Topography of RNA in the ribosome: location of the 5 S RNA residues A_{39} and U_{40} on the central protuberance of the 50 S subunit

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Received 28 March 1985

The internal site of 5 S RNA comprising residues A_{39} and U_{40} has been localized on the *E.coli* 50 S ribosomal subunit by immune electron microscopy. It has been found to be located on the interface side of the central protuberance at the position distinctly apart but very close to the position of the 5 S RNA 3'-end providing evidence for a quite compact folded conformation of the 5 S RNA in situ.

Ribosome *5 S RNA* *Nuclease S1* *Dinitrophenyl hapten* *Immune electron microscopy*

1. INTRODUCTION

Elucidation of the 5 S RNA topography with respect to the other ribosomal components and functional sites is necessary for understanding its role in ribosome functioning. Previously we have mapped the 3',5'-terminal stem of the 5 S RNA on the surface of the 50 S subunit of the *Escherichia coli* ribosome [1].

Here we describe the localization of residues A_{39} and U_{40} of 5 S RNA. These have been found to be located on the interface side of the 50 S subunit central protuberance facing the lateral L1-protuberance, at its upper one-third.

To map these residues the *E. coli* 5 S RNA was cleaved at A_{39} or U_{40} with nuclease S1 and the larger fragment was modified at its 5'-terminal phosphate with 2,4-dinitrophenyl(DNP)-hapten. After reassociation of the unmodified (small) and modified (large) fragments into the whole 5 S RNA molecule (DNP-5 S RNA*), the latter was incorporated into the 50 S ribosomal subunit by in vitro reconstitution. Mapping of the hapten-modified nucleotide residues was done by immune electron microscopy.

2. MATERIALS AND METHODS

5 S RNA was isolated from *E. coli* MRE 600 ribosomes as described in [2]. 100 A_{260} units of 5 S RNA were digested with 2000 units of nuclease S1 (Biolar, USSR) in 2 ml of a buffer containing 20 mM CH_3COONa (pH 4.5), 100 mM NaCl, 2 mM $ZnCl_2$, 4 mM $MgCl_2$ at 25°C for 5 min. The reaction was stopped by phenol extraction of the nuclease and ethanol precipitation of the RNA. The digest was fractionated by polyacrylamide/7 M urea gel electrophoresis, using 5 S RNA and its 41- and 79-nucleotide-long fragments as markers (provided by M. Saarma). Two major products of 5 S RNA hydrolysis of similar length to the markers (denoted further as 'large' and 'small' fragments), detected by UV-shadowing, were eluted from the gel and purified by $CaCl_2$ precipitation [3]. The length of the large fragment was determined from its electrophoretic mobility using 5 S RNA statistical hydrolysate prepared by boiling and the products of U-specific sequence reaction as markers [4].

Modification of the large fragment at the 5'-phosphate group by DNP-ethylenediamine was

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00145793/85/$3.30

carried out as in [5]. 3.2 A_{260} units of the 5 S RNA small fragment and 6.0 A_{260} units of the DNP-modified large fragment were reassociated into DNP-5 S RNA* by slow (1.5 h) cooling in 0.6 ml of buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 200 mM KCl from 60 to 0°C, followed by ethanol precipitation [6]. DNP-5 S RNA* and its complexes with anti-DNP were analyzed by polyacrylamide gel electrophoresis in a buffer with 40 mM Tris, 10 mM acetate, 1 mM EDTA, pH 7.3. The 5 S RNA A→B form interconversion was carried out according to [7].

50 S ribosomal subunits were reconstituted from DNP-5 S RNA*, 23 S RNA and total 50 S subunit protein (TP50) basically as in [8] with 23 S RNA isolated from 70 S ribosomes as recommended in [9].

Reconstituted 50 S subunits were purified as described [10]. The preparation of antibodies specific to DNP-derivatives (anti-DNP) and selection of the optimal antibody:ribosome ratio were performed as described in [5] and [11], respectively. Incubation of 50 S subunits with anti-DNP was carried out for 1.5 h at 0°C in a buffer with 10 mM Tris-HCl (pH 7.3), 5 mM $Mg(CH_3COO)_2$ and 100 mM NH_4Cl. 50 S·IgG·50 S 'dimers' were isolated by sucrose gradient centrifugation (5–20%) in the same buffer using a Beckman SW-41 rotor (21000 rpm, 13 h, 3°C). Electron microscopy was performed as described in [11].

3. RESULTS

The conditions of free 5 S RNA partial digestion with nuclease S1 have been found that result in the appearance of the two major products of hydrolysis. Both isolated fragments have electrophoretic mobilities on 15% polyacrylamide/7 M urea gel similar to those of 5 S RNA fragments 1–41 and 42–120 produced by ribonuclease T_1 partial digestion (fig.1). This correlates with the published data that primary nuclease S1 breaks are located in the hairpin loop involving residues C_{35} to C_{47} [12]. Both fragments turn out to be nonhomogeneous. As has been shown on sequencing gel, the preparation of the large fragment contains two polynucleotide chains comprising residues A_{39}–U_{120} and residues U_{40}–U_{120} in equal amounts (not shown). The average length of the small fragment has been estimated as 37–39 nucleotide residues.

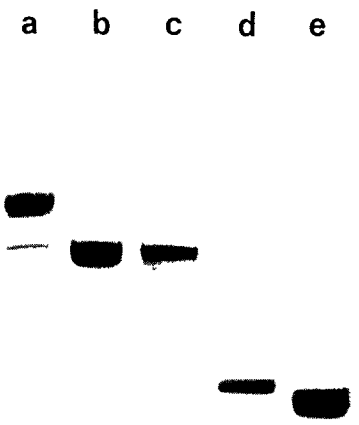

Fig.1. Analysis of RNA by 15% polyacrylamide/7 M urea gel electrophoresis: (a) 5 S RNA, (b and e, respectively) large and small fragments produced by nuclease S1 partial digestion of 5 S RNA, (c and d, respectively) 5 S RNA fragments 42–120 and 1–41 produced by ribonuclease T_1 partial digestion.

The product quantitatively formed after annealing the mixture of small and large 5 S RNA fragments in the presence of 10 mM Mg^{2+} has an electrophoretic mobility equal to that of the 5 S RNA A-form under nondenaturing conditions. An identical product is formed after annealing the mixture of small and DNP-modified large fragments, part of the reassociated DNP-5 S RNA* having been aggregated in this case (fig.2a).

To estimate the extent of the large fragment modification by DNP-ethylenediamine, the reassociated DNP-5 S RNA* was incubated with anti-DNP and the reaction mixture was analyzed by polyacrylamide gel electrophoresis under nondenaturing conditions (fig.2d–g). The specificity of the immunocomplexes thus obtained has been demonstrated by the complete inhibition of their formation when free hapten is added to the reaction mixture (fig.2e). As seen from fig.2f and g the extent of 5 S RNA modification in the DNP-5 S RNA* preparation exceeds 80%.

The subunits reconstituted from 23 S RNA, TP50 and a 2-fold molar excess of DNP-5 S RNA*

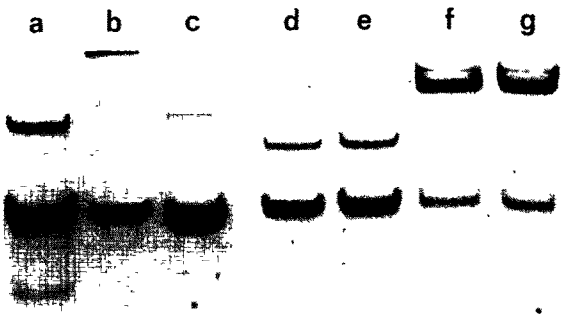

Fig.2. 7.5% polyacrylamide gel electrophoresis under nondenaturing conditions: (a and d) DNP-5 S RNA*, (b) 5 S RNA A-form, (c) 5 S RNA B-form, (e) 2.5 μg DNP-5 S RNA* + 30 μg anti-DNP + DNP-glycine (5 mM final concentration), (f) 2.5 μg DNP-5 S RNA* + 30 μg anti-DNP, (g) 2.5 μg DNP-5 S RNA + 70 μg anti-DNP.

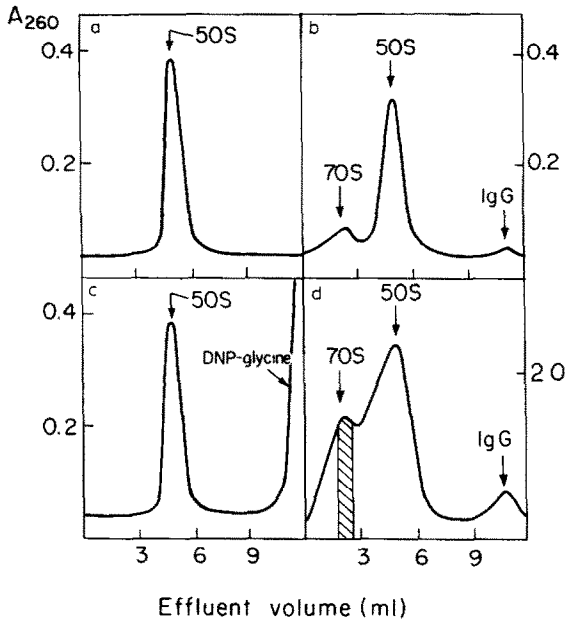

Fig.3. Sedimentation of reconstituted 50 S subunits with DNP-modified DNP-5 S RNA* treated with anti-DNP in a 5–20% sucrose gradient. (a) Modified 50 S subunits (1 A_{260} unit) in the absence of antibodies, (b) with 35 μg anti-DNP, (c) with 35 μg anti-DNP + DNP-glycine (10 mM final concentration), (d) large-scale preparation of dimers: 10 A_{260} units of DNP-modified 50 S subunits + 350 μg anti-DNP; the shaded region indicates fractions used in electron microscopy analysis.

were purified by sucrose gradient centrifugation. The purified subunits gave a symmetrical homogeneous peak in the sucrose gradient, their sedimentation pattern being similar to that of the control 50 S subunits (fig.3a).

To prove that DNP-5 S RNA* had been integrated into the 50 S subunits during reconstitution, the total RNA was isolated from them under mild conditions (according to [2]) and analyzed by polyacrylamide gel electrophoresis. Electrophoresis under nondenaturing conditions demonstrated the presence of 5 S RNA in the sample (not shown). Electrophoresis in 7 M urea revealed the presence of large and small 5 S RNA fragments in the same preparation, the intact 5 S RNA being absent (not shown).

The sedimentation pattern of modified 50 S subunits incubated with anti-DNP is shown in fig.3b. The 50 S·IgG·50 S complexes appear as a peak with a mean of ~70 S. The formation of 50 S subunit dimers is specific, since the incubation of modified 50 S subunits with anti-DNP in the presence of free hapten does not produce the dimer peak and the sedimentation pattern becomes similar to that of the control subunits (fig.3c). For large scale isolation of the 50 S·IgG·50 S complexes the concentrations of components were elevated 10-fold. This resulted in an increase of the relative amount of the dimer fraction (fig.3d).

A general view of the preparation from the dimer fraction is shown in fig.4a. The preparation contains a considerable amount of the 50 S·IgG·50 S dimers. Electron microscopy images of both the single reconstituted 50 S subunits and those in the 50 S·IgG·50 S complexes did not differ from often-described and well-known images of the intact 50 S subunits [13–15].

A gallery of the 50 S·IgG·50 S dimers and single 50 S·IgG complexes is presented in fig.4b. Here the 50 S subunits are in one of the two main

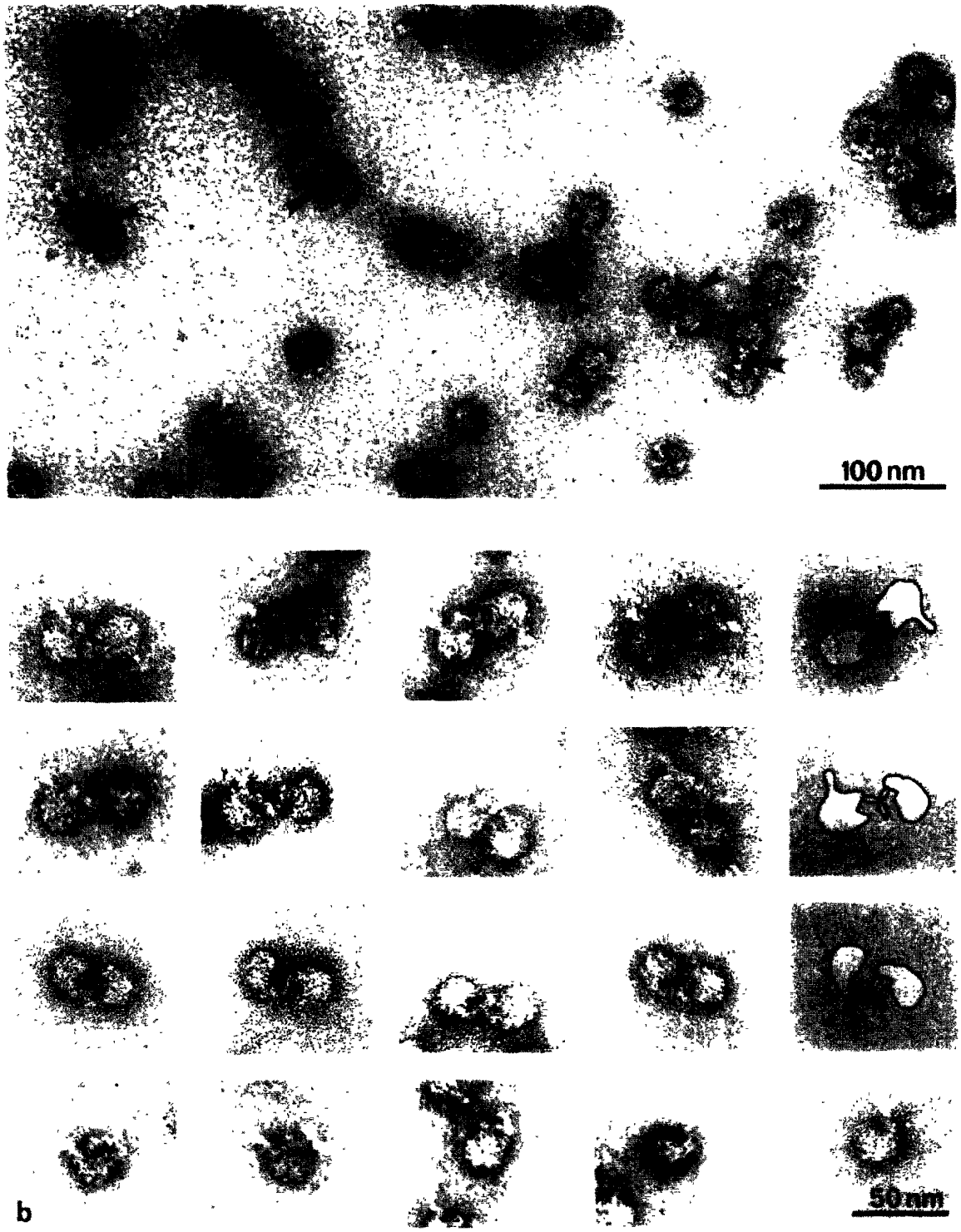

Fig.4. Electron micrographs of the 50 S subunits reconstituted from DNP-5 S RNA*, 23 S RNA and TP50 after reaction with anti-DNP. (a) General view of the preparation from the dimer fraction (fig.3d); arrowheads indicate antibodies in 50 S · IgG · 50 S complexes. (b) Large ribosomal subunits linked with anti-DNP in characteristic projections schematically shown in the right frames. The bottom row shows single subunits with attached antibody molecules.

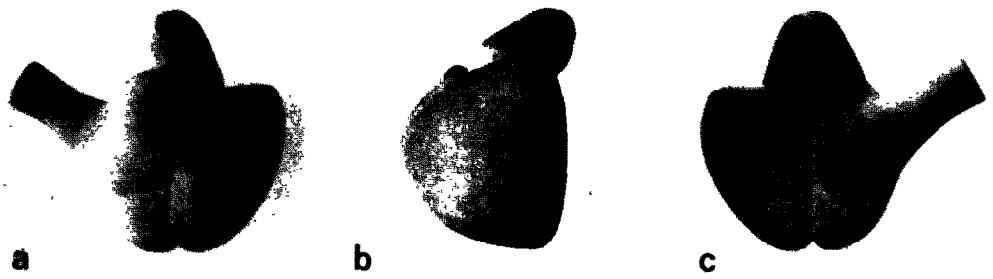

Fig.5. Location of the 5 S RNA residues A_{39} and U_{40} on the 50 S subunit. The position of the residues is denoted by filled circles on the (a) exterior, (b) lateral and (c) interface sides of the 50 S subunit model [15]. The shaded circles indicate the position of the 5 S RNA 3′-end [1].

projections: the crown or the kidney ones. Analysis of 80 such complexes showed that, firstly, the 50 S subunit is always bound with only one antibody molecule, as in all cases we observed (review [16]); secondly, the binding site is always situated on a unique region of the subunit surface. In the crown projection, the antibody binding site is observed on the central protuberance at approx. one-third down from its top, and displaced from the middle towards the lateral L1-protuberance. In the lateral or kidney projection the binding site is observed on the interface side of the central protuberance.

Our interpretation of the micrographs of the immunocomplexes is presented in fig.5. The position of the 5 S RNA residues A_{39} and U_{40} is denoted by filled circles on the exterior (a), lateral (b) and interface (c) sides of the 50 S subunit model [15]. The position of the 5 S RNA 3′-end mapped by us earlier [1] is shown by shaded circles for comparison. Thus, the 3′-end and the residues A_{39} and U_{40} are located on the opposite (exterior and interface) sides of the central protuberance, i.e., at sites which are distinctly apart. At the same time both these sites are situated at the upper one-third of the central protuberance at a distance of approx. 50 Å from each other.

4. DISCUSSION

In previous studies, immune electron microscopy has been applied for localization of terminal nucleotide residues both of rRNA and synthetic mRNA on the surface of ribosomal subunits [16]. Here, for the first time, we used this approach for mapping an internal site of rRNA. For this purpose we prepared 5 S RNA (DNP-5 S RNA*) which contained either a single break (between residues C_{38} and A_{39} or A_{39} and U_{40}) or a gap of 1–2 nucleotide residues in this particular region and a DNP-hapten group attached to the 5′-phosphate group of A_{39} or U_{40} resulting from the breaks. This modified single-stranded region of 5 S RNA does not seem to be responsible both for incorporation of 5 S RNA into the 50 S subunit and its function as demonstrated by chemical modification [17] and partial enzymatic digestion (see [18]). In particular, it was shown that ribosomes reconstituted with the 5 S RNA carrying a deletion of C_{42} to A_{46} or C_{42} to A_{52} retain their ability to translate phage MS2 RNA [19].

In agreement with these results we have found that DNP-5 S RNA* has the same electrophoretic mobility as the native 5 S RNA A-form. It can be readily incorporated into the 50 S subunit. Furthermore, the 50 S subunits with DNP-5 S RNA* form very stable complexes with anti-DNP, the antibody being attached to a single point of the ribosomal surface. All these data provide a strong basis to suggest the correct position of DNP-5 S RNA* in the 50 S subunits.

The 5 S RNA region near the hairpin loop involving residues C_{34}–C_{47} was found to be essential for binding of protein L18, one of the 3 known 5 S RNA-binding proteins [6,20]. Recently L18 has been mapped near the top of the 50 S subunit central protuberance, on its interface side [21]. This is in very good agreement with the location site of the 5 S RNA residues A_{39}/U_{40} identified here.

Two main conclusions can be drawn from the

results of this study. Firstly, the 5 S RNA molecule is located near the other functional sites of a ribosome, indicating the possibility of its direct involvement in ribosomal functioning. Secondly, the 5 S RNA has, in situ, a quite compact folded conformation. Indeed, we have found that the distance between the 5 S RNA 3′-end and residues A_{39}/U_{40} does not exceed 50 Å, whereas one can see from the secondary structure model of *E. coli* 5 S RNA (see [22]) that these two points are separated by 3 double-helical regions and in an extended form of 5 S RNA they should be at least 120 Å apart. Other data [23–25] also suggest the possible folding of 5 S RNA, at least in its free state.

ACKNOWLEDGEMENTS

We are grateful to Professor A.S. Spirin for valuable discussions, to Dr G.G. Dikhanov for kindly providing us with the DNP-specific antiserum and to Dr A.S. Mankin for advice in RNA sequencing.

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