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RNA-protein cross-linking in Escherichia coli 30S ribosomal subunits: a method for the direct analysis of the RNA regions involved in the cross-links

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ABSTRACT

A prerequisite for topographical studies on ribosomal subunits involving RNA-protein cross-linking is that the cross-linking sites on the RNA should be determined. Methodology is presented which offers a solution to this problem, using as a test system 30S subunits in which protein S7 has been cross-linked to the 16S RNA by ultraviolet irradiation. The method is based on a gel separation system in the presence of a non-ionic detergent. When a ribonucleoprotein fragment containing RNA-protein cross-links is applied to this system, non-cross-linked protein is removed, and simultaneously the cross-linked RNA-protein complex is separated from non-cross-linked RNA. Oligonucleotide analysis of the S7-RNA complex isolated in this manner showed it to consist of a region of RNA from sections P-A of the 16S RNA. A single characteristic oligonucleotide was absent from this region, and it was tentatively concluded that this missing oligonucleotide contains the actual site of cross-linking.

INTRODUCTION

The application of RNA-protein cross-linking techniques to the study of the structural organization of the Escherichia coli ribosome is a relatively new field, but nevertheless several methods have already been reported for inducing this type of cross-linking. The techniques that have been aplied to intact ribosomal subunits include irradiation with ultra-violet light (1-3), reaction with formaldehyde (4), and reaction with bi-functional chemical reagents (5). In addition, it has been demonstrated that various proteins can be linked to the 3'-terminus of the ribosomal RNA via periodate oxidation of the 3'-terminal ribose, followed by Schiff's base formation with protein amino groups and subsequent stabilization by borohydride reduction (6,7).

The proteins involved in these cross-linking reactions have to a large extent been identified, but this is not sufficient information for conclusions to be drawn concerning the topographical organization of protein and RNA in the ribosomal subunits. The ribosome is a highly complex structure, and in particular many of the proteins have very elongated shapes, as revealed by immune electron microscopy, neutron scattering, and a number of physico-chemical studies (reviewed in ref. 8). It is therefore clear that useful structural information will only be gained from this type of cross-linking study if the actual sites of cross-linking on both protein and RNA can be determined.

This paper is concerned with the latter part of this problem, namely the identification of cross-linking sites on the ribosomal RNA. In the case of the 3'-terminal oxidation of the RNA mentioned above, the point of attachment to the RNA is self-evident from the nature of the reaction, but in the other cases, where cross-linking to the RNA has occurred internally, suitable methodology for such an analysis has so far been lacking. A partial localization has been made of the site of attachment of protein S7 to 16S RNA following ultraviolet irradiation and mild nuclease digestion of intact 30S subunits (9), as well as similar localizations of the sites of attachment of proteins S4 and S20, following irradiation of single protein-16S RNA complexes (10). The problem in making a more precise localization of the cross-linking site lies in the fact that, at some stage after the cross-linking reaction and partial digestion of the RNA, the cross-linked complex must be de-proteinized to dissociate unreacted protein. This leaves a mixture of ribosomal RNA fragments, of which only some will contain the cross-linked protein. If the deproteinization and separation of these products is attempted by gel electrophoresis in the presence of dodecyl sulphate, as is usually the case (9,10), then a complex profile is obtained which is virtually impossible to analyse successfully, particularly if the cross-linked products are present in low amounts relative to the free RNA fragments. This difficulty results from the fact that in the presence of dodecyl sulphate, protein-free and protein-containing RNA fragments are intermingled in the gel.

In order to overcome this problem, we have developed a gel system which has the unique property of dissociating protein from RNA, while at the same time being able to separate free RNA fragments cleanly from cross-linked RNA-protein complexes. We have applied this system to the analysis of the point of cross-linkage of protein S7 to 16S RNA, which, as mentioned above, is induced by ultraviolet irradiation of intact 30S subunits. This simple cross-linking method was chosen for these experiments, since S7 is the only protein which is cross-linked to any extent to RNA under mild irradiation conditions (1). The complex between S7 and fragments of 16S RNA, which was isolated on the new gel system after mild digestion of the irradiated subunits, was subjected to fingerprint analysis. This showed that the point of attachment of the protein lies within a short well-defined region of the 16S RNA, the probable site of cross-linkage being indicated by the clear absence of a single characteristic T_1-oligonucleotide.

MATERIALS AND METHODS

Preparation, Irradiation, and Hydrolysis of Ribosomal Subunits

Ammonium chloride-washed 30S ribosomal subunits from *E. coli* strain MRE 600, either double-labelled with ^3H in the RNA and ^{14}C in the protein moiety, or single-labelled with ^{32}P in the RNA moiety, were prepared as described previously (9,11). Aliquots containing 10 A_{260} units were irradiated for 20 min with ultraviolet light in 2 ml of a buffer containing 5 mM magnesium acetate, 50 mM KCl and 10 mM Tris-HCl pH 7.8 as in ref. 1. The irradiated subunits were concentrated by ethanol precipitation followed by suspension in 5 mM magnesium acetate, 20 mM KCl, 2 M urea and 10 mM Tris-HCl pH 7.8 at a concentration of ca. 30 A_{260} units/ml, and were then treated with ribonuclease T_1 (17 enzyme units per A_{260} unit ribosomes). The products of hydrolysis were separated on a 5% polyacrylamide gel run at pH 6, all these operations being carried out exactly as has been previously described in detail (1,9,12). Gel slices containing the ribonucleoprotein fragment "Band III" (12) were used for the subsequent experiments.

Application of the "Triton Gel System"

Polyacrylamide slab gels were prepared with the following composition: 6% acrylamide, 0.3% methylene-bis-acrylamide, 0.4% dimethylaminopropionitrile, 50 mM Tris-HCl pH 7.8, 0.2% Triton X-100, 2.5 mM EDTA, 200 mM LiCl, 7 M urea, and 0.03% ammonium persulphate. The reservoir buffer consisted of 50 mM Tris -citric acid pH 8.8, 0.2% Triton X-100 and 2.5 mM EDTA. Gel slices containing Band III from single or double-labelled irradiated and unirradiated subunits were loaded onto this gel, and electrophoresis was allowed to continue for 24 or 48 hrs at 100 volts, following which the gel was sliced and analysed for radioactivity.

Analysis of Cross-linked S7

Appropriate gel slices from the Triton gel containing ^{32}P-labelled RNA-protein complexes were pooled, homogenized through a syringe, and stirred for 4 hr with 1 ml of 10 mM Tris-HCl pH 7.8, 0.05% sodium dodecyl sulphate, 1 mM EDTA and 6 mM 2-mercaptoethanol. The acrylamide residue was centrifuged off, washed with 0.5 ml of water, and the combined eluate and washings were made 100 mM in sodium acetate. 50 µg of unlabelled carrier RNA was added, followed by 3.5 vol. ethanol, and the mixture was allowed to stand overnight at -20°. The precipitated material (containing the RNA-protein cross-linked complex) was centrifuged off and redissolved in 0.3 ml of the dodecyl sulphate buffer above. 4 A_{260} units of unlabelled 30S subunits were added, together with 10 µl of ribonuclease A (1 mg/ml) and 5µl of ribonuclease T_1 (100,000 units/ml). The mixture was incubated at 37° for 30 min to digest the RNA as far as possible, and was then reprecipitated by addition of 5 vol. ethanol at -20° overnight. After centrifugation, the pellet was dissolved in 20 µl of Sarkosyl gel reservoir buffer, incubated a second time with ribonucleases as above, and loaded onto a 17-23% polyacrylamide Sarkosyl gradient gel, exactly as described (1). After electrophoresis, the gel was stained to reveal the positions of the unlabelled proteins, sliced, and the ^{32}P-radioactivity in the region of protein S7 determined.

Oligonucleotide Analysis of S7-RNA Cross-linked Complexes

^{32}P-labelled RNA-protein complexes were extracted from the Triton gel as described above. After standing at -20° overnight, the precipitated material was redissolved in 0.1 ml of 10 mM Tris-HCl pH 7.8, 1% dodecyl sulphate, 100 mM sodium acetate, and the solution was centrifuged to remove remaining traces of polyacrylamide. The RNA-protein complex was precipitated by addition of 3 vol. ethanol for 3 hr at -20°, centrifuged off, washed twice with 80% ethanol and dried in a dessicator. The complex thus isolated (containing 40,000-70,000 Cerenkov counts/min) was digested with ribonuclease T_1 and applied to the two-dimensional fingerprint system of Sanger et al (13) on DEAE paper exactly as described (12). After electrophoresis and autoradiography, the oligonucleotide spots were cut out from the fingerprint and their radioactive content measured. Oligonucleotides requiring secondary digestion were eluted from the DEAE paper with 3 M triethylammonium carbonate, pH 10. The eluates were lyophilized to remove the triethylammonium carbonate, and were then digested with ribonuclease A and applied to small polyethyleneimine plates, using the two-dimensional separation system of Volckaert and Fiers (14). The digestion products were located by autoradiography with the help of Kodak intensifying screens.

RESULTS AND DISCUSSION

We have demonstrated in previous publications that 30S ribosomal subunits can be readily split by mild T_1-ribonuclease digestion in the presence of 2 M urea into two well-defined ribonucleoprotein fragments, the smaller of which ("Band III") contains proteins S7, S9, S10, S14 and S19, together with RNA comprising about 450 nucleotides from sections 0' - A of the 16S RNA (12). Further, it was shown (9) that this hydrolysis behaviour was to a first approximation not disturbed by ultraviolet irradiation of the 30S subunits under mild conditions which lead to the cross-linking to 16S RNA of S7 alone (1). When Band III was isolated from irradiated subunits and deproteinized on dodecyl sulphate polyacrylamide gels, the RNA fractions thus obtained were shown to contain the cross-linked protein S7 (9),

indicating that the point of cross-linkage lies in these same sections O' - A of the RNA.

However, when a more precise localization was attempted by similar experiments on gels containing urea in addition to dodecyl sulphate, in order to reveal hidden breaks and thus to liberate smaller pieces of RNA, a clear result was not obtained (9). This was due to the problem of separation between protein-bound and free RNA fragments, which was mentioned in the Introduction and which has already been discussed previously (4,9). The mobility of the RNA fragments in the gel is affected by the presence of cross-linked protein to an extent which cannot be predicted, except to say that the mobility shift caused by the protein becomes obviously greater as the RNA fragments become smaller, thereby effectively hindering a precise localization of the cross-linking site in the presence of a complex mixture of large amounts of the free RNA fragments. In this case there was the additional complication that the urea-dodecyl sulphate gel profiles of Band III RNA from irradiated subunits were not directly comparable to those from un-irradiated subunits (in contrast to the corresponding profiles on dodecyl sulphate gels minus urea (9)), and this is at least partly due to the formation of intra-RNA cross-links (C. Zwieb and R. Brimacombe, unpublished results).

Since this type of situation is likely to be fairly typical of any RNA-protein cross-linking experiment, there is a clear need for a system which has the property of separating free RNA from RNA-protein complexes. This should be possible on gels which do not contain dodecyl sulphate, where the relatively basic ribosomal protein is able to exert its positive charge (cf. 15), but this leaves the problem of how to remove non-cross-linked protein from the complex, as mentioned in the Introduction. In our hands, once the RNA-protein complexes have been exposed to dodecyl sulphate to deproteinize, they tend to aggregate irreversibly when the dodecyl sulphate is removed. Various other de-proteinizing methods, such as acetic acid extration (16), were also unsatisfactory, as the behaviour of the complex in such systems is very unpredictable and depends on the length of the piece of RNA attached to the protein. The

use of protein-specific antibodies (17) also does not offer a simple solution.

The best answer to this problem would be a dodecyl sulphate-free gel system which deproteinizes in situ, at the same time as separating the cross-linked complexes from free RNA. The polyacrylamide gel system which we have devised for this purpose contains a mixture of 8 M urea, 200 mM LiCl and 0.2% Triton X 100 (a non-ionic detergent), and the detailed composition of the gel is given in Materials and Methods. While this mixture would almost certainly not cause complete deproteinization of ribosomal subunits in a test-tube, the conditions of continually displaced equilibrium which pertain in an electrophoretic separation do allow deproteinization to occur. Trials with intact ^{3}H- and ^{14}C-labelled 30S subunits on 3% gels of this type demonstrated that all the protein remained at or near the gel origin, whereas the RNA moved as a reasonably sharp peak into the gel (data not shown). This gel system will be referred to for convenience as the "Triton system".

To investigate the behaviour of cross-linked RNA-protein complexes in this system, irradiated and unirradiated double-labelled 30S subunits were treated with ribonuclease T_1, and the hydrolysates separated on a 5% polyacrylamide gel at pH 6, exactly as described (9, and see Materials and Methods). Gel slices containing ribonucleoprotein Band III from both reaction mixtures were loaded directly onto a 6% "Triton" gel, and the radioactive profiles obtained from this gel are illustrated in Fig. 1.

The gel profile of Band III from the unirradiated 30S sample (Fig. 1A) shows, as expected, that all the ^{14}C-protein remains near the gel origin, and no protein radioactivity was found in the peak of ^{3}H-RNA. The peaks of ^{3}H and ^{14}C are well separated, with no significant radioactivity in the intervening region. In contrast, the profile from the irradiated sample (Fig. 1B) shows a broad peak containing both protein and RNA, lying (as indicated by the arrows) between the two main peaks. This is presumed to be the S7-RNA cross-linked complex, and it should be noted that the RNA content of this peak is of the order of 10% of that in the main RNA peak. This is roughly in

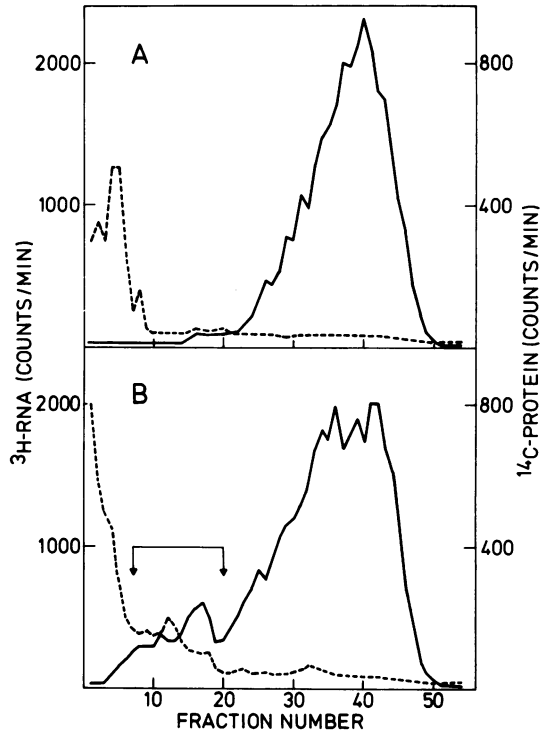


Figure 1: Separations in the Triton gel system.

Gel slices containing double-labelled ribonucleoprotein fragment Band III were applied to the gel, and electrophoresis was continued for 24 hr. Direction of electrophoresis is from left to right and the fractions are 1.7 mm slices of the gel. (———) 3H-RNA; (- - -) 14C-protein. A: Band III from unirradiated 30S subunits. B: Band III from irradiated subunits. The arrows indicate the region presumed to contain the cross-linked RNA-protein complex.

accordance with expectation, given that these irradiation conditions lead to cross-linking of 15-20% of the S7 to the RNA (1,9), and that not all the RNA sequences contained in RNA from Band III are represented in the cross-linked complex (see later). Resolution in the Triton system at this gel concentration is not quite sufficient to separate the characteristic RNA sub-fragments found in Band III from one another (cf. 12). However, in similar experiments with ^{32}P-labelled RNA, which were analysed by autoradiography instead of by slicing and counting the gel, distinct bands of RNA could be seen (data not shown).

In order to demonstrate positively the presence of cross-linked S7 in the central region of the Triton gel, similar experiments were made using ^{32}P-labelled subunits. In this case the gels were allowed to run further to improve the separation, and typical profiles of the upper part of the Triton gels thus obtained are shown in Fig. 2, the main peaks of RNA being off the right-hand side of the diagram. As in Fig. 1, only Band III RNA from the irradiated sample (and not from the control) showed radioactivity in the presumed RNA-protein complex region of the gel, i.e. in fractions ca. 10 to 30, corresponding to the arrowed region of Fig. 1B.

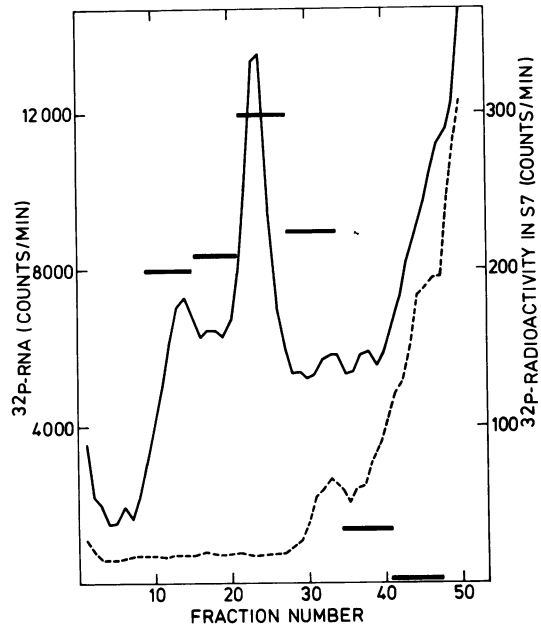


Figure 2: Isolation of cross-linked ^{32}P-labelled S7-RNA complex.

Gel slices containing irradiated or unirradiated ^{32}P-labelled Band III were applied to a Triton gel as in Fig. 1. Electrophoresis was continued for 48 hr. (———) RNA from irradiated Band III; (- - -) RNA from unirradiated Band III. Gel slices were pooled as indicated by the horizontal bars, and were analysed for the presence of cross-linked S7 (see text and Fig. 3). The height of each bar indicates the amount of 32P-labelled S7-oligonucleotide complex found, according to the scale on the right side of the diagram.

Gel slices were pooled as indicated by the horizontal bars in Fig. 2, and the RNA-protein complexes were extracted, digested with ribonucleases A and T_1, and applied to a 17-23% polyacrylamide gel containing Sarkosyl (1), as described in Materials and Methods. After running, the gels were sliced and analysed for ^{32}P-radioactivity in the region of protein S7. Typical results of such analyses are shown in Fig. 3, one from the peak of the RNA-protein complex region (fractions 21-27, Fig. 2), and one from the free RNA region (fractions 41-47, Fig. 2). In the former case, a peak of radioactivity can be seen, running a little slower than S7 in the gel (cf. 1, 9), which corresponds to the ^{32}P-oligonucleotide-S7 cross-linked complex. No significant radioactivity is seen in the latter case, indicating both the absence of cross-linked S7, and demonstrating that the free digested ^{32}P-RNA does not interfere with the analysis.

The results of these S7-oligonucleotide analyses are plotted back into Fig. 2, the height of each horizontal bar indicating the amount of ^{32}P-radioactivity found in each case in the protein-oligonucleotide complex (Fig. 3). The data show clearly that cross-linked S7 is present only in the expected region of the gel (fractions 8-35, Fig. 2). This result was entirely reproducible, and no S7-oligonucleotide complex could be detected in similar analyses of the bulk of the free RNA peak (off the right-hand side of Fig. 2). Further, the amount of radioactivity recovered in the S7-oligonucleotide complexes (ca. 300 counts/min from a pool of ca. 50,000 counts/min prior to digestion) was of a reasonable order of magnitude, allowing for experimental losses, and taking into account our earlier finding that the oligonucleotide remaining attached to S7 is about 5 nucleotides in length (1).

It should also be noted that there is of course no possibility of confusing cross-linked S7 with free protein S7 by this method of analysis. The cross-linked S7 runs in a different position in the Sarkosyl gel (Fig. 3 and see ref. 1), and the free S7 is in any case unlabelled. Any contamination by free S7 (which is possible, as indicated by the gel profiles from double-labelled subunits, Fig. 1) is therefore irrelevant to the analysis.

The results described above demonstrate that the Triton gel

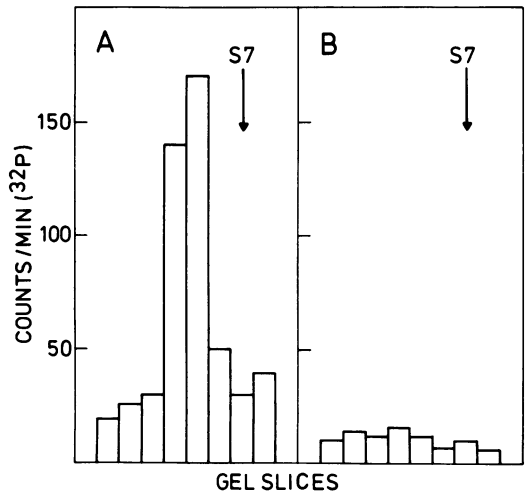


Figure 3: Analysis of ^{32}P-labelled S7-oligonucleotide complex.

Triton gel slices (Fig. 2) were pooled, and their contents extracted and applied to Sarkosyl gels as described in Materials and Methods. The position of free S7 is indicated by the arrow, and the histograms show the 32P-radioactivity found in 3 mm slices of the Sarkosyl gel in this region. A: Analysis from Triton gel slices 21 - 27 (Fig. 2). B: Similar analysis from slices 41 - 47.

system has the required capability of deproteinizing in situ, and of separating cross-linked RNA-protein complexes from free RNA. The peaks of RNA-protein complex in the gel are fairly broad (Fig. 2), and it is not to be expected that they contain homogeneous fragments of the 16S RNA. More likely, these peaks consist of families of RNA fragments of varying lengths, the degree of heterogeneity depending on how far the irradiation procedure has disturbed the detailed hydrolysis behaviour of the 30S subunits (12 and cf. 9). It is also not to be expected that these fragments will be separated in the Triton gel on a simple size basis. RNA-protein complexes with a very high RNA content would be expected to move slowly on account of their size, but RNA-protein complexes with a very low RNA content should also move slowly, due to the relatively greater braking effect of the positively charged protein; fragments of intermediate RNA content should move the fastest. However all the RNA fragments will have the common feature that each must encompass the point (or points) of cross-linkage of S7 to the RNA.

It can therefore be predicted that an oligonucleotide analysis of the RNA-protein complexes from an experiment such as that of Fig. 2 would show a fingerprint in which only those oligonucleotides closest to the point of cross-linkage would be present in high yield. The yield of other oligonucleotides should progressively decrease in relation to their distance from the cross-linking point, reflecting the heterogenous population of fragments. Further, since even after digestion with both ribonuclease A and T_1 an oligonucleotide approximately five residues long remains attached to the irradiated S7-RNA complex (1), it might be possible to discern the actual site of cross-linking by the absence of several nucleotide residues.

Accordingly, ^{32}P-labelled S7-RNA complexes were isolated as above, and suitable slices from the Triton gel (corresponding to the four sample pools rich in cross-linked S7, which are depicted by the horizontal bars in Fig. 2) were extracted, digested with ribonuclease T_1, and subjected to fingerprint analysis as described in Materials and Methods. The results are given in Fig. 4 and Table 1. Fig. 4 (left) shows a ribonuclease T_1 fingerprint obtained from the main peak of the S7-RNA complex (corresponding to fractions 21-27 in Fig. 2), and the same fingerprint is shown diagrammatically in Fig. 4 (right), with the oligonucleotide spots numbered according to the system of Uchida et al. (18 and cf. ref. 12). As predicted above, the fingerprint is obviously heterogeneous, but with several spots appearing at a noticeably higher intensity. All the spots were cut out from the fingerprint, and the relative molarities of the oligonucleotides determined. In ambiguous cases the oligonucleotides were eluted, and subjected to a secondary digestion with ribonuclease A, using the two-dimensional thin-layer system of Volckaert and Fiers (14) to separate the products, as described in Materials and Methods.

Table 1 gives the results of these analyses, averaged over three independent experiments, for the characteristic oligonucleotides in sections O'-A of the 16S RNA (i.e. the region of RNA contained in Band III (12)), arranged in the order in which they occur in the sequence (19,20). The Table shows that a few oligonucleotides from section O' and D are present in small but

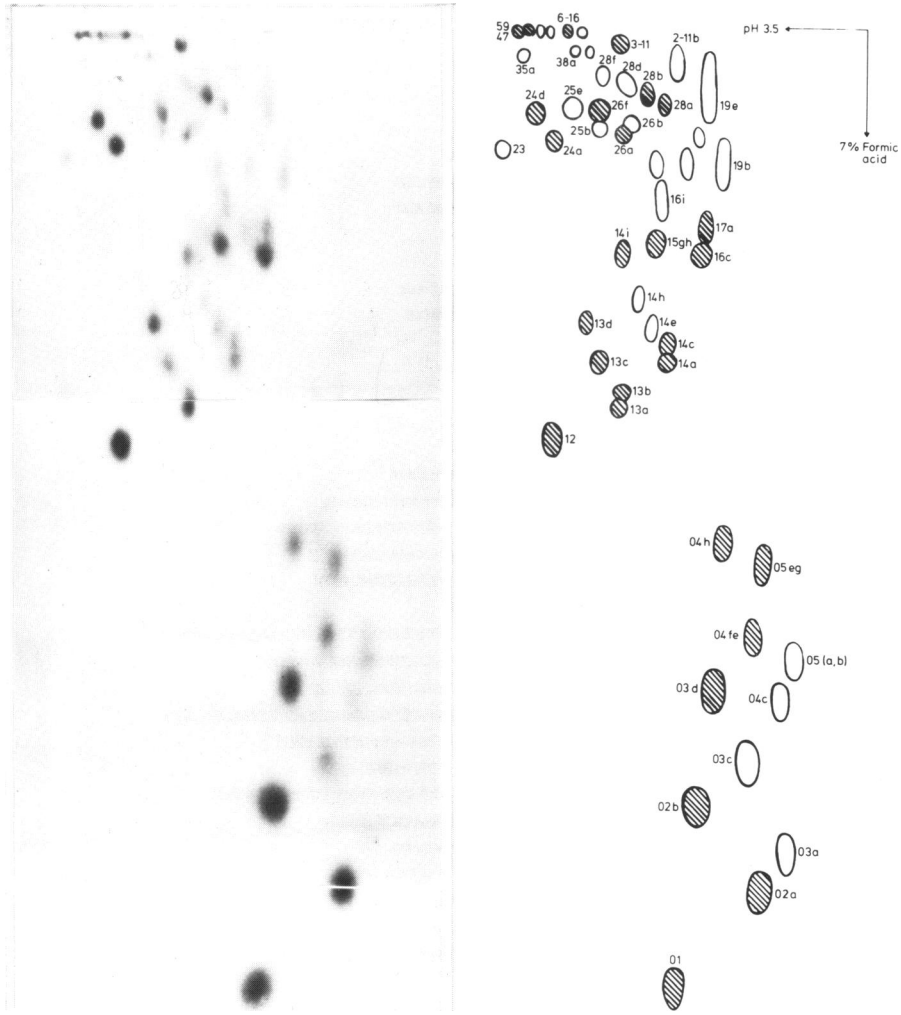
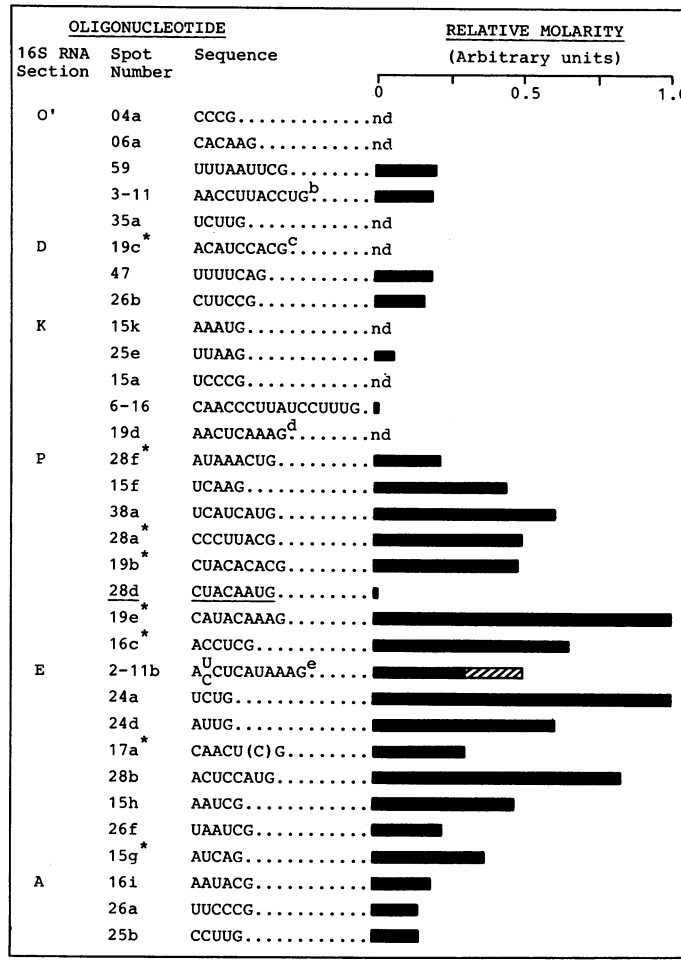


Figure 4: Fingerprint of the cross-linked S7-RNA complex.

The two-dimensional electrophoresis was made as described in Materials and Methods, with the direction of the first dimension from right to left, and that of the second from top to bottom. The oligonucleotide spots are numbered (right side of diagram) according to the system of Uchida et al. (18, and cf. 12), with the strongest spots emphasized by shading.

significant amounts, but that the strongest oligonucleotide spots arise exclusively from sections P-A. Further, one oligonucleotide, namely C-U-A-C-A-A-U-G (28d) from near the middle of

Table 1: Oligonucleotide analysis of the cross-linked S7-RNA[a]



a. The oligonucleotides are listed in the order in which they occur in 16S RNA (19,20), and the spot numbers correspond to those in Fig. 4. The horizontal bars indicate the relative molarities of each oligonucleotide; "nd" denotes not detected. The missing spot 28d (section P) is underlined. Asterisks denote oligonucleotides whose sequence has been modified in the latest 16S RNA sequence (20).

b. This spot gave A-A-C, A-C, and A-U, A-A-A-G on digestion with ribonuclease A, indicating contamination with the U-rich form of spot 2-11b (section E).

c. Not separated from 19b (section P), but no A-U was found in the ribonuclease A digest of the latter.

d. Not separated from 19e (section P), but no A-A-C was found in the ribonuclease A digest of the latter.

e. The cross-hatched part of the horizontal bar denotes the contribution made by the U-rich form of this oligonucleotide, which runs with spot 3-11 (see text and footnote "b").

this region, is almost entirely absent. This oligonucleotide was clearly separated from its nearest neighbouring spot (28b, A-C-U-C-C-A-U-G) in control fingerprints of unirradiated RNA from ribonucleoprotein Band III (cf. Fig. 4), and was also distinguishable from the latter by digestion with ribonuclease A, which yielded A-U but not A-A-U from spot 28b. It should be noted here that the sequences of 28b and 28d have been exchanged with respect to our previous work (12) and the nomenclature of Uchida et al. (18). It is clear from both the results of ribonuclease A digestion and the positions of the two oligonucleotides on the fingerprint (Fig. 4), that the oligonucleotide containing the A-rich sequence (C-U-A-C-A-A-U-G) corresponds to the spot which is named 28d in Fig. 4, and not to 28b.

The yield of oligonucleotides from section P was variable in the different experiments (e.g. spots 28f, 38a and 28a are absent or very weak in the example shown in Fig. 4), reflecting a variation in the length of the S7-RNA fragments at their 5'-end, but in every case spot 28d was virtually absent. This was also observed in fingerprints from other S7-RNA regions of the Triton gel (corresponding to fractions 8 to 20 in Fig. 2), although the peak region (fractions 21-27) gave the clearest results.

The amounts of intervening short non-characteristic or non-separated oligonucleotides (Fig. 4) were consistent with the results found for the characteristic oligonucleotides (Table 1), but it should be noted that there are some anomalies with respect to the ordering of these short oligonucleotides in the published sequence (20) of the 16S RNA in this region (A. Ross and R. Brimacombe, unpublished results). No characteristic oligonucleotides further towards the 3'-end of the 16S RNA than C-C-U-U-G (middle of section A) were present in the S7-RNA, and no oligonucleotides from other regions of the RNA outside sections O'-A were observed (cf. ref. 12), with the exception of A-A-A-G (04h) which possibly arises from overdigestion of spots such as 2-11b. The latter spot (see Table 1) was always found in rather lower yield than expected in our experiments, both with irradiated and unirradiated RNA. Oligonucleotide 17a was also reproducibly found in low yield in the S7-RNA. It should

be noted that the yield of 2-11b (A-C-C-U-C-A-U-A-A-A-G) has been supplemented in Table 1 by the other form of this oligonucleotide A-U-C-U-C-A-U-A-A-A-G (see ref. 19), which runs together with spot 3-11 (A-A-C-C-U-U-A-C-C-U-G) on the fingerprints, but which can be distinguished from the latter by digestion with ribonuclease A. Spots 19b and 19c, and 19d and 19e, were also distinguished by ribonuclease A digestion.

The results of Table 1 show that the principle site of cross-linking of S7 to RNA lies in the area between sections P and the 5'-half of A, confirming our earlier tentative conclusion (9). Results from experiments where the S7-RNA fragments were somewhat shorter (such as that depicted in Fig. 4) allow the 5'-half of section P to be discounted, leaving a region of about 180 nucleotides stretching from the middle of section P to the middle of A. The small amounts of oligonucleotides from sections O' and D could indicate a minor second site of cross-linking to protein S7, but the presence of these oligonucleotides is more likely a result of the ultraviolet-induced RNA-RNA cross-link, which is known to form to some extent between sections O'-D and P-A under these irradiation conditions (C. Zwieb and R. Brimacombe, unpublished results). The minor unidentified oligonucleotide spots remaining at the start-line of the second dimension of the fingerprint (Fig. 4) could arise from RNA-RNA cross-links, and experiments are in progress to determine the point or points of intra-RNA cross-linking. The anomalously low yield of oligonucleotide 17a (Table 1) may also result from this type of reaction.

Such side-reactions cannot however reasonably account for the reproducible absence of oligonucleotide 28d from all the S7-RNA fractions. This oligonucleotide lies between oligonucleotides 19b and 19e in the 16S RNA (20), but is separated from them by short non-characteristic sequences, namely U-G, G, and C-G. We tentatively conclude therefore that the actual site of cross-linking to S7 lies either within 28d itself or within these short adjacent sequences. In accordance with this conclusion are tests which have shown that an S7-oligonucleotide complex, obtained after total digestion with ribonuclease T_1, can be isolated on a dodecyl sulphate polyacrylamide gel, and that this

complex yields A-C, C, U, and G upon further digestion with ribonuclease A. These products are consistent with a cross-linking point in oligonucleotide 28d, and the experiments will be reported in detail elsewhere.

As mentioned in the Introduction, the ultraviolet irradiation system (1,9) was chosen for these experiments, because the RNA-protein cross-linking reaction can be largely restricted to a single protein, namely S7. The results described here show that a clear analysis of the RNA region in the neighbourhood of the cross-link could be obtained, despite the rather heterogeneous nature of the S7-RNA fragments resulting from the irradiation procedure. For the more complex (and more usual) situation where several proteins are simultaneously cross-linked to the RNA (e.g. ref. 5), a further purification step or steps will obviously be necessary. Preliminary tests have shown that RNA-protein complexes isolated on the Triton gel system can be purified by a second electrophoresis step, and, more important, that these complexes can be 5'-labelled with ATP and polynucleotide kinase. This opens the possibility of direct determination of the protein cross-linking point within the ribosomal RNA fragments, by application of the gel sequencing methodology which we have recently reported (21).

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