in genetic screens. The suppression of such stop mutations based on a growth assay provides a simple (yet non-quantitative) means to carry out high throughput analysis of a large numbers of candidates. Once mutants that have a suppressor

phenotype are identified, a number of reporter systems have been developed that can provide a more quantitative measure of the level of readthrough (stop codon suppression) associated with a particular mutation.^{1–7} The major limitation to this approach is the inability to interpret nonsense suppression at a mechanistic level. When an in-frame stop codon reaches the A site of a ribosome, a competition exists between the termination and elongation machineries. This leads to two distinct mechanisms by which readthrough can occur. First, a general decrease in the fidelity of the elongation machinery at a termination codon can result in a nonsense suppression phenotype. Alternatively, mutations that directly reduce the efficiency of the translation termination machinery can also produce

Abbreviations used: SM, synthetic minimal; Ram, ribosomal ambiguity; GAC, GTPase-activating center.

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the same readthrough phenotype. Given that these two distinct mechanisms can elicit a readthrough phenotype, the availability of *in vivo* assays that could discriminate between defects in termination efficiency and elongation fidelity would increase our understanding of how various mutations or drugs confer a readthrough phenotype.

Translation elongation is normally a highly accurate process. This accuracy relies not only on the proper codon–anticodon interaction, but also on an energy-dependent tRNA selection and discrimination process that is catalyzed by the elongation factor EF-Tu in bacteria and eEF1A in eukaryotes.⁸ The role of EF-Tu is critical for the rejection of near-cognate tRNAs (whose anticodon binds to a codon with a single base mismatch) while non-cognate tRNAs (those with two or more mismatches) are discriminated against primarily on the basis of the initial codon–anticodon interaction. However, even with these stringent mechanisms in place to ensure a high degree of fidelity, some errors are made during translation elongation. These are generally thought to involve the misincorporation of amino acid residues that are brought to the A site by a near-cognate tRNA. Surprisingly, few *in vivo* studies have been carried out that directly measure elongation fidelity, and most have been limited to bacterial systems.^{9–11} Such approaches generally relied on a reporter enzyme that had been inactivated by a single point mutation. In such a system, a stimulation of enzymatic activity was assumed to reflect a decrease in the accuracy of the elongation process.^{12,13}

In the current study, we developed a misincorporation reporter system to better understand whether the suppression of stop codons in the simple eukaryote *Saccharomyces cerevisiae* is caused by a decrease in the accuracy of translation elongation or a reduction in the efficiency of translation termination. We chose to utilize firefly luciferase to develop this misincorporation reporter system due to the availability of its crystal structure and the many genetic studies previously carried out with this protein.^{14–21} In those studies, it was shown that the His residue encoded at position 245 (His245 (CAC)) and the Lys residue encoded at position 529 (Lys529 (AAA)) each play an important role in the enzymatic activity of firefly luciferase,^{15–17} probably because these residues properly orient the substrate and stabilize the transition state of the enzyme. It was previously reported that Lys529 is essential for enzymatic activity,¹⁷ while His245 is important, but not absolutely essential.^{15,16} To identify the most sensitive form of this misincorporation reporter system, we systematically introduced a series of near-cognate missense mutations at these two codons. Cells expressing each mutant protein were then grown in the presence or absence of paromomycin to reduce elongation fidelity, and firefly luciferase assays were carried out to determine the level of activity that could be restored by misincorporation. Interestingly, we found that the stimulation of luciferase activity was markedly

allele specific, with the mutant encoding an arginine residue at position 245 (Arg245 (CGC)) showing a 12.5-fold stimulation of enzymatic activity in the presence of paromomycin. Using this misincorporation reporter system in combination with a previously described readthrough reporter system, we show that different perturbations of ribosomal function influence the readthrough of stop codons in distinct ways.

Results

Paromomycin induces misincorporation capable of restoring firefly luciferase activity at only a subset of near-cognate mutants

The basic features of the misincorporation reporter system developed here are similar to the previously described dual luciferase readthrough reporter constructs.^{4–6} It contains an upstream *Renilla* luciferase gene followed by a firefly luciferase gene under the transcriptional control of the PGK promoter (Figure 1(a)). These open reading frames are separated by an in-frame linker sequence, resulting in the synthesis of both enzymes as a single polypeptide chain. The *Renilla* luciferase gene serves as an internal normalization control for both mRNA abundance and the efficiency of translation initiation, since translation of both enzymes originates from the same translation initiation signal.

The misincorporation of an amino acid during translation elongation is thought to occur when a codon located in the ribosomal A site mispairs with the anticodon of a near-cognate aminoacyl tRNA. Little is known about the misreading frequencies associated with different near-cognate tRNAs for a given codon. However, it is likely that many factors,

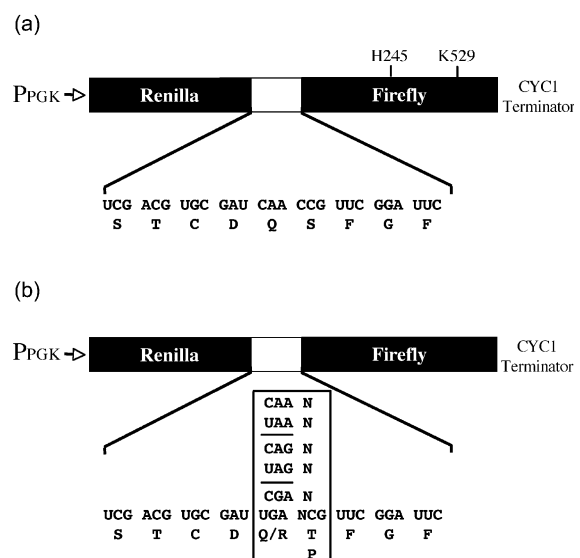


Figure 1. Dual luciferase reporter constructs: (a) misincorporation reporter; (b) readthrough reporter.

Table 1. Wild-type and mutant derivatives of firefly luciferase

Firefly position	Codon ^a	Plasmid name
245	<u>CAC</u> (His)	pDB688
245	AAC (Asn)	pDB864
245	GAC (Asp)	pDB870
245	UAC (Tyr)	pDB871
245	CCC (Pro)	pDB867
245	CGC (Arg)	pDB868
245	CUC (Leu)	pDB869
245	CAA (Gln)	pDB865
245	CAG (Gln)	pDB866
529	<u>AAA</u> (Lys)	pDB688
529	<u>CAA</u> (Gln)	pDB872
529	GAA (Glu)	pDB873
529	ACA (Thr)	pDB828
529	AGA (Arg)	pDB829
529	AUA (Ile)	pDB830
529	AAC (Asn)	pDB825
529	AAU (Asn)	pDB827

^a Wild-type codon at each position is underlined.

such as the concentration of the near-cognate tRNA in the cell and the specific base-pairing mismatch involved, can influence this process. The primary objective of this study was to develop a system that could provide an estimate of the level of misincorporation during translation elongation. However, due to our poor understanding of the general rules that influence misreading, we initially undertook a comprehensive approach to the introduction of mutations in firefly luciferase. The codons encoding the functionally important residues His245 and Lys529 were each changed to all possible codons that allowed the misincorporation of the wild-type amino acid through the mispairing of a near-cognate aminoacyl tRNA. This generated a series of mutant reporter constructs (Table 1). The initial level of firefly luciferase activity obtained with each

mutant protein was corrected for small differences in the activity of the co-translated *Renilla* luciferase and expressed as the percentage of wild-type firefly luciferase activity (referred to as percentage misreading) (Table 2). These assays confirmed that each mutation resulted in a large reduction of firefly luciferase activity under normal conditions. The background level of activity remaining in each of the luciferase mutants could be caused by a number of mechanisms. These include residual enzymatic activity associated with the cognate amino acid incorporated at the mutated codon, misincorporation of a near-cognate amino acid that partially restores activity at the mutated codon, or the mischarging of the cognate tRNA because of an aminoacyl-tRNA synthetase error. Since the ability of paromomycin to increase the misincorporation of near-cognate aminoacyl-tRNAs during translation elongation is firmly established,^{22–24} we next grew strains expressing each of these constructs in the presence of paromomycin to stimulate misreading. Surprisingly, we found that paromomycin induced a significant stimulation of firefly luciferase activity for only a small subset of these mutants. The most significant increase (12.5-fold) was found with the Arg245 (CGC) mutant. For the rest of the mutants, an increase in firefly luciferase activity of more than twofold was observed only for the Asp245 (GAC) mutant (2.9-fold), the Leu245 (CUC) mutant (2.2-fold), the Gln245 (CAG) mutant (4.4-fold), and the Asn529 (AAU) mutant (4.3-fold).

In order to correlate the increase in firefly luciferase activity to the paromomycin concentration in the culture medium, the luciferase activity of the Arg245 (CGC) mutant was measured in cells grown in the presence of increasing paromomycin levels (Figure 2). A linear correlation was observed between increasing paromomycin concentration

Table 2. Effect of paromomycin on misreading during translation elongation

Firefly mutation ^a	Percentage misreading ^b		Fold change ^c
	– Paromomycin	+ Paromomycin ^d	
245 CAC	100	100	–
245 AAC	7.7 ± 1	9.3 ± 1.6	1.2
245 <u>GAC</u>	0.007 ± 0.001	0.02 ± 0.003	<u>2.9</u>
245 <u>UAC</u>	1.4 ± 0.16	1.0 ± 0.09	<u>0.7</u>
245 <u>CCC</u>	0.6 ± 0.1	0.7 ± 0.1	1.2
245 <u>CGC</u>	0.2 ± 0.05	2.5 ± 0.26	<u>12.5</u>
245 <u>CUC</u>	0.12 ± 0.012	0.27 ± 0.025	<u>2.2</u>
245 <u>CAA</u>	0.15 ± 0.017	0.17 ± 0.008	1.1
245 CAG	0.16 ± 0.02	0.7 ± 0.2	<u>4.4</u>
529 AAA	100	100	–
529 CAA	0.05 ± 0.006	0.06 ± 0.01	1.2
529 <u>GAA</u>	0.012 ± 0.001	0.023 ± 0.005	1.9
529 <u>ACA</u>	0.045 ± 0.005	0.05 ± 0.01	1.1
529 <u>AGA</u>	0.032 ± 0.006	0.04 ± 0.006	1.2
529 <u>AUA</u>	0.05 ± 0.008	0.06 ± 0.01	1.2
529 <u>AAC</u>	0.035 ± 0.005	0.05 ± 0.008	1.4
529 <u>AAU</u>	0.023 ± 0.002	0.1 ± 0.017	<u>4.3</u>

^a Mutated nucleotides are underlined.

^b Percentage misreading is expressed as mean ± standard deviation.

^c Changes ≥ 2-fold that yielded a statistically significant *P*-value (<0.05) using the Mann–Whitney test are underlined.

^d Paromomycin concentration is 200 µg/ml.

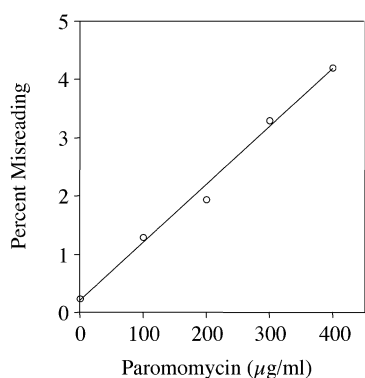


Figure 2. Elongation fidelity errors increase as a function of paromomycin concentration. The data were generated using the Arg245 (CGC) near-cognate mutant of firefly luciferase. The data (percentage misreading) are expressed as firefly/*Renilla* luciferase activity and expressed as the percentage of the same ratio measured in a strain that carried the wild-type His245 (CAC) firefly luciferase construct.

and the restoration of firefly activity, with a 17-fold stimulation observed in cells grown in the presence of 400 µg/ml of paromomycin. We found that the presence of higher paromomycin concentrations led to a significant decrease in growth rate. Due to the large increase in firefly luciferase activity associated with the Arg245 (CGC) mutant observed following exposure to paromomycin, we used this reporter construct to measure changes in elongation fidelity in subsequent experiments.

Paromomycin can also be used to induce the suppression of stop codons (termed readthrough), since it reduces elongation fidelity. The results presented above demonstrate that the reduction in fidelity caused by paromomycin induces misreading during translation elongation in a manner that clearly favors some misincorporation events over others (Table 2). Therefore, it is possible that a bias also exists in the way paromomycin induces readthrough of stop codons. To explore this possibility, we used a readthrough assay to examine how paromomycin influences termination efficiency at different termination signals. Various studies have shown that both the stop codon and the first base that follows it play an important role in determining

the efficiency of translation termination. This extended signal has previously been referred to as the “tetranucleotide termination signal”^{3,25,26}. Here, we used dual luciferase reporter plasmids containing various tetranucleotide termination signals (each of the stop codons followed by an A or a C) to measure readthrough levels^{4,27} (Figure 1(b)). Using this reporter system, we found that 200 µg/ml of paromomycin causes a large increase in readthrough at each of the tetranucleotide termination signals assayed (Table 3), with increases in firefly luciferase activity ranging from 14.5-fold at the UGAA signal to 37.9-fold at the UAGC signal. This large, general increase in readthrough was in marked contrast to the smaller, codon-dependent misincorporation observed in cells grown in the presence of the same paromomycin concentration (Table 2).

Misincorporation errors that restore firefly activity are mediated by near-cognate tRNAs

The results above indicate that paromomycin can induce misreading during translation elongation, which leads to a large increase in luciferase activity with the Arg245 (CGC) mutant firefly construct. Aminoglycosides such as paromomycin are thought to decrease translation fidelity by diminishing the ability of the elongation machinery to distinguish between a cognate and a near-cognate aminoacyl-tRNA.²⁸ This makes it likely that the paromomycin-dependent stimulation of mutant firefly luciferase activity in our misincorporation assay is mediated by the mispairing of a near-cognate aminoacyl-tRNA. In the misincorporation reporter with the greatest increase in activity, the His codon (CAC) at position 245 was changed to an Arg codon (CGC). For the wild-type His residue to be incorporated at this position, the Arg codon must be mispaired with a near-cognate histidinyI-tRNA with a GUG anticodon. This codon-anticodon interaction would involve a single G·U mismatch at the second position. If this reasoning is correct, a firefly luciferase mutant with a synonymous Arg codon at position 245 that is not a near-cognate for this histidinyI-tRNA (such as CGA) should not respond to the presence of paromomycin with an increase in luciferase activity.

We observed several significant differences in

Table 3. Effect of paromomycin on the readthrough of termination codons

Stop signal	Percentage readthrough ^a		Fold change
	–Paromomycin	+Paromomycin ^b	
UAAA	0.068 ± 0.01	1.2 ± 0.26	17.6
UAAC	0.12 ± 0.03	2.2 ± 0.15	18.3
UAGA	0.06 ± 0.005	0.95 ± 0.15	15.8
UAGC	0.07 ± 0.01	2.1 ± 0.2	30.0
UGAA	0.11 ± 0.002	1.6 ± 0.19	14.5
UGAC	0.27 ± 0.64	5.5 ± 0.27	20.4

^a Percentage readthrough is expressed as mean ± standard deviation.

^b Paromomycin concentration is 200 µg/ml.

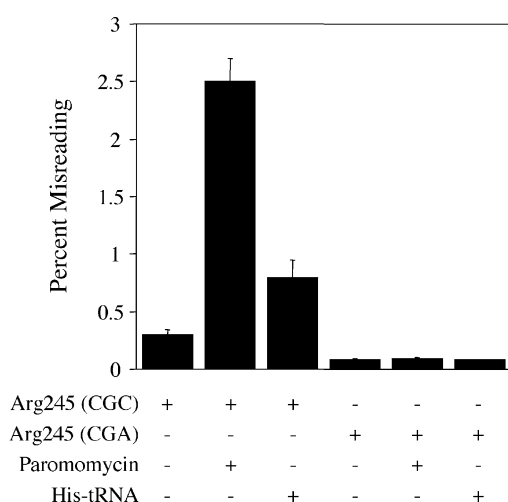


Figure 3. Misreading during translation elongation at near-cognate and non-cognate codons at position 245 of firefly luciferase. Firefly luciferase residue His245 (CAC) was mutated to CGC and CGA. The level of misreading was calculated in a wild-type strain, a wild-type strain grown in 200 μ g/ml of paromomycin, and a strain overexpressing a histidine tRNA from a high copy vector. The data (percentage misreading) are expressed as firefly/*Renilla* luciferase activity and expressed as the percentage of the same ratio measured in a strain that carried the wild-type His245 (CAC) firefly luciferase construct.

misreading between the Arg245 (CGC) and Arg245 (CGA) codons. First, we noted a significant (3.5-fold) difference in basal firefly activity between these two mutants in the absence of paromomycin (Figure 3). This suggests that the intrinsic level of misreading in this yeast strain can incorporate His at the near-cognate Arg245 (CGC) codon at a detectable frequency. The 3.5-fold lower luciferase activity observed with the Arg245 (CGA) mutant suggests that histidine is not incorporated in this case because it would require two base-pairing mismatches with the histidyl-tRNA anticodon. The residual activity observed with the Arg245 (CGA) mutant is probably due to residual intrinsic activity associated with this mutant and/or a low level of tRNA charging errors. Furthermore, paromomycin stimulated a large increase in luciferase activity in a strain expressing the Arg245 (CGC) mutant, but not in a strain expressing the Arg245 (CGA) mutant. These results strongly suggest that the paromomycin-induced stimulation of luciferase activity observed in the CGC mutant is caused by misincorporation during translation elongation *via* a near-cognate tRNA interaction. Consistent with this model, overexpression of a histidyl-tRNA with a GUG anticodon caused a threefold increase in luciferase activity in a strain expressing the Arg245 (CGC) reporter in the absence of paromomycin, but did not elicit any increase in a strain expressing the Arg245 (CGA) reporter enzyme (Figure 3).

A combination of readthrough and misincorporation assays can discriminate between defects in the translation termination and elongation steps

Readthrough assays can be used to directly measure the efficiency of translation termination when characterizing mutations in known termination factors.^{27–30} However, an increased level of readthrough does not necessarily indicate a defect in the termination machinery, since a decrease in the fidelity of translation elongation can also result in the suppression of stop codons.³¹ When the mechanism underlying the readthrough phenotype associated with a mutation is not well understood, it would be useful to assess the contribution of a defect in elongation fidelity to that phenotype. To test the validity of this reasoning, a wild-type strain was grown in the presence of paromomycin to reduce the fidelity of translation elongation. Similarly, a strain with a mutation in the termination factor eRF3 (eRF3-H348Q) was used as a control for a specific defect in termination efficiency. The eRF3-H348Q mutation maps in the GTPase domain of eRF3, and leads to a reduced efficiency of GTP hydrolysis and increased readthrough at many termination signals.²⁷ Here, we used a dual luciferase reporter plasmid containing the UGAC termination signal to measure readthrough levels^{4,27} (Figure 1(b)). For our misincorporation reporter we again used the Arg245 (CGC) firefly mutant described above. Using the readthrough reporter plasmids, we found that paromomycin caused an 18-fold increase in readthrough of the UGAC termination signal, while the H348Q-eRF3 mutation resulted in a 14-fold increase in readthrough at the same termination signal (Figure 4(a)). Similarly, analysis of elongation fidelity revealed that paromomycin increased amino acid misreading by 11-fold (Figure 4(b)). These results demonstrate that paromomycin impairs elongation fidelity, and suggests that its effect on termination may result primarily (if not exclusively) from this elongation fidelity defect. In contrast, elongation fidelity was completely normal in the H348Q-eRF3 strain. These results confirm that the readthrough phenotype associated with this strain is exclusively caused by a defect in the efficiency of translation termination.

A mutation in r-protein S9B increases misincorporation and readthrough

The results described above indicate that the combined use of the misincorporation and readthrough assays can provide new insights into the molecular defects that lead to translational readthrough. To further assess how selected ribosomal mutants cause translational readthrough, we first used a mutant allele of the r-protein S9B. The r-protein S9B is encoded by the *SUP46* gene, which was identified because mutations in this gene result in an omnipotent suppressor phenotype, where suppression occurs at all three stop

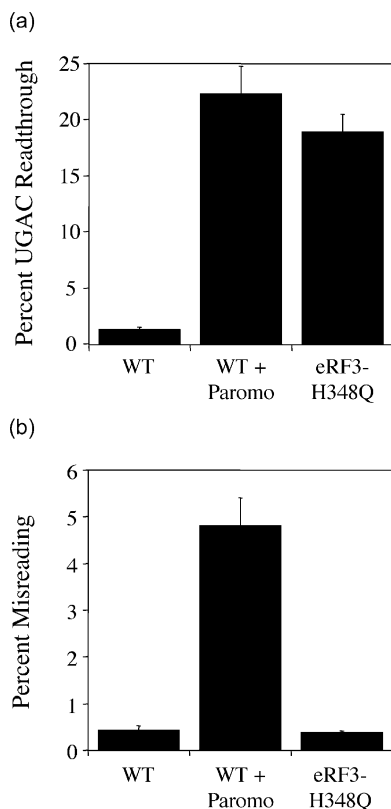


Figure 4. Effect of 200 $\mu\text{g}/\text{ml}$ of paromomycin or the eRF3-H348Q mutation on (a) suppression of a UGAC stop signal and (b) misreading of the Arg245 (CGC) mutant during translation elongation. The eRF3-H348Q mutant was expressed from a centromeric vector under the transcriptional control of its own promoter in a strain carrying a knockout of the eRF3 gene (*sup35 Δ*).

codons.^{32,33} Prior work demonstrated that a *SUP46* mutation also causes an elongation fidelity defect.³⁴ Here, we constructed the S9B-D94N mutant, which corresponds to the previously described *SUP46-1* allele.³³

Genes encoding S9B-WT or S9B-D94N were each subcloned into a low copy plasmid under the transcriptional control of the S9B promoter. These plasmids were then transformed, along with a readthrough or misincorporation reporter plasmid, into a yeast strain carrying a disruption of the endogenous S9B gene. Initial characterization indicated that the S9B disruption conferred a slow-growth phenotype relative to the strain expressing wild-type S9B from the plasmid (data not shown). We next examined elongation fidelity at each of the His245 firefly luciferase mutants in the S9B-WT and S9B-D94N strains (Table 4). We found that the S9B-D94N mutation restored partial firefly luciferase activity at only a subset of the luciferase mutants. The greatest stimulation was observed with the Arg245 (CGC) mutant (7.3-fold), the Asp245 (GAC) mutant (2.8-fold), and the Gln245 (CAG) mutant (1.8-fold) (Table 4). Interestingly, these three near-cognate codons had also been found to be the most

sensitive to misreading in wild-type cells grown in the presence of paromomycin (see Table 2). We next examined the effect of the S9B-D94N mutation on readthrough of a subset of tetranucleotide termination signals (the three stop codons followed by either A or C). We found that the S9B-D94N mutation increased readthrough at all the tetranucleotide termination signals (Table 5), ranging from 5.9 and 6.4-fold increases at UAGA and UGAA, respectively to a 10.5-fold increase at the UAAA termination signal. Together, these results confirm that the S9B-D94N mutation also induces selective misreading, as well as a more general readthrough phenotype.

Depletion of the r-protein L12 causes a small increase in misincorporation and a larger increase in readthrough

We next examined how the perturbation of another ribosomal protein influenced eukaryotic elongation fidelity and termination. The ribosomal GTPase domain in the large ribosomal subunit contains two important RNA structures. These include the sarcin-ricin loop (SRL) in domain VI of the 25 S rRNA and a double hairpin in domain II known as the GTPase-activating center (GAC).^{35,36} Two proteins, L12 (homologous to *Escherichia coli* L11) and P0 (homologous to *E. coli* L10), bind overlapping sites in domain II of the 25 S rRNA of yeast. Furthermore, a protein tetramer known as the stalk, made up of the dimers $P1_2$ - $P2_2$ (homologous to *E. coli* L7/L12), interacts with the ribosome *via* P0.³⁷ It was previously shown that L12 plays an important role in maintaining the integrity of the GTPase domain of yeast ribosomes by influencing both the RNA structure³⁸ and the protein composition of the stalk.³⁹ Since the contribution of the eukaryotic GTPase domain to elongation fidelity or termination efficiency has never been addressed *in vivo*, we next chose to deplete L12 in yeast and measure the effect on each of these ribosomal functions.

The *rpL12A* and *rpL12B* genes encode r-protein L12 in *S. cerevisiae*. It was previously shown that a strain carrying disruptions of both *rpL12* genes is viable, but exhibits a severe slow-growth phenotype.³⁹ We disrupted both *rPL12A* and *rPL12B* as described³⁹ and generated two strains. The first strain carried disruptions of both *rpL12* genes, along with a centromeric plasmid that expressed an HA-tagged *rpL12A* gene under the transcriptional control of the *GAL10* promoter. The second strain also carried disruptions of both *rpL12* genes, as well as a centromeric plasmid with an HA-tagged *rpL12A* gene under the transcriptional control of the *rpL12A* promoter.

Readthrough or misincorporation reporter plasmids were introduced into each of these strains, and they were then grown for several generations in synthetic minimal (SM) medium containing galactose as carbon source. They were then shifted to SM glucose medium, which represses transcription of

Table 4. Effect of the S9-D94N mutation on misreading during translation elongation

Firefly mutation ^a	Percentage misreading ^b		Fold change ^c
	S9-WT	S9-D94N	
245 CAC	100	100	–
245 AAC	7.3 ± 0.9	9.5 ± 1.6	1.3
245 <u>C</u> AC	0.005 ± 0.0004	0.014 ± 0.001	<u>2.8</u>
245 <u>U</u> AC	1.0 ± 0.03	1.6 ± 0.16	<u>1.6</u>
245 <u>C</u> CC	0.41 ± 0.02	0.38 ± 0.1	0.9
245 <u>C</u> GC	0.26 ± 0.03	1.9 ± 0.15	<u>7.3</u>
245 <u>C</u> UC	0.18 ± 0.04	0.24 ± 0.02	<u>1.3</u>
245 <u>C</u> AA	0.24 ± 0.01	0.24 ± 0.05	1
245 <u>C</u> AG	0.15 ± 0.01	0.27 ± 0.1	<u>1.8</u>

^a Mutated nucleotides are underlined.

^b Percentage misreading is expressed as mean ± standard deviation.

^c Changes ≥ 1.8-fold that yielded a statistically significant *P*-value (<0.05) using the Mann–Whitney test are underlined.

rpL12A from the *GAL10* promoter, but not from the *rpL12A* promoter. After growth for four generations with glucose as carbon source, we found that the steady-state level of HA-L12A expressed from the *GAL10* promoter was reduced to 15% of the level observed when *rpL12A* was expressed from the L12A promoter (Figure 5). Because the antibodies used in the Western blot analysis recognized the HA epitope tag, L12 was not detected in a wild-type strain that carried only untagged, genomic *rpL12A* and *rpL12B*. When we examined the fidelity of translation elongation four generations after shifting the carbon source to glucose, we observed a 2.2-fold increase in firefly luciferase activity for the Arg245 (CGC) firefly luciferase mutant in the strain expressing *rpL12A* from the *GAL10* promoter, but did not detect a significant increase in activity for any of the other firefly luciferase mutants at position 245 (Table 6). While the general level of misincorporation observed under these conditions was surprisingly small, these results demonstrate that the Arg245 (CGC) reporter is generally prone to misreading induced by various methods. We next examined how L12 depletion influenced the efficiency of translation termination at six different tetranucleotide termination signals. We found a significant increase in readthrough at all six stop signals examined following L12 depletion by the same conditions used above (Table 7), indicating that the 85% reduction in the concentration of L12 caused a general decrease in the efficiency of translation termination that ranged from 3.2-fold (UAGC) to 5.8-fold (UGAA). These data indicate

that L12 (and consequently the GTPase region of the ribosome) is involved in the fidelity of translation elongation in the eukaryotic ribosome, and a portion of the translational readthrough associated with L12 depletion may be caused by this defect. However, since the magnitude of the increase in readthrough was significantly larger than the decrease in elongation fidelity, these results also suggest that the GTPase region of the ribosome plays a direct role in translation termination.

The loss of Upf1p does not induce misincorporation

It was previously shown that mutations in *UPF1* cause a readthrough phenotype.^{4,40} This phenotype has been interpreted as a defect in termination efficiency, since the product of *UPF1* has been shown to interact with the termination factors eRF1 and eRF3.⁴⁰ To determine the possible contribution of an elongation fidelity defect to the readthrough phenotype resulting from a loss of *UPF1* expression, we monitored both readthrough and misincorporation in a *upf1Δ* strain. As previously described,⁴ we observed a 2.5-fold increase in readthrough at a UGAC termination signal in a *upf1Δ* strain relative to a wild-type strain (Figure 6(a)). In contrast, we did not observe any change in elongation fidelity using the Arg245 (CGC) misincorporation reporter (Figure 6(b)). These results suggest that the readthrough phenotype associated with the loss of Upf1p is specifically caused by a termination defect,

Table 5. Effect of the S9-D94N mutation on readthrough of termination codons

Stop signal	Percentage readthrough ^a		Fold change
	S9-WT	S9-D94N	
UAAA	0.09 ± 0.01	0.97 ± 0.03	10.8
UAAC	0.21 ± 0.04	1.6 ± 0.14	7.6
UAGA	0.08 ± 0.01	0.51 ± 0.1	6.4
UAGC	0.19 ± 0.006	1.6 ± 0.18	8.4
UGAA	0.17 ± 0.02	1.0 ± 0.1	5.9
UGAC	0.44 ± 0.06	3.0 ± 0.28	6.8

^a Percentage readthrough is expressed as mean ± standard deviation.

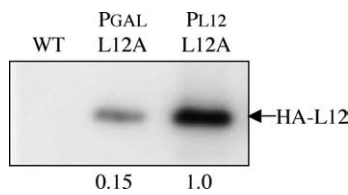


Figure 5. Steady-state levels of HA-tagged L12A are reduced in cells grown in media containing glucose as carbon source when the *rpL12A* gene is expressed from a *GAL10* promoter in a *rpL12AΔ/rpL12BΔ* strain. Western blot showing expression levels of HA-tagged L12A expressed from either the *GAL10* promoter (PGAL) or the *rpL12A* promoter (PL12) from a centromeric plasmid in a *rpL12AΔ/rpL12BΔ* strain. The strains were grown for four generations in glucose minimal media to inhibit expression from the *GAL10* promoter. No band corresponding to HA-tagged L12 was observed in a strain carrying a genomic copy of non-HA-tagged L12 (WT). Twenty-five microgram of total protein was loaded in each lane.

and is not associated with a defect in elongation fidelity.

Discussion

Some misreading events are favored over others during translation elongation

The primary objective of this study was to develop an *in vivo* reporter system that would allow us to determine the extent that defects in elongation fidelity contribute to the suppression of translation termination signals in the eukaryote *S. cerevisiae*. However, while developing this system we also made interesting observations about the tendency of different codons to undergo near-cognate mispairing in this organism. We found that 200 $\mu\text{g}/\text{ml}$ of paromomycin stimulated firefly luciferase activity more than twofold at only five of the 15 near-cognate missense mutations tested. While firefly luciferase activity was stimulated

12.5-fold at the Arg245 (CGC) codon, the other four near-cognate codons that showed the highest level of elongation fidelity errors were only 2.2-fold to 4.4-fold above normal. We observed a less than twofold stimulation of luciferase activity at the other ten near-cognate codons, indicating that misincorporation was surprisingly rare. These results suggest that fidelity errors induced by paromomycin during translation elongation do not occur equally at all sense codons.

We found that paromomycin generally induced a larger increase in our readthrough reporters than we observed using the misincorporation reporters. This probably results from the fact that the insertion of any amino acid at a nonsense codon will allow the translation of a fully active firefly luciferase protein to proceed, while only a subset of the potential amino acid residues inserted in the missense misincorporation assay will allow the restoration of complete (or even partial) firefly luciferase activity. While the lack of response from some of the misincorporation reporters may simply be due to the misincorporation of a subset of amino acid residues by near-cognate tRNAs that do not restore enzymatic activity, these results suggest that paromomycin may induce errors much more selectively during translation elongation than during translation termination. This observation could have important implications for therapeutic strategies that propose to use aminoglycosides to suppress premature stop mutations that cause genetic diseases,^{41–48} since fidelity errors during translation elongation would be considered an undesirable side-effect.

The largest paromomycin-induced increase in firefly luciferase activity observed in the current study was the 12.5-fold stimulation at the Arg245 (CGC) near-cognate codon. A misreading event that incorporates the wild-type histidine residue at this codon must be mediated by an interaction with a near-cognate histidinyI-tRNA^{His} carrying a GUG anticodon, since that anticodon is present in the histidinyI-tRNAs encoded by all seven tRNA^{His} genes in yeast.⁴⁹ However, the mere availability of a near-cognate histidinyI-tRNA^{His} is not sufficient to

Table 6. Effect of L12 depletion on misreading during translation elongation

Firefly mutation ^a	Percentage misreading ^b		Fold change ^c
	PL12-L12A	PGAL-L12A	
245 CAC	100	100	–
245 AAC	9.8 \pm 0.8	10.9 \pm 0.6	1.1
245 <u>G</u> AC	0.03 \pm 0.002	0.025 \pm 0.003	0.8
245 <u>U</u> AC	2.0 \pm 0.3	1.8 \pm 0.13	0.9
245 <u>C</u> CC	0.74 \pm 0.14	0.96 \pm 0.06	1.3
245 <u>C</u> GC	0.3 \pm 0.05	0.65 \pm 0.1	<u>2.2</u>
245 <u>C</u> UC	0.27 \pm 0.04	0.3 \pm 0.02	1.1
245 <u>C</u> AA	0.27 \pm 0.05	0.23 \pm 0.03	0.85
245 <u>C</u> AG	0.27 \pm 0.02	0.25 \pm 0.02	0.9

^a Mutated nucleotides are underlined.

^b Percentage misreading is expressed as mean \pm standard deviation.

^c Changes \geq 2-fold that yielded a statistically significant *P*-value (<0.05) using the Mann–Whitney test are underlined.

Table 7. Effect of L12 depletion on readthrough during translation termination

Stop signal	Percentage readthrough ^a		Fold change
	PL12-L12A	PGAL-L12A	
UAAA	0.21 ± 0.04	1.1 ± 0.13	5.2
UAAC	0.45 ± 0.01	2.3 ± 0.21	5.1
UAGA	0.21 ± 0.03	0.93 ± 0.19	4.4
UAGC	0.34 ± 0.06	1.1 ± 0.09	3.2
UGAA	0.45 ± 0.12	2.6 ± 0.21	5.8
UGAC	1.3 ± 0.18	6.0 ± 1.1	4.6

^a Percentage readthrough is expressed as mean ± standard deviation.

accomplish the incorporation of this amino acid, since a paromomycin-dependent stimulation of firefly luciferase activity was not observed for most of the other near-cognate mutants at position 245 described in Table 1. This indicates that other considerations must also play an important role in the misreading process, such as the relative concentrations of near-cognate *versus* cognate tRNAs, and differences in the tendency of various mismatches to result in the misincorporation event. The mismatch between a CGC codon and a GUG anticodon that resulted in the largest (12.5-fold) increase in misreading in the presence of paromomycin involves a G·U interaction at the second position. However, the G·U mismatch alone is not sufficient to account for this level of misreading, since other mutants that were completely insensitive to paromomycin-induced misreading could have utilized the same mismatch for an activity-restoring misincorporation event (for example, the His245 (UAC) firefly luciferase mutant could bind a histidinyl-tRNA^{His} carrying the GUG anticodon through a G·U interaction at the first position). Another potential factor in the high error rate at the Arg245 (CGC) near-cognate codon is the fact that the cognate arginyl-tRNA^{Arg} for the CGC codon is relatively rare in yeast.⁴⁹ However, other firefly luciferase mutants with a near-cognate codon

recognized by a rare cognate tRNA were unresponsive to paromomycin (for example, the Leu245 (CUC) firefly luciferase mutant codon is recognized by a rare leucyl-tRNA^{Leu}). These results suggest that the high level of misincorporation observed with the Arg245 (CGC) near-cognate codon occurs because several factors contribute simultaneously to a reduction in elongation fidelity.

A broad level of amino acid tolerance at the mutated codon may provide a better estimate of misincorporation during elongation

The requirement for a Lys at position 529 for firefly luciferase activity appears to be quite strict, since the basal activity was ≤0.05% of wild-type activity for seven different near-cognate codons (encoding six different amino acid residues). This stringent requirement could explain why most of the near-cognate codons at position 529 were unresponsive to paromomycin, since most misincorporation events would not provide a positive signal. In contrast, Branchini *et al.* reported that several amino acid substitutions for His245 of firefly luciferase retain partial activity.^{15–17} Our results showing that some near-cognate mutations provide basal levels of firefly luciferase activity as high as 7.7% of wild-type (see Table 2) are consistent with

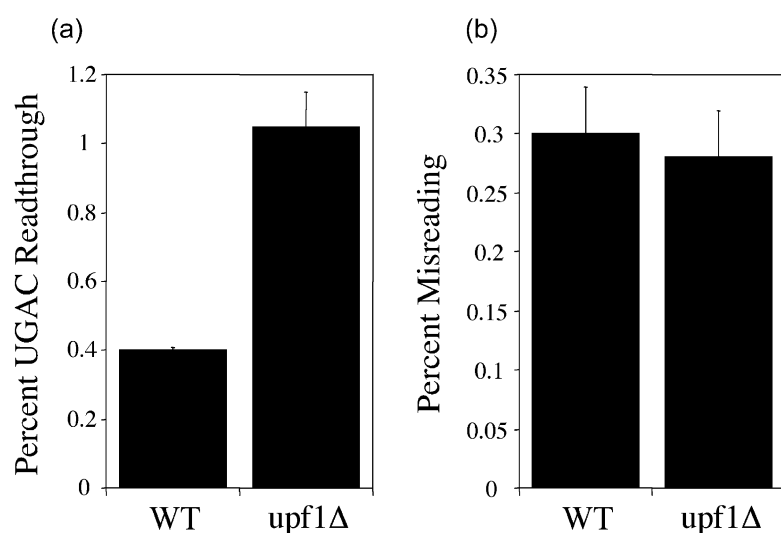


Figure 6. Effects of disrupting the *UPF1* gene (*upf1Δ*) on (a) suppression of a UGAC stop signal and (b) misreading during translation elongation.

that conclusion. In the context of this observation, it is striking that four of the five near-cognate codons that yielded the largest stimulation in the presence of paromomycin were at position 245. This included the Arg245 (CGC) codon, which showed the largest stimulation observed in our study. While the misincorporation of the wild-type amino acid (in this case, His) should provide the largest increase in activity, the ability of more than one near-cognate aminoacyl-tRNA to yield a significant increase in firefly luciferase activity may provide an additive increase in activity. In cases where the misincorporation of the wild-type amino acid is infrequent (as appears to be the case for most of the position 529 mutants), such an additive mechanism could provide a better indication of fidelity errors than a reporter enzyme that regained activity only when the original wild-type amino acid is incorporated. The only limitation to such a mechanism is that the amino acid encoded by the starting near-cognate codon under basal conditions must provide less enzymatic activity than a number of other misincorporated residues so an increase in activity can be observed. In fact, the ideal elongation misincorporation reporter system would contain a single amino acid change that inactivated the enzyme, while all other misincorporated amino acid residues at that position would completely restore enzymatic activity. In such a situation, a positive signal would be obtained for every misincorporation event.

A previous study by Stansfield *et al.*³⁴ also used a near-cognate approach to develop an *in vivo* misincorporation reporter in *S. cerevisiae*. A gene encoding a type III chloramphenicol acetyl transferase (CAT_{III}) enzyme with a mutation at the His195 (CAC) codon was used as the reporter enzyme. They found that the Tyr195 (UAC) mutation greatly inhibited CAT_{III} enzymatic activity, and the CAT_{III} activity of that mutant protein could be stimulated 50-fold by paromomycin. One of the firefly luciferase position 245 mutants characterized in the present study carried an identical CAC to UAC mutation, but we did not observe any stimulation of firefly luciferase activity of this mutant with paromomycin. While His195 is thought to be extremely important for CAT_{III} enzyme activity, at least one other amino acid (glutamic acid) was shown to provide some residual activity.⁵⁰ Since the His245 position of firefly luciferase is relatively tolerant to substitution (as discussed above), it would be reasonable to expect that a number of misincorporation events should be capable of restoring partial activity to the corresponding firefly luciferase His245 (UAC) mutant. However, we did not observe any increase in firefly luciferase activity in the presence of paromomycin. This striking disparity between these two reporter systems may be attributable to differences in the ability of specific amino acid residues encoded by near-cognate tRNAs to provide partial activity in these two reporter systems. Alternatively, it could be caused by differences in the sequence surrounding the

histidine codon that influence the efficiency of misreading or to other unknown differences in the genetic background of the strains.

Paromomycin, L12 depletion, and the S9B-D94N mutation induce misincorporation with similar codon specificities

As discussed above, our results indicate that paromomycin causes a partial restoration of luciferase activity at only a subset of the position 245 and position 529 firefly luciferase mutants. The highest stimulation is observed at the Arg245 (CGC) mutant, followed by the Gln245 (CAG), Asn529 (AAU), Asp245 (GAC), and Leu245 (CUC) mutants (Table 2). A very low frequency of fidelity errors was detected at the other ten near-cognate missense mutations examined. It is possible that these differences in the level of misincorporation at different codons are caused by an intrinsic property of the codon itself, or by some aspect of the mechanism by which paromomycin induces misreading. We attempted to distinguish between these possibilities by determining the pattern of firefly luciferase activity induced by conditions that reduced elongation fidelity other than paromomycin. In a strain carrying the S9B-D94N allele, we found that the greatest restoration of firefly luciferase activity was observed at the Arg245 (CGC) mutant, followed by the Asp245 (GAC) and Gln245 (CAG) mutants (Table 4). This indicates that the near-cognate codon pattern for misincorporation associated with this mutant r-protein was quite similar to the pattern observed in the presence of paromomycin. While the level of misincorporation that occurred upon L12 depletion was much lower, the largest increase in luciferase activity also occurred with the Arg245 (CGC) mutant. Together, these results suggest that the codon bias observed in these experiments is due to a common property of the decoding machinery that recognizes these specific codons, rather than unique factors associated with each condition. As discussed above, these common properties could include the relative codon affinity for cognate *versus* near-cognate tRNAs, or the relative abundance of cognate *versus* near-cognate tRNAs.

The relative effects on readthrough and misincorporation differ as a function of the mechanistic defect

Aminoacyl-tRNAs enter the A site of the ribosome during translation elongation in a ternary complex with EF-Tu and GTP (or eEF1A and GTP in eukaryotes). A cognate codon-anticodon interaction stimulates rapid GTP hydrolysis by EF-Tu, while a near-cognate codon-anticodon interaction stimulates GTP hydrolysis at a much slower rate. Consequently, near-cognate aminoacyl-tRNAs dissociate with high frequency before GTPase activation. If tRNA dissociation does not occur, GTP hydrolysis is followed by the departure of EF-Tu

and GDP, which allows the tRNA to undergo a conformational change (accommodation) that gives the aminoacyl-tRNA one last chance to dissociate before peptide bond formation occurs.^{8,51,52} Therefore, accuracy is achieved with two potential dissociation steps separated by irreversible GTP hydrolysis.

It has been proposed that a codon interaction with its cognate tRNA also induces the 30 S subunit of the *E. coli* ribosome to undergo a conformational change from an open to a closed form (the productive state).^{53,54} The transition to this closed form is believed to accelerate both the rate of GTPase activation of EF-Tu and tRNA accommodation. The 30 S subunit remains in an open form when the A site is empty or contains a near-cognate tRNA. Paromomycin has been shown to stimulate the formation of the closed form even in the presence of near-cognate tRNA in the A site; hence, paromomycin may increase misincorporation by allowing a near-cognate tRNA to be recognized as a cognate tRNA. Transition of the 30 S subunit conformation to the closed form involves the disruption of various interactions at the interface between r-proteins S4 and S5 in the *E. coli* ribosome.²⁸ In addition, mutations that confer the ribosomal ambiguity (Ram) phenotype to *E. coli* ribosomes, characterized by a reduced level of elongation fidelity, map to the S4/S5 interface. It has been hypothesized that changes in the S4/S5 interaction in Ram mutants facilitates the transition of the 30 S subunit to the closed form, causing a reduction in the accuracy of translation elongation. The yeast r-protein S9B shares significant homology (28% identity and 37% similarity) with r-protein S4 from *E. coli*, suggesting that these proteins may be functional homologues. Consistent with this possibility, both the current work and a prior study³⁴ found that S9B mutations reduce elongation fidelity in yeast. Based on its homology with r-protein S4 of *E. coli*, it is possible that the S9B-D94N mutation analyzed in our study also favors the formation of a closed state in the 40 S subunit of the yeast ribosome.

The yeast r-protein L12 is the homologue of r-protein L11 in *E. coli*. L12 binds to a double hairpin in domain II of the 25 S rRNA of yeast known as the GTPase-activating center (GAC),^{35,36} and is required to maintain the structural integrity of the GTPase domain within the 60 S subunit of the

eukaryotic ribosome.³⁹ In this study we observed a small, but significant, increase in misincorporation using the Arg245 (CGC) reporter as a result of L12 depletion (Table 6). This change in elongation fidelity is consistent with previous structural work showing that the GTPase domain of the ribosome plays a role in both decoding⁵⁵ and translocation⁵⁶ during the eukaryotic translation elongation process. Furthermore, *in vitro* studies have directly implicated the GTPase domain of the bacterial ribosome in the control of elongation fidelity⁵⁷ and in the stimulation of the GTPase activity of the elongation factors EF-Tu and EF-G.⁵⁸ The *in vivo* data presented here, together with prior structural and *in vitro* data, suggest that this region of the eukaryotic ribosome plays a role in elongation fidelity. It is likely that L12 depletion decreases fidelity by limiting the efficiency of GTPase activation of eEF1A (the eukaryotic homolog of EF-Tu). The termination factor eRF3 also possesses GTPase activity and has significant homology to eEF1A.^{59,60} It was also recently shown that the GTPase activity of eRF3 plays an important role in the termination process *in vivo*.²⁷ This suggests that L12 depletion may also reduce the ability of the ribosome to activate the GTPase activity of eRF3.

We have shown that exposure to paromomycin, depletion of r-protein L12 and expression of the S9B-D94N mutant r-protein are each capable of decreasing elongation fidelity in a similar codon-specific pattern. In Table 8, we have summarized these data by comparing the relative effects on readthrough and misincorporation. For comparative purposes, we included the results obtained with the UGAC readthrough reporter and the Arg245 (CGC) misincorporation reporter for each condition. In addition, by calculating the ratio of the change in readthrough *versus* misincorporation we were able to compare the relative effect of each condition on these two processes. We found that the increase in readthrough caused by paromomycin or L12 depletion surpassed the increase in misincorporation measured with the Arg245 (CGC) mutant by 1.6-fold and 2.1-fold, respectively, while the effects of expressing the S9B-D94N protein on readthrough and misincorporation were similar. These results suggest that paromomycin and L12 depletion may both have a more severe affect on termination efficiency than the S9B-D94N mutation. By analogy with mechanisms proposed for the

Table 8. Relative effects on readthrough and fidelity

Translation condition	Fold increase		Ratio: readthrough/ misincorporation
	Readthrough ^a	Misincorporation ^b	
WT	1.0	1.0	1.0
Paromomycin	20.4	12.5	1.6
L12 Depletion	4.6	2.2	2.1
S9-D94N	6.8	7.3	0.9

^a Readthrough at the UGAC termination signal.

^b Percentage misreading of the Arg245 (CGC) mutation.

E. coli ribosome, both paromomycin and S9B mutants may stimulate misreading during translation elongation by predisposing the 40 S ribosomal subunit to undergo a transition between the open and the closed conformations.²⁸ To our knowledge, the possible role that this conformational change plays during translation termination has not previously been addressed. Our results suggest that expression of the S9B-D94N protein only stimulates misreading during the elongation process, and does not have a direct effect on termination. In contrast, our results suggest that the readthrough phenotype associated with both L12 depletion and paromomycin results from not only a decrease in elongation fidelity, but also an additional effect on termination efficiency. Further studies will be required to test the validity of this proposal.

The readthrough phenotype observed in a *upf1Δ* strain is caused by a decrease in the efficiency of translation termination

The Upf proteins are the major effectors of the nonsense-mediated mRNA decay (NMD) pathway. It has been suggested that Upf1p links recognition of a nonsense mutation with the degradation of the mRNA molecule carrying that mutation. Consistent with this hypothesis, Upf1p has been shown to interact with the translation termination factors eRF1 and eRF3. Previous work demonstrated that Upf1p influences the translation termination process, since some mutations in the *UPF1* gene cause a readthrough phenotype.^{4,40,61} This readthrough phenotype could be caused by a direct effect on termination efficiency or a more general decrease in elongation fidelity. Our results indicate that the absence of Upf1p causes an increase in readthrough without affecting the level of misincorporation. This observation suggests that Upf1p plays a role in translation termination, but not translation elongation. While it is not clear how Upf1 enhances the efficiency of termination, it could function in the stabilization of the termination complex through its known interactions with eRF1 and eRF3.

Materials and Methods

Strains and growth conditions

The *S. cerevisiae* strains used in this study were 614 (*MATa leu2-3, 112 his3-11, 15 trp1-1 ura3-1 ade1-14 [psi⁻]*), YDB498 (*MATa leu2-3, 112 his3-11, 15 trp1-1 ura3-1 ade1-14 sup35::HIS3 [psi⁻]*), YDB510 (*MAT α leu2-3, 112 his3-11, 15 trp1-1 ura3-1 ade1-14 [psi⁻]*), YDB629 (*MATa leu2-3, 112 his3-11, 15 trp1-1 ura3-1 ade1-14 rpL12A::HIS3 rpL12B::LEU2 [psi⁻]*), YDB415 (*MATa leu2-3, 112 his3-11, 15 trp1-1 ura3-1 ade1-14 upf1::TRP1 [psi⁻]*), BY4741 (*MATa his3 Δ 1, leu2 Δ 0, met15 Δ 0, ura3 Δ 0 [psi⁻]*), 6961 (*MATa his3 Δ 1, leu2 Δ 0, met15 Δ 0, ura3 Δ 0, rpS9B::HIS3 [psi⁻]*). Strains YDB498, YDB629, and YDB415 were derived from 614 using standard yeast genetic techniques. YDB510 is isogenic to 614. Finally, strain BY4741 (wild-type) and the

isogenic rpS9B Δ strain 6961 were purchased from Open Biosystems.

Plasmids

Mutations in firefly luciferase were introduced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and the plasmid p2luc⁵ was used as a template for PCR. A BstEII/NotI fragment from p2luc carrying each mutation was used to replace the corresponding BstEII/NotI fragment from pDB688 (CTY775/luc CAAA).⁴ This resulted in the series of mutants described in Table 1. For the over-expression of histidine tRNAs, the tRNA gene [*tH(GUG)E1*] was PCR-amplified from yeast genomic DNA and cloned in the XbaI and KpnI sites of the high copy vector YEplac181. For the expression of HA-tagged L12A from its endogenous promoter, the *rpL12A* gene was PCR-amplified from yeast genomic DNA while adding XbaI sites at both ends and an N terminal HA tag. It was then subcloned into the XbaI site of the centromeric plasmid vector YCplac22. The *rpL12A* promoter was PCR-amplified from yeast genomic DNA with terminal BamHI and KpnI sites and subcloned into *HA-rpL12A/YCplac22*. For expression of HA-tagged L12A from a *GAL10* promoter, a Sall/BamHI fragment from *HA-L12A/YCplac22* carrying the intact gene was subcloned in *pGAL10/YCplac22*. The plasmid *rpL12A/pYES2* was made by subcloning a BamHI/SphI fragment from *HA-rpL12A/YCplac22* into pYES2. This plasmid was then used to maintain r-protein L12 expression while disrupting the *rpL12A rpL12B* genes. For the disruption of *rpL12B*, primers were used to amplify a region comprising approximately 500 nucleotides downstream of the *rpL12B* gene while adding KpnI sites from yeast genomic DNA. The amplified fragment was subcloned into YCplac22, making 3' L12B/YCplac22. The *LEU2* gene from *Candida glabrata* was PCR-amplified while adding BamHI and SmaI sites and subcloned into 3' L12B/YCplac22 upstream of the *rpL12B* region, making LEU2-3' L12B/YCplac22. Finally, a fragment containing approximately 500 nucleotides of DNA upstream of *rpL12B* was PCR-amplified while adding PstI and BamHI sites, and this DNA fragment was subcloned in LEU2/3' L12B/YCplac22 upstream of the *LEU2* gene, resulting in pDB852. The *rpS9B* gene, together with its promoter and transcriptional terminator, was PCR-amplified directly from genomic DNA while adding BamHI and XbaI sites and subcloned into pRS315. The mutation D94N (GAT to AAT) was introduced into in *rpS9B* using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and *rpS9B/pRS315* as a template for PCR. Plasmids pDB663 and pDB670 for the expression of eRF3-WT and eRF3-H348Q, respectively, were generated as described.²⁷

Dual luciferase assays

The dual luciferase reporter plasmids used to measure the efficiency of translation termination and the fidelity of elongation in yeast were adapted from plasmids previously used to monitor the efficiency of translation termination in mammalian cells^{5,6} as recently described.⁴ Dual luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega). Briefly, yeast strains were transformed with the indicated dual luciferase reporter plasmids. Approximately 10⁴ cells from each strain were assayed for luminescence using a Berthold Lumat LB9507 luminometer. Assays were done in quadruplicate, and the data are expressed as the mean \pm SD. Percentage readthrough in each strain

was expressed as the firefly/*Renilla* luciferase activity (nonsense) divided by the firefly/*Renilla* luciferase activity (sense)×100. For further details, see Keeling *et al.*⁴ The percentage of elongation misincorporation was expressed as mutant firefly/*Renilla* luciferase activity as a percentage of wild-type firefly/*Renilla* luciferase activity.

Gene disruptions

Yeast strain YDB498 was generated as described.²⁷ To generate the *rpL12AΔ*/*rpL12BΔ* strain, *rpL12AΔ* was disrupted in a 614×YDB510 diploid by removal of the entire ORF and the insertion of the *HIS3* gene from *C. glabrata* using a PCR-based gene deletion approach⁶² with plasmid pH4 as template.⁶³ This procedure resulted in strain YDB522. The correct genomic integration event in His⁺ transformants was verified by PCR and Southern blot analysis. For the disruption of *rpL12B*, a DNA fragment comprising 500 nucleotides of DNA homologous to the genome immediately upstream of *rpL12B* followed by the *LEU2* gene from *C. glabrata* and by 500 nucleotides of DNA homologous to DNA immediately downstream of *rpL12B* was PCR-amplified using pDB852 as a template. The disruption fragment generated was transformed in YDB522, and Leu⁺ transformants were screened for the correct integration event by PCR and Southern blot. The double KO strain was transformed with *rpL12A*/pYES2 and induced to sporulate. A *rpL12AΔ*/*rpL12BΔ* haploid strain was verified by PCR and Southern blot analysis in Leu⁺, His⁺, Ura⁺ spores. We found that Leu⁺, His⁺, Ura-spores (lacking the *rpL12A*/pYES2 plasmid) grew very slowly, consistent with the previously reported slow growth phenotype associate with a L12 *rpL12A* *rpL12B* double KO. Yeast strain YDB415 was generated as described.⁴

Preparation of cell lysates for Western blot analysis

After culturing each strain for several generations, cell growth was terminated by the addition of 10% trichloroacetic acid. After incubating the acid precipitates for 30 minutes on ice, the cells were collected by a brief centrifugation in a microfuge. The cells were washed with ice-cold acetone, dried, re-suspended in lysis buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1% (w/v) SDS) and lysed by mechanical agitation with glass beads. The samples were then boiled, cleared by a brief centrifugation in a microfuge, and the supernatant was subjected to SDS-PAGE and Western blotting.

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References

1. Firoozan, M., Grant, C. M., Duarte, J. A. & Tuite, M. F. (1991). Quantitation of readthrough of termination codons in yeast using a novel gene fusion assay. *Yeast*, **7**, 173–183.
2. Fearon, K., McClendon, V., Bonetti, B. & Bedwell, D. M. (1994). Premature translation termination mutations are efficiently suppressed in a highly conserved region of yeast Ste6p, a member of the ATP-binding cassette (ABC) transporter family. *J. Biol. Chem.* **269**, 17802–17808.
3. Bonetti, B., Fu, L., Moon, J. & Bedwell, D. M. (1995). The efficiency of translation termination is determined by a synergistic interplay between upstream and downstream sequences in *Saccharomyces cerevisiae*. *J. Mol. Biol.* **251**, 334–345.
4. Keeling, K. M., Lanier, J., Du, M., Salas-Marco, J., Gao, L., Kaenjak-Angeletti, A. & Bedwell, D. M. (2004). Leaky termination at premature stop codons antagonizes nonsense-mediated mRNA decay in *S. cerevisiae*. *RNA*, **10**, 691–703.
5. Grentzmann, G., Ingram, J. A., Kelly, P. J., Gesteland, R. F. & Atkins, J. F. (1998). A dual-luciferase reporter system for studying recoding signals. *RNA*, **4**, 479–486.
6. Howard, M. T., Shirts, B. H., Petros, L. M., Flanigan, K. M., Gesteland, R. F. & Atkins, J. F. (2000). Sequence specificity of aminoglycoside-induced stop codon readthrough: potential implications for treatment of Duchenne muscular dystrophy. *Ann. Neurol.* **48**, 164–169.
7. Bidou, L., Hatin, I., Perez, N., Allamand, V., Panthier, J. J. & Rousset, J. P. (2004). Premature stop codons involved in muscular dystrophies show a broad spectrum of readthrough efficiencies in response to gentamicin treatment. *Gene Ther.* **11**, 619–627.
8. Rodnina, M. V. & Wintermeyer, W. (2001). Fidelity of aminoacyl-tRNA selection on the ribosome: kinetic and structural mechanisms. *Annu. Rev. Biochem.* **70**, 415–435.
9. Hall, B. & Gallant, J. (1972). Defective translation in RC-cells. *Nature New Biol.* **237**, 131–135.
10. Branscomb, E. W. & Galas, D. J. (1975). Progressive decrease in protein synthesis accuracy induced by streptomycin in *Escherichia coli*. *Nature*, **254**, 161–163.
11. Hughes, D. (1991). Error-prone EF-Tu reduces *in vivo* enzyme activity and cellular growth rate. *Mol. Microbiol.* **5**, 623–630.
12. Toth, M. J., Murgola, E. J. & Schimmel, P. (1988). Evidence for a unique first position codon-anticodon mismatch *in vivo*. *J. Mol. Biol.* **201**, 451–454.
13. Cornut, B. & Willson, R. C. (1991). Measurement of translational accuracy *in vivo*: missense reporting using inactive enzyme mutants. *Biochimie*, **73**, 1567–1572.
14. Viviani, V., Uchida, A., Suenaga, N., Ryufuku, M. & Ohmiya, Y. (2001). Thr226 is a key residue for bioluminescence spectra determination in beetle luciferases. *Biochem. Biophys. Res. Commun.* **280**, 1286–1291.
15. Branchini, B. R., Magyar, R. A., Murtiashaw, M. H., Anderson, S. M. & Zimmer, M. (1998). Site-directed mutagenesis of histidine 245 in firefly luciferase: a proposed model of the active site. *Biochemistry*, **37**, 15311–15319.
16. Branchini, B. R., Magyar, R. A., Murtiashaw, M. H., Anderson, S. M., Helgerson, L. C. & Zimmer, M. (1999). Site-directed mutagenesis of firefly luciferase active site amino acids: a proposed model for bioluminescence color. *Biochemistry*, **38**, 13223–13230.
17. Branchini, B. R., Murtiashaw, M. H., Magyar, R. A. & Anderson, S. M. (2000). The role of lysine 529, a conserved residue of the acyl-adenylate-forming enzyme superfamily, in firefly luciferase. *Biochemistry*, **39**, 5433–5440.

18. Branchini, B. R., Magyar, R. A., Murtiashaw, M. H. & Portier, N. C. (2001). The role of active site residue arginine 218 in firefly luciferase bioluminescence. *Biochemistry*, **40**, 2410–2418.
19. Ugarova, N. N. & Brovko, L. Y. (2002). Protein structure and bioluminescent spectra for firefly bioluminescence. *Luminescence*, **17**, 321–330.
20. Hirokawa, K., Kajiyama, N. & Murakami, S. (2002). Improved practical usefulness of firefly luciferase by gene chimerization and random mutagenesis. *Biochim. Biophys. Acta*, **1597**, 271–279.
21. Ohmiya, Y. & Tsuji, F. I. (1997). Mutagenesis of firefly luciferase shows that cysteine residues are not required for bioluminescence activity. *FEBS Letters*, **404**, 115–117.
22. Davies, J., Gilbert, W. & Gorini, L. (1964). Streptomycin, suppression, and the code. *Proc. Natl Acad. Sci. USA*, **51**, 883–890.
23. Palmer, E., Wilhelm, J. M. & Sherman, F. (1979). Phenotypic suppression of nonsense mutants in yeast by aminoglycoside antibiotics. *Nature*, **277**, 148–150.
24. Singh, A., Ursic, D. & Davies, J. (1979). Phenotypic suppression and misreading *Saccharomyces cerevisiae*. *Nature*, **277**, 146–148.
25. Brown, C. M., Stockwell, P. A., Trotman, C. N. & Tate, W. P. (1990). Sequence analysis suggests that tetranucleotides signal the termination of protein synthesis in eukaryotes. *Nucl. Acids Res.* **18**, 6339–6345.
26. McCaughan, K. K., Brown, C. M., Dalphin, M. E., Berry, M. J. & Tate, W. P. (1995). Translational termination efficiency in mammals is influenced by the base following the stop codon. *Proc. Natl Acad. Sci. USA*, **92**, 5431–5435.
27. Salas-Marco, J. & Bedwell, D. M. (2004). GTP hydrolysis by eRF3 facilitates stop codon decoding during eukaryotic translation termination. *Mol. Cell Biol.* **24**, 7769–7778.
28. Ogle, J. M., Carter, A. P. & Ramakrishnan, V. (2003). Insights into the decoding mechanism from recent ribosome structures. *Trends Biochem. Sci.* **28**, 259–266.
29. Bertram, G., Bell, H. A., Ritchie, D. W., Fullerton, G. & Stansfield, I. (2000). Terminating eukaryote translation: domain 1 of release factor eRF1 functions in stop codon recognition. *RNA*, **6**, 1236–1247.
30. Eurwilaichitr, L., Graves, F. M., Stansfield, I. & Tuite, M. F. (1999). The C-terminus of eRF1 defines a functionally important domain for translation termination in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **32**, 485–496.
31. Carr-Schmid, A., Durko, N., Cavallius, J., Merrick, W. C. & Kinzy, T. G. (1999). Mutations in a GTP-binding motif of eukaryotic elongation factor 1A reduce both translational fidelity and the requirement for nucleotide exchange. *J. Biol. Chem.* **274**, 30297–30302.
32. Ono, B. I., Stewart, J. W. & Sherman, F. (1981). Serine insertion caused by the ribosomal suppressor SUP46 in yeast. *J. Mol. Biol.* **147**, 373–379.
33. Vincent, A. & Liebman, S. W. (1992). The yeast omnipotent suppressor SUP46 encodes a ribosomal protein which is a functional and structural homolog of the *Escherichia coli* S4 ram protein. *Genetics*, **132**, 375–386.
34. Stansfield, I., Jones, K. M., Herbert, P., Lewendon, A., Shaw, W. V. & Tuite, M. F. (1998). Missense translation errors in *Saccharomyces cerevisiae*. *J. Mol. Biol.* **282**, 13–24.
35. Schmidt, F. J., Thompson, J., Lee, K., Dijk, J. & Cundliffe, E. (1981). The binding site for ribosomal protein L11 within 23 S ribosomal RNA of *Escherichia coli*. *J. Biol. Chem.* **256**, 12301–12305.
36. Egebjerg, J., Douthwaite, S. R., Liljas, A. & Garrett, R. A. (1990). Characterization of the binding sites of protein L11 and the L10.(L12)₄ pentameric complex in the GTPase domain of 23 S ribosomal RNA from *Escherichia coli*. *J. Mol. Biol.* **213**, 275–288.
37. Wilson, K. S. & Noller, H. F. (1998). Molecular movement inside the translational engine. *Cell*, **92**, 337–349.
38. Uchiumi, T. & Kominami, R. (1997). Binding of mammalian ribosomal protein complex P0.P1.P2 and protein L12 to the GTPase-associated domain of 28 S ribosomal RNA and effect on the accessibility to anti-28 S RNA autoantibody. *J. Biol. Chem.* **272**, 3302–3308.
39. Briones, E., Briones, C., Remacha, M. & Ballesta, J. P. (1998). The GTPase center protein L12 is required for correct ribosomal stalk assembly but not for *Saccharomyces cerevisiae* viability. *J. Biol. Chem.* **273**, 31956–31961.
40. Wang, W., Czaplinski, K., Rao, Y. & Peltz, S. W. (2001). The role of Upf proteins in modulating the translation read-through of nonsense-containing transcripts. *EMBO J.* **20**, 880–890.
41. Howard, M., Frizzell, R. A. & Bedwell, D. M. (1996). Aminoglycoside antibiotics restore CFTR function by overcoming premature stop mutations. *Nature Med.* **2**, 467–469.
42. Bedwell, D. M., Kaenjak, A., Benos, D. J., Bebok, Z., Bubien, J. K., Hong, J. *et al.* (1997). Suppression of a CFTR premature stop mutation in a bronchial epithelial cell line. *Nature Med.* **3**, 1280–1284.
43. Keeling, K. M., Brooks, D. A., Hopwood, J. J., Li, P., Thompson, J. N. & Bedwell, D. M. (2001). Gentamicin-mediated suppression of Hurler syndrome stop mutations restores a low level of alpha-L-iduronidase activity and reduces lysosomal glycosaminoglycan accumulation. *Hum. Mol. Genet.* **10**, 291–299.
44. Keeling, K. M. & Bedwell, D. M. (2002). Clinically relevant aminoglycosides can suppress disease-associated premature stop mutations in the IDUA and P53 cDNAs in a mammalian translation system. *J. Mol. Med.* **80**, 367–376.
45. Clancy, J. P., Bebok, Z., Ruiz, F., King, C., Jones, J., Walker, L. *et al.* (2001). Evidence that systemic gentamicin suppresses premature stop mutations in patients with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* **163**, 1683–1692.
46. Wilson, W. A. & Roach, P. J. (2002). Nutrient-regulated protein kinases in budding yeast. *Cell*, **111**, 155–158.
47. Wilschanski, M., Yahav, Y., Yaacov, Y., Blau, H., Bentur, L., Rivlin, J. *et al.* (2003). Gentamicin-induced correction of CFTR function in patients with cystic fibrosis and CFTR stop mutations. *New Engl. J. Med.* **349**, 1433–1441.
48. Wilschanski, M., Famini, C., Blau, H., Rivlin, J., Augarten, A., Avital, A. *et al.* (2000). A pilot study of the effect of gentamicin on nasal potential difference measurements in cystic fibrosis patients carrying stop mutations. *Am. J. Respir. Crit. Care Med.* **161**, 860–865.
49. Percudani, R., Pavesi, A. & Ottonello, S. (1997). Transfer RNA gene redundancy and translational selection in *Saccharomyces cerevisiae*. *J. Mol. Biol.* **268**, 322–330.
50. Lewendon, A., Murray, I. A., Shaw, W. V., Gibbs, M. R. & Leslie, A. G. (1994). Replacement of catalytic

- histidine-195 of chloramphenicol acetyltransferase: evidence for a general base role for glutamate. *Biochemistry*, **33**, 1944–1950.
51. Yarus, M., Valle, M. & Frank, J. (2003). A twisted tRNA intermediate sets the threshold for decoding. *RNA*, **9**, 384–385.
52. Valle, M., Sengupta, J., Swami, N. K., Grassucci, R. A., Burkhardt, N. *et al.* (2002). Cryo-EM reveals an active role for aminoacyl-tRNA in the accommodation process. *EMBO J.* **21**, 3557–3567.
53. Ogle, J. M., Brodersen, D. E., Clemons, W. M., Jr, Tarry, M. J., Carter, A. P. & Ramakrishnan, V. (2001). Recognition of cognate transfer RNA by the 30 S ribosomal subunit. *Science*, **292**, 897–902.
54. Ogle, J. M., Murphy, F. V., Tarry, M. J. & Ramakrishnan, V. (2002). Selection of tRNA by the ribosome requires a transition from an open to a closed form. *Cell*, **111**, 721–732.
55. Frank, J., Sengupta, J., Gao, H., Li, W., Valle, M., Zavialov, A. & Ehrenberg, M. (2005). The role of tRNA as a molecular spring in decoding, accommodation, and peptidyl transfer. *FEBS Letters*, **579**, 959–962.
56. Gomez-Lorenzo, M. G., Spahn, C. M., Agrawal, R. K., Grassucci, R. A., Penczek, P., Chakraburty, K. *et al.* (2000). Three-dimensional cryo-electron microscopy localization of EF2 in the *Saccharomyces cerevisiae* 80 S ribosome at 17.5 Å resolution. *EMBO J.* **19**, 2710–2718.
57. Kirsebom, L. A. & Isaksson, L. A. (1985). Involvement of ribosomal protein L7/L12 in control of translational accuracy. *Proc. Natl Acad. Sci. USA*, **82**, 717–721.
58. Mohr, D., Wintermeyer, W. & Rodnina, M. V. (2002). GTPase activation of elongation factors Tu and G on the ribosome. *Biochemistry*, **41**, 12520–12528.
59. Frolova, L., Le Goff, X., Zhouravleva, G., Davydova, E., Philippe, M. & Kisselev, L. (1996). Eukaryotic polypeptide chain release factor eRF3 is an eRF1- and ribosome-dependent guanosine triphosphatase. *RNA*, **2**, 334–341.
60. Kong, C., Ito, K., Walsh, M. A., Wada, M., Liu, Y., Kumar, S. *et al.* (2004). Crystal structure and functional analysis of the eukaryotic class II release factor eRF3 from *S. pombe*. *Mol. Cell*. **14**, 233–245.
61. Maderazo, A. B., He, F., Mangus, D. A. & Jacobson, A. (2000). Upf1p control of nonsense mRNA translation is regulated by Nmd2p and Upf3p. *Mol. Cell. Biol.* **20**, 4591–4603.
62. Longtine, M. S., McKenzie, A., III, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A. *et al.* (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast*, **14**, 953–961.
63. Kitada, K., Yamaguchi, E. & Arisawa, M. (1995). Cloning of the *Candida glabrata* TRP1 and HIS3 genes, and construction of their disruptant strains by sequential integrative transformation. *Gene*, **165**, 203–206.

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