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The influence of base identity and base pairing on the function of the α-sarcin loop of 23S rRNA

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ABSTRACT

The α-sarcin loop of large subunit rRNAs is one of the sites of interaction of elongation factors with the ribosome, and the target of the cytotoxins α-sarcin and ricin. Using a genetic selection for increased frameshifting in a reporter gene, we have isolated a C→U mutation at position 2666 in the α-sarcin loop. In the NMR-derived structure of the loop, bases equivalent to 2666 and 2654 are paired via a non-canonical base pairing interaction. Each of the three base substitutions at C2666 and A2654 was constructed by site-directed mutagenesis of a plasmid borne copy of the *rrnB* operon of *Escherichia coli*. Only the C2666→U and A2654→G mutations that resulted in the formation of canonical A-U and C-G base pairs respectively, increased the levels of stop codon readthrough and frameshifting. The effects of different base pair combinations at positions 2666 and 2654 on ribosome function were then tested by constructing and analyzing all possible base combinations at these sites. All A→G base substitution mutations at position 2654 and C→U substitutions at position 2666 increased the levels of translational errors. However, these effects were greatest when G2654 and U2666 had the potential to engage in standard Watson–Crick base pairing interactions. These data indicate that base identity as well as base pairing interactions are important for the function of this essential component of the large subunit rRNA.

INTRODUCTION.

The α-sarcin loop of large subunit rRNAs is among the most highly conserved of all RNA sequences and has been identified as one of the sites in the ribosome that interacts with elongation factors. The bacterial elongation factors Tu and G, and the eukaryotic factor, EF2, protect several bases in this loop from chemical modification (1,2). A variety of plant and fungal toxins, including α-sarcin and ricin also interact with this region of rRNA; these toxins cleave or depurinate specific bases within the loop and abolish all factor-dependent translational events (3).

Elongation factor Tu is responsible for the delivery of aminoacyl tRNA to the ribosomal A site. The elongation factor also has a role in the selection of the correct tRNA by the ribosome; several mutations in EF-Tu and its eukaryotic equivalent, EF1α, have been isolated that decrease the fidelity of translation (4,5). In addition, mutations in ribosomal proteins S12 and L7/L12 that affect the fidelity of translation, alter EF-Tu–ribosome interactions (6). Mutations in the α-sarcin loop have also been shown to influence the fidelity of translation through their effects on EF-Tu–ribosome interactions. A G→C mutation at position 2661, the site of cleavage by the toxin α-sarcin, increased the fidelity of translation by decreasing the affinity of EF-Tu for the ribosome (7,8). Conversely, mutations at position 2658 (*Escherichia coli* numbering) in yeast 28S rRNA have been isolated that cause suppression of nonsense and frameshift mutations (9).

The structure of a 23mer oligonucleotide corresponding to the α-sarcin loop of rat 28S rRNA has recently been solved by NMR spectroscopy (10). The structure of the RNA fragment is characterized by a series of non-canonical base pairings within the loop. In this paper, we describe the isolation of mutations at one non-standard base pairing site in the α-sarcin loop that increase frameshifting and readthrough errors during translation. These mutations are at positions A2654 and C2666. As part of our analysis, we have constructed all 15 possible base combinations at these two positions. Replacement of C2666 with U, or A2654 with G resulted in elevated levels of stop codon readthrough and frameshifting. While the greatest effects were seen with G-C, G-U or A-U base pair combinations, the U-A and C-G mutants had no effect. These results indicate that the identity of individual bases, as well as the type of base pairings within the α-sarcin loop, are important for the function of this universally conserved element of the large subunit rRNA.

MATERIALS AND METHODS

Bacterial strains and plasmids

Strain MC126 [F^- Δ(Lac-Pro) thi^- trpE91 $recA^-$ srl^-] was used for the isolation of rRNA suppressors of the *trpE91* frameshift mutation. Plasmid pMO11 is derived from the pSC101-based plasmid, pHSG575 (11) and contains the intact *rrnB* operon under the control of the native P_1P_2 promoters. In plasmid pLK35, the rrnB operon is transcribed from the inducible λP_L promoter. In the presence of the thermolabile λcI repressor, transcription of the *rrnB* operon is induced by a temperature shift to 42°C. Strain MC140 [F^- Δ(Lac-Pro) thi^- $recA^-$ srl^-] transformed with pLG857 (encoding the temperature-sensitive λcI_{857} repressor) was used as a host for pLK35-derived plasmids. The pSG and pLM series of lacZ

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mutant plasmids contain nonsense or frameshift mutations in the 5′ end of the lacZ coding region (12). The dut⁻ung⁻ strain, CJ236 was used to make uracil-containing DNA for site directed mutagenesis. XL1 was used as a host for M13 phages. Bacteria were routinely cultivated in LB medium, supplemented appropriately with antibiotics (200 μg/ml of ampicillin, 5μg/ml of neomycin and 12.5 μg/ml of tetracycline).

Mutagenesis

Mutagenesis of MC126 pMO11 with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine was carried out as described by Miller (13). Site directed mutagenesis was performed as described in Kunkel *et al.* (14), using an M13 clone carrying the *Eco*RI–*Bam*HI fragment of plasmid pLK35, encoding the 3′ half of 23S rRNA.

Isolation and analysis of rRNA

Ribosomes were extracted from logarithmically-growing cells following a 90 min induction of transcription of mutant rRNA. 30S and 50S subunits were separated from 70S ribosomes and polysomes as described by Tapprich and Dahlberg (7). Fractions were collected by displacement with 50% sucrose and precipitated with ethanol. RNA was extracted with phenol and phenol/chloroform, and precipitated with ethanol. The amount of plasmid-encoded rRNA in each fraction was determined by primer extension as described by Sigmund *et al.* (15) and the bands corresponding to plasmid-encoded and chromosomally encoded rRNA extension products were quantitated using a Fuji phosphorimager. Because of problems associated with stalling of the reverse transcriptase within the α-sarcin loop, an allele-specific priming site in the 1360 region of 23S rRNA (16) was used as a marker for plasmid-encoded rRNA expression in the primer extension experiments. The growth rates and suppressor activities of α-sarcin loop mutants carrying the 1360 allele-specific priming site did not differ from mutants carrying a wild-type 1360 region (data not shown).

Growth rate determinations and β-galactosidase assays

Growth rates of strains carrying pLK35-derived plasmids were determined by diluting overnight cultures into fresh LB medium supplemented with ampicillin (200 μg/ml) and neomycin (50 μg/ml), incubating the cultures in a shaking water bath at 42°C and monitoring the increases in turbidity with a Klett–Summerson colorimeter. Our previous measurements of doubling times of strains carrying the wild-type pLK35 plasmid (or equivalent derivatives) have shown that this value ranged between 38 ± 2 min and 45 ± 3 min (12,17,18). Doubling times of strains contained within this range of values were not considered to be significantly different. β-Galactosidase activities of logarithmically-growing MC140 pLG857 cells carrying pLK35-derived plasmids and any of the pSG or pLM series of lacZ mutants were determined after transcription of mutant rRNA had been induced for 2.5 h by growth at 42°C. Assays were carried out as described (17).

RESULTS

Isolation and identification of frameshift suppressors in the α-sarcin loop of 23S rRNA

Plasmid encoded rRNA suppressors of the *trpE91* frameshift mutation were isolated as described (18). Briefly, strain MC126, carrying the *rrnB*-containing, chloramphenicol-resistant plasmid, pMO11, was mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and tryptophan-independent revertants were isolated by plating the mutagenized culture directly on minimal medium lacking tryptophan. Plasmid-borne suppressors were separated from chromosomal suppressors and *trpE* revertants by isolating plasmid DNA from pooled Trp^+ colonies, transforming MC126 with this DNA and selecting for chloramphenicol resistance and tryptophan-independence. A further round of transformations, using plasmid DNA isolated from individual Trp^+ isolates, confirmed that the suppressors were plasmid-encoded. Many different classes of suppressors were isolated in these experiments, based on the efficiency of suppression and other growth characteristics (described in refs 12 and 18). A class of very weak suppressors isolated in these mutagenesis experiments is analyzed here. Strain MC126 containing these suppressors took 7–10 days to grow on minimal medium and, in contrast to strong rRNA suppressors, the mutant plasmids had no effect on the growth rate of the cell, when grown in rich (non-selective) medium. In 10 mutagenesis experiments, five independent isolates of this class of weak suppressor were isolated.

The suppressor mutations were localized to 23S rRNA by constructing a *Stu*I deletion in the suppressor-containing plasmids. This 371 bp deletion inactivates 16S rRNA but does not affect the function of 23S rRNA. As the suppressor phenotype was retained in each of the deletion derivatives, it was concluded that the mutations were in 23S rRNA. The suppressor mutations were identified by cloning the 3′ end of 23S rRNA (*Sph*I–*Bam*HI fragments) from each suppressor containing plasmid into M13mp19 and sequencing the coding region in its entirety. All five independent isolates of this class of weak suppressor were found to contain a C→U mutation at position 2666 in 23S rRNA (Fig. 1).

Mutations at position 2666 in the α-sarcin loop

Position 2666 lies in the highly conserved α-sarcin loop of 23S rRNA that is involved in ribosome–EF-Tu and EF–G interactions. To examine the effect of other mutations at position 2666 on ribosome function, all three mutations were constructed by site directed mutagenesis and the mutant rRNAs were expressed in plasmid pLK35 where the *rrnB* operon is transcribed from the inducible λP_L promoter. As can be seen from Table 1, none of the mutations at position 2666 had any significant effect on growth rate. The effects of the rRNA mutations on translational fidelity were assessed by transforming strains containing *lacZ* nonsense or frameshift mutations with pLK35-derivatives carrying the rRNA mutations and measuring β-galactosidase activities in these strains. The results, presented in Table 1, showed that only the C→U mutation at position 2666 had any significant effect on stop codon readthrough or frameshifting. Little or no effect was observed with the other mutations at position 2666. In eukaryotic rRNAs, an adenosine residue is present at the position equivalent to C2666 in *E.coli* 23S rRNA. In the NMR-derived structure of the eukaryotic α-sarcin loop, the positions equivalent to positions 2666 and 2654 are paired via a non-canonical A-A pair (10). The lack of effect on fidelity of the C→A and C→G substitutions at positions 2666 raised the possibility that the effect of the C→U mutation was due to the creation of a Watson–Crick pair between positions A2654 and U2666. This possibility was tested by constructing all three mutations at position 2654.

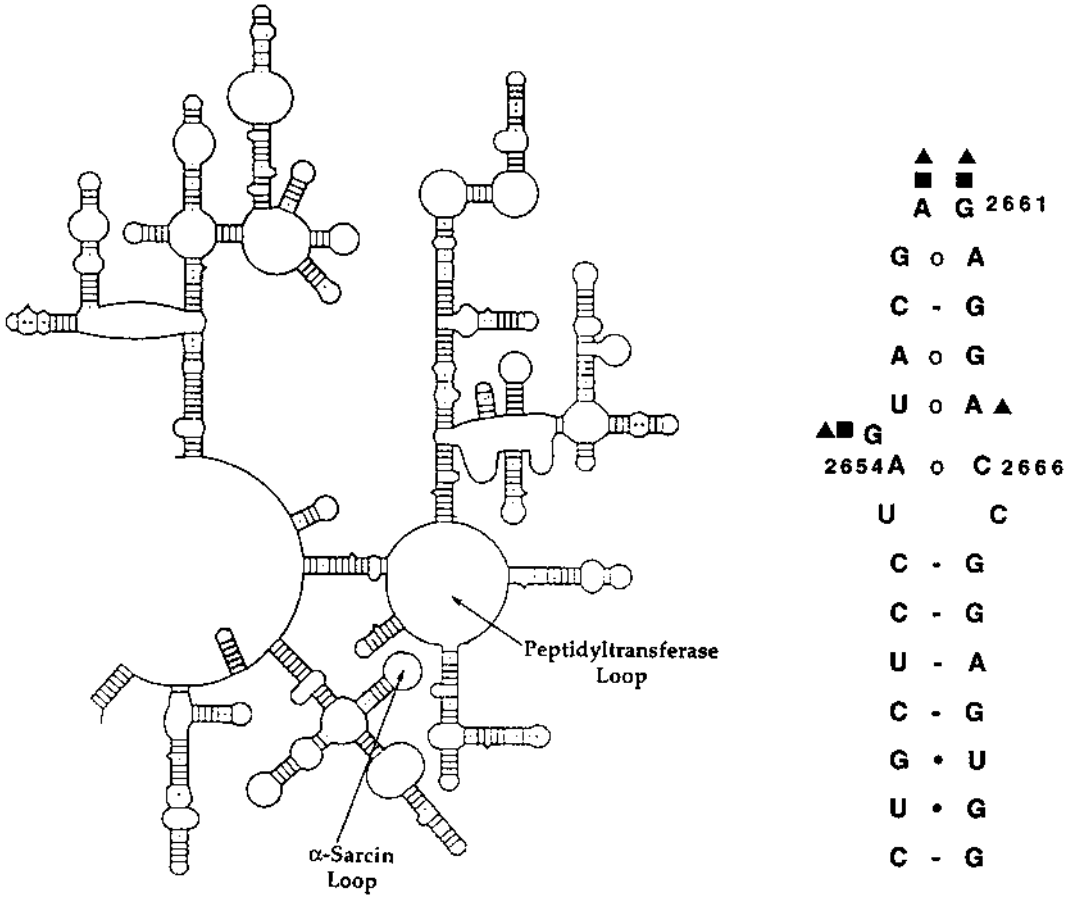

Figure 1. Secondary structure of the 3′ half of 23S rRNA (left hand panel) and a proposed secondary structure of the α-sarcin loop of *E.coli* 23S rRNA (right hand panel), based on the NMR-derived structure of the rat cytoplasmic sarcin/ricin loop (10). The symbols (–) and (●) indicate standard, Watson–Crick and G-U wobble pairings respectively, while open symbols (○) indicate non-canonical pairings. EF-Tu- and EF-G-dependent footprints are denoted by the symbols ▲ and ■ respectively.

Table 1. Effects of mutations at positions 2654 and 2666 on growth rate, stop codon readthrough and frameshifting

rRNA mutation	2654–2666 base pair	Doubling time (min)	Units of β-galactosidase activity (lacZ mutants) pSG853 (UAA)	pSG3/4 (UGA)	pSG163 (UAG)	pSG12DP (–1)	pLM161 (–1)	pLM211 (+1)
Wild-type	A-C	41 ± 1	6.4 ± 0.6	79.8 ± 7.8	33.2 ± 0.1	82.0 ± 1.9	15.1 ± 3.0	58.1 ± 5.8
U2666	A-U	39 ± 1	19.6 ± 0.8	130.0 ± 11.4	47.2 ± 0.7	229.6 ± 0.7	59.4 ± 4.2	178.8 ± 32.8
G2666	A-G	44 ± 3	5.9 ± 0.5	84.5 ± 4.2	33.5 ± 1.0	99.5 ± 2.1	23.7 ± 1.3	94.4 ± 5.1
A2666	A-A	46 ± 1	8.0 ± 1.1	93.7 ± 14.3	36.5 ± 3.5	117.8 ± 4.8	14.6 ± 0.1	63.9 ± 5.1
C2654	C-C	41 ± 2	4.2 ± 0.3	80.2 ± 6.5	33.2 ± 2.0	61.4 ± 3.1	12.6 ± 0.7	61.3 ± 2.0
U2654	U-C	40 ± 1	5.8 ± 0.7	91.6 ± 8.1	32.0 ± 1.5	111.4 ± 3.0	22.2 ± 1.7	78.3 ± 4.8
G2654	G-C	41 ± 2	24.5 ± 2.1	145.0 ± 14.8	66.0 ± 2.3	382.8 ± 11.9	80.8 ± 3.1	495.3 ± 58.4
G2654/U2666	G-U	39 ± 1	23.3 ± 2.2	141.3 ± 20.4	56.5 ± 3.4	315.6 ± 3.1	82.9 ± 1.1	223.3 ± 19.4
G2654/G2666	G-G	45 ± 2	18.3 ± 1.7	128.2 ± 12.1	36.6 ± 1.7	171.3 ± 11.2	43.3 ± 0.8	153.4 ± 16.2
C2654/U2666	C-U	41 ± 1	17.5 ± 1.2	121.3 ± 15.4	36.8 ± 0.8	161.3 ± 7.1	40.4 ± 1.5	163.3 ± 7.0
C2654/G2666	C-G	42 ± 2	7.2 ± 0.8	103.8 ± 9.5	31.2 ± 2.3	94.0 ± 5.4	16.9 ± 1.5	71.3 ± 3.4
U2654/A2666	U-A	42 ± 1	7.6 ± 0.4	98.2 ± 9.1	30.9 ± 0.7	91.2 ± 6.6	16.6 ± 1.3	75.2 ± 6.1
U2654/G2666	U-G	38 ± 1	9.1 ± 0.8	96.3 ± 10.0	30.6 ± 0.7	153.9 ± 9.8	30.4 ± 2.4	100.7 ± 12.0
U2654/U2666	U-U	42 ± 1	8.7 ± 0.3	108.3 ± 8.6	30.1 ± 0.8	108.3 ± 6.0	26.2 ± 2.1	75.5 ± 2.1
C2654/A2666	C-A	44 ± 3	8.9 ± 0.6	104.2 ± 11.8	30.1 ± 1.1	100.3 ± 3.8	28.1 ± 2.5	75.9 ± 9.3
G2654/A2666	G-A	38 ± 1	18.9 ± 0.4	138.2 ± 3.1	35.9 ± 0.6	213.8 ± 1.1	59.8 ± 2.9	178.7 ± 11.0

Values for stop codon readthrough and frameshifting are expressed in Miller units of β-galactosidase activity (13). β-Galactosidase activities were measured after induction of transcription of plasmid-encoded rRNA at 42°C for 150 min. Growth rates of strains expressing mutant rRNA were measured after induction of transcription of plasmid-encoded rRNA at 42°C. Each value for β-galactosidase activity and each growth rate determination is the mean value of three to five independent measurements ± one standard error.

Mutations at position 2654 in the α-sarcin loop

The three possible mutations at A2654 were constructed by site-directed mutagenesis and were expressed in plasmid pLK35. The data presented in Table 1 show that only the A→G mutation, that was predicted to create a G-C base pair with C2666, had any significant effect on codon readthrough or frameshifting. Only minor increases in readthrough and frameshifting levels were observed with the A→U mutation, while in some instances, the A→C mutation appeared to have a mildly restrictive effect on fidelity. This suggested that different base substitutions at the same position in the α-sarcin loop could have opposite effects on decoding. Similar, differential effects on decoding have been reported for different base substitutions at position C1054 and the base paired positions C1409-G1491 in the small subunit rRNA (19,20). Primer extension analysis of rRNAs from selected single base substitution mutants showed that both the error-promoting U2666 and G2654 mutations (A-U and G-C base pairs) and the phenotypically silent C2654 and G2666 mutations (C-C and A-G base pairs) that had no effect on fidelity were equally well represented in 50S subunits, 70S ribosomes and the polyribosome pools (Table 2).

Table 2. Distribution of mutant RNA in 50S subunits, 70S ribosomes and polysomes

rRNA mutant	% Plasmid-encoded rRNA		
	50S	70S	Polysomes
Wild-type[a]	55.2 ± 1.9	49.6 ± 3.9	47.8 ± 6.1
U2666	48.8 ± 2.4	50.3 ± 2.3	42.7 ± 1.2
G2666	44.6 ± 8.6	48.9 ± 0.8	45.5 ± 2.1
G2654	55.7 ± 3.5	51.6 ± 3.6	45.8 ± 6.5
C2654	51.6 ± 5.3	51.6 ± 5.5	45.1 ± 5.4
G2654/G2666	49.9 ± 5.2	52.2 ± 4.5	45.0 ± 5.0
C2654/U2666	56.3 ± 6.2	60.6 ± 7.2	49.0 ± 3.7
G2654/U2666	53.5 ± 2.3	54.1 ± 1.6	45.5 ± 7.5
C2654/G2666	38.5 ± 4.8	36.8 ± 3.2	35.1 ± 0.8

[a]All plasmid-encoded rRNAs used in these primer extension assays carried the allele-specific priming site in the 1360 region of 23S rRNA (16). Relative proportions of plasmid-encoded and chromosomally encoded rRNAs were determined by the primer extension method of Sigmund *et al.* (15), using a primer complementary to nucleotides 1389–1369 of 23S rRNA. Values represent the means of three to five independent primer extension assays ± one standard error.

With the exception of the G2654 and U2666 mutations that affected decoding fidelity, none of the six single base substitutions at positions 2654 and 2666 had the potential to create a standard base pair across the loop. Consequently, these data did not allow us to distinguish between the contributions of base identity and base pairing to ribosome function. To address these two differing possibilities, a series of double base mutations at A2654 and C2666 were constructed, that were predicted to maintain or disrupt standard base pairing interactions.

2654–2666 double mutants

The nine possible double base mutations at positions 2654 and 2666 were constructed by site directed mutagenesis. As was seen with the single base mutations, none of the double base mutations had any substantial effects on cell growth rate (Table 1) and only some of the mutations had effects on stop codon readthrough and frameshifting. The data in Table 1 show that all of the mutations with a G at position 2654 promoted frameshifting and readthrough errors. These levels of errors were highest when G2654 was paired with C2666 or U2666 (G2654 and G2654-U2666 mutations) and considerably lower in the G2654-G2666 and G2654-A2666 mutants. However, analysis of the C2654-G2666 and U2654-A2666 double mutants showed that mere base-pairing potential alone was insufficient to affect decoding fidelity, as both of these mutants displayed wild type levels of frameshifting and readthrough. The C2654-G2666 mutant rRNA was present in slightly reduced levels in all ribosome fractions (Table 2). Similar results were observed with mutations at G2663 and G2664, by Marchant and Hartley (21). This suggests that mutations in the α-sarcin loop may also affect the assembly and/or stability of the 50S subunit. Nevertheless, in the selected mutants examined, both the error-enhancing and silent mutations were well represented in the functioning polyribosome pools (Table 2). Together, these data showed that the presence of a G at position 2654 altered the fidelity of decoding, but that this effect was augmented when G2654 had the potential to form a G-C canonical base pair or G-U wobble pair with the base at position 2666.

A similar pattern was observed with the N2654-U2666 series of mutations. All of these mutations had some effects on both readthrough and frameshifting. This effect was greatest in the G2654-U2666 mutant. However, in this mutant, some of the effects were probably due to the presence of a G at position 2654. The A2654-U2666 mutant also promoted high levels of frameshifting and readthrough, while the C2654-U2666 mutant was less effective in promoting miscoding, and only small increases in β-galactosidase activities were observed with the U2654-U2666 mutant. In summary, a U at position 2666 promoted misreading errors and this effect was greatest when U2666 had the potential to engage in canonical base pairing with the base at position 2654.

The data presented in the preceding sections showed that both the identity of the bases at positions 2654 and 2666, as well as base-pairing interactions affected the function of the α-sarcin loop. The remaining two double base mutations, U2654-G2666 and C2654-A2666 both showed small, but significant effects on readthrough and frameshifting. The levels seen with the U2654-G2666 mutant were higher than observed in any of the other U2654 or G2666 mutants (U2654-C2666, U2654-A2666, A2654-G2666 and C2654-G2666 mutants, respectively). Similar observations were made for the C2654-A2666 mutant. This suggests that, even in mutants not containing G2654 or U2666, particular combinations of bases at these two positions can affect the function of the α-sarcin loop, and supports our proposal that both the type of base-pairing interactions as well as the identity of the individual bases are important for ribosome function.

DISCUSSION

The data presented here show that the accuracy of tRNA selection by the ribosome can be reduced by alterations to the primary and secondary structure of the α-sarcin loop of 23S rRNA. Previous analyses of another α-sarcin loop mutation showed that a G→C transversion at position 2661 had the opposite effect on tRNA selection, and increased the accuracy of translation by affecting EF-Tu–ribosome interactions (7,22). Mutations in elongation factor Tu have also been isolated as suppressors of the *trpE91*

frameshift and other frameshift and nonsense mutations (4). Together, these observations suggest that the mutations at positions 2654 and 2666 in the α-sarcin loop influence the accuracy of tRNA selection and reading frame maintenance by affecting the interaction of the elongation factor with the ribosome.

The binding of aminoacyl tRNA to the ribosome begins with the formation of an initial, labile complex of EF-Tu•aminoacyl tRNA•GTP and the ribosome that precedes the codon recognition step. Cognate, codon–anticodon interaction is necessary to support the subsequent rapid GTP hydrolysis step and accommodation of the aminoacyl tRNA in the ribosomal A site (23,24). Recent *in vitro* studies showed that codon–anticodon interaction induces a conformational change in EF-Tu that precedes GTP hydrolysis (23). The ribosomal components responsible for signaling to EF-Tu are at present unknown, but genetic and biochemical studies have implicated the 530 loop in 16S rRNA and the α-sarcin loop in 23S rRNA in this process (8,17,25). Elaborate *in vitro* analyses of ribosomes carrying the error-restrictive C2661 mutation indicated that the increased accuracy was associated with a reduced binding affinity and an enhanced selection of cognate ternary complexes by mutant ribosomes during the initial selection phase of tRNA binding (8). The G2654 and U2666 mutations described here have the opposite effects on accuracy and so, might be predicted to increase the non-specific affinity of ternary complexes for mutant ribosomes at the expense of the codon–anticodon interaction, leading to misincorporation and frameshift errors.

Elongation factors Tu and G display mutually exclusive binding to ribosomes and protect an overlapping set of bases in the α-sarcin loop from chemical modification (Fig. 1; ref. 1). These data, together with the differing sequence requirements for α-sarcin and ricin recognition, led to the elaboration of a model for the sequential interaction of the elongation factors with the ribosome during elongation (26). The model proposes that the α-sarcin loop oscillates between EF-Tu- and EF-G-interacting conformations. In the context of this model, the mutations described here might affect the inter conversion of these two conformers and, consequently, the interaction of both elongation factors with the ribosome. Furthermore, the effects of base pairing between positions 2654 and 2666 could be due to increased stability of one of the possible loop conformations that promotes EF-Tu-dependent GTP hydrolysis and accommodation of tRNA in the ribosomal A site.

The NMR-derived structure of the α-sarcin loop is based on the sequence of the rat cytoplasmic 28S rRNA, that contains A residues at both 2654 and 2666. These NMR experiments provided clear evidence for a non-canonical A-A pairing between these two positions in 28S rRNA. The data presented here provide evidence for interaction between the equivalent two positions in *E.coli* 23S rRNA *in vivo*, and suggest that an analogous, non-canonical A-C pairing may exist at these positions in wild-type *E.coli* 23S rRNA.

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