Mutations in the peptidyl transferase region of *E.coli* 23S rRNA affecting translational accuracy

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ABSTRACT

We have produced mutations in a cloned *Escherichia coli* 23S rRNA gene at positions G2252 and G2253. These sites are protected in chemical footprinting studies by the 3' terminal CCA of P site-bound tRNA. Three possible base changes were introduced at each position and the mutations produced a range of effects on growth rate and translational accuracy. Growth of cells bearing mutations at 2252 was severely compromised while the only mutation at 2253 causing a marked reduction in growth rate was a G to C transversion. Most of the mutations affected translational accuracy, causing increased readthrough of UGA, UAG and UAA nonsense mutations as well as +1 and –1 frameshifting in a *lacZ* reporter gene *in vivo*. C2253 was shown to act as a suppressor of a UGA nonsense mutation at codon 243 of the *trpA* gene. The C2253 mutation was also found not to interact with alleles of *rpsL* coding for restrictive forms of ribosomal protein S12. These results provide further evidence that nucleotides localized to the P site in the 50S ribosomal subunit influence the accuracy of decoding in the ribosomal A site.

INTRODUCTION

The correct translation of mRNAs requires precisely coordinated interactions among the macromolecular components of the protein synthetic apparatus. In addition to accurate codon–anticodon pairing, proper interactions of tRNAs with aminoacyl tRNA synthetases, tRNA modifying enzymes, elongation factors, and 30S and 50S ribosomal subunits are necessary to ensure optimum fidelity.

A number of ribosomal components and translation factors which influence the accuracy of decoding have been identified by mutational studies. Ribosomal proteins of both the small and large ribosomal subunits have been found which decrease (1, 2, 3), and increase fidelity (1, 4). Mutations in elongation factor EF-Tu increase missense errors and suppress nonsense and frameshift mutations (5, 6, 7). Similarly, there are mutations in rRNA which decrease (8, 9, 10, 11) and increase (12, 13) accuracy.

Our interest in translational fidelity has focused primarily on the role of rRNAs. With the application of chemical footprinting and crosslinking, a number of nucleotide residues in rRNA have been identified which either directly or indirectly interact with the other components of the translational machinery (14, 15, 16). Of particular interest to this report are those sites in the peptidyl transferase region of 23S rRNA which have been implicated in rRNA–tRNA interactions by chemical footprinting experiments (17). Mutations at two of these positions, U2555 (11) and G2583 (12) affect translational accuracy. Another site of interest in the peptidyl transferase region is two highly conserved G residues at positions 2252 and 2253. Footprinting of these residues by P site-bound N-acetyl-Phe-tRNA^{Phe} was dependent upon the presence of the 3' terminal CA but not on the terminal A residue of the tRNA; these footprints were also obtained with an aminoacyl-tRNA-derived oligonucleotide fragment which included the 3' terminal CCA and aminoacyl moiety (18). These findings suggested a functional and possibly direct interaction between the terminal CCA of tRNA and these positions of 23S rRNA. Recently, a mutation at position C74 in the terminal CCA of tRNA^{Val} was isolated as a frameshift suppressor and was found to increase readthrough of nonsense mutations downstream of valine codons (19). The mutant tRNA^{Val} affected decoding in the A site when bound to the P site or in some instances when bound to the E site. The CCA mutations may produce the suppressor phenotype by perturbing the interaction of the CCA terminus with G2252 or G2253 of 23S rRNA. If this is so, decreased translational accuracy should be among the defects caused by mutations at one or both of these positions in 23S rRNA.

We have tested this prediction by introducing mutations at G2252 and G2253, and have characterized the effects of these mutations on readthrough of nonsense and frameshift mutations in *lacZ* and on suppression of nonsense and frameshift mutations in *trpA*. Consistent with the proposed interaction between the CCA terminus of P site-bound tRNA and these nucleotides in 23S rRNA, two mutations at position 2253 and all three mutations at 2252 were found to decrease translational accuracy in a *lacZ* reporter gene, while C2253 was shown to also act as a suppressor of a UGA nonsense mutation in *trpA*. These results provide further evidence that nucleotide residues in rRNA, believed to interact with tRNAs, influence the accuracy of translation.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *rrm* mutants were analyzed in EF41, F⁻ $\Delta(lac-pro)$ *thi recA1*, an F⁻ Gal⁺ Rec⁻ derivative of CSH41 (20). MC127, F⁻ $\Delta(lac-pro)$ *thi recA56 srl::Tn10* (10), and *rpsL* mutants (21) were also derived from CSH41. M13 subclones were manipulated in XL-1 and uracil-containing M13 DNA (kindly provided by William Tappich) was prepared in the *ung dut* strain RZ1032. F'128 carries the wild type *lac-pro* region of the *E. coli* chromosome (22).

Plasmid pMO10 contains the entire *rrmB* operon in a kanamycin resistant pSC101 based vector (23). The *rrmB* operon is transcribed constitutively from the P₁P₂ promoter. Plasmid pGQ7 carries the *rrmB* operon transcribed from the inducible phage λ P_L promoter in pBR322 and confers ampicillin resistance. pLG857 contains the phage λ cI857 allele in the kanamycin resistant plasmid pLG339 (23). pSG25 is a tetracycline resistant plasmid derived from pACYC184 and contains the *lacZ* gene transcribed constitutively from the P_{tac} promoter in $\Delta(lac-pro)$ strains. Mutant pSGlac constructs were derived from this plasmid (11). Plasmid p415 is a pBR322 derived *lacZ* construct bearing a UGA nonsense mutation (19).

β -galactosidase assays and growth rate determination

β -galactosidase activity was assayed by the method of Miller with modifications (24). EF41 containing pMO10 and pSG25 (or mutant derivatives) was grown at 37°C to mid log phase in LB medium containing 50 μ g/ml kanamycin and 12.5 μ g/ml tetracycline and assayed. EF41 containing pGQ7, pLG857 and pSG25 (or mutant derivatives) was grown overnight at 30°C in LB containing 100 μ g/ml ampicillin, and 12.5 μ g/ml tetracycline, diluted into the same medium, incubated at 42°C for 150 minutes and assayed.

Growth rates were determined using a Klett–Summerson photometer. Cells were prepared as described above, grown at 37°C (pMO10) or 42°C (pGQ7).

In vivo suppression of mutations in *trpA* and *trpE*

Nonsense and missense suppression was tested in a series of strains with deletions extending into the chromosomal *trp* operon and a conjugative episome containing the *cysB trp tonB* region with nonsense or missense mutations in *trpA* (kindly provided by E.J. Murgola). Frameshift suppression was tested in several strains bearing frameshift mutations in the chromosomal *trpA* or *trpE* gene.

The *trp* mutants were transformed with plasmid pMO10 or pMO10 bearing the C2253 mutation and selected on LB plus 50 μ g/ml kanamycin. Suppression was assayed by streaking several transformants of each plasmid for single colonies on M9 minimal medium (20) containing 50 μ g/ml kanamycin and supplemented with 0.1% casamino acids. To monitor maintenance of the episome in the *trpA* nonsense and missense mutants, the same colonies were also streaked on the same medium supplemented with .002% indole, which supports the growth of *trpA* mutants. Restreaks were incubated at 37°C.

Polysome preparation and primer extension analysis

Cultures of EF41 containing pLG857 and pGQ7 (bearing mutations at position 2252 of 23S rRNA) were grown overnight at 30°C and diluted into fresh LB media containing kanamycin and ampicillin. Mutant rRNA was expressed by incubation at 42°C for 2.5 hrs prior to harvesting. Cultures of EF41 containing

plasmid pMO10 (bearing mutations at position 2253 of 23S rRNA) were grown to mid-log phase at 37°C. Cells were harvested by a freeze-thaw lysozyme treatment (25) and lysates were fractionated by centrifugation through linear 10–40% sucrose gradients for 18 hours at 18,000 rpm in an SW28 rotor. Subunit, 70S and polysome fractions were collected, rRNA from each fraction was extracted and then analyzed by primer extension (26) using a 5' ³²P end-labeled oligodeoxynucleotide primer complementary to positions 2254–2274 of 23S rRNA. Extension products were quantitated by densitometry.

RESULTS

Construction of rRNA mutants and their effects on growth rate

The *EcoRI*–*Bam*HI fragment containing the 3' portion of the *rrmB* 23S rRNA gene was cloned into M13mp18 and oligonucleotide directed mutations were produced at either position 2252 or 2253. Mutant 23S rRNA gene fragments were introduced into plasmid pGQ7 as an *EcoRI*–*Esp*II fragment or into plasmid pMO10 as an *Sph*I fragment. The sites of the mutations in the secondary structure model of 23S rRNA are indicated in Figure 1.

All mutations were initially introduced into plasmid pGQ7 which contained the λ P_L promoter repressed at 30°C by a thermolabile mutant λ cI repressor expressed from plasmid pLG857. Transcription of the *rrm* operon was induced by incubation at 42°C. Cells containing mutant plasmids were tested for their ability to form single colonies by streaking on plates and incubating at 30°C and 42°C. All three mutations at position 2252 produced severe growth defects and did not form uniform

Figure 1. The secondary structure model of domain V of *E. coli* 23S rRNA. The 2252/2253 stem loop structure is also shown in expanded form to indicate the sites of mutations examined in this study.

single colonies at 42°C. All subsequent analyses of the 2252 mutations were done in pGQ7. None of the mutations at position 2253 exhibited such deleterious effects and only C2253 caused a noticeable reduction in colony size. Therefore these three mutations were transferred to pMO10 and expressed constitutively from the P₁P₂ promoter.

Growth rates of mutants in liquid culture are shown in Table 1. The 2252 mutants were monitored at 42°C during induction of mutant rRNA expression. Because of the deleterious nature of these mutations, the accumulation of mutant 23S rRNA resulted in non-logarithmic growth and steady-state growth rates could not be determined. Mutation of G2253 to A had no detectable effect on growth rate despite the high level of phylogenetic conservation of this nucleotide. Transversion to U resulted in a small but detectable growth rate reduction while C2253 had a more substantial effect.

Distribution of mutant 23S rRNAs in ribosome fractions

Polysomes, 70S ribosomes and free 50S subunits were prepared (25) and the proportion of mutant rRNA in each fraction was determined by primer extension analysis (26). The results are presented in Table 1. rRNA bearing any of the three mutations at position 2252 was present in polysomes but in reduced amounts (25–30%) relative to its abundance in 70S and 50S fractions. In contrast, 23S rRNA bearing a C or U transversion mutation at position 2253 was found to constitute about 50% of 23S rRNA in both polysomes and free 50S subunits. Surprisingly, the A2253 mutant rRNA, which produced neither growth nor accuracy defects, was incorporated into polysomes at lower levels (32%) than the C or U2253 mutant rRNA. The reason for this difference is unknown. However, this mutant rRNA is not highly enriched in free 50S subunits or 70S ribosomes, consistent with the absence of a growth defect. This lower level is also unlikely to mask accuracy defects.

Mutations at position 2252 of 23S rRNA increase readthrough of nonsense and frameshift mutations in *lacZ*

The ability of ribosomes to read through nonsense and frameshift mutations in *lacZ* has been used as a measure of translational accuracy (10, 11, 13). In order to quantitate the effects of

mutations at 2252, EF41 containing wild type or mutant pGQ7 and mutant versions of the *lacZ* reporter plasmid pSG25 were grown at 30°C overnight. Transcription of mutant rRNA was then induced by shifting temperature to 42°C and after 2.5 hours the cells had accumulated 25 to 30% mutant 23S rRNA in polysomes (see above and in Table 1.). At this point β -galactosidase activity was measured. The results, presented in Table 2, show that all three rRNA mutants at 2252 displayed increased nonsense and frameshift readthrough, although U2252 generally produced the highest levels. No pattern of specificity for either stop codon identity or direction of frameshifting was apparent. We conclude that these rRNA mutations produce a

Table 2. Effects of mutations at position 2252 of 23S rRNA on readthrough of nonsense and frameshift mutations in *lacZ*

<i>lacZ</i> mutant	β -galactosidase activity (Miller units)			
	G2252 (WT)	A2252	C2252	U2252
pSG3/4 (UGA)	161.5 $\pm$ 5.8	188.4 $\pm$ 13.9	200.3 $\pm$ 8.0	304.3 $\pm$ 2.3
pSG12-6 (UAG)	15.6 $\pm$ 0.4	22.5 $\pm$ 0.8	23.7 $\pm$ 2.5	81.2 $\pm$ 3.1
pSG163 (UAG)	36.2 $\pm$ 1.3	46.5 $\pm$ 2.0	46.4 $\pm$ 1.2	63.2 $\pm$ 3.7
pSG853 (UAA)	7.1 $\pm$ 0.2	10.6 $\pm$ 0.2	9.2 $\pm$ 0.6	15.2 $\pm$ 0.6
pSG12DP (-1)	84.7 $\pm$ 1.6	87.4 $\pm$ 1.6	89.0 $\pm$ 1.8	97.0 $\pm$ 2.2
pSGlac10 (-1)	6.0 $\pm$ 0.1	11.6 $\pm$ 0.6	12.5 $\pm$ 0.2	15.9 $\pm$ 0.7
pSGlac7 (+1)	77.9 $\pm$ 2.3	147.9 $\pm$ 5.1	146.7 $\pm$ 5.7	164.0 $\pm$ 16.5
pSG25 (WT <i>lacZ</i>)	6,847.8 $\pm$ 288.8	6,261.9 $\pm$ 465.2	6,570.7 $\pm$ 396.0	8,166.0 $\pm$ 608.0
F128 (WT <i>lacZ</i>)	2,106.0 $\pm$ 110.1	1,150.2 $\pm$ 95.0	1,089.5 $\pm$ 107.7	874.0 $\pm$ 22.3

Values for β -galactosidase activity are given as Miller units (24). Each value represents the mean and standard deviation of at least three experiments. Cultures bearing mutations at 2252 were incubated at 42°C for 2.5 hrs to induce mutant rRNA expression after which assays were performed.

Table 3. Effects of mutations at position 2253 of 23S rRNA on readthrough of nonsense and frameshift mutations in *lacZ*

<i>lacZ</i> mutant	β -Galactosidase activity (Miller units)			
	G2253 (WT)	A2253	C2253	U2253
pSG3/4 (UGA)	55.6 $\pm$ 1.4	65.2 $\pm$ 3.7	167.4 $\pm$ 6.8	115.8 $\pm$ 0.6
pSG12-6 (UAG)	7.2 $\pm$ 0.2	7.4 $\pm$ 0.2	12.9 $\pm$ 0.6	11.3 $\pm$ 0.4
pSG627 (UAA)	0.9 $\pm$ 0.1	1.1 $\pm$ 0.1	2.6 $\pm$ 0.1	1.9 $\pm$ 0.04
pSG12DP (-1)	54.1 $\pm$ 2.3	69.1 $\pm$ 1.3	260.6 $\pm$ 5.6	83.1 $\pm$ 2.3
pSGlac7 (+1)	75.9 $\pm$ 2.4	76.8 $\pm$ 1.2	131.8 $\pm$ 3.3	108.6 $\pm$ 2.7
pSG25 (WT <i>lacZ</i>)	5,216.2 $\pm$ 54.1	4,644.7 $\pm$ 193.6	n.d.	4,805.3 $\pm$ 537.5
F128 (WT <i>lacZ</i>)	1653.9 $\pm$ 252.6	1539.8 $\pm$ 218.2	966.4 $\pm$ 123.3	1060.0 $\pm$ 56.5

Values for β -galactosidase activity are given in Miller units (24). Each value represents the mean and standard deviation of at least three experiments. Cultures bearing mutations at 2253 were grown at 37°C to mid-log phase and assayed for enzyme activity. nd; not determined.

Table 1. Growth rates and distribution of mutant 23S rRNA in 50S subunits, 70S ribosomes and polysomes

rRNA Mutant	Doubling Time (min)	Mutant 23S rRNA (percent of total)		
		50S	70S	Polysomes
A2252	nd	75 $\pm$ 7	56 $\pm$ 2	32 $\pm$ 2
C2252	nd	61 $\pm$ 2	48 $\pm$ 4	32 $\pm$ 2
U2252	nd	39 $\pm$ 1	40 $\pm$ 3	24 $\pm$ 1
G2253 (WT)	45 $\pm$ 3			
A2253	47 $\pm$ 4	36 $\pm$ 4	50 $\pm$ 1	32 $\pm$ 2
C2253	71 $\pm$ 4	50 $\pm$ 2	61 $\pm$ 2	52 $\pm$ 4
U2253	52 $\pm$ 5	53 $\pm$ 3	80 $\pm$ 6	46 $\pm$ 1

Doubling times as presented are the mean and standard deviation of three independent measurements. Distribution of mutant rRNA was determined by primer extension analysis of RNA from three independent cultures of each mutant. nd; not determined.

Table 4. Effects of C2253 on growth rate of restrictive *rpsL* mutants

Strains ^a	Doubling Time (min.)		Ratio C/G ^b
	G2253 (WT)	C2253	
<i>rpsL</i> ⁺	43 ± 3	59 ± 2	1.37
<i>rpsL2224</i>	41 ± 5	54 ± 2	1.32
<i>rpsL2222</i>	53 ± 1	68 ± 14	1.28

^aAll *rpsL* alleles were examined in an MC127 background. *rpsL2224* is a moderately restrictive allele whereas *rpsL2222* is a highly restrictive allele (21).

^bThe Ratio C/G is the ratio of doubling times of the C2253 mutant to G2253

Table 5. Effects of C2253 mutation on readthrough of a UGA codon in *lacZ* in restrictive *rpsL* mutants expressed in Miller units

Strain ^b	F' 128 (WT <i>lacZ</i>)		<i>lacZ</i> (UGA) ^a		Ratio C/G ^c
	G2253(WT)	C2253	G2253(WT)	C2253	
<i>rpsL</i> ⁺	2333.4 ± 134.2	2060.9 ± 26.7	80.3 ± 6.9	181.0 ± 33.2	2.3
<i>rpsL2224</i>	4119.2 ± 260.2	2827.3 ± 190.5	22.3 ± 1.1	54.8 ± 7.4	2.9
<i>rpsL2222</i>	2708.6 ± 76.6	3179.2 ± 166.8	7.7 ± 1.2	22.3 ± 4.2	2.5

^aThe mutant *lacZ* gene is carried on a pBR322 based construct, p415 (19).

^bAll *rpsL* alleles were examined in an MC127 background.

^cRatio C/G is the ration of UGA readthrough by C2253 mutant to the readthrough by wild type G2253.

general defect in accuracy, as opposed to a defect in recognition of specific termination codons.

Both pSG25 and F'128 were used to determine the level of expression of wild type *lacZ* in the presence of the rRNA mutants. While no substantial differences were seen with the pSG25 construct, all three mutants showed an approximately 50% reduction in expression from the F' encoded *lacZ* gene. We interpret these results to indicate that the mutant ribosomes are impaired in some translational function and are also interfering with wild type ribosomes on the limiting *lac* messenger RNA in the F' strain. In cells containing pSG25, this difference is not seen as the much higher levels of *lac* mRNA are essentially saturating.

Mutations at position 2253 of 23S rRNA increase readthrough of nonsense and frameshift mutations in *lacZ*

Mutations at position 2253 were analyzed in plasmid pMO10 for nonsense and frameshift readthrough. Constitutive expression from the canonical P₁P₂ *rrn* promoter represents the preferred condition for characterization of rRNA mutants as analysis is performed under steady state conditions. EF41 containing *rrn* mutant plasmids and mutant derivatives of pSG25 were grown to mid log phase, and assayed for β -galactosidase activity. As shown in Table 3, the A2253 mutation gave no substantial increase in either nonsense or frameshift readthrough. This is consistent with the absence of a growth defect associated with this mutant.

The U2253 and C2253 mutants produced significantly higher levels of β -galactosidase activity from the mutant *lacZ* constructs. The levels of readthrough correlated with the growth defects associated with each mutant. C2253, which produced the most substantial increase in doubling time, gave the highest levels while U2253 was found to be intermediate in both growth rate and readthrough. As was observed with the 2252 mutations, mutations at 2253 caused a general defect in translational accuracy.

The levels of β -galactosidase produced from F'128 were reduced in mutants with substantial growth defects. In particular, U2253 and C2253 produced approximately two-thirds the activity of either G2252 or A2253. Expression of very high levels of β -galactosidase from pSG25 appeared to be somewhat toxic in combination with C2253, as judged by aberrant colony morphology and high variability in enzyme assays. Therefore, reliable measurements of β -galactosidase activity could not be obtained.

In vivo suppression of nonsense mutations in *trpA* by C2253

We were interested in determining if the levels of nonsense readthrough and frameshifting observed in β -galactosidase assays were sufficient to produce either nonsense, missense or frameshift suppressor phenotypes. A series of strains bearing nonsense, missense, or frameshift mutations in *trpA* and one strain bearing a frameshift mutation in *trpE* were transformed with either wild type pMO10 or with pMO10 bearing the C2253 mutation. The C2253 mutation was then tested for a suppressor phenotype by assessing its ability to promote growth of the *trpA* or *trpE* mutants on tryptophan-free minimal media. The C2253 mutation was chosen as it could be expressed constitutively and produced the most significant readthrough of nonsense mutations in *lacZ* as quantitated in enzyme assays.

Several single colonies from each transformation were tested for *in vivo* suppression by streaking onto M9 minimal media supplemented with casamino acids and 50 μ g/ml kanamycin and incubated at 37°C for up to one week.

C2253 was found to cause suppression of a UGA nonsense mutation at codon 243. The UGA243 mutation is somewhat leaky, such that after three days incubation at 37°C, wild type cells produce small visible colonies. The substantially larger size of colonies of the C2253 mutants clearly demonstrated their suppressor activity. C2253 did not suppress other *trpA* nonsense mutations including UAG15, UAA15, UGA211, UAG211, UAA211, UGA234, UAG234, UAA234, UAG243 or UAA243. Suppression of a nonsense mutation in *trpA* provided an independent demonstration of the effects of the C2253 mutation on translational accuracy.

UGA codons are normally read at low levels in wild type cells by UGG-decoding tRNA^{Trp} (27) while UAG and UAA codons are read at substantially lower levels by CAG- and CAA-decoding tRNA^{Gln} (28). Of the four codon positions in *trpA* tested, only at codon 243 does insertion of tryptophan produce functional tryptophan synthetase, and only at codons 15 and 243 does insertion of glutamine result in functional enzyme (29). The exclusive suppression of *trpA*(UGA243) is consistent with the *lacZ* nonsense readthrough data which indicated that the C2253 mutation merely amplifies the intrinsic readthrough of all three nonsense codons. The failure to detect suppression of UAG or UAA mutations at codons 15 and 243 was probably due to the lower level of natural readthrough of UAG and UAA codons (30).

No missense suppression was detected either at codon 211 (UGG, CGA, AGA, AGG, AAA and GAA were tested) or at

codon 234 (UGC, AGA, AAA and AAG were tested). While nonsense suppression requires that a non-cognate aminoacyl-tRNA compete with release factor, missense suppression requires the competition of a non-cognate, suppressing aminoacyl-tRNA with a cognate, non-suppressing aminoacyl-tRNA. It could therefore be argued that the levels of miscoding required to allow missense suppression might be beyond the level of misreading induced by C2253, and that the absence of observed missense suppression does not exclude increased misincorporation at sense codons.

C2253 was also tested for its ability to act as a suppressor of frameshift mutations in the *trp* operon. No suppression was detected of the +1 frameshift mutations *trpA8* and *trpA46*, or of the -1 frameshift mutations *trpA9813* and *trpE91*.

Effects of restrictive mutations in the gene coding for ribosomal protein S12 on growth rate and misreading by C2253 mutant ribosomes

To examine the possible relationship between increased misreading and growth defects caused by the mutations at position 2253, the C2253 mutation was expressed in strains with restrictive alleles of *rpsL*, coding for ribosomal protein S12, and growth rates and levels of readthrough of a *lacZ* UGA mutation were determined. Plasmid pMO10 (either wild type or with the C2253 mutation) was introduced into MC127 bearing either wild type *rpsL* or restrictive *rpsL* alleles conferring different degrees of increased translational accuracy. Accuracy was assayed as readthrough of a UGA mutation in *lacZ* on a pBR322 derived construct. The results are shown in Tables 4 and 5.

In all three *rpsL* backgrounds, introduction of the C2253 mutation caused a similar fold-increase in UGA readthrough. Doubling times also increased 1.3 to 1.4 fold, even in an *rpsL* background in which the net level of readthrough is less than in wild type cells. Expression of C2253 results in a similar fold increase in doubling time regardless of the net level of translational accuracy. These observations argue that other aspects of ribosome performance are affected by this mutation in addition to its effect on translational accuracy.

DISCUSSION

The results presented here provide further evidence that 23S rRNA is involved in determining the accuracy of translation. The finding that mutations at positions 2252 and 2253, footprinted by P site-bound tRNA, affect the discrimination of aminoacyl-tRNA in the A site emphasizes the complex nature of the decoding process. These mutations in the peptidyl transferase region caused increased frameshifting and readthrough of nonsense codons and also had a variety of effects on growth rate. All three base changes at position 2252 were lethal after prolonged expression at high levels, while mutations at position 2253 could be expressed constitutively. A G to C transversion at 2253 was error prone yet sufficiently functional to act as a suppressor of a UGA nonsense codon at one position in *trpA* *in vivo*. The only mutation examined in this study which affected neither growth rate nor translational fidelity was a G to A transition at position 2253. Interestingly, two examples of phylogenetic variation exist for this position, in *Chlamydomonas reinhardtii* mitochondria (31) and *Apis mellifera* mitochondria (32). Both bear an A at the position homologous to 2253 in *E. coli*.

Mutations at the phylogenetically conserved bases 2252 and 2253, candidates for interaction with the CCA terminus of tRNA,

might be expected to produce pleiotropic effects on tRNA binding, peptide bond formation and translocation in addition to the observed effects on translational accuracy. In fact, the growth rate defects caused by the mutations examined in this study cannot be attributed exclusively to decreased accuracy, since the introduction of restrictive *rpsL* alleles into the C2253 mutant reduced UGA readthrough to below wild type levels but did not rescue the growth defect. Ribosomes bearing a GG to CC double mutation at positions 2252 and 2253 have been found to exhibit a significantly reduced rate of peptide bond formation *in vitro* (K.Lieberman and A.E.Dahlberg, submitted). Mutations at other conserved bases in the peptidyl transferase region have been shown to have profound effects on growth rate and viability (33).

Mutations in EF-Tu and in ribosomal components thought to interact with EF-Tu have previously been found to affect translational accuracy. Gentamicin resistance mutations in *rplF*, the gene coding for ribosomal protein L6, produce a restrictive or hyperaccurate phenotype (4), while mutations in *rplL*, coding for ribosomal protein L7/L12, have a *ram*, or ambiguity phenotype (3). Transversion mutations in 23S rRNA at position G2661 in the α -sarcin loop of the EF-Tu binding site have been discovered to increase the efficiency of proofreading (13) whereas mutations in the 530 loop of 16S rRNA decrease fidelity (10). Mutations in *rplL*, the α -sarcin loop of 23S rRNA and the 530 loop of 16S rRNA are thought to perturb the interaction of the ribosome with EF-Tu during the proofreading step.

Mutations in the peptidyl transferase region of 23S rRNA which affect fidelity may represent a functionally distinct group which exerts its effects on accuracy through interactions with tRNA. Mutations at position G2583 were shown by Saarma and Remme (12) to decrease missense errors in *in vitro*, while a mutation at U2555 has been selected as a frameshift suppressor (11). U2584 and U2555 are protected in chemical footprinting experiments by the CCA terminus of P site-bound N-acetyl-Phe-tRNA^{Phe} and A site-bound aminoacyl-tRNA^{Phe}, respectively (17). It should also be noted that A site-bound aminoacyl-tRNA footprints C2254 (17) which is adjacent to the nucleotides examined in this study. If G2252 and G2253 directly contact the CCA terminus of P site-bound tRNA, the functional requirements of peptide bond formation place these nucleotides in close proximity to the A site and to the CCA terminus of aminoacyl-tRNA. It is therefore unlikely that mutations at 2252 or 2253 affect P site interactions in isolation without affecting interactions between the ribosome and A site-bound aminoacyl-tRNA. The accuracy defects we have observed may be attributable either to disruption of the proposed interaction between G2252/G2253 and the CCA terminus of peptidyl-tRNA in the P site, or by directly influencing events occurring in the A site. These two possibilities could be distinguished by the rescue of accuracy defects at specific nonsense or frameshift mutations by compensatory mutations in the appropriate tRNA bound to the P site.

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