Wheat Germ Cytoplasmic Ribosomes

STRUCTURE OF RIBOSOMAL SUBUNITS AND LOCALIZATION OF N\textsuperscript{6},N\textsuperscript{6}-DIMETHYLADENOSONE BY IMMUNOELECTRON MICROSCOPY*

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Cytoplasmic ribosomes have been isolated from wheat germ, and the structure of ribosomal subunits has been examined by electron microscopy of negatively stained preparations. Small (40 S) subunits show structural features generally regarded as characteristic of eukaryotic particles, while large (60 S) subunits show shapes that are equally well described by models of prokaryotic 50 S particles. Small subunit 18 S RNA contains 2 residues of N\textsuperscript{6},N\textsuperscript{6}-dimethyladenosine 19 and 20 residues from the 3'-end (Hagenbüchle, O., Santer, M., Steitz, J. A., and Mans, R. J. (1978) Cell 13, 551–563). Nucleoside analysis by high performance liquid chromatography shows no other residues of this component in the RNA. Anti-dimethyladenosine immunoglobulins were reacted with wheat germ 40 S subunits, and the resulting complexes were studied by electron microscopy in order to localize the nucleoside. In about 90% of the complexes observed, antibody-subunit contact was consistent with a single binding site. We place the localization of the 3'-end of the RNA (14), suggest the availability of the 3'-terminal segment of 18 S RNA and localize the dimethyladenosine residues near the end of the platform of the 40 S particle.

Protein synthesis in all cells occurs on ribosomes whose overall size and shape are similar, regardless of the source (1, 2). The Escherichia coli ribosome has been extensively studied by electron microscopy. There is fairly good agreement among the morphological models that have been proposed (3), much is known about the distribution of proteins within each subunit (1, 3, 4), and a limited amount of information on RNA conformation within the subunits is available (2, 5). Study of the structure of ribosomes from other sources is at a comparatively early stage. There is controversy on the interpretation of specific features seen in micrographs (2, 4, 6). A few proteins of rat liver ribosomes have been localized (7, 8), but essentially nothing is known about RNA folding. However, many rRNA (or cDNA) sequences have been determined; there is significant evolutionary conservation of primary structure and of proposed secondary structures (5, 9), and two successive residues of N\textsuperscript{6},N\textsuperscript{6}-dimethyladenosine occur near the 3'-end in almost all species (5, 10, 11).

This work is concerned with the structure of ribosomes from wheat germ, a common source of experimental materials for in vitro protein biosynthesis. In wheat germ 18 S rRNA the dimethyladenosine residues occur 19 and 20 nucleotides from the 3' terminus (10). Antibodies to dimethyladenosine have allowed localization of the modified residues in E. coli (12) and pea chloroplast (13) 30 S subunits and, along with the localization of the 3'-end of the RNA (14), suggest the placement of the message-positioning Shine and Dalgarno (15) sequence of prokaryotic rRNA on the subunit platform. In this paper we describe the preparation of wheat germ ribosomal subunits and their characterization by electron microscopy. We then demonstrate the availability of the 3'-terminal segment of 18 S RNA and localize the dimethyladenosine residues near the end of the platform of the 40 S particle.

MATERIALS AND METHODS

Preparation of Ribosomes and Ribosomal Subunits—Wheat germ 80 S ribosomes were prepared by a modification of the method of Treadwell et al. (16). Fifteen grams of raw wheat germ (Richards Food Corp.) were ground in a mortar on ice with an equal volume of sand for 10 min and mixed with 150 ml of extraction buffer (6 mM KHCO\textsubscript{3}, 90 mM KCl, 4 mM magnesium acetate, 2 mM CaCl\textsubscript{2}, 6 mM 2-mercaptoethanol, 10% v/v glycerol). After 12 min of centrifugation at 15,000 rpm in a Sorvall SS-34 rotor, the fat layer was removed from the top and the supernatant was decanted and filtered through two layers of cheesecloth. 0.1 volume of 0.1 M magnesium acetate and 0.1 volume of 1 M Tris-HCl, pH 7.8, were added to the filtrate, and the solution was centrifuged as above for 15 min. The upper about seven-eighths of the supernatant was filtered through cheese cloth, layered over 1.5-ml pads of 20% sucrose in extraction buffer, and centrifuged in a Beckman 65 rotor for 90 min at 60,000 rpm. The resulting pellets were resuspended in buffer (20 mM Hepes-KOH, pH 7.6, 150 mM KCl, 5 mM magnesium acetate, 6 mM 2-mercaptoethanol, and 10% glycerol) to give an A\textsubscript{260} of about 800. If not used immediately, aliquots of the suspension were quick-frozen and stored at −20 or −70°C.

Ribosomal subunits were prepared by a modification of the method of Spremulli et al. (17, 18). Aliquots of 120 A\textsubscript{260} units (about 10 mg) of ribosomes were diluted with 10 volumes of dissociation buffer (150 mM KCl, 1 mM magnesium acetate, 0.1 mM EDTA, 6 mM 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.7), containing 5% sucrose. After incubation at 30°C for 5 min, the samples were applied to 56 ml of 15-30% linear sucrose gradients in dissociation buffer and centrifuged in a Beckman SW 25.2 rotor at 19,000 rpm for 16 h at 10°C. Appropriate fractions were pooled, and the 40 and 60 S subunit preparations were dialyzed overnight at 4°C against 50 mM KCl, 5 mM MgCl\textsubscript{2}, 0.1 mM EDTA, 6 mM 2-mercaptoethanol, 10% glycerol, 50 mM Tris-HCl, pH 7.7, and then centrifuged for 3 h at 60,000 rpm in a Beckman 65 rotor at 4°C. The resulting pellets were resuspended at an A\textsubscript{260} of about 120–250 in 5 mM Mg(OAc)\textsubscript{2}, 120 mM KCl, 8 mM 2-mercaptoethanol, 10% glycerol, 20 mM Hepes-KOH, pH 7.6. If not

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1The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; m\textsuperscript{6}Ado, N\textsuperscript{6},N\textsuperscript{6}-dimethyladenosine; m\textsuperscript{6}Ado, N\textsuperscript{6}-monomethyladenosine; HPLC, high performance liquid chromatography.
used immediately, the suspensions were quick-frozen in small aliquots and stored at ~20 °C. E. coli (strain PR7) ribosomes and ribosomal subunits were isolated as previously described (12).

**Analysis of Nucleosides**—RNA was isolated from purified wheat germ 40 S and E. coli 30 S subunits by phenol extraction (19). The RNA was hydrolyzed to nucleosides (20) with bacterial alkaline phosphatase ribonuclease A and ribonuclease T$_1$ (Sigma) and snake venom phosphodiesterase (Worthington). Nucleosides from the hydrolysis of 0.2–1 A$_{260}$ units of RNA were quantitated by HPLC on a Supelco 5 micron reversed-phase column (250 × 4.6 mm) using a modification of the method of Buck et al. (21). A flow rate of 2 ml/min was used in a gradient from buffer A (0.25 M ammonium acetate, pH 6.0) to buffer B (40:60 water/acetone) as follows: initial conditions, 100% A; at 30 min, 100% A; at 45 min, 25% A via Waters curve 8; at 50 min, 100% B via Waters curve 6. Absorbance was monitored at 260 nm, and the data were integrated with a Shimadzu C-R3A Chromatopac. Purified nucleosides used as standards were purchased from Pharmacia LKB Biotechnology Inc.

**Preparation and Characterization of Antibodies**—Antibodies were induced in rabbits by toe pad injection of a covalent m$_3$Ado-protein conjugate. The procedures used in the synthesis and characterization of nucleoside conjugates, immunization, blood collection, serum preparation and antibody purification, and characterization have been described (12, 14).

**Synthetic Oligodeoxynucleotides**—Sequences complementary to the 3' terminus of wheat germ 18 S rRNA and E. coli 16 S rRNA were synthesized using $\beta$-cyanoethyl phosphoramidites (22) and a Vega Biotechnologies/Dupont Coder 300 automated synthesizer. Oligomers were purified by ion exchange HPLC using a Bio-Rad TSK-DAE5PW column with a Waters chromatograph; products were eluted with a hyperbolic gradient (curve 7) of 0–1 M NaCl in 20 mM Tris-HCl, pH 7.5. Salts were removed by dialysis against water (Spectrapore 1 dialysis tubing), and the oligonucleotides were concentrated in a Speed-Vac (Savant Instruments). Radiolabeling at the 5' terminus and analysis of oligodeoxynucleotide binding with a nitrocellulose filter assay have been described (23). Poly(U)-stimulated binding of radiolabeled phenylalanyl tRNAPh$'$ followed Zamir et al. (24).

**Electron Microscopy**—For the characterization of ribosomal subunit preparations, particles were diluted to an A$_{260}$ of 0.1–0.2 either in 5 mM magnesium acetate, 50 mM KCl, 20 mM Hepes-KOH, pH 7.6, or in 200 mM NH$_4$Cl, 10 mM Tris-HCl, pH 7.4, containing 1–5 mM MgCl$_2$. Antibody-subunit complexes were formed by overnight incubation at 4 °C of 0.25 A$_{260}$ units (about 17 pmol) of purified 40 S ribosomal subunits with 1–4 eq of anti-dimethyladenosine IgG (measured as $[^3H]$nucleoside binding capacity) in 30–50 µl of 200 mM NH$_4$Cl, 10 mM Tris-HCl, pH 7.4, containing 1–5 mM MgCl$_2$. Unreacted antibodies were removed by size exclusion HPLC on a Beckman Sphugel-TSK 3000 SW column (7.5 × 300 mm) at a flow rate of 1 ml/min. Samples were prepared for electron microscopy by negative staining with 1% uranyl acetate according to the double carbon technique (25). Electron micrographs were obtained with a JEOL 100B microscope operated at 80 kV and a magnification of approximately 69,000.

**RESULTS**

**Characterization of Wheat Germ 40 S Subunits**—Wheat germ 40 S subunits were isolated by a variety of procedures.
negatively stained with 1% uranyl acetate, and observed by electron microscopy. Our isolation procedure yielded particles we considered to be satisfactory in purity and apparent structural integrity. A field of such particles is shown in Fig. 1. The subunits appear quite homogeneous, and distinctive and reproducible features can be seen in the micrographs. In order to define an antibody binding site on a three-dimensional model, it is necessary to evaluate complexes in more than one two-dimensional projection. Therefore, several hundred micrographs were studied in order to define the most common and reproducible projections.

Most of the images of 40 S subunits could be clearly identified with the three representative projections (intermediate, quasi-symmetric, and asymmetric) first described by Lake (26) for E. coli 30 S subunits and since applied to subunits from many sources including eukaryotes (1). We will use the Lake nomenclature (1, 2, 26) in our description of wheat germ subunits. The subunits appear to be adsorbed to the carbon layer in a selective way so that the asymmetric view predominates; approximately 70–80% of the images represent this projection. Most of the remaining images fall into the other two categories. Images of the wheat germ 40 S subunit arranged according to characteristic views are shown in Fig. 2. In the intermediate view, row A, a region of accumulated stain, defines a cleft which separates the platform from the body; both enantiomorphic projections are shown, while the interpretive drawing represents frames 1, 2, and 5. The quasi-symmetric view, row B, is characterized by a line of approximate mirror symmetry, which corresponds to the long axis, and a line of stain that separates the upper and lower segments. In the quasi-symmetric and intermediate views the wheat germ 40 S subunit appears only slightly different from the bacterial or chloroplast 30 S ribosomal subunit. The asymmetric view is shown in its two enantiomorphic projections in rows C and D. These images emphasize features considered characteristic of eukaryotic 40 S subunits: a "bill" protruding from the head, an apparent split in the platform, and additional material or lobes at the base (1–3, 6). These images and the interpretative drawings at the end of each row are consistent with previous observations of other eukaryotic 40 S subunits.

Characterization of 60 S Subunits—A number of methods of preparation of 60 S subunits were evaluated. A micrograph of particles purified by two rounds of sucrose gradient sedimentation and concentration by ultracentrifugation, steps commonly used in subunit purification, is shown in Fig. 3A. Although in about 100 micrographs at least 90% of the particles appeared to be 60 S subunits, only 1–2% of the images showed a clearly defined stalk, the most useful reference feature for interpretation of micrographs and identification of the different projections. Moreover, the 60 S population did not appear very well preserved or homogeneous. If minimal handling of the ribosomes was emphasized the apparent quality of the subunit was improved. Freshly isolated 80 S ribosomes were dissociated, sedimented through sucrose gradients, and the 60 S fraction was directly prepared for microscopy. Fig. 3B shows a field of such subunits. In more than 100 micrographs about two-thirds of the particles were clearly defined 60 S subunits; the majority of the others were 40 S subunits plus some 80 S ribosomes. Even though a stalk is identifiable in a relatively low percentage of the large subunit images (about 10%), the 60 S subunits appear to be a rather homogeneous population.

The gallery of 60 S subunits in Fig. 4 is arranged to show characteristic projections that we consistently observed. They will be described using the Lake nomenclature (1, 2, 26). Row A shows both enantiomorphic projections of one of the quasi-symmetric views, characterized by three protuberances: the
FIG. 4. Selected images of wheat germ 60 S subunits. Particles are shown in various quasi-symmetric projections (rows A–E) and the asymmetric projection (row F). Bar length, 100 nm.

stalk, the headlike central protuberance, and the shoulder. In this projection of the subunit the stalk is seen at about a 45° angle from the vertical axis. Row B shows both enantiomorphs of a quasi-symmetric view which corresponds to a different orientation of the particle in space; the stalk projects horizontally with respect to the central protuberance. Rows C and D show images that lack the stalk. In row C the particles appear symmetric about the vertical axis. They could correspond to either of the two views described above. In the images in row D, a region of accumulated stain can be seen between the central protuberance and the shoulder. These images were observed quite frequently in the micrographs. They could represent a projection in which the subunit orientation is such that the stalk is obscured by the particle. Row E shows another quasi-symmetric projection of the particle in which the stalk is clearly identifiable. Row F shows the asymmetric view.

Our interpretation of the structure of wheat germ 60 S particles is consistent with a common basic morphology of large ribosomal subunits; they resemble E. coli 50 S particles in overall shape but show a deeper notch between the central protuberance and the body, and/or the protuberance is at a different (sharper) angle. Consistent with observations of Boublik et al. (6) we do not see the "eukaryotic lobe" or the "bulge" described by Lake (1, 2).

Quantitation of Base-methylated Adenosines in Wheat Germ 18 S rRNA—Wheat germ 18 S rRNA and E. coli 16 S rRNA, used as a control, were hydrolyzed to nucleosides and analyzed by reversed-phase HPLC. The integrated A_{260} was used to determine the amounts of each nucleoside in the hydrolysates. A level of 1.65 mol of m^6Ado/mol of RNA was found in wheat germ 18 S rRNA, and E. coli 16 S rRNA hydrolysates gave 1.7 mol of m^6Ado/mol of RNA. Wheat germ 18 S rRNA was also found to contain N^6-monomethyladenosine at about 1 mol of modified nucleoside/mol of rRNA. No m^7Ado was detected in E. coli 16 S rRNA hydrolysates.

Binding of a Complementary Oligodeoxynucleotide to the 40 S Ribosomal Subunit—The oligonucleotide dCAATGATCCTTC, which complements the 3'-terminal nucleotides of wheat germ small subunit RNA (10), was chemically synthesized, purified by HPLC, and labeled with ^32P at its 5'-end. Varying amounts of oligomer were incubated with a fixed quantity of 40 S subunits, and oligonucleotide-subunit complexes were quantitated. Binding reached a plateau of about 0.3 mol of oligomer/mol of 40 S in the presence of a 2-fold molar excess of nucleotide; a similar level of binding of a 3'-terminal complementary oligonucleotide was observed with E. coli 30 S subunits (see also Ref. 23), and in each instance the level of oligodeoxynucleotide binding was slightly greater than the poly(U)-stimulated binding of [3H]phenylalanyl tRNA_em to active 30 S subunits. We interpret the results to indicate that the 3'-terminal region of 18 S rRNA is available for binding on the surface of the 40 S subunit.

Localization of Dimethyladenosine—Purified wheat germ 40 S ribosomal subunits were incubated with anti-m^6Ado IgG. After removal of unreacted antibodies by size exclusion HPLC, the ribosomal subunits were negatively stained and examined in the electron microscope. Two types of antibody-subunit complexes were observed: monomers, in which a single subunit has one IgG molecule bound, and dimers, in which two subunits are linked by an antibody. Fig. 5 shows fields in which monomers (panel A) or dimers (panel B) are indicated. Although uncomplexed wheat germ 40 S subunits...
FIG. 5. Electron micrographs of wheat germ 40 S subunits complexed with antibodies to N\textsuperscript{6},N\textsuperscript{6}-dimethyladenosine. A, arrowheads indicate subunit-antibody monomeric complexes; B, arrowheads indicate antibody-linked subunit dimers. Bar length, 100 nm.

preferentially orient with respect to the carbon layer in the asymmetric projection, in antibody-subunit complexes the particles are most commonly seen in the quasi-symmetric projection. Bound antibody appears to introduce a constraint on the way the particles are adsorbed to the carbon layer.

The location of dimethyladenosine in the 40 S particle was approximated by analysis of the apparent point of contact of antibody with the subunit in each characteristic projection. Only those complexes that showed an identifiable antibody in contact with a structurally intact subunit were used in the analysis. About 70 micrographs containing approximately $7 \times 10^4$ small subunits were used, and 549 antibody-subunit interactions were identified and evaluated. In 429 monomers 88% of the contact points were consistent with a single binding site. In antibody-linked subunit dimers, 90% of the contact points were consistent with the same binding site, as described below.

A gallery of ribosomal subunit-antibody monomers is shown in Fig. 6. The subunits are arranged according to the characteristic projections. In the intermediate view, row A, the antibody molecules are seen attached at or near the end of the platform. The cleft between the subunit body and the platform was often obscured, but the lack of basal lobes and the bill allows differentiation of this view from the asymmetric projection. In the asymmetric view, row B, antibodies bind to the convex side of the particle. This projection was particularly useful in placement of the binding site on the platform far from the bill. In the quasi-symmetric view, rows C and D, the point of antibody contact is at or slightly below the partition between the upper and lower sections of the subunit. These images were useful in placing the contact site on the vertical axis, even when the antibody approach is from the top (e.g. row D, frames 1 and 2).

Fig. 7 shows a gallery of antibody-linked subunit dimers in which each characteristic projection is represented. Antibody contact occurs at the same site described above. For example, in row A, frame 2, and row C, frame 3, the IgG molecule is

FIG. 6. Selected images of monomeric antibody-40 S complexes. Subunits are shown in the intermediate view (row A), the asymmetric view (row B), and the quasi-symmetric view (row C). An interpretive drawing is below each frame. Bar length, 100 nm.

FIG. 7. Selected images of dimeric subunit-antibody complexes. Bar length, 100 nm.
with the loss of the stalk from the structural characteristics seen in micro-methods can have on observed structures and the need for integrity upon purification noted by Boublik and Ramagopal treatment with NH$_\text{4}$Cl and ethanol (27) or the loss of ribosome reducibility of images). The disappearance of the stalk of the purification of characteristic features (e.g. the stalk) and apparent quality (e.g. reproducibility of images). The disappearance of the stalk of the wheat germ 60 S particle upon centrifugation can be compared with the loss of the stalk from the E. coli 50 S subunit upon treatment with NH$_\text{4}$Cl and ethanol (27) or the loss of ribosome integrity upon purification noted by Boublik and Ramagopal (28). Our results re-emphasize the effects that preparation methods can have on observed structures and the need for caution in evaluating structural characteristics seen in micrographs, as stressed by van Heel and Stößler-Melilcke (29).

**DISCUSSION**

**Ribosome and Subunit Preparation and Characterization**—Several purification schemes were tested to obtain preparations of wheat germ ribosomal subunits suitable for study by electron microscopy, i.e. that appeared homogeneous and structurally intact. No single purification procedure was acceptable (by this criterion) for the preparation of both 40 and 60 S subunits. Highly purified 40 S subunits were fully satisfactory for the studies reported here. In contrast, extensive purification of 60 S subunits resulted in the loss of characteristic features (e.g. the stalk) and apparent quality (e.g. reproducibility of images). The disappearance of the stalk of the wheat germ 60 S particle upon centrifugation can be compared with the loss of the stalk from the E. coli 50 S subunit upon treatment with NH$_\text{4}$Cl and ethanol (27) or the loss of ribosome integrity upon purification noted by Boublik and Ramagopal (28). Our results re-emphasize the effects that preparation methods can have on observed structures and the need for caution in evaluating structural characteristics seen in micrographs, as stressed by van Heel and Stößler-Melilcke (29).

**Nucleotide Analysis and Availability**—HPLC analysis of wheat germ small subunit RNA showed no more than 2 m'Ado residues/molecule. Terminal sequence analysis (10) showed that 2 residues of m'Ado occur 19 and 20 nucleotides from the 3'-end. Hence, these must be the only residues of this modified component in the RNA. Binding of an oligodeoxyribonucleotide complementary to the 3'-terminal sequence suggests that this region of the RNA is on the subunit surface and likely to be available for interaction with antibody. The equivalent region of E. coli 16 S RNA is similarly capable of binding a complementary oligomer (23, 30) and functions in the alignment of mRNA on the small subunit (15). Although a similar message placement function in eukaryotes has been postulated, it is not clear that the 3'-region of 18 S functions in this manner (31); our data indicate only that such a function remains possible.

**Localization of Dimethyladenosine in 40 S subunits**—Antibodies of well established specificity (12) were used to place N$^\text{6}$,N$^\text{6}$-dimethyladenosine on the wheat germ 40 S subunit. This antibody preparation strongly binds the modified nucleoside, and interaction with unmethylated (or monomethylated) adenosine is negligible. (Since wheat germ 18 S RNA contains 1 m'Ado residue, a possible interference of the monomethylated compound had to be taken into account.) Our interpretation of the electron micrographs of 40 S subunits reacted with anti-N$^\text{6}$,N$^\text{6}$-dimethyladenosine IgG is that in the great majority of the complexes (about 90%) the antibody is bound at a single area at or near the end of the platform. This placement of m'Ado within the wheat germ 40 S subunit shows the modified nucleosides in a conserved position in an eukaryotic cytoplasmic particle relative to their location in either E. coli or chloroplast ribosomes (12, 13). The two adjacent N$^\text{6}$,N$^\text{6}$-dimethyladenosine residues that are nearly universally conserved in the sequence at the 3'-end of small subunit RNA thus appear to be very highly conserved in the three-dimensional structure of the ribosome as well.

The location of the m'Ado residues in all three types of ribosomes is on the platform of the small subunit, i.e. in an area of the particle that is involved in subunit-subunit interaction and in translation. Several studies have attempted to determine a specific function of the m'Ado residues (11); the methyl groups have minor effects on the interaction of the ribosomes with molecules involved in the initiation cycle and upon accuracy in translation in vivo. Distinct characteristics are seen when conformational and thermodynamic effects of the methylated groups on RNA secondary structure are investigated, and it is postulated that the methylated bases, both through their hydrophobicity and their effect on RNA conformation, may control the "fine tuning" of the initiation process. The location of the m'Ado residues in wheat germ 40 S particles virtually assures a platform location for the 3'-terminal sequence of the 18 S rRNA; cDNA binding shows that sequence to be at the subunit surface and further suggests some role in the function of the small subunit.

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