REPORT

Contribution of 16S rRNA nucleotides forming the 30S subunit A and P sites to translation in *Escherichia coli*

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ABSTRACT

Many contacts between the ribosome and its principal substrates, tRNA and mRNA, involve universally conserved rRNA nucleotides, implying their functional importance in translation. Here, we measure the *in vivo* translation activity conferred by substitution of each 16S rRNA base believed to contribute to the A or P site. We find that the 30S P site is generally more tolerant of mutation than the 30S A site. In the A site, A1493C or any substitution of G530 or A1492 results in complete loss of translation activity, while A1493U and A1493G decrease translation activity by >20-fold. Among the P-site nucleotides, A1339 is most critical; any mutation of A1339 confers a >18-fold decrease in translation activity. Regarding all other P-site bases, ribosomes harboring at least one substitution retain considerable activity, >10% that of control ribosomes. Moreover, several sets of multiple substitutions within the 30S P site fail to inactivate the ribosome. The robust nature of the 30S P site indicates that its interaction with the codon–anticodon helix is less stringent than that of the 30S A site. In addition, we show that G1338A suppresses phenotypes conferred by m²G966A and several multiple P-site substitutions, suggesting that adenine at position 1338 can stabilize tRNA interaction in the P site.

Keywords: translation; ribosome; tRNA–rRNA interactions; A site; P site

INTRODUCTION

During protein synthesis, the tRNA substrates move through three distinct binding sites of the ribosome, the aminoacyl (A), peptidyl (P), and exit (E) site. These sites lie at the subunit interface, where the small (30S) subunit contacts the anticodon stem–loop (ASL) regions of the tRNAs and the large (50S) subunit contacts the D- and T-stem–loops, acceptor stems, and universally conserved CCA-3' ends of the tRNAs. A growing body of evidence suggests that the A, P, and E sites of each subunit can act independently during the translation cycle (Moazed and Noller 1989; Odom et al. 1990; Valle et al. 2003; Blanchard et al. 2004). When the ternary complex, comprised of aminoacyl-tRNA (aa-tRNA), elongation factor Tu (EF-Tu), and GTP, encounters the elongating ribosome, the ASL portion of aa-tRNA interacts first with the 30S subunit A site (Moazed and Noller 1989). Cognate codon–anti-

codon interaction stimulates: (1) rapid GTP hydrolysis, which causes a structural rearrangement of EF-Tu and its release of aa-tRNA, and (2) subsequent movement of the acceptor end of aa-tRNA into the 50S subunit A site (Berchtold et al. 1993; Abel et al. 1996; Polekhina et al. 1996; Pape et al. 1998, 1999; Ogle et al. 2003). Once aa-tRNA is in the A site of both subunits (termed the A/A state), peptide bond formation occurs, resulting in transfer of the peptidyl group from the P-site tRNA to the A-site tRNA. Following peptidyl transfer, elongation factor G (EF-G) interacts with the complex and catalyzes hydrolysis of GTP and translocation of the tRNAs to the adjacent ribosomal sites (Rodnina et al. 2001). Translocation of the tRNAs within the ribosome is believed to occur in a step-wise fashion. The newly deacylated tRNA and newly formed peptidyl-tRNA move first with respect to the 50S subunit into the P/E and A/P hybrid states, respectively. Then, the ASL portions of the tRNAs, paired to the mRNA, move with respect to the 30S subunit. This results in a posttranslocation complex that contains deacylated tRNA in the E/E state, peptidyl-tRNA in the P/P state, and a vacant A site, available for the next round of elongation.

Recent X-ray crystal structures of 70S ribosome complexes have identified numerous interactions between the

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ribosome and its primary substrates, tRNA and mRNA (Cate et al. 1999; Yusupov et al. 2001; Yusupova et al. 2001). Insights into the atomic details of many of these interactions have come from structures of the isolated subunits containing fragments, analogs, or molecular mimics of tRNA (Carter et al. 2000; Nissen et al. 2000; Ogle et al. 2001; Schmeing et al. 2002, 2003; Bashan et al. 2003). In the 30S subunit, the binding pockets for the ASLs are formed at the junction of the major structural domains: the head, body, and platform. Messenger RNA interacts within a channel that wraps around the neck of the subunit, and a 45° kink in the mRNA backbone is observed between the A and P codons that allows base pairing with tRNA in each site. The 30S A and P sites are composed predominantly of rRNA, although ribosomal protein S12 forms a contact to the A codon, and the C-terminal tails of S9 and S13 contact the P-site tRNA. Many of the ribosomal interactions with tRNA or mRNA involve functional groups of conserved 16S rRNA bases. Indeed, of the 10 16S bases observed to contact tRNA or mRNA, eight are >99% conserved in all phylogenetic domains of life (Table 1) (Cannone et al. 2002), implying the functional importance of specific contacts made by these bases during translation. However, the degree to which many of these interactions contribute to translation is unknown.

Here, we use ribosomes with altered specificity in translation initiation to test the relative importance for gene expression of each 16S rRNA base believed to contribute to the 30S subunit A or P site. Our results indicate that the

functions of the 30S A site depend on several strictly required tRNA–rRNA interactions, whereas the functions of the 30S P site involve a larger number of less critical tRNA–rRNA interactions.

RESULTS AND DISCUSSION

Construction of an *Escherichia coli* strain to quantify translation activity of ribosomes containing 16S rRNA mutations

De Boer and colleagues have shown that ribosomes containing an alternative anti-Shine-Dalgarno (ASD) sequence at the 3' terminus of 16S rRNA have altered specificity in the initiation of translation in vivo (Hui and de Boer 1987). These “specialized” ribosomes efficiently translate reporter mRNA containing the complementary Shine-Dalgarno (SD) sequence but are unable to efficiently translate endogenous mRNA. Thus, translation activity conferred by mutations in the 16S rRNA can be quantified in these cells, which are uncompromised for growth. Based on the work of Cunningham (Lee et al. 1996), we constructed an *E. coli* strain, KLF10, harboring the *lacZ* gene with the alternative SD sequence 5'-ATCCC-3' in single copy on a recombinant λ prophage (see Materials and Methods). We then engineered plasmid pKF207, which contains the 16S rRNA gene with the complementary ASD sequence 5'-GGGGT-3'. Expression of the specialized 16S rRNA from pKF207 in KLF10 cells resulted in a >50-fold increase in β -galactosidase activity. This strain has an advantage over those described previously in that the reporter gene and the specialized 16S rRNA gene are unlinked.

Mutations of the decoding center nucleotides G530, A1492, and A1493 confer loss of translation activity

Mutations were made at positions corresponding to each 16S rRNA nucleotide (nt) believed to contribute to the 30S A or P site (Table 1), and activities of the corresponding ribosomes were measured in vivo. In general, the 30S A site was more sensitive to mutation than the 30S P site (Fig. 1). Because synthesis of functional β -galactosidase requires that the ribosome successfully complete >1000 rounds of elongation, our assay is predicted to be particularly sensitive to mutations that confer elongation defects. The contribution of the targeted nucleotides to translation

TABLE 1. Summary of 16S rRNA nucleotides observed by X-ray crystallography to interact with tRNA or mRNA in the A and P sites of the 30S subunit

Nucleotide	Conservation ^a	Relevant contacts ^b
<i>A site</i>		
G530	B, A, E	nt 35 of A-tRNA, nt 3 of A codon, and A1492 of 16S rRNA
C1054	B, A, E	nt 34 of A-tRNA
A1492	B, A, E	nt 2 of A codon and G530 of 16S rRNA
A1493	B, A, E	bp 1–36 of the codon–anticodon helix
<i>P site</i>		
A790 ^c	B, A, E	bk of P-tRNA at position 38
G926	B, A, E	phosphate of nt 1 of P codon (mRNA)
m ² G966	b	bk of P-tRNA at position 34
G1338	B, A, E	nt 41 of P-tRNA
A1339	B, A, E	nt 40 of P-tRNA
C1400	b, a, e	nt 34 of P-tRNA

^aUppercase letters B, A, and E denote >99% conservation in the phylogenetic domains *Bacteria*, *Archaea*, and *Eukarya*, respectively; lowercase letters indicate >95% conservation. Letter omission indicates that <95% conservation is observed in the corresponding domain.

^bAssignments of P-site interactions are based predominantly on the structures of ribosome complexes at medium resolution containing natural tRNA and mRNA (Yusupov et al. 2001; Yusupova et al. 2001). Assignments of A-site interactions are based on the atomic resolution structure of the 30S subunit containing a synthetic anticodon stem–loop in the A site (Ogle et al. 2001).

^cInteraction between A790 base and P-site tRNA mimic observed in the atomic resolution 30S subunit structure (Carter et al. 2000).

(bk) Backbone; (nt) nucleotide; (bp) base pair.

FIGURE 1. In vivo translation activity conferred by systematic mutagenesis of 16S rRNA nucleotides contributing to the 30S A or P site. In this genetic system, translation of *lacZ* depends on the activity of the mutagenized ribosomes. The specific activity of β -galactosidase was quantified for each strain and normalized to the wild-type control strain, KLF10(pKF207). The 16S rRNA expressed in strain KLF10(pKF207) lacks mutations aside from the altered ASD. In each case, the data represent the mean $\pm$ standard deviation calculated from three or more independent experiments.

initiation is more difficult to deduce. For example, mutations that specifically decrease the fidelity of initiation would not be expected to confer a decrease in β -galactosidase activity.

Of all the nucleotides targeted, only A1492 and G530 were found to be absolutely crucial for translation in vivo (Fig. 1). Any substitution at either position resulted in background levels of β -galactosidase activity. Mutation A1493C also abolished translation, whereas ribosomes harboring A1493G and A1493U retained residual activity, approximately two to threefold above background, a result corroborated by inspection of colonies on X-gal plates adjacent to those of the vector control strain (data not shown).

Important insights concerning the role of G530, A1492, and A1493 in the mechanism of aa-tRNA selection have recently been obtained from structural studies of the 30S subunit with or without an ASL bound to the A site (Ogle et al. 2001, 2002). These studies provide evidence that when cognate aa-tRNA interacts with the ribosome, a conformational rearrangement occurs in the 30S A site. A1492 and A1493 flip out of helix 44, G530 moves from a *syn* to an *anti* conformation, and each of these three bases interacts with the minor groove of the codon-anticodon helix. A1493 forms a Type I A-minor interaction with codon-anticodon base pair 1:36, while A1492 and G530 interact with one another and with the minor groove of codon-anticodon base pair 2:35. A1492 forms additional interactions with ribosomal protein S12, and G530 makes an additional contact with nucleotide 3 of the A codon. This rearrangement

of the 30S A site is proposed to monitor the geometry of the codon-anticodon helix and thereby discriminate against incorporation of non-cognate aminoacyl-tRNA during protein synthesis (Ogle et al. 2001, 2002).

We consider it most likely that ribosomes harboring base substitutions of G530, A1492, or A1493 are defective in aa-tRNA selection, making them inactive or marginally active in our in vivo translation assay. The fact that G530, A1492, and A1493 act together to recognize the codon-anticodon helix, and mutation of any of these nucleotides is highly detrimental to translation, implies a functional interdependence of these bases during decoding.

In addition to their role in aa-tRNA selection, G530 and A1493 have been implicated in the mechanism of translocation. ASL analogs of tRNA containing 2'-deoxy or 2'-fluoro substitutions at positions 33, 35, or 36 are specifically defective in their ability to undergo EF-G-dependent translocation from the A

to P site (Phelps et al. 2002). Because the 2'-hydroxyl groups of tRNA nt 35 and 36 donate hydrogen bonds to G530 and A1493, respectively, it was suggested that these tRNA-rRNA contacts play a role in the mechanism of translocation. These observations present the possibility that reduced translocation rates in ribosomes harboring mutations of G530 and/or A1493 may contribute to the loss of translation activity seen in this study.

We considered the possibility that mutations at positions 530, 1492, and 1493 decreased translation indirectly by causing a defect in ribosome assembly. This scenario seemed unlikely, because each of these mutations confers a dominant lethal phenotype in a different genetic context (Powers and Noller 1990; Yoshizawa et al. 1999). In an effort to rule out this possibility, we grew several strains in the presence of arabinose to induce expression of the mutant 16S rRNA, fractionated the corresponding lysates by sedimentation through sucrose gradients, and used primer extension to determine the relative amount of mutant 16S rRNA in the 30S, 70S, and polysome fractions. Mutation C1192U was included in this analysis for comparative purposes. C1192U does not confer decreased translation activity (data not shown), but the mutation allows the specialized ribosomes to be distinguished from the endogenous wild-type ribosomes by primer extension. For each A-site mutation analyzed (A1492U, A1492C, A1493U, A1493C, G530C, and G530A), mutant 16S rRNA was detected in the 30S fraction (Fig. 2). In no case was there evidence for partially assembled or degraded small subunits

FIGURE 2. Detection of mutant 16S rRNA in ribosomal fractions separated by sucrose gradient sedimentation. In each panel, primer extension products templated from wild-type and mutant 16S rRNA are indicated by arrowheads. Control reactions include those in which template was omitted (–RNA) and those in which purified 16S rRNA from wild-type *E. coli* strain MRE600 (wt 16S rRNA) was used as template. Ribosomal fractions (as indicated) were analyzed from strains expressing 16S rRNA containing mutation A1492U (A), A1492C (B), A1493U (C), A1493C (D), G530A (E), G530C (F), or C1192U (G).

from the A_{260} trace of the sucrose gradient or primer extension analysis of its top fraction (Fig. 2; data not shown). By phosphorimager analysis, we estimated the percentage of 30S subunits carrying a 16S rRNA mutation, and this value was high for A1492U (38%) and A1493U (52%); intermediate for A1492C (11%), G530A (15%), G530C (12%), and C1192U (9%); and low for A1493C (3%). For A1492U, A1492C, A1493U, G530A, and G530C, the percentage of 30S subunits carrying a mutation was comparable to or greater than that observed for C1192U, arguing against the idea that loss of translation activity conferred by these mutations stems primarily from assembly defects. In the case of A1493C, considerably lower levels of 30S subunits were detected, consistent with a primary defect in assembly or stability. However, it should be noted that these data are correlative in nature and provide no direct evidence that decreased translation conferred by A1493C is caused by defective assembly. Mutant 16S rRNA was also detected in the 70S monosome fraction for A1492C (5%), G530A (11%), and G530C (4%), and in the polysome

fraction for G530A (8%) and G530C (3%) (Fig. 2). Under identical conditions, highly active C1192U ribosomes accounted for 9%, 7%, and 5% of the total 30S, 70S, and polysome pools, respectively. Among inactive mutant ribosomes, the differences in sedimentation profiles were unanticipated and may reflect allele-specific defects in translation initiation or ribosome turnover. Further experiments will be necessary to investigate these possibilities.

Compared with mutations at positions 530, 1492, and 1493, mutation of A-site nucleotide C1054 decreased translation to a much lesser degree (Fig. 1). C1054 packs against the ribose of tRNA nt 34, which pairs with the third base of the A codon (Ogle et al. 2001). Clearly, any constraints provided by specific interaction of C1054 with this “wobble pair” are not crucial for translation elongation.

Relative tolerance of the 30S subunit P site to mutation

Structures of the 70S ribosomal complex at 5.5 Å resolution have revealed six regions of contact between the ribosome

and tRNA or mRNA in the 30S P site (Yusupov et al. 2001). At this resolution, 16S rRNA nucleotides involved in these contacts can be assigned, but details of the interactions at the atomic level remain ambiguous. Structures of the isolated 30S subunit have been solved at atomic resolution (Wimberly et al. 2000; Pioletti et al. 2001), but in these crystals, the P site is occupied by the stem-tetraloop of helix 6 of an adjacent subunit in the lattice. A comparison of the position and interactions of this stem-tetraloop in the 30S structure to that of the P-site tRNA within the 70S ribosome complex suggests that the helix 6 stem-tetraloop mimics P-site tRNA closely but not identically (Carter et al. 2000).

Contacts to the codon and anticodon loop made by individual P-site nucleotides appear to involve less buried surface area than contacts made by A-site nucleotides A1492, A1493, and G530, an observation reflected in this study by the relative tolerance of ribosomes to mutation of these P-site nucleotides. In the 30S crystal structure, A790 packs against the backbone of nt 37–38 of the P-site tRNA mimic in an interaction involving one hydrogen bond, but interaction between base A790 and native P-site tRNA is not apparent in the 70S structure (Carter et al. 2000; Yusupov et al. 2001). Mutation of A790 to any other nucleotide decreased translation from 1.2- to 4.9-fold (Fig. 1), indicating that interaction of A790 with tRNA is not critical for translation. These results are consistent with a previous study in which active ribosomes harboring multiple mutations of the 790 loop were selected, and isolates carrying A790C or A790G with one or more additional substitutions were obtained (Lee et al. 1997).

Base m²G966 contacts the backbone of tRNA nt 34 in the 70S crystal structure and has been identified by modification interference experiments as important for tRNA binding to the P site (von Ahlsen and Noller 1995; Yusupov et al. 2001). Base substitutions of m²G966 conferred decreased translation activity in vivo, but in each case, the mutant ribosomes retained >10% activity (Fig. 1). These data indicate that m²G966, the least-conserved base in this analysis (Table 1), contributes to P-site function but is not essential for translation.

C1400 stacks with base 34 of tRNA in the P site. Consistent with previous experimental data and the natural occurrence of U1400 (Hui et al. 1988; Cannone et al. 2002), mutation C1400U had little effect on translation in vivo (approximately twofold decrease), while the A and G substitutions reduced translation by ~12-fold and ~20-fold, respectively (Fig. 1). Because stacking interactions involving purines are energetically more favorable than those involving only pyrimidines (Saenger 1984), one possibility is that inhibition of translation in ribosomes harboring C1400A or C1400G results from a decreased dissociation rate of the P-site ASL during translocation, although this hypothesis remains to be tested.

The only 16S rRNA base that appears to contact the P codon is G926, which donates two hydrogen bonds to the

phosphate group of nt 1 of the P codon in the 30S subunit crystal structure (Carter et al. 2000). Kethoxal modification of G926 strongly inhibits P-site tRNA binding (von Ahlsen and Noller 1995), which presumably results from destabilization of the P codon. However, reconstituted ribosomes containing a deletion of G926 exhibit only about a threefold reduction in tRNA binding (Ericson et al. 1995), suggesting that modification of G926 with the bulky kethoxal group may cause a steric block that prevents suitable positioning of the P codon (von Ahlsen and Noller 1995). Here, we show that any base substitution of G926 results in about an eightfold decrease in translation activity (Fig. 1). These data provide evidence that the contact between G926 and mRNA contributes to but is not required for P-site function. The latter conclusion was confirmed by the observation that ribosomes harboring mutations at position 926 are able to support cell growth (Vila-Sanjurjo et al. 1999).

The ribosome makes a number of contacts with the anticodon stem in the 30S P site (Carter et al. 2000; Yusupov et al. 2001). Among these, universally conserved bases G1338 and A1339 interact with the minor groove of the P-site tRNA stem. Mutation G1338A did not significantly decrease translation, whereas pyrimidine substitutions G1338C and G1338U decreased translation by 11-fold and 4.5-fold, respectively (Fig. 1). By contrast, any substitution of A1339 reduced translation by ~18-fold, to levels about threefold above background. This residual activity appeared significant on X-gal indicator plates and was most apparent in the case of A1339C (data not shown). Thus, of all P-site mutations, those of A1339 conferred the most substantial defects in translation but did not completely inactivate the ribosome. In independent experiments, similar decreases in translation activity were observed after mutagenesis of G1338 and A1339 (L. Lancaster and H. Noller, pers. comm.).

In the 30S subunit structure (Carter et al. 2000), A1339 forms a Type I A-minor interaction with base pair 30–40 of the P-site tRNA mimic. The adjacent G1338 forms a Type II minor interaction with tRNA nt 41. The ability of a docking G to substitute for the more typical A in Type II minor motifs has been predicted based on experimental and phylogenetic analyses (Doherty et al. 2001). Our data are consistent with the idea that these interactions also occur in the P site during translation in vivo. The fact that A1339 was found to be the most critical P-site nucleotide suggests that its interaction contributes substantially to stabilization of the P-site tRNA, and Type I A-minor interactions are among the most energetically favorable RNA base triple interactions known (Doherty et al. 2001). Mutation G1338A results in highly active ribosomes, suggesting that either G1338 or A1338 can interact productively with P-site tRNA, as would be expected for a Type II minor interaction.

Additional ribosomal contacts to the P-tRNA anticodon stem include those of 16S rRNA nt 1229–1230, which are involved in backbone–backbone packing with tRNA nt

28–30, and interactions made by the C-terminal tails of ribosomal proteins S9 and S13. Although the importance of the 1229–1230 contacts remain unclear, *E. coli* strains with chromosomal deletions that correspond to these C-terminal tails have been constructed, and both strains exhibit <20% reduction in growth rate (Hoang et al. 2004). Thus, S9 and S13, like several P-site bases analyzed in this study, appear to each contribute in a relatively small way to P-site function.

G1338A suppresses phenotypes conferred by other P-site mutations

We also tested whether ribosomes containing two or more base substitutions within the 30S P site retained translation activity (Fig. 3). Six of the 10 double mutants constructed retained >13% activity. By contrast, ribosomes harboring any P-site substitution in addition to A1339C exhibited activity near background. Among double mutants that retained substantial activity, those containing G1338A were the most active. Although the G1338A mutation decreased the activity of ribosomes containing either A790C or C1400U by 22% or 66%, respectively, G1338A suppressed the defect conferred by m²G966A, causing about a twofold increase in *lacZ* expression. Similar approximately twofold increases of activity were observed when G1338A was introduced into double or triple mutants harboring A790C m²G966A, m²G966A C1400U, or A790C m²G966A C1400U. Suppression by G1338A does not depend on the m²G966A mutation, because phenotypes

conferred by the double mutation 790C 1400U or the single mutation A1339G are also suppressed by G1338A (Fig. 3; L. Lancaster and H. Noller, pers. comm.). These results suggest that G1338A can stabilize tRNA interaction in the P site, thereby compensating for loss of contacts caused by other P-site substitutions. Consistent with this interpretation, G1338A was found to stabilize the interaction of fMet-tRNA^{fMet} with the 30S P site in the presence of excess IF3 (L. Lancaster and H. Noller, pers. comm.). Remarkably, ribosomes containing G1338A and up to three additional mutations within the 30S P site retain some activity in vivo (Fig. 3), indicating that this site can tolerate extensive alteration without complete loss of function.

CONCLUSIONS

In this study, the translation activity conferred by each substitution of each 16S rRNA base believed to contribute to the A or P site was measured. The 30S A site was found to be more sensitive to mutation than the 30S P site. We suggest that these data reflect a fundamental difference in the nature of the two binding sites. Interaction of tRNA with the 30S A site induces a rearrangement of G530, A1492, and A1493 to form the binding pocket around the codon–anticodon helix. It has been proposed that this induced-fit interaction acts to ensure high fidelity during the tRNA selection step of protein synthesis (Ogle et al. 2003). A functional interdependence of bases G530, A1492, and A1493 during tRNA selection could explain why each of these nucleotides is critical to translation. By contrast,

FIGURE 3. Effect of double, triple, and quadruple mutations of the 30S P site on translation activity. As in Figure 1, the specific activity of β -galactosidase was quantified for each strain and normalized to the wild-type control strain, KLF10(pKF207). In each case, the data represent the mean $\pm$ standard deviation calculated from three or more independent experiments.

the more robust nature of the 30S P site suggests that its interaction with tRNA and mRNA is less complex. No P-site nucleotide targeted in this study was found to be absolutely crucial, and most nucleotides were inferred to play a relatively minor role in translation elongation. We suggest that each of the numerous ribosomal elements that compose the 30S P site contribute in a fairly independent manner to stabilize tRNA or mRNA. Finally, mutation G1338A was found to suppress phenotypes conferred by m²G966A and several multiple P-site substitutions, suggesting that adenine at position 1338 can stabilize tRNA interaction in the P site.

MATERIALS AND METHODS

Strain KLF10 [F– *ara* Δ(*gpt-lac*)5 λ(ΦSD_{5'}-ATCCC-3'-*lacZ*) Δ(*recA-srl*)306 *srlR301::Tn10*] was constructed in several steps. A DNA fragment containing a variant of the *P_{ant}* promoter (Moyle et al. 1991) and the altered SD sequence 5'-ATCCC-3' (Lee et al. 1996) was generated by annealing oligonucleotide #3 (5'-GGAATTCAC TAGTTTGAATGAATGAAGCACTCTACTATATTCTTAATAGG TCC-3') with #5 (5'-CGGGATCCATTTCTCGAGGGATATGAT AGTCAAACAGGACCTATTAAG-3') and extending with Sequenase (USB Corporation) and dNTPs (Rossi et al. 1982). This fragment was digested with EcoRI and BamHI and cloned upstream of *lacZ* in pRS552, and the resulting fusion was transferred to λRS45 by homologous recombination in vivo (Simons et al. 1987). The recombinant λ phage was then used to lysogenize strain CSH142 (Miller 1992), generating strain KLF3, which was confirmed to contain a single prophage (Powell et al. 1994). Finally, Δ(*recA-srl*)306 *srlR301::Tn10* was moved into KLF3 by P1 transduction to generate strain KLF10.

Plasmid pKF207 is pBAD18 (Guzman et al. 1995) containing the gene encoding 16S rRNA with the altered ASD 5'-GGGGU-3' (Lee et al. 1996). To mutagenize the 3' end of the 16S rRNA gene, we used recombinant PCR. Oligonucleotide #11 (5'-CGGTGAAT ACGTTCCCGGG-3') and mutagenic oligonucleotide #10 (5'-GC TTCTTTAAGGTAACCCCATGATCCAACCGC-3') were used to amplify a fragment of the *rrnB* operon from position 1660 to 1846 (relative to the P1 promoter transcriptional start site) that contained the altered ASD sequence at near its 3' end. Mutagenic oligonucleotide #9 (5'-GCGGTTGGATCATGGGGTTACCTTAA AGAAGC-3') and oligonucleotide #12 (5'-TGAAAGGCGGTG TCCTGG-3') were used to amplify a fragment of the *rrnB* operon from position 1814 to 2042 that contained the altered ASD sequence near its 5' end. These two DNA fragments (1660–1846 and 1814–2042) were purified and combined to template amplification of the recombinant PCR product (bp 1660 to 2042) using oligonucleotides #11 and #12. The recombinant product was purified, digested with BsrGI and XbaI, and cloned into pLK50, a derivative of pL-*rrnB* (Gourse et al. 1985), replacing the corresponding wild-type fragment. The intact specialized 16S gene was then subcloned as a KpnI/XbaI fragment into pBAD18, and the Spc^R mutation was reverted (U1192C) to generate pKF207. Mutations were then engineered into the specialized 16S rRNA gene of pKF207 as described (Fredrick et al. 2000).

Each mutant variant of plasmid pKF207 was introduced into strain KLF10 to determine the translation activity of the corre-

sponding mutant ribosomes. To quantify translation activity, 2 μL of saturated culture of each strain was diluted into 1 mL of Luria Broth (LB) containing 100 μg/mL ampicillin and 5 mM L-arabinose and grown for 6 h at 37°C. Cells were washed once in Z buffer (100 mM sodium phosphate [pH 7.0], 10 mM KCl, 10 mM MgSO₄), and the specific β-galactosidase activity was measured as described (Miller 1992), except that cells were permeabilized using the reagent P-BER (Pierce). Under these conditions, the specific β-galactosidase activity was 56.5 ± 11.6 Miller Units for KLF10(pKF207) and 1.02 ± 0.61 Miller Units for KLF10(pBAD18).

Lysates from strains expressing 16S rRNA with A1492U, A1492C, A1493U, A1493C, G530A, or G530C were analyzed for the presence of mutant 16S rRNA in 30S, 70S, and polysome fractions. For each strain, a saturated culture was diluted 500-fold into 50 mL LB containing 100 μg/mL ampicillin and 5 mM L-arabinose and grown for 6 h at 37°C. Cells were quickly cooled by pouring the culture over crushed ice, harvested by centrifugation, resuspended in 0.5 mL chilled lysis buffer (10 mM Tris-HCl pH 7.8, 15 mM MgCl₂, 1 mg/mL lysozyme), and frozen in a bath of dry ice and ethanol. From these cells, lysates were prepared and fractionated by sedimentation through 10%–40% sucrose gradients as described (Fredrick et al. 2000). The top fraction of the gradient and fractions corresponding the 30S, 70S, and polysome peaks were collected, and RNA was extracted from each fraction as described (Merryman and Noller 1998). To determine the relative amount of mutant 16S rRNA in each fraction, we adapted a primer extension method developed by Morgan and coworkers (Sigmund et al. 1988). Primers were designed to anneal to 16S rRNA at a position 3' of the mutation site such that primer extension in the presence of a specific dideoxynucleotide triphosphate would result in distinct products that reflect the fraction of templates containing the mutation. Briefly, each primer was 5' end-labeled using γ-[³²P]-ATP and T4 polynucleotide kinase (NEB) and purified from free γ-[³²P]-ATP by Sephadex G-15 (Amersham Biosciences) chromatography. In a 10-μL reaction containing 50 mM HEPES (pH 7.6) and 100 mM KCl, labeled primer was annealed to ~1.5 pmol 16S rRNA by heating the reaction to 95°C for 1 min and then allowing it to cool slowly. After a brief centrifugation to recover condensation, 10 μL of 2X extension mix (260 mM Tris-HCl [pH 8.5], 20 mM MgCl₂, 20 mM DTT, 8 U AMV reverse transcriptase [Seikagaku America], 340 μM of the appropriate dideoxynucleotide triphosphate, and 340 μM of each other deoxynucleotide triphosphate) was added and the reaction was incubated for 10 min at 42°C. Finally, the primer extension products were precipitated with ethanol in the presence of glycogen (5 μg), dissolved in loading solution (95% formamide, 20 mM EDTA, 0.05% xylene cyanol FF, and 0.05% bromophenol blue), and resolved by denaturing 20% PAGE. To detect A1492U or A1493U, primer #1494 (5'-CGGTTACCTTGTT ACGA-3') was extended in the presence of ddATP, dCTP, dGTP, and dTTP. To detect A1492C or A1493C, primer #1495 (5'-CT ACGGTTACCTTGTTACG-3') was extended in the presence of ddGTP, dATP, dCTP, and dTTP. To detect G530A, primer #531 (5'-CTTGCACCCTCCGTATT-3') was extended in the presence of ddTTP, dATP, dCTP, and dGTP. To detect G530C, primer #535 (5'-AACGCTTGCACCCTCCG-3') was extended in the presence of ddGTP, dATP, dCTP, and dTTP. To detect C1192U, primer #1194 (5'-AGGGCCATGATGACTTG-3') was extended in the presence of ddGTP, dATP, dCTP, and dTTP.

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REFERENCES

- Abel, K., Yoder, M.D., Hilgenfeld, R., and Jurnak, F. 1996. An a to b conformational switch in EF-Tu. *Structure* **4**: 1153–1159.
- Bashan, A., Agmon, I., Zarivach, R., Schlunzen, F., Harms, J., Berisio, R., Bartels, H., Franceschi, F., Auerbach, T., Hansen, H.A.S., et al. 2003. Structural basis of the ribosomal machinery for peptide bond formation, translocation, and nascent chain progression. *Mol. Cell* **11**: 91–102.
- Berchtold, H., Reshetnikova, L., Reiser, C.O.A., Schirmer, N.K., Sprinzl, M., and Hilgenfeld, R. 1993. Crystal structure of active elongation factor Tu reveals major domain rearrangements. *Nature* **365**: 126–132.
- Blanchard, S.C., Kim, H.D., Gonzalez, R.J., Puglisi, J.D., and Chu, S. 2004. tRNA dynamics on the ribosome during translation. *Proc. Natl. Acad. Sci.* **101**: 12893–12898.
- Cannone, J.J., Subramanian, S., Schnare, M.N., Collett, J.R., D'Souza, L.M., Du, Y., Feng, B., Lin, N., Madabusi, L.V., Muller, K.M., et al. 2002. The comparative RNA web (CRW) site: An online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. *BMC Bioinformatics* **3**: 15.
- Carter, A.P., Clemons, W.M., Brodersen, D.E., Morgan-Warren, R.J., Wimberly, B.T., and Ramakrishnan, V. 2000. Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature* **407**: 340–348.
- Cate, J.H., Yusupov, M.M., Yusupova, G.Z., Earnest, T.N., and Noller, H.F. 1999. X-ray crystal structures of 70S ribosome functional complexes. *Science* **285**: 2095–2104.
- Doherty, E.A., Batey, R.T., Masquida, B., and Doudna, J.A. 2001. A universal model of helix packing in RNA. *Nat. Struct. Biol.* **8**: 339–343.
- Ericson, G., Minchew, P., and Wollenzien, P. 1995. Structural changes in base-paired region 28 in 16S rRNA close to the decoding region of the 30S ribosomal subunit are correlated to changes in tRNA binding. *J. Mol. Biol.* **250**: 407–419.
- Fredrick, K., Dunny, G.M., and Noller, H.F. 2000. Tagging ribosomal protein S7 allows rapid identification of mutants defective in assembly and function of 30 S subunits. *J. Mol. Biol.* **298**: 379–394.
- Gourse, R.L., Takebe, Y., Sharrock, R.A., and Nomura, M. 1985. Feedback regulation of rRNA and tRNA synthesis and accumulation of free ribosomes after conditional expression of rRNA genes. *Proc. Natl. Acad. Sci.* **82**: 1069–1073.
- Guzman, L., Berlin, D., Carson, M.J., and Beckwith, J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.* **177**: 4121–4130.
- Hoang, L., Fredrick, K., and Noller, H.F. 2004. Creating ribosomes with an all-RNA 30S subunit. *Proc. Natl. Acad. Sci.* **101**: 12439–12443.
- Hui, A. and de Boer, H.A. 1987. Specialized ribosome system: Preferential translation of a single mRNA species by a subpopulation of mutated ribosomes in *Escherichia coli*. *Proc. Natl. Acad. Sci.* **84**: 4762–4766.
- Hui, A.S., Eaton, D.H., and de Boer, H.A. 1988. Mutagenesis at the mRNA decoding site in the 16S ribosomal RNA using the specialized ribosome system in *Escherichia coli*. *EMBO J.* **7**: 4383–4388.
- Lee, K., Holland-Staley, C.A., and Cunningham, P.R. 1996. Genetic analysis of the Shine-Dalgarno interaction: Selection of alternative functional mRNA–rRNA combinations. *RNA* **2**: 1270–1285.
- Lee, K., Varma, S., SantaLucia, J., and Cunningham, P.R. 1997. In vivo determination of RNA structure–function relationships: Analysis of the 790 loop in ribosomal RNA. *J. Mol. Biol.* **269**: 732–743.
- Merryman, C. and Noller, H.F. 1998. Footprinting and modification–interference analysis of binding sites on RNA. In *RNA: protein interactions* (ed. C.W.J. Smith), pp. 237–253. Oxford University Press, Oxford, United Kingdom.
- Miller, J.H. 1992. *A short course in bacterial genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Moazed, D. and Noller, H.F. 1989. Intermediate states in the movement of transfer RNA in the ribosome. *Nature* **342**: 142–148.
- Moyle, H., Waldburger, C., and Susskind, M.M. 1991. Hierarchies of base pair preferences in the P22 *ant* promoter. *J. Bacteriol.* **173**: 1944–1950.
- Nissen, P., Hansen, J., Ban, N., Moore, P.B., and Steitz, T.A. 2000. The structural basis of ribosome activity in peptide bond synthesis. *Science* **289**: 920–930.
- Odom, O.W., Picking, W.D., and Hardesty, B. 1990. Movement of tRNA but not the nascent peptide during peptide bond formation on ribosomes. *Biochemistry* **29**: 10734–10744.
- Ogle, J.M., Brodersen, D.E., Clemons, W.M., Tarry, M.J., Carter, A.P., and Ramakrishnan, V. 2001. Recognition of cognate transfer RNA by the 30S ribosomal subunit. *Science* **292**: 897–902.
- Ogle, J.M., Murphy, F.V., Tarry, M.J., and Ramakrishnan, V. 2002. Selection of tRNA by the ribosome requires a transition from an open to a closed form. *Cell* **111**: 721–732.
- Ogle, J.M., Carter, A.P., and Ramakrishnan, V. 2003. Insights into the decoding mechanism from recent ribosome structures. *Trends Biochem. Sci.* **28**: 259–266.
- Pape, T., Wintermeyer, W., and Rodnina, M.V. 1998. Complete kinetic mechanism of elongation factor Tu-dependent binding of aminoacyl-tRNA to the A site of the *E. coli* ribosome. *EMBO J.* **17**: 7490–7497.
- . 1999. Induced fit in initial selection and proofreading of aminoacyl-tRNA on the ribosome. *EMBO J.* **18**: 3800–3807.
- Phelps, S.S., Jerinic, O., and Joseph, S. 2002. Universally conserved interactions between the ribosome and the anticodon stem-loop of A site tRNA important for translocation. *Mol. Cell* **10**: 799–807.
- Pioletti, M., Schlunzen, F., Harms, J., Zarivach, R., Gluhmann, M., Avila, H., Bashan, A., Bartels, H., Auerbach, T., Jacobi, C., et al. 2001. Crystal structures of complexes of the small ribosomal subunit with tetracycline, edeine and IF3. *EMBO J.* **20**: 1829–1839.
- Polekhina, G., Thirup, S., Kjeldgaard, M., Nissen, P., Lippmann, C., and Nyborg, J. 1996. Helix unwinding in the effector region of elongation factor EF-Tu-GDP. *Structure* **4**: 1141–1151.
- Powell, B.S., Court, D.L., Nakamura, Y., Rivas, M.P., and Turnbough, C.L. 1994. Rapid confirmation of single copy λ prophage integration by PCR. *Nucleic Acids Res.* **22**: 5765–5766.
- Powers, T. and Noller, H.F. 1990. Dominant lethal mutations in a conserved loop in 16S rRNA. *Proc. Natl. Acad. Sci.* **87**: 1042–1046.
- Rodnina, M.V., Semenov, Y.P., Savelsbergh, A., Katunin, V.I., Peske, F., Wilden, B., and Wintermeyer, W. 2001. Mechanism of tRNA translocation on the ribosome. *Mol. Biol.* **35**: 559–568.
- Rossi, J.J., Kierzek, R., Huang, T., Walker, P.A., and Itakura, K. 1982. An alternate method for synthesis of double-stranded DNA segments. *J. Biol. Chem.* **257**: 9226–9229.
- Saenger, W. 1984. *Principles of nucleic acid structure*. Springer-Verlag, New York.
- Schmeing, T.M., Seila, A.C., Hansen, J.L., Freeborn, B., Soukup, J.K., Scaringe, S.A., Strobel, S.A., Moore, P.B., and Steitz, T.A. 2002. A pre-translocational intermediate in protein synthesis observed in crystals of enzymatically active 50S subunits. *Nat. Struct. Biol.* **9**: 225–230.
- Schmeing, T.M., Moore, P.B., and Steitz, T.A. 2003. Structures of deacylated tRNA mimics bound to the E site of the large ribosomal subunit. *RNA* **9**: 1345–1352.
- Sigmund, C.D., Ettayebi, M., Borden, A., and Morgan, E.A. 1988. Antibiotic resistance mutations in ribosomal RNA genes of *Escherichia coli*. *Meth. Enzymol.* **164**: 673–690.

- Simons, R.W., Houman, F., and Kleckner, N. 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene* **53**: 85–96.
- Valle, M., Zavialov, A., Sengupta, J., Rawat, U., Ehrenberg, M., and Frank, J. 2003. Locking and unlocking of ribosomal motions. *Cell* **114**: 123–134.
- Vila-Sanjurjo, A., Squires, C.L., and Dahlberg, A.E. 1999. Isolation of kasugamycin resistant mutants in the 16S ribosomal RNA of *Escherichia coli*. *J. Mol. Biol.* **293**: 1–8.
- von Ahsen, U. and Noller, H.F. 1995. Identification of bases in 16S rRNA essential for tRNA binding at the 30S ribosomal P site. *Science* **267**: 234–237.
- Wimberly, B.T., Brodersen, D.E., Clemons, W.M., Morgan-Warren, R.J., Carter, A.P., Vornrhein, C., Hartsch, T., and Ramakrishnan, V. 2000. Structure of the 30S ribosomal subunit. *Nature* **407**: 327–339.
- Yoshizawa, S., Fourmy, D., and Puglisi, J.D. 1999. Recognition of the codon–anticodon helix by ribosomal RNA. *Science* **285**: 1722–1725.
- Yusupov, M., Yusupova, G., Baucom, A., Lieberman, K., Earnest, T.N., Cate, J.H., and Noller, H.F. 2001. Crystal structure of the ribosome at 5.5 Å resolution. *Science* **292**: 883–896.
- Yusupova, G.Z., Yusupov, M., Cate, J.H.D., and Noller, H.F. 2001. The path of messenger RNA through the ribosome. *Cell* **106**: 233–241.

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