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Ribosome structure: Localization of 3′ end of RNA in small subunit by immunoelectronmicroscopy

(ribosomal RNA/ribosome topography/RNA modification)

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ABSTRACT **The 3′ end of the RNA in the 30S ribosomal subunit of *Escherichia coli* has been modified by oxidation with sodium periodate and conjugation with the (mono) dinitrophenyl derivative of ethylenediamine. Antibodies, induced with dinitrophenyl-bovine serum albumin, interact with the modified ribosomal subunits. Electron micrographs of negatively stained antibody–subunit complexes show individual ribosomal subunits to which a single antibody molecule is bound and subunit dimers cross-linked by an IgG molecule. The modified 3′ terminus has been localized to a single site on the upper portion of the platform region of the 30S subunit. This location is consistent with earlier placements of proteins that react with the 3′ end of the RNA.**

The small subunit of the *Escherichia coli* ribosome is composed of 21 different proteins and a single molecule of RNA. The structure of each of the proteins is under study (reviewed in refs. 1–3), and the sequence of the RNA has been determined in two laboratories (4, 5). Much effort has also been devoted to the determination of the overall structure of the particle and to the definition of the physical and functional relationships of its components (see refs. 1 and 2). Immunoelectromicroscopy—the visualization in electron micrographs of antibody-linked biological structures—has been of particular value in the localization of the individual proteins of each ribosomal subunit (6–12) and in the identification of a ribosomal neighborhood involved in the initiation of protein synthesis (7, 8). Immunoelectronmicroscopy has also proven useful in localizing specific nucleotides; it has been used to map the N^6-dimethyladenosine of the 30S ribosomal subunit (13) and to determine the site of adenylylation of *E. coli* glutamine synthetase (14).

In this paper we are concerned with the localization of the 3′ end of the 16S ribosomal RNA in the small ribosomal subunit. This segment of the RNA is functionally important in the recognition and binding of mRNA for the initiation of protein synthesis (15, 16). Its localization has thus far been implied; it has been chemically crosslinked to ribosomal proteins (17, 18) and initiation factor IF-3 (19), which in turn have been localized directly (7, 8, 12) or after crosslinking to other ribosomal proteins (20). Our approach is more direct. The 3′ end of the RNA (in the ribosomal subunit) was oxidized with sodium periodate, and the resulting dialdehyde was conjugated with a dinitrophenol derivative. Antidinitrophenyl (anti-DNP) antibodies were then used to localize the DNP-modified terminus in electron micrographs of subunit–antibody complexes. Preliminary reports of this work have been presented (21, 22).

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MATERIALS AND METHODS

Methods used in the production of DNP-bovine serum albumin (23) and in immunization, blood collection, and antibody characterization (24) have been described. A membrane filter assay (13, 14) was used to measure antibody binding of ε-[3,5-^{3}H]DNP-lysine (Amersham). Ribosomes and ribosomal subunits were isolated from *E. coli* strain Q13 as described (13, 25).

The (mono) dinitrophenyl derivative of ethylenediamine was prepared as follows: 4.8 μmol of 1-fluoro-2,4-dinitro[^{3}H]benzene (Amersham, 200 mCi/mmol; 1Ci = 3.7×10^{10} becquerels) was dissolved in 100 μl of ethanol. One hundred microliters of $NaHCO_3$ (0.37 M, pH 8.8) and 390 μmol of ethylenediamine in 150 μl of H_2O (pH 8.4) were added, and the mixture was shaken for 11 hr at 37°C in the dark and then dried by rotary evaporation. The solid was dispersed in butanol (300 μl) plus NH_4OH (40 μl), and the product was isolated by repeated extraction with 100-μl portions of butanol and subsequent electrophoresis on Whatman no. 1 paper in 7% formic acid, pH 1.9. The product was eluted with 0.01 M HCl, dried by rotary evaporation, and further purified by gel filtration on a 1.2 × 30 cm column of Sephadex G-10. The product (3.5 μmol) was diluted with nonradioactive DNP-ethylenediamine (Calbiochem) as appropriate.

Purified 30S ribosomal units (1.2 nmol) were dissolved in 250 μl of cold buffer (10 mM Hepes/40 mM NaCl/0.1 mM EDTA/3 mM magnesium acetate at pH 7.35) containing 590 nmol of [^{3}H]DNP-ethylenediamine (*ca.* 10 mCi/mmol). Cold 0.1 M sodium periodate (25 μl) was added, and the mixture was incubated at 0°C in the dark for 15 min. Then 30 μl of 0.1 M sodium borohydride (in 0.01 M NaOH) was added, and incubation was continued for an additional 10 min. As a control, mock-modified ribosomal subunits were treated in the same way, except the sodium periodate was replaced by an equivalent volume of buffer.

DNP-modified ribosomal subunits were freed of excess reagents by precipitation with ethanol (26), followed by sedimentation in 5–20% sucrose gradients (Spinco SW27 rotor, 26,000 rpm for 10.5 hr) in buffer II (10 mM Tris·HCl/30 mM NH_4Cl/6 mM 2-mercaptoethanol/1 mM magnesium acetate at pH 7.5). Subunits were concentrated from gradient fractions by ethanol/$MgCl_2$ precipitation and stored −30°C in buffer I (10 mM Tris· HCl/30 mM NH_4Cl/6 mM 2-mercaptoethanol/10 mM magnesium acetate at pH 7.5).

Ribosomal proteins were extracted with acetic acid and analyzed by polyacrylamide gel electrophoresis by using a variation of the first dimension in the system described by Howard and Traut (27). RNA was isolated by phenol extraction (25) and hydrolyzed with 1% (wt/wt) pancreatic ribonuclease (Worth-

Abbreviation: DNP, 2,4-dinitrophenyl.

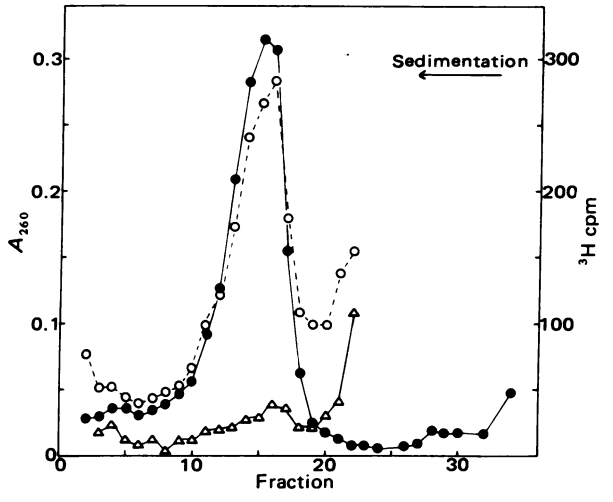

FIG. 1. Sedimentation of DNP-modified ribosomal subunits. Subunits were oxidized with sodium periodate (or mock-oxidized), conjugated with [^{3}H]DNP-ethylenediamine, and precipitated with ethanol prior to centrifugation. Samples (1 ml) were sedimented through 34-ml gradients (5–20% sucrose) in buffer II (Spinco SW 27.1 rotor, 26,000 rpm, 10.5 hr). Measurements of absorbance of DNP-modified subunits (●), ^{3}H content of modified subunits (○), and ^{3}H content of mock-modified subunits (△) were made on 1:33 dilutions of gradient fractions.

ington) for 30 min at 37°C in 20 μl of 0.1 M imidazole·HCl, pH 7.0. Hydrolysis products were separated by thin-layer chromatography on Eastman 13255 cellulose sheets by using *n*-propanol/NH_3/H_2O (6:3:1, vol/vol).

For electron microscopy, *ca.* 50–70 pmol of modified or mock-modified subunits were incubated with 0.5–4 equivalents of anti-DNP antibodies in a total volume of 40–80 μl of buffer (buffer I supplemented with NH_4Cl to a total level of 200 mM). Excess globulins were removed by Sepharose 6B chromatography (13). Samples were prepared for microscopy by negative staining with 1% uranyl acetate by using the method of Valentine *et al.* (28) as modified by Lake and Kahan (7). Electron micrographs were obtained with a JEOL 100B microscope at 80 kV and a magnification of 66,000.

RESULTS

Antibody Production and Characterization. Anti-DNP antibodies were induced in rabbits by injection of a bovine albumin–DNP complex, and an IgG fraction was characterized by measurement of inhibition of ε-[3,5-^{3}H]DNP lysine binding in a membrane filter assay. Inhibition of radioligand binding by nonradioactive dinitrophenol, ε-DNP lysine, or DNP derivatives of ethylenediamine or hexanediamine was seen at 0.01–10 μM. The midpoint of the inhibition curve for DNP-ethylenediamine, the derivative used in ribosome labeling, was at less than 1 μM. This indicates that formation of an acceptably stable complex of antibody with DNP-modified ribosomal subunits, suitable for immunoelectronmicroscopy, is possible.

Modification of the Ribosomal RNA. Small (30S) ribosomal subunits were oxidized with sodium periodate and incubated with large excess of [^{3}H]DNP-ethylenediamine, and the addition product was stabilized by reduction with sodium borohydride. As a control, an equivalent quantity of nonoxidized subunits were mock-modified with the DNP derivative and borohydride. Each subunit preparation was purified by ethanol precipitation and sedimentation in sucrose gradients as shown in Fig. 1. In the preparations used for electron microscopy the amount of DNP derivative associated with oxidized subunits

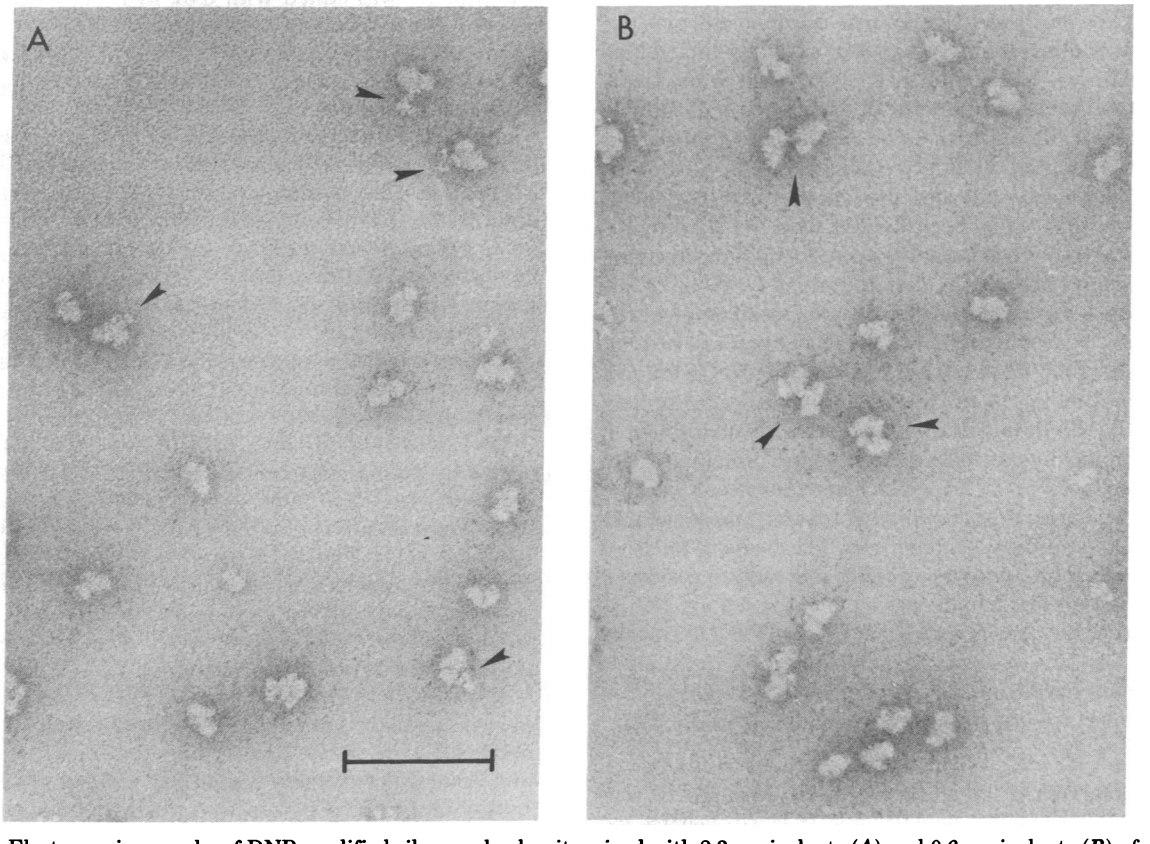

FIG. 2. Electron micrographs of DNP-modified ribosomal subunits mixed with 2.3 equivalents (*A*) and 0.6 equivalents (*B*) of anti-DNP antibodies. Subunit–antibody complexes are indicated by arrows. Bar length: 1000 Å.

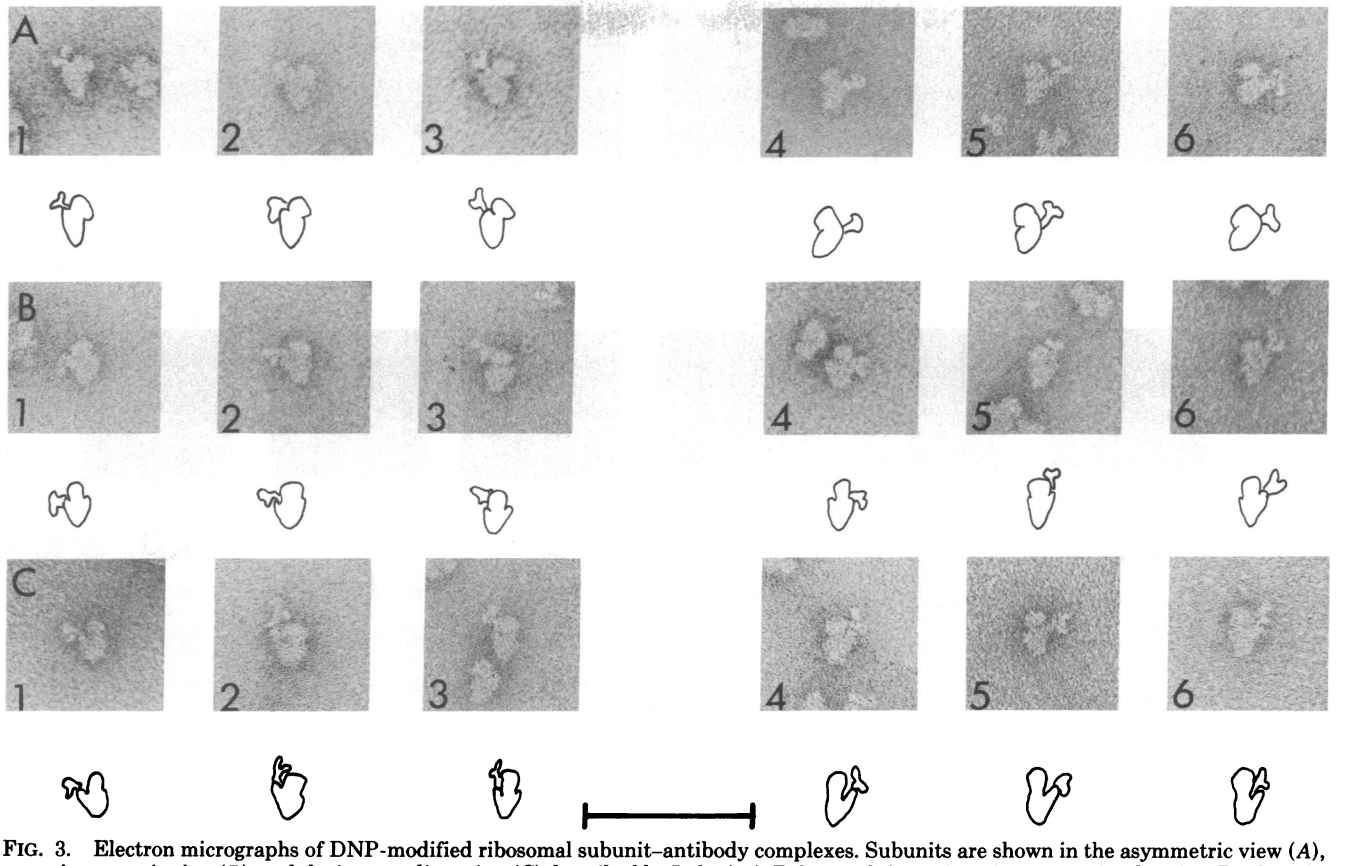

FIG. 3. Electron micrographs of DNP-modified ribosomal subunit–antibody complexes. Subunits are shown in the asymmetric view (*A*), the quasisymmetric view (*B*), and the intermediate view (*C*) described by Lake (29). Below each frame is an interpretative drawing. Bar length: 1000 Å.

ranged from 0.7 to 1.2 mol per mol of ribosome, whereas DNP derivative associated with mock-modified subunits was <0.15 mol per mol of ribosome. Incorporation of DNP derivative was not significantly affected if the magnesium ion concentration of the reaction mixture was varied in the range 1–10 mM, but a 1:10 reduction in the concentration of the DNP derivative resulted in a barely detectable level of incorporation.

[^{3}H]DNP-modified ribosomal subunits were dissociated into protein and RNA fractions. No measurable amount of radioactivity was associated with any ribosomal protein band upon one-dimensional polyacrylamide gel electrophoresis. In contrast, ^{3}H from the DNP derivative was clearly associated with the ribosomal RNA fraction. RNA from DNP-modified subunits was treated with pancreatic RNase, and the hydrolysis products were fractionated by thin-layer chromatography. Tritium was found quantitatively (at least 85%) associated with material migrating as the DNP-adenosine derivative expected from hydrolysis of the 3′-terminal —CpUpUpA sequence of the RNA (4, 5). No significant radioactivity was found in areas of the chromatogram corresponding to the migration of nucleotides (<4%) or uncomplexed DNP-ethylenediamine (<4%).

Electron Microscopy. Chemical modification of the ribosomal subunits did not alter their appearance in electron micrographs of negatively stained preparations. Mock-modified subunits and DNP-modified subunits were indistinguishable from each other and appeared typical of ribosome structures previously observed by these techniques. In each instance, characteristic images representing different orientations of the particle about its long axis were identified and related to the model of the ribosomal subunit proposed by Lake (29).

DNP-modified (or an equivalent quantity of mock-modified) subunits were incubated with 0.5–4 equivalents of antibodies, as estimated from DNP-lysine binding capacity. Excess globulins were removed by gel filtration, and antibody–subunit complexes were examined by electron microscopy. Mock-modified subunits were not seen bound with antibody in our micrographs. In contrast, complexes of antibodies with DNP-modified ribosomal subunits were readily observed. Examples are presented in Fig. 2. When an excess of antibody was present, ribosomal subunits were frequently seen to bind a single IgG molecule (Fig. 2*A*). When antibody levels were lower, pairs of subunits crosslinked by a single IgG could be identified (Fig. 2*B*). The location of the DNP-modified 3′ end of the RNA was approximated from the apparent point of contact between antibody and ribosomal subunit in each of several orientations of the subunit about its long axis. About 125 micrographs were examined, and 350 antibody–ribosome complexes were evaluated. At least 88% of the interpreted images were consistent with the localization described below.

Fig. 3 shows a gallery of individual antibody–subunit complexes in the major views described by Lake (29). Subunits in the asymmetric view, identified by a concave and a convex side, inevitably showed antibody attachment at the convex side regardless of the orientation of the subunit (Fig. 3*A*; contrast panels 1–3 with panels 4–6). In some cases, the antibody may be partially hidden by the subunit (e.g., panels 1 and 2). Antibody attachment to subunits in the quasisymmetric orientation was at or slightly above the partition separating the upper and lower parts of the subunit (Fig. 3*B*). Antibody was frequently partially obscured by the ribosome in these views (e.g., panels 1, 3, 4, and 5).

We interpret the site of antibody attachment to be directly

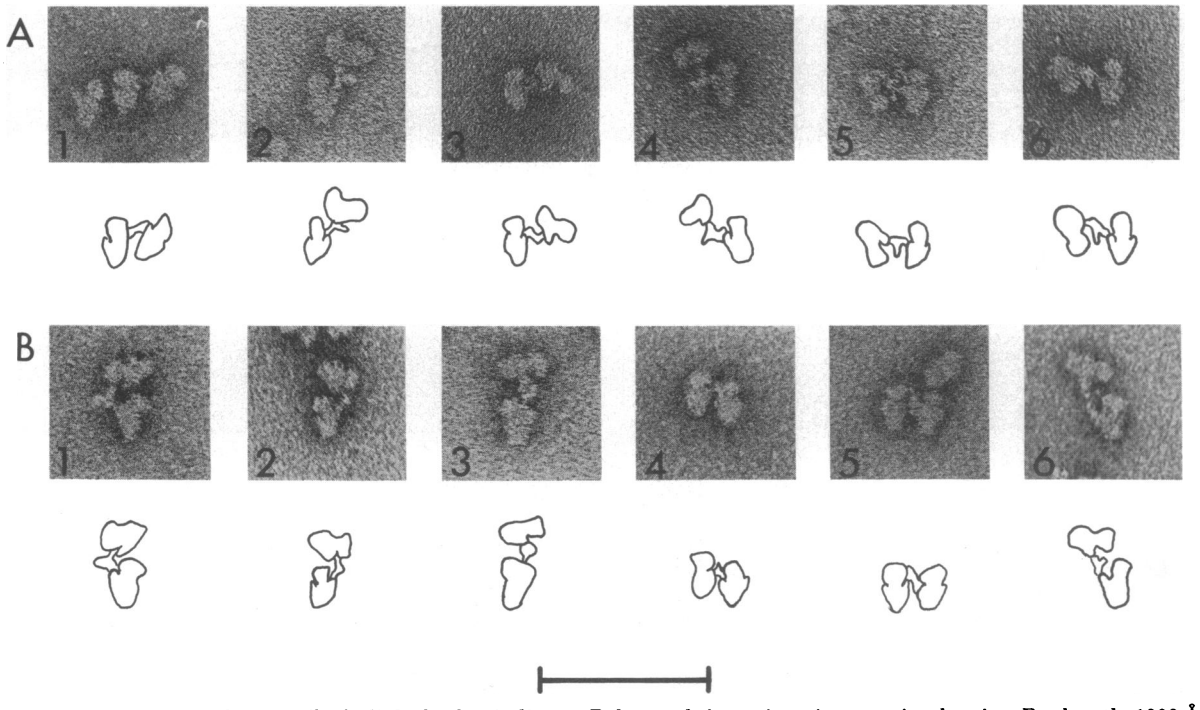

FIG. 4. Electron micrographs of antibody-linked subunit dimers. Below each frame is an interpretive drawing. Bar length: 1000 Å.

behind (or in front of) the subunit in this orientation (see Fig. 5). Hence, the unobscured part of the IgG molecule could lie to either side of the particle (Fig. 3*B*, panels 1–3 compared to panels 4–6), and the actual contact point could be hidden. Subunits in the intermediate view show a cleft that separates a platform from the body of the ribosome; antibody attachment to these particles was always to the subunit platform, at or near the end of the structure (Fig. 3*C*). The resolution obtained in these micrographs is insufficient to clearly localize the binding site to either the inner or outer surface of the platform.

Fig. 4 presents a gallery of antibody-linked subunit dimers in which each of the subunit orientations described above can be seen. The site of antibody binding is to the convex surface of subunits in the asymmetric view (row A, panels 2, 4, and 6; row B, panels 1, 2, and 3) or to the platform in the intermediate view (row A, panels 4 and 5; row B, panels 3 and 6). All of our observations are consistent with a single binding site located on the upper part of the platform of the 30S ribosomal subunit. Our interpretation is shown in Fig. 5.

DISCUSSION

The periodate sensitivity of the 3′-terminal nucleoside of RNA has frequently been exploited in probes of RNA structure. Oxidation with periodate and amine-catalyzed base elimination is the basis of a stepwise method of RNA sequence analysis (30–32). The 3′ end of oxidized RNA has been chemically labeled with a radioactive marker, either through reduction with [^{3}H]borohydride (33) or by labeling with, for example, hydrazine derivatives (34–36) or dimedone (37). Within the 30S subunit, the 16S ribosomal RNA is also susceptible to oxidation with periodate. Oxidized RNA has previously been crosslinked to ribosomal proteins S1 and S21 (17, 18) and to initiation factor IF3 (19). Recently, Schreiber *et al.* (36) have coupled a fluorescent semicarbazide to the 16S RNA within the ribosomal subunit. Thus, it seems reasonable that the oxidation and coupling procedures used in this work should result in the specific covalent modification of the 3′ terminus of the ribosomal RNA. Our evidence indicates that this is the case; radioactive DNP derivative is bound to RNA but not protein. In addition, the derivative remains bound to the expected 3′-terminal nucleoside, the only nucleoside released upon RNase hydrolysis of the RNA. Binding at internal nucleotides or at new ends created by possible nucleolytic cleavage of the RNA is improbable because DNP is not found associated with any phosphate-containing materials and because most nucleases would leave a 3′-phosphate end upon cleavage. Moreover, the single characteristic localization of the modified terminus seen in our micrographs argues against significant secondary modes of binding of the DNP derivative.

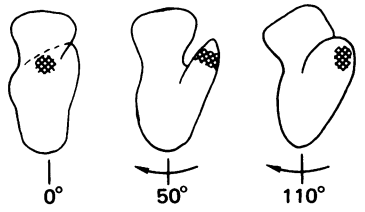

FIG. 5. Localization of the 3′ end of RNA in DNP-modified 30S ribosomal subunits. The shaded area in the Lake (29) model indicates the binding site of anti-DNP immunoglobulins.

We interpret our micrographs to indicate a unique localization of the 3′ end of the RNA on the upper portion of the subunit platform (Fig. 5). This placement is consistent with several earlier observations. Ribosomal proteins S1 and S21, which have been crosslinked to the 3′ end of the RNA (17, 18), are localized near each other and near the end of the platform (8). Initiation factor IF3, to which the oxidized 3′ end of the RNA has also been linked (19), has been itself crosslinked to ribosomal proteins S1, S11, S12, S13, S19, and S21 (20); antigenic determinants of each of these proteins have been localized either on the subunit platform or on nearby parts of the upper portion of the subunit (7, 8). Finally, the modified nucleoside N^6-dimethyladenosine, 24 and 25 residues from the 3′ end of the RNA, has been localized on the subunit platform (13). The mRNA-complementary sequence of the 16S RNA occurs between the dimethyladenosine residues and the 3′ end (15). Our observations thus support the suggestion that the RNA and

proteins of this section of the subunit comprise a ribosomal neighborhood involved in the initiation of protein synthesis (1, 7, 8, 20).

Our micrographs can also be interpreted with respect to the subunit model proposed by Vasiliev (38); the 3′ end of the ribosomal RNA would be placed near the point of the triangular body of the 30S particle. The model of the 30S subunit proposed by Tischendorf *et al.* (10–12) is more symmetric and lacks the platform characteristic of the Lake model. Nevertheless, these workers also localized antigenic determinants of proteins S1 and S21 to nearby sites on the upper part of the structure (summarized in refs. 1 and 2). In our micrographs, interpretation of those views most compatible with the Tischendorf *et al.* model places the 3′ terminus near the site reported for protein S21 (11).

We believe our data are sufficient to localize the 3′ end of the RNA in this ribosomal subunit. It is likely that the same experimental approach should be applicable to localization of the 3′ end of the RNA in both small and large ribosomal subunits from other sources. In a more general context, immunoelectronmicroscropic localization of sites of specific chemical modification of macromolecular complexes should be widely applicable.

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