DNA hybridization electron microscopy: Ribosomal RNA nucleotides 1392–1407 are exposed in the cleft of the small subunit

(ribosome structure/oligodeoxyribonucleotides/rRNA function/peptidyl site/phylogenetic comparisons)

MELANIE I. OAKES, MICHAEL W. CLARK, ERIC HENDERSON, AND JAMES A. LAKE

Molecular Biology Institute and Department of Biology, University of California, Los Angeles, CA 90024

ABSTRACT   The ribosomal sequence corresponding to *Escherichia coli* 16S rRNA nucleotides 1392–1407 (the "1400 region") is phylogenetically conserved and is in a functionally important region of the subunit. Using the technique of DNA hybridization electron microscopy, we have mapped this sequence on the surface of the small ribosomal subunit. In this procedure a synthetic oligodeoxynucleotide probe, complementary to a specific rRNA sequence and carrying an attached marker molecule, is hybridized to ribosomal subunits in order to determine the dimensional site of attachment. In the *E. coli* ribosome, the 1400 region is located at the level of the neck, near the cleft and most likely on the head of the small subunit. The related sequence in yeast 18S rRNA, nucleotides 1618–1633, is located in the topological equivalent of the *E. coli* site. The location of this region, which has been crosslinked to the anticodon of a peptidyl-site-bound tRNA, indicates that this part of the cleft of the small subunit has a similar three-dimensional organization in phylogenetically diverse organisms and suggests that it is the site of the codon–anticodon interaction.

The ribosomal sequence corresponding to *Escherichia coli* 16S rRNA nucleotides 1392–1407, the "1400 region," is highly conserved phylogenetically and significant functionally. This sequence probably is exposed in active small subunits, since within this region, nucleotide G_{1405} can be modified by kethoxal (1–3). The region also appears to be adjacent to an anticodon binding site, since C_{1400} can be crosslinked to the "wobble" nucleotide of a modified tRNA bound to the peptidyl tRNA binding (P) site (4). In yeast 18S rRNA the identical sequence is found at positions 1618–1633. The yeast 18S rRNA exhibits primary and secondary structural homology to *E. coli* 16S rRNA and has a similar reactivity with kethoxal (2). This suggested that the 1400-region-like rRNA sequence in both these subunits could be located at similar three-dimensional sites.

Relatively little is known concerning the location of the 1400 sequence and, indeed, little is known of the tertiary structure of the ribosomal RNAs. Locations have been determined for the 5' and 3' ends of rRNA and for several modified nucleotides of 16S rRNA (5–11). Other locations have been surmised from immunoelectron microscopy of ribosomal proteins that have been crosslinked to, or shown to protect, particular domains, sequences, or even nucleotides in rRNA (reviewed in ref. 12). It is clear, however, for the majority of sequences, like the 1400 region, that a general method for mapping specific rRNA nucleotides is needed.

We have devised a general method for determining the location of the 1400 sequence that can, in principle, be applied to many rRNA regions. DNA probes are constructed that will specifically hybridize to complementary regions of rRNA, and the DNA is labeled with biotin in order to detect the probe binding sites by electron microscopy. The strong binding of avidin to biotin and the ability to visualize avidin directly by electron microscopy make biotin a useful label. Using this technique, we find that nucleotides 1392–1407 on the *E. coli* small subunit and nucleotides 1618–1633 on the yeast ribosomal subunit are located in topologically equivalent sites. Both sites are near the cleft of the small subunit at the level of the neck and most probably on the head rather than on the platform. This location, which has been crosslinked to the anticodon of a P-site-bound tRNA (4), strongly suggests that the cleft of the small subunit is the location of the ribosomal decoding site.

MATERIALS AND METHODS

Reagent grade chemicals were used for all experiments. Terminal deoxynucleotidyltransferase was from Bethesda Research Laboratories; T4 polynucleotide kinase, from P-L Biochemicals; ^{125}I-labeled avidin, from New England Nuclear; 5-[*N*-(*N*-biotinyl-ε-aminocaproyl)-3-aminoallyl]-2'-deoxyuridine 5'-triphosphate (biotin-dUTP), from Enzo Biochemicals (New York); and avidin DN, from Vector Laboratories (Burlingame, CA).

Purification of Ribosomes. *E. coli* (strains Q13 and MRE600) ribosomes and subunits were prepared according to Clark (13). Yeast [*Saccharomyces cerevisiae* strain RNase⁻3aleu2, kindly supplied by A. E. Dahlberg (Brown University, Providence, RI)] were grown in Ym-1 medium at room temperature (14). Yeast ribosomes and subunits were purified according to the protocols of Lozano *et al.* (15) and Battaner and Vazquez (16), with some modifications. Subunits were pooled and pelleted by centrifugation.

Synthesis and Purification of DNA Probes. DNA was synthesized by the phosphoramidite method (17), using an Applied Biosystems (Foster City, CA) model 380A DNA synthesizer. The probes were electrophoresed in a 20% acrylamide/7 M urea gel. DNA probe was eluted from slices of the gel and dried by evaporation on a Vortex. When required, DNA probes were end-labeled by use of [γ-³²P]ATP and T4 polynucleotide kinase or were biotinylated by use of Bio-dUTP and terminal deoxynucleotidyltransferase essentially as described (18). The 1400-region probe sequence was 5' TGACGGGCGGTGTGAAC 3' and the mismatch-probe sequence was 5' CATTGCACTAACCTCCTGATGTCC 3'.

Hybridization and Analysis of Binding of ³²P-Labeled DNA to Small Ribosomal Subunits. Heat-activated small subunits (75 μg of protein) were incubated with ³²P-labeled DNA for 30 min at 30°C in a reaction volume of 10 μl. The buffer for *E. coli* subunits was 20 mM Tris·HCl/10 mM MgCl₂/200 mM NH₄Cl, pH 7.5 (buffer A). The buffer for yeast subunits was

Abbreviation: P site, peptidyl-tRNA binding site.

20 mM Tris·HCl/10 mM MgCl$_2$/200 mM KCl, pH 7.5 (buffer B). After incubation, the reaction mixture was diluted to 200 μl with buffer A or B and loaded onto a prespun column (19) to separate subunits from unhybridized DNA. The eluant was loaded onto 5-ml sucrose gradients that contained 15–30% sucrose in buffer A or buffer B. Gradients were centrifuged in a VTi65 rotor (Beckman) for 45 min at 48,000 rpm at 4°C and monitored at 254 nm using an ISCO fractionator. Fractions were collected at constant time intervals. For scintillation counting, 2 ml of 3a70B scintillation fluid (Research Products International, Mount Prospect, IL) was added to each fraction.

Analysis of Binding of ^{125}I-Labeled Avidin. To monitor avidin binding, we followed the protocol used for the analysis of ^{32}P-labeled DNA, except that after incubation of the DNA probe and subunits, ^{125}I-labeled avidin [69 μCi/μg (1 Ci = 37 GBq), diluted 1:25 with avidin DN] was added and the incubation was continued for 30 min at 30°C. The mixture was then diluted to 200 μl with buffer A or buffer B, for *E. coli* or yeast subunits, respectively, and prepurified by centrifugational chromatography (19) before sucrose density centrifugation.

Electron Microscopy. Ribosomal subunits (345 μg) were incubated with the biotinylated probe for 30 min at 30°C in a reaction volume of 10 μl in buffer A or B, for *E. coli* or yeast subunits, respectively. The subunits were in a 10-fold molar excess to DNA. Avidin DN was then added to the mixture and the incubation continued for 30 min at 30°C. The samples were diluted to 200 μl and loaded onto sucrose gradients for density centrifugation as described above. Sucrose was removed from the dimer (or monomer—not shown) fraction by centrifugational chromatography (19). The eluant was negatively stained using the double-layer carbon technique (20) and micrographs were obtained with a Philips 400 electron microscope.

RESULTS

Purification and Biotinylation of the 1400-Region Probe. The 1400-region DNA probe was purified from contaminating shorter oligomers by polyacrylamide gel electrophoresis. After purification, the ^{32}P-labeled probe ran as a single radioactive band in a polyacrylamide gel (Fig. 1, lane A). Upon biotinylation, biotin-dUMPs were added in a bimodal distribution to the 3' end of the probe. The most strongly labeled bands corresponded to additions of two and of more than four biotinylated nucleotides (Fig. 1, lane B). The

reaction was essentially complete, with >80% of the probe molecules labeled with at least one biotin-dUMP nucleotide. We find that these biotinylated probes are stable for several months at −80°C and can be used for subsequent reactions without further purification.

The 1400-Region Probe Hybridizes to Ribosomal Small Subunits from *E. coli* and from Yeast. ^{32}P-labeled, biotinylated probe readily hybridizes to small ribosomal subunits and forms a complex that is relatively stable during sucrose density centrifugation (Fig. 2). In DNA-probe excess, up to 60% of both prokaryotic and eukaryotic small subunits can be labeled with probe. In contrast, under these conditions, no significant probe hybridizes to *E. coli* large subunits (data not shown). This indicates that the 1400 sequence is sufficiently exposed for significant DNA binding to occur and that this region can be mapped. Our initial attempts to attach avidin to the biotinylated DNA–ribosomal subunit complexes were unsuccessful.

Avidin can, however, be bound to the small subunit through the biotin-DNA if the 3' end of the probe is extended. When a biotinylated probe was synthesized with an additional 10 deoxycytidylate nucleotides at its 3' end (i.e., sequence 5' TGACGGGCGGTGTGAACCCCCCCCCCCC 3'), avidin binding was increased to levels that were comparable to the stoichiometry of DNA binding. Further, the avidin binding obtained with the extended probe was strictly dependent on the presence of the probe. In the absence of a DNA probe, no stable avidin binding to either 30S or 40S small subunits occurred (Figs. 3A and 4A, respectively). Addition of a biotinylated DNA probe with a sequence that was not complementary to 16S or 18S rRNA did not result in significant ^{125}I-labeled avidin sedimenting with the subunits (Figs. 3B and 4B). Only hybridization of biotinylated 1400-region probe resulted in the binding of avidin to the subunits. This binding was accompanied by the appearance of a second, faster-sedimenting radioactive peak (Figs. 3C and 4C). Electron microscopy showed that the faster-sedimenting peak principally corresponded to two subunits linked by one or more avidin tetramers. This peak also contained contaminating unlabeled small subunits from the trailing edge of the monomer peak (see Figs. 5A and 6A). Electron microscopy showed that the slower-sedimenting radioactive peak corresponded to single subunits labeled with avidin.
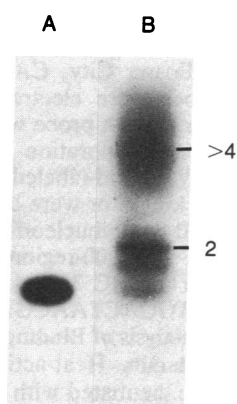

FIG. 1. Polyacrylamide gel electrophoresis of the purified 1400-region probe before (lane A) and after (lane B) biotinylation. As indicated at right, a major component contains two 3'-terminal biotin-dUMP units, but molecules with three or more additions are also present.
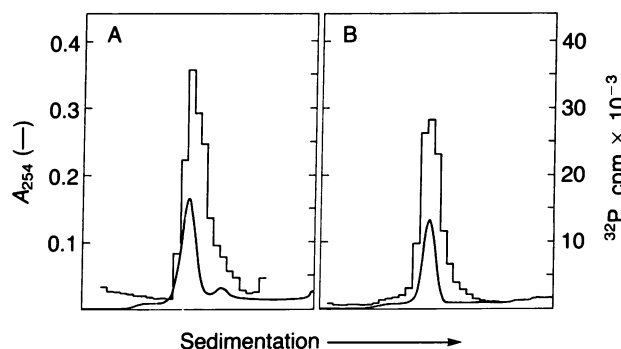

FIG. 2. The 1400-region probe binds tightly to both *E. coli* (A) and *S. cerevisiae* (B) small ribosomal subunits. Small subunits hybridized with DNA probes were centrifuged in sucrose gradients. Radioactivity at the left of each panel (i.e., at the top of the gradients) corresponds to DNA released from subunits during centrifugation. The ^{32}P-labeled, biotinylated 1400-region DNA probe was incubated with heat-activated subunits at subunit/DNA molar ratios of 20:1. The specific activity of the probe was determined by thin-layer chromatography on PEI-cellulose and was 8.5 μCi/μg. The results indicated that 64% and 40% of the probe molecules bound to 30S (*E. coli*) and 40S (yeast) subunits, respectively, and that DNA binding was stable during centrifugation.
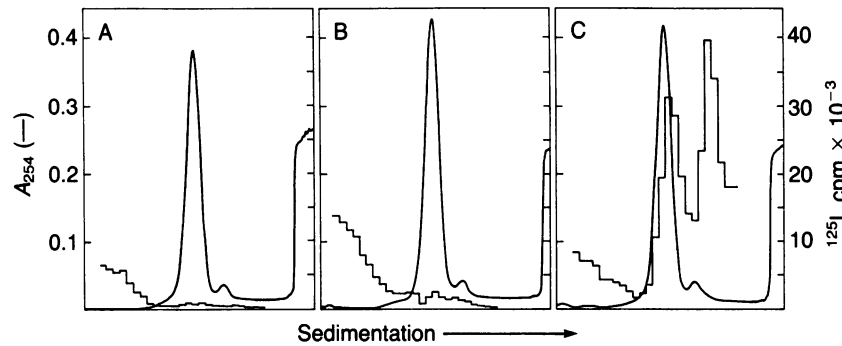
FIG. 3. The binding of ^{125}I-labeled avidin to *E. coli* ribosomal subunits in the presence or absence of DNA, analyzed by sucrose density gradient centrifugation. The specific activity of the ^{125}I-labeled avidin was 14.2 μCi/μg. (*A*) In the absence of biotinylated DNA probes, avidin did not bind to the 30S ribosomal subunits. (*B*) In the presence of the mismatched (noncomplementary) DNA probe, no binding to subunits occurred. (*C*) Two radioactive peaks were present in the presence of the 1400-region probe. The DNA/ribosome molar ratio was 0.05, hence no significant changes in optical density occurred in *C*.

When dimers and monomers from the faster- and slower-sedimenting peaks were examined by electron microscopy, it was found that chains of avidin were bound to 30S and 40S ribosomal subunits (Figs. 5 and 6) in the vicinity of the small-subunit neck. Consistent with the addition of multiple biotins to the DNA probes, and with the tetravalency of the avidin complex, chains as long as four avidins could be identified. These provided images of subunits in both the intermediate ($+40°$) and the asymmetric ($+90°$) projections of the small subunit (21). In the asymmetric projection of the subunit, avidin chains bind at the level of the neck between the head and the body in the vicinity of the platform and head. In the intermediate projection, avidin chains bind to the head at the level of the neck of the subunit in both prokaryotic and eukaryotic subunits (Figs. 5*D* and 6*D*). Our data suggest that the probe is probably attached to the head rather than to the platform. Monomeric subunits were more useful for this discrimination than were dimers. In particular, see the monomer subunits in Fig. 5 *D* (third frame) and *E* (first and third frames) and in Fig. 6*E* (third frame). We cannot entirely rule out attachment of the probe to the platform, since the separation between the head and the platform ($\approx$30 Å) is not much greater than the point-to-point resolution ($\approx$20 Å) estimated for our images (22), although the data clearly favor binding to the head.

DISCUSSION

During the past decade a general idea of ribosome structure has emerged. Through mapping studies with antibody probes, we have learned the locations of many ribosomal proteins (22, 23); of some of the ligand binding sites, such as the elongation factor EF-Tu and EF-G sites (26, 27); and of

the ends of the rRNAs (5–11). With the development of rapid sequencing techniques and of ways to reliably determine secondary structures for the ribosomal RNAs, a technique is needed for mapping specific rRNA sequences in three dimensions. DNA hybridization electron microscopy is such a technique. It is well suited for phylogenetic studies because it does not require purification of ribosomal components and the subsequent preparation of antibodies against them. In addition, unlike techniques using antibodies, probes may be synthesized in days rather than months.

The 1400 region is a unique part of ribosomal rRNA. It is conserved even among organisms from different kingdoms. The region is functionally important and is located at an exposed site on the exterior of the small subunit. Our mapping studies indicate that this region is at a topologically complex part of the small subunit. It is located near the cleft and at the back of, and approximately one-half of the way up, the head of the small subunit. In this position it is approximately at the level of, or just slightly below, the top of the platform. The platform may prevent access to the 1400 region. Although the 1400 probe can easily hybridize to its complementary sequence, the larger avidin tetramer can only react with a bound probe that has been extended (10 nucleotides). Probes directed against other regions of 16S rRNA have not required extension (unpublished results).

Adjacent to the 1400 sequence are several regions that are labeled in Fig. 7. These include the penultimate rRNA helix (nucleotides 1409–1445/1457–1491 or the "cleft anchor") and the linker (nucleotides 1492–1505) between the cleft anchor and the dimethyladenosine helix (1506–1515/1520–1529). The 1400 sequence is separated by the cleft from the dimethyladenosine helix and the 3' end of 16S rRNA (both are on the platform; refs. 5, 6, and 10) although all three
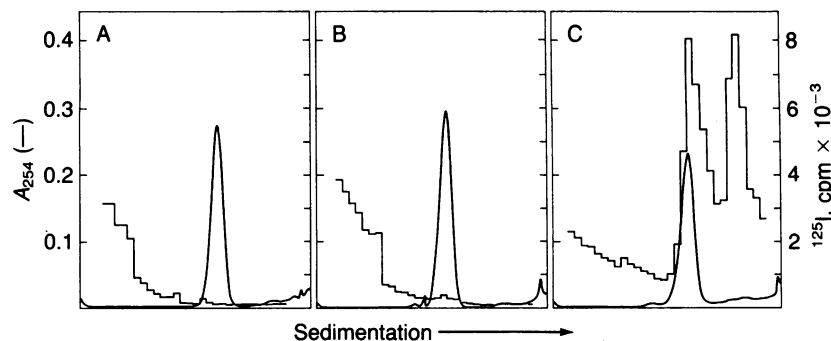

FIG. 4. The binding of ^{125}I-labeled avidin to *S. cerevisiae* 40S ribosomal subunits in the presence or absence of DNA probes, analyzed by sucrose density gradient centrifugation. Specific activity of the ^{125}I-labeled avidin was 2.53 μCi/μg. For other conditions, see legend to Fig. 3.
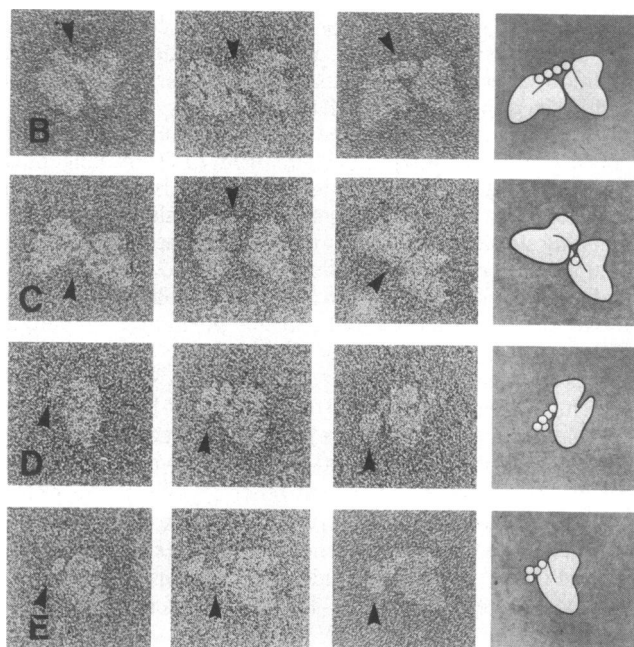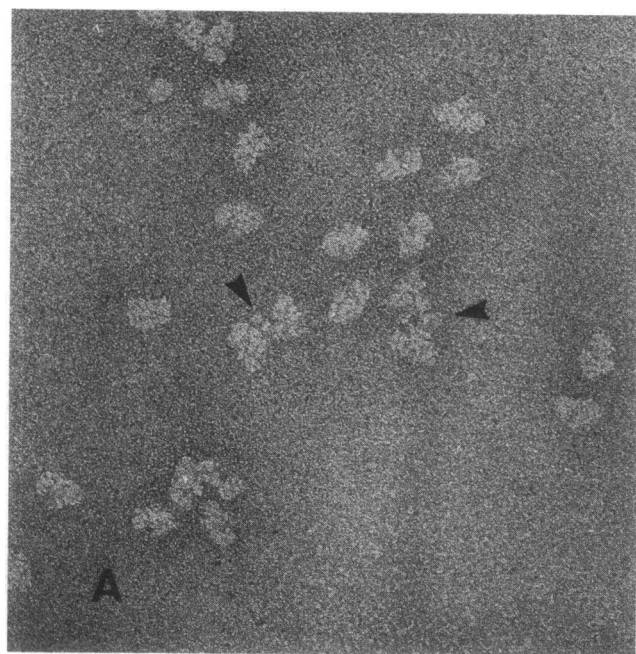
FIG. 5. Electron micrographs of eubacterial (*E. coli*) small subunits after hybridization with the 1400-region probe and labeling with avidin. (*A*) A field of small subunits with avidin molecules connecting pairs of subunits (arrowheads). (×150,000.) (*B–E*) Pairs of subunits are shown in the galleries in *B* and *C*, and monomers in the intermediate and asymmetric projection are shown in *D* and *E*, respectively. The last figure of each row is a schematic diagram of the adjacent micrograph. (×250,000.)
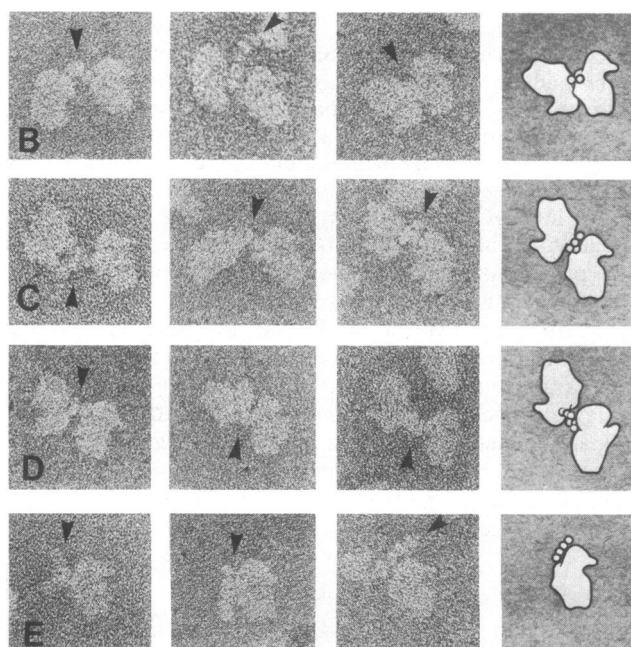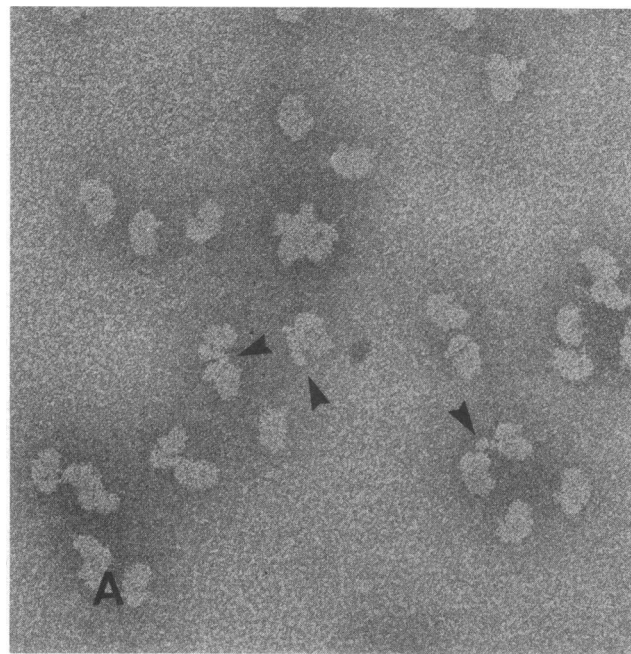
FIG. 6. Electron micrographs of eukaryotic (*S. cerevisiae*) small subunits and their reaction with the 1400-region probe. (*A*) A field of small subunits with avidin molecules indicated by arrowheads. (×150,000.) (*B–E*) Pairs of subunits in the asymmetric projection are shown in the galleries in *B* and *C*, and pairs in the intermediate projection are shown in *D*. Monomers in the asymmetric projection are shown in *E*. The last figure of each row is a schematic diagram of the adjacent micrograph. (×250,000.)

regions are in the same rRNA secondary structural domain. From the locations of the 1400 sequence on the head and the 3' end of 16S rRNA on the platform, however, we predict that the proximal base pairs of the cleft anchor line the base of the ribosomal cleft as shown in Fig. 7, since otherwise, rRNA would need to extend across the cleft. Noller and Lake (12) have argued previously that the distal end of the helix might be contained with the eukaryotic lobes that are found at the bottom of 40S subunits.

Localization of the 1400 region indirectly positions the P site. In particular, in accord with the crosslinking results of Prince *et al.* (nucleotide C_{1400} can be crosslinked directly to

the anticodon of a tRNA bound at the ribosomal P site; ref. 4), we position the P site as shown in Fig. 7. In this orientation, the codon contacts the platform, the wobble nucleotide of the P-site-bound tRNA (at the tip of the anticodon loop) can be crosslinked to C_{1400} of the rRNA, and the acceptor stem of the tRNA faces the peptidyltransferase center of the large subunit. This is consistent with data that suggest that the platform in the vicinity of the cleft is the site of the codon–anticodon interaction (23). Included in these data are immunolocalization of proteins (21) identified in mRNA crosslinking studies, localizations of the 3' and 5' ends of bound mRNA analogues (24), and immunolocaliza-
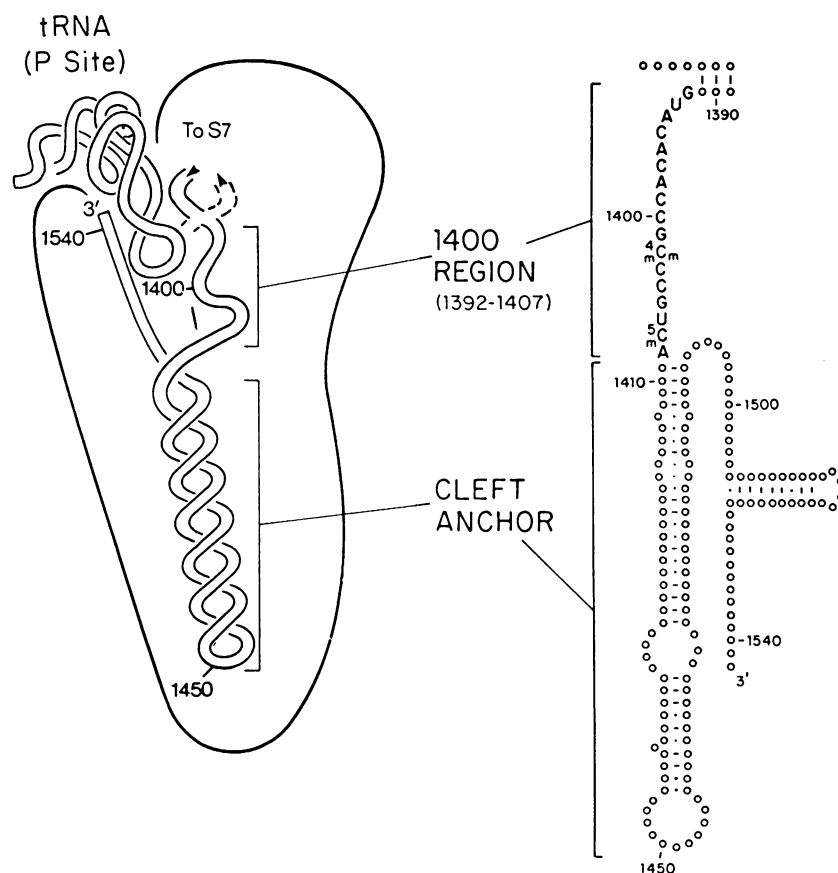
Biochemistry: Oakes *et al.*

Proc. Natl. Acad. Sci. USA 83 (1986) 279

FIG. 7. Diagrammatic representation of the location of *E. coli* rRNA sequence 1392–1407. (*Left*) The locations inferred for the penultimate helix, the cleft anchor, and for the P site are shown together with the 1400 site. (*Right*) The 3'-terminal domain of the 16S secondary structure.

tion of the anticodon of a modified tRNA (25). Thus our localization of the 1400 region appears to be consistent with a body of data on the functioning of the head–cleft–platform region of the small ribosomal subunit.

DNA hybridization electron microscopy promises to be a useful and needed technique for mapping the locations of specific RNA sequences. It is easily applied to complexes from phylogenetically diverse organisms and the structural information it provides nicely complements the emerging knowledge of rRNA primary and secondary structure. We hope that this method will be applied to other molecular complexes in addition to ribosomes.

1. Noller, H. F. (1974) *Biochemistry* **13**, 4694–4702.
2. Chapman, N. M. & Noller, H. F. (1979) *J. Mol. Biol.* **109**, 131–149.
3. Vassilenko, S. K., Carbon, P., Ebel, J. P. & Ehresmann, C. (1981) *J. Mol. Biol.* **152**, 699–719.
4. Prince, J. B., Taylor, B. H., Thurlow, D. L., Ofengand, J. & Zimmerman, R. A. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5450–5454.
5. Shatsky, I. N., Mochalova, M. S., Kojoriharova, M. S., Bogdanov, A. A. & Vasiliev, V. D. (1979) *J. Mol. Biol.* **133**, 505–515.
6. Olsen, H. M. & Glitz, D. G. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1054–1058.
7. Shatsky, I. N., Evstafieva, A. G., Bystrova, T. F., Bogdanov, A. A. & Vasiliev, V. D. (1980) *FEBS Lett.* **121**, 97–100.
8. Stoffler-Meilicke, M., Stoffler, G., Odom, O. W., Zinn, A., Kramer, G. & Hardesty, B. (1982) *Proc. Natl. Acad. Sci. USA* **78**, 5538–5542.
9. Clark, M. W. & Lake, J. A. (1983) *J. Bacteriol.* **157**, 971–974.
10. Politz, S. M. & Glitz, D. G. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1468–1972.
11. Trempe, M. R., Ohgi, K. & Glitz, D. G. (1982) *J. Biol. Chem.* **257**, 9822–9829.
12. Noller, H. F. & Lake, J. A. (1984) in *Membrane Structure and Function*, ed. Bittar, E. (Wiley, New York), pp. 217–297.
13. Clark, M. W. (1982) Dissertation (Univ. of California, Los Angeles), pp. 102–106.
14. Harnwell, L. H. (1967) *J. Bacteriol.* **93**, 1662–1670.
15. Lozano, A., Sandoval, A. & Heredia, C. F. (1974) *Methods Enzymol.* **30**, 254–261.
16. Battaner, E. & Vazquez, D. (1971) *Methods Enzymol.* **20**, 446–449.
17. Beaucage, S. L. & Caruthers, M. H. (1981) *Tetrahedron Lett.* **22**, 1859–1864.
18. Bollun, F. J. (1974) in *The Enzymes*, ed. Boyer, P. D. (Academic, New York), Vol. 10, pp. 148–198.
19. Langer, J. A., Jurnak, F. & Lake, J. A. (1984) *Biochemistry* **23**, 6171–6178.
20. Lake, J. A. (1979) *Methods Enzymol.* **61**, 250–257.
21. Lake, J. A. & Kahan, L. (1975) *J. Mol. Biol.* **99**, 631–644.
22. Lake, J. A. (1981) *Sci. Am.* **245**, 84–97.
23. Lake, J. A. (1985) *Annu. Rev. Biochem.* **54**, 507–530.
24. Evstafieva, A. G., Shatsky, I. N., Bogdanov, A. A., Semenkov, V. P. & Vasiliev, V. D. (1983) *EMBO J.* **2**, 799–804.
25. Gornicki, P., Nurse, K., Hellmann, W., Boublik, M. & Ofengand, J. (1984) *J. Biol. Chem.* **259**, 10493–10498.
26. Langer, J. A. & Lake, J. A. *J. Mol. Biol.*, in press.
27. Girsovich, A. S., Kurtschaliov, T. V., Ovchinnikov, Yu., & Vasiliev, V. D. (1981) *FEBS Lett.* **130**, 54–59.