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A single base mutation at position 2661 in *E.coli* 23S ribosomal RNA affects the binding of ternary complex to the ribosome

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A single base substitution mutation from guanine to cytosine was constructed at position 2661 of *Escherichia coli* 23S rRNA and cloned into the *rrnB* operon of the multi-copy plasmid pKK3535. The mutant plasmid was transformed into *E.coli* to determine the effect of the mutation on cell growth as well as the structural and functional properties of the mutant ribosomes *in vivo* and *in vitro*. The results show that the mutant ribosomes have a slower elongation rate and an altered affinity for EF-Tu-tRNA-GTP ternary complex. This supports previous findings which indicated that position 2661 is part of a region of 23S rRNA that forms a recognition site for binding of the ternary complex in the ribosomal A site. Combinations of the 2661 mutation with various mutations in ribosomal protein S12 also demonstrate that elements of both ribosomal subunits work in concert to form this binding site.

Key words: EF-Tu/mutagenesis/translation/tRNA

Introduction

Many regions of rRNA participate directly in translation, particularly the phylogenetically conserved single-stranded sequences (see Noller, 1984 and Dahlberg, 1989 for reviews). One such region, which has long been recognized as critically important for the proper functioning of the ribosome, is the single-stranded loop that contains the site of action for the cytotoxins, alpha sarcin and ricin (Wool, 1984). In *Escherichia coli*, this loop is located between positions 2653 and 2667 of 23S rRNA.

Alpha sarcin and ricin act on single nucleotides in the rRNA of the large subunit of both prokaryotes and eukaryotes. In *E.coli*, alpha sarcin produces a single cleavage between positions 2661 and 2662 (Hausner *et al.* 1987; Endo and Tsurugi, 1988) while ricin depurinates the adenine at position 2660 (Endo and Tsurugi, 1988). The corresponding bases are also modified in eukaryotes (Endo and Wool, 1982; Chan and Wool, 1983; Endo *et al.*, 1987; Endo and Tsurugi, 1987). The action of these enzymes renders the ribosomes completely inactive in translation (Sperti *et al.*, 1973; Montanaro *et al.*, 1973; Fernandez-Puentes and Vasquez, 1977; Schindler and Davies, 1977; Conde *et al.*, 1978; Hobden and Cundliffe, 1978). The remarkable specificity of these cytotoxins for single nucleotides and their effects on ribosome activity have been valuable tools for identifying a region of rRNA that participates in protein synthesis (Wool, 1984; Hausner *et al.*, 1987).

In this study, site-directed mutagenesis was used to investigate the function of the rRNA in the loop containing the target sites for alpha sarcin and ricin. A single base transversion of G to C was constructed at position 2661 of *E.coli* 23S rRNA, the alpha sarcin site, and the structural and functional characteristics of the mutant ribosomes were investigated. The mutation at position 2661 of 23S rRNA was introduced into the *rrnB* operon carried on the high copy plasmid pKK3535 (Brosius *et al.*, 1981). The mutant plasmid was transformed into *E.coli* to determine the effect of the mutation on cell growth as well as the structural and functional properties of the mutant ribosomes *in vivo* and *in vitro*. The results of the studies support previous findings which showed that the 2660 region of 23S rRNA is involved in the recognition and binding of EF-Tu-tRNA-GTP ternary complexes (Hausner *et al.*, 1987; Moazed *et al.*, 1988). In addition, modulation of the effects of the 2661 mutation by mutations in ribosomal protein S12 suggests that elements on both ribosomal subunits work in concert to select ternary complexes during translation.

Mutations in ribosomal protein S12 which confer streptomycin resistance can be classified according to their effects on the interaction between the ribosome and ternary complexes (Bohman *et al.*, 1984). Restrictive S12 mutations are characterized by slow elongation rates *in vivo* and more aggressive proofreading *in vitro* (Ruusala *et al.*, 1984). The latter can be attributed to an increased K_m for ternary complexes (Bohman *et al.*, 1984). Non-restrictive S12 mutations are still streptomycin resistant but do not display the slower elongation rate or an altered interaction with ternary complexes. Effects on ribosome function by the mutation at position 2661 in 23S rRNA could be detected only when combined with restrictive S12 mutations. Interestingly, the combination of restrictive S12 and 2661C increased the affinity of ternary complexes for the ribosome, an effect which is opposite to the effect of restrictive S12 mutations alone.

Results

Construction of the rRNA mutation

The single base mutation (G to C) at position 2661 of the 23S rRNA gene was constructed by oligonucleotide directed mutagenesis using the *Eco*RI–*Bam*HI fragment of *rrnB* cloned into M13. Following mutagenesis the *Pvu*II-*Esp*I subfragment containing the mutation was cloned into the expression vector pKK3535 to form the mutant plasmid pKK2661C. The mutant construct was analyzed by restriction digestion to ensure that the rRNA gene was intact, and the DNA region around position 2661 was sequenced to confirm that the mutant plasmid was identical to the wild-type plasmid with the exception that cytosine replaces guanine at position 2661.

Expression of mutant rRNA and incorporation into ribosomes

The expression, processing and assembly characteristics of plasmid-encoded mutant rRNA were analyzed using ^{32}P-labeled maxicells and by sequencing the rRNA from ribosomal pools using reverse transcriptase. Analysis of labeled rRNA from maxicells by agarose–acrylamide composite gel electrophoresis confirmed that the processing and assembly of mutant rRNA was identical to wild-type rRNA (data not shown). Reverse transcriptase was used to determine the percentage of mutant rRNA present in total cellular RNA, 50S ribosomal subunits, 70S ribosomes and polyribosomes isolated from HB101 and DH1 cells containing pKK2661C or pKK3535 (as a control). The rRNAs extracted from these samples were sequenced and the relative band intensities in the G and C tracks at position 2661 were quantitated to determine the level of mutant rRNA. For both HB101 and DH1 the expression of mutant rRNA from the plasmid, as revealed by the ratio of mutant rRNA in the total RNA preparation, was ~65%. This ratio was also found in the rRNA from 50S subunits, 70S ribosomes and polyribosomes. Thus there was no apparent block in the expression of mutant rRNA, in the assembly of mutant rRNA into ribosomal particles or in the uptake of mutant ribosomes into polyribosomes.

Maxicell 70S particles contain a high proportion of mutant 23S rRNA

A significant difference between mutant and wild-type ribosomal components was evident when the maxicell ribosomes were analyzed on two dimensional composite gels. In these gels 70S ribosomes were separated from free 50S and 30S subunits in the first dimension and the rRNAs associated with each of these components were separated in the second dimension. In agreement with the rRNA sequencing data the mutant 23S rRNA was assembled into 50S subunits and these subunits associated with 30S subunits to form 70S ribosomes (Figure 1). However, the distribution of plasmid-encoded mutant rRNA is greater in 70S than 50S particles, when compared with plasmid-encoded wild-type RNA. This effect is noticeable in the first dimension where the mutant preparation showed a greater proportion of rRNA in the 70S ribosomes than did the wild-type control. The effect is confirmed when the second dimension RNA pattern is analyzed. In the wild-type preparation the competition between labeled plasmid-encoded and unlabeled chromosome-encoded subunits produces an even distribution of labeled 23S rRNA in the 50S and 70S regions. In contrast the majority of labeled 23S rRNA in the mutant preparation is present in the 70S ribosomes. The 50S subunits carrying the 2661 mutation would appear to either compete favorably with unlabeled, chromosome-encoded, wild-type 50S subunits for the 30S subunit pool or form a more stable 70S complex. The enhancement of mutant 23S rRNA in 70S ribosomes is accentuated in the maxicells since they have a relative deficiency of 30S subunits (Stark *et al.*, 1982). This difference was not observed in non-maxicell ribosome preparations analyzed by RNA sequencing.

Growth rate of transformed cells depends on the nature of ribosomal protein S12

The growth rate of cells containing the plasmid pKK2661C varied widely depending on the genetic background of the host (Table I). This difference was first noted for the strains DH1 and HB101. In DH1 the mutation at position 2661 had no effect on growth rate while in HB101 the mutation was extremely deleterious to cell growth. Since one genetic difference between these two strains resides in ribosomal protein S12, where DH1 is wild-type and HB101 contains a restrictive *rpsL* mutation, the mutant plasmid was transformed into the isogenic series of *rpsL* strains UK327, UK328 and UK235. No reduction in growth rate was found for the non-restrictive strain UK327, growth was significantly inhibited in the slightly restrictive strain UK328 and the 2661 mutation was lethal in the restrictive strain UK235.

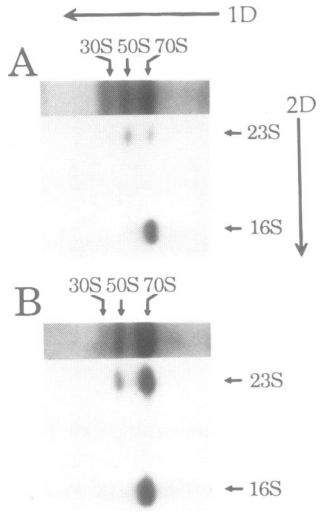


Fig. 1. Two-dimensional gel electrophoresis of ^{32}P-labeled maxicell lysates. Aliquots of maxicell lysate (10^5 c.p.m.) were electrophoresed in the first dimension for 6 h at 150 V in a 2.75% acrylamide–0.5% agarose gel in 25 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 30 mM KCl at 3°C. Proteins were removed from the ribosomes by soaking the gel slice in 90 mM Tris–borate, pH 8.2, 1 mM EDTA, 0.1% SDS and then the sample was electrophoresed in the second dimension in a 3% acrylamide–0.5% agarose gel for 16 h at 250 V at 3°C in the same buffer without the SDS. The first dimension (top, right to left) separated 70S ribosomes, 50S subunits and 30S subunits in wild type (**A**) and mutant (**B**) lysates. The second dimension (top to bottom) identified the rRNA species associated with the ribosomal particles.

Table I. The effect of restrictive S12 mutations on cell growth rate in the presence of the 2661 mutation

Cell strain	S12 genotype	S12 phenotype	Plasmid	Growth rate (min)
DH1	wild-type	wild-type	pKK3535	46 ± 3
			pKK2661C	47 ± 3
UK328	*rpsL226*	non-restrictive	pKK3535	58 ± 5
			pKK2661C	55 ± 8
UK327	*rpsL224*	slightly restrictive	pKK3535	52 ± 5
			pKK2661C	72 ± 9
HB101	*rpsL20*	restrictive	pKK3535	55 ± 3
			pKK2661C	90 ± 10
UK235	*rpsL282*	restrictive	pKK3535	58 ± 8
			pKK2661C	lethal

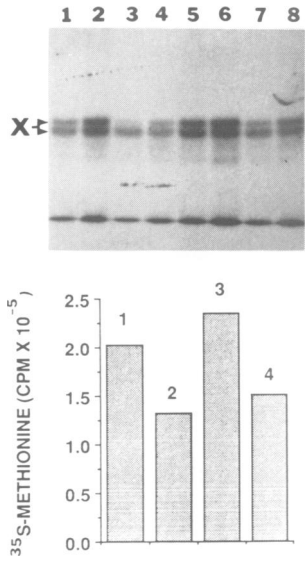


Fig. 2. *In vitro* synthesis of β-lactamase in a transcription/translation system. The ^{35}S-labeled products of *in vitro* protein synthesis, directed by transcripts of pBR322, were electrophoresed on an SDS gel. Aliquots of the synthesis reactions were also removed and precipitated with TCA to quantitate the level of synthesis. The autoradiogram of the gel shows the β-lactamase (X) produced using two different concentrations of ribosomes (6 pmol and 12 pmol) from two independent preparations of wild-type (lanes 1,2 and 5,6) and mutant (lanes 3,4 and 7,8) ribosomes. The level of [^{35}S]methionine incorporation by ribosome samples of lanes 2,4, 6 and 8 are represented as 1,2,3 and 4 respectively in the graph.

In vitro functional assays of mutant ribosomes

The effect of the 2661 mutation in a restrictive background was examined by *in vitro* translation assays utilizing ribosomes isolated from HB101 cells containing pKK2661C or pKK3535 (as a control). In each of these assays the ribosomes with the 2661 mutation showed a decrease in the overall rate of elongation. For example, the level of β-lactamase production in a transcription/translation system by the preparation of mutant ribosomes was ~60% of that produced by wild-type ribosomes (Figure 2). However, when the ribosomes were placed in assays which measure dipeptide and tripeptide formation, no difference between mutant and wild-type ribosomes could be detected in the rate of formation of the first or second peptide bond (Figure 3).

From the results of the dipeptide and tripeptide assays it appeared that the 2661 mutation was not inhibiting the initiation of protein synthesis, but there was a significant reduction in the overall rate of protein synthesis (as seen in the transcription/translation assay). To investigate this in more detail, a poly(U) directed translation system was employed. In this system it was easier to control the input of mRNA and this allowed the incorporation levels to be assayed from conditions of ribosome excess to ribosome limiting. The results of these experiments are shown in Figure 4A. Once again the mutant ribosome preparation incorporated amino acids less efficiently than the wild-type ribosomes, and this was true for every concentation of mRNA tested. However, the relative level of polypeptide synthesis by mutant and wild-type ribosomes was not identical for each mRNA concentration (Figure 4B). At low concentrations of mRNA, when ribosomes were in excess, the level of synthesis by mutant ribosomes was much lower

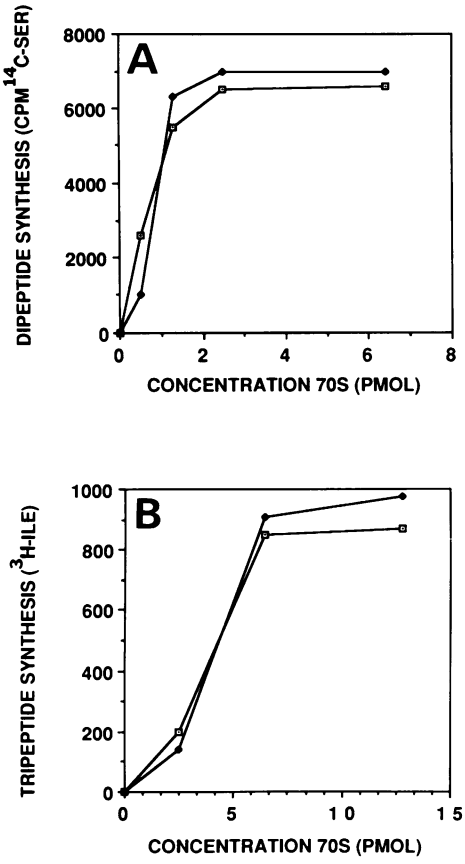


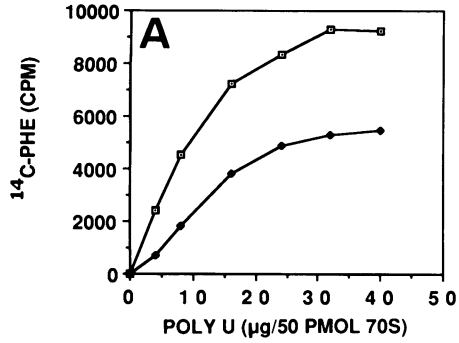
Fig. 3. Synthesis of dipeptides and tripeptides in a DNA-directed *in vitro* translation assay. The formation of dipeptides (**A**) and tripeptides (**B**) were assayed using increasing concentrations of 70S ribosomes isolated from HB101 cells containing pKK3535 (♦) and pKK2661C (□).

than the level of synthesis by the wild-type ribosomes (30%). As the mRNA concentration increased so did the relative level of incorporation by the mutant ribosomes. The level plateaued when the ribosome concentration became limiting. Interestingly. the ratio of mutant incorporation to wild-type incorporation at the plateau was 58%, the same as was found in the transcription/translation assay.

Finally, the elongation rates of mutant and wild-type ribosomes were measured in a ribosome limiting, poly(U) translation system which approximates *in vivo* translation rates (Ehrenberg and Kurland, 1988). For these assays the elongation rates of ribosomes isolated from both HB101 and DH1 were compared. The results (shown in Figure 5) are consistent with the *in vitro* data in Figures 2 and 4 as well as the growth rate data. Mutant ribosomes from HB101 showed a reduced elongation rate (66% of wild-type). The elongation rate for mutant ribosomes from DH1, the non-restrictive strain, was identical to that for wild-type ribosomes from DH1.

Increased level of tRNA in mutant ribosomes

The structure of the mutant ribosomes and mutant rRNA, as determined by sucrose gradients and agarose–acrylamide composite gels, were not significantly different from wild-type ribosomes and wild-type rRNA. However, because there were significant functional differences the protein and nucleic acid compositions of the mutant 70S ribosomes were



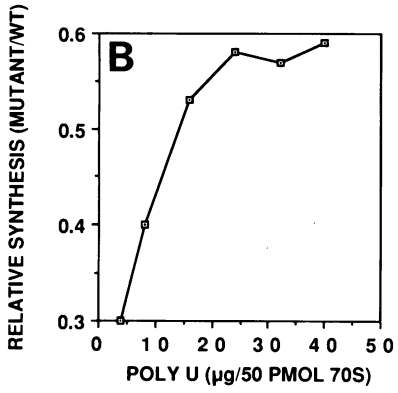
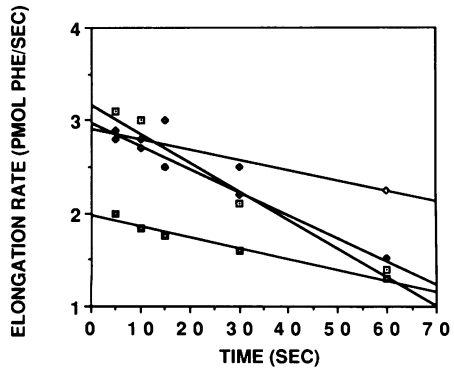


Fig. 4. *In vitro* protein synthesis as a function of mRNA [poly(U)] concentration. [^{14}C]phenylalanine incorporation into TCA precipitable polypeptides was measured over a range of poly(U) concentrations using 70S ribosomes isolated from HB101 cells containing pKK3535 (□) and pKK2661C (◆) (**A**). The poly(U) concentration is in µg per 50 pmol of 70S ribosomes per assay. The ratio of incorporation (pKK2661C/pKK3535) for each concentration of poly(U) is plotted in (**B**).



Elongation Rates
(pmol phe/ribosome/sec)

pKK3535 HB101	(▫)	3.1
pKK3535 DH1	(◆)	3.0
pKK2661C HB101	(▪)	2.0
pKK2661C DH1	(◆)	3.0

Fig. 5. Comparison of elongation rates of mutant and wild-type ribosomes in restrictive and non-restrictive cells. The elongation rate of 70S ribosomes isolated from HB101 (restrictive) and DH1 (non-restrictive) cells containing either pKK3535 or pKK2661C were measured in the poly(U) directed *in vitro* translation system of Kurland (Ehrenberg and Kurland, 1988). The elongation rate was calculated by extrapolating the rate function to zero time.

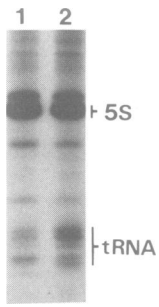


Fig. 6. tRNA associated with mutant and wild-type 70S ribosomes. RNA in HB101 cells containing pKK3535 or pKK2661C was labeled *in vivo* with ^{32}P. The RNA associated with 70S ribosomes was extracted and electrophoresed for 8 h on a 6% polyacrylamide gel containing 8 M urea. Note the greater amount of tRNA associated with mutant (lane 2) than wild-type (lane 1) ribosomes.

investigated in more detail. The nucleic acids in HB101 cells containing the mutant and wild-type plasmid were labeled *in vivo* with ^{32}P and then the 70S ribosomes were isolated by sucrose gradient centrifugation. The labeled RNA from the 70S ribosomes was extracted and analyzed by polyacrylamide gel electrophoresis. The only significant difference between mutant and wild-type RNAs was an increase in the level of tRNA bound to the mutant ribosomes (Figure 6). Densitometric scanning of the tRNA region revealed that the mutant 70S ribosomes contained approximately three times more tRNA than the wild-type 70S ribosomes.

Increased level of EF-Tu in mutant 70S ribosomes

The protein complements of mutant and wild-type 70S ribosomes were analyzed by SDS−PAGE. No differences in the concentrations or the mobilities of ribosomal proteins were noted. Similarly there were no differences between mutant and wild-type 50S proteins (data not shown). However, in the 70S preparations there was a noticeable difference in the concentration of a protein which co-migrated with EF-Tu. The concentration of EF-Tu in the 70S ribosomes was determined by Western blot analysis. The mutant and wild-type ribosomes were prepared by two methods: ribosomes from a cell lysate were washed by pelleting through 0.25 M NH_4Cl buffer or through a 10/30% sucrose cushion containing 0.25 M NH_4Cl. The two preparations differed with respect to the stringency of factor removal. The protein complements from these preparations were separated by SDS−PAGE, stained and then probed for EF-Tu using anti-EF-Tu IgG. Inspection of the stained gel confirmed that equivalent quantities of wild-type and mutant ribosomes were loaded onto the gel. The Western blot analysis (Figure 7) shows that a greater amount of EF-Tu was associated with the mutant ribosomes. The effect is most easily demonstrated with ribosomes prepared by method 2: the sucrose, salt-washed preparations (lanes 6 and 7), where no EF-Tu remained bound to wild-type ribosomes but a significant amount of the factor remained bound to the mutant ribosomes. A comparison of the intensities of the EF-Tu bands in lanes 1−4 with the EF-Tu standard (shown in lane 5) indicated that the concentration of EF-Tu bound to mutant ribosomes was increased ~3-fold. This is the same level of increase found for the tRNA bound to the mutant ribosomes.

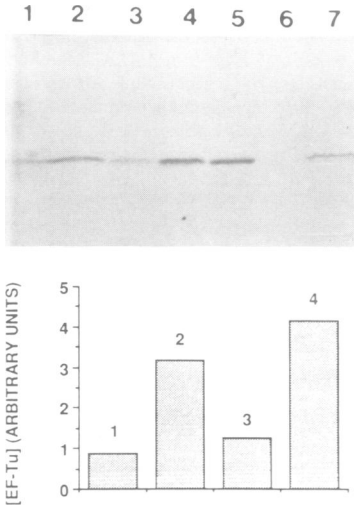


Fig. 7. Western blot analysis of EF-Tu bound to 70S ribosomes. The protein component of 70S ribosomes from HB101 cells containing either pKK3535 or pKK2661C was separated by SDS–PAGE, electrotransferred to nitrocellulose and probed for EF-Tu using anti-EF-Tu-IgG. Lanes 1 and 3 show the level of EF-Tu bound to 20 μg and 30 μg of wild-type 70S ribosomes respectively. Lanes 2 and 4 show the level of EF-Tu bound to 20 μg and 30 μg of mutant 70S ribosomes respectively. Lane 5 is 50 ng of purified EF-Tu-GDP. Lanes 6 and 7 demonstrate the level of EF-Tu bound to 50 μg of wild-type (lane 6) and mutant (lane 7) 70S ribosomes which had been pelleted through a high-salt sucrose cushion. Band intensities of samples 1–4 were quantitated by scanning and are plotted in the graph.

Discussion

The single-stranded rRNA region surrounding the alpha sarcin cleavage site is vitally important to the proper functioning of the ribosome. Early evidence in eukaryotic systems (see Terao *et al.*, 1988; Wool, 1984 and references cited therein) and more recently with prokaryotes (Hausner *et al.*, 1987) have shown that altering this region with the cytotoxic enzymes alpha sarcin and ricin completely inactivates the ribosome. The striking sequence conservation of the single-strand loop throughout the prokaryotic and eukaryotic kingdoms (Gutell and Fox, 1988) serves to confirm the importance of maintaining the structure of the region for proper function. Although clues to the function of this region have existed for some time, only more recently has it been possible to probe the function of rRNA in more detail. In the present study we have altered this structure by substituting a single base (2661 G to C) near the center of the conserved loop. The single base change had a deleterious effect on the functional capabilities of ribosomes derived from cells which also contained a restrictive mutation in ribosomal protein S12. Somewhat unexpectedly however, the rRNA mutation did not affect the function of ribosomes with wild-type S12 to any detectable extent.

The functional abnormalities of the mutant ribosomes from the restrictive strain, HB101, were apparent in the *in vitro* translation assays. Both poly(U) and natural mRNA translation systems showed that the mutant ribosomes were synthesizing polypeptides, but did so at a reduced rate. When the mRNA was in excess (ribosome limiting conditions) the mutant ribosome preparation incorporated amino acids at ~60% the rate of wild-type ribosomes. Since the mutant preparation contained ~35% wild-type ribosomes the actual rate of synthesis by mutant ribosomes was only ~25% that of wild-type ribosomes.

The *in vitro* results also offer some insight into how the 2661 mutation reduces the growth rate of the cell. When the mRNA concentration was lowered in the poly(U) system (ribosome excess) the translation rate was lower than when the mRNA was in excess (ribosome limiting). This behavior is indicative of a situation where both mutant and wild-type ribosomes were translating a single mRNA molecule and the mutant ribosomes were impeding the progress of the wild-type ribosomes behind them in the polyribosome. An alternative interpretation is that at low mRNA concentrations the mutant ribosomes were completely inactive, unable to form a peptide bond, and only the wild-type ribosomes were translating. This latter possibility is inconsistent with the results of the dipeptide assay which showed no deficiency by the mutant ribosomes and is further weakened by the finding that mutant ribosomes were found in high concentrations in the polyribosome fraction of the cell. We conclude that the mutant ribosomes were slowing the progress of the wild-type ribosomes in polysomes, thereby slowing the growth of the cell.

Analysis of the composition of the mutant ribosomes from HB101 indicated that both tRNA and EF-Tu were more abundant in the mutant 70S ribosomes than in the wild-type ribosomes. The increase in both components was approximately equal indicating an enhanced association between the mutant ribosomes and the EF-Tu-tRNA complex. It appears that the 2661 mutation alters the ribosomes such that the normal ternary complex cycle is impeded at the A site. An enhanced stability of EF-Tu-tRNA complex on the mutant ribosomes would reduce the rate of translation and also account for the favored inclusion of mutant 50S subunits in the 70S ribosomes in the maxicell analysis. Elongation factor G (EF-G) as well as EF-Tu has been shown to protect bases in the 2661 region of 23S rRNA from chemical modification (Moazed *et al.*, 1988). Since EF-Tu and EF-G are known to compete for binding on the 70S ribosome, one could also interpret the increased level of EF-Tu on mutant ribosomes as indicating that the 2661 mutation is decreasing the affinity of the ribosome for EF-G. Thus the mutation may indirectly increase the availability of the ribosome for EF-Tu rather than directly enhance the stability of EF-Tu-tRNA complexes. We cannot rule out this possibility.

The effects of the 2661 mutation, slow elongation rate and perturbed ternary complex interaction, were detected only when the 2661 mutation was expressed in cells that contain a restrictive S12 mutation. The role of S12 in modulating the effects of the 2661 mutation was most clearly demonstrated by transforming pKK2661C into the isogenic *rpsL* strains UK327, UK328 and UK235. The effect of pKK2661C in these strains varied from wild-type growth rate to lethality and correlated directly with the extent to which each particular S12 mutation perturbs the ternary complex interaction. It is important to note that whereas restrictive S12 mutations alone destabilize the ternary complex (Bohman *et al.*, 1984), the combination of 2661C and restrictive S12 stabilized the ternary complex. The molecular basis for this effect is not understood. However, both S12 and the 23S rRNA region around position 2661 have been shown to be involved in ternary complex interactions. Cleavage of 23S rRNA with alpha sarcin specifically

blocks the elongation factor dependent functions of the ribosome (Hausner *et al.*, 1987). In addition, ternary complex binding protects 2661G from modification by chemical probes (Moazed *et al.*, 1988). Detailed kinetic studies have demonstrated that restrictive S12 mutations alter ternary complex interactions (Bohman *et al.*, 1984; Ruusala *et al.*, 1984). The present study reinforces these findings and indicates that elements on both the 30S and 50S subunits monitor the binding of ternary complex in a coordinate fashion.

Pleiotropic effects associated with mutations in ribosomal protein S12 have been demonstrated previously for tRNA and EF-Tu mutations. The EF-Tu mutation Ar (Duisterwinkel *et al.*, 1981), which stabilizes ternary complex interaction (Tapio and Kurland, 1986), compensates for the destabilization of ternary complex by restrictive S12 mutations (Tapio and Isaksson, 1988). A mutation in *miaA* which reduces tRNA modification generates streptomycin dependence when combined with a restrictive S12 mutation (Petrullo *et al.*, 1983). The mutations in *miaA* and *rpsL* both reduce elongation rates by destabilizing ternary complex interaction. The combination requires streptomycin to increase ternary complex stability, thereby decreasing proofreading and increasing elongation rates to a tolerable level (Diaz *et al.*, 1986). These pleiotropic effects help to define the involvement of S12 in ternary complex interaction. The present combination of 2661C and restrictive S12 shows that components of both ribosomal subunits work coordinately to define the proper binding site for the ternary complex. This suggests a link between the two subunits involving S12 on the 30S subunit and the 2660 loop on the 50S subunit. Together these elements contribute to a critical recognition and binding site for the ternary complex in the A site ribosomal elongation.

Materials and methods

Mutagenesis and expression

The single base substitution mutation was constructed by oligonucleotide directed mutagenesis in M13 as described by Zoller and Smith (1982). The *Eco*RI–*Bam*HI fragment from *rrnB* was cloned into M13mp18 and the template strand was isolated from the *ung*$^-$ *dut*$^-$ strain RZ1032 to increase the frequency of mutagenesis (Kunkel, 1985). Isolated transformants from the mutagenesis were screened by DNA sequencing (Sanger *et al.*, 1977) and the *Pvu*II–*Esp*I subfragment containing the mutation was cloned into the expression vector pKK3535 (Brosius *et al.*, 1981) to create the mutant plasmid pKK2661C. The mutation in pKK2661C was confirmed by sequencing the plasmid directly (Chen and Seeburg, 1985). The level of mutant 23S rRNA expressed from pKK2661C and the incorporation of mutant rRNA into 50S subunits, 70S ribosomes and polyribosomes was determined by sequencing the rRNA using reverse transcriptase (Vester and Garrett 1988; Tapprich *et al.*, 1989).

Maxicells

Plasmid-encoded rRNA was labeled with ^{32}P in maxicells as described by Jemiolo *et al.* (1988). Ribosomal particles and rRNA were analyzed by one dimensional and two dimensional electrophoresis on agarose–acrylamide composite gels (Stark *et al.*, 1982).

Ribosomes and ribosomal subunits

Plasmid-containing cells, growing in 500 ml of LB media with 200 μg/ml ampicillin were harvested in log phase (A_{600} = 0.6) and the cells were broken by grinding with alumina in 20 mM Tris–HCl (pH 7.6), 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT. Alumina and cell debris were cleared by consecutive centrifugation at 10 000 and 16 000 *g* for 15 min and 90 min respectively. Ribosomes were pelleted from the cleared supernatant by centrifugation at 50 000 r.p.m. for 3 h at 4°C in a Beckman Ti60 rotor. Ribosomal pellets were resuspended in 20 mM Tris–HCl, pH 7.6, 0.5 M NH_4Cl, 10 mM $MgCl_2$, 1 mM DTT, cleared of insoluble material by low speed centrifugation, repelleted, resuspended and then centrifuged for 16 h at 35 000 r.p.m. in a Ti60 rotor through a 20 ml 10%/30% sucrose cushion in the same buffer. The final ribosome pellets were dissolved in 20 mM Tris–HCl, pH 7.6, 60 mM KCl, 6 mM $MgCl_2$ 1 mM DTT and stored frozen in small aliquots at −70 C. Tight couple 70S ribosomes were isolated by fractionating a 5–30% sucrose gradient in the same buffer which was centrifuged for 14 h at 20 000 r.p.m. in a Beckman SW28 rotor. The ribosomal particles were recovered from the pooled fractions by pelleting for 16 h at 40 000 r.p.m. in a Beckman Ti60 rotor. Ribosomal subunits were isolated by fractionating a 5–30% sucrose gradient in 25 mM Tris–HCl, pH 7.6, 100 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT which was centrifuged for 16 h at 20 000 r.p.m. in a Beckman SW-28 rotor. Subunits were recovered by pelleting as above. Polyribosomes were prepared by the procedure of Godson and Sinsheimer (1967) and fractionated on a 10–35% sucrose gradient in 25 mM Tris-HCl, pH 7.6, 60 mM NH_2Cl, 10 mM $MgCl_2$, 1 mM DTT by centrifugation for 8 h at 20 000 r.p.m. in a Beckman SW28 rotor.

Analysis of tRNA and EF-Tu bound to 70S ribosomes

Ribosomes prepared from lysates of 25 ml cell cultures were analyzed for bound tRNA and EF-Tu. For the tRNA analysis, RNA was labeled *in vivo* with ^{32}P. Cells were grown to A_{600} = 0.4–0.5 in 25 ml of LB containing 200 μg/ml of ampicillin and then harvested by centrifugation at 4°C. The cells were washed twice with cold ZPM media (Jemiolo *et al.*, 1988) and the final cell pellet was resuspended in 5 ml of the same media. Carrier free [^{32}P]orthophosphate (100 μCi) was added and the culture was shaken at 37°C for 15 min. Unlabeled phosphate was added (to 0.4 mM KH_2PO_4) and the culture shaken for an additional 15 min. Cells were harvested and lysed as described by Jemiolo *et al.* (1988). Ribosomes were isolated from the cell lysate by fractionating on a 5–30% sucrose gradient in 25 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 60 mM NH_4Cl, 1 mM DTT. Labeled RNA from the 70S ribosomes was extracted with phenol and analyzed by gel electrophoresis (Peacock and Dingman, 1967).

For the EF-Tu analysis a 25 ml culture at A_{600} = 0.6 was harvested and lysed as above. The lysate was diluted to 5 ml with 25 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 0.25 M NH_4Cl, 1 mM DTT and the ribosomes pelleted by centrifugation for 8 h at 35 000 r.p.m. at 4°C in a Beckman SW-41 rotor. An identical sample was pelleted through a 10 ml 10%/30% sucrose cushion in the same buffer for 16 h. Ribosomal pellets were resuspended in 25 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 60 mM NH_4Cl, 1 mM DTT. Proteins associated with the ribosomes were solubilized in 60 mM Tris–HCl, pH 6.8, 2% SDS, 0.6 M 2-mercaptoethanol, 10% glycerol, 0.025% bromphenol blue, electrophoresed in a 12.5% SDS–polyacrylamide gel for 6 h at 200 V and then electrophoretically transferred to nitrocellulose filter paper at 125 mA for 16 h in 25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol (Towbin *et al.*, 1979). Proteins on the nitrocellulose filter were reversibly stained with Ponceau S (Salinovich and Montelaro, 1986) before detecting EF-Tu by Western blotting. The filter was blocked with 3% BSA in TS buffer (20 mM Tris–HCl, pH 7.5, 200 mM NaCl, 0.05% Tween 20) for 30 min, washed for 5 min in TS buffer and then incubated with anti-EF-Tu IgG (diluted 1:1000 in TS buffer containing 1% BSA) for 4 h. The filter was washed with TS buffer (10 times for 3 min each), incubated with peroxidase conjugated anti-IgG (diluted 1:2000 in TS buffer containing 1% BSA) for 2 h, then washed again as above. Antibody bound to the filter was stained using 0.5% 4 chloro-1-napthol, 0.15% H_2O_2, 16.5% methanol in TS buffer (without Tween 20).

In vitro protein synthesis

Several different cell-free translation systems were used to assay the functional actvity of the mutant ribosomes. Poly(U) directed incorporation of phenylalanine into TCA precipitable polypeptides was determined by the system of Traub *et al.* (1971). The elongation rate was assayed in a poly(U) directed burst experiment according to Ehrenberg and Kurland (1988). Translation of natural mRNA utilized a highly defined DNA-directed transcription/translation system (Kung *et al.*, 1977; Zaruki-Shultz *et al.*, 1979), and formation of the first and second peptide bonds were assayed in DNA-directed dipeptide (Robakis *et al.*, 1982) and tripeptide (Centiempo *et al.*, 1982) systems. These latter assays were performed by B.Redfield and H.Weissbach.

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