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Mutations at U2555, a tRNA-protected base in 23S rRNA, affect translational fidelity

(rRNA mutations/frameshifting/stop codon readthrough/translational accuracy/ribosomal A site)

MICHAEL O'CONNOR AND ALBERT E. DAHLBERG

Section of Biochemistry, Box G, Brown University, Providence, RI 02912

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ABSTRACT **A plasmid carrying a mutation in the highly conserved base U2555 in *Escherichia coli* 23S rRNA was isolated by selecting for suppression of the −1 frameshift mutation *trpE91*. U2555 is normally protected in chemical footprinting experiments by the aminoacyl residue of A-site-bound tRNA. Substitution of U2555 by adenine or guanine (but not by cytosine) increased readthrough of all three stop codons and +1 and −1 frameshifting. These effects on translational fidelity demonstrate the importance of U2555 for selection of the correct tRNA at the ribosomal A site.**

Translation is a rapid and accurate process, proceeding at a rate of 10–20 amino acids per sec with a misincorporation frequency in the range of 10^{-3}–10^{-4} per amino acid (1, 2). However, the error rate can be altered both by the addition of certain antibiotics and by mutations in several translational components. Streptomycin and other aminoglycoside antibiotics cause misreading of the genetic code, whereas kasugamycin appears to increase the accuracy of translation (3, 4). Mutations in ribosomal proteins S12, S17, and L6 increase fidelity, whereas mutations in S4, S5, L7/L12, elongation factor (EF)-Tu and release factors 1 and 2 decrease the accuracy of protein synthesis (5). Recently, evidence has accumulated from several sources that suggests the direct involvement of rRNA in all stages of translation, including the accuracy of tRNA selection (6). Chemical footprinting experiments have shown that tRNA protects a distinct group of highly conserved residues in rRNA, and the accessibility of some of these residues is altered in ribosomes containing mutant forms of ribosomal proteins S12 or S4, which alter the accuracy of translation (7). Furthermore, a number of mutations have been isolated in rRNA that influence the fidelity of translation (8–10). Thus, the interactions of tRNAs and translation factors with rRNA, as well as with ribosomal proteins, modulate the accuracy of protein synthesis.

During a single round of translation, tRNAs contact a number of bases in 16S and 23S rRNA. Residues in both rRNAs have the potential to influence the interaction of tRNAs with the ribosome and, consequently, the selection of the correct tRNA. In our effort to understand the role of rRNA in the process of tRNA discrimination, we have sought to isolate rRNA mutants that decrease the accuracy of translation. Specifically, we have selected for mutants that cause frameshifting. This particular genetic selection was chosen to enable us to recover mutants that were affected at various stages of the translation cycle. We reasoned that while missense errors and readthrough events occur primarily at the level of binding of noncognate tRNAs to the A site, frameshift events might occur at several stages in the elongation cycle (11, 12). Using this approach, we have isolated a mutation in a highly conserved base in 23S rRNA that increases the levels of frameshifting as well as stimulating readthrough of stop codons. The residue, U2555, is also protected from chemical attack by the binding of aminoacylated tRNA to the A site of the ribosome (13). Taken together, the chemical-protection data and the phenotype of the 2555 mutants described here suggest that interaction between tRNA and this 23S rRNA residue is important for selection of the correct tRNA at the ribosomal A site.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Strain MC85 [F⁻ *thi*⁻ Δ(*lac-pro*) *trpE91*], a derivative of strain CSH41 (14) carrying the *trpE91* frameshift mutation (15), was used for the selection of frameshift suppressors. The kanamycin-resistant plasmid pMO10 was constructed by inserting the *Bam*HI fragment (carrying the entire *rrnB* operon) from plasmid pKK3535 into the *Bam*HI site of the low-copy-number, pSC101-derived plasmid pLG339 (16, 17). Plasmid pMO11 is a chloramphenicol-resistant version of pMO10. All Lac mutants used in this study carried the *lacZ* gene on a tetracycline-resistant plasmid derived from pACYC184. These mutants were identical to those used in a previous study, except that the *lacZ* gene in the series of plasmids used here (the pSGlac series) was transcribed from the strong *tac* promoter (9). The high level of transcription of the *lacZ* gene from this promoter allowed greater sensitivity in the detection of low-level readthrough and frameshift events. Strain MC140 [F⁻ *thi*⁻ Δ(*lac-pro*) *recA*⁻] carrying the λ temperature-sensitive repressor on a neomycin-resistant plasmid (pLG857) (9) was used as a host for all the Lac plasmids and plasmids carrying the *rrnB* operon under control of the λ P_L promoter (18).

Mutagenesis. Strain MC85 pMO10 was mutagenized with UV as described (14) and, after overnight growth, the washed, mutagenized cells were plated on minimal E medium (14) containing glucose, thiamine, proline, and kanamycin (25 mg/liter) to select for *trpE91* suppressors. Site-directed mutagenesis of rDNA was done on a M13mp18 *Eco*RI–*Bam*HI clone carrying the 3′ end of the *rrnB* operon, by using the methods described by Kunkel (19). This M13 clone contained the G2058 mutation conferring resistance to erythromycin (20). Plasmid pLEry contained the intact *rrnB* operon under the control of the λ P_L promoter (18) and also contained the G2058 erythromycin-resistance mutation. This plasmid was constructed by replacing the *Eco*RI–*Bam*HI fragment of pLK35 (21) with the *Eco*RI–*Bam*HI fragment from the M13 clone used for the site-directed mutagenesis experiments described above. Mutant clones from the site-directed mutagenesis were identified by sequencing; the mutant *Eco*RI–*Bam*HI fragments were cloned into pLEry, and the presence of the mutations was reconfirmed by sequencing the intact plasmids. Plasmid DNA extraction, transformations, se-

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Abbreviation: EF, elongation factor.

quencing, and other standard procedures were done as described (22).

β-Galactosidase Assays and Growth-Rate Measurements. Cultures to be assayed were grown to saturation at 30°C in LB medium containing neomycin (50 mg/liter), tetracycline (25 mg/liter), and ampicillin (200 mg/liter). These cultures were diluted 1:50, incubated at 42°C for 150 min, and then assayed as described (9). To determine growth rates of strains containing pLEry and its derivatives, strains were grown to saturation at 30°C in LB medium containing neomycin and ampicillin, diluted into fresh medium, incubated with vigorous shaking at 42°C; the increase in turbidity thereafter was followed by using a Klett–Summerson colorimeter.

RESULTS

Isolation of a rRNA Frameshift Suppressor. *trpE91* is a −1 frameshift mutation in the gene encoding anthranilate synthetase and leads to a tryptophan-requiring phenotype (Fig. 1). Previous studies have shown that this requirement can be suppressed by mutations in a variety of translational components. These mutations allow the translating ribosomes to shift frame within a short, defined sequence and read either a doublet or quintuplet, thereby restoring the correct reading frame (15, 23). rRNA suppressors of this frameshift mutation were isolated by mutagenizing a *trpE91* strain (MC85) containing a low-copy-number plasmid carrying the intact *rrnB* operon (pMO10) and plating the mutagenized culture on minimal medium lacking tryptophan. After approximately 2 weeks of incubation, several hundred TRP^+ colonies had appeared on the minimal medium plates. These colonies were pooled, and plasmid DNA was extracted and used to retransform strain MC85. The transformation mixture was plated on kanamycin-containing minimal medium (lacking tryptophan) to select specifically for plasmid-borne suppressors. Four TRP^+ colonies arose from this transformation. Plasmid DNA was isolated from each of these putative rRNA suppressor-containing strains and again was used to transform strain MC85. This time, the transformation mixture was plated on rich (LB) medium containing kanamycin. The transformants were then screened for coinheritance of the TRP^+ phenotype by streaking on minimal medium. Only one of the original four putative rRNA suppressors was shown to be plasmid borne by this means. This suppressor, pMOUV5, grew extremely slowly on rich medium containing kanamycin, which suggested that some critical aspect of translation was perturbed by the mutation in the rRNA.

The mutation on the plasmid allowing suppression of the *trpE91* frameshift was mapped initially by restriction fragment-exchange experiments between pMOUV5, which was used as a donor of the restriction fragment, and the wild-type plasmid pMO10, which was used as a recipient. These experiments localized the suppressor mutation to the 3′ half of 23S rRNA, downstream of the *Pvu* II site corresponding to base 1761 in 23S rRNA. The 3′ half of the operon (between the *Eco*RI site at base 843 in 23S and the *Bam*HI site at the end of the operon) was subcloned onto M13mp18, and the rRNA-coding region of this fragment was sequenced in its entirety. The sole difference between the mutant and wild-type clones was a T2555 → A base substitution in 23S rRNA.

Mutations at Position 2555 in 23S rRNA. The U2555 nt is located in a highly conserved, 5-base loop close to the peptidyltransferase center in 23S rRNA. This base had previously been thought to be a pseudouridine; however, more recent analysis has shown it to be an unmodified uridine (24, 37) (Fig. 2). This residue is protected from chemical modification upon binding of tRNA to the ribosomal A site (13). To examine the effects of other base substitutions at nt 2555 and to exclude the possibility of the presence of secondary mutations on the mutagenized fragment, all three base substitutions were constructed by site-directed mutagenesis. The M13 clone used for site-directed mutagenesis also contained the A2058 → G base substitution, which confers resistance to erythromycin in the intact operon but which has no effect on the growth rate or translational fidelity of the host strain (unpublished results). The G2058 erythromycin-resistance mutation was included in the site-directed mutagenesis experiments so that plasmid-encoded rRNA could be readily identified by primer-extension analysis (26). M13 clones containing the desired mutations were identified by sequencing, and each mutant was cloned into the erythromycin-resistant, inducible expression vector pLEry, with MC140 pLG857 as a host strain. Upon induction of transcription of mutant rRNA by incubation at 42°C, both G2555 and A2555 displayed severely reduced growth rates (doubling times of 79 ± 6 min and 63 ± 5 min, respectively, compared with a doubling time of 41 ± 2 min for the wild-type pLEry plasmid). In contrast, the C2555 mutant had a growth rate virtually indistinguishable from that of the wild type (43 ± 1 min).

Effects on Fidelity of Mutations at nt 2555. The original mutation in nt 2555 was obtained by selection for increased levels of frameshifting at a specific site in the *trpE* message. The ability of all nt 2555 mutants to affect the levels of misreading and frameshifting at other mRNA sequences was examined. We have previously described (9) the construction of a series of *lacZ*-containing plasmids that contain either a nonsense codon or a frameshift in the *lacZ* coding region. The effect of ribosomal mutations on β-galactosidase levels is a reflection of their effects on translational fidelity. Each strain containing a *lacZ* mutation (containing the $c1^{857}$ repressor on plasmid pLG857) was transformed with each of the pL2555 mutants and, after a 2.5 hr induction of transcription of mutant rRNA, the levels of β-galactosidase were measured. The data presented in Table 1 show that both the G2555 and A2555 mutations allow high levels of readthrough of all three

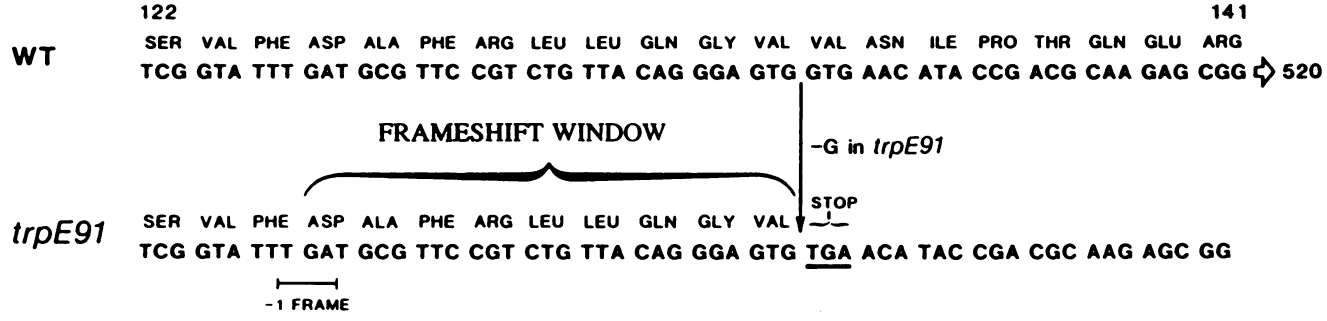

FIG. 1. Sequence of the *trpE91* frameshift mutation and of the corresponding segment of the wild-type (WT) *trpE* gene. The in-frame stop codon created by the deletion of a guanine in *trpE91* and the upstream stop codon in the −1 frame preceding the deletion are underlined. Ribosomes must shift frame within the sequence bounded by these two stop codons to restore translation to the wild-type reading frame.

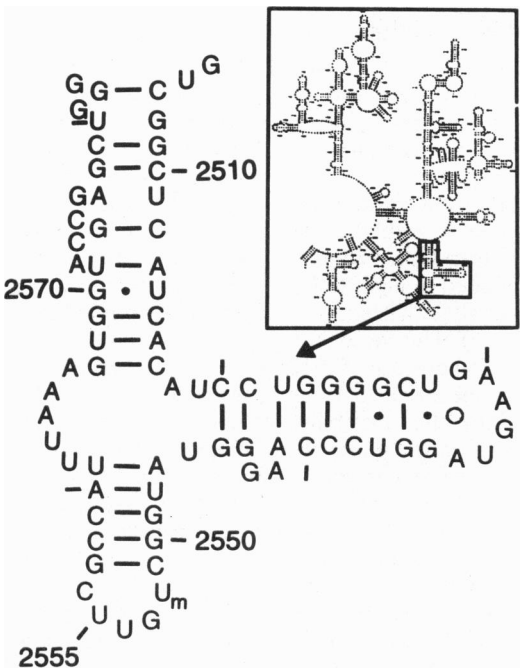

FIG. 2. Sequence and secondary structure of the nt-2555 region of *Escherichia coli* 23S rRNA, enlarged from the model of Gutell and Fox (25). U_m, methylated base.

stop codons and increase the levels of both +1 and −1 frameshifting. By contrast, the C2555 mutation has no effect either on frameshifting or on nonsense-codon readthrough. The levels of misreading and frameshifting seen with the mutants containing G2555 and A2555 correlate well with their growth rates: the more deleterious mutation, G2555, produced significantly higher levels of β-galactosidase with virtually every *lacZ* nonsense and frameshift mutant tested, whereas the mutant containing C2555, which did not affect growth rate, had no effect on translational fidelity.

All three constructed mutations in nt 2555 were also examined for their effects on frameshifting at the *trpE91* frameshift site; this was achieved by expressing these mutants from the constitutive P_1P_2 promoters of the low-copy-number *rrnB*-containing plasmid pMO11, in a *trpE91* strain (MC85) and then testing these rRNA mutant strains for tryptophan independence. The wild-type plasmid and the C2555 mutant showed no suppression of *trpE91*. The mutant containing G2555 could not be stably expressed in pMO11 due to its deleterious effects. The slow-growing G2555 clones that did arise on LB chloramphenicol plates rapidly segregated larger sectors of faster growing cells, which when sequenced proved to contain plasmids of wholly wild-type sequence (U2555) or mixtures of wild-type and mutant plasmids. This result is consistent with the observation above that the G2555 mutation is the most deleterious mutation at this site. The constructed A2555 mutation suppressed the *trpE91* frameshift as efficiently as the original A2555 mutation isolated in pMO10 by UV mutagenesis. This result showed that the U2555 → A base substitution alone was responsible for suppression of *trpE91*. The A2555-containing mutant constructed by site-directed mutagenesis also carried the G2058 erythromycin-resistance mutation. The lack of any difference between the constructed A2555 mutant and the original isolate (which had the wild-type A2058 sequence) renders unlikely the possibility of any synergism between mutant sites 2058 and 2555.

The ratio of plasmid/host-encoded rRNA found in 50S subunits, 70S ribosomes, and polysomes (as determined by primer extension) did not differ significantly between wild-type and mutant strains; this ratio was ≈40–50% in all cases (data not shown). These data indicate that the mutant ribosomes are not impaired in subunit association. The equal distribution of mutant rRNA in subunits, 70S ribosomes, and polysomes together with the lack of specificity seen in the readthrough and frameshifting assays described above suggest that the principal defect in the mutant ribosomes is their reduced ability to discriminate against the binding of non-cognate tRNAs to the A site.

DISCUSSION

In this study, we have used a genetic method to probe the interactions between tRNA and rRNA that determine the selection of the correct ternary complex at the ribosomal A site. The altered residue in 23S rRNA, U2555, is one that has previously been found to be protected by A-site-bound aminoacylated tRNA in footprinting experiments. tRNA-dependent protection from chemicals such as dimethyl sulfate and kethoxal is thought to derive from contacts made between tRNAs and rRNA or from conformational changes induced upon tRNA binding (6). Consequently, these protected bases are believed to be functionally important resi-

Table 1. Effect of mutations at position 2555 in 23S rRNA on frameshifting and stop codon readthrough

	β-Galactosidase activity, units			
lacZ	U2555 (WT)	G2555	C2555	A2555
WT				
pSG25 (WT *lacZ*)	6048.7 ± 329.9	6929.5 ± 494.8	6513.2 ± 253.0	6132.6 ± 847.0
Nonsense mutants				
pSG12-6 UAG	16.6 ± 3.0	49.8 ± 3.4	16.2 ± 2.5	30.5 ± 4.3
pSG163 UAG	33.3 ± 3.1	78.7 ± 3.0	33.3 ± 2.4	58.4 ± 6.0
pSG34-11 UGA	34.2 ± 6.8	225.4 ± 17.2	35.9 ± 6.8	148.2 ± 22.9
pSG3/4 UGA	90.3 ± 7.3	332.0 ± 50.0	86.0 ± 5.7	233.2 ± 30.7
pSG627 UAA	4.2 ± 0.4	10.0 ± 1.1	3.4 ± 0.8	10.5 ± 1.9
pSG853 UAA	6.4 ± 1.9	30.1 ± 5.8	6.0 ± 1.1	25.4 ± 2.0
Frameshift mutants				
pSG12DP (−1)	111.7 ± 14.8	348.3 ± 50.7	97.7 ± 13.4	315.4 ± 36.7
pSG CAUGGA (−1)	11.0 ± 1.0	23.8 ± 2.4	9.6 ± 0.7	21.0 ± 1.6
pSGlac10 (−1)	6.8 ± 0.9	16.9 ± 1.1	7.3 ± 1.6	14.1 ± 0.9
pSGlac7 (+1)	45.4 ± 1.9	106.2 ± 2.3	43.9 ± 1.4	111.4 ± 2.5
pSGCCCU (+1)	18.4 ± 1.8	35.2 ± 1.2	19.9 ± 0.5	34.6 ± 1.9

Values represent Miller units of β-galactosidase activity. Each number represents the average of three to six independent measurements ± SE. β-Galactosidase activities were measured after induction of transcription of plasmid-encoded rRNA at 42°C for 150 min. WT, wild type.

dues in rRNA. Here we present evidence derived from *in vivo* experiments that residue 2555 is functionally important in 23S rRNA; mutations at this position have profound effects on the level of translational fidelity. The genetic and functional data presented here fit precisely with the structure-probing results, and together, they suggest that interaction of the aminoacylated tRNA with U2555 is important for the discrimination between cognate and noncognate tRNAs at the ribosomal A site.

Previous work with 16S rRNA mutants in *E. coli* has shown that mutations at positions in the decoding center of the ribosome, or any of several regions outside it, can affect the fidelity of translation (9, 10, 27, 28). These mutants may affect tRNA–mRNA interaction directly, or the interactions between the ribosome and EF-Tu or release factors during the elongation and termination phases of translation. Mutations in the 2660 region of 23S rRNA decrease misreading by altering the interaction of EF-Tu with the ribosome (29). Recently, mutations at position 2583 in the peptidyltransferase center of the ribosome have been shown to display increased accuracy. This increased accuracy may be due to an altered interaction of the 3′ end of the tRNA with the peptidyltransferase center (30).

When the ribosome binds aminoacylated tRNA complexed with EF-Tu and GTP, codon–anticodon interaction occurs on the 30S subunit where the anticodon stem-loop region of the tRNA protects specific 16S residues from chemical attack. Meanwhile, EF-Tu interacts with the 2660 loop of 23S rRNA and various ribosomal proteins on the 50S subunit (13). EF-Tu shields the 3′ end of the bound tRNA from productive interaction with the peptidyltransferase center of the ribosome during the initial decoding phase of translation (31). The accuracy of translation is enhanced by proofreading, which is associated with the EF-Tu-dependent hydrolysis of GTP (32, 33). After GTP hydrolysis and EF-Tu–GDP dissociation, the bound tRNA protects a group of 23S residues that are not protected in the initial binding of the ternary complex (13). One of these residues is U2555. Thus, a transition exists between the initial interaction of tRNA with the ribosome (as part of a ternary complex) and a subsequent interaction of the same tRNA in the absence of EF-Tu. Both the initial discrimination between correct and incorrect ternary complexes and the subsequent proofreading step(s) must occur before the interaction of the 3′ end of the A-site-bound tRNA with the peptidyltransferase center of the ribosome. Codon–anticodon interaction is confined to the 30S subunit, whereas reactions involving the rest of the ternary complex may be influenced by its interaction with both large and small subunits.

The A2555 and G2555 mutations both lower the discrimination of the ribosome against noncognate tRNAs. This result suggests that these mutations alter the codon–anticodon interaction directly or allow the ribosome to bypass the proofreading step. A direct effect of a 23S rRNA mutation on the decoding site in the small subunit seems unlikely. However, the emergence of the nt-2555 protection after GTP hydrolysis and dissociation of EF-Tu–GDP is compatible with mutations at nt 2555 influencing the degree of proofreading by altering the interactions between the ribosome and EF-Tu. Mutations at nt 2555 could directly increase the nonspecific interactions of the ternary complex with the ribosome at the expense of the codon–anticodon interaction. Alternatively, the mutations might increase the rate of ribosome-dependent GTP hydrolysis by EF-Tu (and the subsequent dissociation of EF-Tu–GDP), thereby decreasing the likelihood of rejection of the bound tRNA before its interaction with the peptidyltransferase center. In either case, these models predict that the mutations at position 2555 influence the interaction between EF-Tu and the ribosome but do so with a loss of discrimination between cognate and noncognate tRNAs.

A second class of models proposes that the nt-2555 mutants have no direct influence on ribosome–EF-Tu interactions but affect the interaction of the 3′ end of the tRNA with the peptidyltransferase center of the ribosome. Increasing the rate at which the 3′ end of a tRNA can interact productively with the peptidyltransferase center will lower the probability of rejection of the bound tRNA (both cognate and noncognate) from the ribosome. A variation on this model is that the mutations at nt 2555 may enhance, or stabilize, the contacts between the 3′ end of the tRNA and the large ribosomal subunit, thus overcoming to some extent, any unfavorable codon–anticodon interaction in the small subunit. During peptide bond formation, the 3′ ends of A- and P-site-bound tRNAs must come into close contact to allow transpeptidation to occur. Structural studies indicate that 2555 loop and the 3′ end of the tRNA are in close association. Chemical protection of U2555 in footprinting studies depends upon the aminoacyl residue at the 3′ end of the tRNA, and protection of an adjacent residue, G2553, depends upon an intact CA end. In addition, two UV-induced crosslinks have been obtained between the 2555 loop and the 1945 region in domain IV of 23S rRNA, the same region to which the terminal adenine of $tRNA^{Phe}$ has been crosslinked (34, 35). Together, these data support the notion that mutations at position 2555 can influence the binding of noncognate tRNAs to the ribosome by facilitating the interaction of 3′ end of A-site-bound tRNAs with the catalytic center of the 50S subunit.

U2555 is a highly conserved nucleotide in all large subunit rRNAs. The strong phylogenetic conservation argues for an important role for this nucleotide. The U → G and U → A changes constructed here had profound effects on ribosome performance *in vivo*. However, the mutant carrying C2555 behaved exactly like the wild-type rRNA. In a small group of archaebacteria, cytosine is found at position 2555 (36). Thus, we observe that the sole variation found at position 2555 in nature is precisely the change that had no effect on ribosome performance *in vivo*.

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