Ribosome Structure

LOCALIZATION OF 7-METHYLGUANOSINE IN THE SMALL SUBUNITS OF ESCHERICHIA COLI AND CHLOROPLAST RIBOSOMES BY IMMUNOELECTRON MICROSCOPY*

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The minor nucleoside 7-methylguanosine occurs in Escherichia coli 16 S ribosomal RNA at a single site. High pressure liquid chromatographic analysis shows that a single residue of 7-methylguanosine is also present in chloroplast 16 S ribosomal RNA, presumably at an analogous position in the sequence. Antibodies to 7-methylguanosine were induced in rabbits and shown to be highly specific for the intact methylated base. These antibodies were reacted with 30 S ribosomal subunits from E. coli and from the chloroplasts of Alaskan peas. These two types of ribosome have been shown to be topographically similar (Trempe, M. R., and Glitz, D. G. (1981) J. Biol. Chem. 256, 11873-11879). Electron microscopy of the subunit-antibody complexes showed similar subunit-IgG monomers and antibody-linked subunit dimers. In greater than 95% of the complexes observed for each type of ribosome, antibody contact was consistent with a single binding site, which places 7-methylguanosine near the junction of the upper one-third and lower two-thirds of the subunit and maximally distant from the platform. The analogous localization in both E. coli and chloroplast 30 S ribosomal subunits lends support to their proposed common evolutionary origin.

All ribosomes are composed of two unlike subunits, both of which contain RNA and protein. In the attempt to define the functional relationships between these components, much work has been devoted to the characterization of the individual proteins and RNA molecules, the determination of their specific functional roles, and the distribution of these components on structural models of the ribosome (for reviews, see Refs. 1-4).

One of the major techniques used in these studies has been immuno-electron microscopy—the visualization in electron micrographs of antibody-linked biological structures. It has been used to map the distribution of ribosomal proteins (summarized in Refs. 5 and 6), to map functional domains such as the ribosomal neighborhoods involved in purumycin (6-9) and tRNA (10) binding, and to investigate the structure of ribosomal RNA (11-13), most frequently in the Escherichia coli ribosome.

Interestingly, the ribosomes from the chloroplasts of green plants are strikingly similar to those of E. coli. The bacterial and chloroplast ribosomes are similar in size, contain similar numbers of proteins and comparable RNA species (23 S, 16 S, and 5 S), and have related mechanisms of translation and responses to antibiotics (for reviews, see Refs. 14 and 15). There is strong homology between the sequences of E. coli 16 S and 23 S ribosomal RNAs and the DNA sequences coding for the corresponding ribosomal RNAs of Zea mays chloroplasts (16, 17). Moreover, electron micrographs of 30 S and 50 S ribosomal subunits from the chloroplasts of Alaskan peas show that their overall topography and localization of N7,N7-dimethyladenosine (18) are very similar to that seen with E. coli ribosomes (11, 19). These facts have been used to argue for the theory that chloroplasts are descended from prokaryotes (particularly cyanobacteria) that became symbiotically associated with eukaryotic cells (20).

In this paper, another minor nucleoside, 7-methylguanosine, has been localized in both E. coli and chloroplast 30 S ribosomal subunits by immuno-electron microscopy. This serves as a probe of the distribution of RNA within the ribosome, provides an additional structural detail for comparison of the two types of ribosome, and extends the molecular evidence for the evolutionary relationship of bacteria and chloroplasts.

MATERIALS AND METHODS

Ribosome and Ribosomal Subunit Preparation—E. coli strain Q13 was cultured in Difco antibiotic No. 3 medium (for unlabeled cells) or the low phosphate medium of Landy et al. (21) supplemented with up to 10 μCi/liter of 32P as orthophosphate (ICN Pharmaceuticals). Cells were harvested in late log phase and ribosomes were isolated as described by Traub et al. (22). Ribosomal subunits were separated by sedimentation for 12 h at 23,500 rpm in a Spinco SW 28 rotor through linear 10-30% (w/v) gradients of sucrose (Schwarz/Mann, ribonuclease-free) in 30 mM NH4Cl, 1 mM Mg-acetate, 6 mM 2-mercaptoethanol, 10 mM Tris, pH 7.5. Subunits were concentrated from gradient fractions by centrifugation for 3 h at 200,000 × g (60,000 rpm in a Beckman Type 65 rotor) and resuspended for immediate use in buffer A (150 mM NH4Cl, 10 mM Mg-acetate, 10 mM Tris, pH 7.5, 6 mM 2-mercaptoethanol) or stored frozen as pellets at −70 °C.

Chloroplasts and chloroplast ribosomes were prepared from Alaskan pea seedlings as previously described (18). Chloroplast ribosomal subunits were separated as above except that the sucrose gradients were prepared with 50 mM NH4Cl, 0.5 mM Mg-acetate, 10 mM Tricine, pH 7.5, 6 mM 2-mercaptoethanol. Purified subunits were stored frozen as pellets at −70 °C, or reconstituted for immediate use in buffer B (150 mM NH4Cl, 2 mM Mg-acetate, 10 mM Tricine, pH 7.5, 6 mM 2-mercaptoethanol).

Quantitation of 7-Methylguanosine in 16 S Ribosomal RNA—RNA was isolated from approximately 0.5 mg of purified 30 S subunits by phenol extraction (22). The RNA was hydrolyzed to nucleosides with snake venom phosphodiesterase, bacterial alkaline phosphatase ( Worthington), bovine pancreatic ribonuclease, and ribonuclease T1 (Sigma) (23). The resulting nucleoside mixture was analyzed using
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FIG. 1. Immunodiffusion of antiserum. Antiserum was placed in the center well. All conjugates were at 1.0 mg/ml.

FIG. 2. Effect of inhibitors on [3H]m7Guo binding by antibodies. Reaction mixtures (250 μl) contained [3H]m7Guo trialcohol (50,000 cpm, 20 pmol), unlabeled nucleosides as indicated, and antibodies in 0.15 M NaCl, 0.01 M Tris, pH 7.2 buffer. Samples were incubated 30 min at 37 °C followed by 60 min at 0 °C and then rapidly passed through Millipore type HA (0.45 μm) filters. The sample tubes and filters were immediately washed with three 2-ml portions of cold buffer and 4H was measured. Inhibitors used were: C, m7Guo; ○, mGMP; ▲, ring-opened mGuo; Δ, Guo; ▲, GMP.

RESULTS

Antibody Production and Characterization

The m7Guo-albumin conjugate used for immunization was calculated to contain 14 mol of nucleoside per mol of albumin and exhibited the absorption spectrum characteristic of intact 7-methylguanosine (29). A qualitative determination of the antibody response induced with this immunogen was obtained by immunodiffusion analysis (Fig. 1). Both m7Guo-BSA and BSA are precipitated, indicating that some of the antibodies are carrier-specific. The strong precipitation of m7Guo-RSA, however, demonstrates that there are hapten-specific antibodies in the population. Precipitation of Guo-BSA is due to carrier-specific antibodies, since the Guo-BSA precipitin band does not cross the BSA band. Also, Guo-RSA is not precipitated.

A quantitative determination of antibody specificity was obtained in ligand binding assays: [3H]m7Guo trialcohol bind-
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enzymatically hydrolyzed to nucleosides. Fig. 3 shows the high pressure liquid chromatography trace obtained from 0.2 nmol of the hydrolyzed chloroplast RNA. The 7-methylguanosine peak occurs at 8.2 min and gives a value of 0.66 mol of m'Guo/mol of RNA. The comparable hydrolysate of E. coli 16 S ribosomal RNA gave a value of 0.7 mol of m'Guo/mol of RNA. Each result is somewhat lower than the expected value of 1 mol of m'Guo/mol of RNA, perhaps due to the slightly alkaline conditions used during extraction of the RNA from the ribosome under which the nucleoside is susceptible to ring hydrolysis (29).

Interaction of Antibodies with 30 S Ribosomal Subunits

Anti-nucleoside antibodies (or nonimmune rabbit IgG) were allowed to react with 32P-labeled E. coli 30 S ribosomal subunits and then precipitated by means of a second antibody, goat anti-rabbit γ-globulin. In control experiments, precipitation of active and inactivated (31, 32) subunits by anti-m'Ado IgG was highly dependent on the Mg2+ concentration and subunit conformation, in accord with previous results (30). In contrast, subunit precipitation with anti-m'Guo immunoglobulins was essentially independent of these factors; active and inactivated subunits were precipitated almost equally at 1–10 mM Mg2+ (approximately 10% of the 32P present, under conditions which minimized nonspecific precipitation but were suboptimal for subunit-antibody interactions). The quantity of subunits precipitated by anti-m'Guo IgG was approximately 75% of that seen in parallel experiments with anti-m'Ado immunoglobulins at 10 mM Mg2+ (30). It was concluded that a significant and specific subunit-anti-

Electron Microscopy of Ribosomal Subunit-Antibody Complexes

E. coli—Anti-m'Guo antibodies were reacted with E. coli 30 S ribosomal subunits. After partial purification by gel filtration, the subunit-antibody complexes were negatively contrasted and viewed by electron microscopy. Two types of complex were seen: 1) monomers, in which only one antigen-binding site of an IgG molecule had reacted with the m'Guo in the ribosomal subunit; and 2) antibody-linked subunit dimers, in which both antigen-binding sites of an IgG molecule were filled, resulting in an antibody bridge between two ribosomal subunits. In Fig. 4, both types of complex are illustrated: A shows a field of monomer structures and B shows antibody-linked dimers. In competition experiments to test the specificity of the interaction, antibodies were added to reaction mixtures containing ribosomal subunits and 5 × 10−3 M 7-methylguanosine (a 10-fold excess relative to 30 S subunits). Electron micrographs showed 33 antibody-subunit interactions in a total of 550 clearly identified small ribosomal subunits. A parallel reaction mixture, in which competing 7-methylguanosine was not included, showed 128 antibody-subunit interactions in a total of 550 well defined subunits. Hence, inclusion of 7-methylguanosine specifically eliminated approximately 75% of the complexes such as were scored in the localization experiments. Moreover, complex formation is not seen when nonimmune IgG is used in place of the anti-nucleoside antibodies.

![Fig. 4. Electron micrographs of E. coli 30S ribosomal subunits complexed with anti-7-methylguanosine immunoglobulins. A, subunit-antibody monomers. B, antibody-linked subunit dimers. Complexes are indicated by arrows. Bar length, 1000 Å.](image-url)
**FIG. 5.** Electron micrographs of *E. coli* 30 S ribosomal subunit-antibody monomer complexes. Subunits are shown in the intermediate (50°) view (Row A), the asymmetric (110°) view (Row B), and the quasisymmetric (0°) view (Row C) as described by Lake (19). Below each frame is an interpretive drawing. *Bar length*, 1000 Å.

**FIG. 6.** Electron micrographs of antibody-linked *E. coli* 30 S ribosomal subunit dimers. Below each frame is an interpretive drawing. *Bar length*, 1000 Å.
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By analysis of the apparent point of contact of antibody with the ribosomal subunit in each of the characteristic subunit orientations, the location of the 7-methylguanosine residue within the ribosome could be approximated. About 140 micrographs showing $5 \times 10^5$ E. coli small ribosomal subunits were examined, and 974 antibody-subunit interactions were clearly identified and evaluated. In 613 monomer structures, all except 23 (3.8%) were consistent with a single binding site. In antibody-linked subunit dimers, all except 5 (1.4%) of the 361 contact points were consistent with the same binding site as described below.

In Fig. 5, a gallery of E. coli 30 S ribosomal subunit-antibody monomer structures arranged in the characteristic subunit projections is shown. In the intermediate (50°) view (Row A), the antibody molecules are seen attached near the junction between the upper one-third and lower two-thirds of the subunit and directly across the subunit from the platform. This is seen in both orientations of the subunit. In the asymmetric (110°) view (Row B), the point of antibody contact is consistently seen on the concave side of the ribosomal subunit, regardless of subunit orientation with respect to the carbon film. In the quasisymmetric (0°) view (Row C), the site of antibody attachment to the subunit is near the partition between the upper and lower section of the particle. In this view, the antibody can be seen lying either to the left or the

**Fig. 7.** Localization of 7-methylguanosine in 30 S ribosomal subunits. The shaded areas indicate the binding site of anti-m'Guo antibodies to the E. coli 30 S ribosomal subunit (Row A) and to the chloroplast 30 S ribosomal subunit (Row B). Subunits are drawn in the quasisymmetric (0°), intermediate (50°), and asymmetric (110°) views as described by Lake (19). Rotation is clockwise about the long axis of the subunit.

**Fig. 8.** Electron micrographs of chloroplast 30 S ribosomal subunits complexed with anti-7-methylguanosine immunoglobulins. **A,** subunit-antibody monomers. **B,** antibody-linked subunit dimers. Complexes are indicated by arrows. Bar length, 1000 Å.
Fig. 9. Electron micrographs of chloroplast 30 S ribosomal subunit-antibody monomer complexes. Subunits are shown in the intermediate (50°) view (Row A), the asymmetric (110°) view (Row B), and the quasisymmetric (0°) view (Row C) as described by Lake (19) for E. coli 30 S ribosomal subunits. Below each frame is an interpretative drawing. Bar length, 1000 Å.

Fig. 10. Electron micrographs of antibody-linked chloroplast 30 S ribosomal subunit dimers. Below each frame is an interpretive drawing. Bar length, 1000 Å.
right of the subunit.

A gallery of antibody-linked E. coli ribosomal subunit dimers is shown in Fig. 6, in which all of the views described for the monomer structures can be seen. Again, the site of antibody contact is on the concave side of the ribosomal subunit in the asymmetric views (see Row B, frames 2 and 5) and is maximally distant from the platform as seen in the intermediate views (see Row A, frames 1 and 2; Row B, frames 2 and 4). These data are consistent with a single binding site, which is shown in Fig. 7A.

Chloroplast—Small ribosomal subunits from chloroplasts were complexed with antibodies to 7-methylguanosine and then examined using the methods described. Once again, electron micrographs showed both types of antibody-subunit complex: 1) monomers, as shown in Fig. 8A and antibody-linked subunit dimers, as shown in Fig. 8B. In about 100 micrographs showing 103 chloroplast 30 S ribosomal subunits, 1277 of these antibody-subunit interactions were clearly identified and evaluated. In 448 monomer structures, all except 22 (4.3%) were consistent with a single binding site. In antibody-linked subunit dimers, all except 11 (1.3%) of the 829 contact points were consistent with the same binding site as described below.

A gallery of chloroplast small ribosomal subunit-antibody monomers is presented in Fig. 9. Again, the point of antibody contact with the ribosomal subunit appears to be near the division between the upper and lower regions of the subunit. The antibody can be seen lying either to the left or the right of the subunit in the quasisymmetric (0°) view (Row C), but it is always on the concave side of the subunit in the asymmetric (110°) view (Row B). In the intermediate (50°) view (Row A), antibody contact with the subunit is always on the side opposite the platform. Each of these views can also be seen in the antibody-linked chloroplast ribosomal subunit dimers shown in Fig. 10. For example, antibody contact is seen on the concave side of the subunit in the asymmetric views (see Row B, frames 2 and 3) and maximally distant from the platform in the intermediate views (see Row A, frames 4 and 5). In all cases, the point of antibody contact is at or near the junction between the major sections of the subunit. These observations are again consistent with a single binding site as depicted in Fig. 7B. Hence, the structural similarity of E. coli and chloroplast ribosomal subunits extends not only to their nearly identical overall morphology and N6,N6-dimethylenosine localization (18), but also includes placement of the single residue of 7-methylguanosine.

DISCUSSION

Of primary importance in the technique of immunoelectron microscopy is a clear demonstration of the specificity of the antibodies used. Our results (Figs. 1 and 2) show this antibody preparation to be highly specific for 7-methylguanosine. In order to halve the binding of [3H]m5'Guo trialcohol, a concentration of guanosine is required that is at least 104 times higher than that required for 7-methylguanosine. The intact methylated base appears to be dominant in ligand interaction with the antibody, since N-methyl derivatives lacking the purine ring had essentially no effect on radioligand binding, and approximately 500 times more ring-opened m'Guo than intact m'Guo is needed to halve binding. This specificity for the intact nucleoside is comparable to that reported by others (29, 35), while the affinity is similar to that of other anti-nucleoside antibodies used in immunoelectron microscopy (11, 36). The ability of the antibodies to recognize m'Guo within the ribosomal subunit is demonstrated in the second antibody precipitation experiments, and the specificity of this interaction is indicated by the prevention of complex formation by free m'Guo as seen in electron micrographs.

A single residue of m'Guo has been placed at position 526 of the E. coli 16 S ribosomal RNA (37, 38), and our high pressure liquid chromatography analysis (Fig. 3) shows an equivalent amount of m'Guo in chloroplast RNA. In the chloroplast 16 S RNA gene sequence (18), an identical 19-nucleotide segment has been placed in an equivalent element of secondary structure (39), in which the modified guanosine residue would occur at position 474. An appropriate oligonucleotide including a modified Guo residue has been reported (40) in Euglena chloroplast 16 S RNA. Therefore, we think it likely that the m'Guo of chloroplast 16 S RNA occupies a position equivalent to that in E. coli, particularly when localization by immunoelectron microscopy is considered.

The localization of 7-methylguanosine in the E. coli and the chloroplast 30 S ribosomal subunits (Fig. 7) places this residue in an analogous position in each type of ribosome. These results are fully compatible with the hypothesis that chloroplast ribosomes are closely related to and perhaps descended from bacterial ribosomes (20). The 5'-proximal 35% of the E. coli 16 S ribosomal RNA, which includes the 7-methylguanosine at position 526, contains the binding sites for ribosomal proteins S4 (41) and S20 (42). Since the binding of these proteins is crucial for ribosome assembly (43) and this region corresponds to the proposed structural "core" of the 30 S subunit (44), it is very likely that this segment of the E. coli 16 S ribosomal RNA is involved in overall structural organization and ribosomal subunit assembly. Again, the similarity of overall subunit structure (18), along with the 74% sequence homology with E. coli 16 S RNA (16), suggests a comparable role for this section of the chloroplast 16 S ribosomal RNA.

It is perhaps surprising that 7-methylguanosine could so readily be mapped since it falls within the binding site of ribosomal protein S4 which reportedly does not appear on the subunit surface as judged by its inaccessibility to anti-S4 antibodies (45, disputed in Ref. 6). Indeed, the position of S4 as determined by neutron scattering places it slightly below the partition of the upper and lower sections of the 30 S particle (46), near our localization of m'Guo. Gentle ribonuclease treatment of protein S4-16 S RNA complexes, however, shows that the segment containing residues 518-530 is one of four specific excisions of the RNA (41, 47); that is, this section of the RNA is exposed even in the protein-RNA complex.

The immunoelectron microscopic localizations of 7-methylguanosine place it in a section of the 30 S ribosomal subunit for which no specific functional interactions have yet been described. However, several ribosomal proteins for which roles in tRNA binding have been suggested are clustered in this region (5), and the Guo residue at position 530 is highly susceptible to kethoxal modification in 30 S subunits or 70 S ribosomes (48, 49), but not in polysomes in which tRNA and message are bound. 7 This fits well with our placement of the 7-methylguanosine in a region of the 30 S subunit that is closely aligned with what has been defined in the E. coli 50 S subunit as the peptideyltransferase site (5-7) and thus this segment of the 16 S ribosomal RNA may be involved in messenger or tRNA binding. Finally, concurrent experiments using antibodies directed against m'GMP 7 give a similar placement of the nucleotide in E. coli ribosomal subunits.

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