Mutations at Positions 13 and/or 914 in *Escherichia coli* 16S Ribosomal RNA Interfere with the Initiation of Protein Synthesis†

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Received March 28, 1995; Revised Manuscript Received May 16, 1995⊗

ABSTRACT: Mutations at positions 13 (U→A) and/or 914 (A→U) of *Escherichia coli* 16S rRNA severely affect cell growth and protein synthesis, when expressed *in vivo* in a vector encoding an *rrn* operon under control of an inducible promoter. *In vitro* assays using extension inhibition indicate that the mutations interfere with the formation of the 30S translational initiation complex, which can account for their effect on cell growth. The two mutations destabilize an adjacent pseudoknot helix in which bases 17−19 pair to bases 916−918. This was shown by the increased binding of an oligodeoxyribonucleotide probe complementary to one strand of the pseudoknot helix, and by the increased reactivity to kethoxal of base G917 within this helix. These observations suggest that this pseudoknot helix participates in the formation of the 30S translational initiation complex.

Ribosomal RNA plays a central role in the function of the ribosome, and site-directed mutagenesis has been a useful tool for gaining insights into this role (Noller, 1991; Tapprich *et al.*, 1990). We have previously shown that mutations at positions 13 (U→A) and/or 914 (A→U) (see Figure 1) in *Escherichia coli* 16S rRNA impair the binding of streptomycin to the ribosome and increase translational accuracy (Pinard *et al.*, 1993, 1994). This was observed when expressing the mutations in a vector containing an *rrn* operon under control of the thermoinducible λpL promoter. Under these conditions, about 50% of the 70S ribosomes contain the plasmid-encoded rRNA. Attempts to increase the proportion of ribosomes with mutant rRNA by expressing the *rrn* operon under control of the strong constitutive P_1P_2 promoters were unsuccessful. This implied that mutations at positions 13 and 914 are deleterious to the cells when the proportion of ribosomes harboring these mutant rRNAs exceeds 50%.

Mutations in protein S12 also impair streptomycin binding and increase translational accuracy (reviewed in Kurland *et al.*, 1990). However, these mutations are not deleterious although all the ribosomes contain the mutated protein. We therefore hypothesized that mutations at positions 13 and/or 914 of 16S rRNA interfere with another step of protein synthesis. This interference would cause their adverse effect on cell growth. The two mutations are adjacent to a pseudoknot helix formed between the loop of the 5′ terminal hairpin (positions 17−19) and residues 916−918 (see Figure 1). This pseudoknot helix, at the junction of the three major domains of 16S rRNA, is involved in initiation, but its role has not been defined (Brink *et al.*, 1993). In this study, we show that both single and double mutations in 16S rRNA destabilize the pseudoknot helix and decrease the rate of formation of the 30S initiation complex. This suggests that the pseudoknot helix is required for the formation of the 30S initiation complex.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Strain POP2136, the host for plasmids containing the *rrn*B operon under control of the thermoinducible λpL promoter, and POP2136SM, a streptomycin-resistant derivative of POP2136 harboring a mutated protein S12, have been described (Pinard *et al.*, 1993, 1994). Plasmid pNO2680 contains the *rrn*B operon under control of the λpL promoter (Gourse *et al.*, 1985). Plasmid pLΔH is a derivative of pNO2680 with a short deletion upstream of the promoter region that removes restriction sites *Eco*R1 and *Hin*dIII. Plasmid pLΔH1192 is a derivative of pLΔH with a C→U mutation at position 1192 in the 16S rRNA that confers spectinomycin resistance (Sigmund *et al.*, 1984). Derivatives of pLΔH1192, with a mutation at position 13 (U→A), at position 914 (A→U), or at both positions 13 and 914 in 16S rRNA, are named pL13A, pL914U, and pL13A-914U, respectively (Pinard *et al.*, 1993, 1994). Plasmid pSTL102 is a derivative of pKK3535 containing the *rrn*B operon under control of its constitutive promoters plus selectable markers for spectinomycin resistance (U1192 in 16S rRNA) and erythromycin resistance (G2058 in 23S rRNA) (Triman *et al.*, 1989). Plasmid pSTL102/III is a derivative of pSTL102 with a specific priming site (priming site III) in the 1015 region of 16S rRNA (Powers & Noller, 1993). This plasmid was a generous gift from R. Samaha and H. Noller, University of California, Santa Cruz, CA. Plasmid pLRCAT is a derivative of pBluescript SK⁻ (Stratagene), in which a *Taq*I/*Taq*I fragment containing the chloramphenicol acetyltransferase gene (CAT)[1] from plasmid pACY184 (New England Biolabs) has been inserted in the *Cla*I site of pBluescript. Plasmid pRS170, which contains gene 32 of T4 bacteriophage under control of the T7 phage promoter (Hartz et al., 1988), was a generous gift from S. Ringquist and L. Gold, University of Colorado, CO. These two latter plasmids were used to generate messenger RNA for extension inhibition assays.

† Supported by a grant from the Medical Research Council of Canada. R.P. is the recipient of a studentship from the Fonds pour la Formation des Chercheurs et l'Aide à la Recherche (F.C.A.R.).

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1995.

[1] Abbreviations: kethoxal, 2-keto-3-ethoxybutyraldehyde; HPLC, high performance liquid chromatography; CAT, chloramphenicol acetyltransferase; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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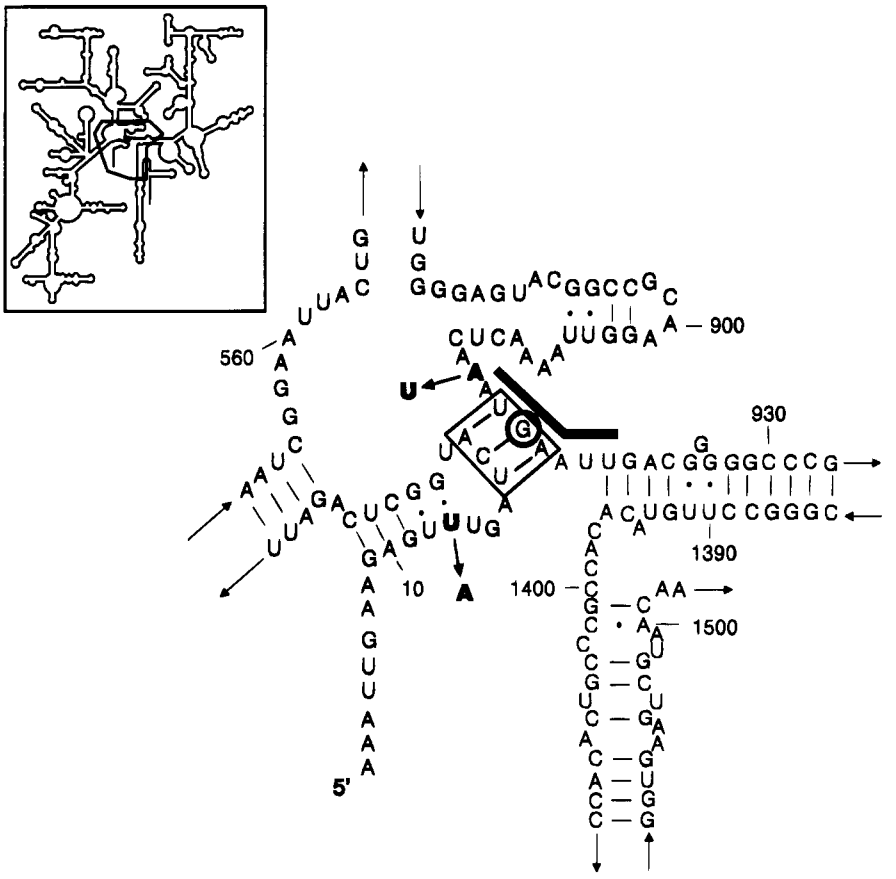

FIGURE 1: Portion of the 16S rRNA, showing the central pseudoknot (boxed) formed by the pairing of residues 17−19 and 916−918. The secondary structure is according to Gutell (1993). Positions 13 and 914 where mutations 13U→A and 914A→U were introduced are in boldface type. The bar indicates the 915−921 region, which was probed with a complementary oligodeoxyribonucleotide. G917 which was probed with kethoxal is circled. The inset shows the skeleton of the 16S rRNA.

Isolation of Ribosomes and Ribosomal Subunits. Ribosomes were obtained by a standard lysis procedure from slow-cooled cultures of POP2136 cells transformed with pLΔ1192 or its mutant derivatives and from POP2136SM (Tapprich *et al.*, 1989). Initiation factor-free 30S subunits were obtained by sucrose gradient centrifugation of dissociated run-off ribosomes in a low magnesium buffer, following standard procedures (Melançon *et al.*, 1984). The protein composition of the mutated 30S subunits was analyzed by HPLC, according to the method of Kerlavage *et al.* (1984).

Effect of the Mutations in 16S rRNA on Cell Growth. POP2136 cells containing either pLΔH, pLΔH1192, or its mutant derivatives (pL13A, pL914U, and pL13A-914U) were grown overnight and used to inoculate LB medium containing 0.1% glucose, prewarmed to 42 °C. At midlog phase, 120 μg/mL of spectinomycin was added to the cultures and the cells were grown to late log phase (absorbance of about 0.8 at 550 nm).

Effect of the Mutations in 16S rRNA on Protein Synthesis and Analysis of the Synthesized Proteins by Gel Electrophoresis. POP2136 cells transformed with pLΔH, pLΔH1192, or its mutant derivatives were grown overnight at 30 °C, and a 30 μL volume from these cultures was used to inoculate 10 mL of MOPS medium supplemented with all amino acids except methionine (Van Bogelen & Neidhardt, 1990). Cells were grown at 42 °C to midlog phase, at which point 120 μg/mL spectinomycin was added, and 5 min later, 25 μCi of [^{35}S]methionine (NEN Express ^{35}S Protein Labeling Mix, 1175 Ci/mmol, 7.9 mCi/mL) was added. The reaction proceeded for 75 min at 42 °C. Samples of 1 mL were taken every 15 min; the cells were rapidly cooled, pelleted, resuspended in a Tris-HCl buffer (pH 7.8), and lysed by freezing and thawing three times. Cell debris was then pelleted, and the proteins in the supernatant were precipitated with trichloroacetic acid. The amount of [^{35}S] incorporation into protein was quantitated by filtration through 934-AH glass microfiber filters (Whatman).

The synthesized proteins were also analyzed by one-dimensional polyacrylamide gel electrophoresis. Total cellular proteins were labeled as described above, except that 100 μCi of [^{35}S]methionine was used. The reaction proceeded for 20 min at 42 °C, 0.2 mM of cold methionine was added, and cells were grown for an additional 40 min. Labeled proteins were extracted as described above and fractionated on 12% polyacrylamide gels in the presence of sodium dodecyl sulfate (Laemmli, 1970).

Synthesis of Messenger RNAs. CAT mRNA and T4 gene 32 mRNA was synthesized *in vitro* from the *Bam*HI-linearized plasmid pLRCAT and from *Pvu*II-cut plasmid pRS170, respectively, using T7 RNA polymerase. *In vitro* transcription was performed using the RiboMAX Large Scale RNA Production System (Promega). The mRNAs were purified on 8% polyacrylamide gels, and the transcripts were excised from the gel and purified by passage through a reverse-phase NENsorb column (New England Nuclear), followed by precipitation with ethanol. The precipitate was resuspended in a Tris−EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4), and the RNA concentration was

determined, assuming that 1 A_{260} unit corresponds to a concentration of 40 μg/mL of RNA.

Extension Inhibition Assays. Extension inhibition assays (toeprinting) using CAT and T4 gene 32 mRNAs were carried out as described by Hartz *et al.* (1988), with DNA primers complementary to nucleotides 76−96 and 60−80 downstream from the initiation codon of the CAT mRNA and the T4 gene 32 mRNA, respectively. The oligonucleotide primers were synthesized with a Pharmacia LKB Gene Assembler and purified by electrophoresis on a 20% polyacrylamide gel. After annealing the 5′-[^{32}P]-end-labeled primer to the mRNA, primer extension was performed with AMV reverse transcriptase for 15 min at 42 °C, in 10 μL of standard buffer (10 mM Tris−acetate, pH 7.4, 60 mM NH_4Cl, 10 mM magnesium acetate, and 6 mM 2-mercaptoethanol), containing 1.5 pmol of mRNA, 10 pmol of heat-reactivated 30S subunits, and 1 nmol of $tRNA_f^{met}$ (Boehringer). DNA extension fragments were separated on 6% polyacrylamide gels and analyzed by autoradiography. Toeprint results were quantitated by measuring band intensities of the full-length and the truncated (toeprint) transcripts using an LKB UltroScan XL laser densitometer.

Binding of an Oligodeoxyribonucleotide Probe to Ribosomal Particles. An oligodeoxyribonucleotide probe complementary to sequence 915−921 of 16S rRNA was synthesized and purified as described above. Heat-reactivated 30S subunits (0.66 A_{260} unit) were incubated with a 20-fold excess of 5′-[^{32}P]-end-labeled probes (specific activity of about 2000 cpm/pmol) for 4 h at 4 °C in 50 μL of binding buffer (10 mM Tris-HCl, pH 7.4, 10 mM magnesium chloride, 0.5 mM EDTA, 60 mM KCl, and 6 mM 2-mercaptoethanol). Binding of the DNA probe was assessed by nitrocellulose filtration using Millipore GSWP 0.22 μm filters, following the procedure of Weller and Hill (1992). The specificity of hybridization of the probe was controlled by RNase H treatment and analysis of the digestion products by gel electrophoresis, as described (Weller & Hill, 1992).

Chemical Modifications. Allele-specific probing was used to assess the reactivity of the plasmid-encoded rRNA in 30S subunits using a specific priming site in the 1015 region (priming site III) (see Powers & Noller, 1993). This priming site was introduced into pLΔH1192 and its mutant derivatives, pL13A, pL914U, and pL13A-914U, by replacing the *ApaI*−*XbaI* fragment with its counterpart from pSTL102/III containing priming site III. Reactivated 30S subunits (14 pmol) were chemically modified with kethoxal (United States Biochemical) in 200 μL of modification buffer (70 mM Hepes−KOH, pH 7.8, 10 mM magnesium chloride, 0.5 mM EDTA, 150 mM KCl, and 1 mM dithiothreitol), containing 5 μL of kethoxal from a stock solution at 37 mg/mL in 20% ethanol. The mixture was incubated at 37 °C for 10 min. After stopping the reaction, the rRNA was extracted and analyzed by primer extension and electrophoresis on 8% acrylamide sequencing gels, as described (Stern *et al.*, 1988a). Autoradiographs were scanned with an LKB UltroScan XL laser densitometer.

RESULTS

Effect of the Mutations on Cell Growth Rates and Protein Synthesis. Figure 2A compares the growth rate of POP2136 cells, transformed either with the wild-type plasmid containing the *rrn* operon (pLΔH1192, where a substitution at position 1192 of 16S rRNA confers spectinomycin resistance to ribosomes containing plasmid-encoded rRNA) or with its three mutant derivatives pL13A, pL914U, and pL13A-914U, harboring respectively a mutation at position 13, at position 914, and at both positions 13 and 914 in the 16S rRNA gene. Cell growth was examined after inducing transcription of the plasmid-encoded *rrn* genes in the presence of spectinomycin, which selectively inactivates ribosomes containing the chromosomally-encoded rRNA. Cells transformed with pLΔH, which lacks the spectinomycin marker, were used as a negative control. Cell growth was not affected by the rRNA mutations in the absence of spectinomycin (not

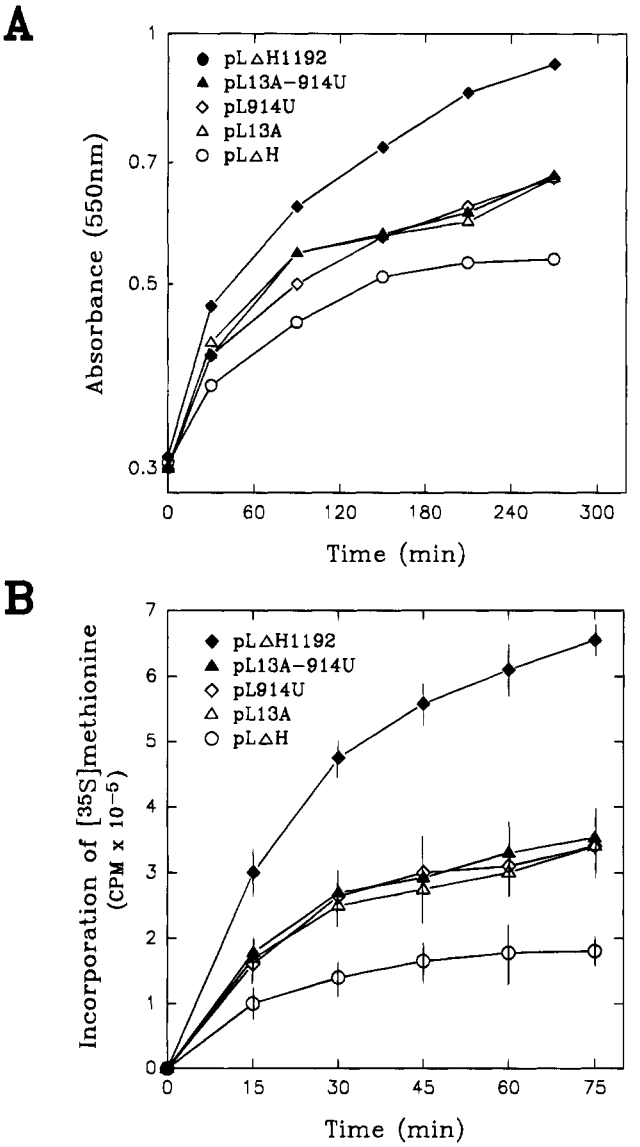

FIGURE 2: (A) Growth curves at 42 °C in the presence of spectinomycin of POP2136 cells transformed with wild-type plasmid pLΔH1192 or its derivatives mutated in the 16S rRNA gene at position 13 (pL13A), at position 914 (pL914U), and at both positions 13 and 914 (pL13A-914U). Cells transformed with pLΔH were used as a negative control. Time 0 corresponds to the addition of spectinomycin at midlog phase. Cell growth was monitored by measuring the absorbance at 550 nm. Four independent assays were carried out. The figure is representative of one experiment. (B) Incorporation of [^{35}S]methionine in total cellular proteins as a function of time. The cells were processed as described in Materials and Methods. [^{35}S] incorporation into protein was quantitated by nitrocellulose filtration. Results are the means of three independent assays. Standard deviations are indicated by error bars.

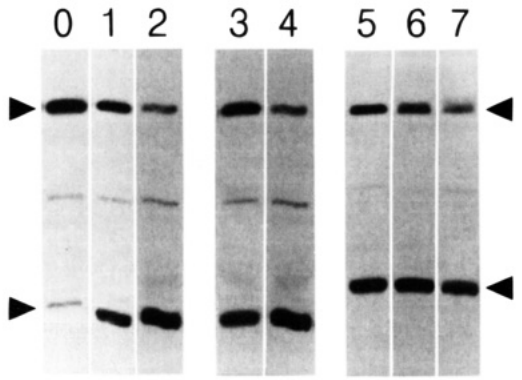

FIGURE 3: Example of autoradiographs showing toeprinting assays with CAT mRNA, obtained with wild-type and mutant 30S subunits, as described in Materials and Methods. The arrowheads indicate the truncated transcript (toeprint signal), corresponding to the inhibition of extension (the lower band), and the full-length transcript (the topmost band). Lane 0: control lacking 30S subunit; lane 1: mutant 13A; lanes 2, 4, and 6: wild-type; lane 3: mutant 914U; lane 5: mutant 13A-914U; lane 7: mutant with altered protein S12. The autoradiographs corresponding to lanes 0−4 and lanes 5−7, respectively, were obtained in two independent experiments. The toeprint signal corresponds to nucleotide 16 of the mRNA, as determined from sequencing lanes run in parallel with dideoxynucleotides (not shown). Nucleotide 1 corresponds to the first base of the initiation triplet.

shown), but was severely affected in its presence, when protein synthesis exclusively depends on the mutant ribosomes. The single and double mutations affected cell growth to the same extent. [^{35}S]Methionine incorporation into total cellular protein was strongly decreased in cells transformed with mutant plasmids in the presence of spectinomycin (Figure 2B), confirming the observations obtained when examining cell growth. When synthesized proteins were analyzed by gel electrophoresis, identical patterns of fractionation were seen with cells transformed with wild-type and mutant plasmids (data not shown). This indicates that the mutations have no selective effect on the synthesis of individual proteins.

Effect of the Mutations on the Initiation of Translation. The extension inhibition assay (toeprinting) developped by Hartz *et al.* (1988) was used to probe the formation of the initiation complex with wild-type and mutated 30S subunits. In the toeprinting assay, a ternary complex formed between mRNA, the 30S subunit, and the initiator tRNA ($tRNA_f^{met}$) inhibits the extension of a DNA primer hybridized downstream from the initiation site. This inhibition causes the production of a truncated extension product, the toeprint. The signal of the truncated extension product divided by the sum of this signal plus that of the uninhibited extension product is the relative toeprint, which reflects the rate of formation of the 30S initiation complex under the conditions of the assays (Spedding *et al.*, 1993).

The 30S subunits used in the toeprinting assays were obtained by dissociating 70S ribosomes, which were isolated from bacteria transformed with pLΔH1192 or its three derivatives with a single or double mutation in the 16S rRNA gene. We have previously shown by primer extension that the proportion of ribosomes with plasmid-encoded rRNA was about 50%, when bacteria were transformed with these vectors (Pinard *et al.*, 1994). Figure 3 presents examples of toeprints obtained with wild-type 30S subunits and 30S subunits containing mutated 16S rRNA. As a means of comparison, 30S subunits with a mutated protein S12 were also included in the assays. The toeprint signals were decreased to a similar extent with 30S subunits having a single or a double mutation in 16S rRNA, which indicates that the rate of formation of the 30S initiation complex is slowed down. The observed effects were small but reproducible and significant, taking into account that only 50% of the ribosomal particles contain the mutant RNA (Table 1). In contrast, no decrease was seen with 30S subunits in which protein S12 is mutated. HPLC analysis of the 30S subunits with mutated 16S rRNA revealed a full complement of proteins (data not shown), excluding the possibility that the absence of one ribosomal protein could be responsible for the altered toeprints.

Effect of the Mutations on the Structure of the Central Pseudoknot. As mentioned in the Introduction, the mutations which we introduced into the 16S rRNA are near a central pseudoknot helix (17−19/916−918), that is required at some step during initiation (Brink *et al.*, 1993). We investigated whether these mutations could affect the structure of the pseudoknot helix, by assessing the binding of an oligodeoxyribonucleotide probe complementary to the 915−921 region, which encompasses one strand of the pseudoknot helix. Binding of the probe was performed under saturating conditions by adding a 20-fold molar excess of probe per 30S subunit, and was monitored by nitrocellulose filtration. Either the ribosomal particles used were wild-type or they contained mutated 16S rRNA or mutated protein S12. The specificity of hybridization of the probe to its complementary site was controlled by RNase H treatment. Only one major cleavage site was observed, and the resulting fragments were of the expected size (data not shown). The results of the binding studies show that the target is weakly accessible: only 3% of the wild-type 30S subunits bound the probe (Table 2). This weak binding was expected, since the target forms a secondary structure. However, the binding was significantly and reproducibly increased with 30S subunits containing mutated 16S rRNA. A similar increase was observed with the single and double mutants, whereas no change was observed with 30S subunits having the mutated protein S12. This suggests that the mutations at positions 13 and 914 destabilize the pseudoknot helix, thereby increasing the accessibility of the 915−921 region to the probe. An alternative procedure was used to assess whether mutations at positions 13 and 914 affect the structure of the pseudoknot helix. The accessibility of base G917 within this helix was monitored with kethoxal in mutant and wild-type 30S subunits and analyzed by extension of a primer specific for plasmid-encoded rRNA. We found that kethoxal reacts very weakly with G917 in wild-type 30S subunits, in agreement with Moazed *et al.* (1986), and consistent with its involvement in a base-paired structure. The reactivity of G917 to kethoxal was increased in 30S subunits with mutated 16S rRNA, but not in 30S subunits with the mutated protein S12 (Figure 4 and Table 3). The effect was significant and reproducible, and it confirms that the two 16S rRNA mutations destabilize the pseudoknot structure. The reactivity of G917 to kethoxal was increased to the same extent with the single or double mutants, in line with our studies on the binding of a probe complementary to the 915−921 region. This may reflect the fact that the distortion induced by each single mutation brings the pseudoknot helix to an identical state.

Table 1: Formation of the Initiation Complex with Wild-Type and Mutant 30S Subunits[a]

origin of the 30S subunits	mutations	relative toeprints with CAT mRNA	relative toeprints with T4 gene 32 mRNA
POP2136/pLΔH1192 (wild-type)	none	0.77 ± 0.04	0.35 ± 0.04
POP2136SM	protein S12	0.75 ± 0.05	0.33 ± 0.03
POP2136/pL13A	13U→A in 16S rRNA	0.56 ± 0.04	0.22 ± 0.05
POP2136/pL914U	914A→U in 16S rRNA	0.58 ± 0.04	0.23 ± 0.05
POP2136/pL13A-914U	13U→A and 914A→U in 16S rRNA	0.55 ± 0.03	0.21 ± 0.04

[a] Relative toeprints correspond to the intensity of the truncated transcript band (toeprint signal) divided by the sum of the intensities of the truncated and full-length transcript bands, measured by densitometric scanning of autoradiographs. Results are the means and the standard deviation of ten independent assays for the CAT mRNA and six independent assays for the T4 gene 32 mRNA.

Table 2: Binding to the 30S Subunit of a Probe Complementary to the 915−921 Region of 16S rRNA[a]

characteristics of the 30S subunits	binding of the probe to the mutated 30S subunits relative to the wild-type
mutation in protein S12	0.98 ± 0.05
mutation 13U→A in 16S rRNA	1.27 ± 0.11
mutation 914A→U in 16S rRNA	1.26 ± 0.12
mutations 13U→A and 914A→U in 16S rRNA	1.30 ± 0.13

[a] Binding of the probe was assessed with 30S subunits that either were wild-type or contained mutated protein S12 or mutated 16S rRNA. Results are the means and the standard deviation of eight to ten independent experiments. In each experiment, a value of 1.00 was arbitrarily assigned to the extent of the probe binding to the wild-type 30S subunits, which had a mean value of 0.03 ± 0.01 mol/mol. Binding in the absence of the ribosomal particles was subtracted from the data.

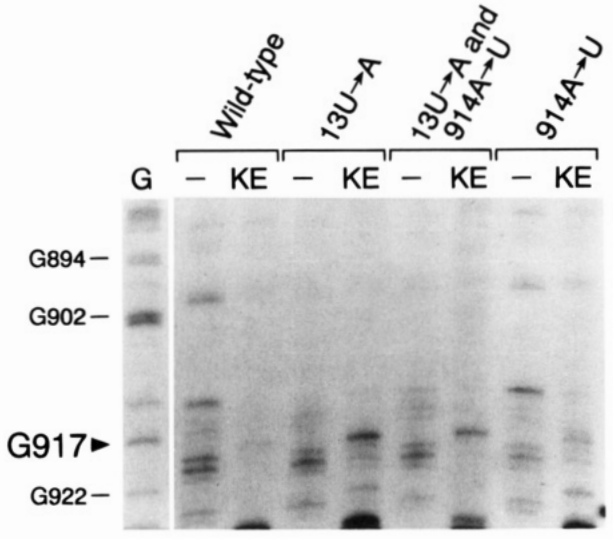

FIGURE 4: Example of an autoradiograph showing the chemical probing of 30S subunits with kethoxal in the 917 region of 16S rRNA. The characteristics of the 30S subunits are indicated on the top. G is a dideoxy sequencing lane using ddCTP and refers to the sequence of 16S rRNA −, unmodified RNA; KE, kethoxal-modified RNA. The arrowhead points to G917.

DISCUSSION

In this study, we show that mutations 13 (U→A) and/or 914 (A→U) in *E. coli* 16S rRNA severely affect cell growth when protein synthesis is carried out exclusively by the mutant ribosomes. Extension inhibition assays revealed that the two mutations decrease the rate of formation of the 30S initiation complex. This effect was not observed when protein S12 was mutated. We have previously shown that the proportion of 30S subunits with plasmid-encoded rRNA bearing either mutation was increased in the free 30S subunits

Table 3: Reactivity of G917 of 16S rRNA to Kethoxal[a]

characteristics of the 30S subunits	band intensity of G917 in mutated 30S subunits relative to wild-type
mutation in protein S12	1.03 ± 0.11
mutation 13U→A in 16S rRNA	1.45 ± 0.15
mutation 914A→U in 16S rRNA	1.41 ± 0.13
mutations 13U→A and 914A→U in 16S rRNA	1.38 ± 0.17

[a] Band intensities were measured by densitometric scanning of autoradiographs and normalized to intensities of control bands. The values are the means and the standard deviation of four independent experiments. A value of 1.00 was arbitrarily assigned to the reactivity of G917 in wild-type 30S subunits.

but decreased in the polysomes of cell lysates, when compared to 30S subunits with plasmid-encoded wild-type rRNA. This must reflect the lower capacity of the mutated 30S subunits to participate in the formation of initiation complexes. Using a poly(U)-directed assay which monitors the activity of ribosomes in the elongation step of protein synthesis, we also found that the mutations at positions 13 and/or 914 did not affect the elongation activity of the ribosomes (Pinard *et al.*, 1994). We therefore conclude that it is a defect in the initiation step that accounts for the adverse effect of the mutations on cell growth.

Structural studies indicate that the two mutations in 16S rRNA destabilize the adjacent pseudoknot helix formed by the pairing of bases 17−19 with bases 916−918. This was shown by the increased binding of a DNA probe complementary to one strand of the helix and the increased reactivity to kethoxal of G917 within the helix. The double mutation had the same effect as each single mutation. The structure of the pseudoknot was not affected when protein S12 was mutated. Bases 17−19 are located in the loop of the 5′ terminal hairpin. Mutation 13 (U → A) perturbs the base pair which closes the loop, and it is therefore expected that it affects the structure of this loop. Mutation 914 (A → U) is located two bases upstream from the 916−918 strand, in a large internal loop whose structure is ill-defined. Without knowing the detailed structure of this loop, it is difficult to understand how this mutation affects the pseudoknot helix. Since 30S subunits with mutations disrupting this pseudoknot helix were absent from the 70S and the polysome fractions in cell lysates, Brink *et al.* (1993) concluded that the pseudoknot helix must be present in order to complete translation initiation. We observe here that mutations which destabilize the pseudoknot helix decrease the rate of formation of the 30S translation initiation complex. We therefore propose that the pseudoknot helix is directly involved in the formation of this complex. The formation of the 30S translational initiation complex is known to occur in two

steps. In a reversible first step, a preinitiation complex is formed between the mRNA, the 30S subunit, and the initiator tRNA. During the irreversible second step, the 30S subunit undergoes a conformational rearrangement to the final initiation complex, and it is the rate of formation of this final complex that is detected by the toeprint analysis. The rearrangement enables the aligning and base-pairing of the codon−anticodon sequences in the decoding center (reviewed in Gualerzi & Pon, 1990). In the current models of the 30S subunit (Stern *et al.*, 1988b; Brimacombe *et al.*, 1988; Malhotra & Harvey, 1994), the pseudoknot helix has been located at the 50S subunit interface, near the decoding center. It is likely that it contributes to the conformational rearrangement leading to the final initiation complex.

We suggest that the 17−19/916−918 pseudoknot helix does not exist in the ribosomes during the elongation of protein synthesis; if the pseudoknot was required for this step, the activity of the mutant ribosomes would also have been affected during elongation. From a compilation of sequence data, we proposed earlier that this pseudoknot helix could be dissolved in 70S ribosomes and replaced with an alternative pseudoknot structure where residues 12−16 could pair to residues 911−915 (Leclerc & Brakier-Gingras, 1991). The results of the present study do not support this hypothesis. Indeed, the double mutation (13U→A and 914A→U), which is expected to restore base-pairing in the proposed alternative helix, affects the function and the structure of the ribosome exactly like each single mutation. Kössel *et al.* (1990) also proposed from a compilation of sequence data that the 17−19/916−918 pseudoknot helix could melt in the 70S ribosomes and that a novel helix could be formed between residues 14−18 and 1530−1534. However, in a more recent analysis using a larger sequence data base (Maidak *et al.*, 1994), we could not substantiate this proposal. When the 50S subunit associates with the 30S subunit, some bases and phosphates become more protected in the 900−920 region of 16S rRNA, and this increased protection likely results from a conformational rearrangement rather than from shielding by the 50S subunit (Moazed & Noller, 1987; Baudin *et al.*, 1989). The pseudoknot helix could be disrupted during this rearrangement, but the detailed nature of the changes that occur remains to be uncovered.

ACKNOWLEDGMENT

We thank François Dragon, Michael Laughrea, Steve Lodmell and Donna Michnick for stimulating discussions and critical reading of the manuscript.

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BI9506987