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Protein binding sites on *Escherichia coli* 16S RNA; RNA regions that are protected by proteins S7, S14 and S19 in the presence or absence of protein S9

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SUMMARY

^{14}C-labelled proteins from E. coli 30S ribosomal subunits were isolated by HPLC, and selected groups of these proteins were reconstituted with ^{32}P-labelled 16S RNA. The isolated reconstituted particles were partially digested with ribonuclease A, and the RNA fragments protected by the proteins were separated by gel electrophoresis and subjected to sequence analysis. Protein S7 alone gave no protected fragments, but S7 together with S14 and S19 protected an RNA region comprising the sequences 936-965, 972-1030, 1208-1262 and 1285-1379 of the 16S RNA. Addition of increasing amounts of protein S9 to the S7/S14/S19 particle resulted in a parallel increase in the protection of the hairpin loop between bases 1262 and 1285. The results are discussed in terms of the three-dimensional folding of 16S RNA in the 30S subunit.

INTRODUCTION

The "classical" approach to the study of RNA-protein interactions in the ribosome is the "bind and chew" method, in which a single protein is bound to the ribosomal RNA, the complex thus formed is then digested with ribonuclease, and the protected RNA fragments are subsequently identified by sequence analysis. In the case of the Escherichia coli 16S RNA this approach has yielded a considerable amount of data for the 5'-proximal half of the molecule, involving proteins S4, S8, S15, S17 and S20 (reviewed in ref. 1). On the other hand, the corresponding study of the 3'-proximal region has been less encouraging. Protein S7 is the only "primary binding protein" which attaches to this half of the molecule, and attempts to localize its binding site have not been successful (1). It seems likely that the protein binds to a region of the RNA with a complex and sensitive tertiary structure, and that binding of protein S7 alone is not sufficient to prevent this structure from falling apart under nuclease attack. This in

turn suggests that selective addition of other proteins might "stabilize" the binding site, and thus allow the differential protective effects of various proteins or groups of proteins to be studied. Experiments of this nature have already been successfully performed with E. coli ribosomal proteins, most notably in the case of proteins S6, S8, S15 and S18 with 16S RNA (2), and of proteins L5, L18 and L25 with 5S RNA (3).

Protein S7 has been shown by immune electron microscopy (e.g. refs. 4,5) and neutron scattering studies (6) to lie in the "head" region of the 30S subunit, together with proteins S3, S9, S10, S13, S14 and S19. The same proteins (without S3 and S13) were also found fifteen years ago in a ribonucleoprotein particle isolated from the 30S subunit by nuclease digestion (7), and the corresponding RNA region was shown to comprise bases ca. 920 to 1390 of the 16S molecule (8). If the physical locations (4-6) of these "head" proteins are compared with their locations in the assembly map (9) of the 30S subunit, then a number of protein groupings as potential candidates for a "differential protection study" become apparent. In this paper we describe our first experiments in this area, in which we have identified a binding region within the 16S RNA for proteins S7, S14 and S19, in the presence or absence of protein S9.

These experiments are intended to complement our current RNA-protein and intra-RNA cross-linking studies with the E. coli 30S subunit (e.g. refs. 10-13). Cross-linking is a purely topographical approach, which serves to pinpoint sites of contact or neighbourhood within the RNA or between the individual proteins and the RNA, whereas the protection studies indicate RNA regions where an extensive physical interaction with the proteins occurs. Taken together, the results of both approaches give an increasingly convincing picture of the three-dimensional folding of the 16S RNA in the "head" region of the 30S subunit.

MATERIALS AND METHODS

Isolation of ^{32}P-labelled 16S RNA: 200 ml cultures of E. coli strain MRE 600 were grown in the presence of 10 mCi ^{32}P-orthophosphate, and the 30S ribosomal subunits isolated by the method of Stiege et al (14), omitting the dialysis steps. The 30S sub-

units were layered on the top of 7.5 -30% sucrose gradients in the presence of 0.1% SDS, 2 mM EDTA, 10 mM Tris-HCl pH 7.8, 4 mM 2-mercaptoethanol, and were centrifuged at 25,000 rpm for 19 h at 10° in a Beckman SW 27 rotor. Ribosomal proteins remain at the top of these gradients. Fractions containing ^{32}P-labelled 16S RNA were pooled, and the RNA was precipitated with ethanol, collected by low-speed centrifugation, and finally re-dissolved in 4 mM magnesium acetate, 2 mM EDTA, 10 mM Tris-HCl pH 7.8. The yield of RNA at this stage was ca. 10 A_{260} units, 5 x 10^8 counts/min total.

Preparation of ^{14}C-labelled ribosomal proteins: 1 litre cultures of E. coli strain MRE 600 or A19 were grown in minimal medium in the presence of 0.25 mCi amino acid mixture (Amersham, CFB 104), and the 30S ribosomal subunits isolated by the procedure of ref. 7. The 30S subunits (ca. 180 A_{260} units, 5 x 10^7 counts/min total) were diluted 1:3 with unlabelled 30S subunits, and the proteins were extracted by the acetic acid/magnesium method of Hardy et al (15). After extensive dialysis against 2% acetic acid, the proteins were concentrated in vacuo, and then separated by HPLC using a Vydac TR-RP column (C18, particle diameter 5 µm, pore size 300 Å, column dimensions 250 mm x 4.6 mm) as described by Kamp et al (16). Four column runs were made, each with 25% of the protein sample. The columns were eluted with 0.1% trifluoracetic acid containing a gradient of 2-propanol, as in the latter publication (16). Column eluates were monitored at 220 nm, and 0.5 ml fractions were collected. The identity and purity of the proteins in the various column fractions were tested by two-dimensional gel electrophoresis in the system of Geyl et al (17). For this purpose an aliquot of the appropriate protein fraction was mixed with total unlabelled proteins from 2 A_{260} units of 30S subunits, and applied to the gel. After electrophoresis, the gels were stained with Coomassie blue, and the stained spot were cut out and counted in a toluene scintillation fluid containing 0.5% PPO and 8% Soluene 350 (Packard). Appropriate HPLC column fractions were pooled on the basis of the gel analyses, evaporated to dryness, taken up in 10% acetic acid, and stored frozen. Since the proteins were labelled with a uniform mixture of amino acids, the concentrations of the isolated pro-

teins in terms of their molar equivalence to 16S RNA could readily be calculated from the molecular weights of the individual proteins (18) and the specific radioactivity (^{14}C-counts/min per A_{260} unit) of the 30S subunits prior to the acetic acid extraction (see above).

Reconstitution (cf. ref. 9) and isolation of protein-RNA complexes: Selected ^{14}C-proteins in 10% acetic acid (above) were combined as required for the individual experiments in 1:1 molar ratios and lyophilized. The lyophilized residues were dissolved in a small volume of 20 mM Tris-HCl pH 7.5, 4 mM $MgCl_2$, 400 mM NH_4Cl, 4 mM 2-mercaptoethanol, 6 M urea, and were dialysed first overnight against this buffer and then for three hours against the same buffer minus urea. Next, the magnesium concentration was raised to 20 mM, and the protein solution was mixed with ^{32}P-labelled 16S RNA (see above), the later solution also having been adjusted to the same ionic conditions as the dialysed protein solution. The proteins and RNA were mixed so as to give a protein:RNA ratio of 1.5:1.0, a typical reaction mixture containing 3 A_{260} units of RNA in a final volume of 250 µl. The mixtures were incubated for 30 min at 40°, then for 15 min at room temperature. After cooling on ice, the solutions were layered onto 10 - 30% sucrose gradients in 10 mM triethanolamine-HCl pH 7.8, 60 mM KCl, 10 mM magnesium acetate, and centrifuged at 25,000 rpm for 20 hr at 4° (Beckman SW 40 rotor). Fractions containing the reconstituted complex were pooled, divided into two aliquots and precipitated with ethanol. The first aliquot, corresponding to one-third of the total sample, was dissolved in 100 µl of 0.1% SDS, 2 mM EDTA, 10 mM Tris-HCl pH 7.8, for analysis of the protein content of the complex. For this purpose, 2 A_{260} units of unlabelled 30S subunits were added, and the RNA moiety was fully digested by incubation with ribonucleases A and T_1, followed by ethanol precipitation to remove oligonucleotides, as described in ref. 19. The proteins were then separated on the two-dimensional system of Geyl et al (17), the gels being stained and the protein spots analysed for ^{14}C-radioactivity, exactly as described above. The second aliquot of the reconstituted complex (two-thirds of the sample) was dissolved in 50 µl of 10 mM magnesium acetate, 10 mM Tris-HCl pH 7.8 for partial digestion of the RNA (below).

Partial digestion of the reconstituted complexes, and analysis of RNA fragments: Ribonuclease A (1.5 μg per A_{260} unit RNA) was added to the solution of RNA-protein complex, and the mixture was kept at room temperature for 15 min, then chilled on ice. The partially digested samples were loaded onto "non-denaturing" 8% polyacrylamide slab gels (20 cm long), containing 50 mM Tris-HCl pH 7.8 and 10 mM magnesium acetate (cf. refs. 2,20). The reservoir buffer contained 50 mM Tris-citric acid pH 8.8 and 10 mM magnesium acetate (cf. ref. 21). After electrophoresis overnight at 120 V the gels were autoradiographed, and bands corresponding to protected RNA fragment complexes (see Results) were cut out. The RNA was extracted from the crushed gel slices with SDS buffer in the presence of phenol (8), the yields in each extract being of the order of 1 - 2 x 10^5 counts/min of ^{32}P at this stage. An aliquot of each sample (ca. 30,000 counts/min) was set aside for oligonucleotide analysis, and the remainder was applied to a 7% polyacrylamide gel containing 0.1% SDS and 7 M urea (8), in order to separate the individual RNA fragments. After electrophoresis, the gels were autoradiographed and the RNA extracted as outlined above. The isolated RNA fragments, as well as the aliquots of each sample held back before the final electrophoresis step, were subjected to total digestion with either ribonuclease A or ribonuclease T_1, followed by separation of the oligonucleotides on polyethyleneimine cellulose plates according to the method of Volckaert and Fiers (22). Secondary digestions of the oligonucleotides, and subsequent fitting of the oligonucleotide data to the 16S RNA sequence of Brosius et al (23) were carried out using our standard procedures (24).

RESULTS

A partial reconstitution study of the 30S ribosomal subunit requires a source of pure isolated ribosomal proteins, and HPLC is the obvious separation method to choose for this purpose. The question must first be answered, however, as to whether the proteins isolated by a particular HPLC procedure are capable of re-associating with 16S RNA to form active ribosomal subunits. Accordingly, *E. coli* 30S ribosomal proteins - in this case unlabelled - were separated on an HPLC column as described in

Materials and Methods, and the entire eluate from the column was combined and reconstituted with 16S RNA. The activity of the reconstituted particle was tested in the poly(U) system of Dohme and Nierhaus (25). It was found that if the column eluate was dialysed directly into reconstitution buffer, then no active subunits were formed at all. If on the other hand the column eluate was lyophilized, taken up in acetic acid, lyophilized again, and dissolved in a urea-containing buffer before finally dialysing into reconstitution buffer (as described in Materials and Methods), then the reconstituted subunits showed a reasonable and acceptable level of activity. This activity was approximately 40% of that observed in control (native) 30S subunits, the somewhat low value being possibly at least partly due to the fact that protein S1 was never recovered from the HPLC columns. No significant difference in activity was observed when phenol-extracted 16S RNA was used for the reconstitution instead of RNA isolated by our preferred method of centrifugation through sucrose gradients in the presence of SDS (Materials and Methods).

A typical HPLC elution profile for the 30S ribosomal proteins is illustrated in Fig. 1, and an example of the two-dimensional gel separation of the proteins (17) is shown in Fig. 2. The latter Figure is included to underscore the fact that all the proteins relevant to this paper are clearly separated in this gel system. As described in Materials and Methods, the proteins were labelled with ^{14}C-amino acids in the principal reconstitution experiments, and were analysed on the gels in the presence of total unlabelled protein. By measuring the radioactivity in each stained protein spot on such gels, the identity of the proteins in the individual HPLC column fractions could be unambiguously determined and any cross-contamination with other proteins quantitatively assessed.

The proteins with which this study is concerned are S7, S9, S14 and S19. It can be seen from the HPLC profile of Fig. 1 that S14 and S19 are well separated on the column (elution times ca. 45 and 55 min, respectively), and this was confirmed by the gel analysis (cf. Fig. 2). Protein S7 on the other hand appeared as two distinct peaks in the column eluate, one of which was well separated (elution time ca 75 min), the other appearing together

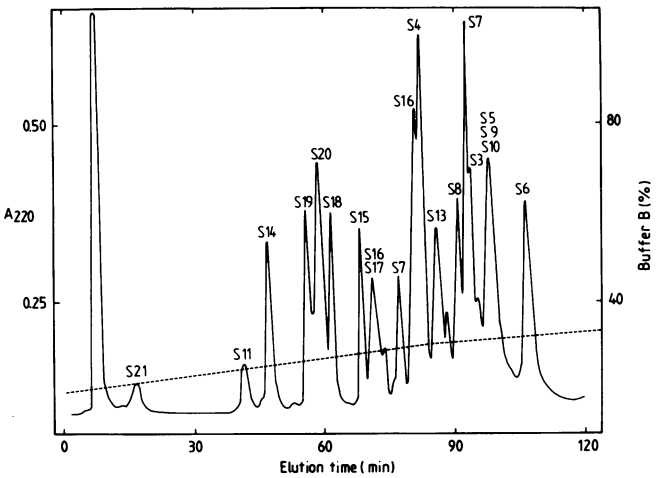

Figure 1: HPLC elution profile of 30S ribosomal proteins (cf. ref. 16). Identities of the proteins in each column fraction were determined by two-dimensional gel electrophoresis (Fig. 2). The dotted line indicates the concentration of "Buffer B" (0.1% trifluoracetic acid in 2-propanol) in the elution gradient.

with S3 (elution time 95 min) rather close to the peak containing S5, S9 and S10 (elution time 100 min), in the example shown in Fig. 1. The reason for there being two S7 peaks is not known to us, but both peaks showed identical mobilities on the two-dimensional gels, as well as identical reconstitution properties. The HPLC elution profile in the region of the second S7 peak (elution time 95 min) was somewhat variable, and in other column runs S7 and S9 appeared together in a single peak in this area.

The profile shown in Fig. 1 is of 30S proteins from E. coli strain MRE 600, in which protein S7 is smaller than its counterpart in strain A19 (26). Protein S7 from strain A19 therefore elutes at a different position from the HPLC column (also as two peaks), and by using A19 proteins we were able to isolate a relatively pure fraction of protein S9. (The MRE 600 version of protein S7 was however found to give more reproducible binding to the 16S RNA than the corresponding A19 protein). Thus from a series of HPLC runs we could isolate pure S14, S19 and S7, an S7-S9 mixture, and finally almost pure S9. The complexes discussed below were reconstituted with this set of proteins.

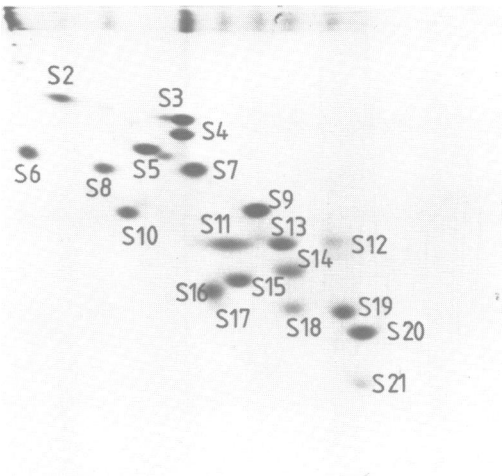

Figure 2: Separation of 30S ribosomal proteins in the gel system of ref. 17. Direction of electrophoresis is from left to right (first dimension) and from top to bottom (second dimension).

In the assembly map of the 30S subunit (9), S7 binds directly to the 16S RNA, and the respective binding of S9 or S19 is dependent on the presence of S7. S14 binding depends on the presence of S19, and is also influenced by S9. From neutron scattering data (6), S9 lies close to S7 in the 30S subunit, proteins S19 and S14 lying near to S9 but on the far side of the "head" of the subunit relative to S7. Immune electron microscopic studies (4) place S19 somewhat closer to S7, a location which is supported by the isolation and identification of a cross-link between proteins S19 and S13 both in E. coli and Bacillus stearothermophilus (27).

Using the HPLC-isolated proteins, we found that, although S7 (from MRE 600) bound well to the 16S RNA, no significant binding of S9 occurred in the presence of S7 alone. S14 and S19 together were efficiently bound in the presence of S7, and this particle was also able to bind protein S9. The reconstituted particles were isolated on sucrose gradients, followed by protein anlysis on the two-dimensional gel system of Fig. 2, as described in Materials and Methods. The use of ^{14}C-labelled proteins enabled the stoichiometry of the proteins relative to each other in the

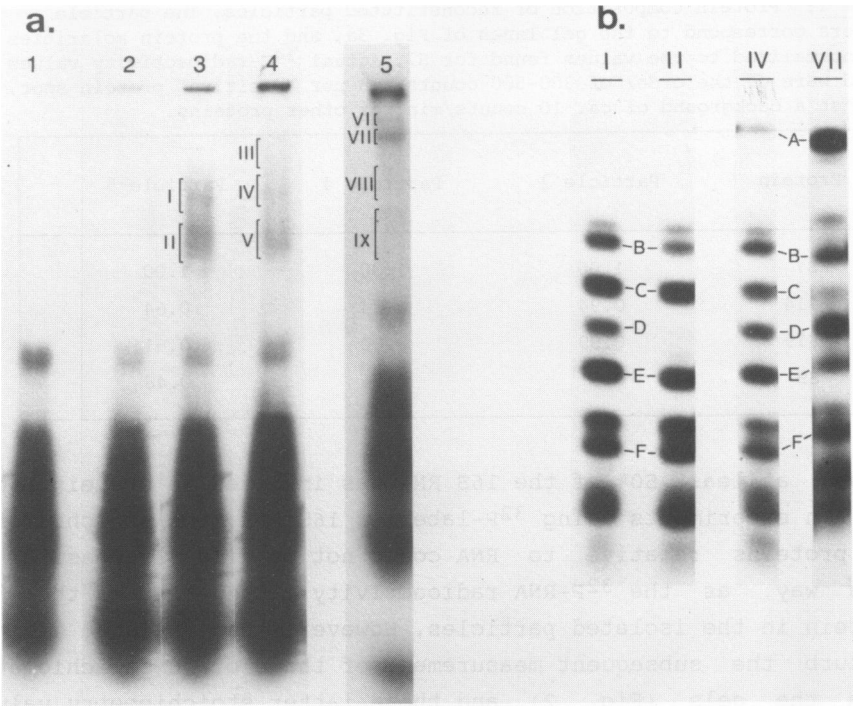

Figure 3: Separation of protected RNA fragments on polyacrylamide gels. (a): Partial ribonuclease A digests of reconstituted particles on gels containing magnesium. Lane 1, partially digested 16S RNA. Lane 2, a corresponding digest of a reconstituted particle containing S7 alone. Lanes 3 to 5, corresponding digests of particles containing S7, S14, S19 together with 0.0, 0.26 and 0.48 molar equivalents of protein S9, respectively (cf. Table 1). Roman numerals indicate the fractions taken for further analysis on SDS-urea gels. (b): Examples of the SDS-urea gel separations of the RNA fragment complexes from (a). The roman numerals correspond to those in (a), and the letters indicate the RNA fragments discussed in the text. Direction of electrophoresis is from top to bottom.

particles to be accurately determined from the gels in each case (cf. Table 1, below). Furthermore, in preliminary tests where ^{3}H-labelled or unlabelled RNA was used in place of ^{32}P-labelled RNA, the stoichiometry of the proteins relative to 16S RNA could also be calculated, by comparing the total protein radioactivity in the isolated particle with the optical density (260 nm) of the RNA present in the complex. These measurements showed that, at molar ratios of protein:RNA of 1.5:1.0 in the reconstitution mix-

Table 1: Protein composition of reconstituted particles. The particle numbers correspond to the gel lanes of Fig. 3a, and the protein molarities are normalized to the values found for S7. Actual ^{14}C-radioactivity values found were of the order of 300-500 counts/min per "positive" protein spot, against a background of ca. 10 counts/min for other proteins.

Protein	Particle 3	Particle 4	Particle 5
S7	1.00	1.00	1.00
S14	0.94	0.74	0.64
S19	0.89	0.80	0.61
S9	0.0	0.26	0.48

tures, at least 50% of the 16S RNA was involved in protein binding. In experiments using ^{32}P-labelled 16S RNA, the stoichiometry of proteins relative to RNA could not be directly measured in this way, as the ^{32}P-RNA radioactivity masked that of the ^{14}C-protein in the isolated particles. However, the ^{32}P-label did not disturb the subsequent measurement of the protein stoichiometry from the gels (Fig. 2), and these latter stoichiometry values were precisely equivalent to those found in the corresponding preliminary experiments with non-labelled or ^{3}H-labelled RNA.

The ^{32}P-labelled reconstituted particles were subjected to partial digestion with ribonuclease A, in order to analyse the RNA regions protected by the proteins. A series of experiments was performed, and examples which typify the results obtained are illustrated in Fig. 3. Fig. 3a shows the separation of the RNA from the hydrolysed particles on polyacrylamide gels in the presence of magnesium (cf. refs. 2,20). Lane 1 is a control with 16S RNA alone, and lane 2 is a 16S RNA-S7 complex, where it can be seen that no protected fragments relative to the lane 1 control are formed. Lanes 3, 4 and 5 are from reconstituted particles containing S7, S14 and S19, with increasing amounts of protein S9, the corresponding protein stoichiometries in the particles after sucrose gradient isolation being shown in Table 1. "Particle 3" (lane 3) was prepared with S7, S14 and S19 alone, and "particle 4" (lane 4) was prepared using the S7-S9 mixture from the HPLC column (see above). "Particle 5" (lane 5)

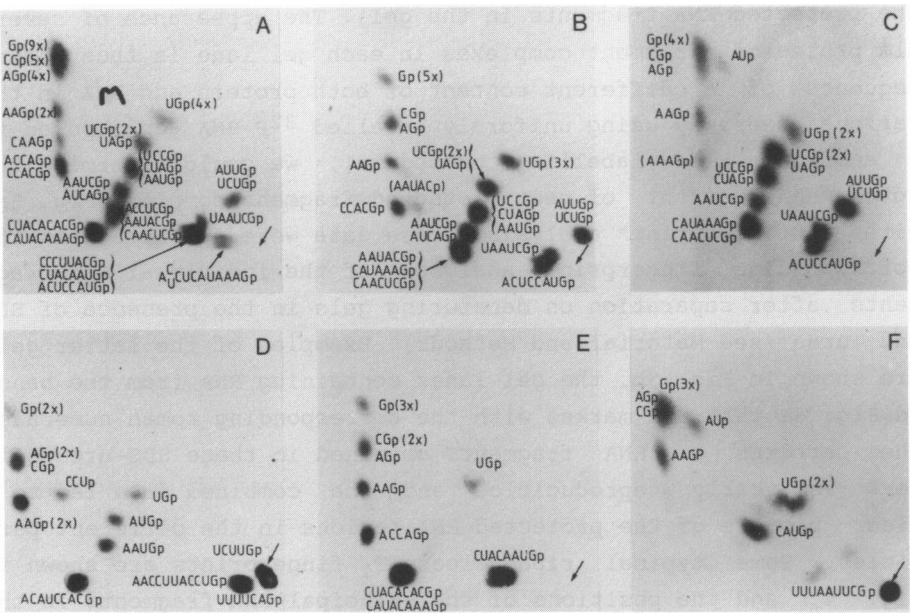

Figure 4: Examples of ribonuclease T_1 fingerprints (22) of the isolated RNA fragments. The letters A to F correspond to those assigned to the fragments on the gels of Fig. 3b. The first dimension runs from right to left, and the second dimension from bottom to top, the arrows indicating the sample application points. Identities and molarities of the oligonucleotides are shown. The "M"-shaped symbol in (A) indicates the position of a xylene cyanol dye marker.

was prepared using the almost "pure" S9 fraction, which was in fact slightly contaminated with protein S5. Not surprisingly (cf. the assembly map (9)) the S5 was not incorporated into the complex, and no proteins other than S7, S9, S14 and S19 were found in significant amounts in the two-dimensional gel analyses of any of the particles (Table 1).

Lanes 3, 4 and 5 of Fig. 3a all show the appearance of several protected RNA fragment complexes. In our first experiments we attempted to determine the protein compositions of the individual bands in the gels, but these tests - with the help of the ^{14}C-labelled proteins - showed clearly that the proteins bleed off continuously from the RNA fragments during the course of the electrophoresis (in contrast to the usual assumption in experiments of this nature, namely that the proteins remain bound to

the protected RNA fragments in the gel). The appearance of several protected fragment complexes in each gel lane is thus a consequence of a different content of both protein and RNA in the various bands. By using uniformly labelled ^{32}P-RNA (as opposed to a subsequent end-labelling procedure), we could determine the total RNA content of each protected fragment complex (Fig. 3a) with a "fingerprint" analysis. These data were compared with the corresponding fingerprint analyses of the individual RNA fragments after separation on denaturing gels in the presence of SDS and urea (see Material and Methods). Examples of the latter gels are shown in Fig. 3b, the gel lanes containing RNA from the bands in Fig. 3a that are marked with the corresponding roman numerals. The patterns of RNA fragments obtained in these SDS-urea gels were remarkably reproducible, and the combined data led to a clear picture of the protected RNA regions in the different particles. Some typical ribonuclease T_1 fingerprints are shown in Fig. 4, and the positions of the principal RNA fragments in the 16S RNA (23) are illustrated in Fig. 5. The interpretation of the data in terms of the secondary structure of the 3'-region of the 16S RNA (28) is presented in Fig. 6, (from which the reader can also follow the detailed sequence of the RNA, for comparison with the oligonucleotide data of Fig. 4).

The protected RNA region from particle 3 (containing S7, S14 and S19 only) consisted of two bands (I and II in Fig. 3a), which both gave almost identical fragment patterns in the SDS-urea gel (Fig. 3b). The only difference was the appearance in complex I of the RNA fragment D (Fig. 3b). The 5'-end of this fragment was defined by the absence from the fingerprint (Fig. 4D) of the ribonuclease T_1 oligonucleotide mCAACGp (positions 967-971), although the AAGp at positions 974-976 was present. Thus, since the fragments were generated by partial digestion of the reconstituted particles with ribonuclease A (which cuts after U or C residues), the actual 5'-terminus must have been either position G-971 or G-973. The coresponding 3'-terminus of the fragment was within the T_1-oligonucleotide CCUUCGp (pos. 1027-1032), either at position 1029 or 1030, as indicated by the presence of the oligonucleotide CCUp in the example shown in Fig. 4D.

The RNA fragments common to both complexes I and II, namely

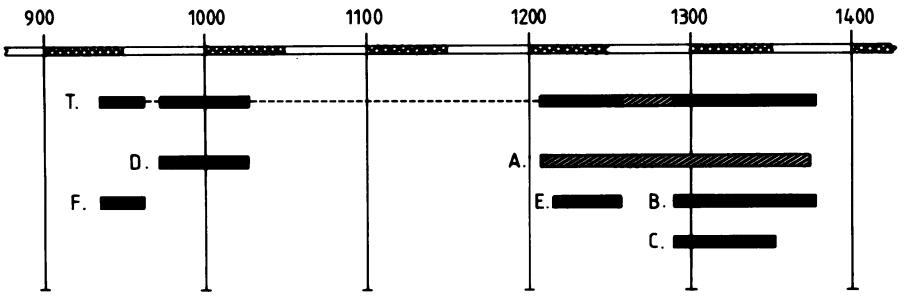

Figure 5: Locations of the RNA fragments in the 16S RNA sequence. The letters correspond to those assigned to the fragments in Fig. 3b, and the horizontal bars indicate the sequence positions found in each case. "T" denotes the total RNA found in the complexes before separation of the individual fragments on SDS-urea gels. Cross-hatching indicates sequence regions or fragments found only in the presence of protein S9 (in particles 4 and 5, see text). The sequence is numbered from the 5'-end.

fragments B, C, E and F, had the following compositions (cf. Figs. 4, 5 and 6). Fragment B had a 5'-terminus at position 1284 within the T_1-oligonucleotide A$^{C}_{U}$CUCAUAAAGp (pos. 1280-1290), as indicated by the presence of CAUAAAGp on the fingerprint. The corresponding 3'-terminus was at position 1378, as shown by the oligonucleotide AAUACp (pos. 1374-1378). Fragment C covered the same RNA region, but was slightly shorter, its 5'-terminus being heterogeneously at positions 1284 and 1287, as indicated by the presence of both CAUAAAGp (cf. fragment B) and also AAAGp (pos. 1287-1290, Fig. 4C). The 3'-terminus of this fragment was at position 1358, defined by the absence of AUCAGp (pos. 1357-1361) but the presence of AUp (pos. 1357-1358). In fragment E the first characteristic T_1-oligonucleotide was ACCAGp (pos. 1216-1220), indicating a 5'-terminus at G-1215, following C-1214 (cf. fragment A, below). The 3'-terminus was at position 1262, as shown by the presence of GACp (pos. 1260-1262) in a ribonuclease A fingerprint of fragment E, ACCUCGp (pos. 1261-1266) being absent from the ribonuclease T_1 fingerprint. Fragment F had a 5'-terminus within the oligonucleotide CACAAGp (pos. 934-939), usually at position 937, as indicated by AAGp (pos. 937-939) in the fingerprint (Fig. 4F). The 3'-terminus of this fragment was at position 965, defined by the presence of AUp (pos. 964-965). The other unmarked fragments in lanes I and II of Fig. 3b were heterogeneous,

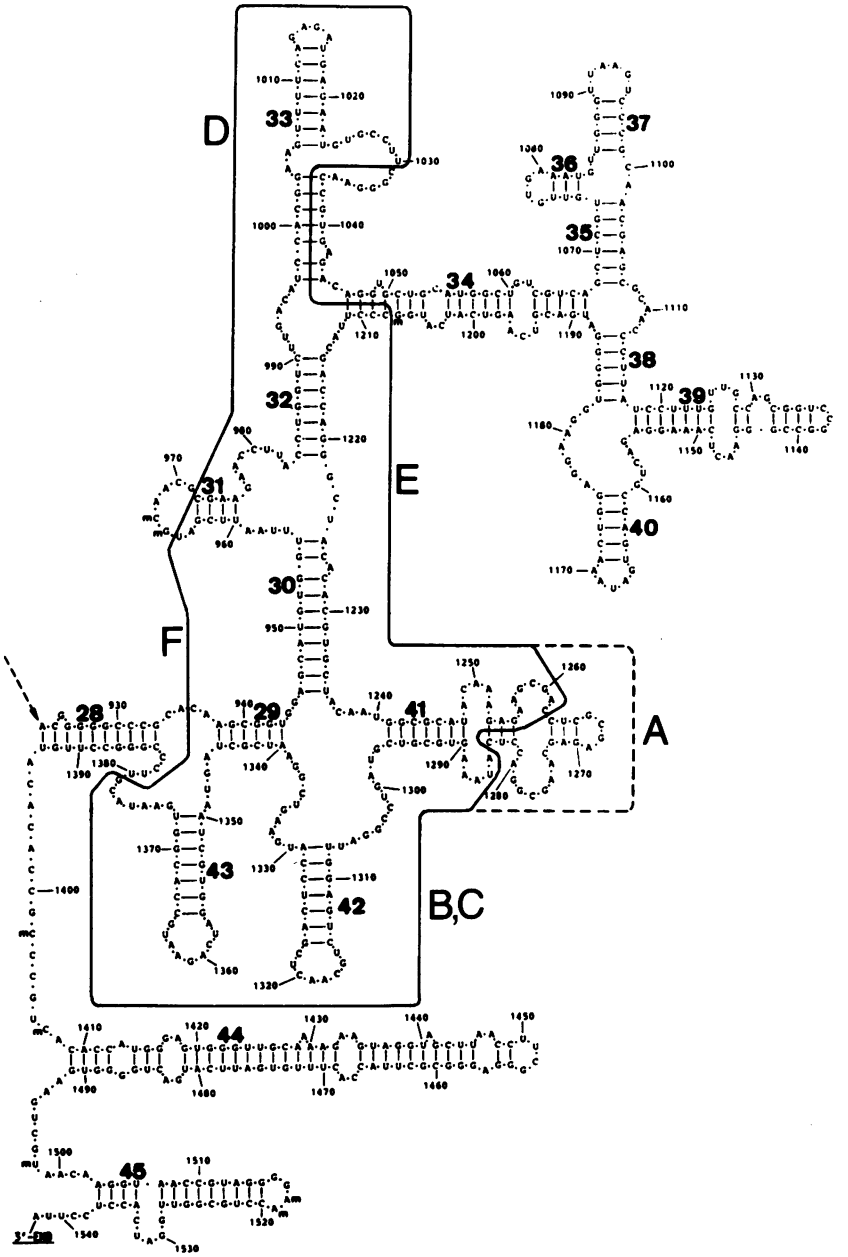

Figure 6: Secondary structure of the 3'-half of 16S RNA, slightly modified from the structure of ref. 28. The solid line indicates the RNA region protected by proteins S7, S14 and S19, the dotted line showing the additional protection in the presence of protein S9. The letters A to F are included to facilitate comparison with Figs. 4 and 5.

and contained shorter versions of the fragments B to F just described. The corresponding fingerprints of the total RNA from fragment complexes I and II showed them to contain equimolar proportions of the RNA regions corresponding to the sub-fragments B to F (with or without region D, respectively), and no other sequence regions of the 16S RNA were present (Fig. 5).

When protein S9 was present in the reconstituted particles, even in low amounts (particle 4, Fig. 3a), a new band began to be visible in the SDS-urea gels (lane IV, Fig. 3b). This RNA band (fragment A) corresponded to a continuous sequence covering the region of fragments E and B, together with the intervening loop-end (bases 1263-1284). The 5'-terminus of the fragment was a few bases farther along the RNA, as indicated by the presence in the fingerprint (Fig. 4A) of CCCUUACGp (pos. 1208-1215) but the absence of UCAUCAUGp (pos. 1199-1206). The corresponding 3'-terminus was at approximately position 1380, since AAUACGp (pos. 1374-1379) was present, but UUCCCGp (pos. 1380-1386) absent. Fragment A encompasses the heterogeneous T_1-oligonucleotide $A^{C}_{U}CUCAUAAAGp$ (pos. 1280-1290, see ref. 29), but interestingly only the version with U at position 1281 could be seen in fingerprints of fragment A from particle 4, although the C-containing version is usually present to the extent of about 60% in E. coli strain MRE 600. This suggests that the latter version is more susceptible to nuclease cleavage in the reconstituted particles.

The RNA components of fragment complex V from particle 4 (Fig. 3a) were identical to those of complex II from particle 3. In complex III from particle 4 (which has no counterpart in particle 3) the fraction of fragment A present was noticeably larger than in complex IV and this trend was further pronounced in the reconstituted paticle 5, which contained substantially greater amounts of protein S9 (Table 1). In the principal RNA fragment complex from this particle (fraction VII, Fig. 3a), fragment A was the strongest band on the SDS-urea gel (lane VII, Fig. 3b). This was also the case in the RNA from complexes VI and VIII from the same particle (Fig. 3a). Again, the fingerprints of the total RNA from complexes III to V and VI to IX (before separation of the individual fragments on the SDS-urea gels) confirmed this analysis (cf. Fig. 5), showing an increasing molar

content of the RNA region between bases 1263 and 1284, parallel to the increasing content of protein S9.

DISCUSSION

Three facts emerge clearly from the results described above. First, no protected 16S RNA fragment are observed in nuclease digests of reconstituted particles containing protein S7 alone, in agreement with earlier results (1). Secondly, as suggested in the Introduction, addition of other proteins does enable a set of protected fragments to be isolated, and the RNA protected by proteins S7, S14 and S19 corresponds to a well-defined region of the secondary structure of the 16S RNA, covering about half of the RNA from the "head" region of the 30S subunit (Fig. 6). Thirdly, addition of protein S9 to this particle results in the further protection of the end of hairpin loop 41 (Fig. 6). Although the protein stoichiometry in the S9-containing particles was not so satisfactorily equimolar as that of the particle containing S7, S14 and S19 alone (cf. Table 1), the S9-mediated extra protection was clearly related to the dosage of protein S9 present in the particles.

In fact, molar quantities of S9 were only found in reconstituted particles which also contained proteins S3 and S10 (our unpublished results). The protected RNA region in these particles covered the whole RNA region from bases ca. 920 to 1390 (cf. Fig. 6), except that helices 36 and 37 were missing. The latter particle therefore corresponds very closely to the ribonucleoprotein particle mentioned in the Introduction (7,8), which was isolated from nuclease digests of the 30S subunits; UUAAGp (in the loop end of helix 37) was also missing in this case (8). Preliminary results with reconstituted particles containing other groups of 30S ribosomal proteins suggest that further application of this approach will lead to a detailed description of the "differential protection" effects of a numer of proteins.

The protection of helix 41 by protein S9 fits very well with our recent cross-linking data. Protein S9 is cross-linked by the bi-functional reagent "APAI" to positions 1130-1131 in helix 39 (ref. 11, cf. Fig. 6), and positions 1125-1127 were found to be cross-linked to positions 1280-1281 in helix 41 in an intra-RNA cross-link induced by ultraviolet irradiation either of the 30S

subunit (12) or of growing E. coli cultures (13). Thus, helix 39 is in the immediate vicinity of helix 41, protein S9 being cross-linked to the former helix, and causing nuclease protection of the latter. Further cross-links to proteins S9, S7 and S19 (11,30,31) lie within the region of RNA protected by S7, S14 and S19 (Fig. 6), and all these data can be accommodated in our model for the three-dimensional folding of the 16S RNA in the 30S subunit (32).

A further point of interest is that the region protected by proteins S7, S14 and S19 shows a remarkable similarity to an RNA domain which is present in the small subunit of mammalian mitochondrial ribosomes (33). In the 12S RNA from these ribosomes, helices corresponding precisely to helices 28 to 32 and 42 - 43 in the E. coli structure are found, but the region corresponding to helices 33 to 40 is substantially deleted (33), and half of helix 41 is also missing (cf. Fig. 6). This is one of several examples where helices that are remote from one another in the secondary structure appear to constitute discrete domains or sub-structures in the three-dimensional organization of the 30S subunit, and this topic will be discussed in more detail elsewhere (32).

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