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Covalent cross-linking of poly(A) to *Escherichia coli* ribosomes, and localization of the cross-link site within the 16S RNA

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ABSTRACT

Poly(A) can be cross-linked to E. coli 70S ribosomes in the presence of $tRNA^{Lys}$ by mild ultraviolet irradiation. The cross-linking reaction is exclusively with the 30S subunit, and involves primarily the RNA moiety. Following a partial nuclease digestion, cross-linked complexes containing poly(A) and fragments of the 16S RNA were isolated by affinity chromatography on oligo(dT)-cellulose. The complexes were purified by gel electrophoresis and subjected to oligonucleotide analysis, which revealed a single cross-link site within positions 1394-1399 of the 16S RNA. The same pattern of cross-linking, at about one-fifth of the intensity, was observed in the absence of $tRNA^{Lys}$. The cross-link site to poly(A), together with other sites in the 16S RNA that have been implicated in ribosomal function, is discussed in the framework of our recent model for the three-dimensional structure of 16S RNA; all of the functional sites are clustered together in two distinct groups in the model.

INTRODUCTION

A large amount of effort has been devoted to the study of the sites on the ribosome which interact with ligands such as tRNA or mRNA during the course of protein biosynthesis. In the past, most of this effort has been directed towards an analysis of the ribosomal proteins concerned in the binding of tRNA (see ref. 1 for review) or mRNA (e.g. 2,3). However, attention is also being increasingly focussed on the role of ribosomal RNA in these processes, and in the case of tRNA it has been shown that the anticodon loop of the latter can be efficiently cross-linked to position C-1400 of the E. coli 16S RNA (4) or to the equivalent position in the rRNA of other organisms (5). The location of this site on the 30S ribosomal subunit has been determined electron microscopically (6). In another study, a detailed analysis was made of the bases in E. coli 16S RNA that are shielded by bound $tRNA^{Phe}$ in the presence or absence of poly(U) message (7).

In the case of mRNA, the "Shine-Dalgarno" interaction (8) with the 3'-terminus of the 16S RNA at the initiation of protein synthesis is now a gen-

erally accepted principle, but, in contrast, the available information with regard to the location of mRNA on the ribosome during peptide chain elongation is les detailed. The binding region of mRNA on the 30S subunit has been located by immune electron microscopy (9), and there is also electron microscopic evidence that a poly(U) mRNA molecule makes a "U-turn" in the ribosome (10). It is further known that the ribosome shields approximately 50 nucleotides of the message from nuclease attack (e.g. 11). However, apart from an older report which identified the sites of covalent attachment of an oligo(U) analogues to 16S RNA at positions 462 and 474 in the latter (12), there is no information at the nucleotide level relating to neighbourhoods between mRNA and ribosomal RNA during the elongation process.

In order to gain a deeper insight into this question, we have begun a cross-linking programme to map the path of the mRNA through the translating E. coli ribosome, making use of the intra-RNA and RNA-protein cross-linking methodology which we have already developed for the study of the topography of rRNA and protein within the ribosomal subunits (e.g. 13,14). In our first experiments, which are described in this paper, we have cross-linked a poly-(A) message to the ribosome (cf. ref. 15) in the presence of $tRNA^{Lys}$ using a mild ultraviolet irradiation procedure, and have analysed the products of cross-linking to the 16S RNA. Perhaps surprisingly, only a single site of cross-linking was found, at a position immediately adjacent to the C-1400 residue, which as already mentioned above (4) can be cross-linked to the anticodon loop of tRNA. The same cross-link site was also found when the reaction was carried out in the absence of tRNA. In the three-dimensional model of the 16S RNA which we have recently proposed (16), this position lies deep within the "cleft" of the 30S subunit, between the "head" and the "platform" regions. A further examination of the model shows striking correlations between the positions of (a) these cross-link sites to poly(A) and tRNA, (b) the sites that are shielded by bound tRNA as noted above (7), (c) the nine modified bases in the 16S RNA (17), and (d) the sites of interaction with various antibiotics (18). These correlations are described in the final section of the paper, and have led us to suggest a possible mechanism for the movement of tRNA (and mRNA) through the ribosome.

MATERIALS AND METHODS

Preparation of homogeneous fragments of poly(A) or poly(U). 5 mg of poly(A) or poly(U) (Boehringer, Mannheim) was dissolved in 0.5 ml of 0.05 M NaOH and incubated for 25 min at 37°. The hydrolysate was cooled to 0° and neutralized

by addition of 0.25 ml of 0.1 M HCl together with 50 µl of 1 M Tris-borate buffer pH 8.5 containing 20 mM EDTA. The solution was loaded into a wide (7 cm) slot of a 10% polyacrylamide sequencing gel (20 cm long, 2 mm thick; ref. 19), and the fragments were separated by electrophoresis at 250 V overnight. Narrow bands containing homogeneous polymer fragments (± 1 or 2 nucleotides) with chain lengths in the range from 20 to 70 nucleotides were excised from the gel, and extracted with phenol in the presence of SDS (20). The aqueous phase from the phenol extraction was filtered through a 0.45 µm Millipore filter, and the polynucleotides were precipitated twice with ethanol.

Where required, 50 µg aliquots of the isolated polynucleotides were 5'-end labelled with γ-^{32}P-ATP (100 - 250 µCi, Amersham) and polynucleotide kinase (10 - 50 units, Boehringer), as described by Maxam and Gilbert (19), with the exception that 50 mM Tris-HCl pH 7.8 was used in place of glycine-NaOH pH 9.5. The labelled fragments were re-purified by gel electrophoresis as above. Specific radioactivities obtained were of the order of 6 x 10^6 counts/min/µg.

Preparation of 70S ribosomes. ^{32}P-labelled ribosomes were prepared from 50 ml cultures of E. coli strain MRE 600 by the procedure of Stiege et al (21), with the exception that the magnesium concentration was raised to 10 mM to keep the 70S ribosomes intact. Unlabelled ribosomes were prepared as in ref. 22. In both cases the 70S particles were isolated by centrifugation through 10 - 40% sucrose gradients in 10 mM Tris-HCl pH 7.8, 10 mM magnesium acetate, 100 mM NH_4Cl and 6 mM 2-mercaptoethanol. Fractions containing the ribosomes were precipitated with ethanol, redissolved in 25 mM triethanolamine-HCl pH 7.8, 10 mM magnesium acetate, 50 mM KCl, 6 mM 2-mercaptoethanol, and dialysed extensively against the latter buffer.

Preparation and cross-linking of mRNA-70S complexes. Complexes were prepared containing either ^{32}P-labelled 70S ribosomes and unlabelled poly(A) or poly-(U), or unlabelled 70S ribosomes with ^{32}P-end labelled poly(A) or poly(U) (see above). In each case 5 A_{260} units of 70S ribosomes (ca. 140 pmol) were incubated with a 10-fold molar excess of homogeneous poly(A) or poly(U) fragment, together with a similar 10-fold excess of $tRNA^{Lys}$ (Subriden RNA, Washington) or $tRNA^{Phe}$ (Boehringer), respectively. The incubation was for 15 min at 37° in 50 mM Tris-HCl pH 7.8, 10 mM magnesium acetate, 160 mM NH_4Cl and 4 mM 2-mercaptoethanol, in a total volume of 0.2 ml. In control experiments, the tRNA was omitted. The 70S-mRNA complexes, with or without tRNA, were purified by centrifugation through 10 - 40% sucrose gradients as described above.

Fractions containing the complex were transferred to a Petri dish so as to give a solution depth of 2 mm, and were cross-linked by ultraviolet irradiation for 5 min, as described in ref. 13.

The irradiated complexes were precipitated with ethanol, redissolved in 1 ml of 10 mM Tris-HCl pH 7.8, 0.3 mM magnesium acetate, 50 mM KCl, 6 mM 2-mercaptoethanol, and centrifuged through 10 - 40% sucrose gradients in the same buffer in order to separate the 30S and 50S subunits. Fractions containing the 30S subunit together with the cross-linked poly(A) or poly(U) were precipitated with ethanol. In experiments where the poly(A) or poly(U) was ^{32}P-labelled, these subunits were subjected to a further gradient centrifugation step (using 10 - 30% sucrose gradients in 10 mM Tris-HCl pH 7.8, 1 mM EDTA and 0.05% SDS) in order to measure the amount of ^{32}P-homopolymer that was cross-linked to the 16S RNA. In experiments where the 70S ribosomes carried the ^{32}P-label, the 30S subunit fraction was submitted to a partial digestion with ribonuclease T_1, as the first step in the analysis of the cross-link site on the 16S RNA (see below).

Isolation of fragments of ^{32}P-labelled 16S RNA cross-linked to poly(A). Cross-linked 30S subunit complexes prepared from ^{32}P-labelled 70S ribosomes and poly(A), with or without $tRNA^{Lys}$, were partially digested with ribonuclease T_1 (0.025 units, Sankyo, Tokyo) for 30 min at 37°, in 100 µl of 10 mM Tris-HCl pH 7.8, 1 mM $MgCl_2$ and 20 mM NH_4Cl. The solution was then made 5 mM in EDTA, and the ribonuclease T_1 and ribosomal proteins were destroyed by addition of 50 µg of proteinase K (Merck, Darmstadt), followed by incubation for 10 min at 37° in the presence of 0.25% SDS. The solution was diluted 2-fold, brought to 100 mM in sodium acetate, and extracted with an equal volume of phenol for 1 to 2 hr at room temperature. The RNA in the aqueous phase was precipitated with ethanol and redissolved in 50 µl of water. 450 µl of 10 mM Tris-HCl pH 7.8, 500 mM NaCl, 1 mM EDTA, 0.2% SDS ("binding buffer") was added, and the solution was gently rotated with 50 mg of oligo(dT)-cellulose (Pharmacia, Uppsala) for 1 hr at room temperature. The cellulose was washed ten times with 1 ml of binding buffer, to remove fragments of free ribosomal RNA. The bound cross-linked complexes containing poly(A) were then eluted by washing the cellulose four times with 0.5 ml of 10 mM Tris-HCl pH 7.8, 1 mM EDTA, 0.2% SDS, the first wash being at room temperature and each subsequent wash being carried out for 5 to 10 min at 70°. Eluted fractions containing the ^{32}P-radioactivity were pooled, filtered through a 0.45 µm Millipore filter, and precipitated with ethanol. The precipitate was redissolved and the cross-linked complexes were separated by two-dimensional polyacrylamide gel

electrophoresis in the system of ref. 21, using a 5 - 15% gradient gel as the first dimension and a 10% gel as the second. The complexes were visualized by autoradiography.

Analysis of the cross-link site on 16S RNA. Individual ^{32}P-labelled cross-linked complexes from the two-dimensional gels were extracted, fully digested with ribonuclease T_1, and analysed by the "fingerprint" method on the two-dimensional polyethyleneimine cellulose thin-layer chromatography system of Volckaert and Fiers (23), according to our usual procedures (21). Individual ribonuclease T_1 oligonucleotides on the fingerprints were subjected to a secondary digestion with ribonuclease A, followed by a further two-dimensional thin-layer chromatography step, again using our established procedure (21, 23). The oligonucleotide data were fitted to the 16S RNA sequence of Brosius et al (24). Radioactivity in the individual complexes isolated from the two-dimensional gels was in the range of 2,000 to 20,000 counts/min, or one-fifth of these amounts in the case of complexes prepared in the absence of $tRNA^{Lys}$. In the latter cases, the radioactivity was in general too low to enable secondary digestions to be made.

In some experiments aliquots of the cross-linked complexes (after total digestion with ribonuclease T_1) were re-submitted to the oligo(dT)-cellulose affinity chromatography step. The cellulose was washed six times with the binding buffer (see above) to remove free oligonucleotides, and the residual ribonuclease T_1 oligonucleotide-poly(A) cross-linked complex was eluted at 70° as described above. The eluate was concentrated by ethanol precipitation and subjected to the secondary digestion procedure with ribonuclease A.

RESULTS

In preliminary experiments, complexes were constructed from E. coli 70S ribosomes with either poly(A) and $tRNA^{Lys}$ or poly(U) and $tRNA^{Phe}$. Homopolymers of uniform chain length were used, so as to ensure the formation of discrete spots in any subsequent gel electrophoretic separations of the cross-linked homopolymer-rRNA complexes. According to the requirements of a particular experiment, the complexes were prepared either from uniformly ^{32}P-labelled 70S ribosomes and unlabelled poly(A) or poly(U), or from unlabelled ribosomes and 5'-end ^{32}P-labelled homopolymer. The preparation of these substrates, together with the method for obtaining homopolymers of uniform chain length by mild alkaline hydrolysis followed by gel electrophoresis, are described in Materials and Methods.

Formation of the 70S complex and the properties of the cross-linking

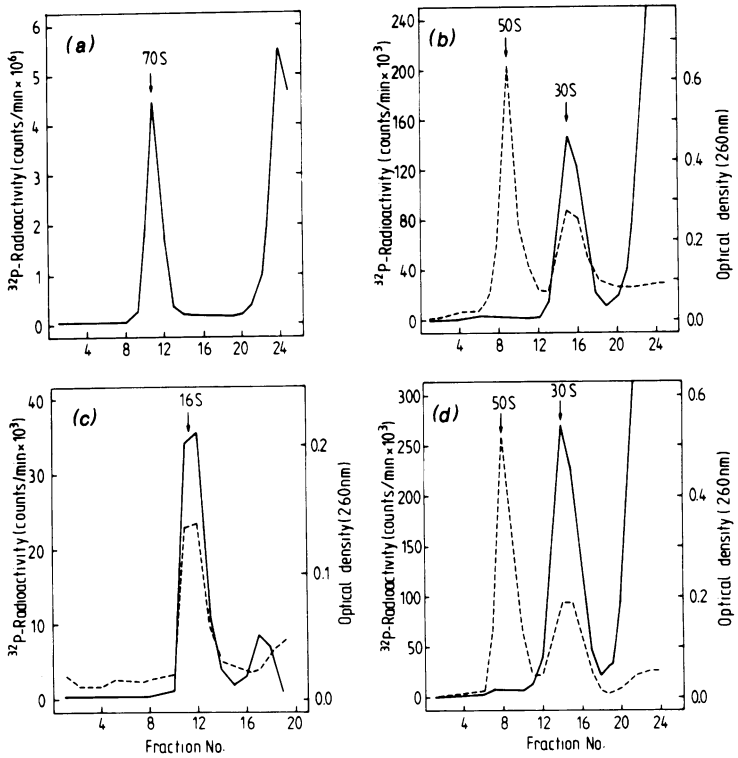

Figure 1: Sucrose gradient separations of ^{32}P-labelled homopolymer-tRNA-ribosome complexes. (a) Purification of the poly(A)-tRNA^{Lys}-70S ribosome complex, on a gradient in 10 mM magnesium. (b) Dissociation of the cross-linked poly(A)-tRNA^{Lys}-70S complex into 50S and 30S subunits, at 0.3 mM magnesium. (c) Dissociation of the 30S subunits containing cross-linked poly(A) from (b) into 16S RNA and proteins, in the presence of SDS. (d) The same as (b), but with poly(U) and tRNA^{Phe} in place of poly(A) and tRNA^{Lys}. In each case the direction of sedimentation is from right to left. ———, ^{32}P-radioactivity; -----, optical density (260 nm).

reaction were assessed by a series of sucrose gradient runs, using labelled homopolymer and unlabelled 70S ribosomes, and some examples of these gradients are shown in Figure 1. The 70S-homopolymer-tRNA complexes were prepared in the presence of a ten-fold molar excess of both homopolymer and tRNA, and after incubation this excess was separated by a first sucrose gradient, in 10 mM magnesium (Fig. 1a). From the specific radioactivity of the homopolymers it could be calculated that ca. 66% of the 70S ribosomes isolated on these gradients had bound a molecule of the messenger RNA in the case of poly(A), and ca. 55% in the case of poly(U). The gradient fractions contain-

ing the 70S complex (Fig. 1a) were the substrate for the cross-linking reaction, which was carried out by a mild ultraviolet irradiation procedure, identical to that previously described for generating intra-RNA cross-links in ribosomes in vivo (13).

After irradiation, the 70S complexes were applied to a second sucrose gradient, this time in 0.3 mM magnesium, so as to separate the ribosomal subunits. It is clear that, both in the case of poly(A) (Fig. 1b) and poly(U) (Fig. 1d), the remaining ^{32}P-radioactivity is associated exclusively with the 30S subunit. If the irradiation step was omitted, no significant amounts of ^{32}P-homopolymer ran with either subunit (not shown), indicating that the observed ^{32}P-radioactivities in the 30S subunits in Figures 1b and 1d do represent the products of a cross-linking reaction. From the amounts of radioactivity associated with the 30S subunits (Figs. 1b, d) it could be calculated that ca. 4% of the subunits carried a cross-linked homopolymer molecule in the case of poly(A), as compared with ca. 11% in the case of poly(U). If tRNA was omitted from the initial incubation, the amount of homopolymer bound to the 70S (cf. Fig. 1a) was significantly reduced, and after irradiation the proportion of this bound homopolymer which remained associated with the 30S subunit (cf. Figs. 1b, d) was approximately one-fifth of that found in the presence of tRNA. The cross-linking reaction was found to be insensitive to the chain length of the homopolymer used, over the range between 20 and 70 nucleotides. In general, however, homopolymers with a length of around 50 nucleotides were used, as this corresponds to the length of messenger which is known to be protected by the 70S ribosome (11).

The cross-linked 30S subunits were dissociated into their constituent RNA and proteins on a final sucrose gradient run in the presence of SDS, and the result showed that in the case of poly(A) approximately 80% of the ^{32}P-label ran with the 16S RNA peak (Fig. 1c). In the case of poly(U) the corresponding value was ca. 50%. The data of Figure 1 thus show that under the conditions described a useful level of cross-linking between poly(A) or poly(U) and the 16S RNA is obtained. Higher levels of cross-linking can of course be reached under more vigorous irradiation conditions, but, in accordance with our usual strategy (e.g. ref. 13), we deliberately kept the cross-linking level low, so as to minimize the danger of artefacts. The specificity of the cross-linking reaction with the protein moiety (the radioactive fraction remaining at the top of the gradient in Figure 1 c) is currently under investigation, but will not be considered further here.

In order to develop methodology for determining the site or sites of

cross-linking on the 16S RNA, 30S subunits containing the cross-linked poly-(A) or poly(U) (from gradients such as those depicted in Figures 1b and 1d) were subjected to various partial nuclease digestion procedures, and, after proteinase K treatment to remove enzyme and ribosomal protein, we attempted to isolate the cross-linked RNA fragments attached to homopolymer by affinity chromatography with complementary homopolymers bound to cellulose. Preliminary tests, again with 5'-end labelled homopolymer and unlabelled ribosomes, revealed two facts which dictated the strategy adopted in the subsequent experiments. First, when the partial nuclease digestion of the cross-linked 30S subunits was made using cobra venom nuclease (which gives the clearest pattern of partial digestion products (25, 21)), the homopolymer was also attacked, resulting in a loss of the ^{32}P-end label. The partial digestions were accordingly made using ribonuclease T_1 instead. Secondly, although cross-linked complexes containing ^{32}P-labelled poly(A) were quantitatively adsorbed onto oligo(dT)-cellulose (see below), a corresponding adsorption of poly(U)-containing complexes onto poly(A)-Sepharose was consistently unsatisfactory. Further tests showed that this was a direct result of the irradiation treatment, and that, even under the mild conditions used, the poly(U) became modified (presumably by U-U dimer formation or uridine hydration) to such an extent that it would not form stable double-helices with the Sepharose-bound poly(A). As a result, a cross-link site analysis of the poly(U)-containing complexes demands a fundamentally different experimental strategy, and in the series of experiments described below we confine our attention to the analysis of the site(s) of cross-linking between poly(A) and 16S RNA.

Cross-linked 30S subunit complexes that had been prepared from ^{32}P-labelled 70S ribosomes and unlabelled poly(A), in the presence or absence of $tRNA^{Lys}$, were partially digested with ribonuclease T_1, treated with proteinase K, and adsorbed onto oligo(dT)-cellulose, as described in Materials and Methods. Oligo(dT)-cellulose was chosen rather than poly(U)-Sepharose for this purpose, as preliminary tests had shown that the former preparation gave the most clearcut separations; as already noted above, cross-linked complexes containing ^{32}P-labelled poly(A) were quantitatively adsorbed to the oligo(dT)-cellulose, whereas on the other hand in the absence of cross-linked poly(A) no significant radioactivity from ^{32}P-labelled 16S RNA fragments remained bound to the matrix after the washing procedure. Table 1 shows the fate of the ^{32}P-radioactivity in the 16S RNA during this affinity chromatography step in a typical cross-linking experiment. The conditions of partial ribonuclease T_1 digestion are difficult to control precisely, and in this particular ex-

Table 1: Affinity chromatography of cross-linked poly(A)-16S RNA fragment complexes on oligo(dT)-cellulose.

Experimental step	^{32}P-radioactivity (counts/min)	
	plus $tRNA^{Lys}$	minus $tRNA^{Lys}$
Cross-linked 30S from sucrose gradient	407 x 10^6	461 x 10^6
Precipitated complexes after T_1-digestion	90 x 10^6	93 x 10^6
Complexes adsorbed onto oligo(dT)-cellulose	246 x 10^3	60 x 10^3
Complexes eluted from oligo(dT)-cellulose	222 x 10^3	49 x 10^3

periment the digestion was relatively vigorous, as evidenced by the high proportion of radioactivity that was not precipitated by ethanol after the digestion (compare the first two lines of Table 1). The radioactivity recovered (220,000 counts/min in the "plus tRNA" column) is however of the correct order of magnitude, if 3 - 4 % of the 16S RNA molecules carried a cross-linked poly(A) molecule (see above), and if the average length of the 16S RNA fragments in the cross-linked complexes was 80 nucleotides (cf. Fig. 4, below). It can be seen from Table 1 (cf. the foregoing discussion) that the radioactivity recovered from complexes formed in the absence of $tRNA^{Lys}$ was approximately one-fifth of that recovered when $tRNA^{Lys}$ was present. The Table also indicates that the elution of the complexes from the oligo(dT)-cellulose was essentially quantitative.

The eluted complexes were separated by two-dimensional gel electrophoresis, and examples of the gels obtained are illustrated in Figure 2. Figure 2a shows the pattern of cross-linked complexes eluted from the oligo(dT)-cellulose (from the same experiment as that of Table 1), whereas Figure 2b is the pattern from an aliquot (containing a similar amount of ^{32}P-radioactivity) of the RNA fragments that were not bound to the cellulose. The latter gel shows a strong diagonal of "free" RNA fragments, with some faint spots lying above this diagonal, which correspond to intra-RNA cross-linked complexes (cf. refs. 13, 21). In contrast, the oligo(dT)-cellulose eluate fraction shows a very characteristic pattern, containing only small amounts of free RNA fragments (Fig. 2a, lower right), the bulk of the radioactivity being present as the poly(A)-rRNA cross-linked complexes. This gel pattern was very reproducible, although the relative intensities of the individual spots varied from one experiment to another, as a result of the variability in the partial digestion with ribonuclease T_1. The corresponding gels from samples cross-linked in the absence of $tRNA^{Lys}$ gave identical patterns, again at one-

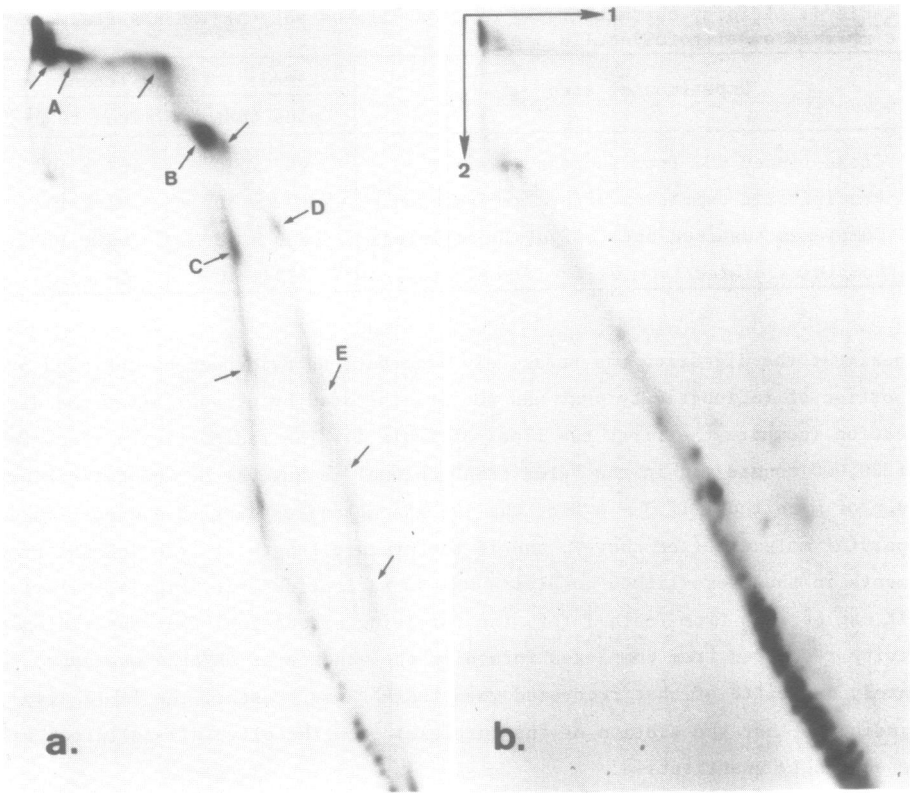

Figure 2: Two-dimensional gel electrophoretic separation (cf. 21) of cross-linked poly(A)-16S RNA fragments, after affinity chromatography on oligo(dT)-cellulose. (a) Autoradiogram of the gel of the poly(A)-containing complexes eluted from the oligo(dT)-cellulose. The ribosomal RNA fragments carry the ^{32}P-label. Arrowed complexes were extracted from the gel for further analysis, the lettered complexes corresponding to those shown in Fig. 4. In the case of smeared spots (e.g. complex E), elongated pieces of gel containing the whole smeared region were extracted. (b) Control gel run in parallel of an aliquot of the RNA fragments that were not adsorbed by the oligo(dT)-cellulose. The size of the aliquot was chosen so as to contain a similar amount of total ^{32}P-radioactivity as the sample applied to gel (a) (cf. Table 1). In both cases the directions of the electrophoretic dimensions are as indicated in (b).

fifth of the intensity. In a control experiment, which was taken as far as the stage of the first dimension gel, the electrophoretic patterns of cross-linked complexes eluted from the oligo(dT)-cellulose were the same from samples prepared with ^{32}P-labelled poly(A) as those from samples with ^{32}P-labelled ribosomes, thus providing further confirmation that the observed complexes in Figure 2a do indeed contain the cross-linked homopolymer.

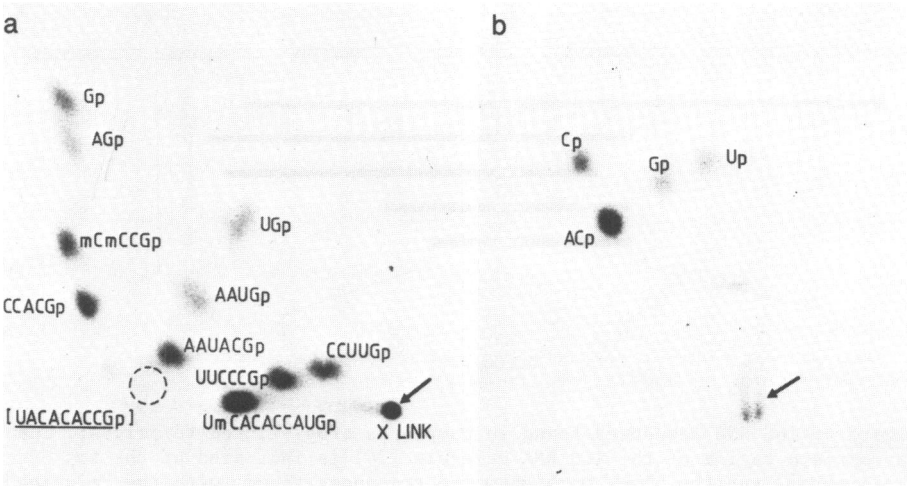

Figure 3: Oligonucleotide analysis of cross-linked poly(A)-16S RNA complexes on polyethyleneimine cellulose plates (23). (a) Ribonuclease T_1 fingerprint of complex E (Fig. 2a). Identities of the oligonucleotides are shown (cf. Fig. 4), "X-link" denoting the residual cross-linked oligonucleotide, and the dotted circle showing the positions where the missing UACACACCGp would normally appear. (b) Ribonuclease A digest of a fully ribonuclease T_1-digested cross-linked poly(A)-RNA complex that had been re-submitted to affinity chromatography on oligo(dT)-cellulose. Identities of the oligonucleotides are marked as in (a). In both photographs the first dimension ran from right to left and the second from bottom to top, arrows denoting the sample application points.

Radioactive spots were excised from the two-dimensional gels (e.g. the spots that are arrowed in Figure 2a), and subjected to total digestion with ribonuclease T_1. The oligonucleotides were separated using the two-dimensional thin-layer chromatography system of Volckaert and Fiers (23), and a typical example of one of the fingerprints obtained in illustrated in Figure 3a. Each oligonucleotide spot was further digested with ribonuclease A (cf. refs. 21, 23), and the oligonucleotide data were fitted to the 16S RNA sequence of Brosius et al (24). The results showed that, without exception, every cross-linked complex arose from the region of the 16S RNA between bases ca. 1300 and 1500, and in every case (including the complexes prepared in the absence of $tRNA^{Lys}$) the oligonucleotide UACACACCGp (positions 1393-1401) was missing from the fingerprint. Some typical fragments are depicted in Figure 4, corresponding to the lettered spots in Figure 2a. The fingerprint shown in Figure 3a corresponds to fragment E in Figure 4, and the oligonucleotides visible in the former figure can be compared with the sequence which is given at

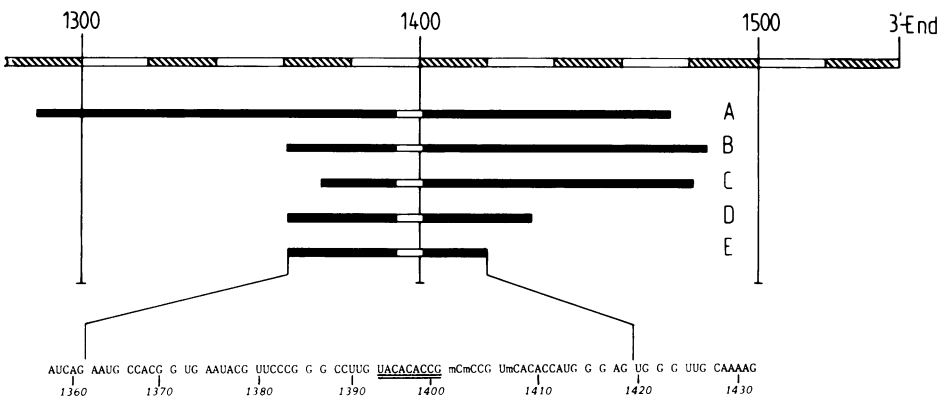

Figure 4: 16S RNA sequences found in complexes cross-linked to poly(A). The appropriate region of the 16S RNA molecule (24) is indicated at the top of the diagram, and the black bars show the sequences found, with the "missing" oligonucleotide at positions 1393-1401. The letters A to E refer to the arrowed spots in Fig. 1a. Complex E is the one whose fingerprint is given in Fig. 3a, and the corresponding detailed sequence region (divided into ribonuclease T_1 oligonucleotides) is included at the bottom of the diagram.

the bottom of Figure 4. It is of interest to note in passing that in Figure 2a the mobilities of the cross-linked complexes in the first gel dimension are inversely proportional to the length of the 16S RNA fragments contained in the complex (cf. Fig. 4), whereas in the second dimension the complexes separate into two distinct near-vertical rows. In the left-hand row (containing complex C, Fig. 2a), it can be seen by comparison with Figure 4 that there is relatively less RNA on the 5'-side of the oligonucleotide UACACACCGp, whereas in the right-hand row (containing complexes D and E) the RNA sequences on the 3'-side of the latter oligonucleotide become progressively shorter.

The universal absence of UACACACCGp (positions 1393-1401) from the fingerprints (Figs. 3 and 4) indicates that this oligonucleotide contains the site of cross-linking to poly(A). In order to confirm this, aliquots of some of the complexes were subjected to a second affinity chromatography step with oligo(dT)-cellulose after total digestion with ribonuclease T_1. In such cases, only the ^{32}P-UACACACCGp-poly(A) complex should be retained by the cellulose and subsequently eluted. (On the fingerprints (cf. Fig. 3a), this complex remains at the origin and is difficult to extract). When the T_1-digested complexes were eluted from the oligo(dT)-cellulose and further digested with ribonuclease A, according to the usual "secondary digestion" procedure (21,23),

the pattern of oligonucleotides shown in Figure 3b was reproducibly observed. This pattern shows approximately equal amounts of Cp, Gp and Up, and a stronger spot corresponding to ACp, consistent with the composition of the "missing" oligonucleotide UACACACCGp. The ACp spot was however not as strong as is usually seen in digests of the latter oligonucleotide (where the ACp spot should have six times the intensity of the three mononucleotide spots), indicating that (since Cp, Gp and Up were all present) the actual site of cross-linking to poly(A) was within one of the three AC sequences between position-1394 and 1399 in the 16S RNA.

DISCUSSION

Purine residues are known to be able to take part in ultraviolet-induced intra-RNA cross-linking reactions (e.g. 26), and we had expected that simple irradiation of a ribosome-poly(A) complex - using a poly(A) template about 50 nucleotides long - would have led to the formation of a number of cross-links at various points along the poly(A) molecule, hence involving a corresponding number of different sites on the 16S RNA, from which the path of the poly(A) through the 30S subunit could ultimately be inferred. The finding of a single highly-specific cross-link site to the 16S RNA was therefore in sharp contrast to this initial expectation, (although we cannot of course exclude that small amounts of other cross-links were present, that were lost in the partial ribonuclease T_1 digestion). Position C-1400 in the 16S RNA has been cross-linked to the anticodon loop of both P-site (4) and A-site (27) bound tRNA, and our poly(A) cross-link to a position between A-1394 and C-1399 in the same immediate neighbourhood supports the view of the latter authors that this highly conserved region of the ribosomal RNA is intimately involved in the decoding process. Furthermore, the finding of the same cross-link site in the absence of added $tRNA^{Lys}$ indicates that the poly(A) template is correctly bound (although about five-fold less efficiently) even without tRNA; the alternative explanation, that 20% of the active 70S ribosomes in our preparations already carried a $tRNA^{Lys}$ molecule, is extremely unlikely. The finding of the same cross-link site in the absence of tRNA also precludes the possibility (already statistically very remote under the deliberately low-yield conditions of cross-linking used) that our observed cross-link is a "double" cross-link involving poly(A) linked to tRNA linked to 16S RNA.

As noted in the Introduction, position C-1400 and the oligonucleotide encompassing the cross-link site to poly(A) lie deep down in the cleft of

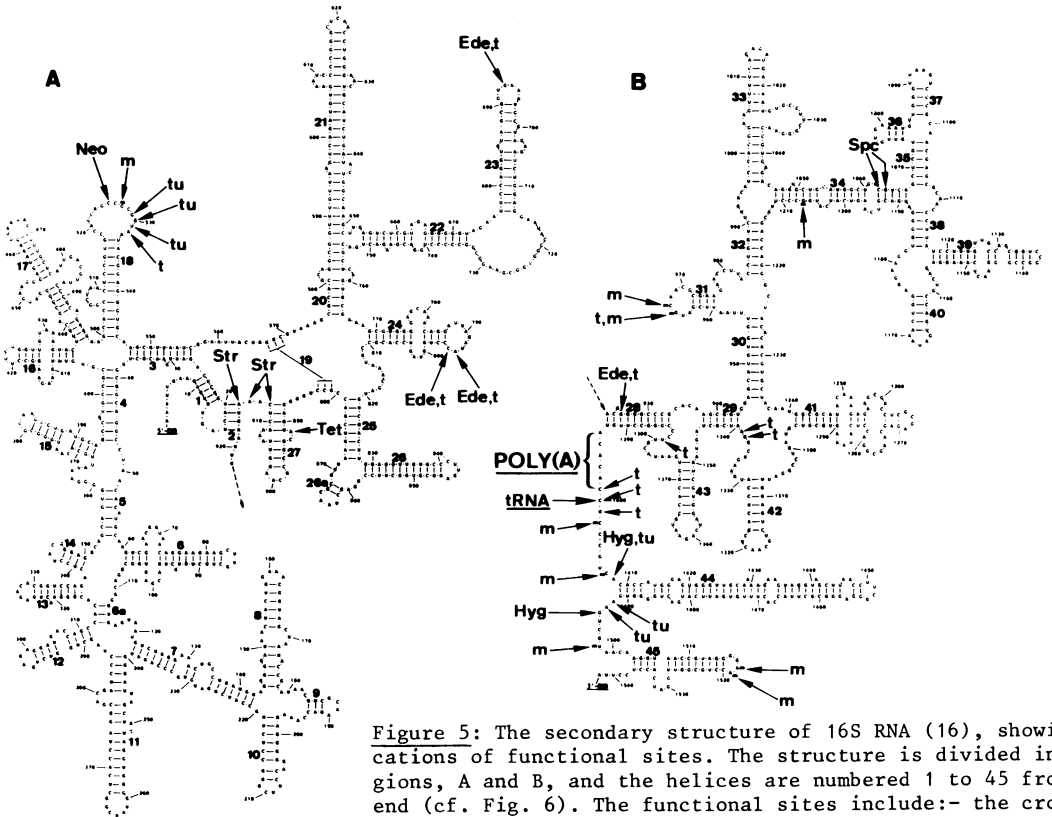

Figure 5: The secondary structure of 16S RNA (16), showing the locations of functional sites. The structure is divided into two regions, A and B, and the helices are numbered 1 to 45 from the 5'-end (cf. Fig. 6). The functional sites include:- the cross-link site to poly(A), underlined (this paper); the cross-link site to tRNA at position 1400, underlined (4); the sites protected by tRNA (7), "tu" denoting poly(U)-dependent protection, "t" protection in the absence of poly(U); sites protected by antibiotics (18), "Neo" denoting neomycin, "Ede" edeine, "Tet" tetracycline, "Str" streptomycin, "Spc" spectinomycin and "Hyg" hygromycin; the modified nucleotides in the 16S RNA itself (17), denoted by "m".

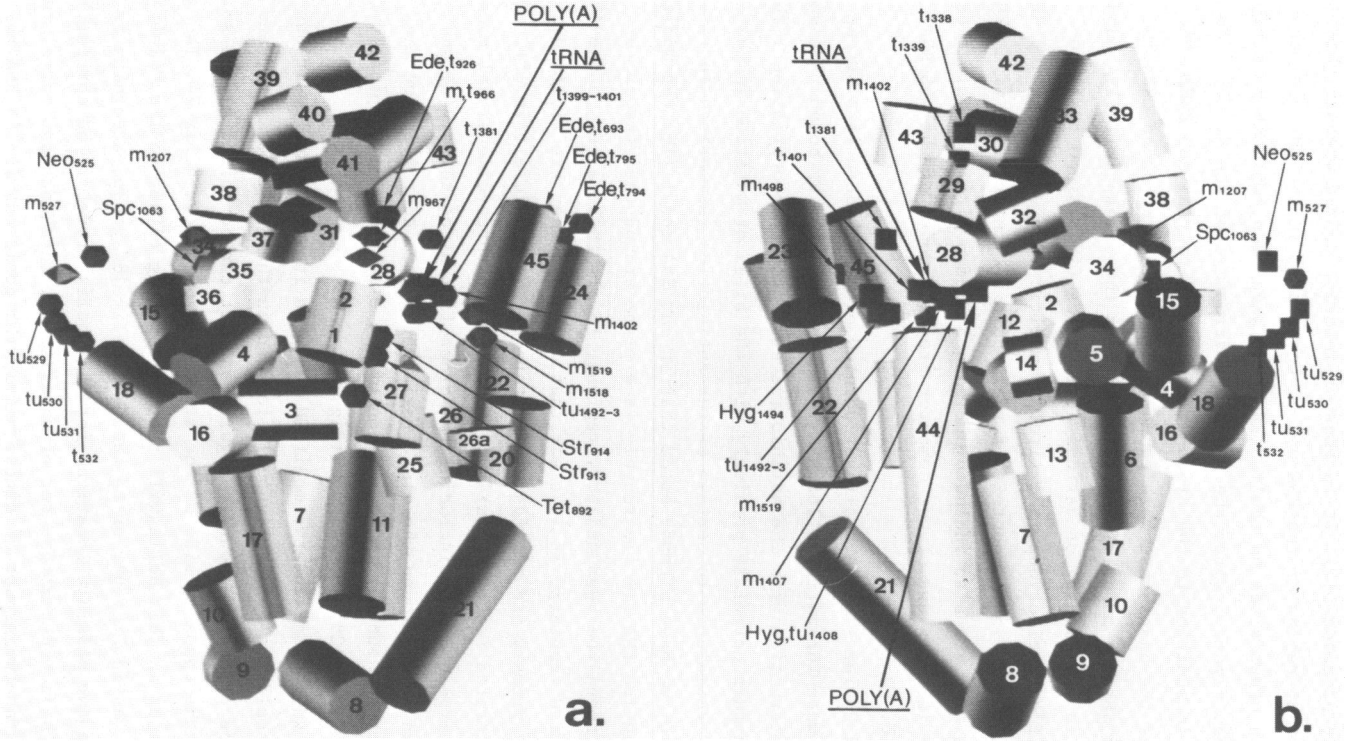

Figure 6: Two views of the computer graphics model of the 16S RNA (cf. 16), generated on the Evans & Sutherland PS 300 system. The cylinders represent the double-helical regions of the 16S RNA, numbered as in Fig. 5, and the small polygons represent the functional sites, also denoted as in Fig. 5. The orientations of the two views are (a) 315° (cf. 16), (b) 130°.

the 30S subunit in the detailed three-dimensional model for the 16S RNA which we have recently proposed (16). It is thus of interest to see whether this obviously important region of the RNA can be correlated in the three-dimensional model with other sites on the 16S RNA that have been implicated in ribosomal function. Figure 5 shows the secondary structure of the E. coli 16S RNA (16), and, in addition to the cross-link sites to poly(A) and tRNA, the figure indicates the bases that were found to be shielded by bound tRNA in the presence or absence of poly(U) (7), as well as the sites similarly found to be involved in the binding of various antibiotics (18). Since tRNA contains many modified nucleotides, the sites of modification within the 16S RNA itself are of interest in the context of their possible interaction with tRNA, and these sites (17) are also included in Figure 5. The positions of all these sites are shown in two views of our computer graphics model of the 16S RNA (16) in Figure 6.

Moazed and Noller (18) have already pointed out that there is a strong correlation between the sites on 16S RNA that are protected by bound tRNA and the sites of interaction with the antibiotics as found by "footprinting". The latter sites in turn correlate precisely with the sites of mutation in the 16S RNA causing resistance to certain of the antibiotics (summarized in ref. 18). Furthermore, we have already noted that in our RNA model (16) the modified bases in the 16S RNA all lie roughly in a plane around the "throat" of the subunit, between the head and body regions. If all these sites on the 16S RNA are considered together, it is clear that, although they are widely distributed in the primary and secondary structure of the molecule (Fig. 5), they are concentrated into two distinct groups in the three-dimensional model (Fig. 6). The largest group, including the cross-link sites to poly(A) and tRNA, is located in or around the cleft region, the remainder lying on the opposite side of the subunit in the region of the electron microscopically located base m^7G-527 (28) in helix 18 (Fig. 5).

Helix 31, which contains two modified bases in its loop end (positions 966 and 967), could not be unambiguously located in the RNA model (16), and it can either be placed as shown in Figure 6b or it can be turned around so as to emerge on the opposite side of the structure just above helix 34. The two modified bases in this helix will therefore belong either to the "cleft" group of sites (as in Fig. 6), or to the "m^7G/helix 18" group, according as to which of the two possible locations for this helix proves to be correct. It should also be noted that one set of experimental data does not correlate with either of the two groups of sites, namely the positions in 16S RNA re-

ported to be cross-linked to an oligo(U) analogue (12). These positions (G-462 and G-474) are at the end of helix 17 (Fig. 5), which is located at the bottom of the 30S subunit in the RNA model (Fig. 6a). Furthermore, whereas all the other functional sites (Fig. 5) are in highly conserved regions of the secondary structure of the 16S RNA (cf. 29), helix 17 is a non-conserved region. Since the binding of the oligo(U) analogue to 30S subunits was only slightly stimulated by tRNA ^{Phe} (30), it seems likely that the reaction with the analogue was at least in part non-specific, and hence that the sites analysed by Wagner et al (12) may not be relevant.

The location of the poly(A) and tRNA cross-link sites in the cleft of the 30S subunit indicates that the "cleft" group of functional sites (Fig. 6) represents the actual binding region of mRNA and tRNA, with the anticodon loop of the tRNA directed downwards into the cleft, and the mRNA running through the deepest part of the cleft. This is consistent with electron microscopic data (summarized in ref. 6), and with the models of Lake (31) and Ofengand et al (1). The bases shielded by tRNA that are situated in or near to the upper part of the cleft (e.g. at positions 693, 795, 926, 1338, 1381) are in positions in the model (Fig. 6) that are compatible with the dimensions of a tRNA molecule lying in the cleft as just suggested, although it should be noted that at this stage we do not attempt to distinguish between the "A" and "P" tRNA binding sites in the model.

Inspection of the model suggests that the cleft is wide enough - but only just - to accomodate a tRNA anticodon stem-loop structure, and this introduces an intriguing possibility for the mechanism of tRNA (and hence mRNA) movement through the ribosome, and for the involvement of the second group of functional sites mentioned above in the m^7G/helix 18 region (Fig. 6). Moazed and Noller (7) have pointed out that the protection of bases in this area by tRNA is probably an allosteric process, and it is noteworthy that the protection of bases 529 to 531 in helix 18 is poly(U)-dependent, the other poly(U)-dependent protections (bases 1408 and 1492-1493) being in the deepest part of the cleft (Fig. 6). This implies that tRNA binding deep in the cleft in the presence of mRNA causes a "rocking" motion of the head region of the subunit, leading to the allosteric protections in the helix 18 region. Successive cycles of such a rocking motion would be accompanied by a corresponding slight opening and closing of the cleft, which would effectively create a "molecular turnstile", allowing the tRNA molecules to pass through - i.e. be translocated through - the cleft one at a time. Evidence in support of this motion comes from the electron microscopic observation of two conformations

of the "beak-like protuberance" (cf. 32) on the head of archaebacterial 30S ribosomal subunits (M. van Heel, personal communication). The beak-like protuberance correlates with the position of helix 39 in the model (16), which lies on the same side of the subunit as the helix 18 region (Fig. 6). It is also interesting in this context that the two antibiotics which show effects in this latter region - namely neomycin which causes enhanced reactivity at position C-525 in helix 18, and spectinomycin which protects bases C-1063 and G-1064 in helix 34 (ref. 18, cf. Fig. 6) - are both inhibitors of translocation. That is to say, these antibiotics could conceivably act by blocking the motion of the head region. Another functional feature in the "helix 18" area of the 30S subunit is the electron microscopically observed attachment site of elongation factor Tu (33, 34). The binding of this factor, which brings the charged tRNA molecules to the ribosome, could thus trigger or be triggered by a movement of the head of the subunit. This in turn could begin to provide a molecular basis for the allosteric interaction between the tRNA "A" and "E" sites observed by Rheinberger and Nierhaus (35).

Whether or not the postulated rocking motion of the head of the subunit actually occurs remains of course to be proven. More important at the moment is the fact that, although (as already noted) the functionally-related positions that we have discussed here are widely distributed in both the primary and secondary structure of the 16S RNA (Fig. 5), they are nonetheless concentrated into two distinct groups in the three-dimensional structure (Fig. 6). The 16S RNA model (16) thus has begun to provide a framework for describing functional sites at the nucleotide level in three dimensions.

A number of proposals (1, 31, 36) have been made for the relative locations of the tRNA "A" and "P" sites on the 30S subunit. A crucial factor in testing the relative merits of these proposals will now be to determine in which direction the mRNA runs through the cleft in the RNA model. A further important question to settle is the way in which the mRNA is then "wrapped" around the subunit, so as to account for the protection of ca. 50 nucleotides of message by 70S ribosomes or 30S subunits (11), as well as for the observation that the entry and exit sites of the message are at the same location (10). We believe that an extension of the cross-linking approach reported in this paper, using properly phased mRNA analogues carrying affinity labels at appropriate positions such as those used by Stahl and Karpova (3) or Babkina et al (2), but concentrating on the analysis of cross-link sites to the ribosomal RNA rather than to the ribosomal proteins, should provide definitive answers to these questions.

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REFERENCES

1. Ofengand, J., Ciesiolska, J., Denman, R. and Nurse, K. (1986) In "Structure, Function and Genetics of Ribosomes", Hardesty, B. and Kramer, G., Eds., pp. 473-494, Springer-Verlag, New York and Heidelberg.
2. Babkina, G.T., Veniaminova, A.G., Vladimirov, S.N., Karpova, G.G., Yamkovoy, V.I., Berzin, V.A., Gren, E.J. and Cielens, I.E. (1986) FEBS Lett. 202, 340-344.
3. Stahl, J. and Karpova, G.G. (1985) Biomed. Biochim. Acta 44, 1057-1064.
4. Prince, J.B., Taylor, B.H., THurlow, D.L., Ofengand, J. and Zimmermann, R.R. (1982) Proc. Nat. Acad. Sci. USA 79, 5450-5454.
5. Ehresmann, C. and Ofengand, J. (1984) Biochemistry 23, 438-445.
6. Gornicki, P., Nurse, K., Hellmann, W., Boublik, M. and Ofengand, J. (1984) J. Biol. Chem. 259, 10493-10498.
7. Moazed, D. and Noller, H.F. (1986) Cell 47, 985-994.
8. Shine, J. and Dlagarno, L. (1974) Proc. Nat. Acad. Sci. USA 71, 1342-1346.
9. Stöffler, G. and Stöffler-Meilicke, M. (1981) In "International Cell Biology", Schweiger, H.G. Ed.,pp. 93-102, Springer-Verlag, Berlin.
10. Evstafieva, A., Shatsky, I.N., Bogdanov, A.A., Semenkov, Y. and Vasiliev, V.D. (1983) EMBO J. 2, 799-804.
11. Kang, C. and Cantor, C.R. (1985) J. Mol. Biol. 181, 241-251.
12. Wagner, R., Gassen, H.G., Ehresmann, C., Stiegler, P. and Ebel, J.P. (1976) FEBS Lett. 67, 312-315.
13. Stiege, W., Atmadja, J., Zobawa, M. and Brimacombe, R. (1986) J. Mol. Biol. 191, 135-138.
14. Oßwald, M., Greuer, B., Brimacombe, R., Stöffler, G., Bäumert, H. and Fasold, H. (1987) Nucleic Acids Res. 15, 3221-3240.
15. Towbin, H. and Elson, D. (1978) Nucleic Acids Res. 5, 3389-3407.
16. Brimacombe, R., Atmadja, J., Stiege, W. and Schüler, D. (1988) J. Mol. Biol. 199, 115-136.
17. Carbon, P., Ehresmann, C., Ehresmann, B. and Ebel, J.P. (1979) Eur. J. Biochem. 100, 399-410.
18. Moazed, D. and Noller, H.F. (1987) Nature 327, 389-394.
19. Maxam, A.M. and Gilbert, W. (1977) Proc. Nat. Acad. Sci. USA 74, 560-564.
20. Yuki, A. and Brimacombe, R. (1975) Eur. J. Biochem. 56, 23-34.
21. Stiege, W., Zwieb, C. and Brimacombe, R. (1982) Nucleic Acids Res. 10, 7211-7229.
22. Morgan, J. and Brimacombe, R. (1972) Eur. J. Biochem. 29, 542-552.
23. Volckaert, G. and Fiers, W. (1977) Analyt. Biochem. 83, 222-227.
24. Brosius, J., Palmer, M.L., Kennedy, P.J. and Noller, H.F. (1978) Proc. Nat. Acad. Sci. USA 75, 4801-4805.
25. Vassilenko, S.K., Carbon, P., Ebel, J.P. and Ehresmann, C. (1981) J. Mol. Biol. 152, 699-721.
26. Atmadja, J., Brimacombe, R., Blöcker, H. and Frank, R. (1985) Nucleic Acids Res. 13, 6919-6936.
27. Ciesiolska, J., Gornicki, P. and Ofengand, J. (1985) Biochemistry 24, 4931-4938.

28. Trempe, M.R., Oghi, K. and Glitz, D.G. (1982) J. Biol. Chem. 257, 9822-9829.
29. Brimacombe, R., Maly, P. and Zwieb, C. (1983) Progr. Nucleic Acid Res. 28, 1-48.
30. Wagner, R. and Gassen, H.G. (1975) Biochem. Biophys. Res. Commun. 65, 519-529.
31. Lake, J.A. (1977) Proc. Nat. Acad. Sci. USA 74, 1903-1907.
32. Oakes, M., Henderson, E., Scheinman, A., Clark, M. and Lake, J.A. (1986) In "Structure, Function and Genetics of Ribosomes", Hardesty, B. and Kramer, G., Eds., pp. 47-67, Springer-Verlag, New York and Heidelberg.
33. Langer, J.A. and Lake, J.A. (1986) J. Mol. Biol. 187, 617-621.
34. Girshovich, A.S., Bochkareva, E.S. and Vasiliev, V.D. (1986) FEBS Lett. 197, 192-198.
35. Rheinberger, H.J. and Nierhaus, K.H. (1986) J. Biol. Chem. 261, 9133-9139.
36. Spirin, A.S. (1983) FEBS Lett. 156, 217-221.