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Decoding fidelity at the ribosomal A and P sites: influence of mutations in three different regions of the decoding domain in 16S rRNA

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ABSTRACT

The involvement of defined regions of *Escherichia coli* 16S rRNA in the fidelity of decoding has been examined by analyzing the effects of rRNA mutations on misreading errors at the ribosomal A and P sites. Mutations in the 1400–1500 region, the 530 loop and in the 1050/1200 region (helix 34) all caused readthrough of stop codons and frameshifting during elongation and stimulated initiation from non-AUG codons at the initiation of protein synthesis. These results indicate the involvement of all three regions of 16S rRNA in decoding functions at both the A and P sites. The functional similarity of all three mutant classes are consistent with close physical proximity of the 1400–1500 region, the 530 loop and helix 34 and suggest that all three regions of rRNA comprise a decoding domain in the ribosome.

INTRODUCTION

Accurate decoding of mRNA requires the active participation of ribosomal components. The involvement of ribosomal proteins in this process was demonstrated by classical genetic analyses, which implicated several ribosomal proteins in decoding fidelity (1). While no ribosomal RNA mutations were isolated in these studies, evidence from many different sources has identified rRNA as an indispensable component in all steps of translation. The sites of interaction of tRNA, mRNA and other ligands with rRNA have been identified using crosslinking, chemical footprinting and site-directed mutagenesis (reviewed in 2). Among the first tRNA–ribosome crosslinks to be obtained was a zero length crosslink between base 34 of the anticodon of P site-bound tRNA and C1400 of 16S rRNA (3). Many of the subsequent biochemical and genetic studies have corroborated the importance of the 1400–1500 region of the small subunit rRNA in tRNA and antibiotic binding, decoding and subunit–subunit interaction. Moreover, Purohit and Stern (4) have shown that a short oligoribonucleotide that mimics the 1400–1500 region of the small subunit rRNA can interact with aminoglycoside antibiotics and tRNAs in a way that is surprisingly similar to interaction of the intact 16S rRNA molecule with these ligands.

In the light of the wealth of data linking the 1400–1500 region of 16S rRNA with decoding, this region has been termed the decoding center or decoding site. However, other regions of the 16S rRNA molecule also participate in ribosome–tRNA–mRNA interactions (5). Furthermore, site-directed mRNA–rRNA cross-linking has shown that as well as the 1400–1500 region, the 530 loop and the 1050/1200 regions of helix 34 (Fig. 1) are also close to the site of codon–anticodon interaction (6). In order to emphasize the contribution of rRNA nucleotides outside the 1400–1500 region to decoding, the term 'decoding domain' has been used to denote all regions of 16S rRNA that impinge upon decoding (7).

The functional role of particular sites in rRNA have been analyzed by examining the effects of mutations at these sites on defined ribosomal functions (8,9). In the present study, we have analyzed the effects of mutations in three distinct regions of the decoding domain on tRNA selection during the initiation and elongation phases of translation. In common with previously characterized base substitution mutations in the 530 loop and helix 34 (10,11), we have found that a variety of mutations in the 1400–1500 region of 16S rRNA promote stop codon readthrough and frameshifting during elongation. This indicates that these mutations influence tRNA selection at the ribosomal A site. However, mutations in these same three regions of 16S rRNA, as well as the lack of post-transcriptional modification of bases A1518 and A1519, increase the frequency of initiation from non-AUG codons, indicating that P site decoding is also affected in these mutants. Together, our data show that tRNA selection at both the A and P sites is influenced by the same three regions of 16S rRNA and are consistent with the notion that these three regions are in close proximity to the site of codon–anticodon interaction in the ribosome.

MATERIALS AND METHODS

Bacterial strains

Strain M41 and its *recA*$^-$ derivative MC140 were used as the standard wild-type strains (12). Kasugamycin-resistant (*ksgA*)

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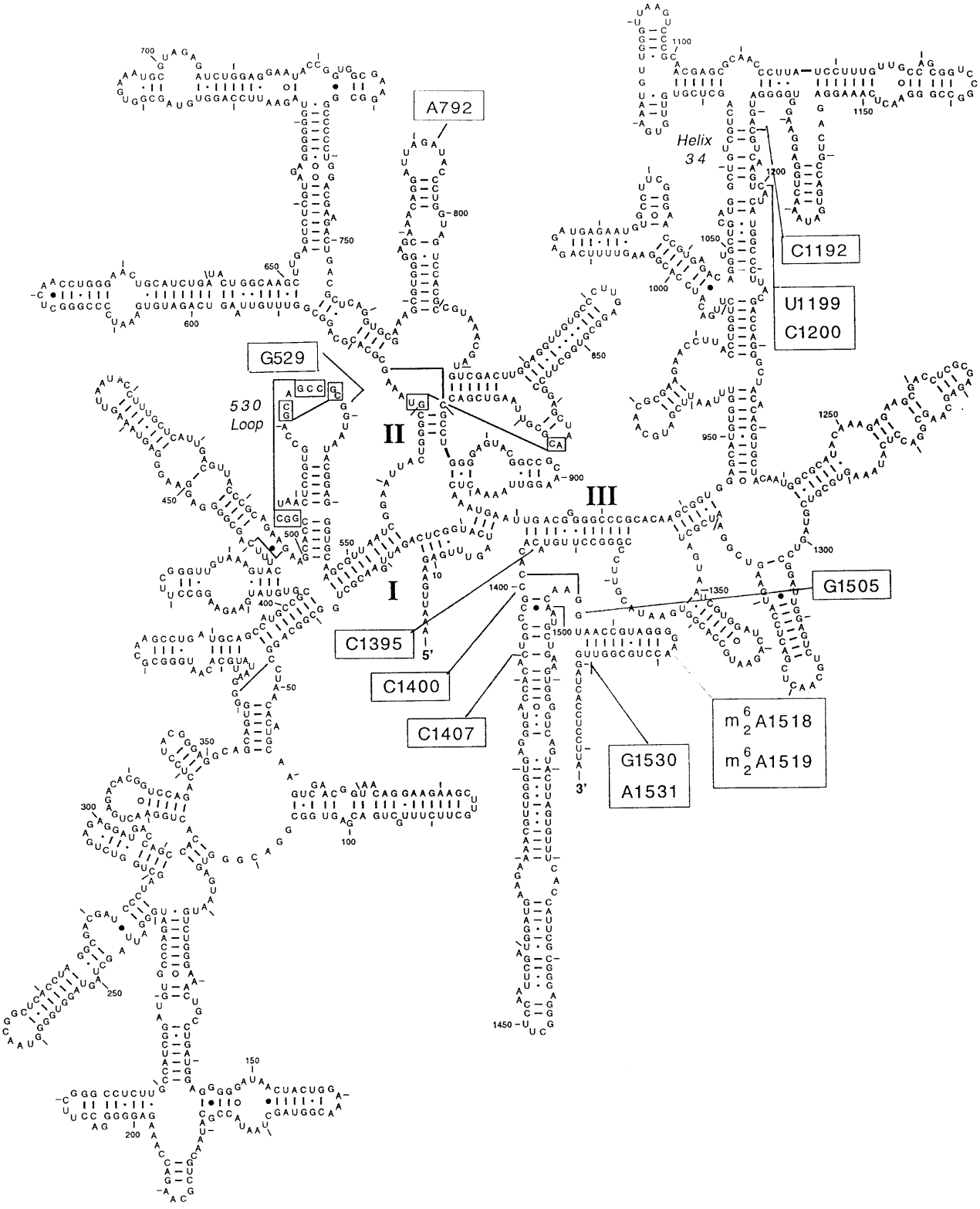

Figure 1. Secondary structure of *E.coli* 16S rRNA (57) showing the sites of mutations and post-transcriptional modification analyzed in this study. The identities of the wild-type bases are indicated.

mutants were obtained from the late Dr Peter van Knippenberg (University of Leiden). The *ksgA19* mutation was transferred into the MC41 genetic background by transducing strain MC86 (MC41 *thr34*::Tn*10*) to threonine independence with phage P1 prepared on the *ksgA19*-containing strain and screening for kasugamycin resistance. The resultant strain was designated MC178.

Table 1. LacZ plasmids used in this study

Plasmid	Relevant feature	Sequence
pSG25	Wild type lacZ	AUG AUU ACG CUA AGC UUG GCA CUG
pSG413	CUG initiation codon	CUG AUU ACG CUA AGC UUG GCA CUG
pSG414	UUG initiation codon	UUG AUU ACG CUA AGC UUG GCA CUG
pSG415	AUC initiation codon	AUC AUU ACG CUA AGC UUG GCA CUG
pSG416	AUA initiation codon	AUA AUU ACG CUA AGC UUG GCA CUG
pSG417	AUU initiation codon	AUU AUU ACG CUA AGC UUG GCA CUG
pSG431	GUG initiation codon	GUG AUU ACG CUA AGC UUG GCA CUG
pSG12-6	UAG mutant	AUG AUU ACG CUA AGC UUU GUU UAG GCC GGC CCU AAU UCA CUG
pSG163	UAG mutant	AUG AUU ACG CUA AGC UUA GGG UAU CUU UAG CUA CGG GGC CCU AAU UCA CUG
pSG627	UAA mutant	AUG AUU ACG CUA AGC UUU GUG GAA UAA GUU AGC GGC CCU AAU UCA CUG
pSG853	UAA mutant	AUG AUU ACG CUA AGC UUU GUC UAA GUU AGC GGC CCU AAU UCA CUG
pSG34-11	UGA mutant	AUG AUU ACG CUA AGC UUU GUG UGA GCC GGC CCU AAU UCA CUG
pSG3/4	UGA mutant	AUG AUU ACG CUA AGC UUA GGG UAU CUU UGA CUA CGA CGG AUC CCC GGG AAU UCA CUG
pSG12DP	–1 frameshift	AUG AUU ACG CUA AGC UUG GG AUA AGG AUC CCC GGG AAU UCA CUG
pSGlac7	+1 frameshift	AUG AUU ACG CUA AGC UUU GUGU AGG GUU AGC GGC CCU AAU UCA CUG

The underlined CUG leucine codon corresponds to codon 7 of the wild type lacZ gene.

rRNA and *lacZ* plasmids

Mutant rRNA was expressed from two different sets of plasmids in this study. In pKK3535, the intact *rrnB* operon is transcribed constitutively from the native P_1P_2 promoters (13). In pNO2680 (14), rRNA is transcribed from the λ P_L promoter. In the presence of the temperature-sensitive λ cI repressor (supplied on the neomycin-resistant, pSC101-derived plasmid pLG857; 12), transcription of plasmid encoded rRNA is repressed at 30°C but can be induced upon shifting the culture to 42°C. A number of single base substitutions in 16S rRNA, including C1395U, ΔC1400, C1407U, G1505U, C1192U and G529U, were carried on pNO2680-derived plasmids (15,16). Mutations at positions A792 and G1530/A1531 were carried on pKK3535-derived plasmids, which contained, in addition, the spectinomycin resistance C1192U mutation (17,18).

The pSG series of plasmids containing frameshift and nonsense mutations in the 5′-end of the *lacZ* gene have been described previously and the relevant sequences are given in Table 1 (11,12). Plasmid pSG25 is derived from pACYC184 and is compatible with both the pSC101-derived plasmid pLG857 encoding the λ repressor and the pBR322-derived, *rrnB*-containing plasmids pNO2680 and pKK3535. The AUG initiation codon in the wild-type *lacZ* plasmid pSG25 is bounded on the 5′- and 3′-sides by *Eco*RI and *Hin*dIII sites respectively. Plasmids containing non-AUG codons were constructed by replacing the 20 bp *Eco*RI–*Hin*dIII fragment with pairs of complementary synthetic oligonucleotides containing AUN or NUG initiation codons. The primary structure of all mutant plasmids was verified by nucleotide sequencing and the relevant sequences are listed in Table 1.

Culture media and growth conditions

Bacteria were routinely cultured in Luria–Bertani (LB) medium. Antibiotics were added as required at the following concentrations: tetracycline, 12.5 μg/ml; neomycin, 50 μg/ml; ampicillin, 250 μg/ml; kasugamycin, 100 μg/ml. β-Galactosidase activities in cells harboring both mutant rRNA and pSG plasmids were measured after dilution of overnight cultures and growth at 42°C (for the pNO2680-derived plasmids) or 37°C (for pKK3535 and its derivatives) for 150 min and assayed as previously described (12).

Isolation and sequencing of β-galactosidase

To isolate β-galactosidase from strains containing a *lacZ* construct and a pNO2680-derived rRNA plasmid, 2 l LB medium were inoculated with 200 ml cells that had been grown overnight at 30°C. Expression of the mutant rRNA was induced by growing these cultures at 42°C for 3–5 h. Cells were harvested and β-galactosidase was isolated, purified and sequenced as described previously (19).

RESULTS

Mutations in the decoding center of 16S rRNA decrease the fidelity of A site decoding

We have previously described the construction of mutations at positions C1395, C1400, C1407 and G1505. Deletion of C1400 and C→U mutations at positions 1395 and 1407 had a dominant lethal phenotype and the mutant rRNAs could only be expressed transiently from an inducible promoter (15). Mutations at G1505 had little effect on cell viability and, when combined with the C1395U, ΔC1400 or C1407U mutations, suppressed lethality of the single base mutations. As part of our analysis of the influence of defined regions of 16S rRNA on decoding functions, we have examined the effects of these mutations on stop codon read-through and frameshifting. Strains carrying plasmids encoding a temperature-sensitive allele of the λ cI repressor and *lacZ* constructs containing stop codons or frameshifts in the 5′ portion of the coding region were transformed with the wild-type rRNA plasmid (pNO2680) and each of the mutant rRNA plasmids and transcription of the mutant rRNA was induced by shifting the cultures to 42°C. The data in Table 2 show that mutations at C1400, C1395, C1407 and G1505 caused a 2- to 5-fold increase in the level of readthrough of stop codons and frameshifting. Readthrough of stop codons occurs by binding of a near-cognate tRNA to the termination triplet in the A site, while frameshifting can be related to both A and P site decoding events (20). These data show that one effect of mutations in the decoding center is to perturb codon–anticodon interactions in the A site and decrease the fidelity of elongating ribosomes.

Table 2. Effects of rRNA mutations on stop codon readthrough and frameshifting

rRNA Mutation	LacZ Plasmids							
	pSG12-6 UAG	pSG163 UAG	pSG627 UAA	pSG853 UAA	pSG34-11 UGA	pSG3/4 UGA	pSG12DP (–1)	pSGlac7 (+1)
pNO2680 (wt)	16 ± 2	37 ± 3	5 ± 1	7 ± 1	35 ± 7	107 ± 14	85 ± 4	56 ± 3
pPL133 (C1407U)	29 ± 1	95 ± 3	9 ± 1	44 ± 5	59 ± 5	224 ± 13	241 ± 22	222 ± 6
pPL111 (C1395U)	28 ± 1	61 ± 12	8 ± 1	22 ± 3	58 ± 4	171 ± 30	241 ± 38	165 ± 13
pPLAM5 (ΔC1400)	28 ± 2	101 ± 2	10 ± 2	44 ± 4	59 ± 2	214 ± 13	255 ± 31	221 ± 2
pPL1301m26 (G1505U)	24 ± 2	62 ± 3	8 ± 1	23 ± 1	52 ± 2	169 ± 23	142 ± 10	123 ± 9
pPLAM5m26 (ΔC1400/G1505U)	30 ± 5	87 ± 3	7 ± 1	24 ± 1	51 ± 4	147 ± 17	256 ± 13	215 ± 13
pPL111m28 (C1395U/G1505U)	29 ± 4	92 ± 3	6 ± 1	28 ± 1	47 ± 6	144 ± 8	257 ± 14	206 ± 11
pPL133m26 (C1407U/G1505U)	26 ± 4	92 ± 2	7 ± 1	30 ± 2	46 ± 5	151 ± 9	235 ± 20	181 ± 11
pNOC1192U	19 ± 1	40 ± 1	5 ± 1	10 ± 1	41 ± 1	135 ± 7	109 ± 3	57 ± 3

Values for stop codon readthrough and frameshifting are expressed in Miller units of β-galactosidase activity (56). Each value for β-galactosidase activity is the result of 3–5 independent experiments. Assay conditions are described in Materials and Methods.

Characterization of ribosomal frameshifting events by protein sequencing

The mechanism of frameshifting promoted by the rRNA mutations described in the preceding section and in our previous work (11,16) was analyzed further by N-terminal sequencing of β-galactosidase isolated from strains carrying selected rRNA mutations and *lacZ* frameshift constructs. Protein sequencing of β-galactosidase isolated from a strain expressing the C1407U rRNA mutation and the pSG12DP –1 *lacZ* frameshift gave the following sequence: M I T (L+R) S L G I R I P (Fig. 2). This suggested that, at the site of frameshifting (UUG GGA UAA), a GGA-decoding $tRNA^{Gly}$ slipped backwards by one base onto the 5′ overlapping GGG glycine codon. This frameshift site, where a string of repetitive nucleotides is bordered on the 3′-side by an in-frame stop codon constitutes a 'shifty stop' and has been studied extensively by Weiss *et al.* (21). Their analyses have uncovered the importance of an overlapping cognate codon in the new reading frame and an adjoining stop codon in the original reading frame. The enhancing effect of the stop codon suggests that tRNA slippage occurs when the $tRNA^{Gly}$ is in the P site and the stop codon is located in the A site. Protein sequencing showed that at position 4, arginine as well as the expected leucine were recovered (Fig. 2). The origin of arginine at this position is unknown, but it may derive from (mis)reading of the CUG leucine codon by a CGG-decoding $tRNA^{Arg}$ via a second position codon–anticodon mismatch.

Frameshifting in the +1 direction was also characterized by protein sequencing. Our previous analyses had shown that the G529U mutation gave a 6-fold increase in +1 frameshifting in the pSGlac7 construct (16). Sequencing of β-galactosidase isolated from a strain expressing the G529U rRNA mutation and the pSGlac7 frameshift gave the sequence M R V S G P N (Fig. 3; protein sequence data not shown). This unexpected sequence suggests that rather than initiating at AUG, translation began at the downstream GUG codon, followed by binding of an AGG-decoding $tRNA^{Arg}$ one base out-of-frame and continuation of translation in the +1 reading frame (Fig. 3). The UAG stop codon that follows the presumptive initiator GUG codon may induce a translational pause that promotes out-of-frame tRNA binding to the A site. The proposed frameshifting mechanism bears some similarity to the programed ribosomal frameshift in the yeast Ty3 element (22). At the junction of the GAG3 and

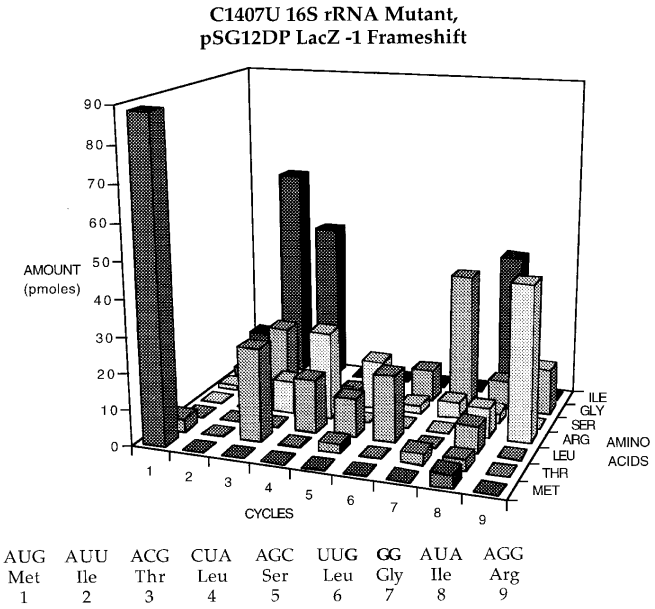

Figure 2. N-terminal sequencing of β-galactosidase purified from a strain carrying the C1407U rRNA mutation and the pSG12DP –1 *lacZ* frameshift construct. The yield in pmol of selected PTH amino acids from the first nine cycles of sequencing is shown. The lower panel depicts an interpretation of this frameshift event obtained by superimposing the protein and mRNA sequences. The GGG codon decoded by backward (–1) slippage of the GGA-decoding $tRNA^{Gly}$ is indicated in bold.

POL3 reading frames in Ty3, the seven base sequence GCG A GUU is decoded as alanine–valine. The low availability of AGU-decoding $tRNA^{Ser}$ has been shown to contribute to the Ty3 frameshifting event. In both the Ty3 frameshift and the proposed frameshift in the pSGlac7 construct, tRNA slippage is not required. Although initiation of translation on the pSGlac7 mRNA apparently occurred at the GUG codon, this *lacZ* construct lacks a recognizable Shine–Dalgarno sequence at an appropriate distance upstream. Consequently, initiation at GUG may require that ribosomes initiate at the *bona fide* AUG codon, terminate at the in-frame UAG codon and re-initiate at the preceding GUG codon by backward scanning (23,24). The various elements of this model are depicted in Figure 3. Both types of frameshifting

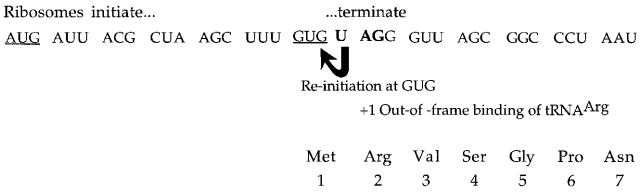

Figure 3. Proposed mechanism of frameshifting (following re-initiation) in the pSGlac7 +1 frameshift construct. The bottom two lines show the protein sequence obtained from N-terminal sequencing of β-galactosidase purified from a strain expressing the G529U rRNA mutation and the pSGlac7 construct.

events characterized here are stimulated by the G517, G529, C1400 region and helix 34 mutations (Table 2; 10,11,16). This suggests that mutations in each of these three regions of 16S rRNA cause tRNA slippage by disrupting tRNA–mRNA contacts in the P site and promote out-of-frame tRNA binding by relaxing the stringency of tRNA selection at the A site.

Effects of decoding domain mutations on codon recognition at the P site

The AUG triplet is overwhelmingly the most common initiation codon in all organisms, GUG and UUG are infrequently used and AUU, AUA and AUC are rarely, if ever, used. The initiator tRNA is unique in that it binds directly to the ribosomal P site. Initiation factors and ribosomal proteins are involved in selection of the correct mRNA initiation codon and tRNA and actively discriminate against non-initiator tRNAs and codons. In an effort to characterize the effects of rRNA mutations on P site function, we have examined their influence on the levels of initiation from non-AUG codons. We reasoned that mutations that affected decoding function in the P site might alter the frequency of initiation from non-AUG initiation triplets. The AUG initiation codon of the wild-type *lacZ* plasmid pSG25 was replaced by GUG, UUG and CUG (pSG431, pSG414 and pSG413 respectively) or AUA, AUC and AUU (pSG416, pSG415 and pSG417 respectively). Decoding of these triplets by $tRNA_f^{Met}$ during initiation from the non-standard initiation codons involves single base codon–anticodon mismatches at the first or third positions, and is thus analogous to readthrough of UGA codons by $tRNA^{Trp}$ or UAA and UAG codons by $tRNA^{Gln}$ in the A site (25–27). The data in Table 3 show that the mutations in the 530 loop (G529U) and helix 34 (U1199C/C1200U and U1199G/C1200G), as well as the mutations in the C1400 region described in the preceding section, all stimulated initiation from each of the non-AUG initiation codons. Only some of the helix 34 mutations promoted miscoding, however. Thus, the C1192U mutation in helix 34, that confers resistance to spectinomycin (28), had no effect on stop codon readthrough, frameshifting or initiation events (Tables 2 and 3), consistent with its lack of effect on cell growth or *in vitro* translation parameters (29). In order to characterize these aberrant initiation events, β-galactosidase was purified from selected strains and subjected to N-terminal sequencing. Sequence analysis of β-galactosidase isolated from a strain carrying the pSG413 *lacZ* construct and the G529U rRNA mutation showed that, as predicted, initiation occurred at the CUG codon (Fig. 4). Similarly, protein sequencing showed that in a strain carrying the pSG415 *lacZ* plasmid and the U1199G/C1200G rRNA mutations, initiation occurred at the predicted AUC codon (Fig. 5). These

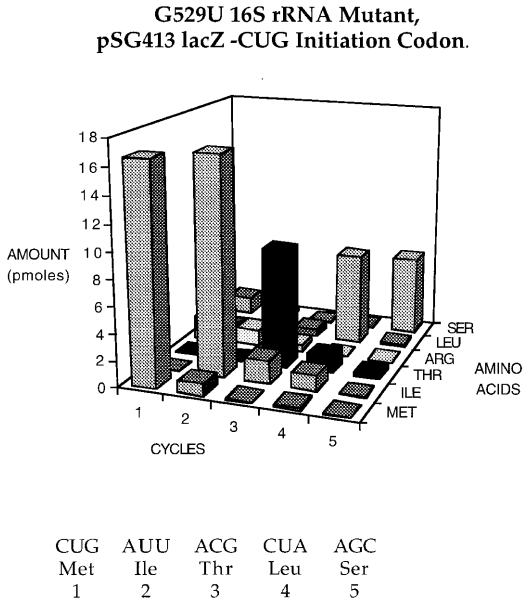

Figure 4. N-Terminal sequencing to determine the initiation codon used in the pSG413 *lacZ* construct. β-Galactosidase was purified from a strain expressing the G529U rRNA mutation and the pSG413 *lacZ* construct. The yield in pmol for selected PTH amino acids analyzed for the first five cycles is shown.

data show clearly that mutations in three different regions of 16S rRNA affect codon–anticodon interactions at the ribosomal P site, while the data presented in the preceding section and in our previous analyses (10,11,16) show that these same regions of rRNA are involved in tRNA selection at the A site and in reading frame maintenance. The similarity of the functional effects of these mutants suggests that the 530 loop, the C1400 region and helix 34 are all involved in modulating tRNA–ribosome interactions at multiple steps during translation. Moreover, the isolation of suppressor/antisuppressor mutations at equivalent positions in yeast mitochondrial and cytoplasmic small subunit rRNAs (under conditions where all the rRNA is mutant) suggests the involvement of these three regions of rRNA in decoding in all organisms (7).

Initiation of translation requires the participation of the three initiation factors IF1, IF2 and IF3. IF3 is involved in selection of the initiator tRNA that binds to the P site of the small subunit. The factor interacts with the anticodon stem–loop region of the tRNA and destabilizes initiation complexes containing non-initiator tRNAs or non-canonical initiation complexes (30). Crosslinking experiments have shown that IF3 interacts with both the central and 3′ minor domains of 16S rRNA (31) and 30S ribosomal subunits containing mutations at positions G791 and A792 in the 790 loop or at G1530/A1531 at the 3′-end of 16S rRNA were shown to be defective in IF3 binding (17,18,32). Because of the influence of these rRNA mutations on ribosome–IF3 interactions, we have analyzed their effects on selection of AUG initiation codons. These data, presented in Table 3, show that the A792U, A792G and G1530A/A1531G mutants do not increase initiation from non-AUG codons. This indicates that decreases in ribosome–IF3 interaction *per se* do not affect the selection of AUG initiation codons, but instead suggests that the rRNA mutations analyzed here affect initiation by decreasing the accuracy of tRNA selection in the ribosomal P site.

Table 3. Effects of rRNA mutations on initiation from non-AUG codons

rRNA plasmid/ mutation	LacZ plasmids initiation codons[a] pSG25-AUG	pSG413-CUG	pSG414-UUG	pSG431-GUG	pSG415-AUC	pSG416-AUA	pSG417-AUU
pNO2680 (wt)	7298 ± 222	115 ± 13	1739 ± 238	3428 ± 39	87 ± 5	63 ± 3	80 ± 5
pPL133 (C1407U)	8592 ± 453	241 ± 45	3707 ± 187	4078 ± 259	333 ± 16	210 ± 4	315 ± 10
pPL111 (C1395U)	9118 ± 445	174 ± 20	3165 ± 162	5403 ± 201	319 ± 6	141 ± 10	191 ± 2
pPLAM5 (ΔC1400)	8679 ± 522	322 ± 4	4026 ± 276	4800 ± 435	394 ± 22	218 ± 18	355 ± 10
pPL1301m26 (G1505U)	7461 ± 428	159 ± 21	2601 ± 143	3647 ± 155	180 ± 10	108 ± 5	147 ± 11
pNOG529U	6487 ± 645	326 ± 11	4161 ± 119	5429 ± 270	478 ± 31	278 ± 96	364 ± 15
pNOU1199C/C1200U	7469 ± 437	574 ± 18	3712 ± 154	4641 ± 475	585 ± 40	365 ± 16	628 ± 27
pNOU1199G/C1200G	9319 ± 568	591 ± 26	4196 ± 159	5169 ± 411	557 ± 16	365 ± 15	629 ± 22
pNOC1192U (wt)[b]	8985 ± 589	120 ± 6	1709 ± 350	3486 ± 168	95 ± 5	68 ± 8	118 ± 4
pKKC1192U (wt)[b]	17 241 ± 2738	161 ± 21	2579 ± 514	13 139 ± 1351	142 ± 3	107 ± 10	161 ± 7
pKKA792U[b]	19 388 ± 1180	214 ± 19	3615 ± 707	12 575 ± 1524	132 ± 32	116 ± 17	178 ± 18
pKKA792G[b]	14 176 ± 2237	142 ± 7	2138 ± 474	n.d.	105 ± 4	90 ± 13	131 ± 9
pKKG1530A/A1531G[b]	n.d.	167 ± 5	2994 ± 79	13 672 ± 453	148 ± 10	130 ± 14	168 ± 35

[a]Numbers represent Miller units of β-galactosidase activity, ± one standard error. Each assay value represents the mean value for 3–5 independent measurements.
[b]Contains, in addition, the U1192 mutation.
n.d., not determined.

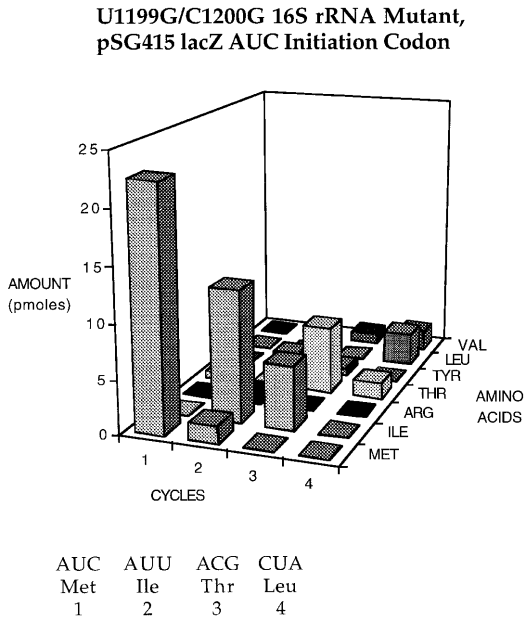

Figure 5. N-Terminal sequencing to determine the initiation codon used in the pSG415 *lacZ* construct, using β-galactosidase purified from a strain expressing the U1199G/C1200G rRNA mutation. The yield in pmol for selected PTH amino acids obtained for the first four cycles is shown.

Interaction of G1505 with other regions of 16S rRNA

Our earlier study indicated that the lethal effects of mutations at positions 1395, 1400 and 1407 were suppressed by a second mutation at position 1505 (15). As part of our characterization of the mechanism of suppression, the translational error levels supported by the C1395U/G1505U, ΔC1400/G1505U and C1407U/G1505U double mutant rRNA combinations were examined. These data (Table 2) showed that, in general, each of the double mutant rRNAs increased frameshifting and stop codon readthrough to the same (or greater) extent than any of the single base mutations and thus indicated that the double mutant rRNAs were active in translation. The suppressor effect of base substitutions at position 1505 is not limited to mutations in the C1400 region, as Jemiolo and colleagues have recovered the G1505A mutation as an intragenic suppressor of two lethal transversions at G1207 (33; D.Jemiolo, personal communication). G1505 lies in a region of rRNA that forms a minor crosslink to IF3 (31). The interaction of G1505U and G791A, another mutation that decreases IF3 binding, was examined by combining both mutations in a pNO2680-derived plasmid. Growth rate measurements of strains expressing the various rRNAs indicated that the wild-type and G1505U mutant had indistinguishable doubling times (relative doubling time = 1), while the G791A/G1505U mutant grew more slowly than the G791A mutant (relative doubling times of 1.8 and 1.3 respectively). Thus, mutations at position 1505 can interact either positively or negatively with deleterious mutations at different positions in 16S rRNA. The negative effect of G1505U on the growth rate of the G791A mutant together with the effects of the G1505U-containing mutations on translational fidelity (Table 2) indicate that suppression of the C1400 region and G1207 mutants by base substitutions at G1505 does not derive from sequestration of the plasmid-encoded rRNAs in an unprocessed or inactive subunit form. A more plausible hypothesis is that positions G791, G1207, C1395, C1400, C1407 and G1505 are all at or near the binding site for an essential ligand, such as IF3, and modulate its affinity for the 30S subunit.

Influence of the *ksgA*-dependent dimethylation of A1518 and A1519 on A and P site decoding

The antibiotic kasugamycin is believed to inhibit protein synthesis by interfering with the ribosomal P site (34). However, an effect

of kasugamycin on A site decoding has also been reported (35). Resistance to the antibiotic can arise through mutations in the KsgA methylase that modifies A1518 and A1519 near the 3′-end of 16S rRNA. Methylase-deficient *ksgA*⁻ strains are defective in subunit–subunit and IF3 interactions and display elevated levels of stop codon readthrough and frameshifting (35). The influence of unmodified A1518/A1519 on decoding at the P site was tested by transforming methylase-deficient *ksgA*⁻ strains with a range of *lacZ* initiation codon constructs. The data presented in Table 4 show that *ksgA*⁻ strains also display elevated levels of initiation from non-AUG codons. These data suggest that in addition to changes in the primary structure, alterations in the pattern of post-transcriptional modification of 16S rRNA can affect decoding fidelity at both the A and P sites.

Table 4. Effects of ksgA-dependent rRNA modification on initiation from non-AUG codons

LacZ plasmids	Strain/mutation	
	MC41, wild type	MC178, ksgA19
pSG25-AUG	25 858 ± 597	27 989 ± 829
pSG413-CUG	508 ± 62	868 ± 36
pSG415-AUC	461 ± 71	870 ± 154
pSG416-AUA	501 ± 42	674 ± 93
pSG417-AUU	535 ± 73	835 ± 140

Numbers represent Miller units of β-galactosidase activity (56), and are the mean values of 3–6 independent measurements, ± one standard error.

DISCUSSION

Chemical protection and crosslinking experiments have defined specific bases in 16S rRNA that are involved in tRNA binding to the ribosomal A, P and E sites and have helped define the orientation of tRNAs on the ribosome (6). The data presented here show that mutations at several of these positions, such as G529, which has been linked to A site function, and C1400, which has been linked to P site function, affected decoding at both the A and P sites. This suggests that mutations in the 530 loop, the 1400–1500 region and mutations in helix 34 all lead to a distortion of the site of codon–anticodon interaction that results in a loss of discrimination between cognate and near-cognate tRNAs at the adjacent A and P sites. The effect of rRNA mutations on tRNA selection might be similar to that provoked by the error-inducing aminoglycoside antibiotics, which are thought to enhance non-specific tRNA–ribosome interactions at the expense of accurate codon–anticodon interactions (36). Weakening of specific mRNA–tRNA contacts would also be consistent with the effects of the rRNA mutations on tRNA slippage that lead to reading frame errors (Table 2 and Fig. 2). In this context, it is significant that the antibiotics streptomycin and neomycin, as well as error-enhancing mutations in ribosomal proteins S4 and S5 and error-restrictive mutations in ribosomal protein S12, have all been shown to affect tRNA–ribosome interactions at both the A and P sites (37). However, an important functional difference between these ribosomal mutants is that while the antibiotics and ribosomal protein mutations had differential effects on tRNA–ribosome interactions at the ribosomal A and P sites, the rRNA mutations described here had the same error-enhancing effect on decoding at both sites.

The 1400–1500 region of 16S rRNA

Both A and P site-bound tRNAs reduce or enhance the reactivities of several nucleotides in the 1400–1500 region towards chemical probes (5) and aminoglycoside antibiotics that perturb decoding protect A1408, G1491 and G1494 (34). Furthermore, it has been shown that positions +4 and +7 on the mRNA can be crosslinked to bases C1402 and C1395 respectively (38). Consequently, the effects of the 1400–1500 region mutations on decoding are likely to derive from direct disruption of tRNA–mRNA–ribosome interactions. Although the nucleotides surrounding C1400 were originally depicted as being single stranded, recent phylogenetic and mutational analyses have suggested that nucleotides adjacent to C1400 are base paired with nucleotides adjacent to A1500 (39; Fig. 1). Thus, C1407 is proposed to be paired with G1494 and genetic evidence has been obtained for the existence of G1401–C1501, C1404–G1497 and G1405–C1496 base pairs (40,41). In the C1407U mutation analyzed here, for instance, a U–G pair has replaced the native C–G pair. Analyses of ribosomes carrying disruptions in tertiary base pairs or deletions in the 1400–1500 region, by Ofengand and co-workers, indicated that initiation, as well as binding of tRNA to A and P sites, was affected by the mutations (40–42). This is consistent with our results, which show that disruption of the decoding center by the C1395U, ΔC1400, C1407U and G1505U mutations influences maintenance of the reading frame and affects tRNA–mRNA–ribosome interactions at the adjacent A and P sites.

Helix 34

The isolation of an apparently UGA-specific suppressor mutation at position C1054 in *E.coli* 16S rRNA led to the elaboration of a model for UGA termination (43). This model proposed that recognition of the UGA termination codon occurred through base pairing between the UGA triplet and either of the tandem UCA triplets at bases 1199–1204 in helix 34. However, subsequent analyses demonstrated that mutations at or adjacent to positions C1054/C1200 caused readthrough of all three stop codons, as well as enhancing frameshifting levels (11). In this study we have demonstrated that the effects of mutations in helix 34 are not limited to the elongation phase of translation, but also affect the fidelity of decoding in the P site during initiation. Another rRNA–mRNA base pairing model for termination was proposed by Tate *et al.* (44), based on crosslinking of the UAA termination triplet to position A1408. However, the data presented here show that the C1407U mutation, which alters the proposed recognition sequence for the termination triplets, not only affected readthrough of all three stop codons and frameshifting, but also affected the fidelity of decoding at initiation. Moreover, mutations at C1395, C1400 and G1505 provoked the same pattern of A site and P site decoding errors as was observed with the C1407U mutation. While these results underline the importance of helix 34 and the C1400 region in reading frame maintenance and tRNA–mRNA interactions in both the A and P sites, they do not support the specific involvement of either of these regions in termination. Consequently, the effects of mutations in both of these regions of 16S rRNA most likely derive from disruption of tRNA–mRNA–ribosome interactions in a general way, rather than from a specific influence on the termination process. Direct evidence linking helix 34 with mRNA–ribosome interactions came from site-directed crosslinking experiments, which showed that while positions +4 and +7 of the mRNA were crosslinked to

C1395 and C1402 respectively, position +6 was crosslinked to U1052 in helix 34 (38,45). In addition tetracycline, an antibiotic that inhibits A site tRNA binding, protects A892 in the central domain and U1052 and C1054 in helix 34 from chemical modification (46). Thus, while there are no tRNA-dependent crosslinks or chemical protections in helix 34, the mRNA crosslinks and antibiotic footprints, together with the genetic data presented here, indicate the involvement of helix 34 in decoding functions at initiation and elongation.

The 530 loop

Genetic evidence linking the 530 loop with decoding function came from isolation of a nonsense suppressor mutation at position G517 in yeast mitochondria (47) and mutations conferring resistance to the error-inducing antibiotic streptomycin in chloroplasts and mycobacteria (48,49). The involvement of the 530 loop in decoding and antibiotic resistance in both *E.coli* and yeast cytoplasmic ribosomes was subsequently established by site-directed mutagenesis of rRNA operons (10,16,50,51). Analysis of a lethal G530A mutation by Powers and Noller (52) suggested that these mutant ribosomes might be specifically defective in EF-Tu–ribosome interactions. In addition, it was found that antibiotics and ribosomal protein mutations that promoted miscoding differentially affected tRNA-dependent protection of G530, but not protection of A1492/A1493 at the A site (53). These findings led to the advancement of a model which proposes that the conformation of the 530 loop responds differentially to cognate versus non-cognate ternary complex binding during initial recognition and proofreading of tRNAs (52,53). According to this model, the 530 loop influences tRNA binding via its effects on ribosome–EF-Tu interaction. However, our results indicate that the 530 loop may play a much more direct role in tRNA–ribosome interaction and at multiple stages of translation. The G529U mutation affects reading frame maintenance and decoding fidelity during both the initiation and elongation phases of translation. Initiator tRNA binding to the ribosomal P site does not involve EF-Tu and at least some of the ribosomal frameshifting events that are influenced by mutations in the 530 loop involve slippage of cognate tRNAs in the P site (rather than mistranslocation of non-cognate tRNAs or out of phase tRNA binding to the A site). These considerations, together with the functional similarity of the G529U mutation to changes in helix 34 and the C1400 region, suggest that all three regions of rRNA comprise a decoding domain and that alterations in any of the three regions have similar effects on tRNA–ribosome interactions. This interpretation is consistent with the mRNA crosslinking data, which show that the 530 loop, helix 34 and the C1400 region all form crosslinks to mRNA between positions +4 and +11 (38,45,54). Also consistent with this arrangement of the decoding domain are the recent data of Heilek and Noller (55), who have determined the rRNA neighborhood of ribosomal protein S5 using a Fe(II)–EDTA cleavage reagent tethered to amino acid 21. Using this reagent, cleavages were observed in the 420 and 530 loops, the central pseudoknot, the 920 and 1400 regions and in helix 34, indicating the close physical proximity of all of these regions of 16S rRNA.

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