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A ribosomal ambiguity mutation in the 530 loop of *E.coli* 16S rRNA

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ABSTRACT

A series of base substitution and deletion mutations were constructed in the highly conserved 530 stem and loop region of *E.coli* 16S rRNA involved in binding of tRNA to the ribosomal A site. Base substitution and deletion of G517 produced significant effects on cell growth rate and translational fidelity, permitting readthrough of UGA, UAG and UAA stop codons as well as stimulating +1 and −1 frameshifting *in vivo*. By contrast, mutations at position 534 had little or no effect on growth rate or translational fidelity. The results demonstrate the importance of G517 in maintaining translational fidelity but do not support a base pairing interaction between G517 and U534.

INTRODUCTION

The 530 region of the *E.coli* small subunit ribosomal RNA (Fig. 1) is one of the most highly conserved sequences in the entire 16S rRNA molecule (1). This strong conservation argues for an important functional role for this segment of RNA in translation. However, the precise function and location on the ribosome of the 530 region remain obscure. Several independent lines of evidence link this region of the 16S rRNA molecule with binding of tRNA to the ribosomal A and P sites. Chemical protection experiments have revealed that A and P site-bound tRNAs (or a tRNA fragment consisting only of the anticodon stem and loop) protect residues 529, 530 and 531, and 532 respectively from attack by various chemicals (2). Aminoglycoside antibiotics of the neomycin class, which lower the ribosome's discrimination against non-cognate tRNAs bound to the A site, enhance the reactivity of residue 525 to chemical probes (3). In addition, several base substitution mutations have been isolated in the 530 loop which confer resistance to streptomycin, an antibiotic which also promotes misreading (4, 5, 6) Finally, Shen and Fox (7) have identified a base substitution at position 517 in the 15S rRNA of the yeast mitochondrion which acts as a suppressor of UAA nonsense codons.

The decoding centre of the ribosome, comprising residues in the 1390–1400 region of 16S rRNA, has been localised to the base of the cleft in the 30S subunit. The 530 region has been localised by DNA hybridization microscopy and immune electron microscopy to the head region of the subunit, on the opposite side of the 30S particle to the decoding centre (8). Since the head region of the ribosome and the cleft are some 70A apart, this has led to the proposal that the altered conformation of the 530 region observed upon binding of tRNA and antibiotics must be due to allostery between the decoding centre and the 530 region (9). Such a view has recently been challenged by Rinke-Appel et al (10) who observed simultaneous crosslinking of mRNA to the 1390–1400 region and to base 532. They propose that the 530 region is much closer to the decoding centre than the interpretations of electron microscopy data suggest and, in fact, may be part of the decoding region itself.

In most secondary structure models of *E.coli* 16S rRNA (11,12), residue G517 is placed at the top of a stem and is proposed to pair with U534, with the intervening residues being bulged out into a loop. However, in the light of more extensive comparative sequence analysis, Gutell, Larsen and Woese (13) have re-drawn this structure with the result that bases 517 and 516 are no longer paired with 534 and 535 on the opposite side of the helix (Fig 1). G517 is an invariant residue while U534 shows considerable variability. In yeast mitochondria, a G-C pair is found at these same positions and the G517→A change which gave rise to the UAA suppressor isolated by Shen and Fox (7) disrupted this potential base pairing interaction.

In this study we have examined the effects of base substitutions at positions 517 and 534 in *E.coli* 16S rRNA on cell growth and the ability of mutant ribosomes to misread. Combinations of mutant bases at positions 517 and 534 also permit one to test the contribution of base pairing between these two residues to ribosome performance *in vivo*. The results indicate that G517 is an important residue for the maintenance of translational fidelity; both base substitutions and deletions increased the level of translational errors. By contrast, base substitutions are readily tolerated at position 534 with little effect on cell growth or

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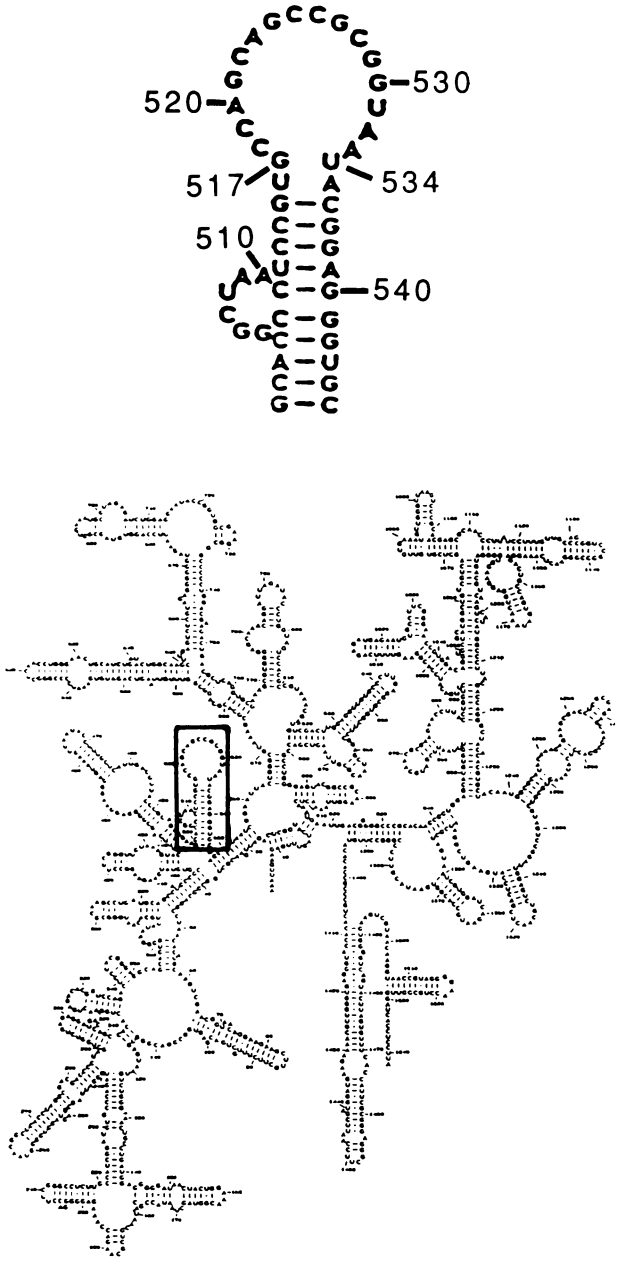

Figure 1. Sequence and secondary structure of *E.coli* 16S rRNA enlarged from the model of Gutell et al. (13).

translational fidelity. The results stress the importance of the primary sequence of this region of rRNA for proper ribosome function but do not support a base pairing interaction between residues 517 and 534.

MATERIALS AND METHODS

Bacterial strains and plasmids

Strain MC127, F^- Δ (lac-pro) thi^- $recA^-$ srl::tn10, a rec-derivative of CSH41 (14) was routinely used as a host for plasmids. MDA6646, F^- ilvB1202 ilvH2202 rbs221 ara^- thi^- Δ(lac-pro) Δgpt $pcnB^-$ ΔrecA, a gift from Dr E.J.Murgola, M.D.Anderson Cancer Center, was used to maintain deleterious mutations at low plasmid copy number. XL1 Blue (Stratagene, Ca.) was used to propagate M13 phages. CJ236 and BW313 (both dut- ung-) were used to prepare uracil-containing DNA. Plasmid pKK3535 (15) contains the intact rrnB operon inserted in pBR322 and pSTL102 (16) is a derivative of pKK3535 and contains the U1192 and G2058 mutations in 16S and 23S rRNA respectively, conferring resistance to spectinomycin and erythromycin. In plasmid pNO2680 (17) the constitutive P_1P_2 promoters of the rrnB operon have been replaced by the leftward promoter of phage lambda, allowing repression of plasmid borne rRNA mutations. Plasmid pcI857 carries the thermolabile lambda repressor allele on a kanamycin/neomycin resistant p15A replicon (18). pLG857 carries the same insert in pLG339, a pSC101 replicon compatible with both p15A and pBR322 replicons (19). All lac Z plasmid mutations used in this study are derivatives of a full length lacZ gene carried on pACYC184, constructed by Dr. Robert Weiss, University of Utah. Many of these lacZ mutations were originally constructed in a pBR322 based version of the same vector (20, 21). Lac mutants were transferred from the pBR322 version to the pACYC184-based plasmid on a HindIII-SacI fragment and their identity confirmed by sequencing. Luria-Bertani (LB) medium supplemented with appropriate antibiotics was used for the routine cultivation of strains. Bacterial growth was followed using a Klett−Summerson Colorimeter.

Construction of rRNA mutations

Single base substitution and deletion mutations at positions 517 and 534 were constructed via site-directed mutagenesis of an M13 KpnI-XbaI clone containing the intact 16S rRNA cistron using synthetic 17 base oligonucleotides (22, 23). Double mutations at positions 517 and 534 were constructed by performing a second round of site-directed mutagenesis on an M13 clone already containing one of the single base mutations. Mutations were identified by sequencing and cloned into the expression vectors pSTL102 or derivatives of pNO2680 and pKK3535 lacking vector HindIII sites, on a 568 base pair HindIII fragment. Mutant plasmids were verified by sequencing and restriction analysis. pKK3535 and pSTL102-based clones were transformed into MC127 and plated on ampicillin plates while pNO2680-derived clones were transformed into MC127 pcI_{857} and plated on media containing ampicillin and neomycin at 30°C.

Analysis of RNA

Total cellular RNA was prepared as described by Tapprich et al. (24). Polysomes, 70S, 50S and 30S fractions were isolated by centrifugation of quick-cooled lysates through linear 10−40% sucrose gradients for 18 hours at 18,000 rpm in a Beckman SW28 rotor and analysed on an ISCO gradient fractionator. RNA was isolated from subunits by phenol extraction and the proportion of mutant RNA quantitated by the primer extension method of Sigmund et al (25), followed by densitometric scanning of the autoradiographs.

Assay of β-galactosidase

β-Galactosidase was assayed according to the method of Miller (14) with the modifications described in Weiss et al (20). Lac mutants containing pKK3535 or pSTL102-derived plasmids were grown in LB medium with ampicillin and chloramphenicol to mid-logarithmic phase at 37°C and were then assayed. LacZ mutant strains containing pLG857, $F'lacI^Q$ and pNO2680-derived plasmids were grown to early logarithmic phase at 30°C and were then shifted to 42°C for 21/2 hours before being assayed. Suppression values for lacZ nonsense and frameshift

mutants were calculated as the units of β-galactosidase activity obtained with the nonsense or frameshift mutant divided by the units obtained with a wild type lacZ gene in the same strain.

RESULTS

Base substitution mutations at 517/534

Base substitution changes at position 517 in 16S rRNA were constructed by site directed mutagenesis and the mutations were initially expressed in pNO2680. This plasmid places the rrnB operon under the control of the P_L promoter of phage lambda. The presence of the thermolabile cI_{857} repressor allele allows expression of rRNA mutations to be induced by incubation at 42°C of a strain carrying both the repressor plasmid and pNO2680. All three base substitution mutations at position 517 were tested and all three had negligible effects on the growth rate of MC127 pcI_{857} at 42°C (data not shown). However, when the same mutations were expressed from the native P_1P_2 promoters of *rrnB* carried on plasmids pKK3535 or pSTL102, significant effects on growth rate were observed (Table 1). Plasmid pSTL102 is derived from pKK3535 and contains base substitution mutations at positions 1192 (in 16S rRNA) and 2058 (in 23S rRNA), which confer resistance to spectinomycin and erythromycin respectively. The addition of spectinomycin to a strain carrying pSTL102 forces the cell to use the plasmid encoded rRNA exclusively. Thus, growth of strains carrying the pSTL517 series of plasmids on spectinomycin is an index of the performance of these mutant ribosomes *in vivo*. Growth of the mutants in the presence of spectinomycin was found to mirror the pattern of growth rates seen in its absence (data not shown). C517 caused a modest reduction in cell growth, U517 caused a more severe reduction in growth while growth of the A517 mutant was severely compromised and this mutant did not grow on spectinomycin. This 'reversal' of spectinomycin resistance by A517 was presumably due to the poor functional activity of the mutant ribosomes, to the extent that they could no longer support cell growth.

Role of G517 and its interaction with U534

A potential G-U pair between 517 and 534 exists in most eubacterial species. Of the base substitution mutations constructed at position 517, the G→A change which replaces a G-U pair with a potentially stronger A-U pair had the most deleterious effect on cell growth. To test the possible contribution of base pairing interactions to this poor growth phenotype, base U534 was changed to C via site directed mutagenesis and expressed directly in pSTL102. This change resulted in the creation of a G-C pair at the top of the helix and should have greater stability than the wild type G-U pair or the mutant A-U pair. Strains carrying pSTL534C had only slightly reduced growth rates and were fully resistant to spectinomycin (Table1). The lack of effect of the C534 change suggested that it may be the identity of the 517 residue which is important rather than its potential to pair with the base at 534. This question was addressed in two ways: we first constructed a double mutant which replaced the G517-U534 pair with a U517-G534 pair. This double mutant left base pairing potential unaltered but reversed the order of the bases. Secondly, we changed U534 to G. This abolished any base pairing potential with G517, but left G517 unaltered. These mutants were constructed as described in Materials and Methods and expressed in pSTL102. The U517-G534 double mutant had a reduced growth rate, similar in extent to the U517 single mutant (Table 1). By contrast, the G534 single mutant had a growth rate virtually indistinguishable from the wild type. While the C517 mutant did not have an appreciable effect on growth rate, we conclude that the G residue at position 517 is important for ribosome function but considerable latitude can be tolerated in the selection of bases at 534. Also, there is no support for a base pairing interaction between 517 and 534. Such a base pairing interaction also is not supported by phylogenetic evidence. While a G is always found at 517, the identity of the base at 534 is particularly variable in the small subunit RNAs from plastids where any nucleotide can be found at this position (13, 26).

The distribution of wild type and mutant rRNA among 30S subunits, 70S ribosomes and polysomes was determined by sucrose density gradient centrifugation and primer extension analysis (25). The mutant and wild type RNAs were found in the same ratios in 30S, 70S and polysome fractions (Table1). These data indicated that mutations at positions 517 or 534 did not affect rRNA processing and did not impair the ribosome's ability to form 70S particles or function in polysomes.

Deletion mutations at position 517

Deletion mutations of 517 and of both 517 and 516 were constructed in addition to the base substitution mutations at 517. These mutations were initially expressed in pNO2680 in a strain carrying the thermolabile cI857 repressor. At 42°C, both deletion mutations had severely prolonged doubling times (Table1). Attempts to express these deletion mutations in pSTL102 with the strong, constitutive P1P2 promoters were unsuccessful, indicating that the deletion mutations were lethal when expressed at high levels. Plasmids such as pBR322, from which pKK3535 and pSTL102 are derived, are maintained at a copy number of about 20 per cell but a host mutation (pcnB) lowers the copy number of pBR322 and its derivatives 16 fold (27, 28). Using a $pcnB^-$ strain, MDA6646, as a host for transformation we were able to express the 517 and 517/516 deletion mutations in pSTL102. Plasmid DNA isolated from these transformants re-transformed MDA6646 at high frequency while no transformants were obtained with MC127 ($pcnB^{wt}$). In a $pcnB^-$ background both wild type pSTL102 and its mutant derivatives were now unable to grow in the presence of spectinomycin. This presumably is due to the reduced gene dosage of plasmid-borne 16S rRNA molecules carrying the U1192 spectinomycin resistance mutation, which also accounts for the viability of the deletion mutations in the $pcnB^-$ background.

Primer extension analyses were carried out using rRNA from strains containing the deletion mutants on an inducible plasmid, (the pNOΔ517 and pNOΔ517/516 plasmids). The results (Table1) showed that in both deletion mutants, approximately equal amounts of mutant and wild type RNA were present in 30S and 70S fractions but that the plasmid-encoded mutant RNAs were preferentially excluded from the polysome pools, particularly in the two base deletion. Primer extensions were also carried out on the same mutants using rRNA extracted from $pcnB^-$ strains carrying the deletion mutants on pSTL102 (which transcribes the rRNA from the constitutive promoters P1P2). Again very little mutant RNA was detected in the polysome pools. However, we found that the mutant RNA was also excluded from 70S particles relative to plasmid-coded, wild type ribosomes. The discrepancy between the inducible and the constitutive system with respect to the level of mutant RNA in 70S particles is unexpected, especially since approximately equal amounts of plasmid-encoded RNA are produced under both conditions. The differences may

Table 1. Growth rates and distribution of plasmid-encoded mutant rRNA in subunits, 70S ribosomes and polysomes

Strain	Plasmid	517/534	Doubling Time (min)	Plasmid-Encoded rRNA (percent of total) 30S	70S	Polysomes
MC127	pSTL102(W.T.)	GU	52 ± 6	91.6 ± 0.5	85.9 ± 7.2	87.0 ± 7.6
	pSTL517C	CU	55 ± 2	92.0 ± 0.8	90.0 ± 5.7	74.2 ± 1.3
	pSTL517U	UU	64 ± 5	88.2 ± 8.9	88.7 ± 7.8	83.8 ± 12.6
	pSTL517A	AU	74 ± 2	92.8 ± 2.8	91.1 ± 4.8	87.0 ± 6.5
	pSTL534C	G C	60 ± 2	87.6 ± 1.8	82.0 ± 0.9	77.8 ± 12.6
	pSTL534G	G G	58 ± 3	94.0 ± 1.3	95.1 ± 2.9	85.8 ± 8.0
	pSTL17U,34G	UG	65 ± 2	93.8 ± 3.2	93.8 ± 0.1	81.7*
MDA6646	pSTL102(W.T.)	GU	41 ± 1	57.4 ± 12.0	58.3 ± 12.4	59.3 ± 6.9
	pSTLΔ517	-U	50 ± 3	57.8 ± 10.4	23.7 ± 12.8	14.0 ± 3.6
	pSTLΔ516/17	-U	55 ± 5	64.4 ± 12.8	23.9 ± 9.1	20.0*
MC127F'	pNO2680(W.T.)	GU	45 ± 3	n.d.	n.d.	n.d.
	pNO517U	UU	n.d.	59.5 ± 2.1	49.4 ± 4.7	59.5*
pLG857	pNOΔ517	-U	95 ± 14	59.2 ± 1.0	50.9 ± 5.4	29.6 ± 4.6
	pNOΔ516/17	-U	73 ± 6	72.1 ± 8.5	49.0 ± 5.4	17.6 ± 3.5

Relative proportions of plasmid-encoded and chromosomally encoded rRNA were determined by 2–6 independent reverse transcription assays, unless otherwise indicated. * indicates the result of a single experiment. n.d., not determined. Reverse transcription assays of RNA from strains carrying the pSTL series of plasmids were carried out using an oligonucleotide which annealed one base downstream of 1192 in 16S rRNA. The pNO series of plasmids was assayed using an oligonucleotide complementary to bases 544–521 in 16S rRNA.

'Wild-type' lacZ, p90.91
AUG ACC AUG ATT ACG CCA AGC UUA GAU UGG GGU AAG GGC CCU AAU UCA CUG

Nonsense Mutants	
p627 UAA	AGC UUU GUG GAA UAA GUU AGC GGC CCU
p853 UAA	AGC UUU GUC UAA GUU AGC GGC CCU
p12–6 UAG	AGC UUU GUU UAG GCC GGC CCU
p163 UAG	AGC UUA GGG UAU CUU UAG CUA CGG GGC CCU
p34–11 UGA	AGC UUU GUG UGA GCC GGC CCU
pCH3/4 UGA	AGC UUA GGG UAU CUU UGA CUA CGA CGG AUC CCC GGG AAU UCA CUG, etc.
Frameshift Mutants	
2p12D(–1)	AGC UUG GG AUA AGG AUC CCC GGG AAU UCA CUG
plac10 (–1)	AGC UUU GUG GUA UA GUU AGC GGC CCU
plac7 (+1)	AGC UUU GUGU AGG GUU AGC GGC CCU
2p991 (–1)	AGC UUU AAG UAC GU AUA GAA GGC CCU
4pCCCU (+1)	AGC UUA GAU CCCU GCU AAC UGG AUC CCC GGG AAU UCA CUG, etc.

Figure 2. Sequences of the 5' end of constructed 'wild type,' nonsense and frameshift lacZ alleles used in this study. The underlined AUG and CUG codons in the wild type sequence correspond to the codons for the initiator methionine and codon #8 respectively of the bona fide wild type lacZ gene of *E.coli*. Only bases surrounding the site of the frameshift or nonsense mutation which differ from the wild type sequence are indicated.

reflect the cell's response to the differing sets of growth conditions under which the plasmid-coded RNA is transcribed, viz. a constitutive level of transcription from the P_1P_2 promoters in the pcnB⁻ strain during growth at 37°C compared with a rapid induction from the strong P_L promoter during growth at 42°C.

Effects on translational fidelity of mutations at 517/534

Mutations at position 517 were first isolated in yeast mitochondria on the basis of their ability to suppress UAA stop codons and were found to be specific for UAA codons in that organism (7). This specificity raised the possibility that this region of 16S rRNA might be involved in termination at UAA stop codons in a manner similar to which the 1199–1204 region of 16S has been implicated in termination at UGA codons (29, 30). Using a lacZ reporter gene, the effects of mutations at positions 517 and 534 on the levels of readthrough of stop codons and frameshifting was examined.

A series of lacZ mutants was constructed as described in Materials and Methods. Each of these mutant derivatives contains either a stop codon or a frameshift mutation at the 5' end of the lacZ gene so that a readthrough or frameshift event is necessary to produce active, full length β-galactosidase (Fig. 2). Analogous systems have been used by others to measure the effects of ribosomal protein (ram) mutants on nonsense readthrough and frameshifting (31, 32). Each lacZ mutant was transformed in turn with each of the ribosomal RNA mutants and β-galactosidase activities were measured. The assay data, presented in Table 2 show that all three base substitutions at position 517 allowed increased levels of readthrough of stop codons and frameshifting. The level of readthrough and frameshifting observed depended both on the sequence of the lacZ construct employed and the identity of the base substitution at 517. Similar results were obtained with mutant rRNAs from pSTL102 and pKK3535. The C517 mutant, which was only mildly affected in growth rate, appeared to be the most permissive of the three substitutions in allowing nonsense readthrough and frameshifting. The other two substitution mutations at position 517, which had more significant growth defects, showed rather low levels of readthrough of few

Table 2. Nonsense readthrough and frameshifting in strains carrying mutant and wild-type rRNA plasmids

	wt	517C	517U	517A	534C	534G	1734UG
Nonsense Readthrough							
p627UAA	6.5 ± 1.5	27.4 ± 4.2	8.2 ± 3.1	10.4 ± 1.0	5.4 ± 0.7	5.4 ± 0.2	6.5 ± 0.5
p853UAA	11.6 ± 2.1	21.5 ± 2.6	23.0 ± 2.7	33.0 ± 5.0	10.1 ± 0.2	9.8 ± 0.2	13.2 ± 1.6
p12-6UAG	7.3 ± 2.2	67.4 ± 25.7	11.8 ± 2.7	8.4 ± 2.0	7.8 ± 2.2	4.2 ± 1.0	17.0 ± 1.8
p163UAG	37.4 ± 8.5	166.1 ± 25.7	78.3 ± 21.3	135.0 ± 15.3	37.9 ± 4.1	35.5 ± 4.3	70.4 ± 1.9
p34-11UGA	13.1 ± 1.8	21.5 ± 1.9	13.9 ± 3.0	14.5 ± 3.6	11.0 ± 1.3	10.3 ± 1.4	10.5 ± 0.7
pCH3/4UGA	89.8 ± 12.9	157.8 ± 18.7	156.3 ± 13.6	156.2 ± 23.9	75.1 ± 8.8	75.9 ± 13.1	139.5 ± 14.4
Frameshifting							
2p12DP (−1)	55.4 ± 7.9	86.8 ± 11.1	101.3 ± 9.8	140.8 ± 21.6	47.6 ± 2.5	46.3 ± 1.1	93.9 ± 11.6
plac10 (−1)	5.1 ± 1.0	15.0 ± 2.8	9.4 ± 1.3	12.3 ± 0.7	5.1 ± 0.4	4.4 ± 0.9	10.4 ± 1.7
plac7 (+1)	14.7 ± 0.6	31.1 ± 5.1	25.8 ± 5.4	24.7 ± 3.8	15.1 ± 0.4	11.6 ± 1.0	26.2 ± 6.1
2p991 (−1)	7.6 ± 1.7	27.6 ± 3.1	10.7 ± 1.8	11.0 ± 1.8	8.1 ± 2.8	10.1 ± 0.4	14.8 ± 1.8
4pCCCU (+1)	20.6 ± 3.4	32.6 ± 1.5	27.6 ± 7.4	38.7 ± 3.6	13.7 ± 0.9	14.0 ± 0.7	23.2 ± 0.5
Wild type lacZ p90.91 (Miller Units)	3,879 ± 412	3,560 ± 284	3,584 ± 156	3,362 ± 460	3,904 ± 496	3,959 ± 137	3,663 ± 164

Values for nonsense readthrough and frameshifting are expressed as suppression $\times 10^4$. Values for the wild type lacZ gene are expressed as (Miller) units of β-galactosidase activity (14). Each value is the result of 3−6 independent experiments. Assay conditions and calculations are described in Materials and Methods.

constructs. In all base substitution mutations examined, the steady-state level of wild type β-galactosidase did not differ from the level supported by non-mutant ribosomes (Table1). This suggests that ribosome drop-off events (33) or multiple shifts in reading frame cannot account for the low levels of readthrough observed with the slow-growing U517 and A517 mutants.

In contrast to the significant translational errors produced by mutations at position 517, translational fidelity was unaltered by the 534 base substitution mutations. Only in the U517 G534 double mutant was any increase in misreading observed. Both the level and specificity of this readthrough was similar to that seen in the U517 single mutant and so suggested that changes at 534, in addition to having little effect on cell growth, did not affect the level of translational accuracy.

The effects of the deletion mutations on fidelity were measured following induction by a shift in temperature of strains carrying the pNO series of plasmids. The data presented in Table 3 show that deletion of base 517 or of 517 and 516 allowed readthrough of stop codons and promoted frameshifting. The levels of readthrough seen with the deletion mutants are approximately the same as those seen with the 517 base substitution mutants. However, since the deletion mutants are not well represented in polysomes, they may be much more potent misreaders than any of the base substitution mutants.

All base substitution mutants were tested for their sensitivity to streptomycin. Unlike other mutants in the 530 loop region (4, 5, 6), none of the 517 or 534 mutants conferred streptomycin resistance.

Table 3. Nonsense readthrough and frameshifting directed by pNO2680 (WT), pNOΔ517 and pNOΔ516/17

	wt	Δ517	Δ516/17
Nonsense Readthrough			
p853UAA	4.1 ± 1.1	7.3 ± 2.9	6.4 ± 1.7
p163UAG	21.7 ± 5.3	34.7 ± 1.1	49.8 ± 9.2
pCH3/4UGA	43.6 ± 6.1	111.0 ± 21.6	95.9 ± 14.2
Frameshifting			
2p12DP (−1)	37.3 ± 4.0	53.9 ± 9.4	74.3 ± 15.9
plac10 (−1)	9.0 ± 1.7	53.1 ± 12.7	38.3 ± 5.1
Wild type lacZ p90.91 (Miller Units)	700 ± 21	245 ± 46	296 ± 10

All plasmids were in MC127F′ lac I^Q Δ ZM15, pLG857. β-galactosidase activities were measured after 2−1/2 hours of induction at 42°C. Values for nonsense readthrough and frameshifting are expressed as suppression $\times 10^4$. Values for the wild type lacZ gene are expressed as (Miller) units of β-galactosidase activity (14).

DISCUSSION

Here we have described the effects of base substitution mutations at positions 517 and 534 as well as deletions of 517 and 516/517 in a highly conserved region of 16S rRNA involved in ribosome A site-tRNA interactions. All base changes at 517 and the deletions had significant effects on cell growth and on translational fidelity as measured *in vivo* using a *lacZ* reporter gene system. Not only did the mutants read through UAA, as originally reported by Shen and Fox (7), but also read through UAG and UGA as well as stimulating +1 and −1 frameshifting. Thus the integrity of the guanosine residue at position 517 is important for maintaining translational fidelity and mutations at this site produce a significant ribosomal ambiguity (ram) phenotype. The ram phenotype has also been observed in another region of 16S rRNA, when a C→U mutation was introduced at position1469 (34). Little or no effect on fidelity was seen with mutations at position 534.

The high degree of translational fidelity achieved by wild type ribosomes is due to a two-step process involving an initial selection of the tRNA followed by a proofreading step. Mutations have been isolated in ribosomal proteins S4, S5, S12, S17, L6 and L7/L12 and in elongation factor Tu which affect the level of translational fidelity (35). The S4, S5 and S12 mutants have been shown specifically to affect the proofreading step while the EFTu mutants are defective both in the initial discrimination step and in the proofreading step (36, 37). Several lines of evidence link these proteins to the 530 region and to the selection of cognate tRNAs at the ribosomal A site. Specific residues in the 530 stem

and loop are protected by S4 and S12 in chemical protection experiments (38). The binding site for elongation factor Tu has been mapped to the neck region of the ribosome by immune electron microscopy, the same region to which the 530 loop has been mapped (39, 40). There is, however, no evidence for direct contact between EFTu and the 530 region. Resistance to the antibiotic streptomycin, which also affects the proofreading step, can be obtained both by mutations in ribosomal protein S12 and at several positions in the 530 loop (4). The data on the 517/534 mutants presented in this paper provide further evidence for the involvement of the 530 region in the maintenance of translational fidelity.

The 517 mutants, like the well-studied S4 (ram) and EFTu mutants, allow readthrough of all three stop codons as well as increasing the frequency of frameshifting (31,41). *In vitro* studies with mutant EFTu and ram ribosomes have shown that they each support high levels of missense errors (36, 42). In the absence of any mutant translational components, a low level of readthrough of stop codons is observed *in vivo*. Readthrough of UGA occurs through recognition of UGA by $tRNA^{TRP}$ via a single base mismatch at the third position of the codon (43) while UAG and UAA stop codons can be misread *in vivo* by CAA and CAG-decoding $tRNA^{GLN}$ through mispairing at the first position of the codon (44, 45). Although direct evidence is lacking, it seems likely that the 517, EFTu and ram mutants merely increase the frequency of these naturally-occurring 'misreading' events at stop codons. Several models can be invoked to explain the effect of increased levels of missense errors on the frequency of frameshifting. Kurland (46) has proposed that distortion of the codon-anticodon geometry (through codon-anticodon mismatches or mutations in the tRNA outside of the anticodon) may enhance the ability of such mismatched tRNAs to shift frame. Support for a coupling between the frequency of missense and frameshift errors comes from recent studies with mutants of EFTu (47). These studies have shown that the frameshift events are coupled to misreading events at downstream stop codons. Sequencing of proteins produced by frameshifting during translation of the MS2 coat protein gene has suggested that doublet decoding events can occur when two bases of the anticodons of certain tRNAs are paired (in-frame) with a non-cognate codon (48). More recent investigatigations into the sequence requirements necessary for high level frameshifting indicates that many originate from re-pairing of a tRNA onto an overlapping, cognate codon (49). The ability of ribosomal mutants to stimulate these different frameshifting events remains to be tested.

Both phylogenetic and mutational analyses have helped to elucidate the structure of the 530 region. Powers and Noller have shown by site directed mutagenesis that a pseudoknot exists by pairing bases 524−526 to bases 505−507 (4). This interaction is thought to be stabilized by ribosomal protein S12. Moreover, certain combinations of bases which disrupt this pseudoknot give rise to streptomycin resistant ribosomes, confirming the importance both of the primary sequence and higher order structure of this region in determining the level of translational fidelity. Phylogenetic analyses by Gutell et al. (13) led to the proposal that neither 517−534 nor 516−535 are base paired. Our data presented here confirm the proposal that bases 517 and 534 are indeed, not paired.

The precise location of the 530 region with respect to other regions of the ribosome remains obscure. Most models of the ribosome place this region at some distance from the decoding center but nonetheless coupled to it allosterically. More recent data, however, place it considerably closer to the site of codon-anticodon interaction (10). Given this uncertainty, several explanations exist for the effects of the 517 mutations on translational fidelity. One possibility is that mutation of the bases which comprise the decoding site enhance the stability the non-specific contacts between tRNAs and the ribosome at the expense of the codon-anticodon interaction and so increase the affinity of non cognate tRNAs for a programmed ribosome. Alternatively, since the 530 region has also been localized to the site of interaction between EFTu and the ribosome, mutations in this region may affect the efficiency of this interaction, decreasing the rate at which non-cognate ternary complexes are discarded from the ribosome. The data obtained with the 517 mutants cannot distinguish between a defect in an initial discrimination or in a subsequent proofreading step. However, given the involvement of ribosomal proteins S4 and S12 in the proofreading step and their location in the vicinity of the 530 region, it would not be surprising to discover that the 517 mutants are defective in proofreading.

Growth rates of ribosomal mutants provide an index of their performance *in vivo*. The C517 mutant is only mildly affected in growth rate but yet has the highest overall misreading levels of the substitution or deletion mutations. This suggests that other steps of translation besides fidelity may be affected in the A517, U517 and 517 deletion mutants. This is clearly the case in the 517 and 516/517 deletion mutations where the level of β-galactosidase obtained with a wild type lacZ plasmid construct in the presence of these mutant ribosomes is only 40% of that obtained with wild type ribosomes (Table 3). Since the polysome fractions from these deletion mutants contain a minority (18−30%) of mutant rRNA, the 60% reduction in β-galactosidase levels suggests that the few mutant ribosomes in the polysome fraction are affecting the progression of wild type ribosomes along the mRNA chain. In contrast the 517 substitution mutations display variable decreases in growth rate, but the mutant rRNAs are equally well represented in 30S, 70S and polysome fractions and they all have approximately the same level of wild type β-galactosidase synthesis (Table1). Thus while we have successfully demonstrated that these mutants have a significant effect on translational fidelity, presumably on the binding of tRNA to the A site, the other effects of the mutants on the process of translation remain to be uncovered.

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