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The conserved 900 stem/loop region in *Escherichia coli* 16S ribosomal RNA is not required for protein synthesis

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ABSTRACT

Plasmid pPM114 carries the *Escherichia coli* 16S ribosomal RNA gene under the control of a T7 promoter. It can generate *in vitro* transcribed 16S rRNA that can be assembled into functional 30S ribosomal subunits. Two deletion mutants were derived from pPM114, by partial or total deletion of the conserved 900 stem/loop region of the 16S rRNA. These mutants, pMGΔ10 and pMGΔ23, respectively lack bases 895 to 904 and 889 to 911 of the 16S rRNA. The amputated 16S rRNA transcripts synthesized from these mutated plasmids were assembled into 30S subunits which were as active under the direction of an artificial or a natural messenger as subunits reconstructed with the full-length 16S rRNA transcript. They also responded as well to the stimulation of misreading by streptomycin, although the deleted region is proximal to the streptomycin binding domain. However, when we attempted to delete the 895-904 or 889-911 region from the 16S rRNA gene in plasmid pKK3535 which carries the *rrn*B operon, no transformants harbouring plasmids with one of these deletions could be recovered. These observations suggest that the 900 stem/loop region of the 16S rRNA is not required for the ribosomal function but is probably essential for important cell regulatory functions.

INTRODUCTION

The 900 stem/loop region at the edge of the central domain of *E. coli* 16S rRNA is highly conserved, which suggests that it is functionally important (1). Furthermore, cross-linking and footprinting studies have indicated that this region is proximal to the streptomycin binding domain (2,3) and it has been shown that a C to U transition at position 912, at the base of the stem of the 900 region, confers resistance to streptomycin (4,5). Noller and his coworkers (6,7) have also shown that specific residues in the 900 region are protected from chemical probes by proteins S4, S5 and S12, three proteins known to be involved in the response of the ribosome to streptomycin and in the control of translational accuracy (8). All these data point to the importance of the 900 region.

We have previously shown that transcription of a plasmid carrying the 16S rRNA gene under the control of a T7 promoter yields a 16S rRNA transcript, which can be assembled into functional 30S ribosomal subunits (9). A similar system has been developed by Ofengand and his collaborators (10). In this study, we have used this

system to further investigate the role of the 900 stem/loop region of the 16S rRNA by introducing deletion mutations into this region. We then assessed the activity of 30S subunits reassembled with the amputated 16S rRNA transcripts generated from the deletion mutants. Our results demonstrate that, although the 900 region is highly conserved and proximal to the binding site of streptomycin, its absence did not prevent the *in vitro* assembly of 30S subunits, which were as active in protein synthesis as 30S subunits reconstructed with the full-length 16S rRNA.

MATERIALS AND METHODS

Bacterial strains, plasmids and phages.

E. coli K12A19, RNaseI$^-$ (11), was the source of 30S and 50S ribosomal subunits and enzymes for cell-free protein synthesis assays. *E. coli* HB101 (12) was plasmid host and phage M13 was grown in *E. coli* JM101 (13). Plasmid pPM114 contains the entire sequence of the 16S rRNA gene under the control of a T7 promoter (Figure 1). It is identical to pPM112 (9), except that two GC pairs have been inserted between the 3' end of the 16S rRNA gene and a flanking *Xba*I site, so that the 3' end of the 16S rRNA gene becomes part of a *Mst*II site, as designed by Krzyzosiak *et al.* (10). The replicative form of phage M13mp19 (13) was obtained from Pharmacia. Plasmid pKK3535, which contains the entire *rrn*B operon cloned into pBR322 (14), was a gift from Dr. H.F. Noller.

Enzymes and chemicals.

T7 RNA polymerase was isolated from *E. coli* BL21 harbouring the plasmid pAR1219, a gift from Dr. F.W. Studier, and purified as described by Ikeda and Richardson (15). Restriction enzymes, T4 DNA ligase, the Klenow fragment of DNA polymerase I, T4 polynucleotide kinase and RNasin were purchased from Pharmacia. RNase-free DNase-free bovine serum albumin and calf intestine alkaline phosphatase were from Boehringer-Mannheim. Streptomycin was from Sigma. [γ -^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq), [^{3}H]phenylalanine (21.2 Ci/mmol), [^{3}H]isoleucine (104 Ci/mmol), [^{3}H]valine (32 Ci/mmol) and [^{3}H]leucine (45 Ci/mmol) were from Amersham; [α-^{32}P]UTP (3700 Ci/mmol) and [α-^{32}P]dCTP (3700 Ci/mmol) were from ICN. Synthetic oligomers were synthesized with an automated Pharmacia gene assembler.

Construction of mutant plasmids.

Deletion mutants were derived from pPM114 by the mismatch oligonucleotide technique (16) with the phosphorothioate method (17,18), using the Amersham *in vitro* oligonucleotide-directed mutagenesis system. Mutagenesis was performed on M13 recombinants carrying the *Hind*III-*Xba*I fragment of the 16S rRNA gene from pPM114 as an insert. This fragment, which encompasses bases 649 to the *Xba*I site flanking the 3' end of the gene, was mutagenized with the synthetic mismatched oligomer

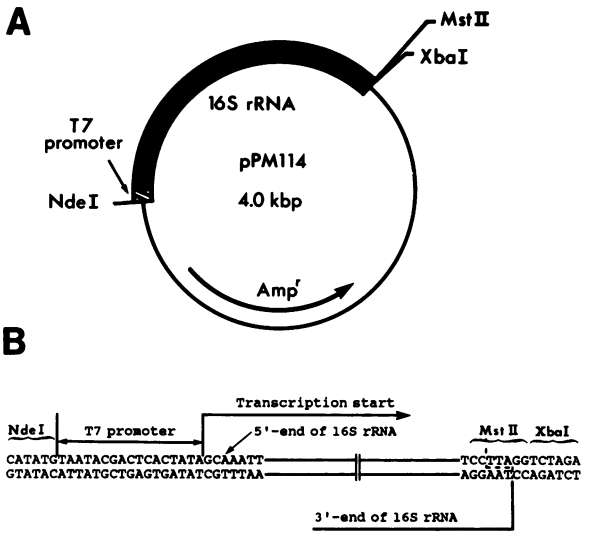

Figure 1: (A) Structure of plasmid pPM114. The thin line represents the pUC18 vector, the filled segment, the 16S rRNA gene and the hatched segment, the T7 promoter. (B) Detailed structure of the 5' and 3' ends of the 16S rRNA gene and of the T7 promoter.

5'-dTTGAGTTTTA↓CGTACTCCCC-3' or 5'-dAATTCATTTG↓CCCCAGGCGGTC-3'. These oligomers are complementary to bases 885 to 914 and 877 to 921, respectively, except for bases 895 to 904 and 889 to 911 (the position of the deleted sequence is indicated by an arrow). After transformation of *E. coli* JM101, phage mutants were identified by the dideoxynucleotide chain terminator method (19). A *Bgl*II-*Xba*I fragment (extending from base 705 to the *Xba*I site flanking the 3' end of the 16S rRNA gene) was excised from the mutant phages and cloned back into pPM114. The mutated region of the deletion derivatives of pPM114 was characterized by restriction mapping and by sequencing (20) to confirm the deletion in the 16S rRNA gene.

In vitro transcription with T7 RNA polymerase and reconstitution assays.

In vitro specific transcription of the 16S rRNA sequence from *Mst*II-linearized pPM114 and its deletion derivatives was carried out following previously described procedures (21,22). Typically, the transcription of 20 μg of DNA yielded about 400 μg of 16S rRNA.

Ribosomes and ribosomal subunits were isolated as described (23). The control 16S rRNA was extracted by phenol treatment and ribosomal proteins were extracted with LiCl-urea (9,24), except that ribosomal proteins were isolated from 70S ribosomes rather than from 30S subunits. This procedure avoids loss of ribosomal proteins during subunit purification. The 16S rRNA transcript or the control native 16S rRNA was reassembled into 30S subunits with ribosomal proteins, using modifica-

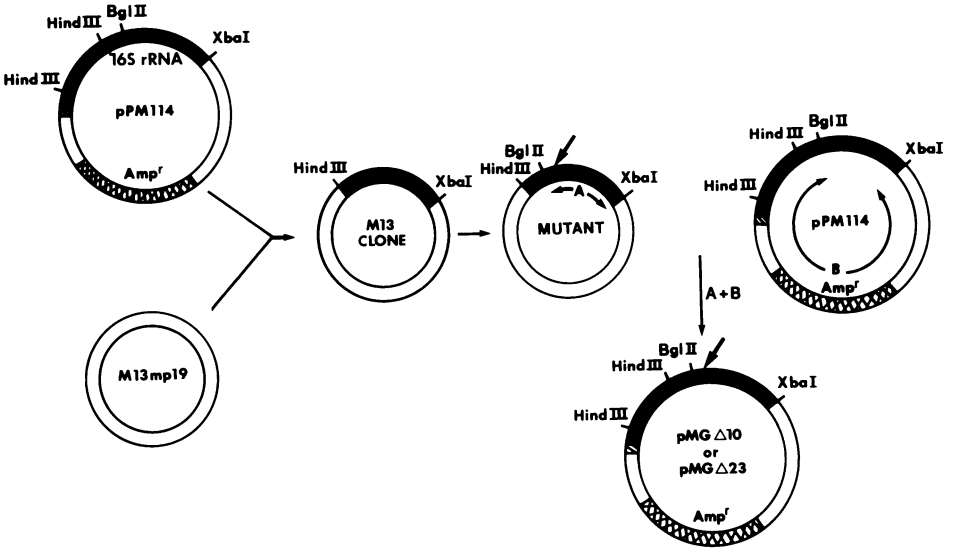

Figure 2: Scheme for the introduction of mutations. The various steps and relevant restriction sites used in the oligonucleotide-directed mutagenesis are indicated (see details in the text). The arrow points to the site of mutation.

tions of previously described procedures (25,26). In a typical reconstitution assay, 1 A_{260} unit of 16S rRNA was mixed with the amount of protein extracted from 9 A_{260} units of 70S ribosomes in 500 μl of reconstitution buffer (30 mM Hepes-KOH, pH 7.4; 26 mM $MgCl_2$; 292 mM KCl; 3 mM spermidine; 6 mM 2-mercaptoethanol). After incubation at 43°C for 2 h, the reconstituted 30S subunits were pelleted by centrifugation overnight at 4°C at 35000 rpm through a 15% (w/v) sucrose cushion in a Beckman SW50.1 rotor.

In vitro protein synthesis.

The basic procedure used for the determination of poly(U)-directed incorporation of phenylalanine was as described (9,27). The incorporation mixture (100 μl) contained 0.16 A_{260} units of 50S subunits and a fivefold molar excess of reconstructed 30S subunits. [^{3}H]Phenylalanine (2.5 Ci/mmol) was present at 20 μM. Misreading assays were as described for poly(U)-directed incorporation of phenylalanine except that the cognate amino acid, [^{12}C]phenylalanine, and the non-cognate, [^{3}H]isoleucine (25 Ci/mmol), were added simultaneously at a concentration of 20 μM and 2 μM, respectively. Polypeptide synthesis programmed with MS2 RNA was performed in a mixture (100 μl) containing: 50 mM Hepes-KOH, pH 7.4; 60 mM NH_4Cl; 8 mM magnesium acetate; 16 mM 2-mercaptoethanol; 1.3 mM ATP; 0.3 mM GTP; 8 mM phosphoenolpyruvate; 1 μg pyruvate kinase; 10 μg leucovorin; 50 μg *E. coli* stripped tRNA; 25 μM each of unlabeled amino acids; 5 μCi each of [^{3}H]leucine and [^{3}H]valine; an

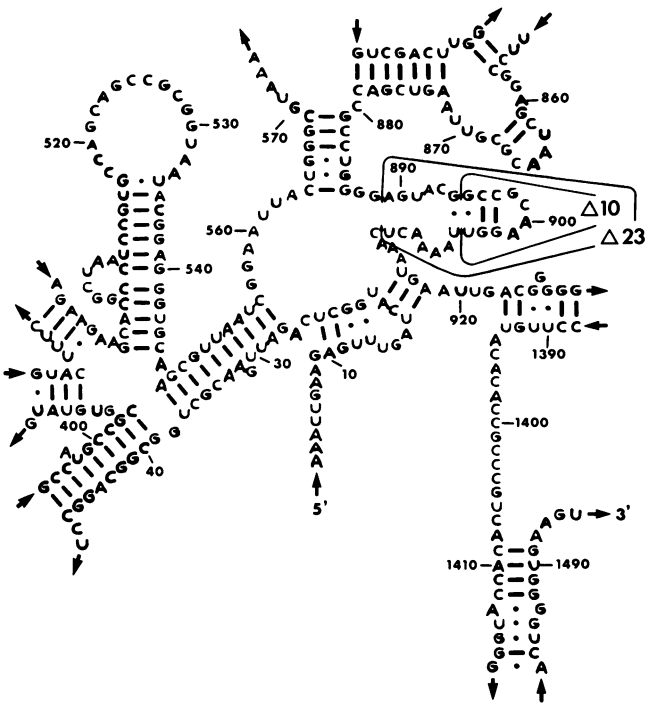

Figure 3: Secondary structure of a portion of *E. coli* 16S rRNA (37). The extent of the deletion in the 16S rRNA transcribed from plasmid pMGΔ10 or pMGΔ23 is indicated by the arrows.

optimal amount (about 300 μg protein) of high-speed postribosomal supernatant (S-150); 25 μg protein from the crude 35-80% $(NH_4)_2SO_4$ fraction of initiation factors (28); 40 μg MS2 RNA prepared by a standard procedure (29); 0.33 A_{260} units of 50S subunits and a fivefold molar excess of reconstructed 30S subunits. For all the protein synthesis assays, the incubation time was as specified in the corresponding Figure and Table.

RESULTS AND DISCUSSION

Deletion mutations were introduced in the 16S rRNA gene carried by pPM114, using the phosphorothioate-based oligonucleotide-directed mutagenesis system. Figure 2 outlines the strategy of site-directed mutagenesis for pMGΔ10 and pMGΔ23, respectively lacking bases 895 to 904 and 889 to 911 of the 16S rRNA gene. The location of the two deletions within the relevant segment of the 16S rRNA secondary structure is shown in Figure 3.

Amputated 16S rRNA transcripts were generated by *in vitro* transcription of

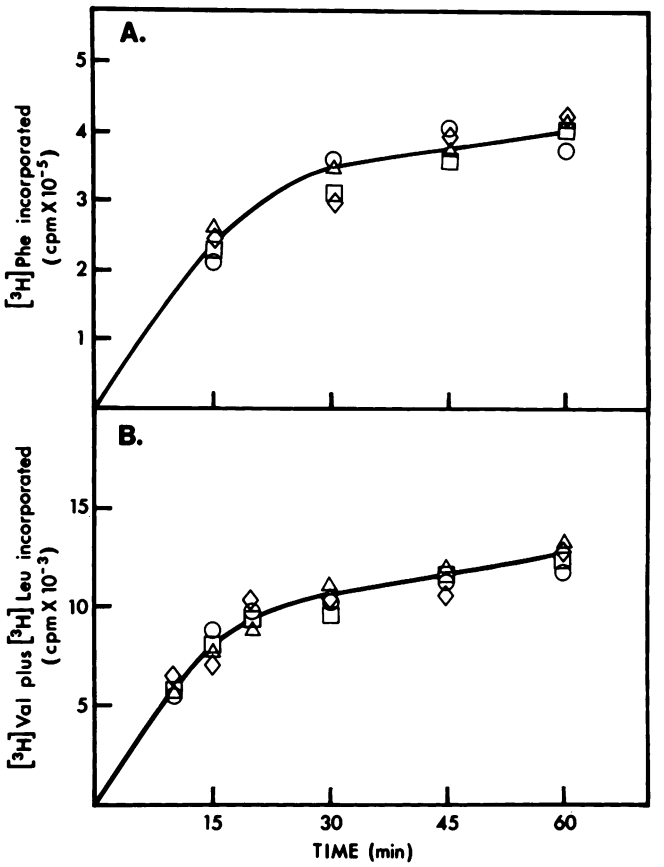

Figure 4: Kinetics of [^{3}H]phenylalanine incorporation under the direction of poly(U) (A) and of [^{3}H]leucine and [^{3}H]valine incorporation under the direction of MS2 RNA (B) with ribosomes containing reconstructed 30S subunits with natural 16S rRNA (○), full-length 16S rRNA transcript (□), amputated 16S rRNA transcript from plasmid pMGΔ10 (△) or pMGΔ23 (◇). The experimental values are the means of three independent experiments. Standard deviations were equal or inferior to ± 15%.

pMGΔ10 and pMGΔ23 and were incubated with ribosomal proteins under the conditions used for the reassembly of 30S subunits. The 30S subunits reconstructed with such transcripts were found to comigrate with native 30S subunits in a sucrose density gradient and the analysis of their protein content by two-dimensional polyacrylamide urea gel electrophoresis (30) revealed that they contained the full complement of 30S proteins (data not shown). It can thus be concluded that the deletion in the 900 region did not perturb the *in vitro* assembly of 30S subunits.

The 30S subunits reconstructed with amputated 16S rRNA transcripts were then

Table I
Stimulation of misreading by streptomycin with ribosomes containing *in vitro* synthesized 16S rRNA

Origin of the 16S rRNA	Poly(U)-directed incorporation of isoleucine (in cpm)	
	Without streptomycin	With streptomycin
Control 16S rRNA (isolated from native 30S subunits)	1845	19 598
In vitro synthesized full-length 16S rRNA	2029	20 844
In vitro synthesized amputated 16S rRNA		
from pMGΔ10	1912	21 296
from pMGΔ23	1868	20 177

Misreading was assessed by measuring the poly(U)-directed incorporation of [^{3}H]isoleucine into trichloroacetic acid-insoluble material. Incubation was for 60 min at 37°C. Streptomycin, when present, was added at a concentration of 0.05 μg per assay. Results are the means of five independent experiments. Standard deviations were equal or inferior to ± 15%.

assayed for their protein synthesis activity under the direction of either an artificial or a natural messenger. This activity was compared to that of 30S subunits reconstructed either with a full-length 16S rRNA transcript or with natural 16S rRNA isolated from native 30S subunits. Time-course studies of the protein-synthesizing activities of these reconstituted particles (Figure 4) showed no difference between the different types of 30S subunits. This suggests that the 900 stem/loop region does not affect the activity of the ribosome.

Since the deleted region has been shown to be proximal to the streptomycin binding domain, it could be involved in the interaction between the drug and the ribosome. Therefore, 30S subunits reconstructed with either the amputated or the full-length 16S rRNA transcripts were assayed for their capacity to misread the message in the presence of streptomycin. Again, the response of the reconstructed 30S subunits was identical, independently of the origin of the 16S rRNA (Table 1).

Our results strongly suggest that the deleted sequence is not essential for ribosomal function, in contrast to what was expected from the high degree of conservation of the 900 region. A similar situation was observed with a conserved GAAC

sequence of 5S rRNA complementary to the GT ψC arm of the tRNA. This sequence was assumed to be involved in the binding of tRNA to the ribosome until Pace and his collaborators demonstrated that the excision of this conserved sequence did not affect the activity of the ribosome (31,32).

We also attempted to introduce the deletions of the 900 region in plasmid pKK3535, which carries the *rrn*B operon under the control of a constitutive tandem promoter (14). In spite of numerous attempts, we could not recover transformants harbouring plasmids with the expected deletions. The transformation process was accompanied by the spontaneous occurrence of huge deletions in the plasmid, implying that the cells do not tolerate the transcription product of the *rrn*B operon lacking the 900 stem/loop region of the 16S rRNA. The inability to isolate mutants with the deletion in the 900 region of 16S rRNA, when expressed in plasmid pKK3535, provides indirect evidence for the importance of this region.

The introduction in the 16S rRNA gene of deletions, even as small as one base pair, has often been shown to drastically affect the growth of cells transformed with the mutated plasmids. The spontaneous occurrence of plasmids with larger deletions than those initially introduced has frequently been observed (33). Deletions of specific portions of the 16S rRNA gene could interfere with the processing of the 16S rRNA transcripts. They could also affect the *in vivo* assembly of the 30S subunits as well as perturb the delicate controls regulating the coordinate synthesis of the components of the ribosome (see 34-36). Dahlberg and his coworkers have developed maxicell systems (34), where the plasmid-encoded *rrn*B operon can be expressed under the control of an inducible promoter while the host-encoded rRNA genes are selectively inactivated. These systems provide a useful approach to further investigate *in vivo* the consequences of deleting the 900 region of the 16S rRNA gene.

In conclusion, this study illustrates the value of using a completely *in vitro* system to investigate the consequences of mutations in the 16S rRNA gene. Our results have shown that the deletion of the 900 region in the 16S rRNA did not affect the activity of the ribosome. Taken together with the fact that it was not possible to select stable derivatives of pKK3535 lacking this region, they indicate that the 900 region of the 16S rRNA is dispensable for the function of the ribosome but is probably essential for important cell regulatory functions. Understanding the nature of these functions will be a main goal of our future experiments.

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