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Conformational analysis of 16 S ribosomal RNA from *Escherichia coli* by scanning transmission electron microscopy

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Digitized images of molecules of 16 S rRNA from *Escherichia coli*, obtained by scanning transmission electron microscopy (STEM), provide quantitative structural information that is lacking in conventional electron micrographs. We have determined the morphology, total molecular mass, mass distribution within individual rRNA molecules and apparent radii of gyration. From the linear density (M/L) we have assessed the number of strands in the structural backbone of rRNA and studied the pattern of branching and folding related to the secondary and tertiary structure of rRNAs under various buffer conditions. Even in reconstitution buffer 16 S RNA did not show any resemblance to the native 30 S subunit.

rRNA; Electron microscopy; RNA structure

1. INTRODUCTION

The conformation of 16 S ribosomal RNA (rRNA) from *Escherichia coli* has been studied extensively using many approaches (for references see [1]). The models proposed for its secondary and tertiary structure were derived from results of indirect methods [2–9]. Conventional transmission electron microscopy (EM), the most direct method for these studies, lacks both contrast and resolution for visualization of free rRNAs in a form suitable for quantitative structural analysis. Spreading of rRNAs under denaturing conditions [10,11], contrasting the RNA molecules with heavy metals, air-drying and exposure to the high electron doses adversely affect the native structure and resolution of the specimen. These drawbacks of conventional EM can be overcome by application of scanning transmission electron microscopy (STEM) [12,13].

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2. MATERIALS AND METHODS

2.1. *Buffers and solutions*

Buffers were of the following compositions: (buffer A) 10 mM Hepes-KOH (pH 7.5), 60 mM KCl, 2 mM $Mg(OAc)_2$, 1 mM DTT; (buffer B) (6000 × diluted buffer A) 1.7 μM Hepes-KOH (pH 7.5), 10 μM KCl, 0.34 μM $Mg(OAc)_2$; (buffer R) 30 mM Hepes-KOH (pH 7.5), 330 mM KCl, 20 mM $Mg(OAc)_2$, 1 mM DTT. All buffers and solutions were prepared and kept under sterile conditions.

2.2. *Ribosomes and their components*

Ribosomes were prepared from frozen *E. coli* MRE 600 cells as described [14]. Ribosomal subunits were separated in 10–38% sucrose gradients in a Beckman zonal Ti15 rotor [15]. 16 S rRNA extracted from the 30 S subunits [16] was purified on 15–30% sucrose gradients, recovered by ethanol precipitation, resuspended in autoclaved distilled water and stored at −80°C. The purity of 16 S rRNA was checked by agarose gel electrophoresis [17].

2.3. *Electron microscopy*

Samples of 16 S rRNA for STEM were prepared under non-denaturing conditions [13,18]. Electron-microscopic imaging of unstained freeze-dried 16 S rRNA molecules was performed with a dedicated STEM [12] at a radiation dose of $< e/Å^2$ and a direct magnification of 125 000× at 40 kV. Mass measurements were carried out as in [19]. The apparent values of radii of gyration (R_G) were calculated from the distribution of the measured intensities of scattered electrons within the individual RNA molecules [20]. The average number of molecules measured in each experiment was between 100 and 200.

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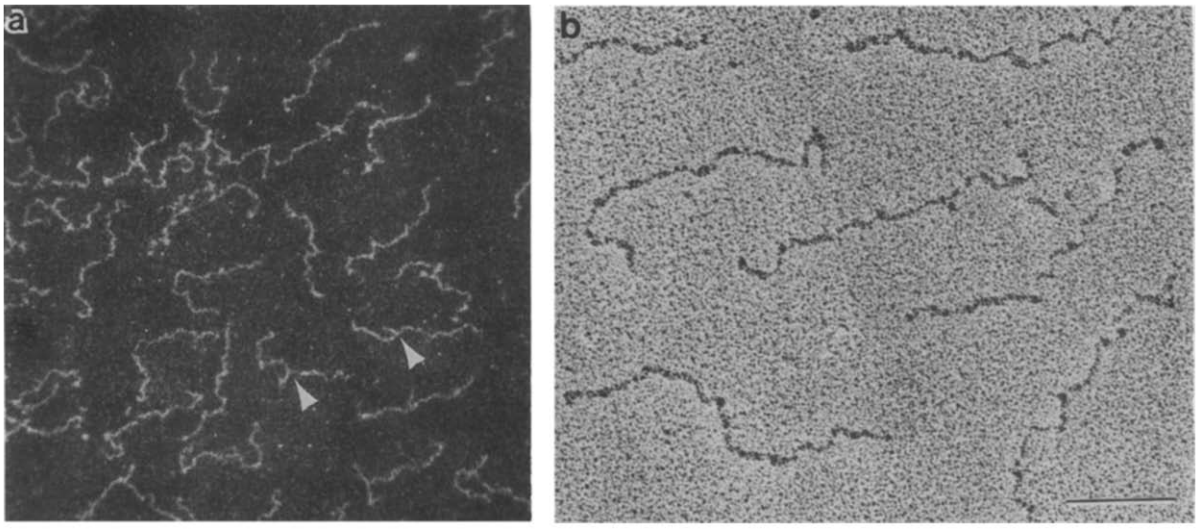

Fig.1. Electron micrographs of *E. coli* 16 S rRNA obtained by (a) STEM, unstained, freeze-dried; (b) conventional BAC-monolayer technique, air-dried, shadowed with PtPd. Deposition in both cases was from water. Arrows point to the protrusion at 2/5th of the RNA molecule. Bar, 0.1 μm.

3. RESULTS AND DISCUSSION

3.1. *Morphology, mass and mass (M/L) distribution in 16 S rRNA*

Fig.1 offers a comparison of 16 S RNA images obtained by STEM (a) and by EM (b) using the routine spreading techniques with benzylalkylammonium chloride (BAC) [11]. The difference in the length of 16 S RNA molecules in EM and in STEM images reflects the mode of specimen deposition on the support carbon film. 16 S rRNA spread on a water hypophase and stretched under denaturing conditions appears as a single strand ~5000 Å long (fig.1b) in agreement with the number of nucleotides (1542), and the internucleotide distance derived from 2.8 Å axial rise per residue for A-RNA [21]. The length of the 16 S RNA molecules in STEM images obtained in the absence of denaturing agents and without any stretching forces is ~1200 Å (fig.1a), only about 1/4 of that in the fully extended state. The difference in thickness is caused by deposition of BAC and tungsten on the RNA molecules. This step obscures the fine structures resolvable on STEM images of unstained freeze-dried RNA molecules (fig.1a), and excludes any mass determinations. The total molecular mass for 16 S rRNA as determined by STEM is 551 ± 22 kDa, in excellent agreement with the theoretical data (550 kDa). The average linear density (*M/L*) for the molecules is about 480 Da/Å. This value is about 4-times higher than that expected for single-stranded RNA in the denatured extended form and indicates a close association of four single-stranded polynucleotide strands in the main backbone of the observed molecules. However, at present there is

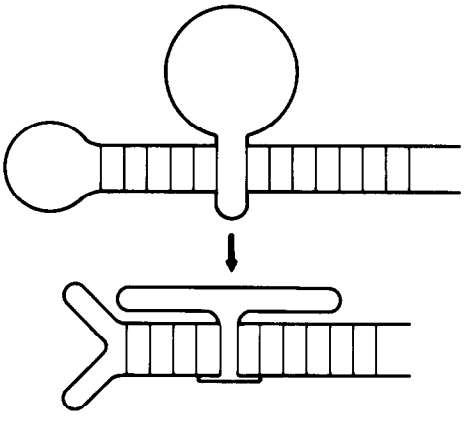

Fig.2. Scheme depicting a possible explanation for shortening of rRNAs and formation of multiple strands in the major backbone of 16 S rRNA by collapse of single-stranded loops and folding around double-stranded stems.

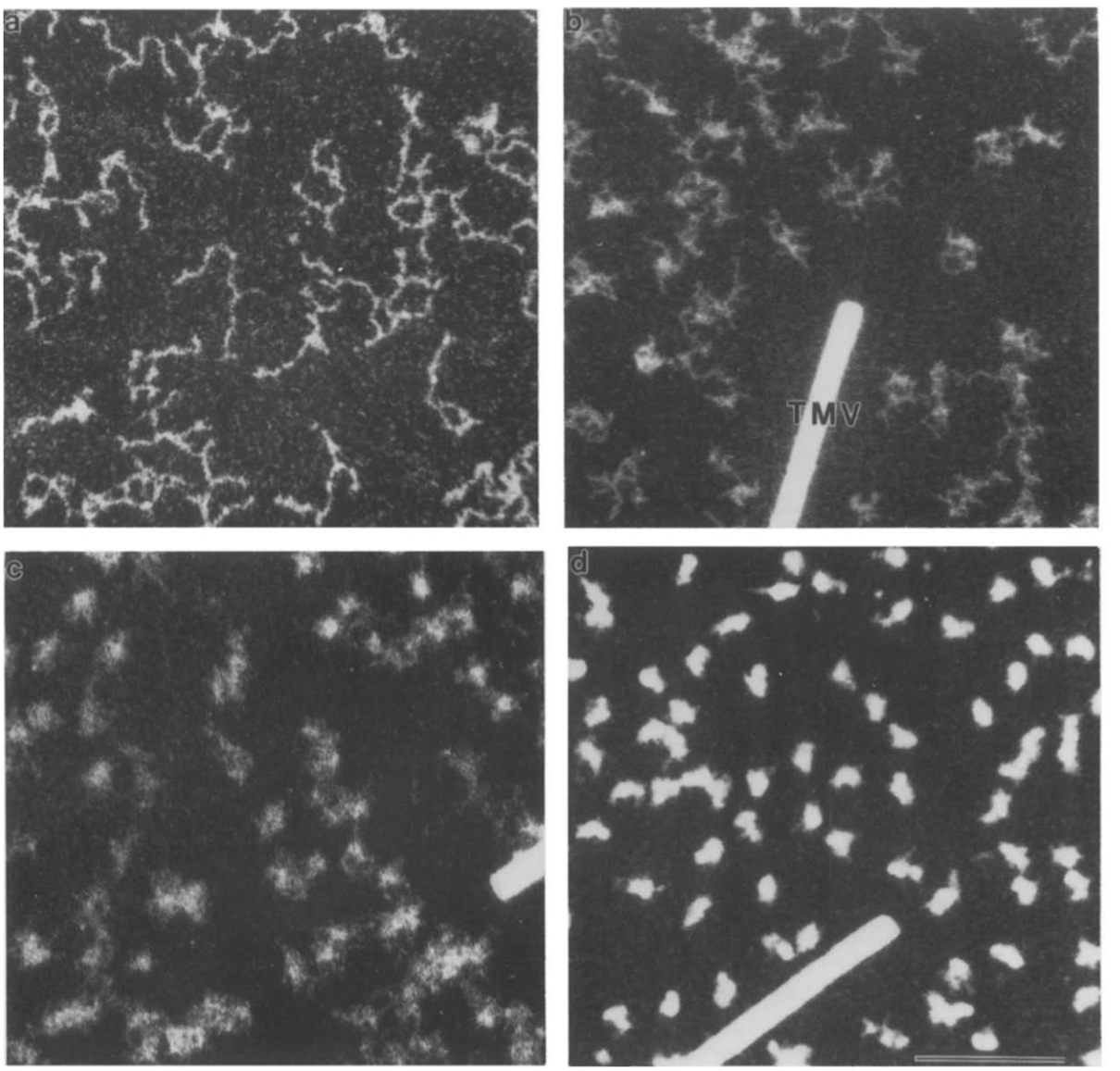

Fig.3. STEM images of *E. coli* 16 S rRNA in (a) buffer B, (b) buffer A, (c) buffer R, (d) 30 S subunits in buffer A. Tobacco mosaic virus (TMV) was used as the standard for mass determinations. Bar, 0.1 μm.

no information on the mutual orientation of the four nucleotide strands as to whether they form coaxial helices [22] and/or are merely a combination of double and single strands in side-by-side position. Also, one should be aware that the images are 2D projections of a 3D structure.

The linear density (480 Da/Å) appears to be evenly distributed along the whole molecule, with the exception of a short bifurcation seen at the 'ends' of some molecules and a small protrusion (arrow in fig.1a), ~80 Å long with a mass of ~60 kDa. Based on the M/L value (750 D/Å), this protrusion could be composed of 6 polynucleotide strands. The asymmetric location of the protrusion at about 2/5th of the length of the 16 S RNA molecule can be used to determine the polarity of

Table 1

Radii of gyration (R_G) of *E. coli* 16 S RNA in various solutions calculated from STEM

Solution	R_G[a] (Å) (±SD)
Water	305 ± 50
Buffer B	213 ± 33
Buffer A	114 ± 20
Buffer R	80 ± 10
Buffer A + 0.5 mM Mg^{2+}	136 ± 20
Buffer A + 10 mM Mg^{2+}	99 ± 10

[a] R_G of 30 S *E. coli* subunit is 68 ± 1.4 Å

the molecule. However, identification of the 3′- and 5′-ends would require linking of a marker to either end of the 16 S RNA molecule.

3.2. *Conformation, radii of gyration and salt-induced conformational transitions*

The secondary structure of 16 S rRNAs of *E. coli* is known to consist of alternating intramolecular double-stranded stems and single-stranded loops of various lengths (for references see [1,9]). The double-stranded segments are sufficiently rigid and stable to be preserved under the non-denaturing conditions of specimen deposition for STEM [13,20]. Freeze-drying and use of a cold stage (−160°C) provide some degree of specimen fixation, not necessarily sufficient for the prevention of a partial collapse of the flexible single-stranded loops. Fig.2 offers a possible explanation for the formation of a 'four-stranded' backbone and for a 'bifurcation' at the end of the rRNA molecule.

From the mass distribution in the digitized images of individual 16 S RNA molecules we have calculated the apparent radius of gyration (R_G), a parameter related to the 3D structure. These results allowed for a direct comparison of the STEM data with those of spectroscopic and hydrodynamic techniques in which the specimens are monitored in a fully hydrated state and under controlled buffer conditions [23,24].

Fig.3 shows examples of the conformational states of 16 S RNA visualized by STEM. 16 S rRNA in very low ionic strength (buffer B, fig.3a) is similar to those molecules deposited in distilled water (fig.1a). Although the molecules remain extended, they are shorter by about 10%. In addition, the branching pattern becomes more complex and the R_G is reduced by about 30% (table 1). Increase in ionic strength induces further RNA coiling (fig.3b) and reduction of R_G. Molecules of 16 S RNA deposited in 'reconstitution' buffer R (fig.3c) appear very tightly coiled. However, even under these conditions their R_G is still substantially larger (80 ± 10 Å) than that of the 30 S subunits ($R_G = 68 \pm 1.4$ Å). These results are in agreement with spectroscopic data [24] indicating that the applied procedure of specimen deposition and freeze-drying did not cause any structural distortion of 16 S RNA. The value of R_G for 16 S RNA determined by STEM in buffer R (table 1) is close to the values of R_G obtained by neutron scattering (84 Å) and X-ray scattering (86 Å) under similar buffer conditions [25]. Apart from being tightly coiled, the 16 S RNA molecules in buffer R do not display any characteristic features which would facilitate direct structural comparison with the proposed models for the tertiary structure of 16 S RNA [9] nor do they show any resemblance to the 30 S subunits (fig.3d). They appeared as tightly packed cores with protruding filaments with no obvious symmetry and no resemblance to the V or Y shapes observed in conventional EM of shadowed and specially treated specimens [25,26].

In summary, we have succeeded in monitoring the conformational transitions that 16 S rRNA undergoes with increasing ionic strength from solution in water up to ribosomal reconstitution buffer. With dedicated STEM we have determined the total molecular mass of 16 S rRNA, its length under non-denaturing conditions and the number of the apparent polynucleotide strands in its major structural backbone. The transition states visible in STEM as coiling of the initially filamentous strand into more compact form were characterized by radii of gyration. Supplementation of STEM results by CD spectroscopic measurements (unpublished) showed that the coiling of free rRNAs is mediated by long-range interactions rather than considerable changes in their secondary structure. STEM images of 16 S rRNA and the values of R_G present clear evidence that the folding of 16 S rRNA even in the reconstitution buffer did not reach the extent of the compactness and similarity to the shape of the native ribosomal subunit, contrary to what has been reported by others [25,26]. A study of the effect of individual ribosomal proteins on the conformation of rRNAs in the

assembly process of *E. coli* ribosomal subunits using the above approach is now in progress.

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