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Structure of Functional A salina – E coli Hybrid Ribosome by Electron Microscopy

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Small 40S Artemia salina and large 50S Escherichia coli ribosomal subunits can be assembled into 73S hybrid monosomes active in model assays for protein synthesis. The reciprocal combination – small 30S E coli and large 60S A salina – fails to form hybrids. The 73S hybrid particles strongly resemble homologous 70S E coli and 80S A salina monosomes. The morphologic differences between the corresponding eukaryotic and prokaryotic ribosomal particles, established by electron microscopy, do not significantly affect the assembly and mutual orientation of 40S A salina and 50S E coli subunits in the heterologous monosome. The fact that the structure of the interface, the supposed site of protein synthesis, is preserved in the active hybrid implies that retention or loss of biologic activity of hybrid ribosomes is determined by the extent of conformational changes in the interface.

Key words: electron microscopy, hybrid ribosome, ribosome structure

Analogy in function in protein synthesis indicates structural similarity of ribosomes of various origins. Supporting evidence for the conformational resemblance of ribosomes has been obtained from reconstitution experiments in which small and large ribosomal subunits from different prokaryotic [1–4] and eukaryotic species [5–7] were assembled into active hybrid monosomes. Lee and Evans [8] combined prokaryotic ribosomal subunits from Escherichia coli with chloroplast ribosomal subunits from the eukaryote Euglena gracilis; only the combination of large (50S) E coli and small (30S) E gracilis subunits yielded 70S hybrid monosomes active in poly(U)-dependent poly(Phe) synthesis. Similar results were reported by Klein and Ochoa [9] on heterologous ribosomes from E coli and Artemia salina; only the hybrids of large prokaryotic and small eukaryotic subunits were active in polyuridylic acid-directed synthesis of N-acetyl-Phe-Phe-tRNA and AUG-directed synthesis of fMet-puromycin. Both model reactions of protein synthesis require a coordinated function of both ribosomal subunits.

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The presented electron microscopy study provides direct evidence of the structural similarity of the active prokaryotic-eukaryotic 73S hybrid ribosome and the homologous 70S E coli and 80S A salina monosomes.

MATERIALS AND METHODS

Ribosomes and Ribosomal Subunits

Eurkaryotic 80S ribosomes and ribosomal subunits were prepared from dehydrated cysts of A salina ("Longlife," Hartz Mountain Corp., Harrison, New Jersey) by the method of Zasloff and Ochoa [10]. Monosomes and ribosomal subunits were suspended in 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH 7.5), 100 mM KCl, 9 mM $MgCl_2$, 0.1 mM Na_2EDTA, 1 mM dithiothreitol (DTT), 5% (w/v) glycerol (buffer I) and stored in a liquid nitrogen refrigerator.

Prokaryotic 70S ribosomes and ribosomal subunits were prepared from E coli strain B as previously described [11]. Ribosomes and subunits were suspended in 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM NH_4Cl, 60 mM KCl (buffer II) and stored in a liquid nitrogen refrigerator.

Hybrid Ribosomes

The prokaryotic-eukaryotic hybrid ribosomes were prepared by a modification of the method described by Klein and Ochoa [9]. The reaction mixture (final volume of 100 μl) consisted of 0.5 A_{260} units of 40S A salina ribosomal subunits, 0.5 A_{260} units of 50S E coli ribosomal subunits, 0.3 mM guanosine triphosphate (GTP), 10 μg polyuridylic acid, 20 pmoles N-acetyl-[^{3}H]phenylalanine-tRNA or [^{14}C]phenylalanine-tRNA, and 10 μg A salina soluble factors prepared by the method of Sierra et al [12]. This mixture was incubated at 25°C for 30 min in 50 mM Tris-HCl (pH 7.5), 25 mM NH_4Cl, 100 mM KCl, 30 mM $MgCl_2$ (buffer III). Samples were then either 1) fixed with 0.5% (w/v) glutaraldehyde (EM grade) for 20 min on ice for sucrose gradient analysis and electron microscopy, 2) filtered directly onto Millipore HA filters (0.45 μm) for binding studies, or 3) heated for 20 min at 90°C in 5% (w/v) trichloroacetic acid and then filtered onto Gelman GN-6 filters for determination of polyphenylalanine synthesis.

Assays

The binding of [^{3}H]AcPhe-tRNA to the hybrid ribosomes was measured by incubating A salina 40S subunits and E coli 50S subunits in the reaction mixture stated above for 30 min at 25°C. Each reaction mixture was then diluted with 2 ml of cold buffer III and washed onto Millipore HA filters (0.45 μm) with three 2-ml volumes of buffer III. The filters were dissolved in 10 ml of Bray's solution and counted in a Beckman LS100 scintillation counter. To determine the release of [^{3}H]AcPhe-tRNA by puromycin, puromycin hydrochloride was added to the reaction mixture (final concentration 2 mM) after 15 min of incubation. The reaction mixture was incubated for an additional 15 min before being assayed by the Millipore filter binding.

Poly(Phe) synthesis was measured by incubating the subunits as described above, but substituting [^{14}C]Phe-tRNA for the [^{3}H]AcPhe-tRNA. After the 30-min incubation, 2 ml of 5% (w/v) trichloroacetic acid were added to the reaction mixture. The mixture was heated at 90°C for 20 minutes and then washed onto Millipore filters three times with 2 mls of 5% trichloroacetic acid (TCA). The filters were dissolved in 10 ml of Bray's solution and counted in a Beckman LS100 scintillation counter.

Electron Microscopy

E coli and A salina monosomes and subunits were prepared for electron microscopy as previously described [13]. The hybrid monosomes were taken from the sucrose gradients as an aliquot of the 73S peak and diluted with five volumes of buffer III to lower the concentration of sucrose. The hybrids were then handled for electron microscopy just as the homologous 70S and 80S ribosomes. A 0.5% aqueous uranyl acetate solution was used as a contrasting solution. The grids were examined on a JEM 100B electron microscope operated at 80V and a direct magnification of 70,000. Printing of the electron micrographs was done with the plate emulsion facing the paper emulsion. The dimensions of the subunits and monosomes were obtained from measurements of highly enlarged (400,000 X) electron images of about 200 ribosomal particles mounted by the sandwich technique [13].

RESULTS AND DISCUSSION

The formation of the prokaryotic-eukaryotic ribosomal hybrids was monitored by sucrose gradient analysis. The hybrid ribosomes had to be fixed with glutaraldehyde [9] because of their sensitivity to the hydrostatic effect of centrifugation. Cross-linking with glutaraldehyde could lead to nonspecific aggregation or dimerization of the subunits into particles with sedimentation coefficients similar to those of hybrid monosomes. To check this possibility, the subunits were incubated in the reaction mixture either individually (Figs. 1A,B) or together (Fig. 1C), fixed with glutaraldehyde, and then separated by sucrose gradient centrifugation. The hybrid monosome peak occurred only when the 40S A salina and the 50S E coli ribosomal subunits were incubated together (Fig. 1C).

The incorporation of AcPhe-tRNA was also monitored on the gradients. The 40S subunit can bind the AcPhe-tRNA in an initiation-type complex, whereas the 50S subunit cannot. The hybrid monosome can bind approximately one molecule of AcPhe-tRNA per particle, indicating that the 50S subunit forms a heterologous monosome with the 40S initiation complex. A reciprocal experiment in which E coli 30S ribosomal subunits were incubated with A salina 60S ribosomal subunits failed to yield any detectable hybrid monosomes (data not shown).

An approximation of the sedimentation coefficient of the hybrid monosome was obtained by centrifuging the hybrid in a sucrose gradient calibrated with 30S, 40S, 50S, 60S, 70S, and 80S ribosomal particles as markers. The value of 73S for the hybrid was obtained by interpolation from a linear regression of the sedimentation coefficient versus the fraction migration distance of the standard peaks into the sucrose gradient. The gradient size and the centrifugation conditions used (see legend to Fig. 1) had properties similar to isokinetic gradients.

The activity of the 73S hybrid monosome in peptide bond formation was tested by Millipore filter assay (Table I). Binding of the AcPhe-tRNA to the hybrid monosome was dependent on the presence of poly(U). As expected, a significant amount of the AcPhe-tRNA was bound to the 40S subunits and none to the 50S. Maximum binding of AcPhe-tRNA occurred with simultaneous incubation of both the 40S and 50S subunits. Approximately 50% of the AcPhe-tRNA could be released by the addition of puromycin to the reaction mixture during the incubation of the 40S and 50S subunits, demonstrating the possibility of translocation and peptide bond formation. Further evidence of peptide bond formation was obtained with Phe-tRNA in the reaction mixture in place of AcPhe-tRNA (Table I). Polyphenylalanine synthesis was optimal when both the 40S and 50S ribosomal subunits and A salina soluble factors were present. These results confirm the experiments of Klein and

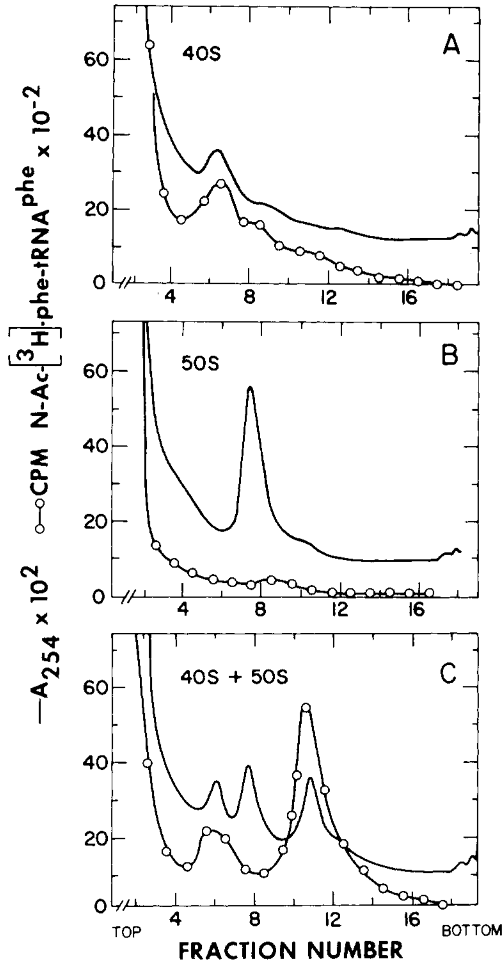


Fig. 1. Sucrose gradient analysis of 40S-50S hybrid monosome formation. The A salina 40S ribosomal subunit and the E coli 50S ribosomal subunit were incubated separately (panels A and B, respectively) and together (panel C) with N-Ac[^{3}H]Phe-tRNA as described in Materials and Methods. After incubation, 5 μl of a glutaraldehyde solution (1 volume 50% glutaraldehyde–1 volume 1 M HEPES (pH 7.5)–0.7 volume 1 N KOH) was added and the samples were incubated on ice for 20 min. The samples were then layered onto a 5-ml 15–30% (w/v) linear sucrose gradient and centrifuged at 50,000 rpm in a Spinco SW65 rotor for 155 min at 4°C. Fractions of 0.3 ml were collected with an ISCO gradient fractionator monitoring the absorbance continually at 254 nm (solid line). A 0.1-ml aliquot of each fraction was added to 0.6 ml of H_2O and the radioactivity determined with 10 ml of Bray's solution (open circles).

Ochoa [9] that the 40S A salina and the 50S E coli ribosomal subunits can interact in a specific manner to permit biologic function and to form small peptides.

The extent of structural similarity of homologous 70S E coli and 80S A salina and heterologous 73S monosomes was studied directly by electron microscopy. Electron micrographs of a field of 73S hybrids (Fig. 2A) demonstrate the overall morphologic similarity to 70S E coli and 80S A salina [14]. The hybrids appear as round-shaped particles with a diameter comparable to that of E coli, approximately 225 Å ± 10Å. A salina monosomes

TABLE I. Biologic Activity of the 40S-50S Hybrid Monosome

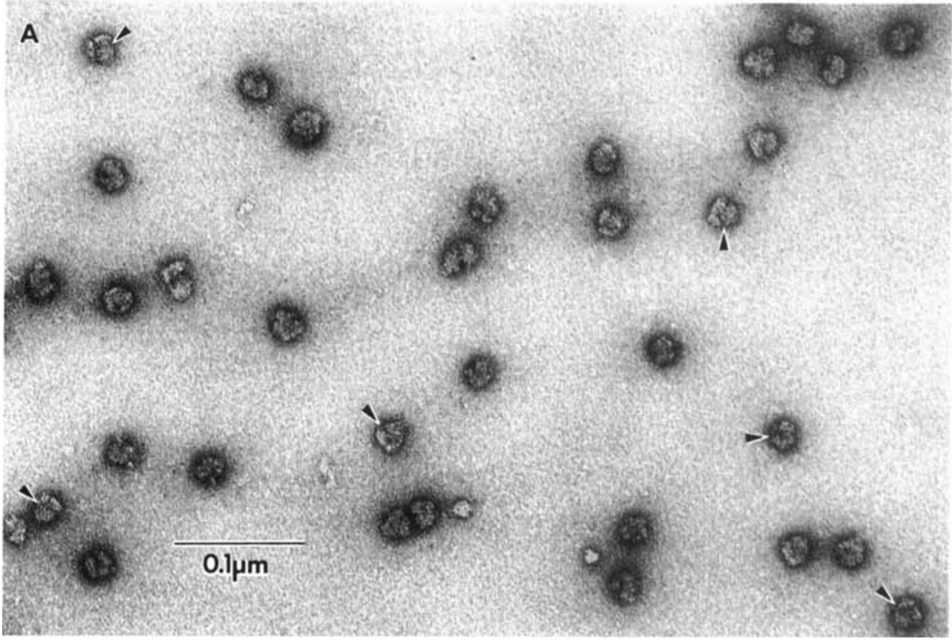
Incubation mixture	Ac[^{3}H]Phe-tRNA[a] bound to filter (pmoles)	[^{14}C]Phe incorporated[b] (pmoles)
Complete (– puromycin)	16.3	10.9
+ puromycin	7.8	–
– soluble factors	6.1	2.6
– 40S	0.4	0.2
– 50S	10.9	0.2
– poly(U)	0.6	0.2

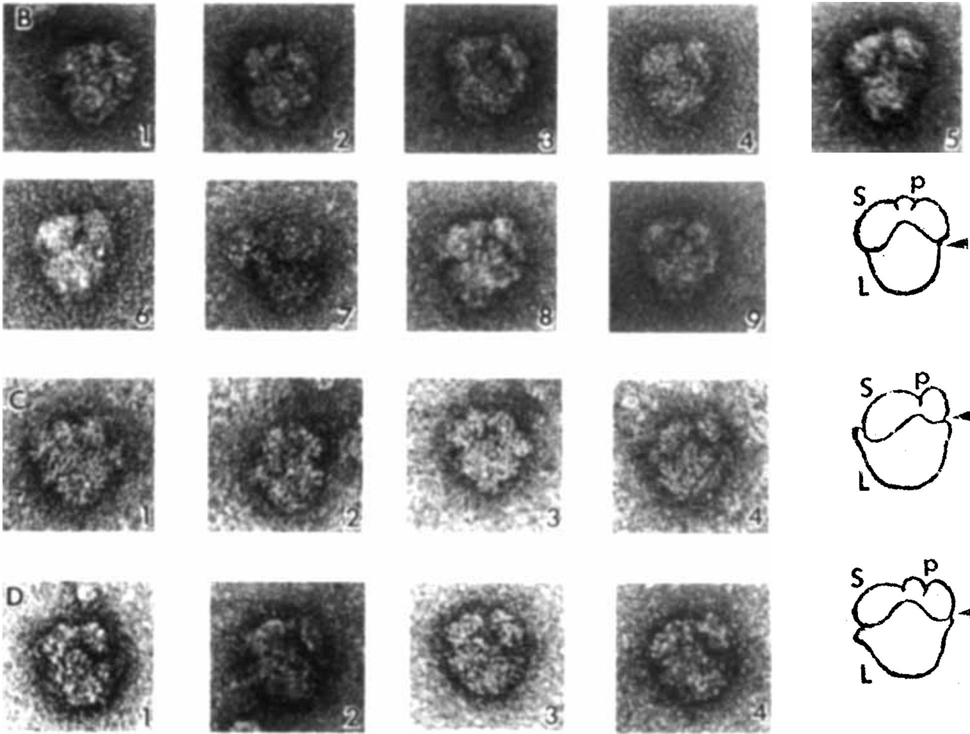
[a]After incubation (see Materials and Methods), the reaction mixture was filtered onto Millipore HA filters and washed three times with 2 ml of buffer III. The filters were then dissolved in 10 ml of Bray's solution and counted in a Beckman LS100 scintillation counter.
[b]After incubation (see Materials and Methods), 1 ml of 5% trichloroacetic acid was added and the samples were heated to 90°C for 20 min. The samples were cooled and the precipitate washed onto Gelman GN-6 filters with three 2-ml volumes of cold 5% trichloroacetic acid. The filters were dissolved in 10 ml of Bray's solution and counted as above.

are considerably larger, 260Å ± 10Å, in agreement with the values of their sedimentation coefficients. The lack of any preferential orientation on the supporting carbon film, in contrast to their subunits [14, 15], might be related to the suppressed asymmetry of the subunits after assembly onto the monosome.

The mutual arrangement of the subunits on the monosome is of particular interest because it determines the interface, the supposed site of protein synthesis. This region can be seen (Fig. 2A) as a dense groove (arrows) of various profiles, depending on the attachment of the monosome to the carbon support. The interface is particularly distinct in the position corresponding to the "crown" view of the large E coli subunit [16]. Selected images of 73S hybrid monosomes, 70S E coli, and 80S A salina in this view (Figs. 2B,C,D), show a striking similarity in the topography of the subunits: the small prolate subunit – 40S A salina on the heterologous 73S and homologous 80S monosomes and 30S E coli on the homologous 70S monosome – is oriented lengthwise in the crown region of the large subunit between the two uneven side crests, with the one-third partition (p) directed toward the more extended left-hand side crest. The 73S hybrid particle appears "heavier" in the top of the crown region due to the larger size of the A salina small subunit compared to that of E coli [14]. The space between the three crests of the large subunit does not show any structures which would imply a preferential site for the attachment of the small subunit. Schematic drawings (inserts in Fig. 2B,C,D) demonstrate the orientation of the large (L) and small (S) subunits which, according to our electron microscopic studies on ribosomes from E coli, A salina [14], slime mold, rat liver, and HeLa cells (unpublished observations) seems to be common to both prokaryotic and eukaryotic ribosomes.

Determination of the structure and topography of ribosomal constituents in the interface is essential for understanding the mechanism of protein synthesis. So far, only an indirect image of the interface can be obtained by conventional transmission electron microscopy. The heavy deposit of staining solution (uranyl acetate), responsible for the high contrast of the interface, obscures all structural details within this region. With this limitation and a lack





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of information on the effect of the association of subunits on their three-dimensional structure, the shape of the interface can be postulated only from the mutual positions of the large and small subunits.

Small subunits of E coli and A salina exhibit considerable structural differences in various corresponding views, except the "frontal" view. Apart from size, the small prokaryotic and eukaryotic subunits in this view are undistinguishable [14]. The similarity in the appearance of the 30S E coli subunit on the homologous 70S monosome, and the 40S A salina subunit on the homologous 80S and heterologous 73S monosomes implies that the complex structural features (eg, the platform of the 30S subunit [17] and the beak-like protrusion of the 40S subunit [14]) are facing the large subunit. Topographic mapping of E coli ribosomes (for reference to various techniques see Weissbach and Pestka [18] and Brinacombe et al [19]) indicates that the majority of ribosomal constituents engaged in protein synthesis are located in the regions which become part of the interface after assembly into a monosome. Since equivalent topographic data on eukaryotic subunits are scarce [20], no analogous conclusions can be drawn for the hybrid ribosome. The discrepancies in the structure of E coli ribosomes, based on electron microscopy observations [13–19], indicate that the interface of even the most closely studied prokaryotic ribosome is still a vaguely defined structure. Tilting experiments, which are in progress in our laboratory, may overcome the limit of views from different angles which are necessary for converting the two-dimensional electron images of ribosomal particles into a three-dimensional structure and ultimately into a model.

Our results give direct evidence that it is possible to form functional 73S hybrid ribosomes from eukaryotic 40S A salina and prokaryotic 50S E coli ribosomal subunits. The morphologic differences between prokaryotic and eukaryotic subunits [14] do not prevent the assembly and the appropriate orientation of these heterologous particles. Conformational changes in the interface may explain why biologic activity of certain combinations of ribosomal subunits is reduced or even abolished while retained or increased for others [2–5, 8, 9].

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Fig. 2. (A) Electron micrographs of an overall field of 73S hybrids (buffer III), contrasted with 0.5% aqueous uranyl acetate. Arrows point to the interface between the subunits. The bar indicates magnification. (B) Selected electron images of 73S A salina–E coli hybrid, (buffer III), (C) 70S E coli (buffer II), and (D) 80S A salina, (buffer I), monosomes in the "crown" view. Magnification 520,000. Note the size discrepancy between the prokaryotic and eukaryotic subunits on the hybrid monosome, evident particularly in B7. This hybrid particle has been rotated by approximately 180° compared to the other monosomes. Schematic drawings demonstrate the arrangement of the ribosomal subunits in the 73S, 70S, and 80S monosomes. S refers to the small, L to the large subunit, p to the "one-third" partition; arrow points to the interface.

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