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Mutations in 16S rRNA in *Escherichia coli* at methyl-modified sites: G966, C967, and G1207

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ABSTRACT

Mutations were constructed at three sites in 16S rRNA in *E. coli* by oligonucleotide-directed mutagenesis, and cloned into the rrnB operon on either pKK3535 or pNO2680. The mutated bases, G966, C967, and G1207, are located in the 3′ major domain of 16S rRNA and are sites post-transcriptionally modified by methylation. We constructed a deletion mutation at C967 (Δ967) and three substitution mutations at each of the following sites: G966, C967, and G1207. By maxicell analysis, we found that all of the mutations were processed normally and incorporated into 30S subunits and 70S ribosomes. We found that Δ967 was a dominant lethal mutation while the substitution mutations at G966 and C967 had no effects on cell growth rate. The mutants C1207 and U1207 were shown to have dominant lethal phenotypes while A1207 had no effect on cell growth rate. These results help to establish the importance of methyl-modified regions to ribosome function.

INTRODUCTION

The site of cellular protein synthesis is the ribosome, a complex macromolecular structure composed of two parts, the large and small subunits. The subunits function together during protein synthesis. They are constructed from specific proteins, termed ribosomal proteins, and ribosomal RNAs (rRNA). It is clear that the rRNAs play a key role in both the structure and function of ribosomes (1).

With the pioneering work of Dahlberg and coworkers (2,3) it is now possible to study rRNA by *in vitro* mutagenesis of rRNA structural genes (for recent review see 4). In *Escherichia coli* (and generally) expression of rRNA genes is a complex process in which transcription, producing a primary transcript, is followed by nucleolytic cleavage and post-transcriptional modifications to produce mature rRNAs. While all of these steps have not been duplicated in vitro, the laboratories of Brakier-Gingras (5) and Ofengand (6) have succeeded in producing mature length rRNA transcripts of the small subunit rRNA that can be incorporated into ribosomal subunits and studied *in vitro*. Interestingly, although this synthetic rRNA lacks post-transcriptional modifications, it is quite functional when assayed *in vitro*. These results seem to indicate that post-transcriptional modifications are not required for protein synthesis.

Post-transcriptional modifications of the small subunit rRNA in *E. coli* result in methylation of 10 specific sites out of the 1542 nucleotide positions in this molecule. These modified sites are located in regions of high sequence conservation and are presumably functional important. To determine the role of modified sites we have constructed mutations at methyl-modified positions by site-directed mutagenesis and have expressed the mutant genes *in vivo*. We have concentrated our studies on two regions of the 16S rRNA molecule: G1207 and G966/C967. We constructed three point mutations at all three sites in addition to a deletion of C967 and have found that certain mutations have a dominant lethal phenotype when expressed in vivo.

MATERIALS AND METHODS

Cell Strains and Plasmids

Plasmid pKK3535 contains the complete rrnB operon of *E. coli* regulated by the ribosomal promoters P1 and P2 (7). Plasmid pNO2680 also contains the rrnB operon but with the phage lambda promoter P_L replacing P1 and P2 (8). Both plasmids are derived from pBR322 and carry an ampicillin-resistance marker. Plasmid pcI857 is a derivative of pACYC177 and carries both a neomycin-resistance marker and a gene for cI857, a temperature-sensitive variant of the phage lambda repressor protein cI (9). *E. coli* strain DH5α (F^-, endA1, hsdR17, supE44, thi-1, recA1, gyrA96, relA1, λ^-, Δ(argF$^-$ laczya)U169, φ80dlacZΔm15) was from BRL. *E. coli* strains CJ236 (dut-1, ung-1, thi-1, relA-1;pCJ105(Cm^r) and MV1190 (Δlac-pro AB), thi, supE, Δ(sr1-recA)306::Tn10(tet^r)[F′:tra D36, proAB, $lacI^qZ\Delta M15$]), and, M13mp18 were from Bio·Rad.

Radiochemicals and Enzymes

Restriction enzymes, Klenow fragment, and bacteriophage T4 DNA ligase were purchased from New England Biolabs or BRL. AMV reverse transcriptase was from Life Sciences. Radiochemicals were from NEN. Oligonucleotides were from Genosys, The Woodlands, TX.

Mutagenesis

A recombinant M13mp18 virus, containing the SalI/XbaI restriction fragment covering the 3′ end of the 16S rRNA gene from pKK3535, was used to infect CJ236 to produce uracil-containing, single-stranded DNA. Mutations were produced by oligonucleotide-directed mutagenesis (10) and cloned into MV1190 to select for mutants according to Kunkel (11,12). Mutations were screened by chain termination sequencing (13).

An ApaI/XbaI fragment from recombinant, mutant virus was cloned between the unique ApaI and XbaI sites in either pKK3535 or pNO2680.

Modified Maxicell Analysis

In order to determine the effects of mutations on rRNA processing and incorporation into ribosomal subunits, cells harboring plasmid borne mutant rRNA genes were analyzed by a modification of the maxicell technique (3,14). DH5α cells with pcI857 and pNO2680 harboring mutations were grown at 42°C for 1 hr to an A_{550nm} = 0.5, irradiated for 75 seconds, allowed to recover for one hour and labelled overnight in low phosphate medium containing 50 μC of ^{32}P. Cells were harvested and lysed. Lysates were analyzed for labelled rRNA in ribosomal subunits by two-dimensional, composite gel electrophoresis, and, RNA was extracted from lysates and analyzed for rRNA processing by composite gel electrophoresis as described (3).

Total RNA Isolation

An overnight culture of cells grown at 30°C in LB broth with 100 mg/ml ampicillin was diluted into fresh culture medium to a final $A_{550\ nm}$ = 0.050 and grown to late log phase ($A_{550\ nm}$ = 1.0). Cells harboring pKK3535 with mutations at position 966, and, cells lacking pcI857 and harboring pNO2680 with nonlethal mutations at position 967 were grown at 37°C. Cells with mutations at 1207 in pNO2680 were grown at 42°C. Cells were harvested by centrifugation, resuspended in TE (10 mM Tris.HCL, 1 mM EDTA, pH = 8.0) and lysed by treatment at 95°C with 3 volumes of a 2:1 mixture of phenol to a solution containing 1% SDS, 100 mM NaCl, 8 mM EDTA, 20 mM TrisHCl, pH = 7.5. Cellular debris was removed by centrifugation at 8,000 RPM for 15 min. The supernatant was extracted with phenol and RNA was precipitated with 2.5 volumes of ethanol at −20°C for 1 hour. The pellet was rinsed with 70% ethanol, vacuum dried and dissolved in 10 mM TrisHCl pH = 7.5, 1.0 mM $MgCl_2$ (15).

Isolation of Ribosomes Containing a Deletion of C967

The deletion mutation , Δ967, was cloned into pNO2680 in DH5α cells harboring pcI857. Cells were grown at 30°C in LB broth with 100 μg/ml ampicillin to midlog and diluted into fresh LB broth at 42°C to $A_{550\ nm}$ = 0.05 and grown to $A_{550\ nm}$ = 0.7. Cells were collected by centrifugation and processed for ribosomes. Small subunit rRNA was isolated by dissociating ribosomes into subunits, separating subunits on a sucrose gradient, removing protein by phenol extraction and concentrating 16S rRNA by ethanol precipitation (16).

RNA Sequencing

Either total RNA or 16S rRNA were used as templates for sequence determination using reverse transcriptase and an end labeled primer in order to detect mutant rRNA in a background of wild type RNA. A primer, complementary to the region from 990 to 970 in 16S rRNA, was employed for mutations at G966 and C967. For mutations at G1207, a primer complementary to the region from 1230 to 1211 in 16S rRNA was used. Primer extension reactions giving standard sequence ladders and strongly terminated primer extension reactions were carried out as

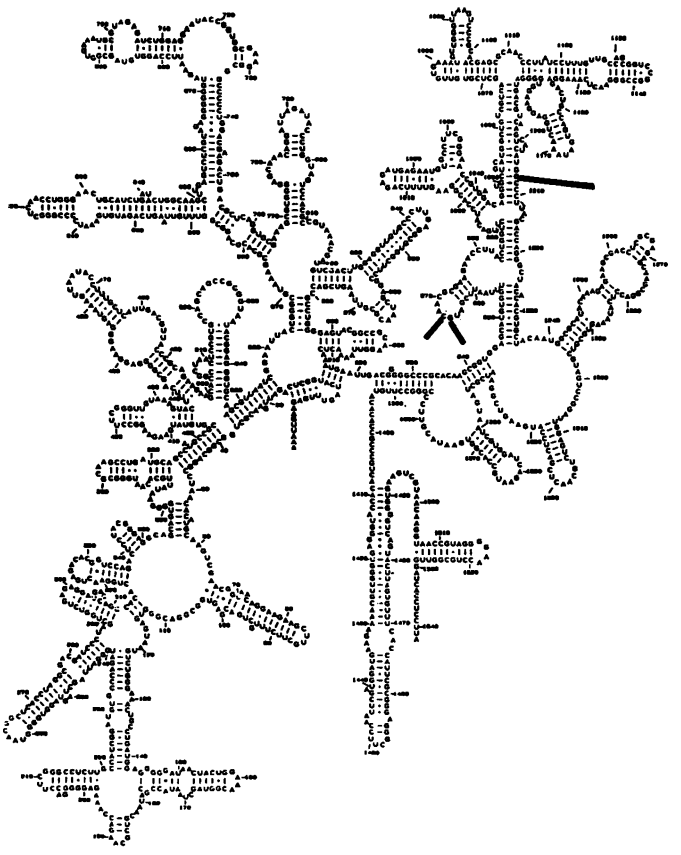

Figure 1. Secondary Structure Map of *E. coli* 16S rRNA. Mutations were constructed in a cloned rrnB operon of *E. coli* by oligonucleotide-directed mutagenesis at three methyl-modified sites in the 16S gene. The sites are located in the 3′ major domain at G966, C967 and G1207. The mutations include three base substitutions at each site in addition to a deletion of C967. The locations of these sites are labelled in the secondary structure map of *E. coli* 16S rRNA.

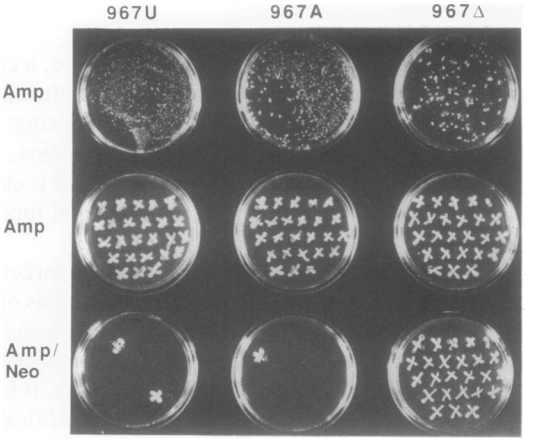

Figure 2. Lethal Phenotype Determination. Three mutations at C967, namely U967, A967 and Δ967, were cloned into pNO2680 and transformed into *E. coli* strain DH5α containing pcI857. Plasmid pNO2680 carries an ampicillin resistance marker and a copy of rrnB regulated by P_L while pcI857 carries a neomycin resistance marker and the temperature sensitive lambda repressor, cI857. Plasmid DNA was isolated from transformed cells grown at 30°C, the permissive temperature for repression of P_L by cI857, and used to retransform DH5α cells (lacking both plasmids). The transformants were selected for ampicillin resistance (top row). The ampicillin resistant cells were then streaked on ampicillin plates to verify ampicillin resistance (middle row) and on ampicillin/neomycin plates to screen for cells transformed by both plasmids (bottom row).

suggested by Morgan and coworkers (17). The strongly terminated primer extension reactions contain three normal deoxynucleotides and one dideoxynucleotide.

RESULTS

Construction of mutations

Mutations at G966, C967 and G1207 were produced by oligonucleotide-directed mutagenesis in a restriction fragment covering the 3′ end of 16S rRNA cloned into M13. For G966, three substitutions were incorporated into the 16S structural gene on the plasmid pKK3535. The mutant plasmids were originally cloned into HB101 and then subcloned into DH5α. A comparison of the efficiency of cloning and growth rate measurements indicated that these mutations were phenotypically silent. Mutations at C967 and G1207 were cloned into pNO2680 in DH5α with pcI857 at 30°C, a permissive temperature for cI repression. Under these conditions the mutations are phenotypically silent. The locations of mutated positions are shown in the secondary-structure map in figure 1.

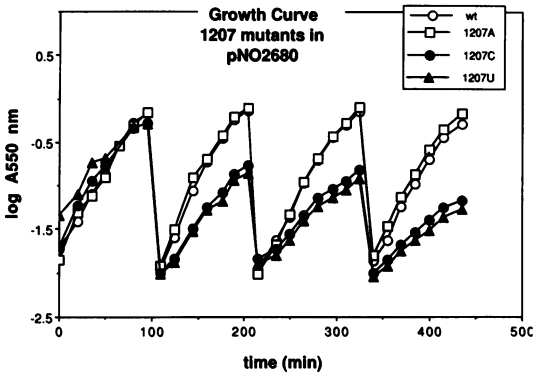

Figure 3. Growth Rate Analysis of 1207 Mutants. Cultures of DH5α (pcI857) containing pNO2680 with either wild type 16S rRNA or substitution mutations at G1207 were grown overnight on LB plates at 30°C and used to inoculate a liquid culture in LB broth with 100 mg/ml ampicillin and grown at 30°C and 42°C. Growth characteristics at 30°C, when transcription is repressed by the lambda repressor cI857, are identical for all four samples (not shown). The 42°C cultures were grown repeatedly to late log phase, diluted into fresh culture medium at 42°C and regrown as shown. Cell growth was monitored by turbidity measurements at 550 nm. The transition mutation A1207 (open squares) exhibits grown characteristics similar to the wild type control (open circles). The transversion mutants, C1207 and U1207 (closed circles and triangles respectively), grow considerably slower than the wild type control but are nearly identical to each other.

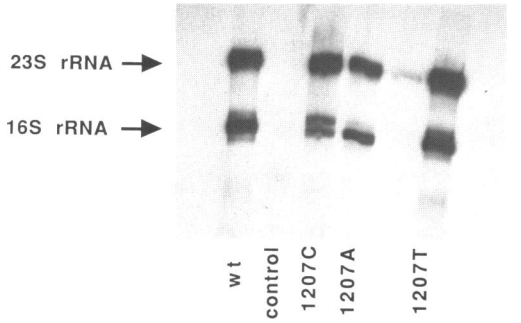

Figure 4. Maxicell Labelling of 1207 Mutants. *E. coli* strain DH5α, harboring pNO2680 with point mutations at G1207 in the 16S gene, were irradiated with ultraviolet light in order to inactivate transcription of host chromosomal rRNA genes. Cells, initially grown at 30°C, were shifted to 42°C in a low phosphate medium containing carrier-free 32P to initiate transcription of plasmid genes and to label transcripts. After a 12 hr. incubation, RNA was isolated and analyzed by electrophoresis. The first lane shows labelled 23S and 16S rRNA from wild type transcripts of plasmid genes. Inactivation of host chromosomal genes by this procedure is shown by the absence of labelled products in the second lane where labelled lysates from cells lacking plasmid rRNA genes are analyzed. Mutants at 1207 appear to be processed into stable, mature rRNA as shown in the last three lanes.

Analysis of Dominant Lethal Phenotype

The deletion mutation, Δ967, and, the transversions of G1207 to C1207 and U1207, were determined to have dominant lethal phenotypes by the following experiment. Plasmid preparations were made from DH5α cells containing pcI857 and pNO2680 with mutations at either G1207 or C967. These plasmid preparations were used to transform DH5α cells (lacking pcI857) to ampicillin resistance, thus selecting for pNO2680 containing the mutations. Transformed cells were screened for neomycin resistance to determine the fraction of cells transformed by both plasmids. The results for mutations at C967 are shown in figure 2. The deletion mutation shows a strong positive selection for neomycin resistance indicating that the ampicillin plasmid alone is lethal to cells. For U967 and A967, only a small fraction of ampicillin-resistant colonies are resistant to neomycin as well, due to random, simultaneous transformation by both plasmids. We tested all mutations at C967 and at G1207 and found that only the deletion mutation, Δ967, and the transversions, U1207 and C1207, had dominant lethal phenotypes.

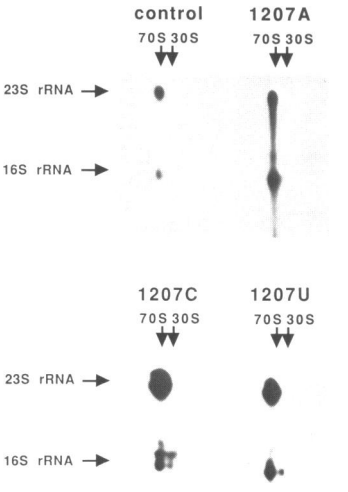

Figure 5. Analysis of Ribosomes Containing Maxicell Labelled rRNA Mutants. Lysates from cells harboring pNO2680 with point mutations at G1207 in the 16S gene were labelled by a modification of the maxicell technique and analyzed by two dimensional gel electrophoresis. In the first dimension, labelled lysates were electrophoresed in the presence of magnesium to separate ribosomes from subunits. Gels were then treated with SDS and EDTA to extract ribosomal proteins and electrophoresed in the second dimension to separate rRNA. The identification of the 70S ribosome band is confirmed by the presence of both 23S and 16S rRNA in this band. It is clear that the three mutations at G1207 are processed into mature rRNA and incorporated into 30S particles capable of interacting with large subunits to form ribosomes. The transversion to C1207 shows two distinct bands in the region of 16S rRNA and both of these bands are found in ribosomes. The lower band is mature 16S rRNA. We are unsure of the identity of the band above 16S but it is not the precursor 17S species because it is found in complete ribosomes as well as small subunits.

Effects of Dominant Lethal Mutant rRNA Transcription on Growth Rate

To determine the effects of expression of mutant 16S rRNA genes on cell growth, cells harboring pcI857 and pNO2680 with mutations in the 16S rRNA gene were grown at 42°C to initiate transcription. Cell growth was followed by turbidity. In the experiment shown in figure 3, mutations at G1207 were analyzed and compared against a wild type control. The transition to A1207 appears to grow with identical characteristics to wild type cells. However, both transversions grow considerably slower than wild type and their growth rates decrease with time. A similar experiment was done for Δ967 except that different colony isolates were grown for one cycle of growth and compared to wild type cells. We found that Δ967 grows at a slower rate than wild type (not shown).

Processing of Mutant rRNA and Incorporation into Ribosomal Subunits

Modified maxicell analysis revealed that all the mutations studied are processed and incorporated into small subunits capable of interacting with large subunits. The results for G1207 mutations are shown in figures 4 and 5. In figure 4, rRNA is analyzed and found to be processed into mature 16S products. For C1207, an additional band slightly larger that the mature 16S rRNA is present. We are unsure of its identity but are certain that it is not a normal precursor like 17S because it appears to be incorporated into ribosomal small subunits. Figure 5 shows incorporation of labelled rRNA into ribosomal particles. It is evident that labelled small subunit rRNA, derived from plasmid borne genes is being incorporated both into 30S subunits and 70S ribosomes.

Accumulation of Mutant rRNA

We analyzed the levels of mutant rRNA and wild type, host coded rRNA by sequencing RNA from cells harboring mutations in the 16S gene on pNO2680. Mutant genes are expressed against a background of host-coded wild type rRNA. Ribosomal RNA with mutations to either U1207 or A1207 were easily detected by standard RNA sequence analysis using total RNA isolated from cells expressing the mutant genes. This is shown in figure 6. However the substitution mutations, U967 and U966, were not detected by a similar analysis (not shown). This was a surprise because maxicell analysis indicated that mutations at either of these two positions did not effect rRNA processing and so we expected to find mutant rRNA. We employed the strongly terminated primer extension reactions in rRNA sequencing using reverse transcriptase to detect low levels of mutant rRNA.

In the first experiment, we isolated ribosomes from DH5α cells expressing Δ967 for several generations. The result are shown in figure 7. The appearance of mutant rRNA in ribosomes is indicated by the additional extension product for rRNA from cells

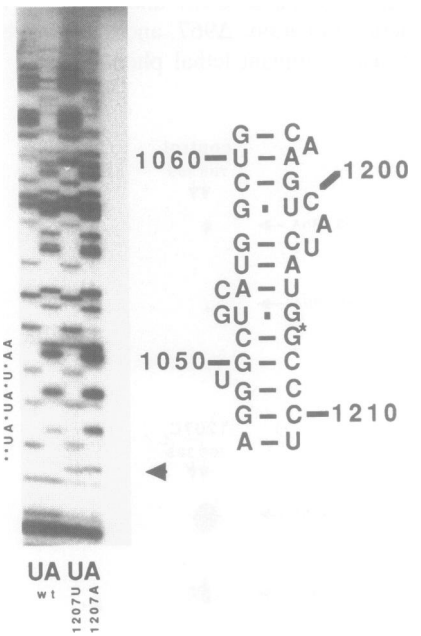

Figure 6. Accumulation of Mutant rRNA. The point mutations, U1207 and A1207, in 16S rRNA were transcribed *in vivo* from pNO2680. Total RNA was isolated from cells grown for 1 hr. at 42°C and used as templates for RNA sequencing with reverse transcriptase. The sequencing reactions were primed with a 32P, end labelled deoxyoligonucleotide complementary to positions 1211 to 1230 of 16S rRNA. The locations of U's and A's are shown for wild type pNO2680 in the first two lanes. The location of U's for the U1207 mutation and the location of A's for the A1207 mutation are shown in the third and fourth lanes respectively. The unlabeled arrow on the right points to position 1207 with A or U mutations. The band immediately below, and prominent in all four lanes, is position 1207 with methylated G. The difference in mobility between position 1207 in wild type versus mutant rRNA is due to band compression (see results). The unextended primer is the intense band on the bottom of the gel. The gel is labelled on the left to show the location of U's and A's and a secondary structure map of this region of 16S rRNA is also shown.

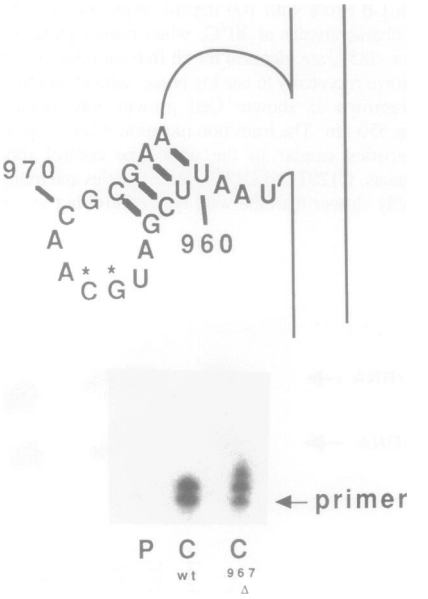

Figure 7. Mutant rRNA Accumulation in Ribosomes. Small subunit rRNA was purified from 70S ribosomes from cells with pNO2680 containing either a wild type 16S gene or a single base deletion of position 967 (Δ967). The rRNA was used as a template for strongly terminated primer extension reactions using an end labelled oligonucleotide complementary to positions 970 to 990 in 16S rRNA. The reaction mixture contained dATP, dCTP, dTTP and *ddGTP* thus chain termination occurs opposite C's in the template. The unextended primer is shown as a faint band in the first lane. The middle lane shows the reaction product using wild type 16S rRNA as a template. As a result of termination at C967, only a single extended product is found. In the lane on the right, rRNA from cells with Δ967 show two extended products corresponding to termination at C967 for wild type 16S rRNA from chromosomal genes and a longer product terminating at C960 from the deletion mutation. A portion of the secondary structure map of this region is shown above the autoradiogram.

containing the mutation. Next, we employed this technique on total RNA isolated from cells expressing U967. The results are shown in figure 8. Analysis of mutations at positions 966 and 967 are complicated by the fact that methyl-modified C967 causes reverse transcriptase to pause at this position giving rise to a band. This is evident by the appearance of bands at C967 in the G lanes of figure 8. The presence of U967 in total RNA is shown by additional bands in both the C and U tracks.

We were surprised by the low levels of mutant rRNA found for the U967 substitution mutation using the strongly terminated primer extension technique. Because we could easily detect 1207 mutant rRNA by conventional RNA sequence analysis (figure 6), we decided to reanalyze A1207 and U1207 by strongly terminated primer extension reactions on total RNA. The results are shown in figure 9. Position G1207 can be identified by the presence of a band at this position in both the U and A tracks in normal RNA sequencing, resulting from reverse transcriptase pausing at this methyl-modified position and shown in lanes 2 and 3. This identification is confirmed by the appearance of a prominent extension product for the strongly terminated G sequence for wild type gene expression (in lane 5). However, it appears that the G1207 termination product experiences band compression. This can be seen by comparing its mobility with those obtained for mutants A1207 and U1207. The location of A1207 and U1207 are shown in lanes 7 and 8. These bands appear to have the correct mobility for position 1207. They are one nucleotide shorter that G1206 (shown in lanes 9 and 10), two nucleotides shorter that U1205 (shown in lane 8) and three nucleotides shorter than A1204 (shown in lanes 6 and 7).

DISCUSSION

The presence of methyl-modified residues in ribosomal RNA has been known for some time yet their function is obscure. In the small subunit rRNA, there are 10 modified positions located in highly conserved regions. Further, these positions appear to be grouped in a three dimensional model of the small subunit near the decoding site (18). That these positions are highly conserved, and that they are located in a functional region of the ribosome, may indicate that they play a critical role in protein synthesis. However, experiments by Brakier-Gingras (5) and by Ofengand (6) have shown that quite functional small subunits can be assembled in vitro from synthetic, unmethylated 16S rRNA. We are attempting to determine the role of modified positions in 16S rRNA in protein synthesis by site-directed mutagenesis.

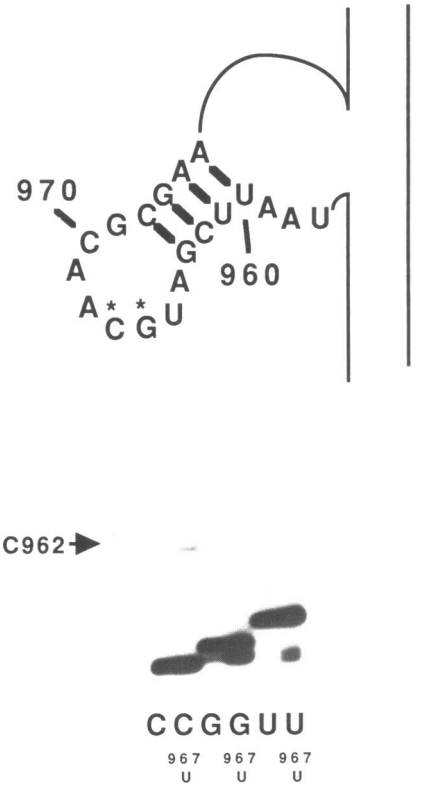

Figure 8. Mutant U967 in rRNA. Total RNA was isolated from cells expressing 16S rRNA from pNO2680 with either a wild type gene or a point mutation, U967. The RNA was used in strongly terminated primer extension reactions to determine the relative amounts of wild type and mutant rRNAs. The reactions revealing the position of C, G and U for cells with a wild type gene are shown in the first, third, and fifth lanes. The corresponding reactions for U967 are in the second, fourth, and sixth lanes. A portion of the secondary structure map with the primary sequence is presented. The presence of U967 is shown by the appearance of a faint C band in lane 2, labelled with an arrow. This band corresponds to a stop at position C962 as a result of strong termination at that position for U967 mutations. Additional evidence for the presence of U967 can be seen in the last two lanes. A strong U stop at position 967 appears in cells expressing U967 (lane 6) and is absent from wild type cells (lane 5). The presence of an additional stop in the G lanes at position 967 is probably due to reverse transcriptase pausing at methylated C967.

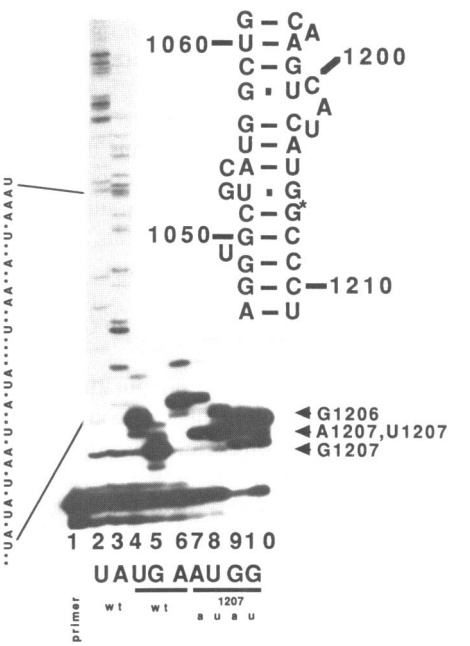

Figure 9. Expression of Mutations at G1207 in 16S rRNA. Point mutations at G1207 in 16S rRNA were transcribed *in vivo* from pNO2680. Total RNA was isolated and used as a template in strongly terminated primer extension reactions using an end labelled deoxyoligonucleotide complementary to positions 1211 to 1230 of 16S rRNA. Lane 1: end labelled primer. Lanes 2 and 3: location of U's and A's by conventional chain termination sequencing using reverse transcriptase and total RNA from cells expressing wild type 16S rRNA. Lanes 4, 5 and 6: the location of U's, G's and A's by strongly terminated primer extension reactions using RNA from cells expressing wild type 16S rRNA genes. Lanes 7 and 9: the location of A's and G's by strongly terminated primer extension reactions using RNA from cells expressing A1207. Lanes 8 and 10: the location of U's and G's by strongly terminated primer extension reactions using RNA from cells expressing U1207. Arrows indicate the positions of G1207, A1207, U1207, and G1206.

Mutations were constructed at three methyl-modified sites in 16S rRNA in *E. coli*. Two of the sites, G966 and C967, are located in the loop of a short hairpin structure. Substitution mutations at either site have no effect on growth rate and appear to be processed normally and incorporated into 30S subunits capable of interacting with 50S subunits to form ribosomes. In contrast, the deletion of C967 has a dominant lethal phenotype when cloned on a high copy-number vector in *E. coli* strain DH5α. This mutation is processed normally and is incorporated into ribosomes. The short stem and loop containing G966 and C967 has several highly conserved positions. In fact, the existence of a base-paired stem is unproven due to a lack of compensatory base changes within the stem. The loop has several conserved bases including C967 which is highly conserved with variations found only in mitochondrial rRNA and a few eubacteria. In contrast, G966 is variable (19).

For the G966/C967 region, our results show that substitution mutations have no apparent effect on either rRNA processing or on cell growth rates. Point mutations at G966 did not produce measurable effects on growth rate even when cloned behind ribosomal RNA promoters on a high copy number plasmid. The post-transcriptional modification that normally occurs at this position, namely methylation of G at the 2 position, must be blocked in these mutations. Furthermore, no adjacent G's are available for modification. Similar observations were made for the substitution mutations at C967. This position is highly conserved, nonetheless, substitutions at this position could be cloned with no apparent growth rate effects. The loop does not contain a C adjacent to position 967 and it is reasonable to conclude that normal base modifications are absent from the 967 substitution mutations. Thus, we are left with the observation that substitutions at either G966 or C967 are phenotypically silent. However, the region is conserved and thus must be important.

During the course of mutagenesis at position C967 we isolated a single-base deletion mutation and to our surprise this deletion proved to be lethal. This mutation was thus cloned behind the regulatable lambda promoter, P_L. When transcription was derepressed by thermal inactivation of a temperature sensitive lambda repressor, cI857, mutant rRNA with C967 deleted could be isolated from 70S ribosomes.

The lethal phenotype of this deletion mutation was discovered by attempting to separate the mutant rrnB-containing, ampicillin resistant plasmid from a compatible plasmid coding for the repressor protein and neomycin resistance. Plasmid DNA preparations from cells harboring both plasmids were used to transform cells to ampicillin resistance. The transformants were then screened and found to all be neomycin resistant as well, indicating that ampicillin resistance alone was lethal. Thus, it was not possible to clone the deletion mutant in the absence of transcriptional repression.

The finding that Δ967 is a dominant lethal mutation is evidences that the region is important in protein synthesis. However it seems unlikely that the phenotype is solely a result of a loss of base modifications in this region because the substitution mutations at G966 and C967 do not show the same lethal phenotype. It is clear that Δ967 rRNA is incorporated into small subunits capable of participating in 70S ribosome formation. Thus, it is possible that the lethal phenotype of Δ967 is a result of a defect in one of the steps of protein synthesis.

In contrast to the G966/C967 region, position G1207 proved far more sensitive to mutagenesis. It is located in a large, irregular helix thought to be involved in termination of translation (20). The sequences both upstream and downstream of this position are highly conserved yet G1207 varies. It appears that G1207 is paired because compensatory base changes are found in archaebacteria, eubacteria, and eukaryotes (19, 21).

We found that the transversions to C1207 and U1207 exhibited dominant lethal phenotypes while the transition to A1207 was phenotypically silent. We showed by maxicell analysis that rRNA from these mutants was processed and incorporated into ribosomes. Using RNA sequencing, we could easily detect the accumulation of both A1207 rRNA and U1207 rRNA in cells conditionally expressing these mutations.

When we measured accumulation of the U967 substitution mutation by RNA sequencing we did find evidence of mutant rRNA production but at levels much lower than those found for G1207 mutations. This may be a reflection of the level of stability of this mutant rRNA.

We have shown here that certain mutations at the methyl-modified regions G966/C967 and G1207 have dramatic effects on cell viability. There are other examples of mutations at methylated sites that effect cell growth. A transition at C1402 was found to be viable yet exhibited a slow growth phenotype when cloned in pKK3535 (22), and, a transition at C1407 was reported to be lethal in pKK3535 (23). The sensitivity of methyl-modified sites to mutagenesis may be a reflection of the importance of these sites to proper ribosome function. Mutagenesis has uncovered a level of complexity not anticipated by sequence analysis. For example, C967 is highly conserved yet only the deletion of this base gives rise to a dominant lethal phenotype. The existance of compensitory base changes at position 1207 indicates that it is involved in a base pair. Although substitution mutations disrupt this pair only transversions were found to be lethal. This differential sensitivity to mutagenesis may be the result of factors other than disruption of a base pair such as alternative secondary structures.

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