Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 5184–5188, September 1982
Biochemistry

Ribosomal protein S4 is an internal protein: Localization by immunoelectron microscopy on protein-deficient subribosomal particles

(ribosome assembly/fidelity/RNA binding protein)

DONALD A. WINKELMANN*, LAWRENCE KAHAN*, AND JAMES A. LAKE†

*Department of Physiological Chemistry, University of Wisconsin–Madison, Madison, Wisconsin 53706; and †Molecular Biology Institute and Department of Biology, University of California, Los Angeles, California 90024

Communicated by Masayasu Nomura, May 27, 1982

ABSTRACT **The location of protein S4 in the small ribosomal subunit has been identified by immunoelectron microscopy. Although intact small subunits are not reactive with antibodies directed against protein S4, subribosomal particles reconstituted without proteins S5 and S12 are reactive. By using these "incomplete" subparticles, we have mapped the position of S4. It is located at a single site on the exterior (cytoplasmic) side of the subunit, at the partition that separates the one-third, or head, from two-thirds, or base, of the subunit. In this location, protein S4 is "beneath" proteins S5 and S12. All three proteins are members of a complex on, or near, the external surface of the small ribosomal subunit that plays an important role in regulation of translational fidelity.**

The technique of immunoelectron microscopy has been of great value in characterizing the surface topography of a number of enzymes and subcellular organelles, including those of the *Escherichia coli* ribosome. Its use has permitted mapping of limited regions of ribosomal proteins and RNA, testing of models of ribosome structure derived from electron micrographs, and localization of functional domains on the ribosome surface (for review, see refs. 1–3; see also refs. 4–8); however, this use has been limited to analysis of the surface topography of the intact organelle. Modification of the technique to make possible locating protein regions normally inaccessible in the intact ribosome would permit the location of proteins that do not have exposed surface antigenic determinants and the study of structural interactions between components that result in the disappearance of those determinants.

Since ribosomes may be assembled *in vitro* in a series of well-characterized protein-deficient subribosomal particles that retain some morphological features of the intact organelle (9), we have investigated the immunoelectron microscopy of specifically constructed reconstituted subribosomal particles to locate a protein that is not immunochemically accessible on the surface of the intact small ribosomal subunit and to confirm the proximal location of the two proteins that render it immunochemically unreactive. We have demonstrated that combination of the reconstitution and immunoelectron microscopy techniques permits the location of proteins within subunits that are not normally exposed on the ribosomal surface.

Antibody preparations specific for ribosomal protein S4 that react with intermediates of small subunit reconstitution but not with intact subunits have been characterized (10, 11). Preliminary experiments had shown us that structurally homogeneous populations of protein-deficient subribosomal particles could be reconstituted, and the morphological similarity of these particles to intact small subunits prompted us to use these S4-specific antibodies to localize S4 on ribosomal subunit reconstitution intermediates. We report here that S4 maps at a single site on the external surface of protein-deficient subribosomal particles at the partition between the upper one-third, or head, and the lower two-thirds, or base, of the subunit. This location is in marked contrast to the highly elongated surface location of S4 previously proposed by us (12) and others (13, 14).

MATERIALS AND METHODS

Purification of *Escherichia coli* (E.) strain PR-C10 and *Bacillus stearothermophilus* (B.) strain 799 ribosomal proteins, preparation of ribosomal [^{3}H]RNA, reconstitution of small ribosomal subunits and assembly intermediates, and assay of their activity were done with minor modification of the methods described by Held *et al.* (15), Higo *et al.* (16), Winkelmann and Kahan (10), and Held and Nomura (17).

S4 antiserum (AS4) and IgG fractions were prepared as described by Winkelmann and Kahan (10). Reconstituted protein-deficient subribosomal particles (referred to hereafter as subparticles) were heat activated for 5 min at 40°C in 10 mM $MgCl_2$/10 mM Tris·HCl, pH 7.8/200 or 30 mM NH_4Cl before reaction with antibody. Antibody binding of [^{3}H]rRNA-labeled ribosome assembly intermediates was measured by using the double antibody "sandwich" precipitation assay described previously (11, 18). For immunoelectron microscopy, subparticles were allowed to react with IgG antibodies in 10 mM $MgCl_2$/10 mM Tris·HCl, pH 7.8/200 mM NH_4Cl for 2 min at 40°C and then for 10 min at 0°C. Dimers and monomers of subparticles were separated from unreacted antibody by fractionation on Sepharose 6B. Isolated antibody–subparticle complexes were examined by electron microscopy. Electron micrographs were taken at an operating voltage of 80 kV on a Philips 400 microscope. Samples were negatively contrasted by the double-layer carbon procedure (19).

RESULTS

The reaction of several S4-specific antibody preparations with *E. coli* small ribosomal subunits and protein-deficient subparticles has been characterized (10). In contrast to earlier work (12–14), these S4 specific IgGs did not react with intact small subunits. However, these antibody preparations did react with intermediates of *in vitro* subunit reconstitution, and it was found that omission of proteins S5 and S12 during subunit reconstitution yielded intermediates that were reactive with AS4 (10, 11). The 30S–S5,S12 subparticle, lacking only proteins S5

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

Abbreviations: AS4, antibodies to ribosomal protein S4; E.S4 and B.S4, ribosomal protein S4 from *E. coli* and *B. stearothermophilus*, respectively.

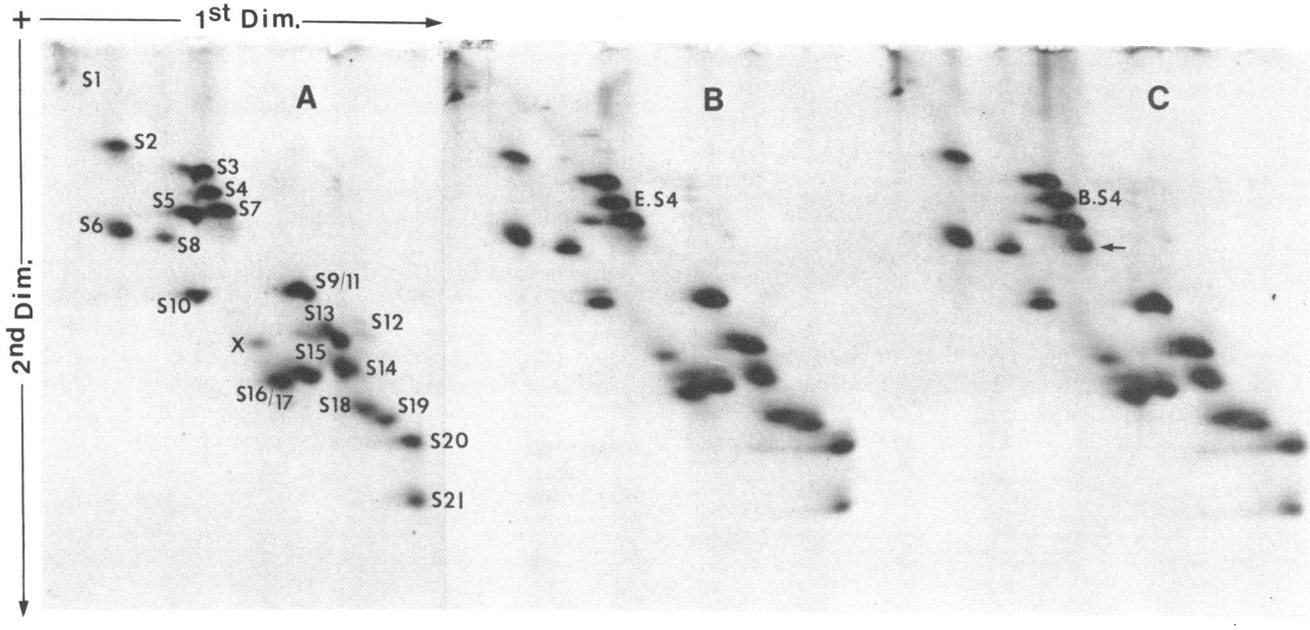

FIG. 1. Two-dimensional gel electrophoresis of proteins from reconstituted small ribosomal subunits and S5,S12-deficient subparticles. (*A*) Reconstituted small ribosomal subunits. (*B*) 30S–S5,S12 subparticles containing E.S4. (*C*) Hybrid 30S–S5,S12 subparticles containing B.S4 instead of E.S4. Isolated ribosomal particles (48 pmol, 0.6 A_{260} units) were digested with ribonuclease, and the proteins were analyzed by using the two-dimensional gel system of Kenny *et al.* (20). (The location of ribonuclease is indicated by the X in *A*). The S12 spot in this two-dimensional gel system is poorly defined, so S12 was also measured immunochemically by using S12 antiserum (18). S12 was absent from the subparticles (*B*) and the hybrid subparticles (*C*) and was present in the reconstituted subunits (*A*) at 80% of the level in native small subunits. A small amount of S5 was present in the subparticles and hybrid subparticles. (*C*) ←, Position of B.S7 present as a contaminant of the B.S4 used. There was at most 20% substitution of E.S7 by B.S7 in these particles as measured by reactivity of the B.S4-substituted particles with S7 antibody.

and S12, is the most nearly complete subparticle that reacts strongly with AS4 antibodies. As assessed by electron microscopy, this subparticle retains the major structural features of the small subunit. Therefore, this subparticle was prepared from a mixture of purified *E. coli* ribosomal proteins and 16S rRNA and isolated by zone sedimentation (10). The protein compositions of the isolated subparticles were analyzed by two-dimensional gel electrophoresis (18), and the particles were found to have the appropriate proteins in amounts comparable with native small subunits (Fig. 1). The inactive subparticles could be completed by addition of the two missing proteins under reconstitution conditions to yield small subunits that were 67% as active as control reconstituted subunits in poly(U)-directed poly(Phe) synthesis (10, 18).

Two types of control experiments were used to determine the specificity of the antibody labeling used to map S4 on isolated subparticles. Antibody binding was demonstrated by a double antibody "sandwich" precipitation assay (Table 1) (11). The antibody reaction was completely inhibited by absorption of the antibody at equivalence with S4, indicating that the reactive antibodies were S4 specific (10). However, this does not demonstrate that S4 is the antigen labeled by antibody on the subparticles. To determine whether the antibody reaction was dependent on the presence of S4 in these subparticles, the effect on the antibody reaction of the replacement of *E. coli* S4 (E.S4) by the noncrossreacting functional analogue from *B. stearothermophilus* (B.S4) was tested. Subparticles lacking S5 and S12 and containing B.S4 rather than E.S4 were prepared and characterized. They were found to have the same composition (with the exception of B.S4) as the E.S4-containing counterparts (Fig. 1) and they could be completed to form subunits having 78% of the poly(U)-dependent poly(Phe)-synthesizing activity of control reconstituted (E.S4) small subunits (18). The substitution of B.S4 resulted in a greater than 80% decrease in the AS4 antibody reaction (Table 1) without affecting the protein composition, the morphology, or the ability to form functionally active subunits from the subparticles (11, 18). We conclude that the antibody reaction is dependent on the presence of E.S4 in these subparticles and that S4 was the antigen labeled by antibody *in situ*.

Electron micrographs of *E. coli* small subunits contrasted by negative staining show that these subunits are characterized by

Table 1. Antibody binding of subribosomal particles

Antibody preparation	Concentration, μM	% antibody-bound subparticles	
		E.S4	B.S4
1-13B	17.7	60	9
	8.8	46	9
	4.4	34	8
2-25B	9.9	72	11
	5.0	53	10
3-21B	9.9	76	3
	5.0	63	4

[^{3}H]RNA-labeled subparticles, 30S–S5,S12, containing either E.S4 or B.S4 were allowed to react with three AS4 antibody preparations (10). AS4 IgG and sufficient nonimmune IgG to give a final concentration of 120 μM were allowed to react with 0.8 pmol of ^{3}H-labeled reconstitution intermediates in 2 μl of 10 mM $MgCl_2$/10 mM Tris·HCl, pH 7.8/135 mM NH_4Cl for 5 min at 40°C and then for 40 min at 0°C. Goat IgG antibody to rabbit IgG (1,460 pmol) was then added, the samples were incubated for 2 min at 24°C, diluted with 500 μl of reaction buffer, and mixed, and the mixtures were centrifuged 1.5 min in a Beckman Microfuge. Radioactivity in the supernatant and in the immunoprecipitate was measured (for details of this assay, see ref. 11). Percent antibody-bound subparticles in the immunoprecipitate was corrected for 6–10% nonspecific precipitation as determined with a nonimmune IgG control. Results are means of two or three determinations. The 30S–S5,S12 contains 16S RNA and proteins S1–S4, S6–S11, and S13–S21 (11).

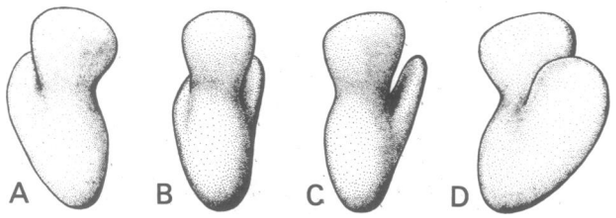

FIG. 2. Diagrams of the small ribosomal subunit. (*A*) Intermediate (−40°) view. (*B*) Quasisymmetric (0°) view. (*C*) Intermediate (50°) view. (*D*) Asymmetric (90°) view.

three regions (21–23), the upper one-third (or head), the lower two-thirds (or base), and the platform. The head and base are separated by a constriction and together form the "body." The platform is attached to the base and is separated from the head by a cleft. Diagrams of our asymmetric model, depicting the four best characterized profiles of the small subunit, are shown in Fig. 2. All of these profiles are present in electron micrographs of the reconstitution intermediates, as well; we have illustrated the binding site (Fig. 3) with micrographs of the asymmetric projection (Fig. 2*D*) and of the projections shown in Fig. 2 *B* and *C* (or views intermediate between them). Together, these projections allow us to locate protein S4 in three dimensions.

Electron micrographs of subparticles allowed to react with AS4 IgG are shown in Fig. 3. In these experiments, 30S–S5,S12 subparticles were allowed to react with three different IgG antibody preparations shown to specifically label S4 *in situ* (see Table 1). After excess antibody was separated from antibody-labeled subparticles and subparticle dimers, the subparticles were negatively stained for electron microscopy. A field of

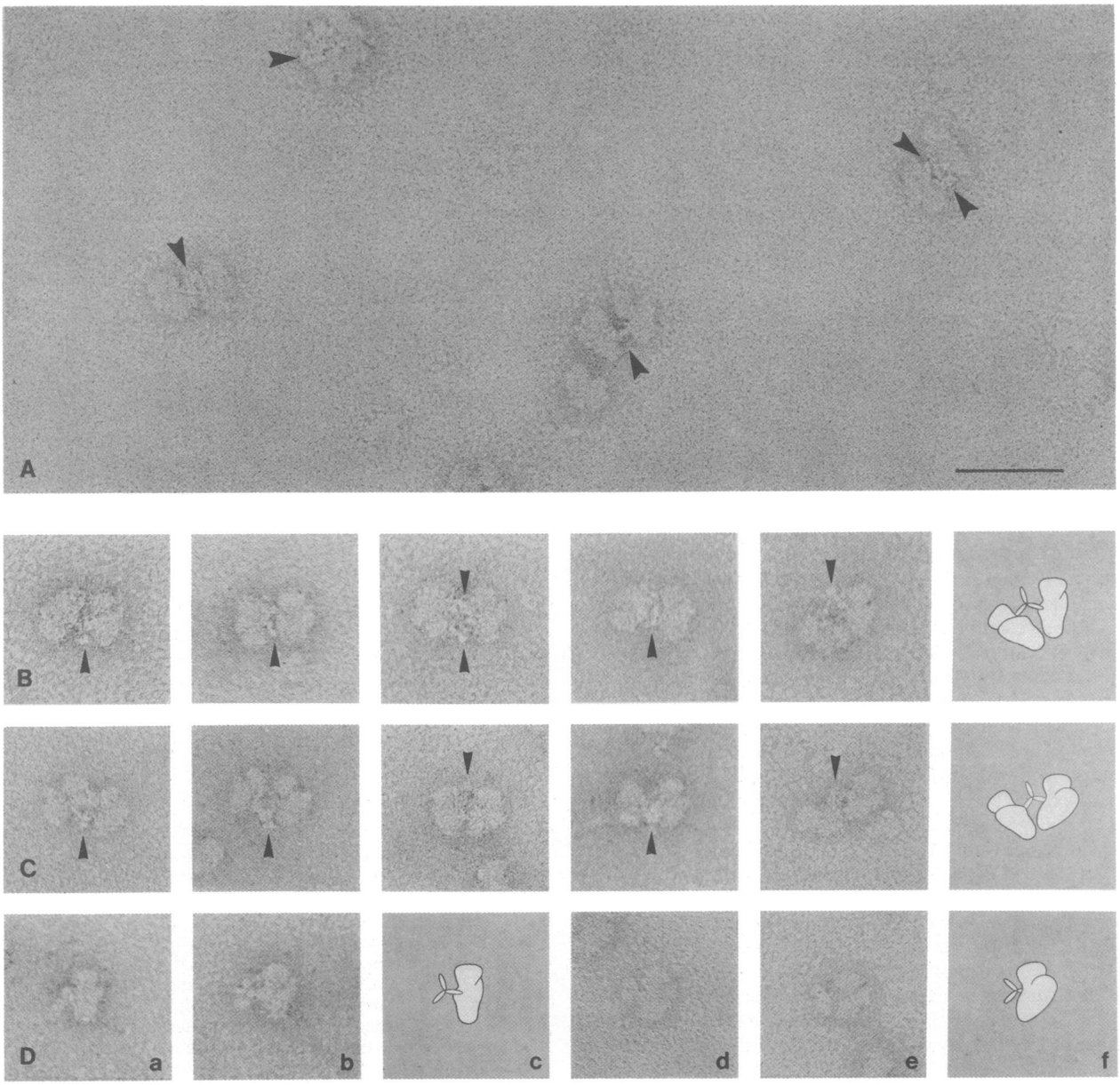

FIG. 3. Electron micrographs of reconstituted subribosomal particles lacking S5 and S12 (30S–S5,S12) allowed to react with antibodies directed against protein S4. (*A*) Field of subparticles. Arrowheads indicate IgG molecules connecting pairs of subparticles. Bar = 500 Å. (*B*) Pairs of subparticles linked by IgGs. The vertical subparticle is approximately in the intermediate orientation. (*C*) Pairs of subparticles in the asymmetric projection (left subunit in *a*, *b*, *d*, and *e*). (*D*) Monomeric subparticles with a single IgG attached are shown in the quasisymmetric projection in *a* and *b* and in the asymmetric projection in *d* and *e*. Interpretive diagrams relate to the micrographs to their immediate left.

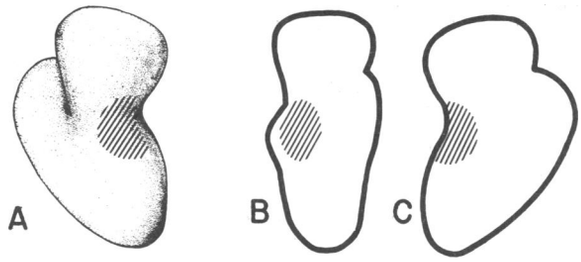

FIG. 4. Diagrams of the location of the S4 antigenic determinant. (*A*) On the surface of the 30S–S5,S12 subparticle. (*B* and *C*) In the quasisymmetric and asymmetric projections of the small subunit, respectively.

30S–S5,S12 subparticles labeled by AS4 IgG is shown in Fig. 3*A*. Galleries of antibody-linked subparticles in the quasisymmetric and intermediate projections (Fig. 3*B*) and in the asymmetric projection (Fig. 3*C*) are shown below the field. Single particles with attached IgGs are shown in both projections in Fig. 3*D*. The location of the antibody binding site is shown diagrammatically in Fig. 4. In the asymmetric projection (Fig. 4*C*), the antibody attachment site is on the concave surface near the constriction between the head and base. In the quasisymmetric projection (Fig. 4*B*), it is on the side opposite the cleft and platform. Hence, from these two projections, we conclude that the antibody attachment site is located on the surface of the subunit shown in Fig. 4*A*. This is the exterior surface of the small subunit—i.e., the surface that faces the cytoplasm in the 70S ribosome. Labeling by AS4 preparations obtained from three different rabbits was confined to a single site on the subparticle.

DISCUSSION

Evidence is presented that ribosomal protein S4 maps at a single region in the interior of the ribosomal subunit and is not exposed to antibody on complete ribosomal subunits.

It should be noted that this result is different from the highly elongated surface localization of S4 originally described by us (12) and others (13, 14). On reexamination of the AS4 preparation used to map S4 (12), we found that the reaction with small subunits was not S4 specific since most of it could be eliminated by previous absorption of the antibody with a small amount of protein S7 (10). The original mapping of S4 (12) should be disregarded.

Others have mapped multiple S4 sites on the surface of the small subunit (13, 14); however, they did not demonstrate that the antibody labeling of subunits was S4 dependent. The similarity of their results to our original incorrect results suggests to us that they also may have mapped a contaminant antibody reaction.

Protein S4 is an assembly protein (17) with several unique properties. Considerable evidence exists to suggest that S4 forms both a physical and a functional complex with other ribosomal components on the small subunit. The position determined for S4 is shown in Fig. 5 together with the small subunit proteins that we have previously mapped. The S4 site overlaps the position in which S5 has previously been mapped on intact small subunits (21) and lies adjacent to the position mapped in preliminary studies for S12 (unpublished results; see also ref. 3). It binds directly to 16S RNA and aids the subsequent incorporation of five other ribosomal proteins, although none strongly. The direct binding to RNA suggests that S4 may function in organizing and stabilizing the RNA in addition to facilitating the binding of other proteins. The general binding region for S4 on 16S RNA is located in the 5′ one-third of the molecule. Specific interaction sites for S4 in this region of 16S RNA have been identified by crosslinking and other methods (for reviews, see refs. 24 and 25) including, most recently, the identification of a homologous binding site on mRNA that is autogenously regulated by S4 (26).

Protein S4, together with S5 and S12, very likely forms a structural domain in the ribosome. Data from a number of experiments are in agreement with our results that S4, S5, and S12 are adjacent in the small subunit. Measurement by neutron scattering of the separation of the centers of mass in the small subunit of the pairs S4-S5 (39.7 Å), S4-S12 (39.9 Å), and S5-S12 (58.7 Å) (27, 28) has shown that the centers of mass of these proteins are in close proximity *in situ*. In addition, various reports of chemical crosslinking of S4-S5 (29–32) and S4-S12 (33, 34) using reagents that bridge short distances (less than 20 Å) also support the conclusion that these proteins are in close proximity *in situ*.

Other data suggest that S4, S5, and S12 form a functional complex. In particular, mutational alterations of both S4 and S5 are known to suppress the streptomycin-dependence phenotype of some S12 mutations (35, 36) and these three proteins appear to cooperate in ribosomal control of translational fidelity (37, 38). The participation of these proteins in the recognition process (for review, see ref. 3) suggests that they form part of the initial binding site for the elongation factor Tu–tRNA complex, the proposed R site (39).

Protein S4 maps at a single site on reconstitution intermediates. Although immunoelectron microscopy cannot prove that S4 is globular, our results provide no evidence to suggest that S4 is highly elongated *in situ* (12–14). In fact, the reactivity of 30S–S5,S12 subparticles with AS4 is comparable with that of the 16S RNA–S4 complex (10), suggesting that, of the ribosomal proteins, only S5 and S12 have a major effect on the exposure of S4 antigenic determinants. A compact globular conformation for S4 in solution (40) and associated with a 13S fragment of ribosomal 16S RNA (41) has been proposed from neutron scattering of S4 and S4–16S rRNA complexes, further suggesting that S4 assumes a compact conformation *in situ*.

Finally, we have demonstrated that the technique of immunoelectron microscopy can be applied to protein-deficient subribosomal particles if antibodies of appropriate reactivity are available. The combination of the technique of immunoelectron microscopy with *in vitro* reconstitution extends immunomapping beyond the arrangement of proteins on the subunit surface and makes possible the location of "internal" proteins in ribosomes and other reconstitutable multicomponent complexes. As the immunoelectron microscopic resolution is improved by

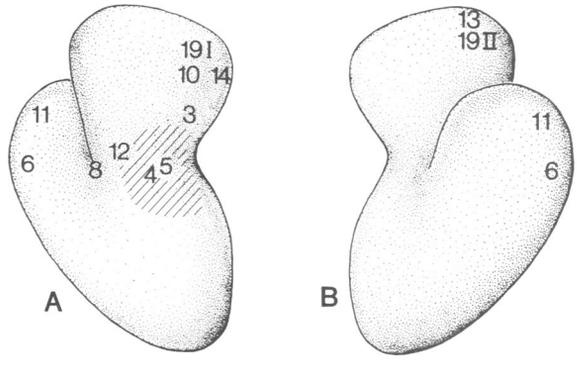

FIG. 5. Locations of small subunit proteins mapped in our previous studies and their relationship to the internal S4 site mapped in this paper (diagonal shading). (*A*) Exterior surface of the small subunit. (*B*) Interface surface of the small subunit.

the use of determinant-specific monoclonal antibodies and improved microscopic analysis, the use of planned reconstructed particles should permit detailed studies of the interior structures of ribosomes and other organelles.

We thank A. Kost, D. Williams, and J. Beyer for excellent electron microscopy and photography. This work was supported by grants from the National Science Foundation (PCM-14718 to J.A.L.) and from the National Institute of General Medical Sciences (GM-22150 to L.K., Training Grant GM-17215, and GM-24034 to J.A.L.).

1. Lake, J. A. (1979) in *Ribosomes*, eds. Chambliss, G., Craven, G., Davies, J., Davis, K., Kahan, L. & Nomura, M. (Univ. Park Press, Baltimore, MD), pp. 207–236.
2. Stöffler, G., Bald, R., Kastner, B., Lührmann, R., Stöffler-Meilicke, M., Tischendorf, G. & Tesche, B. (1979) in *Ribosomes*, eds. Chambliss, G., Craven, G., Davies, J., Davis, K., Kahan, L. & Nomura, M. (Univ. Park Press, Baltimore, MD), pp. 171–206.
3. Lake, J. A. (1979) in *Transfer RNA: Structure, Properties, and Recognition*, eds. Schimmel, P., Söll, D. & Abelson, J. N. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 393–411.
4. Politz, S. M. & Glitz, D. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1468–1472.
5. Strycharz, W. A., Nomura, M. & Lake, J. A. (1978) *J. Mol. Biol.* **126,** 123–140.
6. Keren-zur, M., Boublik, M. & Ofengand, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1054–1058.
7. Shatsky, I. N., Mochalova, L. V., Kojouharova, M. S., Bogdanov, A. A. & Vasiliev, V. D. (1979) *J. Mol. Biol.* **133,** 501–515.
8. Bernabeu, C. & Lake, J. A. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 3111–3115.
9. Vasiliev, V. D., Koteliansky, V. E. & Rezapkin, G. V. (1977) *FEBS Lett.* **79,** 170–174.
10. Winkelmann, D. A. & Kahan, L. (1979) *J. Supramol. Struct.* **10,** 443–455.
11. Winkelmann, D. A. & Kahan, L. (1979) in *Ribosomes*, eds. Chambliss, G., Craven, G., Davies, J., Davis, K., Kahan, L. & Nomura, M. (Univ. Park Press, Baltimore, MD), pp. 255–265.
12. Lake, J. A., Pendergast, M., Kahan, L. & Nomura, M. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 4688–4692.
13. Stöffler, G. & Tischendorf, G. W. (1975) in *Topics in Infectious Disease*, eds. Drews, L. & Hahn, F. E. (Springer, New York), pp. 117–143.
14. Tischendorf, G. W. & Stöffler, G. (1975) *Mol. Gen. Genet.* **142,** 193–208.
15. Held, W. A., Mizushima, S. & Nomura, M. (1973) *J. Biol. Chem.* **248,** 5720–5730.
16. Higo, K., Held, W. A., Kahan, L. & Nomura, M. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 944–948.
17. Held, W. A. & Nomura, M. (1974) *Biochemistry* **12,** 3273–3281.
18. Winkelmann, D. A. (1980) Dissertation (Univ. of Wisconsin–Madison, Madison, WI).
19. Lake, J. A. (1979) *Methods Enzymol.* **61,** 250–257.
20. Kenny, J. W., Lambert, J. M. & Traut, R. R. (1979) *Methods Enzymol.* **59,** 534–550.
21. Lake, J. A. & Kahan, L. (1975) *J. Mol. Biol.* **99,** 631–644.
22. Lake, J. A. (1976) *J. Mol. Biol.* **105,** 131–159.
23. Kahan, L., Winkelmann, D. A. & Lake, J. A. (1981) *J. Mol. Biol.* **145,** 193–214.
24. Noller, H. F. (1979) in *Ribosomes*, eds. Chambliss, G., Craven, Davies, J., Davis, K., Kahan, L. & Nomura, M. (Univ. Park Press, Baltimore, MD), pp. 3–22.
25. Zimmermann, R. A. (1979) in *Ribosomes*, eds. Chambliss, G., Craven, G., Davies, J., Davis, K., Kahan, L. & Nomura, M. (Univ. Park Press, Baltimore, MD), pp. 135–170.
26. Nomura, M., Yates, J. L., Dean, D. & Post, L. E. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 7084–7088.
27. Langer, J. A., Engelman, D. M. & Moore, P. B. (1978) *J. Mol. Biol.* **119,** 463–485.
28. Ramakrishnan, V. R., Yabuki, S., Sillers, I.-Y., Schindler, D. G., Engelman, D. M. & Moore, P. B. (1981) *J. Mol. Biol.* **153,** 739–760.
29. Lutter, L. C., Zeichhardt, H., Kurland, C. G. & Stöffler, G. (1972) *Mol. Gen. Genet.* **119,** 357–366.
30. Peretz, H., Towbin, H. & Elson, D. (1976) *Eur. J. Biochem.* **63,** 83–92.
31. Sommer, A. & Traut, R. R. (1976) *J. Mol. Biol.* **106,** 995–1015.
32. Expert-Bezançon, A., Barritault, D., Milet, M., Guerin, M.-F. & Hayes, D. H. (1977) *J. Mol. Biol.* **112,** 603–629.
33. Sommer, A. & Traut, R. R. (1975) *J. Mol. Biol.* **97,** 471–481.
34. Traut, R. R., Lambert, J. M., Boileau, G. & Kenny, J. W. (1979) in *Ribosomes*, eds. Chambliss, G., Craven, G., Davies, J., Davis, K., Kahan, L. & Nomura, M. (Univ. Park Press, Baltimore, MD), pp. 89–110.
35. Bjare, U. & Gorini, L. (1971) *J. Mol. Biol.* **57,** 423–435.
36. Hasenbank, R., Guthrie, C., Stöffler, G., Wittmann, H. G., Rosen, L. & Apirion, D. (1973) *Mol. Gen. Genet.* **127,** 1–18.
37. Gorini, L. (1971) *Nature (London)* **234,** 261–264.
38. DeWilde, M., Cabezon, T., Villarreal, R., Herzog, A. & Bollen, A. (1975) *Mol. Gen. Genet.* **142,** 19–33.
39. Lake, J. A. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1903–1907.
40. Serdyuk, I. N., Gogia, Z. N., Venyaminov, S. Yu., Khechinashvili, N. N., Bushuev, V. N. & Spirin, A. S. (1980) *J. Mol. Biol.* **137,** 93–107.
41. Serdyuk, I. N., Shpungin, J. L. & Zaccai, G. (1980) *J. Mol. Biol.* **137,** 109–121.