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Evidence for RNA-RNA cross-link formation in Escherichia coli ribosomes

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ABSTRACT

Evidence is presented in three separate cases for the formation of RNA-RNA cross-links in intact E. coli ribosomes and ribosomal subunits. The first case is a cross-link between the 18S and 13S regions of the 23S RNA, induced by ultraviolet irradiation. The second is a cross-link at the subunit interface, generated by the bifunctional reagent bis-(2-chloroethyl)-amine. The third example is a cross-link between sections O'-D and P-A of the 16S RNA, induced as in the first case by ultraviolet irradiation. The RNA-RNA cross-links can be identified as such, despite the complications introduced by concomitant RNA-protein cross-linking reactions. The experiments represent a first attempt to introduce RNA-RNA cross-linking into studies of the topographical organization of the RNA within the ribosome.

INTRODUCTION

The spatial organisation of the RNA within ribosomal subunits is a subject of central importance in studies of ribosome structure, but the problem has up to now not received a great deal of attention. Chemical modification with kethoxal (e.g. 1) has yielded detailed information concerning the accessibility of specific nucleotide residues on the subunit surface, and similar information has to a lesser extent been provided by nuclease digestion experiments (e.g. 2,3). However, in order to obtain direct data on the relative positions of different regions of the RNA chains, another type of approach is required. In this context, some tertiary (or long-range) interactions have been observed between remote regions of the 16S RNA (4-6), but such interactions are only likely to be detectable in cases where a relatively strong complementarity exists between the RNA regions concerned. A more general method would be the introduction of intra-RNA cross-linking

methodology, in an analogous manner to the cross-linking approaches used in the study of protein-protein neighbourhoods or protein-RNA neighbourhoods in ribosomal subunits (reviewed in ref. 7).

It was in fact demonstrated several years ago by Malbon and Parish (8) that intra-RNA cross-links could be generated within E. coli ribosomes by the reagent bis-(2-chloroethyl)-sulphide ("mustard gas"), and, as a by-product of our own experiments on protein-RNA cross-linking (6,9-11), we have on several occasions observed effects which could be attributable to the formation of cross-links within the ribosomal RNA. An analysis of such cross-links would give very useful information on the topographical organization of the ribosomal RNA, and the purpose of this paper is to present some RNA-RNA cross-linking data, in three separate cases. The first example is a cross-link generated within the 23S RNA in the 50S subunit by irradiation with ultraviolet light (cf. ref. 9), and the second is a cross-link generated between 23S and 16S RNA in 70S ribosomes by treatment with the reagent bis-(2-chloroethyl)-amine ("nitrogen mustard") (11). The third example is a cross-link within the 16S RNA in the 30S subunit, also generated by ultraviolet irradiation (cf. ref. 9), and in this latter case we describe the partial localization of the RNA regions concerned.

MATERIALS AND METHODS

Preparation of Ribosomal Subunits

Ammonium chloride-washed ribosomal subunits were prepared from E. coli strain MRE 600 as described (12). The subunits were either labelled in the RNA moiety with ^{3}H or ^{32}P, or else were double-labelled with ^{3}H in the RNA and ^{14}C in the protein moiety (12,13).

Cross-link in 23S RNA

^{3}H- and ^{14}C-labelled 50S subunits (9 A_{260} units per sample) were diluted to a concentration of 4.5 A_{260} units/ml in a buffer consisting of 10 mM magnesium acetate, 50 mM KCl, 6 mM 2-mercaptoethanol and 10 mM Tris-HCl pH 7.8, and were irradiated with ultra-

violet light for various times (up to 10 min) under the conditions previously described (9). The solutions were concentrated by ethanol precipitation followed by resuspension in 100 µl of the same buffer, and were each split into two aliquots of 20 and 80 µl, respectively.

Each 20 µl aliquot was treated with 2 µl of ribonuclease A (0.1 µg/ml) for 1 hr at 25°C, and enzyme was removed by applying the solution to a 3% polyacrylamide-agarose gel containing 1 mM magnesium and 20 mM potassium, at pH 7.8 (14). The gel was run until a bromophenol blue marker had travelled 10 cm, and was then sliced and analysed for radioactivity. Slices containing 50S subunits were applied directly to a second 3% gel, containing in this case 0.1% dodecyl sulphate and 2 mM EDTA (6). This gel was allowed to run a similar distance, and was also sliced and analysed for radioactivity, to determine the proportions of 23S, 18S and 13S RNA. In other experiments (see text), the nuclease treatment was carried out before the irradiation; in such cases the nuclease-treated samples were simply diluted to the appropriate concentration for irradiation, and were then loaded onto the magnesium-containing 3% gel without an ethanol precipitation step.

To analyse the concomitant RNA-protein cross-linking reaction, the second (80 µl) aliquots of the original irradiation mixtures were applied directly to the 3% polyacrylamide-dodecyl sulphate gel system above, and the ^{3}H- and ^{14}C-radioactivity in the gel was carefully measured. The ratio of these isotopes in the 23S RNA peak as compared with a control sample (not irradiated) allows the overall percentage of RNA-protein cross-linking to be estimated, knowing the ratio of ^{3}H:^{14}C in the original subunit preparation (9). The gel slices containing 23S RNA were then extracted into dodecyl sulphate buffer as in ref. 15, and the extracted RNA (plus RNA-protein complex) was precipitated with ethanol overnight in the presence of unlabelled 50S subunits, digested with excess ribonuclease, and applied to a 17 - 23% polyacrylamide Sarkosyl gradient gel, again exactly as described (9, 15). After running, the gel was stained with amido black, and then analysed for ^{14}C-radioactivity in the stained protein bands, in order to gain a further measure of the extent of RNA-protein cross-linking. The yield of cross-linked protein (either for pro-

tein L4 or for the total protein in the gel (see text)) was calculated from this recovered ^{14}C-radioactivity, knowing the amount of ^{3}H-labelled 23S RNA extracted from the dodecyl sulphate gel above, taken together with the ratio of $^{3}H:^{14}C$ in the original subunit preparation (cf. ref. 9).

Cross-link between 16S and 23S RNA

16 A_{260} units of ^{3}H-labelled 50S subunits (26 x 10^6 counts/min total) and 9 A_{260} units of ^{32}P-labelled 30S subunits (3 x 10^6 counts/min total) were dialysed separately into 5 mM magnesium acetate, 50 mM KCl, 6 mM 2-mercaptoethanol, 25 mM triethanolamine-HCl pH 7.8 (buffer A). The dialysed subunits were mixed together (volume ca. 1 ml), the magnesium concentration was raised to 15 mM (buffer B), and the mixture was incubated for 1 hr at 37°C to induce 70S couple formation (cf. ref. 16). At the end of this time, 2 vol. ethanol was added, and the precipitated particles were resuspended at a concentration of 30 A_{260} units/ml in buffer A. Ribonuclease A was added (0.5 mμg per A_{260} unit ribosomes) and the solution was incubated for 1 hr at 25°C (cf. previous section). The 70S particles were next separated on a 10-30% sucrose gradient in buffer B, which was run for 16 hr at 19,000 rpm and 4°C in an SW 27 rotor (16). Fractions containing 70S ribosomes (60-70% of the total material) were pooled, ethanol precipitated, and taken up again in buffer A at a concentration of 12 A_{260} units/ml. The pH was adjusted to 8.5 by addition of 1 M triethanolamine, and 2 A_{260} unit aliquots of the solution were treated with various amounts of a 100 mM solution of bis-(2-chloroethyl)-amine ("nitrogen mustard"), to give final mustard concentrations of 0-20 mM (cf. ref. 11). The mixtures were incubated for 2 hr at 37°C, and were then loaded into slots of a 3-8% polyacrylamide gradient gel containing dodecyl sulphate (10), which was allowed to run until the bromophenol blue marker had run at least 20 cm. The gel was sliced and analysed for ^{32}P- and ^{3}H-radioactivity.

To examine the RNA-protein cross-linking reaction, the starting material consisted of ^{3}H-labelled 30S subunits and ^{3}H- and ^{14}C-labelled 50S subunits (or vice versa). The ribonuclease digestion step was omitted, and the nitrogen mustard incubation (in buffer A adjusted to pH 8.5) was made in this case before appli-

cation to the sucrose gradient, with various concentrations of reagent. After running the gradients, fractions containing 70S ribosomes were pooled, ethanol precipitated, and applied to polyacrylamide gradient gels as above. The RNA peaks from these gels were extracted with dodecyl sulphate buffer, and their cross-linked protein content was analysed (after ribonuclease treatment) in a manner similar to that described in the previous section. In this case however, the digested oligonucleotides were removed by several ethanol precipitations from Sarkosyl buffer (11), and the protein analysis was made on the gel system of Mets and Bogorad (17), as described previously (11).

Cross-link in 16S RNA

4 A_{260} units of ^{32}P-labelled 30S subunits (3 x 10^8 counts/min total) were irradiated with ultraviolet light, hydrolysed with ribonuclease T_1 and separated into ribonucleoprotein fragments on polyacrylamide gels run at pH 6, exactly as described (6,15). Gel slices containing the small ribonucleoprotein fragment "Band III" (together with similar slices from an unirradiated control sample) were applied to a 7% polyacrylamide gel containing dodecyl sulphate and 7 M urea, again as described (13), and the RNA bands were located by autoradiography. Appropriate gel slices were extracted with phenol, and the RNA collected by ethanol precipitation in the presence of unlabelled carrier RNA as described (13), with 100 mM sodium acetate being added to aid the precipitation. The samples were purified by a second ethanol precipitation, and were then digested with ribonuclease T_1 and subjected to oligonucleotide analysis, using the method of Sanger et al. (18), as previously outlined (13). Secondary digestions with ribonuclease A were made on oligonucleotides eluted from the fingerprints, according to the method described by Brownlee (19).

RESULTS AND DISCUSSION

1. RNA-RNA cross-link in 23S RNA

When 50S ribosomal subunits are irradiated with ultraviolet light under mild conditions, an RNA-protein cross-linking reaction takes place, in which the primary targets are proteins L4

and L2 (9). Although this reaction takes place quite rapidly, there is an even faster reaction which involves the formation of an RNA-RNA cross-link between the two halves of the 23S RNA molecule. This can be demonstrated very simply by examining the effect of ultraviolet irradiation upon the ability of the 23S RNA to be split into an 18S and a 13S fragment (20) after mild nuclease digestion. In these experiments, 50S subunits (labelled with ^{3}H in the RNA and ^{14}C in the protein moieties) were irradiated for various times, and aliquots were submitted to nuclease digestion under conditions known to yield the 18S and 13S fragments (cf. 10,20). Nuclease was removed by electrophoresis in magnesium containing polyacrylamide gels (see Materials and Methods and cf. ref. 10), followed by deproteinization and separation of the RNA fragments on gels containing dodecyl sulphate. At the same time, further aliquots of the original irradiated 50S subunits were deproteinized by electrophoresis on dodecyl sulphate gels, and the 23S RNA fractions thus obtained were completely digested with ribonuclease and analysed on Sarkosyl gels for the presence of cross-linked proteins as described in Materials and Methods. The results of typical experiments are shown in Figs. 1 and 2.

Fig. 1 shows the effect of ultraviolet irradiation on the formation of the 18S and 13S fragments. It can be seen that, whereas the RNA in the control sample (Fig. 1A) is almost entirely broken down into the two complementary fragments, the irradiated samples show a progressive increase in the amount of material remaining as 23S RNA, until after 10 min irradiation there is very little fragment formation at all. (The RNA species also become slightly retarded in the gels with increasing irradiation time). It is important to note that a very similar result was obtained when the nuclease treatment was made prior to the irradiation, and therefore the effect on the hydrolysis profile is not merely due to an inability of the nuclease to attack the irradiated subunits under these conditions. These results are summarized in Fig. 2, where the percentage of the RNA remaining as 23S is plotted against irradiation time for both cases, that is to say with nuclease treatment before or after irradiation.

Fig. 2 also shows the corresponding analyses of cross-linked

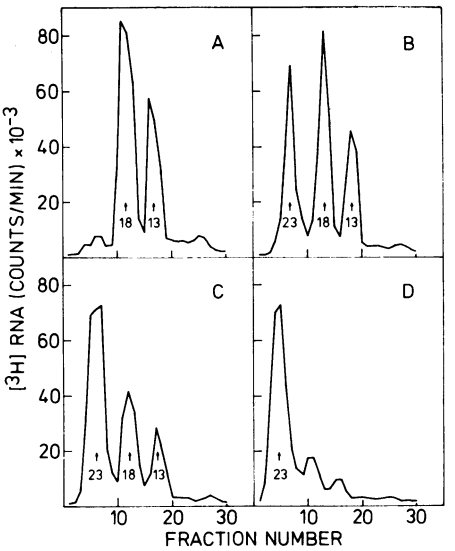

Figure 1: Effect of irradiation of 50S subunits on the formation of 18S and 13S fragments.
The fractions are 1.7 mm gel slices, and the direction of electrophoresis is from left to right. The positions of 23S, 18S, and 13S RNA are indicated. A: Unirradiated control. B,C and D: Samples irradiated for 1 min, 3 min and 10 min, respectively.

protein in the samples, as it is obviously necessary to demonstrate that the observed result (Fig. 1) is not a result of a protein bridge formed between the 18S and 13S species. Analysis was made of the percentage of total 50S protein cross-linked to 23S RNA (estimated both from the $^{3}H:^{14}C$ ratio in the dodecyl sulphate gel and from the ^{14}C-radioactivity in cross-linked protein recovered from the Sarkosyl gel; see Materials and Methods), and also of the percentage of available protein L4 which was cross-linked to RNA (estimated from the Sarkosyl gel). The results of all these analyses are included in Fig. 2. Protein L2 (not shown) is cross-linked to RNA to an extent of about one-third that of L4 (9).

It is clear from Fig. 2 that the L4 cross-linking reaction follows a different kinetic curve to that of the hydrolysis inhibition, and that in any case insufficient L4 (or L2) is cross-linked to the RNA to account for the degree of inhibition. Bearing in mind that formation of a protein bridge between the 18S and 13S species would require two simultaneous RNA-protein cross-

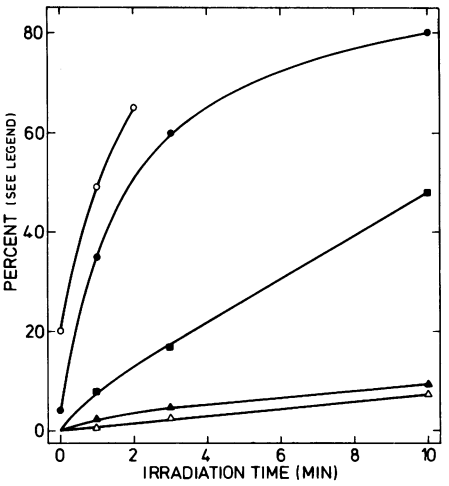

Figure 2: Effect of irradiation of 50S subunits upon various parameters.
●——●: Percentage of RNA remaining as 23S (cf. Fig. 1), with nuclease treatment after irradiation. o——o: The same, but with nuclease treatment before irradiation. (The hydrolysis to 18S and 13S fragments was not quite complete in the control in this case). ■——■: Percentage of protein L4 cross-linked to 23S RNA. △——△: Percentage of total protein cross-linked to 23S RNA, estimated from the 3H:14C ratio in the dodecyl sulphate gel (see Materials and Methods). ▲——▲: The same, estimated from 14C-radioactivity in the Sarkosyl gel.

links to be formed on a single protein, it is also out of the question that the low level of overall "background" RNA-protein cross-linking observed (e.g. 3-4% after 3 min irradiation, Fig. 2) would suffice for non-specific protein bridges to be formed involving various different proteins cross-linked to different individual RNA molecules; although this level of cross-linking corresponds to an average of about one 50S protein per RNA molecule, formation of the second simultaneous cross-link necessary for a protein bridge would be a second order effect of very low probability. We conclude therefore that the observed inhibition of 18S-13S fragment formation is due to an RNA-RNA cross-link between the two halves of the 23S RNA.

2. RNA-RNA cross-link between 16S and 23S RNA

Partially complementary sequences have been observed between 16S and 23S RNA, and interactions between these sequences at the subunit interface have been postulated (e.g. 1,21). However, no direct evidence has so far been published in support of

these hypotheses, and we have therefore attempted to cross-link 16S to 23S RNA within the 70S ribosome. At the same time our intention was to find out whether the 18S or 13S fragment of the 23S RNA was involved in the cross-link. We were unable to detect any cross-linking at the subunit interface with ultraviolet irradiation, and in consequence we have used the symmetrical bifunctional compound bis-(2-chloroethyl)-amine, which we have recently introduced as an RNA-protein cross-linking reagent (11). Just as in the case of the 18S-13S cross-link described above, the experiment divides into two parts, namely the demonstration that a cross-link has occurred, and the analysis of the concomitant RNA-protein cross-linking reaction, in order to exclude the possibility that a protein bridge between the two RNA species is responsible for the observed effect.

To demonstrate the cross-link, 30S subunits (labelled with ^{32}P) were mixed with 50S subunits (labelled in the RNA moiety with ^{3}H) and incubated under conditions for 70S couple formation. The reaction mixture was then subjected to a mild nuclease treatment, and the 70S particles (containing "nicked" RNA) were separated from subunits and ribonuclease by sucrose gradient centrifugation as described in Materials and Methods. The 70S ribosomes thus isolated were treated with various concentrations of the nitrogen mustard reagent, and their RNA content was analysed on gels containing dodecyl sulphate, again as described in Materials and Methods. A typical result is shown in Fig. 3.

The control sample (Fig. 3A) confirms that under these conditions the only point of attack of the ribonuclease is that giving rise to the 18S and 13S fragments of the 23S RNA (cf. ref. 22 and Fig. 1); no significant hydrolysis of 16S RNA occurred. In the cross-linked samples, two distinct effects were observed. The first of these was that at lower concentrations of reagent, the 18S and 13S fragments gradually disappeared (as in Fig. 1), until predominantly a 23S RNA peak remained, besides the 16S RNA. An example of this situation is illustrated in Fig. 3B, at a reagent concentration of 14 mM. Fig. 3B also shows the second effect at an early stage, namely the appearance of a shoulder containing both ^{3}H and ^{32}P, running more slowly than the 23S RNA. As the reagent concentration was raised, this shoulder developed into

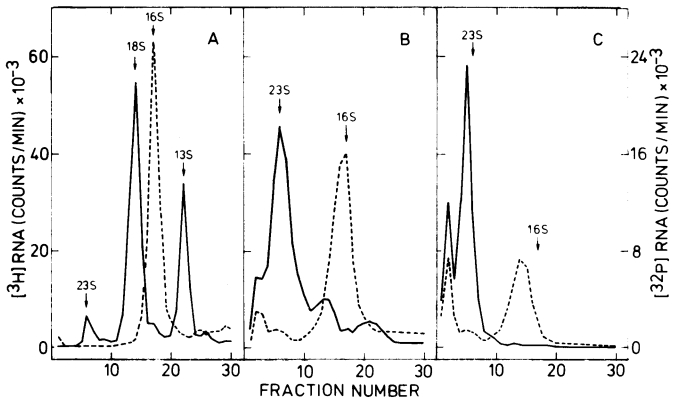

Figure 3: Effect of nitrogen mustard upon 16S and 23S RNA from 70S particles containing a "nicked" 23S RNA.
————: 3H-labelled RNA. – – – –: 32P-labelled RNA. A: Control sample, minus cross-linking reagent. B and C: Samples treated with 14 and 20 mM nitrogen mustard, respectively. The positions of the various RNA species are indicated.

a distinct slow-running peak as illustrated in Fig. 3C, at a reagent concentration of 20 mM. As in Fig. 1, the RNA peaks became slightly retarded in the gel (and also broader in this case) as a result of the cross-linking reaction, but the new double-labelled peak is obviously a cross-linked species containing both 16S and 23S RNA. At higher reagent concentrations, the 23S and 16S peaks both disappeared leaving a single double-labelled species corresponding to the new peak in Fig. 3C.

To examine the possibility of the 16S and 23S RNA molecules being joined by a protein bridge, similar experiments were made using 70S particles in which one subunit was labelled only with ^{3}H in the RNA moiety, and the other with both ^{3}H in the RNA and ^{14}C in the protein moieties. In this case the nuclease digestion was omitted, and the incubation with cross-linking reagent was made prior to separation on the sucrose gradient. The 70S ribosomes were again isolated, and the corresponding RNA fractions were separated on gels as above and analysed for RNA-protein cross-linking as described in the previous section, with the exception that the gel system of Mets and Bogorad (17) was used instead of the Sarkosyl system (see Materials and Methods). The reaction conditions were chosen to give RNA profiles precisely similar to that shown in Fig. 3C.

The overall level of RNA-protein cross-linking in the 70S particle was found to be less than that observed in isolated subunits (11) at the same concentration of nitrogen mustard. Analysis of the ^{14}C-labelled proteins in the cross-linked peak (i.e. the new peak in Fig. 3C) showed, just as described in the previous section, that insufficient RNA-protein cross-linking (ca. 3% of the total protein) had occurred at this level of reaction to account for the observed effect in terms of a protein bridge. This was true both when the 50S proteins were ^{14}C-labelled, and when the 30S proteins were ^{14}C-labelled in the original 70S couple. Again, we conclude that an RNA-RNA cross-link has been formed, between the 16S and 23S RNA. The fact that the 18S-13S fragment formation is almost completely inhibited before this interface cross-link starts to form to a significant extent (Fig. 3B), has so far prevented us from defining which half of the 23S RNA is involved in the reaction.

3. RNA-RNA cross-link in 16S RNA

For the demonstration of the cross-link within 16S RNA, we have used a rather different approach. The RNA-RNA cross-linking in this case takes place concomitantly with the well-established ultraviolet-induced RNA-protein cross-linking reaction involving protein S7 (9). When ultraviolet-irradiated 30S subunits are split into two ribonucleoprotein fragments by mild nuclease digestion, it has been shown that the smaller of the two fragments ("Band III") contains the cross-linked protein (6). In addition, the characteristic pattern of RNA-subfragments from this small ribonucleoprotein particle, in polyacrylamide gels containing dodecyl sulphate and urea, differs slightly from the corresponding pattern derived from unirradiated subunits (13), in that an extra slow-moving RNA band was always observed (6). As will be shown below, this new band contains the RNA-RNA cross-link.

Fig. 4 shows an autoradiograph of the RNA fragments concerned, prepared as described in Materials and Methods from irradiated and unirradiated 30S subunits, and separated on a gel containing 7 M urea. The RNA bands are numbered as in refs. 6 and 13, and the new band (marked "X") can clearly be seen in the irradiated sample, which also shows a reduction in the amounts of bands

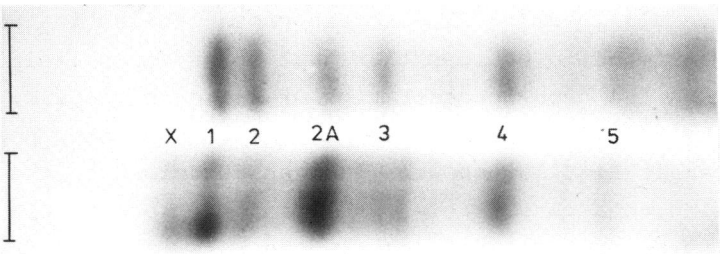

Figure 4: 16S RNA fragments from irradiated and unirradiated subunits on a 7% polyacrylamide gel.
Direction of electrophoresis is from left to right, with the starting point of the gel indicated by the vertical lines. The bands are numbered according to refs. 6 and 13, with the unirradiated sample above, the irradiated sample below.

2 and 3, relative to the control. We have shown (13) that the RNA fragments derived from unirradiated subunits contain a total of about 450 nucleotides, from sections O'-D-E'-K-P-P'-E-A of the 16S RNA (23), near to its 3'-end. In particular, fragment 1 (Fig. 4) contains sections K-A, and fragment 2 sections P-A; fingerprint analyses showed that the characteristic oligonucleotides from the remaining sections (O'-D-E') only appeared in the smaller RNA fragments (principally in fragments 3 and 4), and these oligonucleotides were entirely absent from fragments 1 and 2 (13). In contrast, similar analyses of the RNA fragments from the irradiated subunits (Fig. 4) showed that oligonucleotides from sections O'-D as well as from K-A were present in fragments 1 and 2, and the new band X also contained oligonucleotides from the whole of the O'-A region. A typical example of a ribonuclease T_1 fingerprint from this band is given in Fig. 5 and allows the following conclusions to be drawn.

Firstly, the RNA from band X does not contain an unbroken sequence of RNA from sections O'-A, since oligonucleotide 15K (A-A-A-U-G) from the 5'-end of section K is clearly absent (Fig. 5, and cf. ref. 13). This oligonucleotide was also absent in RNA from unirradiated subunits (13), and it follows that the appearance of band X does not simply reflect a failure of the ribonuclease T_1 to attack at the beginning of section K, in the original hydrolysis of the irradiated subunits. Secondly, as mentioned above, oligonucleotides from the whole of the O'-A region were nevertheless present. Although slightly more complex, the finger-

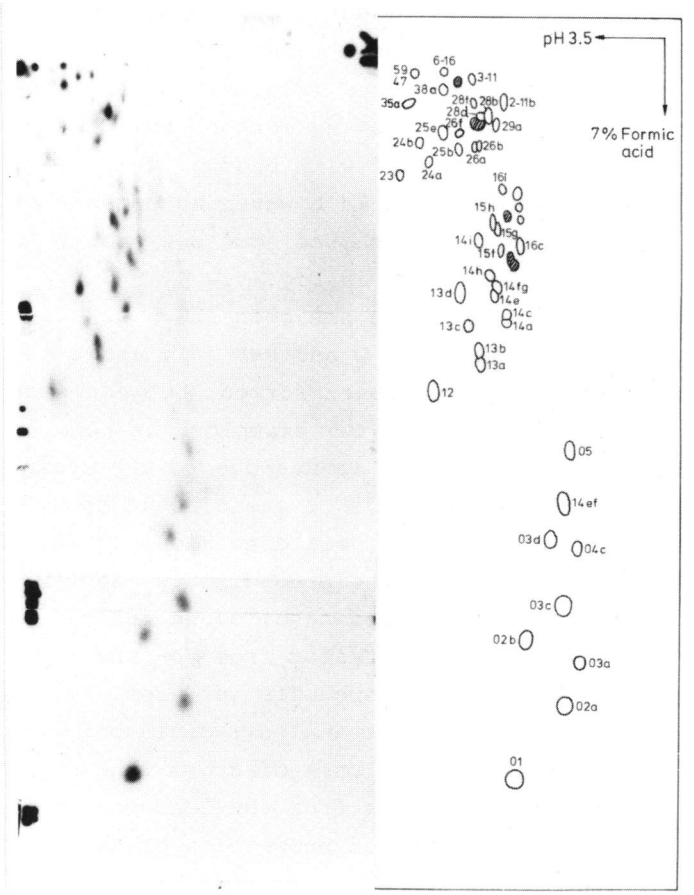

Figure 5: Fingerprint of RNA from band X (Fig. 4).

The electrophoresis was made as described in ref. 18, with the direction of the first dimension from right to left and the second from top to bottom. Principle oligonucleotides are numbered (right side of diagram) according to the system of Uchida et al. (24), and the stronger new spots are indicated by shading. The area of the fingerprint above and to the right of spot 16c was rather anomalous, and the spots in this area were not examined in detail. (The strong spot to the left of spot 59 is the 32P-ink used to mark the fingerprint.)

print closely resembles that of the total RNA from the small ribonucleoprotein fragment "Band III" (13), and examples of some unambiguous characteristic oligonucleotides, whose identities were confirmed where necessary by secondary digestion with ribonuclease A (see Materials and Methods) are :- spot 59 and/or 47 (sections O' and D respectively), spot 3-11 (section O'), spot 6-16

(section K), spots 38a and 29a (section P), spot 2-11b (section E) and spots 16i and 26a (section A) (see Fig. 5). The mobility of band X in the polyacrylamide gel (Fig. 4) is consistent with a nucleotide composition encompassing most of this RNA region (ca. 400 nucleotides, as compared with band 1, which is 300 nucleotides in length (13)). It should however be noted that the fingerprints of RNA from Band X showed some new spots, so far not identified, and these are marked in Fig. 5.

A long-range RNA interaction has been shown to exist in the 30S subunits between sections O'-D and P-A (6), and these data show that here a cross-link has been formed, between sections O'-D and K-A. As in the previous two examples, it remains to be demonstrated that the cross-link is not due to a protein bridge formed by S7, which is the only 30S protein found cross-linked in this region of the RNA under these conditions (9,15). The quantitative arguments used in the preceding two sections of this paper could not be applied in this case, since the yield of band X (Fig. 4) was always markedly greater from ^{3}H- and ^{14}C-labelled subunits than from ^{32}P-labelled subunits (cf. ref. 6), and therefore results from the fingerprint analysis could not be directly compared with a quantitative analysis of cross-linked protein S7 content. This discrepancy results from the tendency of the ^{32}P-labelled samples to show a greater proportion of smaller RNA subfragments (see Fig. 4), with the result that band X was itself often partly broken down into smaller pieces which ran together with bands 1 and 2 in the gel.

However, it has been shown (15) that the RNA cross-linked to protein S7 is characterized by the complete absence of oligonucleotide 28d (C-U-A-C-A-A-U-G) in section P; in the fingerprint of Fig. 5, the spot corresponding to this oligonucleotide is not very clearly separated from the similar octanucleotide 28b (A-C-U-C-C-A-U-G), but two moles of oligonucleotide were found in this area, and the secondary digest showed the presence of both A-A-U and A-U, in addition to U,C,G and A-C. In other words, band X contains both these oligonucleotides, in contrast to the RNA cross-linked to S7 (15). Although band X does contain some cross-linked S7 (data not shown), it follows that it must also contain at least some protein-free RNA, to account for the presence of oligonucleotide 28d. Further, we have recently ana-

lysed the oligopeptide in S7 which is cross-linked to RNA (25), and since only a single short oligopeptide was identified in these experiments, the possibility of a protein bridge being formed between S7 and two regions of the RNA is very remote. Again we conclude, as was previously suggested (6), that band X contains an RNA-RNA cross-link. The small number of unidentified spots on the fingerprint of band X (Fig. 5) may represent products of this cross-link, or may reflect a further cross-link with a short region of RNA from another part of the 16S molecule.

CONCLUSION

RNA-RNA cross-linking is a virtually unexplored field in studies of ribosomal topography, and the experiments described here represent our first attempts to develop suitable technology in this area. The results show that RNA-RNA cross-links can be induced between identifiable RNA fragments or species within the ribosomal particles, and that these cross-links can be distinguished with reasonable certainty from any RNA-protein-RNA bridges which might be formed simultaneously. The next and most important stage in the research will be the precise localization of the RNA sequences concerned in the reactions. This raises a number of technical problems which will not be easy to solve; further work is however in progress.

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