Structural and functional analysis of *Escherichia coli* ribosomes containing small deletions around position 1760 in the 23S ribosomal RNA

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ABSTRACT

Three different small deletions were produced at a single Pvu 2 restriction site in *E. coli* 23S rDNA of plasmid pKK 3535 using exonuclease Bal 31. The deletions were located around position 1760 in 23S rRNA and were characterized by DNA sequencing as well as by direct fingerprinting and S1-mapping of the rRNA. Two of the mutant plasmids, Pvu 2-32 and Pvu 2-33, greatly reduced the growth rate of transformed cells while the third mutant, Pvu 2-14 grew as fast as cells containing the wild-type plasmid pKK 3535. All three mutant 23S rRNAs were incorporated into 50S-like particles and were even found in 70S ribosomes and polysomes *in vivo*. The conformation of mutant 23S rRNA in 50S subunits was probed with a double-strand specific RNase from cobra venom. These analyses revealed changes in the accessibility of cleavage sites near the deletions around position 1760 and in the area around position 800 in all three mutant rRNAs. We suggest, that an altered conformation of the rRNAs at the site of the deletion is responsible for the slow growth of cells containing mutant plasmids Pvu 2-32 and Pvu 2-33.

INTRODUCTION

The use of recombinant DNA technology has become a powerful approach in the elucidation of the structure and function of the ribosome. Using the multicopy plasmid pKK 3535, which has a single copy of the ribosomal *rrnB* operon and has been entirely sequenced (1,2), it is possible to delete regions of DNA of almost any desired size (3). Cells containing such altered plasmids transcribe rDNA containing the expected deletions. The development of a modified maxicell-system for the specific labeling of plasmid coded rRNA (4) has provided a convenient method for studying the effects of mutations on rRNA processing, subunit assembly and ribosome function *in vivo*. Some of the deletions retard or completely prevent processing of precursors of 23S and 16S RNA, while others appear to have no effect on the incorporation of mutant rRNA into particles very similar to unaltered ribosomes (3,4).

The regions of rRNA deleted in these mutants can be analyzed further by forming DNA heteroduplexes between the wild type plasmid and the deletion

plasmid, and modifying the single-stranded regions of the heteroduplex with bisulfite (5). This type of study of 16S rRNA has shown that a single base change can affect significantly the growth rate of cells containing this mutant plasmid (6).

Several groups have suggested that rRNA is not rigid but rather exists in several alternative conformations suggesting an active role in protein synthesis (7,8,9,10,11). Data for alternative conformations (or "switches") come from the identification of RNA-RNA cross-links (8,9) and mainly from the fact that during the analysis of base-paired fragments, using two-dimensional electrophoresis, certain RNA fragments have the potential to interact with several different regions of rRNA in ribosomal subunits (12,13). These studies have been hampered by the fact that it is difficult to fix a substantial amount of ribosomes in a particular conformation. For this reason we chose to analyze mutations of rRNA which might favor one conformation and fix a substantial amount of RNA in one structure. Such an alternative approach might locate regions of rRNA directly involved in conformational changes and would provide evidence in support of a "switch" mechanism.

In this study we selected a site in 23S rRNA near position 1760 which was reported to be involved in interactions with both 5S rRNA (13) and tRNA-fMet (14). We have introduced three different small deletions; one five base deletion and two three base deletions. We have analyzed the structure of the mutant RNAs in 50S subunits by using a double strand specific ribonuclease from cobra venom and present data showing that certain mutant ribosomes are able to bind to mRNA in vivo and, in one case, apparently function quite normally.

MATERIAL AND METHODS

Bacterial Strains E. coli HB 101; MC 1061; CSR 603. Cells were stored in 50% glycerol at -80°C.

Plasmid pKK 3535 was provided by Dr. H. Noller and contains the rrnB operon of E. coli (1,2). Large scale preparation of plasmid DNA was carried out according to standard procedures.

Enzymes Polynucleotide kinase, DNA polymerase (Klenow-fragment), DNA ligase and restriction enzymes were purchased from New England Biolabs and used as recommended by the company except BSA was omitted. Exonuclease Bal 31 was purchased from BRL. Calf alkaline phosphatase and nuclease S1 were from Boeringer, ribonuclease T1 from Sankyo and ribonuclease A from Sigma. The

Figure 1. Construction of the Pvu 2 deletions in pKK 3535

double strand specific RNase isolated from cobra venom was a generous gift from Dr. V. Erdmann.

Construction of deletions Linear, single cut plasmid DNA was prepared by partial digestion of pKK 3535 with Pvu 2 (see Fig. 1), separated on a 0.8% agarose gel and extracted by electroelution. About 10 μ g of linear plasmid DNA was digested in 1 ml of 20 mM Tris.HCl pH 8.1, 12 mM CaCl_2 , 12 mM MgCl_2 , 600 mM NaCl and 1 mM EDTA with 0.06 units of Bal 31 exonuclease at 30°C. Aliquots of 200 μ l were removed after 1, 4, 10 and 40 minutes, placed on ice and extracted with phenol. 1.5 μ g carrier tRNA was added and the DNA was precipitated with 2 volumes of ethanol. The pellet was washed twice with ethanol, dried and dissolved in a small volume of 10 mM Tris.HCl pH 7.8. The DNA was then ligated in a total volume of 30 μ l containing 0.3 μ g of DNA, 50 mM Tris.HCl pH 7.4, 10 mM MgCl_2 , 10 mM DTT, 1 mM spermidine, 1 mM ATP and 1000 units of T4 DNA ligase for 8 hours at 15°C. Competent cells (15) were transformed with 0.1 μ g of DNA and plated on LB agar plates containing 50 μ g ampicillin per ml as described earlier (3). Colonies were picked daily for a period of four days. Small scale preparation of plasmid DNA from single colonies was done as described (3) after which the presence of deletions was determined by using the restriction enzymes Pvu 2 and Ava I.

Gel electrophoresis Plasmids and large DNA fragments were isolated on 0.8% agarose gels containing Tris.acetate, NaCl, EDTA and 1 μ g/ml ethidium bromide. Single cut plasmids used for transformation were visualized using a long-wave UV-transilluminator. Separation of small fragments for sequencing and for the preparation of the hybridization probe against rRNA

was carried out on a 7% acrylamide gel containing Tris, borate and EDTA (16). RNA fragments arising from the digest of 50S subunits with the cobra venom enzyme were separated on a 3% to 15% SDS-urea acrylamide gradient gel as described by Stiege et al (17). The separation of polysomes was achieved on an acrylamide-agarose composite gel as described previously (18).

DNA sequence determination An Ava I fragment from each of the mutant plasmids covering the site of the deletion was isolated on a 7% polyacrylamide gel. The 5'-ends were labeled using alkaline phosphatase, ^{32}P -ATP and polynucleotide kinase and were digested with Mst II creating a fragment labeled on one end only. The sequence was determined using the chemical modification procedure of Maxam and Gilbert (19).

RNA fingerprinting The two dimensional TLC-system of Volkaert and Fiers (20) was used for the separation of oligonucleotides from a complete ribonuclease T1 digest of uniformly labeled 23S rRNA, followed by a secondary analysis with ribonuclease A. A reduced amount of ribonuclease A (0.2 μg in 5 μl of 10 mM Tris.HCl pH 7.8 containing 5 μg of carrier tRNA) had to be used for digestion of the oligo-27-family (see results) to avoid overdigestion.

Isolation of ribosomes and ribosomal RNA specifically labelled in mutant rRNA The maxicell labeling and lysis procedure was used exactly as described by Stark et al (4). Cells were irradiated for 10 seconds and ^{32}P phosphate was added after 5 hours. After a labeling period of 18 hours cells were lysed and loaded on a 10% to 40% sucrose gradient containing 10 mM Mg. acetate, 50 mM Tris.HCl pH 7.8, 50 mM KCl and 6 mM 2-mercaptoethanol. The peak samples of the 50S subunits were pooled and used directly for digestions with the cobra venom enzyme. To isolate rRNA, pooled fractions were precipitated with an equal volume of ethanol, the pellet was dissolved in 0.5% SDS, 2 mM EDTA, 6 mM 2-mercaptoethanol, 10 mM Tris.HCl pH 7.8, extracted twice with phenol and precipitated with ethanol. The RNA was finally dissolved in 10 mM Tris.HCl, 1 mM EDTA, pH 7.8.

Structure analysis of 50S subunits 50S ribosomal subunits isolated from sucrose gradients containing 10 mM Mg. acetate were made 300 mM KCl and 250 μl were incubated for 10 minutes at 37°C. A suitable amount of cobra venom ribonuclease (see results) was added and incubated at 37°C for 1 hour. The digestion was stopped by the addition of 100 μl of 200 mM EDTA, pH 8.0 and heating at 60°C for 5 minutes. The digest was precipitated with ethanol and the pellet dissolved in 200 μl 10 mM Tris.HCl pH 7.8, 10 mM NaCl, 5 mM EDTA, 0.05% SDS. Sodium acetate was added to a final concentration of 100 mM and

the RNA was reprecipitated with ethanol. The RNA pellet was washed with 95% ethanol and dissolved in a small volume of electrophoresis buffer which contained Tris, citrate, SDS and EDTA (17).

Preparation of polysomes Cells containing wild-type or mutant plasmids were grown at 37°C in LB containing 200 µg/ml ampicillin to an A_{660} of 0.5 to 0.6. Erythromycin (300 µg/ml) was added and cells were harvested after 10 minutes at 37°C by quick cooling to 0°C in an ethanol/dry ice bath, and centrifugation at 8000 rpm for 5 minutes. Cells were lysed (8) and aliquots of the lysate were loaded directly on an acrylamide-agarose composite gel or on a 15% to 40% sucrose gradient containing 25 mM Tris.HCl, 10 mM Mg.acetate, 50 mM KCl and 6 mM 2-mercaptoethanol and centrifuged for 15 hours at 12,000 rpm in an SW 27 rotor at 2°C. Fractions containing polysomes or 70S particles were pooled, adjusted to 20 mM MgCl₂, precipitated with an equal volume of 95% ethanol and kept at -70°C for 30 minutes. The ribosome precipitate was dissolved in 25 mM Tris.HCl pH 7.8, 10 mM MgCl₂, 50 mM KCl, and 6 mM 2-mercaptoethanol. RNA was extracted from polysomes or 70S particles with phenol as described above.

S1 mapping of 23S RNA from polysomes and 70S particles A 343 bp fragment of DNA covering the site of the deletions was prepared from pKK 3535 by digestion with Ava I. The ends were labeled by using the Klenow fragment of DNA polymerase and α^{32} P dCTP. Aliquots of 5 ng of labeled DNA were added to 15 µg of RNA isolated from polysomes or 70S ribosomes and ethanol precipitated. The pellet was resuspended in 20 µl hybridization buffer (40 mM PIPES pH 6.4, 1 mM EDTA, 400 mM NaCl, 80% formamide (21)), denatured for 10 minutes at 74°C and renatured at 52°C for 30 minutes. 180 µl aliquots of ice-cold S1-buffer (50 mM Na.acetate pH 4.6, 280 mM NaCl, 4.5 mM ZnSO₄) containing 1250 units S1/ml and 20 µg/ml single stranded carrier DNA were added, incubated at 45°C for 30 minutes and cooled on ice. Then 10 µl of 7.5 M ammonium acetate, 10 µl 200 mM EDTA pH 8, 200 µl of phenol and 5 µg of carrier tRNA were added. After phenol extraction and ethanol precipitation the pellet was washed twice with 95% ethanol and dissolved in a small volume of 8 M urea, 50 mM NaOH, denatured for 1 minute at 90°C and loaded on a 40 cm long 10% polyacrylamide sequencing gel (19).

RESULTS

Isolation of Pvu 2 deletion mutants

We were interested in determining the effects of small deletions at a single site in the ribosomal RNA on the formation, structure and function of

Figure 2. Separation of DNA fragments of mutant and pKK 3535 plasmid DNA after digestion with Ava 1 and Pvu 2 (left) or Ava 1 alone (right) on a 7% polyacrylamide gel.

ribosomes. In order to construct deletions DNA from plasmid pKK 3535, containing the *rrnB* rRNA operon of *E. coli*, was partially digested with the restriction endonuclease Pvu 2. This treatment linearized the plasmid DNA randomly at one of the two Pvu 2 sites (see Fig. 1). The linearized DNA was then digested mildly with Bal 31 exonuclease, ligated and subsequently used to transform competent HB 101 cells. Both slow and fast growing colonies were picked over a period of four days without applying any selection procedure. Plasmid DNAs derived from single colonies were initially characterized by restriction mapping using Pvu 2 and Ava 1 as seen in Figure 2. No deletion mutants were isolated from the one minute Bal 31 treated sample. However three deletion mutants, Pvu2-14, Pvu2-32 and Pvu2-33 were isolated from plasmids treated with Bal 31 for four minutes. Very large deletion mutants of several hundred base pairs (as estimated by restriction digests) were also isolated. We assume that they were produced by a combination of extensive Bal 31 digestion and the loss of sequences during the transformation process. The large deletion mutants were quite stable presumably because their gene products tend not to interfere with vital cell functions. Deletions of intermediate size (10 to 100 base pairs) were never found even though DNA molecules in this size range had been generated by the 10 and 40 minute digestions with Bal 31 as judged by gel electrophoreses. Continued efforts to isolate deletions of intermediate size (5 to 20 bases) were not successful and resulted only in the isolation of another five base deletion (Pvu 2-105) which was identical to Pvu 2-32.

Characterization of Pvu 2 deletion mutants

The deletion mutants Pvu2-14, Pvu2-32 and Pvu2-33 were sequenced using

	1750	1760	1770
pKK 3535	GUCGAAGAU	ACCAGCUGGCUG	CAAC
Pvu2 14	GUCGAAGAUACC	---	UGGCUGCAAC
Pvu2 32	GUCGAAGAU	-----	CUGGCUGCAAC
Pvu2 33	GUCGAAGAUACCA	---	GGCUGCAAC

Figure 3. Sequence of plasmid pKK 3535 in the area of the deletions. The bases which are missing in the mutant plasmids are replaced by dashed lines. Numbers indicate the position of the bases in *E. coli* 23S rRNA.

the Maxam Gilbert procedure and results are shown in Figure 3. All three mutants lack G 1763 plus adjacent bases. Although the deletions are similar in size and position there was a large difference between mutant plasmid Pvu2-14 and the other two plasmids, Pvu2-32 and Pvu2-33, in their effects on the growth rate of transformed cells. The doubling time of CSR 600 cells containing plasmids Pvu2-32 or Pvu2-33 was 80 minutes when grown in LB-medium with 200 µg/ml ampicillin. In contrast the doubling time of cells containing the plasmids pKK 3535 or Pvu2-14 was 49 minutes. This difference in growth rate was clearly plasmid dependent since it was reproduced in HB 101, MC 1061 and CSR 603 cells.

Analysis of plasmid coded 23S rRNA in maxicells

The absence of any obvious effect of plasmid Pvu2-14 on the growth rate of transformed cells made it important to demonstrate that it was actually being transcribed and that mutant 23S rRNA was accumulating in the cells. Transcription was studied in a modified maxicell-system (4) where it has been shown that plasmid coded rRNA can be labeled with ^{32}P in the absence of host coded rRNA transcription. All three mutant plasmids and the wild-type plasmid pKK 3535 were introduced into the UV-sensitive strain CSR 603 via passage through MC 1061. Cells were labeled 18 hours to ensure that the rRNA was fully processed and assembled into particles (if possible) and that no precursor rRNA remained. This was confirmed by examining aliquots of the cell lysates on agarose-acrylamide composite gels (22). The autoradiogram showed that rRNA from all three mutants was processed to mature 23S rRNA at normal rates and was assembled into particles that contained 5S rRNA and migrated as 50S subunits in a composite gel. In addition the 50S subunits bound 30S subunits to form 70S ribosomes, as was determined by two-dimensional gel electrophoresis (22).

Direct evidence that the mutations were actually expressed as rRNA was obtained by RNA fingerprint analysis. Cell lysates were loaded directly

Figure 4. Separation of the RNase A digest of T1 oligonucleotide 28 of wild-type (left) and mutant Pvu2-14 (right) 23S rRNA on the thinlayer system of Volkaert and Fiers (20). The mutant gives rise to a much stronger AU-spot as indicated by the arrow. The minor spots at the right edge of the thin-layer plate are probably due to underdigestion of the T1 oligonucleotide by RNase A.

onto a 10%-40% linear sucrose gradient in 25 mM Tris.HCl pH 7.8, 0.3 mM Mg. acetate, 50 mM KCl, 6 mM 2-mercaptoethanol. Fractions containing 50S subunits were pooled, precipitated with ethanol and the RNA was extracted with phenol in the presence of SDS and EDTA. The 23S rRNA was completely digested with ribonuclease T1 and the resulting oligonucleotides were separated on the two-dimensional TLC-system of Volkaert and Fiers (20). This system separates oligonucleotides primarily according to chain length and uracil content. For example all oligonucleotides seven nucleotides long and containing two uracils (oligonucleotide 27 according to the nomenclature (23)) are well separated from all other oligonucleotides. They run as one single spot on the thin layer plate and the 23S rRNA transcribed from the three base deletion mutant Pvu2-14 should contribute a new oligonucleotide AUACCUG to the 27-family while in pKK 3535 the T1 oligonucleotide in the area of the mutations belongs to the 17-family (AUACCAG) (see Fig. 3). Furthermore digestion of all 27-oligonucleotides of pKK 3535 with RNase A results in 17 U's, 18 C's, 8 G's, 3 AC's, 2 AAU's, AAC, AAAAC, AAAU, AG and AAG. The RNase A digest of the 27-family of Pvu2-14 would give the products listed for pKK 3535 in addition to the extra products C, U, G, AC and AU. The additional C, U, G and AC are not likely to be detected because they increase the amounts of RNase A products of the 27 oligonucleotide family by a relatively small amount. One extra AU however should be detectable in the analysis of Pvu2-14 because AU is not contained in the RNase A digest of the 27-oligonucleotide collection of 23S RNA from pKK 3535. Mild RNase A digestion was necessary to avoid overdigestion and cutting after adenosine, as is described in Material and Methods. Figure 4 shows that Pvu2-14, but not pKK 3535, gives rise to the expected extra AU-spot in the analysis of the T1-oligonucleotides. This is direct proof that 23S rRNA containing the deletion is actually transcribed and assembled into what appears to be perfectly normal ribosomes. Unfortunately an analogous analysis is not applicable to the mutants Pvu2-32 or Pvu2-33 because Pvu2-32 leads to the formation of the rather uncharacteristic oligonucleotide 25 AUCUG and Pvu2-

Figure 5. Autoradiogram of RNA fragments after digestion of 50S ribosomal subunits with the cobra venom enzyme and separation on a 3%-15% polyacrylamide gradient gel containing SDS and urea. RNA transcribed from wild-type plasmid pKK 3535 and the three Pvu 2 mutants were specifically labeled in maxicells. The origin and the position of 5S rRNA (120 bases) are indicated. RNA fragments a, b1, and b2 are located at the marked positions.

33 actually would form the same T1-oligonucleotide 17 (AUACCAG) as pKK 3535.

The presence of the deletions and their location in 23S rRNA were also identified using a more indirect approach. Unlabeled total 23S rRNA was isolated and probed with a ^{32}P -labeled DNA fragment covering the site of the deletion. Digestion of the hybrid with nuclease S1 showed that all of the deletions were present at the expected position in 23S rRNA. This will be described in more detail later in conjunction with the analysis of mutant ribosomes on polysomes.

Digestion of 23S rRNA in 50S subunits using double strand specific RNase from cobra venom

Plasmid coded rRNA was labeled uniformly with ^{32}P in maxicells as

described above and 50S subunits were isolated from sucrose gradients containing 10 mM Mg acetate. The ribosomal profile was similar for all three mutants and pKK 3535. As expected no label was detected in 70S ribosomes or ribosomal subunits from cells containing pBR 322. A surplus of labeled 50S subunits was produced by cells containing mutant or wild-type plasmid and this observation is consistent with previous reports (5). Therefore sufficient radioactive material could be isolated directly from the 50S subunit peak for structural analysis by digestion with the cobra venom RNase. This was fortunate because isolation of 50S subunits on sucrose gradients containing 0.3 mM Mg acetate and concentration by ethanol precipitation always resulted in a substantial amount of breakdown of the RNA (data not shown). Samples of 50S subunits were adjusted to the appropriate buffer (see Material and Methods), and activated by a 10 minute incubation at 37°C. An amount of cobra venom enzyme was added sufficient to produce fragments ranging in size from approximately ten to a few hundred bases. Fragments were separated on a 3% to 15% polyacrylamide gradient gel containing SDS and 7 M urea, in order to disrupt all base pairing. The autoradiogram of a typical fragment pattern is shown in Figure 5. Bands of interest were cut out, the RNA was extracted and then subjected to fingerprint analysis using RNase T1 and secondary analysis of the T1-oligonucleotides with RNase A as described above. Figure 5 shows that a very distinct fragment pattern is produced and that this pattern is very similar for 50S subunits isolated from cells with pKK 3535 and deletion mutant plasmids. The pattern of sharp bands is mainly the result of protection of the rRNA by ribosomal proteins. A digest of naked RNA under conditions identical to those above created a large series of RNA fragments all of which were smaller than 50 bases (data not shown, see also (24), and Stiege, W., personal communication). Therefore we conclude that most, if not all of the proteins are present in the 50S-like subunits which contain mutant 23S rRNA with small deletions around position 1760.

The few differences in the fragment pattern between mutant and wild-type ribosomes are very clear. The amount of fragment a was about five-fold less in all three mutants than in wild-type ribosomes. Fragments b1 and b2 were not present at all in the mutants. Fingerprint analyses of these fragments yielded the following information:

Fragment a: The T1 fingerprint of fragment a from unaltered ribosomes is shown in Figure 6. After secondary analysis of each spot with RNase A the location of this fragment in 23S rRNA could be determined unambiguously.

Figure 6. Fingerprint analysis of a RNase T1 digest of fragment a. See text for explanation.

Its 5'-end is defined at position 1583 by the unusual oligonucleotide pAUCAGp due to the fact that the cobra venom enzyme produces a 5'-phosphate in contrast to RNase T1 (24). No cleavage by the cobra venom enzyme has been reported at this position but rather in the complimentary strand in the secondary structure of 23S rRNA (25,26,27). The 3'-end of fragment a is defined by a known cobra venom cut at position 1725 (25) which is consistent with our results. The yield of a fragment migrating like fragment a in the mutants was about 20% of the wild-type and fingerprint analysis confirmed that it actually was fragment a.

Fragment b2: Figure 7 shows the T1-fingerprint of fragment b2. Clearly no AAG was detected thus locating the 5'-end somewhere between bases 708 and 712 because we are not sure if we can account for oligonucleotide 23 UUG at position 712. The fragment ends between positions 827 and 831 because no AG was found in the RNase A digest of spot 13.

Fragment b1: Fragment b2 is a subfragment of fragment b1. Fragment b1 also starts at a position between 708 and 712 but has an extended 3'-end which is defined by missing oligonucleotide 28 UCAUCCCG at position 884 and by the

Figure 7. Fingerprint analysis of a RNase T1 digest of fragment b2. See text for explanation.

presence of oligonucleotide 04 CAAG at position 879. (The fingerprint of fragment b1 is not shown.)

Sl-mapping shows that mutant 23S rRNAs are in polysomes

To answer the question of whether ribosomes containing the described small deletions in 23S RNA were able to combine with mRNA *in vivo*, we analyzed polysomes from exponentially growing CSR 603 cells, containing either pKK 3535 or one of the three deletion-plasmids. Erythromycin was added prior to harvesting cells to increase the yield of polysomes (14). Cell lysates were loaded on sucrose gradients (see Material and Methods) and fractions containing polysomes or 70S ribosomes were pooled. After concentration by ethanol precipitation aliquots of each sample were separated on a composite gel containing 10 mM MgCl₂ to confirm that no cross contamination had occurred (Fig. 8). The RNA of each sample was extracted with phenol in SDS and EDTA and equivalent amounts of RNA from 70S ribosomes or polysomes were used for hybridization to a ³²P end-labeled Ava I-DNA fragment. The fragment, 343 base pairs long, was isolated from pKK 3535 and included the site of the deletion in 23S rRNA (see Material and Methods).

Figure 8. Staining pattern of polysomes (lane 1) and 70S ribosomes isolated from a sucrose gradient and separated on an agarose polyacrylamide composite gel (see Materials and Methods). The x2, x3, x4 and x5 indicate disome, trisome, tetrasome and pentasome-size polysomes.

Using a 10% polyacrylamide gel containing 7 M urea we had no difficulty in detecting a labeled band of DNA of the expected size with RNA from polysomes or 70S ribosomes of Pvu2-32 (Pvu2-32 contains the 5 base deletion which was sufficient to produce a single strand DNA bulge in the fragment for cleavage by nuclease S1). No bands were detected in the other two mutants. However by using a long sequencing-type gel we resolved the strong band in Pvu2-32 into a series of five bands and were also able to detect a weak band in the reactions where we were using RNA from the three base deletions Pvu2-14 and Pvu2-33 (see Fig. 9). This difference in the band intensity between the five base deletion Pvu2-32 and the two three base deletions Pvu2-14 and Pvu2-33 clearly reflects the difficulty encountered by nuclease S1 in cutting the deletion loop containing only three bases. It is

Figure 9. Autoradiogram of nuclease S1-digested, endlabeled DNA which was hybridized to various rRNA preparations from polysomes (p) or 70S ribosomes (r) of pKK 3535 and the various Pvu2 deletion plasmids (lanes 14, 32, and 33, respectively.) Samples were separated on a 40 cm long 10% polyacrylamide sequencing type gel. In the region of interest one base differences are resolved. The autoradiogram is overexposed to show the more intense bands in Pvu 2-14 and Pvu 2-33. m indicates the 238 bp marker which was produced by digestion of the 343 bp Ava 1 fragment with Pvu 2 (see also Fig. 2).

important to point out that with each plasmid the 70S and polysomal rRNAs gave identical results. Using equal amounts of RNA either from 70S or from polysomes, DNA bands of equal intensities were produced after digestion with ribonuclease S1. This indicates that the ribosomes containing mutant 23S rRNA were associated with mRNA to the same extent as were wild-type ribosomes. In addition the S1-mapping experiment shows clearly that the deletions in the plasmid DNA were correctly transcribed into 23S rRNA with deletions at exactly the expected positions.

DISCUSSION

We were interested in producing small deletion mutations in a region of 23S rRNA at position 1760, which is believed to be functionally important due to its reported association with 5S rRNA (13). The opposite strand of RNA in the secondary structure of 23S RNA (bases 1985-2001) shows extensive complementarity with fMetRNA (28). Similar RNA-RNA interactions can be drawn using eucaryotic cytoplasmic rRNA and initiator tRNA sequences including yeast (Zwieb, unpublished results) in support of these interactions. Our initial intent was to isolate a large series of small deletions in this region, taking advantage of the fact that a Pvu 2 restriction site is located in pKK 3535 corresponding to position 1760 in 23S rRNA, and only one other site exists in the plasmid at position 10,175 (see Fig. 1). To our surprise we could isolate only three different small 23S RNA deletions. Two of the deletions, namely Pvu2-14 and Pvu2-33 were three base deletions at positions 1762-1764 and 1763-1765 respectively. The third deletion, Pvu2-32, is a five base deletion centered at position 1761. We were unable to isolate additional small deletions (1-5 bases) or any deletions of somewhat larger size (5-20 bases) in the region of interest. The five base deletion Pvu 2-32, however, was independently isolated twice indicating a strong selection against most of the deletions once they were introduced into the cells.

While only a very small number of deletions at position 1760 were tolerated by transformed cells, these mutant plasmids were transcribed and the rRNA was processed to mature 23S rRNA. In addition the rRNA was assembled into 50S subunits capable of forming 70S-like particles. Mutant ribosomes were not depleted in their content of 5S RNA as determined by two dimensional gel electrophoretic analysis (14,22). The amount of labeled 5S rRNA found in mutant 70S ribosomes and 50S subunits was unaltered when compared to the wild type ribosomes. In addition it is quite likely that

all of the ribosomal proteins were present, as judged from the digestion patterns of 50S subunits with the cobra venom enzyme. Most interesting was the finding that all mutant ribosomes were capable of interacting with messenger RNA in vivo, during exponential cell growth. This was shown indirectly by S1-mapping since polysomes could not be isolated using the maxicell-labeling procedure (4). We found the expected enhancement of radioactive bands even with the three base deletions (see Fig. 9) by using a sequencing type gel which is capable of resolving one base differences in the region of interest. Under conditions of excess rRNA over labeled DNA probe, analysis of equal amounts of RNA from 70S and polysomes showed that mutant ribosomes are equally distributed in these two samples. Thus they appear to compete equally well with normal 50S subunits for the 30S-mRNA initiation complex and presumably move along the mRNA. We have not quantitated the amount of mutant rRNA present in the cells, but previous estimates indicate that once the plasmid is established in the cell approximately equivalent amounts of rRNA are synthesized from the plasmid and the seven host *rrn*-operons (5). The ratio of wild-type to mutant ribosomes is not indicated by the S1-nuclease experiment since the ability of the enzyme to cut even the five base deletion heteroduplex is certainly very limited.

The effects of the Pvu2 mutant plasmids on the growth of transformed cells was quite remarkable. There was no difference in the growth rate of cells with the three base deletion mutant, Pvu2-14, or the wild-type pKK 3535 plasmid. On the other hand, cells containing mutants Pvu2-32 and Pvu2-33 had identical, reduced growth rates. Since plasmid coded rRNAs of the wild-type and mutants appear to be produced in equivalent amounts and compete equally well for mRNA, we consider the differences in growth rate to reflect differences in function of the mutant ribosomes. The functional differences have not yet been identified by in vitro studies but we did attempt to identify structural differences between the mutant ribosomes. The secondary structures of the rRNA in 50S subunits were probed using cobra venom RNase. This approach proved successful in demonstrating a difference between the mutants and wild-type 23S rRNA (Fig. 5) but, to our surprise, all three mutants rRNAs were similar. The yield of fragment a (positions 1583-1725) was reduced five-fold. Fingerprint analysis showed that it is very close to the site of the deletions. This suggested that a slightly altered cutting mode was created by the deletions but present in all three mutants. On the other hand, fragment b, which was not detected in any

Figure 10. Possible base pairing scheme of 23S rRNA according to (27) and of the deletion mutants Pvu2-14, 32 and 33. Bases at position 1985-1989 are part of the hypothetical interaction with tRNA_{Met} (1-1' (14)). The bases at positions 1756-1768 in 23S rRNA are able to interact with bases at positions 69-79 in 5S rRNA (2-2' (8)). Possible interactions between bases 29,30/54,55, 37-40/75-78 and 70,71/106,107 are not included in this secondary structure model of 5S rRNA. The black triangle indicates the cutting site of the cobra venom RNase.

mutant RNA digest, comes from a region at positions 710-830 in the primary structure of the RNA. Such a change in the RNase cutting mode could have been caused by a major conformational change in the overall structure of the ribosome as a result of the deletion, which might support the idea of structural shifts during translation. It seems more likely, however, that the two regions of RNA, 1583 to 1725 and 710 to 830, are actually quite close to each other in the tertiary structure of the RNA because a covalent cross-link has been found between bases 763 and 1567 using the cross-linking reagent bis-(2-chloroethyl)-methylamine (17). The altered tertiary structure in the mutants may have created a new cleavage site within band b, thus accounting for the loss of this band. In spite of the fact that all of

these changes can be attributed to the deletion mutations around 1760 in 23S rRNA, they do not explain the striking differences between the mutants in their effect on growth rate and ribosome function.

The location of the deletion mutants in the secondary structure model of 23S rRNA (27) is diagrammed in Figure 10. The hypothetical interactions between 23S rRNA, 5S rRNA and fMet-tRNA suggest that this is a functionally important region of the rRNA. A comparison of the two deletions of three bases, Pvu2-14 and Pvu2-33, shows that they are displaced by only a single base and they lack two bases in common, and yet they differ remarkably in their effect on function. While the positioning of the three base deletion is very critical, shortening of the region per se (1762-1765) by three bases is tolerated in Pvu2-14. The intramolecular base pairing of Pvu2-14 23S rRNA need not be disrupted by the deletion since the deleted C 1764 could be replaced by C 1761, which is single stranded in the secondary structure. On the other hand there is no compensation for the loss of the A-U base pair (1987-1761) in Pvu2-33. This might be the essential difference between these two mutants especially since they both lost just one potential base pair with 5S rRNA. The five base deletion in Pvu2-32 did not result in any loss of intramolecular base pairs in 23S rRNA. In this case, however, five bases may be too great a loss to permit retention of function. In addition four of these bases can form potential pairs with 5S rRNA. It is interesting to note that while the structural changes in 23S rRNA in Pvu2-32 and Pvu2-33 might appear to be quite different, their effects on retarding the cell growth rate are identical. This suggests that they both may be disrupting the same mechanism in protein synthesis. However, more detailed studies will be necessary before this can be determined.

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