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EFFECT OF REMOVAL OF 160 NUCLEOTIDES FROM THE 3' END OF ESCHERICHIA COLI 16S rRNA ON THE RECONSTITUTION AND ACTIVITY OF 30S RIBOSOMES

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SUMMARY. E. coli 16S rRNA deprived of 160 nucleotides from its 3' end was obtained by digestion with polynucleotide phosphorylase. Such rRNA was used for the reconstitution of 30S subunits and the resulting particles contained all proteins present in native 30S ribosomes. Their sedimentation coefficient was estimated as 26.5S. Poly AUG-dependent binding of fMet-tRNA to subunits reconstituted with shortened rRNA was the same as to 30S particles reconstituted with the native 16S rRNA. Subunits reconstituted with shortened rRNA were also active in poly U-dependent phenylalanine incorporation; however, their activity reached only 50% of that obtained with 30S subunits reconstituted with native 16S rRNA.

The results of recent experiments show the importance of the 3' end of 16S rRNA in the functioning of E. coli 30S ribosomal subunits (1-5). It is accepted, that the nucleotide sequence near the 3' end of 16S rRNA base-pairs with initiation regions on mRNAs thus playing a crucial role in the first step of initiation of protein synthesis (1, 2). However, there are some reports that an intact 3' end of 16S rRNA is apparently not essential to confer stability and specificity to the mRNA binding (6-10). Cleavage of the 3'-terminal 50-nucleotide fragment of 16S rRNA with colicin E3 or cloacin DF13 inhibits neither the binding of poly U or MS2 RNA to 30S subunits nor the ApUpG-dependent binding of fMet-tRNA. Cloacin DF13-treated 30S subunits carried out normal initiation with MS2 RNA but failed to bind the subsequent aminoacyl-tRNAs resulting in an arrest of elongation.

Experiments with mild nuclease digestion of the 30S subunit indicated that the 3' end of 16S rRNA (approximately 150 nucleotides) is easily accessible

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for degradation and is not involved in strong interactions either with ribosomal proteins or with other regions of the RNA (11). This is consistent with results of Laughrea and Moore (12) who showed that removal of colicin E3 RNA fragment from 30S particles does not cause dissociation of proteins S1 and S21, which were thought to be bound to this fragment (13, 14).

To investigate the involvement of the 3' end region of 16S rRNA in the E. coli 30S subunit structure and function, we removed a limited number of nucleotide residues from this region by polynucleotide phosphorylase digestion. Such RNA was used for reconstitution of 30S subunits and the functional properties of these subunits were tested.

MATERIALS AND METHODS

Phosphorolysis of 16S rRNA. Polynucleotide phosphorylase (PNPase, E.C.2.7.7.8.) from E. coli B was prepared according to Portier et al. (15) except that DE-Sephadex A-50 chromatography was applied before chromatography on Sephadex G-200 and final concentration of active fractions was achieved on a small DE-52 column. The enzyme was dialyzed against 20 mM Tris-HCl (pH 7.5) and stored in liquid nitrogen. PNPase was free of endonucleolytic activity; when phage f2 RNA was incubated with PNPase for 1 h at 37°C in an incubation mixture without phosphate ions and subjected to gel electrophoresis, the mobility of PNPase-treated RNA was identical with that of native f2 RNA.

16S rRNA from E. coli MRE 600 was prepared as described (16). Phosphorolysis of 16S rRNA was performed in a mixture containing 0.28 mg/ml of 16S rRNA and 1.06 mg/ml of PNPase (10-fold molar excess over RNA) in 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$ and 10 mM K_2HPO_4. The mixture was incubated for 20 min at 10°C. 16S rRNA with digested off 3' end fragment was recovered by phenol extraction and ethanol precipitation. The RNA was purified by two consecutive sucrose gradient centrifugations (5-20%) for 21 h at 4°C in a Spinco SW 27.1 rotor at 25,000 rpm.

Gel electrophoresis of RNA. Electrophoresis of RNA was performed on 2.5% polyacrylamide slab gels containing 0.5% agarose. Buffer in the gel and the running buffer contained 40 mM Tris-acetate (pH 7.2), 33 mM sodium acetate, 1 mM EDTA. Formamide electrophoresis on a 5% gel was carried out according to Maniatis et al. (17). Brome Mosaic Virus RNA 1 (M.W. 1.09×10^6), 2 (M.W. 0.99×10^6), 3 (M.W. 0.75×10^6), 4 (M.W. 0.28×10^6) and 16S rRNA (M.W. 0.53×10^6) were used as molecular weight markers.

Isolation of ribosomal subunits. 30S and 50S subunits were prepared from E. coli MRE 600 ribosomes washed with 1 M NH_4Cl as described previously (18). The 1 M NH_4Cl ribosomal wash served as a source of initiation factors.

Reconstitution of 30S subunits. E. coli 30S ribosomal proteins were isolated by extraction of subunits with 2 M LiCl and 4 M urea (19). Reconstitution of 30S subunits was performed as described in ref. 19 with the use of about 1 mg of native or phosphorolysed 16S rRNA.

Two-dimensional gel electrophoresis of proteins from reconstituted subunits was performed according to Howard and Traut (20).

Activity assays of reconstituted subunits. Poly AUG-dependent binding of [^{3}H]fMet-tRNA to reconstituted subunits was carried out as described previously (18). Poly U-dependent [^{14}C]phenylalanine incorporation was performed in the mixture described in (21), with addition of E. coli postribosomal supernatant and reconstituted subunits.

RESULTS AND DISCUSSION

Polynucleotide phosphorylase is an exonuclease which phosphorolyses polynucleotides by a processive mechanism starting at the 3' end (22). In spite of that, only about one half of the RNA population was attacked by the enzyme. Electrophoretic analysis of PNPase-treated 16S rRNA showed two distinct bands: one in the position of intact rRNA and another which migrated slightly faster. This pattern of phosphorolysis was reproducible under our experimental conditions. The limited digestion could be explained by some obstacle in the structure of rRNA which impairs the movement of PNPase along the RNA chain.

Phosphorolysed RNA was purified by two successive centrifugations through sucrose gradients. Such purification yielded an almost homogenous population of shortened 16S rRNA (Fig. 1B). The molecular weight of this RNA was estimated by formamide gel electrophoresis as being 475,000 daltons. From the difference in molecular weight of the intact (M.W. 0.53×10^{6} daltons, ref. 23) and phosphorolysed RNA it was calculated that the native RNA chain was shortened by a sequence of about 160 nucleotides.

Phosphorolysed 16S rRNA was mixed with proteins isolated from 30S particles and the reconstitution of 30S subunits was performed (19). The yield of reconstituted particles was the same as that obtained when native 16S rRNA

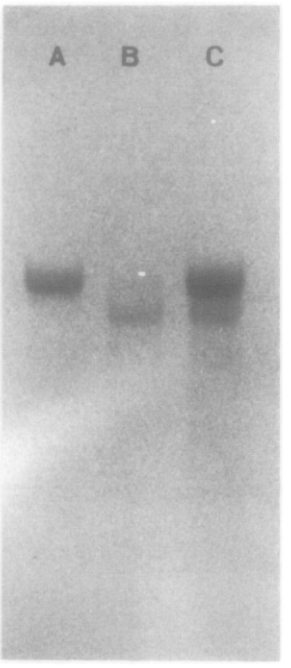

FIG. 1. Gel electrophoresis of purified phosphorolysed 16S rRNA. After 2.5 h of electrophoresis at 70 mA at room temperature, the gel was stained in 0.1% toluidine blue. Lane A, 3 μg of native 16S rRNA; lane B, 1.4 μg of phosphorolysed 16S rRNA purified as described in Materials and Methods; lane C, mixture of 1.8 μg phosphorolysed 16S rRNA and 3 μg of native 16S rRNA.

was used. Sedimentation analysis of the material recovered from the reconstitution mixtures containing native or phosphorolysed 16S rRNA showed that 30S particles reconstituted in the presence of shortened 16S rRNA sedimented at 26.5S, while native subunits and those reconstituted in the presence of intact 16S rRNA sedimented at 30.5S and 30.25S, respectively.

Two-dimensional polyacrylamide gel electrophoresis of proteins from 30S particles reconstituted with native or phosphorolysed 16S rRNA revealed that in both cases the protein patterns were identical and all of the 30S proteins were present (Fig. 2), in agreement with the results of Laughrea and Moore (12). These results indicate that the 160 nucleotides long fragment from the 3' end of 16S rRNA is not essential for maintaining the structural organization of 30S particles.

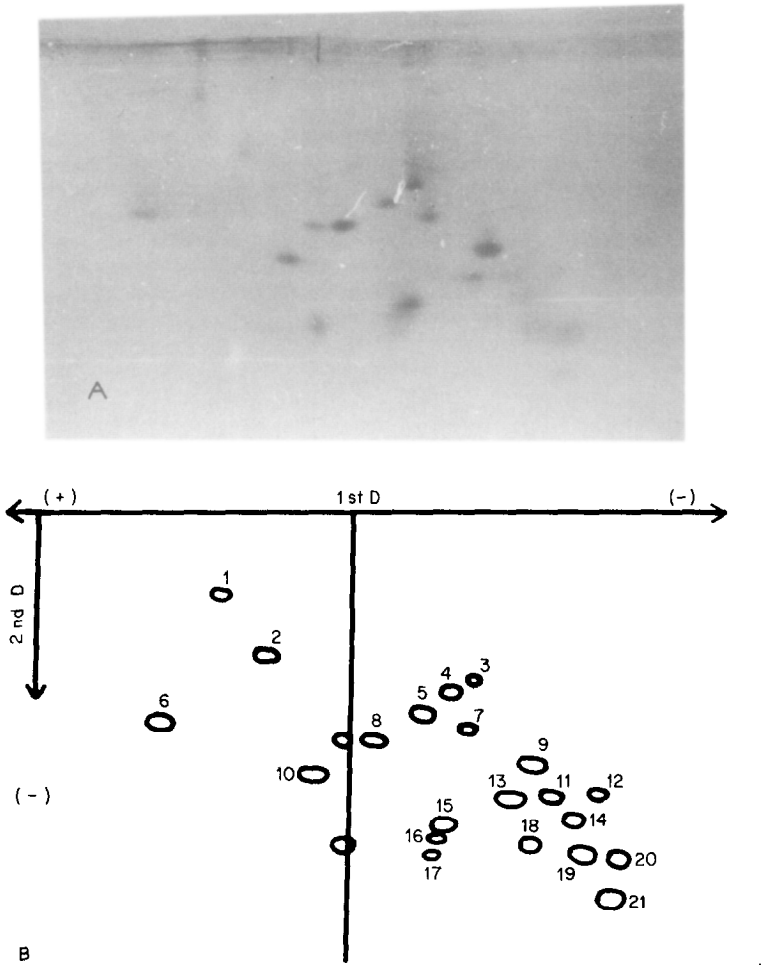

FIG. 2. Electrophoretic pattern of proteins from 30S subunits reconstituted with 16S rRNA deprived of 160 nucleotides from 3' end. Proteins obtained from 300 μg of 30S subunits were loaded on two short first-dimensional gels run in opposite directions. A - gel photograph; B - tracings of A.

Further experiments have been performed to study the effect of removal of 160 nucleotides from the 3' end of 16S rRNA on the function of 30S subunits. For this purpose poly AUG-dependent binding of fMet-tRNA to subunits containing shortened 16S rRNA was tested (Table 1). In the complete system, with poly AUG and crude initiation factors, similar amounts of fMet-tRNA were bound to both types of 30S subunits. The puromycin reaction indicated that initiator tRNA was positioned at the "P" site of the ribosomes (data not shown).

Table 1. Poly AUG-dependent binding of [^{3}H]fMet-tRNA to reconstituted subunits

30S subunits	[^{3}H]fMet-tRNA bound (cpm)		
	complete system	-poly AUG	-IF
30S reconstituted with native 16S rRNA	1416	314	670
30S reconstituted with phosphorolysed 16S rRNA	1444	466	679

The incubation mixture (50 µl) contained 26 µg of 30S subunits, 52 µg of 50S subunits, 34 µg of crude initiation factors (IF), 10 µg of poly AUG and 32 µg of [^{3}H]fMet-tRNA. Other components of the incubation mixture were as described in ref. 18.

30S subunits reconstituted with 16S rRNA deprived of its 3' end were still active in the poly U-dependent phenylalanine incorporation (Fig. 3). However, their activity reached only 50% of that of 30S subunits reconstituted with native 16S rRNA.

These results indicate that 16S rRNA shortened by 160 nucleotides from the 3' end is fully active in the reconstitution of 30S subunits, forming parti-

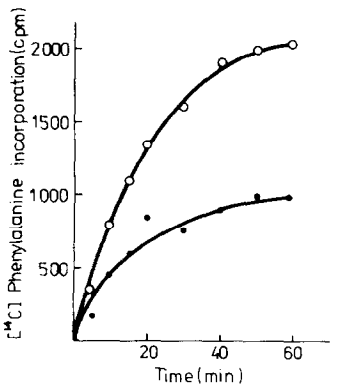

FIG. 3. [^{14}C]Phenylalanine incorporation with reconstituted 30S subunits. Incubation mixtures (25 µl) contained 26 µg of 30S subunits, 52 µg of 50S native subunits, 40 µg of poly U, 60 µg of the postribosomal supernatant, 25 µg of RNA. Other components of the incubation mixtures were as described in ref. 21. Incubation was carried out at 37°C. At indicated times, two microlitre aliquots were withdrawn and hot TCA-insoluble radioactive products were determined.

cles which contain all the proteins (including S1 and S21). The particles retain their activity in the poly AUG-dependent binding of fMet-tRNA as well as in the synthesis of polyphenylalanine.

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