Proc. Natl. Acad. Sci. USA
Vol. 75, No. 3, pp. 1389–1393, March 1978
Cell Biology

Localization of eukaryotic initiation factor 3 on native small ribosomal subunits

(protein synthesis/electron microscopy/ribosomes)

IVAN EMANUILOV*†, DAVID D. SABATINI*, JAMES A. LAKE‡, AND CHRISTOPH FREIENSTEIN*

* Department of Cell Biology, New York University School of Medicine, 550 First Avenue, New York, New York 10016; and ‡ Institute of Molecular Biology and Department of Biology, University of California, Los Angeles, California 90024

Communicated by George E. Palade, December 13, 1977

ABSTRACT **The localization of eukaryotic initiation factor 3 (eIF-3) on native small ribosomal subunits has been established by electron microscopy through a comparison of native small ribosomal subunits with derived subunits and with native subunits stripped of eIF-3. Small subunits derived from reticulocyte ribosomes by the puromycin/KCl method are seen in electron micrographs as elongated particles, divided by a heavily stained partition into approximately one-third and two-third domains. Most particles (60–70%) observed in electron micrographs of native small subunit preparations resemble derived small subunits, but have an additional mass attached to one side, thus producing profiles with a three-lobed appearance. The mass measures approximately 160 × 100 × 60 Å, and its particle weight is estimated to be about one-third to one-half that of a 40S subunit. The site of attachment of the additional mass is located on a prominence extending from the central part of the small subunit and is separated by a cleft from the smaller third of the subunit. The remaining particles in preparations of native subunits resemble the profiles seen in electron micrographs of derived subunits. After removal of eIF-3 by treatment with high concentrations of salt, profiles observed in electron micrographs of washed, native subunits were indistinguishable from those of derived subunits. Since removal of eIF-3 coincided with removal of a mass of the correct molecular weight, subunits with the three-lobed appearance are identified as native small subunits carrying eIF-3.**

Preparations of native small ribosomal subunits from rabbit reticulocytes are able to carry out the initiation steps of protein synthesis when added to an *in vitro* system containing globin mRNA, derived large ribosomal subunits, and the components of the pH 5 enzyme fraction (1, 2). This ability is due to the presence of initiation factors, particularly to a large protein complex consisting of at least 10 polypeptides (3) which is attached to the native subunits. When isolated, the large protein complex corresponds in its sedimentation value and in the molecular weights of its components to eukarytoic initiation factor (eIF-3) (3–8). The large S value (15–17 S) and the estimated molecular weight (greater than 500,000) of the complex prompted us to attempt its localization by electron microscopy in preparations of native small ribosomal subunits.

METHODS

Preparation of Native Small Ribosomal Subunits. Native small ribosomal subunits were prepared from rabbit reticulocytes as described (1). In brief, blood from anemic rabbits (9) was collected in the presence of cycloheximide. Further operations were performed at 1–4°C. Washed reticulocytes were lysed in two volumes of solution A (10 mM Tris·HCl, pH 7.5/10 mM KCl/1.5 mM $MgCl_2$/2 mM dithiothreitol) and then disrupted with three strokes in a Potter–Elvehjem homogenizer. A supernatant obtained by centrifugation of the lysate for 10 min at 20,000 × g_{av} was recentrifuged twice: first for 45 min to remove polysomes and ribosome monomers and then for 5 hr at 105,000 × g_{av} in a Ti60 rotor. The 5-hr sediment, containing the bulk of the native ribosomal subunits, was resuspended in solution A at a concentration of 120 A_{260} units/ml; 0.5-ml aliquots were layered onto 12 ml of 10%–40% sucrose density gradients containing 20 mM triethanolamine·HCl, pH 7.5/90 mM KCl/3 mM $MgCl_2$/2 mM dithiothreitol, which were centrifuged at 190,000 × g_{av} for 5 hr in an SW41 rotor of a Beckman centrifuge. Gradients were fractionated with an ISCO gradient analyzer; regions corresponding to the subunit peaks were collected; and the subunits were sedimented by centrifugation for 10 hr at 105,000 × g_{av}. Pellets were stored at −80°C.

Preparation of Derived Small Ribosomal Subunits. Derived small subunits were prepared from reticulocyte ribosomes by the puromycin/KCl method (10).

Electron Microscopy. Ribosomal subunits were resuspended in solution A at a concentration of approximately 0.3 A_{260} unit/ml and negatively contrasted at 0°C by a double carbon layer modification (11) of the method of Valentine *et al.* (12), with 2% wt/vol aqueous uranyl acetate. Electron micrographs were taken at an operating voltage of 80 kV on a Philips 301 microscope equipped with a liquid nitrogen cold trap.

RESULTS

A field of derived small ribosomal subunits prepared by the puromycin/KCl method is shown in Fig. 1 *upper*. As described earlier (13), profiles of derived small subunits appear elongated and are divided by a darker staining partition into approximately one-third and two-thirds domains. Most images could be regarded as approximating one or another of three characteristic views (Fig. 1 *upper*, arrows) or their enantiomorphs. Selected images of these views are presented in Fig. 1 *lower*. In these images the smaller domain of each subunit was oriented toward the top and is referred to in the description as the upper third. One of the typical views is characterized by a quasi-symmetric profile with an approximate line of mirror symmetry (Fig. 1*a*). By analogy with small subunits from *Escherichia coli* (14), the angle of projection corresponding to this view will be referred to as 0°. Another view, which results in asymmetric profiles, would then correspond to a projection after a rotation

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: eIF-3, eukaryotic initiation factor 3 (nomenclature proposed at the International Symposium on Protein Synthesis, National Institutes of Health, October 18, 1976); solution A, 10 mM Tris·HCl, pH 7.5/10 mM KCl/1.5 mm $mgCl_2$/2 mM dithiothreitol.

† Present Address: Central Electron Microscopy Laboratory, Medical Academy, Sofia 1431, Bulgaria.

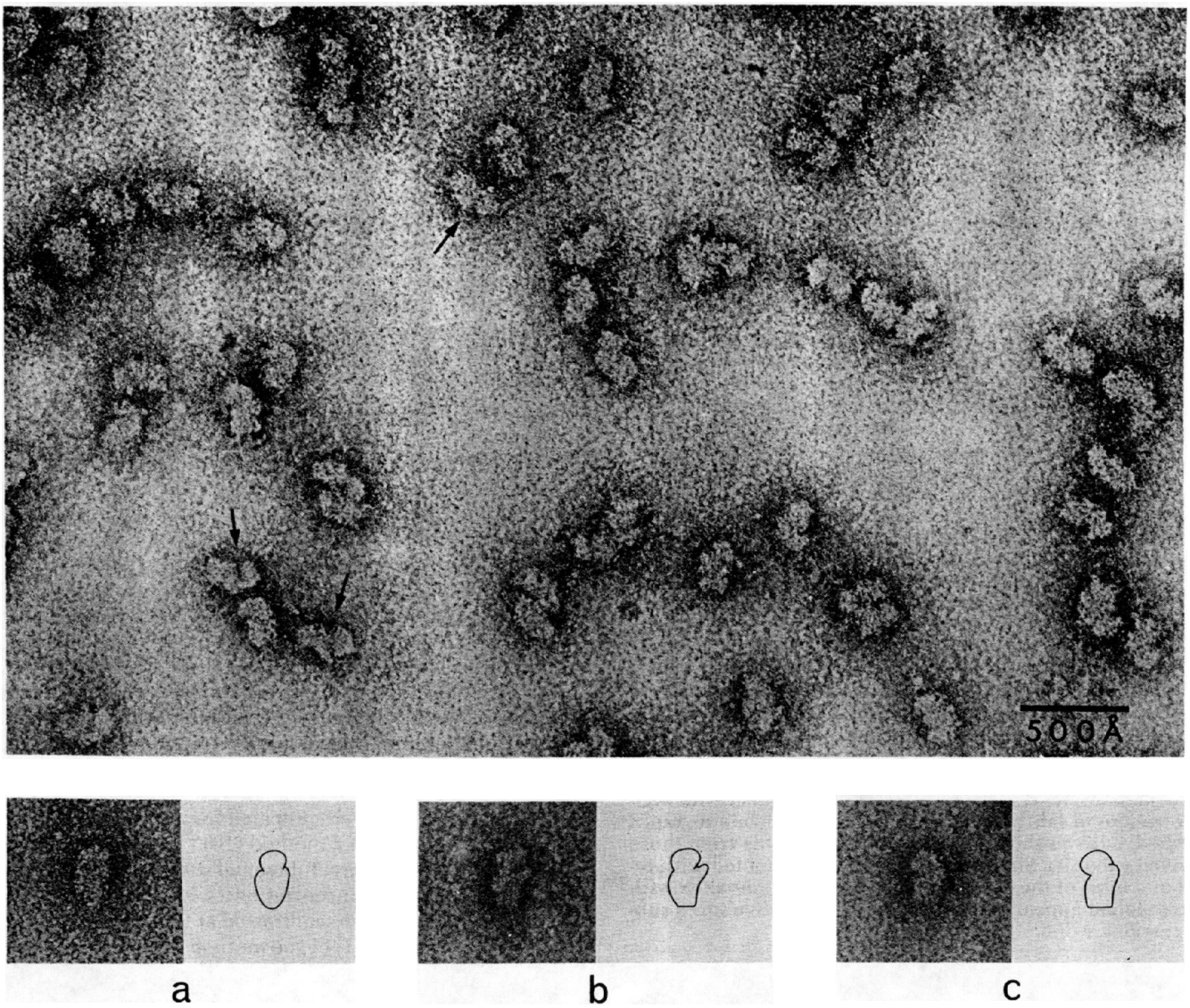

FIG. 1. (*Upper*) Field of negatively stained small ribosomal subunits derived from polysomes by dissociation with puromycin/KCl. Most images are divided by a darker staining partition into one-third and two-thirds portions. Three different characteristic subunit profiles are marked by arrows. (*Lower*) Selected profiles of each kind and their schematic representations. (*a*) Quasi-symmetric profile; the angle of projection that gives this type of image is defined for reference as 0°. (*b*) View that results from a projection intermediate (45°) between those shown in (*a*) and (*c*). (*c*) Asymmetric profile that results from a projection approximately orthogonal to that in (*a*).

of approximately 45° on the vertical axis (Fig. 1*b*). Images of this kind are referred to as "intermediate" and are characterized by a prominence in the contour that extends out from the uppermost portion of the "two-thirds" region of the subunit. A third type of profile, also asymmetric, is shown in Fig. 1*c*. These profiles are significantly wider than those of the other views and are characterized by concave and convex sides. They are interpreted as corresponding to a view that is approximately orthogonal (90°) to that resulting in quasi-symmetric profiles (0°).

Fields like the one shown in Fig. 2*a* are characteristic of samples of native small subunits. Approximately 60%–70% of the particle in these fields, depending on the particular preparation, showed a three-lobed appearance resulting from the apposition of an additional large mass on one side of each subunit (Fig. 2*a*, arrowhead). Subunit profiles of this type were observed mainly in views that corresponded to the 45° asymmetric projection of the derived subunits. The remaining (30%–40%) profiles were similar to those seen in preparations of derived subunits. Frequently, in samples of native subunits, particles of both types showed small and variable amounts of heterogeneous material attached to different regions. A gallery of selected images of native small subunits with the additional mass attached is shown in Fig. 2*b* in an orientation corresponding to the intermediate projection (approximately 45°). In these images the particle profiles show the additional mass apposed to the central part of the subunit, which bears the prominence or platform. A gallery of images of native subunits corresponding to the 90° projection is shown in Fig. 2*c*. In this orientation the additional mass is attached to the central portion of the convex side of the subunit. In general, in these images (Fig. 2*c*) more of the mass is exposed than in the intermediate projections (Fig. 2*b*), in which the profile of the mass frequently overlaps that of the subunit. Although the degree of contact between the subunit and the additional mass is variable, in the 90° projection the mass does not appear to be attached to the smaller one-third domain. This possibility, however, cannot be completely excluded. The size and shape of the additional mass are somewhat heterogeneous, although in most instances its profile appears elongated. From measurements of the mass

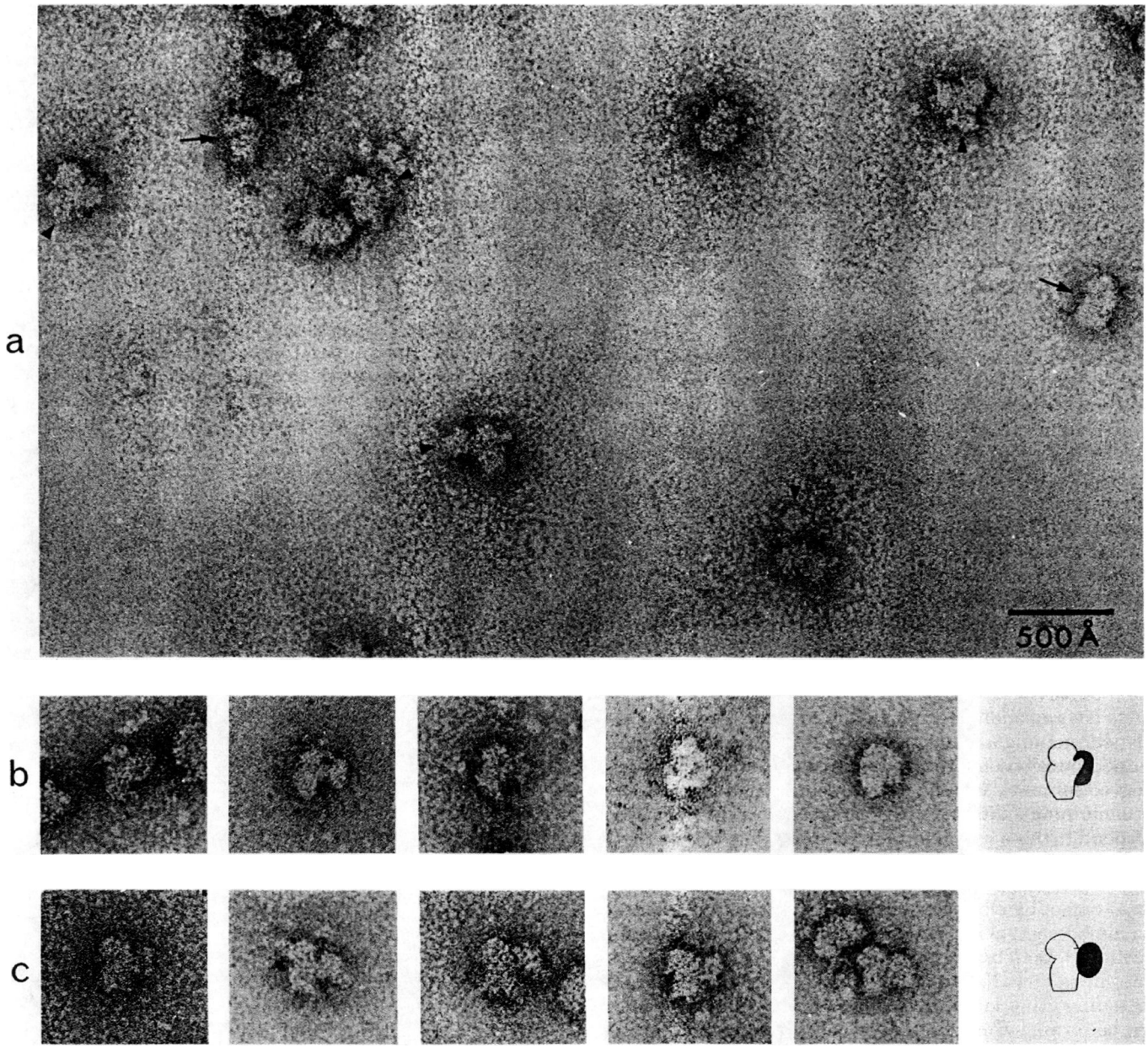

FIG. 2. (*a*) Field of negatively stained native small ribosomal subunits. Two types of particles can be distinguished. Particles of type 1 (arrows) give profiles identical to those of derived small subunits. Those of type 2 (arrowheads) show a large mass attached to the central portion of the subunits. (*b* and *c*) Galleries of selected images of native small subunits of type 2. These are usually observed in views that correspond to the intermediate (*b*) and approximately orthogonal (*c*) asymmetric projections.

profiles (approximately 160 × 100 × 60 Å) an approximate volume of 800,000 Å^3 can be calculated and a molecular weight of 650,000 estimated, assuming a weight of 0.81/Å^3 (based on an average specific volume of 0.74 cm^3/g for hydrated protein).

Treatment of the native subunits used in these studies with high concentrations of salt resulted in removal of a 15S protein complex and a 4S fraction. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of this 15S protein complex (described in ref. 3) led to its identification as eIF-3. Profiles observed in electron micrographs of the subunits stripped by high salt concentrations were indistinguishable from profiles of derived small subunits shown in Fig. 1.

DISCUSSION

Electron microscopy of preparations of native small ribosomal subunits from rabbit reticulocytes allows the recognition of two types of particles. The first is characterized by elongated profiles and appears identical to the 40S subunits derived from polysomes dissociated with puromycin/KCl. Each profile of this type can be classified as approximating one of three main views: two approximately orthogonal to each other and one intermediate between these. The second, and more frequent, type of particle in preparations of native small subunits, however, is characterized by an additional large mass attached to one side of the subunit, so that images have a three-lobed appearance. Removal of the large mass from these subunits coincided with removal of eIF-3 by treatment of native subunits with high concentrations of salt.

The morphological heterogeneity in the population of native subunits is consistent with previous biochemical observations (3, 15–20). Native small subunits have a lower density and a higher protein content than derived 40S subunits (16, 17). Native small subunits from Ehrlich ascites cells contain two

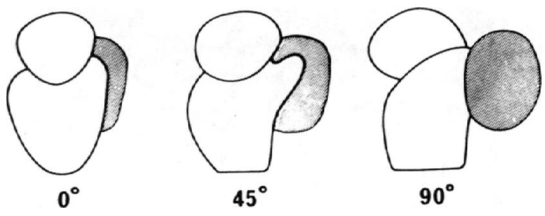

FIG. 3. Schematic representation of a model of native small ribosomal subunits of type 2. The place of attachment of eIF-3 (shaded area) is located on the prominence at the uppermost portion of the lower two-thirds region of the subunit. Profiles corresponding to the same characteristic views described in Fig. 1 *a*, *b*, and *c* are shown.

particle populations which, after fixation in aldehyde, band isopycnically at different densities, 1.49 and 1.40 g/cm^3. From these observations it was suggested that a larger set of additional proteins is attached to the lighter particles (16, 17). The heterogeneity of native small subunits has also become apparent in sedimentation profiles of samples treated with EDTA (3). This chelating agent did not remove the additional proteins from the native particles, but caused conformational changes that allowed the resolution of two distinct types of subunits on the basis of their sedimentation coefficients. It was also possible to show that the faster sedimenting subunits contained a complex of nonribosomal proteins which, if separated, had a sedimentation coefficient of 15 S (3). Hence, the faster sedimenting particles, having the lower isopycnic density, most likely correspond to native subunits that in electron micrographs were found to have an additional mass attached. Analysis of the more slowly sedimenting native small subunits (3) showed that these contained nonribosomal polypeptides and were not identical to derived subunits. The slowly sedimenting native small subunits containing a different set of nonribosomal proteins may correspond to those which in electron micrographs showed no apparent mass attached. However, the additional polypeptides are of low molecular weight and their presence would be difficult to detect by electron microscopy if their attachment to the subunit occcurs at independent regions or without formation of a complex. Both types of native 40S subunits are believed to be intermediates in the initiation steps of protein synthesis; the faster sedimenting subunits with the additional mass attached would be formed after the more slowly sedimenting subunits (16, 17, 20).

Since removal of eIF-3 from native subunits by treatment with high concentrations of salt coincided with removal of a mass of the correct molecular weight, we conclude that the additional mass in native subunits represents eIF-3. This factor has been shown to consist of at least 10 different polypeptides associated in a linkage that is resistant to high salt concentrations (3, 6, 7, 21, 22). Although our observations indicate that the initiation factor is bound to a single, discrete region of the small subunit, as schematically depicted in Fig. 3, considerable size heterogeneity of the additional mass was observed by electron microscopy. Estimates of the molecular weight based on measurements in electron micrographs ranged from 400,000 to 700,000. This size variability could reflect instability of the complex under the conditions of specimen preparation, a natural heterogeneity due to stepwise assembly-disassembly, or alternative assembly pathways of the multicomponent factor during the process of initiation. Biochemical heterogeneity is consistent with the wide range of different molecular weight estimates for eIF-3 (480,000–780,000) that has been reported (3, 6–8, 21, 22). It is also possible that, at least in part, the variable appearance of the population of native subunits with the additional mass attached results from the association of the small subunit with other factors or molecules (e.g., tRNA and messenger ribonucleoprotein) that participate in protein synthesis and may bind to the subunit near the site for eIF-3.

In addition to the mass that most probably represents the eIF-3 complex bound to a characteristic region of the small subunit, smaller amounts of amorphous material were frequently observed attached to other regions of the subunit profiles. It is not possible, however, to determine if this represented still other specific factors involved in initiation or only extraneous proteins contaminating the native subunits. The size heterogeneity of the eIF-3 complex itself and the presence of material attached to different regions of the subunits could account for the considerable heterogeneity in buoyant densities observed in some preparations of native small ribosomal subunits. Up to five classes have been recognized in samples of native small subunits obtained from mouse ascites tumor cells (18, 19).

eIF-3 is absolutely required for translation of natural RNAs (3, 4, 6–8, 21). It is assumed that its main function is in promoting binding of RNA to the 40S subunit (5, 23), and it has been suggested that a small piece of RNA that has been reported to be an intrinsic component of eIF-3 may participate in this process (5, 24). Our observations, indicating that eIF-3 is attached to the prominence in the middle region of the 40S subunit, adjacent to the cleft, suggest that this area of the subunit is a functional site that plays an important role in initiation. eIF-3 prevents the binding of 60S subunits to 40S subunits and strongly enhances the dissociation of inactive monosomes (20, 23). This effect of eIF-3 may be a direct one, i.e., eIF-3 may interfere with subunit association because the area of its attachment to the 40S subunit is located at the interface between the subunits. If this is the case, then the region to which eIF-3 binds in the subunits most probably is, in active ribosomes, the interface between the subunits, where the mRNA is believed to be located during translation (13). The fact that an additional mass is not observed in small subunits that may form initiation complexes within polysomes or in subunits of monomeric ribosomes (13) suggests that the factor dissociates from the small subunit before association with the large subunit.

Prokaryotic small subunits show several structural features that are similar to features of eukaryotic small subunits; both are elongated in shape and are divided into one-third and two-thirds domains. A prominence in the form of a platform has been described in 30S subunits (25) similar to that observed in eukaryotic 40S subunits, although this feature is less pronounced than in prokaryotic subunits (14, 26). Because of these similarities, it is of interest to relate the binding site of eIF-3 in 40S subunits to those sites of the 30S subunit where initiation factors are thought to be localized. Prokaryotic initiation factors can be efficiently crosslinked to 30S subunit proteins (27) that have been mapped in a region composed of the platform, the cleft, and the upper one third in the vicinity of the platform (25). Since eIF-3 is attached to the prominence of the 40S subunit, corresponding to the platform of the 30S subunit but apparently not to the smaller one third, binding of initiation factors to 40S and 30S subunits seems to occur in related but not identical regions.

We thank Mrs. Myrna E. Cort for preparation of this manuscript, and Mr. Miguel Nievas and Ms. Maryanne Pendergast for their work on the illustration. This work was supported by Grants GM 20277, GM 24034, and AG 00378 from the National Institutes of Health and PCM 76-14718 from the National Science Foundation. J.L. received support from the Irma T. Hirschl Foundation while a member of the Cell

Biology Department at New York University Medical School. C.F. received support from the Deutsche Forschungsgemeinschaft.

1. Freienstein, C. & Blobel, G. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 3435–3439.
2. Sundkvist, I. C., McKeehan, W. L., Schreier, M. H. & Staehelin, T. (1974) *J. Biol. Chem.* **249**, 6512–6516.
3. Freienstein, C. & Blobel, G. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3392–3396.
4. Prichard, P. M., Gilbert, J. M., Shafritz, D. A. & Anderson, W. F. (1970) *Nature* **226**, 511–514.
5. Heywood, S. M. (1970) *Proc. Natl. Acad. Sci. USA* **67**, 1782–1788.
6. Schreier, M. H. & Staehelin, T. (1973) *Nature New Biol.* **242**, 35–38.
7. Sundkvist, I. C. & Staehelin, T. (1975) *J. Mol. Biol.* **99**, 401–418.
8. Strycharz, W. A., Ranki, M. & Dahl, H. M. (1974) *Eur. J. Biochem.* **48**, 303–310.
9. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G. & Lowy, P. H. (1952) *J. Biol. Chem.* **196**, 669–694.
10. Blobel, G. & Sabatini, D. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 390–394.
11. Lake, J. A., Pendergast, M., Kahan, L. & Nomura, M. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 4688–4692.
12. Valentine, R. C., Shapiro, B. M. & Stadtman, E. R. (1968) *Biochemistry* **7**, 2143–2152.
13. Nonomura, Y., Blobel, G. & Sabatini, D. (1971) *J. Mol. Biol.* **60**, 303–324.
14. Lake, J. A. (1976) *J. Mol. Biol.* **105**, 131–159.
15. Baglioni, C. (1972) *Biochim. Biophys. Acta* **287**, 189–193.
16. Hirsch, C. A., Cox, M. A., van Venrooij, W. J. W. & Henshaw, E. C. (1973) *J. Biol. Chem.* **248**, 4377–4385.
17. Ayuso-Partilla, M., Henshaw, E. C. & Hirsch, C. A. (1973) *J. Biol. Chem.* **248**, 4386–4393.
18. Sameshima, M. & Izawa, M. (1975) *Biochim. Biophys. Acta* **378**, 405–414.
19. van Venrooij, W. J. W., Janssen, A. P. M., Hoeymakers, J. H. & de Man, B. M. (1976) *Eur. J. Biochem.* **64**, 429–435.
20. Freienstein, C. M. & Blobel, G. (1976) *J. Cell Biol.* **70**, 185a.
21. Safer, B., Adams, S. L., Kemper, W. M., Berry, K. W., Lloyd, M. & Merrick, W. C. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2584–2588.
22. Benne, R. & Hershey, J. W. B. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3005–3009.
23. Kaempfer, R. & Kaufman, J. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3317–3321.
24. Heywood, S. M., Kennedy, D. S. & Bester, A. J. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 2482–2431.
25. Lake, J. A. & Kahan, L. (1975) *J. Mol. Biol.* **99**, 631–644.
26. Vasiliev, V. O. (1974) *Acta Biol. Med. Ger.* **33**, 779–793.
27. Bollen, A., Heimark, R. L., Cozzone, A., Traut, R. R. & Hershey, J. W. B. (1975) *J. Biol. Chem.* **250**, 4310–4314.