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Involvement of bases 787–795 of *Escherichia coli* 16S ribosomal RNA in ribosomal subunit association

(oligodeoxynucleotide probes/hybridization/RNase H/RNA sequencing)

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ABSTRACT **A nine-base DNA oligomer [d(GTATCTAAT)] was used to probe the accessibility and function of bases in the region 787–795 of *Escherichia coli* 16S rRNA. Hybridization of the cDNA [d(GTATCTAAT)] to 16S rRNA *in situ* was carried out by binding the probe to intact 30S ribosomal subunits. Nitrocellulose filter binding showed that cDNA hybridization saturated with increasing probe concentration, suggesting that the probe was binding to a discrete site or sites. RNase H digestion of the rRNA under the DNA·rRNA hybrid and sequencing of the resultant RNA fragments verified that the cDNA probe bound specifically to the 787–795 region. Hybridization experiments using the cDNA probe showed that bases in the 787–795 region of 16S rRNA are exposed on the surface of 30S subunits. The functional role of bases 787–795 was then tested by assaying various ribosomal activities with the cDNA in place. Results of these functional assays demonstrate that this 16S rRNA region is directly involved in the association of 30S and 50S subunits.**

The functional involvement of rRNA in the protein translation cycle is becoming increasingly apparent (1). Already well documented is the role rRNA plays in mRNA initiation (2) and tRNA binding (3–6). In addition, some rRNA sites have been implicated in the peptidyltransferase center (7), and subunit–subunit interactions (8–10).

Exposed regions of the rRNA have been probed using chemical modification and nuclease digestion techniques. Examples include guanine-specific kethoxal (11), adenine-specific dimethyl sulfate (12), and base-specific ribonuclease T1 (10). A kethoxal-reactive site, guanosine-791, in the 30S subunit was found to be protected from modification upon 70S ribosome formation (13), suggesting that this site might be utilized in subunit association. A subsequent kethoxal modification study showed that when this base was modified, the ability of the subunits to associate was impaired (8).

Additional evidence that the stem and loop structure surrounding guanosine-791 is accessible was shown by mapping the rRNA binding sites of the 16S central-domain-binding proteins (14). These results, coupled with those from chemical modification studies suggest that the single-strand loop 787–795 is exposed, most likely in the interface region where the 50S subunit binds to the 30S subunit, and this rRNA region is apparently functional during ribosomal subunit association.

We have recently begun probing various rRNA regions using chemically synthesized DNA oligomers that are complementary to specific regions of the rRNA. Successful hybridization of these cDNA probes to rRNA *in situ* demonstrates the accessibility of the RNA region on the surface of the ribosome. The function of the exposed region can also be assayed by observing disruption of discrete ribosomal functions such as subunit association, tRNA binding, or mRNA binding with the cDNA in place. Work with cDNA probes by Mankin *et al.* (15) has identified a surface-exposed region of rRNA near the 5′ end of 16S rRNA. A somewhat more extensive cDNA probe study on the 3′ end of 16S rRNA has been carried out by Backendorf *et al.* (16).

To investigate the location and function of the rRNA region near guanosine-791, we have used an oligonucleotide complementary to bases 787–795 of the 16S rRNA [d(GTATCTAAT)]. We have demonstrated that the probe binds to the 30S subunit and that the binding is specific. Confirmation that the binding occurred at the region around base 791 was made by digesting the rRNA under the probe with RNase H and sequencing the 5′ end of the smaller fragment for a short distance away from the duplexed region. Finally, the functional role of this region in subunit association and protein translation was assessed by testing these functions in the presence and absence of bound probe.

MATERIALS AND METHODS

Ribosomes were prepared from *Escherichia coli* strain MRE 600 by the methods of Hill *et al.* (17). Ribosomal subunits were then purified using zonal centrifugation as outlined by Tam and Hill (18). The purified subunits were stored in small aliquots at −70°C.

The DNA probe [d(GTATCTAAT)] was chemically synthesized using phosphotriester chemistry on a Bachem DNA synthesizer. Synthetic DNA from which the blocking groups had been removed was purified on a 2.0 × 40 cm Sephadex G-25 column followed by gel electrophoresis and elution of the full length oligomer from the gel (19). Oligomers were stored until use at −70°C. Purified DNA probe was 5′-end labeled using [γ-^{32}P]ATP (New England Nuclear) and T4 polynucleotide kinase (Bethesda Research Laboratories) according to the method of Chaconas and van de Sande (20) with the omission of the dephosphorylation step.

The binding of the probe to ribosomal subunits was carried out by incubating 30S, 50S, or 70S ribosomes with 5′-^{32}P-end-labeled probe for 0.5–4 hr at 0°C in 50–200 μl binding buffer (10 mM Tris·HCl, pH 7.4/60 mM KCl/10 mM $MgCl_2$). For binding assays, the reaction mixtures were filtered through nitrocellulose filters and washed five times with 1-ml aliquots of binding buffer. Filters were dried, and the radioactivity of the complexed subunits was determined using liquid scintillation counting. Binding reactions were also assayed using sucrose gradient centrifugation. In this experiment binding reaction mixtures were layered onto a 4-ml 5–20% (wt/vol) sucrose gradient in binding buffer. The gradients were then centrifuged in a Beckman SW 60 rotor at 54,000 rpm for 3 hr 20 min at 4°C. Gradient fractions were assayed for 30S migration spectrophotometrically at 260 nm, and probe migration was monitored by counting the fractions using liquid scintillation.

To determine the exact site of cDNA probe base pairing, the heteroduplex probe–30S complex was incubated with

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RNase H, and the digestion products were analyzed. The conditions were similar to those outlined by Donis-Keller (21) and Mankin *et al.* (15). Digestion reaction mixtures (20 μl) containing 10–20 μg of 30S subunits, 10–20 μg of cDNA probe and 1–3 units of RNase H (P-L Biochemicals) in 40 mM Tris·HCl (pH 7.9), 10 mM $MgCl_2$, 60 mM KCl, and 1 mM dithiothreitol were incubated at 4°C for 18 hr. RNA was extracted and purified by using three consecutive extractions with buffer-equilibrated phenol, by precipitating with 2.5 vol of 95% ethanol at −70°C for 1 hr, and by pelleting by centrifugation. RNA pellets were resuspended in 20 μl of 8 M urea, 0.025% bromphenol blue, and 0.025% xylene cyanol and electrophoresed for 6 hr at 12.5 mA in a 4% polyacrylamide gel containing 7 M urea, 89 mM Tris·borate (pH 8.3), and 2 mM EDTA. RNase H fragment bands were observed by UV shadowing, eluted from the gel (22), labeled at their 5′ ends with ^{32}P (20), and partially sequenced after digestion with T1 and U2 ribonucleases (P-L Biochemicals) by electrophoresis on a 20% acrylamide sequencing gel (23).

Association of 50S subunits with 30S subunits to form active 70S ribosomes was performed by using a slight modification of the technique outlined by Herr and Noller (9). Equimolar quantities of 30S and 50S subunits were incubated in 20 mM Tris·HCl (pH 7.4), 60 mM KCl, 10 mM $MgCl_2$ for 50 min at 37°C. Samples were chilled and centrifuged through a 13-ml 5–20% (wt/vol) sucrose gradient in the same buffer for 4.5 hr at 37,000 rpm in a Beckman SW 41 rotor at 4°C. Gradient fractions were assayed spectrophotometrically to determine the positions of the 70S, 50S, and 30S species.

The translational activity of ribosomes was determined by measuring poly(U)-directed [^{14}C]phenylalanine incorporation into trichloroacetic acid (TCA) precipitable polypeptides. The *in vitro* protein synthesis protocol was adapted from Traub *et al.* (24).

RESULTS

Binding of the cDNA probe to 30S subunits was demonstrated by two techniques. In the gradient assay (Fig. 1), binding was indicated by the comigration of ^{32}P-end-labeled probe and the 30S subunits. However, the fraction of 30S subunits carrying bound probe was quite low, presumably due to the nonequilibrium conditions for the binding of the probe during centrifugation.

A more sensitive and analytical technique is the filter-binding assay (Fig. 2). In this experiment reaction mixtures containing ^{32}P-end-labeled cDNA and 30S subunits were filtered through nitrocellulose. Results from several filter-binding experiments showed that the probe reproducibly saturated the 30S binding site(s) when 40–50% of the subunits were complexed. The same level of binding was found for 30S subunits that had been dialyzed and stored in 1 mM $MgCl_2$ as was found for 30S subunits that had been activated by incubation at 37°C for 10 min in 10 mM $MgCl_2$ (25).

It is shown in Figs. 1 and 2 that the oligonucleotide probe bound to 30S subunits. The fact that binding saturated with increasing probe concentration (Fig. 2) strongly suggests that the probe was binding to a specific site(s) on the 30S subunit. The relative affinity of the probe for the 30S binding site(s) was indicated by incubating the probe with 50S subunits (Fig. 2). Despite several single-stranded regions on the 23S rRNA with five or more bases complementary to the probe, 50S subunits bound the probe only about half as effectively as 30S subunits. These results suggest that the probe is specific for a given site(s) and binds with high affinity to 30S subunits. However, to this point there was no evidence that the probe–30S interaction occurred at the 787–795 region of 16S rRNA.

To provide this evidence RNase H was used to digest the rRNA under the heteroduplex region. Fig. 3 shows the RNA fragments obtained from RNase H digestion. As expected, two major digestion products were found, both of them quite close together. This was to be expected since a break in the 790 region should produce one fragment containing about 790 bases and the other containing about 750 bases. The control experiments show a slight amount of 790 and 750 base fragments were present in untreated 30S subunits (Fig. 3, lane 4). This suggests that the 790 region of 16S rRNA was

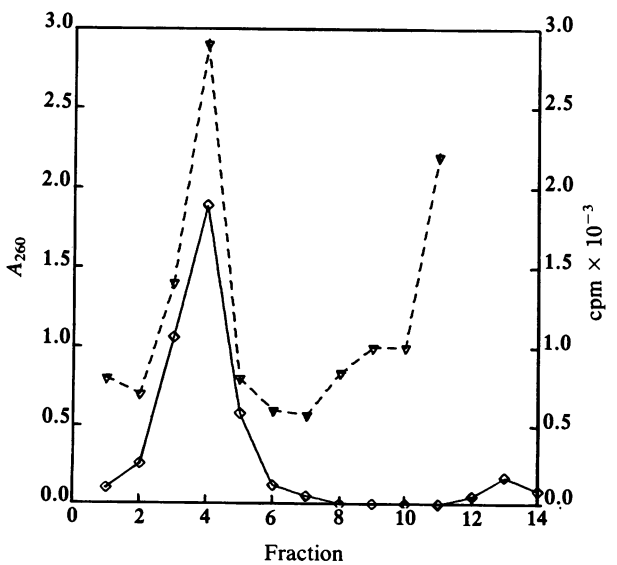

FIG. 1. Sucrose gradient analysis of cDNA probe binding to 30S subunits. 30S subunits (200 pmol) were incubated with 2000 pmol of ^{32}P-labeled probe for 30 min at 0°C in 200 μl of binding buffer (10 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/60 mM KCl). The reaction was layered on a 5–20% (wt/vol) sucrose gradient in binding buffer and centrifuged at 54,000 rpm for 3.3 hr in a SW 60 rotor. Gradient fractions were assayed for probe location by counting small aliquots (▽---▽). 30S migration was monitored by reading the absorbance at 260 nm (◇—◇). Sedimentation is from right to left.

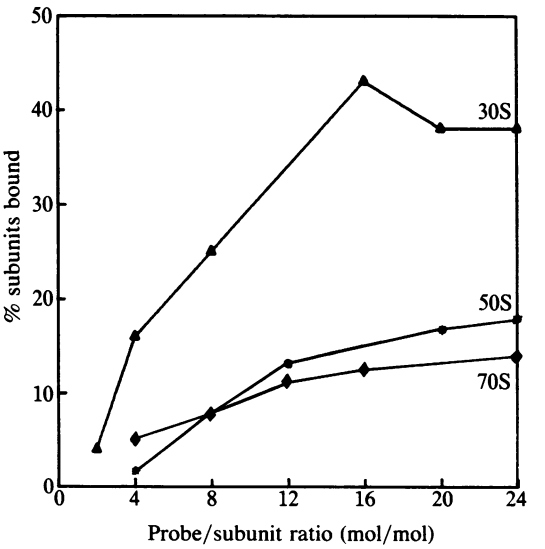

FIG. 2. Nitrocellulose filter assay of cDNA probe binding to 30S, 50S, and tight-couple 70S ribosomes. cDNA probe was labeled at the 5′ end with ^{32}P and incubated with 30S subunits for 30 min at 0°C in 50 μl of binding buffer. 50S and tight-couple 70S ribosomes were incubated under identical conditions but for 2 hr. The reaction mixtures contained 25 pmol of ribosomes and various concentrations of probe. Following incubation, the reaction mixtures were filtered through nitrocellulose and washed five times with 1 ml of binding buffer. The filters were dried, and the radioactivity was quantitated. Labeled probe (1 pmol) was 780 cpm.

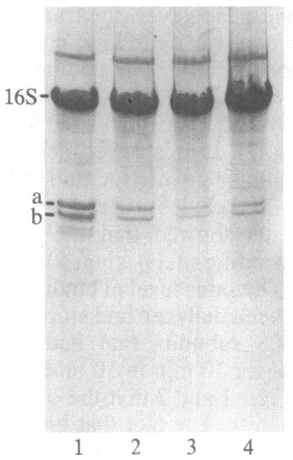

FIG. 3. RNase H digestion of probe–30S complex. Probe (20 μg) was incubated with 20 μg of 30S subunits and 3.5 units of RNase H in a 20-μl reaction mixture containing 40 mM Tris·HCl, pH 7.9/10 mM $MgCl_2$/60 mM KCl/1 mM dithiothreitol. After 18 hr at 4°C the reactions were diluted to 50 μl with buffer and extracted three times with buffer-equilibrated phenol. RNA in the aqueous phase was precipitated with 3 vol of cold 95% EtOH, pelleted, and resuspended in 20 μl of 8 M urea, 0.025% bromphenol blue and 0.025% xylene cyanol. The samples were electrophoresed on a 4% polyacrylamide gel containing 7 M urea, 89 mM Tris·borate (pH 8.3), and 2 mM EDTA for 6 hr at 12.5 mA. Lane 1 shows the products of a complete reaction. The remaining lanes represent controls showing the reaction products observed when RNase H (lane 2), the probe (lane 3), or both (lane 4) are omitted. RNA fragments produced by RNase H digestion are labeled a and b.

exposed to endogenous nuclease during ribosome preparation. The population of fragments was not increased upon RNase H addition (lane 3), but the addition of the probe by itself caused enhanced cleavage in the 790 region (lane 2). It seems the probe directed a slight amount of RNA digestion in the area where it bound.

The digestion products, labeled a and b, were excised and eluted from the gel. The purified products were then 5′-end labeled, partially digested with ribonuclease, and electrophoresed to ascertain the sequence. Fragment a gave the pattern shown in Fig. 4. Consistent with cleavage in the 787–795 region, this larger fragment gave the sequence corresponding to the 5′ end of 16S rRNA. Under the conditions used for electrophoresis, the first three bases from the 5′ end were not present.

Fragment b was treated identically, and the results are shown in Fig. 5. Despite secondary bands, indicating that RNase H clipped at several points under the probe, the most prominent bands show that the sequence matches that found starting at base 798 in 16S rRNA. Once again, three nucleotides were lost under the experimental conditions used. Nonetheless it is clear that the primary clip of RNase was at base 795, directly below the 3′ end of the probe, which verifies that the probe was binding to the 787–795 region.

To investigate the function of the probed area, the probe·30S subunit complexes were tested for their ability to form 70S ribosomes. Fig. 6 shows that the presence of the probe greatly diminished the ability of the subunits to form 70S ribosomes. In fact, the reduction of association was essentially equal to the percentage of the subunits bound by probe.

The migration of the probe in the subunit association experiment is shown in Fig. 7. Note that a large majority of the probe migrated with the 30S subunits, not the 70S ribosomes or the 50S subunits, confirming that the probe bound preferentially to the 30S subunits.

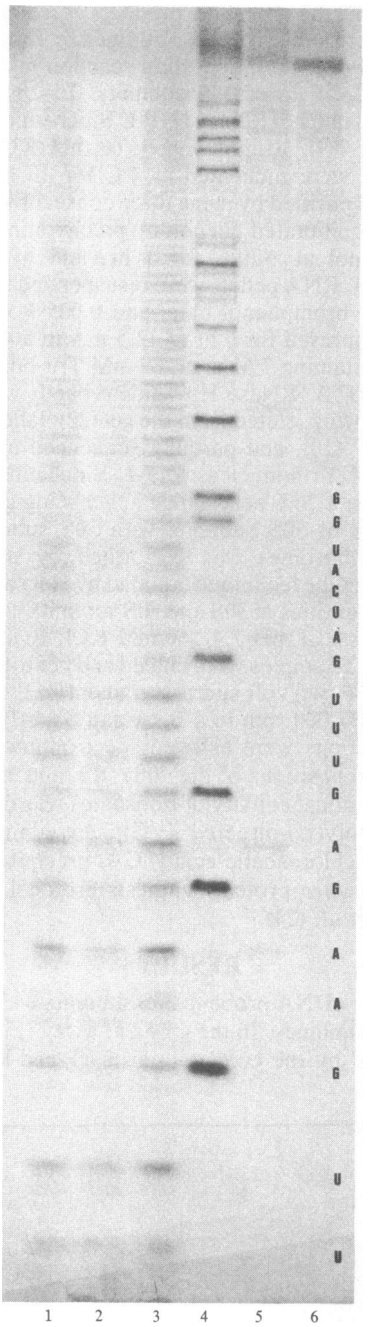

FIG. 4. Sequence of RNase H fragment a. Purified RNase H fragment a was labeled at the 5′ end with [γ-^{32}P]ATP by using polynucleotide kinase. The labeled RNA was partially digested with ribonuclease T1 and U2. Digestion at every base was carried out in alkaline conditions. Digested RNA was electrophoresed on a 0.35 mm thick 20% polyacrylamide gel for 5 hr at 1600 V. T1 digestion, lane 4; U2 digestion, lane 5; alkaline hydrolysis, lane 3.

The probe was then incubated with 70S tight-couple ribosomes to ascertain whether the complementary region was available in associated ribosomes. As shown in Fig. 2, there was very little binding of the probe to tight-couple 70S ribosomes. It can be inferred from the probe·50S data of Fig. 2 that probe binding to tight-couple 70S ribosomes was largely due to sites on the 50S subunit. These results suggest that the 50S subunit shielded the 787–795 region of 16S rRNA in the 70S ribosome. The results shown in Fig. 7 corroborate this finding as well.

The translational activity of the probe-30S complexes was tested. Reaction mixtures containing probe showed diminished protein translation (1205 cpm of [^{14}C]phenylalanine was

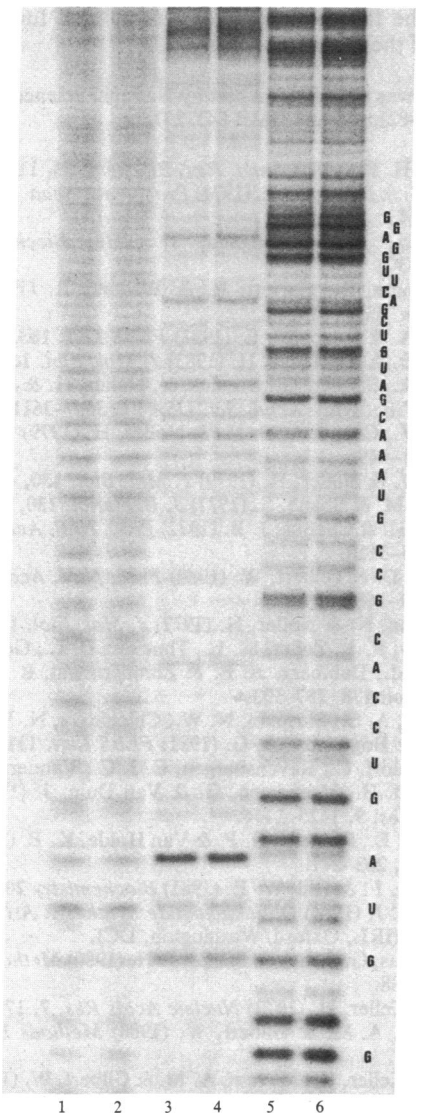

FIG. 5. Sequence of RNase H fragment b. RNase H fragment b was 5'-end labeled, digested, and electrophoresed as outlined in the legend of Fig. 4. T1 digestion, lanes 5 and 6; U2 digestion, lanes 3 and 4; and alkaline digestion ladder, lanes 1 and 2.

incorporated in the control, while 832 cpm was incorporated in the presence of the probe) suggesting that the disruption of the association of the subunits markedly diminished their ability to translate protein. As in the subunit association experiment, the extent of inhibition was comparable to the amount of complex formed.

DISCUSSION

The results of this study clearly show that oligonucleotides complementary to specific rRNA sequences can be used to investigate the location and the functions of rRNA regions on intact ribosomal subunits. Specifically it is shown that the cDNA probe [d(GTATCTAAT)] hybridizes to bases 787–795 of 16S rRNA *in situ* and that this region is necessary for subunit association and for protein translation.

These results are in complete agreement with the accepted models for the secondary structure of 16S rRNA in which bases 787–795 are shown to be a single-strand region in a loop at the end of a moderately long stem (see ref. 1). That this region is accessible was initially suggested by Chapman and Noller (13). Careful kethoxal modification studies later

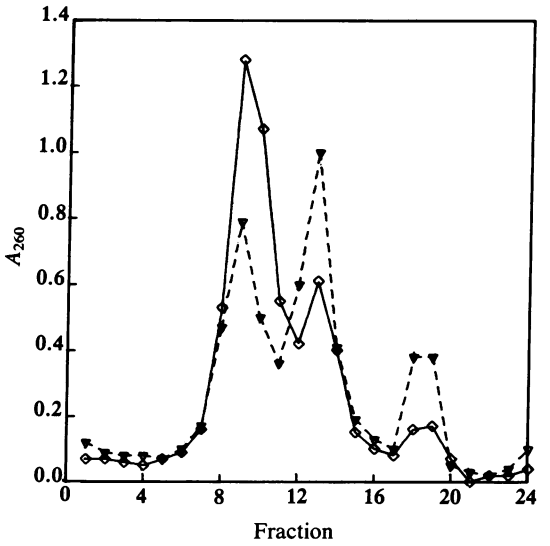

FIG. 6. 70S ribosome formation in the presence of cDNA probe. 50S and 30S subunits were combined under conditions favoring 70S ribosome formation in the presence of the probe. 50S and 30S subunits (100 pmol of each) were incubated at 37°C with 1500 pmol of ^{32}P-labeled cDNA for 50 min in 100 μl of binding buffer. The reaction was layered onto a 5–20% (wt/vol) sucrose gradient in binding buffer and centrifuged at 37,000 rpm for 4.5 hr at 4°C in a SW 41 rotor. 70S, 50S, and 30S species were located by monitoring gradient fractions spectrophotometrically at 260 nm. 70S ribosome formation without cDNA probe (◇—◇). Subunit association in the presence of probe (▽---▽). Sedimentation is from right to left.

showed guanosine-791 was important for subunit association (8). Our results confirm these studies and provide more extensive evidence of the necessity of this site for subunit–subunit interaction.

Site-specific cDNA probes, such as [d(GTATCTAAT)], have the distinct advantage of being able to modify a single site. Thus the function of a single site can be directly assayed without modifying other regions of the rRNA. It is possible that the presence of the cDNA probe itself may cause secondary effects, thereby altering the function of nearby

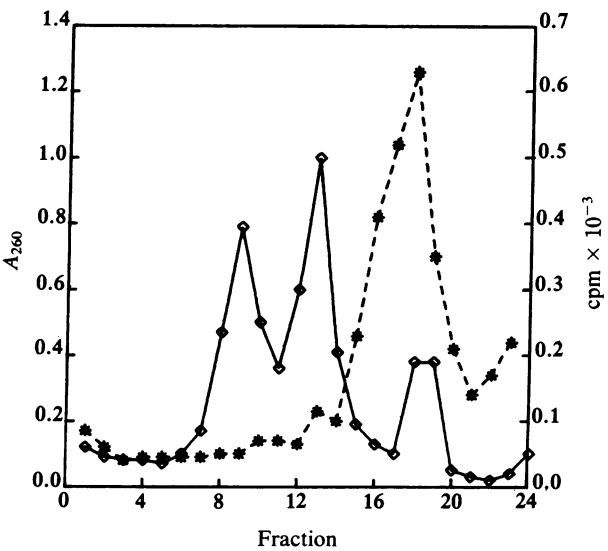

FIG. 7. Sedimentation of cDNA probe in the sucrose gradient used to generate the curves in Fig. 6. The migration of cDNA probe in the gradient was monitored by counting small aliquots of each fraction. Probe (*---*). 70S, 50S, and 30S ribosomes (identical to dashed line curve in Fig. 6, (◇—◇). Sedimentation is from right to left.

regions rather than the region in question. It would be difficult to distinguish between primary and secondary effects.

It is interesting to note that in all cDNA·30S hybridization tests to date only 40–50% of the subunits present bind the probe. This behavior has been reported by Backendorf *et al.* (16) as well. Explanations for this less than complete binding include conformational heterogeneity in the 30S subunits, protein complement heterogeneity, or low association constants. There are not yet sufficient data to distinguish between these possibilities.

The fact that the cDNA·30S complexes apparently cannot participate in protein translation is to be expected if the probe remains bound during the incubation period. However, it appears that there may be some disassociation and reassociation of the probe and the 30S subunit. Nonetheless, the overall effect is to reduce the effectiveness of the 30S subunits in the translational process.

The results of these cDNA experiments strongly suggest that bases 787–795 are located in the interface region where 30S and 50S subunits interact. As mentioned before, the central domain binding proteins S6, S8, S15, and S18 have been mapped on the 16S rRNA and encompass nearly all of the rRNA between bases 771–818 and some regions near bases 620 and 690 (14). Using the model given, the stem and loop between bases 771 and 818 appears to protrude from the proteins surrounding it, making it an ideal location for subunit interaction.

Neutron-scattering has shown that the four central domain binding proteins (S6, S8, S15, and S18) all cluster in the lower to middle platform region of the 30S subunit (26). These positions are further corroborated by crosslinking and immunohistochemical electron microscopic studies (27). The findings of Gregory *et al.* (14) would then place the stem-loop region containing bases 787–795 in this vicinity as well.

The placement of the 787–795 region on the platform is in complete agreement with all the proposed models of the coupled subunits given by electron microscopists (27) in which the platform region of the 30S subunit is shown to be adjacent to the 50S subunit. From these models it could be postulated that the stem-loop region (bases 771–818) must emerge from the side of the platform distal from the cleft.

Thus the combined results of chemical modification, nuclease digestion, neutron scattering, electron microscopy, and site-specific cDNA studies can be coupled to give a detailed structural–functional model of a specific working site on the ribosome. Without doubt the combination of these techniques and others will provide additional definitive results in the future to unravel the detailed functions and structure of the ribosome.

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