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# Localisation of a series of intra-RNA cross-links in 16S RNA, induced by ultraviolet irradiation of Escherichia coli 30S ribosomal subunits

Christian Zwieb and Richard Brimacombe

Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann, Berlin-Dahlem, GFR

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ABSTRACT

Mild ultraviolet irradiation of E. coli ribosomal subunits leads to the formation of a number of intra-RNA cross-links, in addition to the RNA-protein cross-links already reported (see refs. 9, 10). After partial ribonuclease digestion of the RNA from irradiated subunits, complexes containing these intra-RNA cross-links can be isolated on a two-dimensional gel electrophoresis system, and subjected to sequence analysis. A series of these cross-linked complexes is described, and the cross-linked RNA regions are compared with the secondary structure derived for 16S RNA (see refs. 6, 7).

## INTRODUCTION

In 1971 it was demonstrated by Malbon and Parish (1) that sulphur mustards could be used to generate intra-RNA cross-links within ribosomal subunits. However, since that time there has been a long lag in the application of this type of cross-linking approach, despite its obvious importance for studies on the structural organisation of the ribosomal RNA in situ. The reason for this delay is primarily that the localisation of the sites of cross-linking within the RNA chains is technically very difficult, such localisations being essential if useful topographical information is to be gained.

Wagner and Garrett (2) have described a reversible RNA-RNA cross-linking reagent with which they were able to pin-point a site of cross-linking in the double-helical stem region of 5S RNA from E. coli, but their technology has not yet been applied to the much more complex 16S and 23S RNA molecules. More recently, Cantor and his co-workers have used photo-activatable psoralens to induce intra-RNA cross-links in isolated 16S RNA or 30S subunits (3,4), and have attempted to localise the cross-links by

electron microscopy. In this case however the accuracy of the measurements is severely limited by the resolving power of the electron microscope.

Two years ago (5) we reported that simple ultraviolet irradiation could be used to generate intra-RNA cross-links within E. coli 30S or 50S subunits, but at that time we were unable to localise the cross-links. Here we describe a solution to this problem, based on a two-dimensional gel electrophoresis system which has the property of separating fragments of free RNA from those containing an RNA-RNA cross-link. This gel system is a slight modification of the one already reported for the analysis of interacting sequences in ribosomal RNA (6), and the application of the system has enabled us to localise a series of cross-links within the 16S RNA, induced by mild irradiation of 30S subunits. All of the cross-links so far identified appear to be within helical domains of the secondary structure which we have proposed for the 16S RNA (6,7).

## MATERIALS AND METHODS

$^{32}$P-labelled 30S subunits were prepared from E. coli MRE600 as described (8), with the exceptions that no ammonium chloride wash was made, and that the isolated subunits were subjected to an activation dialysis as in ref. 9 (cf. ref. 7). The subunits (ca. 5 $A_{260}$ units, 5 x $10^8$ counts/min) were irradiated with ultraviolet light at a concentration of 5 $A_{260}$ units/ml in 50 mM KCl, 5 mM magnesium acetate, 10 mM Tris-HCl pH 7.8, under the conditions previously reported (9,10), except that the time of irradiation was reduced to 7 min. After concentration by ethanol precipitation, the irradiated subunits were applied to a 7.5 - 30% sucrose gradient containing 0.1% dodecyl sulphate, 0.3 mM magnesium acetate, 10 mM NaCl, 10 mM Tris-HCl pH 7.8, as described (6). After centrifugation, gradient fractions containing 16S RNA were pooled, precipitated with ethanol to concentrate, and taken up in 50 µl of this same dodecyl sulphate buffer. The RNA concentration was adjusted to 100 $A_{260}$ units/ml by addition of a suitable amount of unlabelled carrier RNA, and the solution was treated with ribonuclease $T_1$ (60 units per $A_{260}$ unit of RNA) for 30 min at 37°.

The ribonuclease hydrolysis was stopped by digestion with proteinase K (10 mg/ml), the enzyme being added in 2 x 5 µl aliquots, with incubation for 15 min at 37° after each addition. The hydrolysate was then applied to the two-dimensional gel system of ref. 6, using a 5 - 20% polyacrylamide gradient in the dodecyl sulphate-EDTA buffer system (11) as first dimension, and a 20% polyacrylamide gel in the buffer system of Maxam and Gilbert (12) as second dimension. Before polymerising into the second dimension, the gel strips from the first dimension were soaked in second dimension reservoir buffer containing 5% 2-mercaptoethanol for 30 min at 37°. Two second dimension gels were used for each first dimension sample strip, exactly as in ref. 6.

After electrophoresis, the gels were autoradiographed, spots corresponding to cross-linked RNA fragments (see text) were cut out, and the RNA was extracted with phenol, then precipitated with ethanol as described (8), in the presence of 40 µg carrier RNA. The isolated RNA was fully digested with ribonuclease $T_1$, using double the normal concentration of enzyme (8 µg per sample; cf. ref. 8), and during a 15 min incubation with the nuclease the temperature was slowly raised from 37° to 60°, this incubation being followed by a further 15 min at 60° to complete the hydrolysis. The samples were lyophilized, and the oligonucleotides separated on polyethyleneimine plates in the "mini-fingerprint" system of Volckaert and Fiers (13), as described (14). In order to separate material remaining at the origin after the second dimension, a strip was cut from each polyethyleneimine plate encompassing the sample application point and following the direction of the second dimension. These strips were subjected to a further chromatographic elution, using 3 M urea and 0.75 M ammonium bicarbonate pH 7 as solvent. After drying, the strips were rinsed in 70% ethanol, taped back onto the same plates from which they had been cut, and each whole plate was then subjected to autoradiography. The oligonucleotides were eluted, digested with ribonuclease A, and the products separated on polyethyleneimine plates in the "double-digestion system" of Volckaert and Fiers (13), exactly as described (7).

## RESULTS AND DISCUSSION

The chief problem in identifying intra-RNA cross-links in a large molecule such as 16S RNA is one of separation. Some type of partial hydrolysis must be introduced, in order to generate RNA fragments of an identifiable size encompassing the cross-link, and these fragments must be separated from the complex mixture of free RNA fragments. This problem becomes considerably worse when the intra-RNA cross-links are only present in low yield, and in the case of cross-links induced into intact ribosomal subunits there is a further complication due to the concomitant RNA-protein cross-linking reaction, which is usually unavoidable.

The solution to this problem which we describe here has evolved as a by-product of our analysis of secondary structure in 16S RNA (6,7). This analysis involves a two-dimensional gel electrophoresis system in which sets of interacting RNA fragments are first separated under non-denaturing conditions, and are then dissociated in a second gel dimension containing urea. Gel patterns from this system show a "diagonal" consisting of non-dissociable pieces of RNA, whereas the dissociated pairs of fragments run as families lying below the diagonal (6,7). The area above the diagonal is entirely free of radioactivity (cf. Fig. 1 in ref. 7), but, if a cross-linked RNA digest is applied to the system, then radioactive spots appear in this otherwise clear area of the gel. We will show that these spots contain the intra-RNA cross-links.

30S subunits were irradiated with ultraviolet light, with an irradiation time approximately one-half of that used in our previous experiments (9), where we were concerned with the analysis of the specific cross-link formed under these conditions between protein S7 and 16S RNA within the 30S subunit. RNA was isolated from the irradiated subunits, partially digested with ribonuclease $T_1$, and applied to the two-dimensional gel system, as described in Materials and Methods. The clarity of the gel patterns was improved by soaking the first-dimension gel strips in buffer containing 2-mercaptoethanol before applying to the second dimension (see Materials and Methods). The appropriate area of a typical two-dimensional gel pattern is shown in Fig. 1, the key diagram on the right of the Figure indicating the radio-

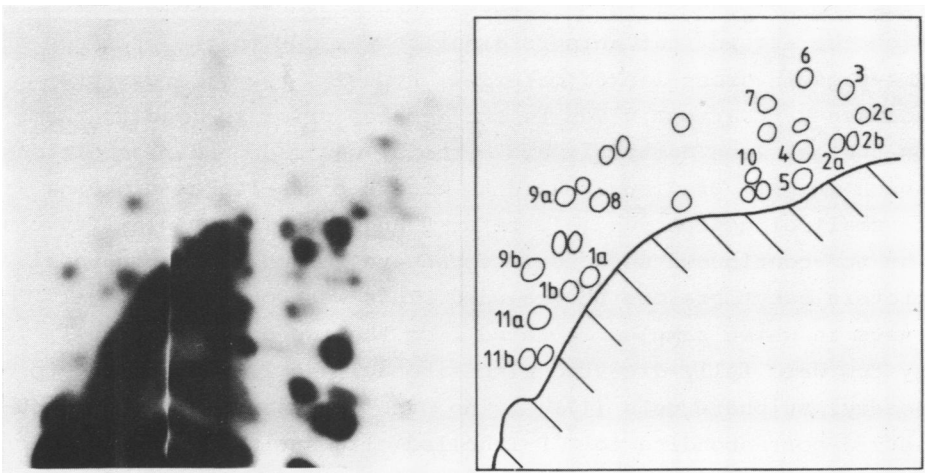



Figure 1: Two-dimensional gel separation of cross-linked RNA complexes. Direction of electrophoresis is from right to left, then from top to bottom. The Figure is a composite of the two second dimension gels used for each sample (see Materials and Methods). The key diagram on the right indicates the "diagonal" (solid line, cf. ref. 6), and also the spots which were not observed in a parallel non-irradiated control sample. The numbered spots are those discussed in the text (cf. Figs. 3 and 4).

active spots which were entirely absent from a parallel non-irradiated control sample. This pattern of spots was quite reproducible (in particular the strong triangle of spots comprising number 9b, 1a and 8), although it varied in some details from one experiment to another. The evidence that these extra spots in the irradiated sample correspond to RNA-RNA cross-linked fragments (and not to RNA-protein cross-linked material) is as follows.

Firstly, oligonucleotide analysis showed that most of the spots contained non-contiguous sequences of RNA, often from RNA regions widely separated in the primary structure of the 16S molecule (15,16). In any particular spot, the sequence regions involved were usually of different lengths, which precludes the possibility of a chance co-migration of two or more independent RNA fragments.

Secondly, the mobility of the spots in the second-dimension gel was considerably slower than that which would be expected

from the combined length of the sequences they contained. It might be argued that this retardation was due to the presence of RNA-protein cross-linked material. However, the vigorous proteinase K treatment given to the samples prior to loading them on the gel (see Materials and Methods) was made under conditions which we have previously used to digest cross-linked proteins to small oligopeptides (cf. refs. 9 and 14). The possibility that the non-contiguous sequences found are joined via a ribosomal protein can therefore be excluded. This was confirmed by gel tests in which samples isolated from the two-dimensional gel system were fully digested with ribonuclease $T_1$ and applied to dodecyl sulphate gels (17); in no case could any material be observed corresponding to $^{32}P$-labelled oligonucleotide-protein (or large peptide) complexes (cf. refs. 9 and 14). The retardation in the second gel dimension is therefore a consequence of the intra-RNA cross-link, the basis of the separation being presumably that, under the strong denaturing conditions of the second dimension, the cross-linked fragments from an "octopus-like" structure which cannot migrate freely into the gel.

Thirdly, the oligonucleotide analysis usually showed a "gap" in each sequence component, and in every case showed at least one extra oligonucleotide, which, upon subsequent digestion with ribonuclease A, gave an anomalous product. These products had chromatographic mobilities very different from those of the cross-linked oligopeptide-oligonucleotide complexes found in our previous studies (9,14), but the mobilities were consistent with those expected from short covalently-linked oligonucleotides, i.e. the digestion products from an RNA-RNA cross-link.

The key diagram on the right of Fig. 1 indicates spots which gave a clearly-interpretable and reproducible oligonucleotide analysis. The complexes are numbered from 1 to 11, and in cases where two spots gave an almost identical analysis, they are distinguished by letters (e.g. 1a, 1b). The differences between these closely related complexes reflect either slight variations in the fragment lengths or local variations at the site of the cross-link, and will not be discussed further.

Fig. 2 shows two examples of the ribonuclease $T_1$ fingerprints obtained from the cross-linked complexes, in the one case

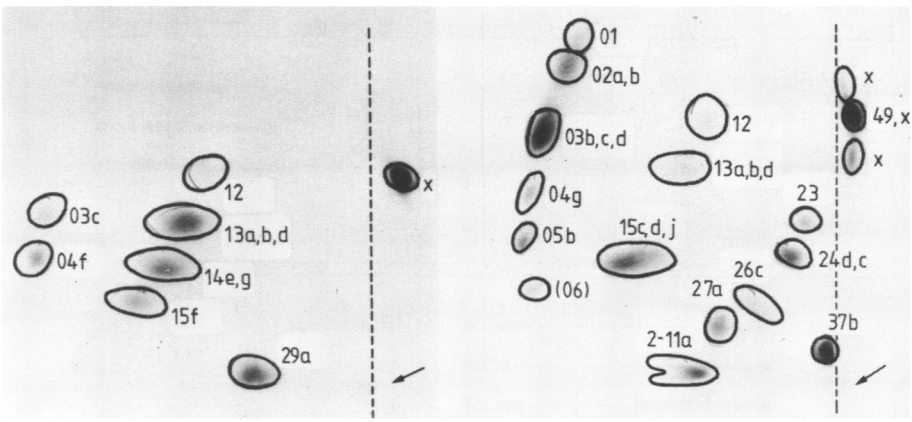



Figure 2: Examples of ribonuclease T1 fingerprints of the cross-linked complexes. Direction of chromatography is from right to left, then from bottom to top. The arrow denotes the sample application point, and the dotted line indicates the strip which was subjected to the third chromatographic dimension (see Materials and Methods). The oligonucleotides are numbered according to Uchida et al (18), the spots marked "X" containing the cross-linked oligonucleotides (see text). The fingerprints correspond to complexes 9 (cf. Fig. 3) and 3, respectively.

from a rather simple product (complex 9, Fig. 1), and in the other from a more complicated one (complex 3). In our experiments with ultraviolet-induced RNA-protein cross-links, the cross-linked oligonucleotide-oligopeptide product showed a high mobility in the first chromatographic dimension (14). Here, in contrast, the cross-linked products remained at the sample application point in the normal "mini-fingerprint" system (13), and for this reason the third chromatographic dimension was introduced (see Fig. 2) as described in Materials and Methods. This served to purify the cross-linked oligonucleotides (marked X in Fig. 2; see later for discussion) from other large oligonucleotides or small amounts of undigested material, and greatly facilitated the sequence analysis. In every case, all the oligonucleotides were eluted from the plates, digested with ribonuclease A, and the products separated in the "double-digestion" system of Volckaert and Fiers (13). The oligonucleotide sequences found were then fitted to the 16S RNA sequence (15,16), and the combined results are summarised in Fig. 3, which also illustrates

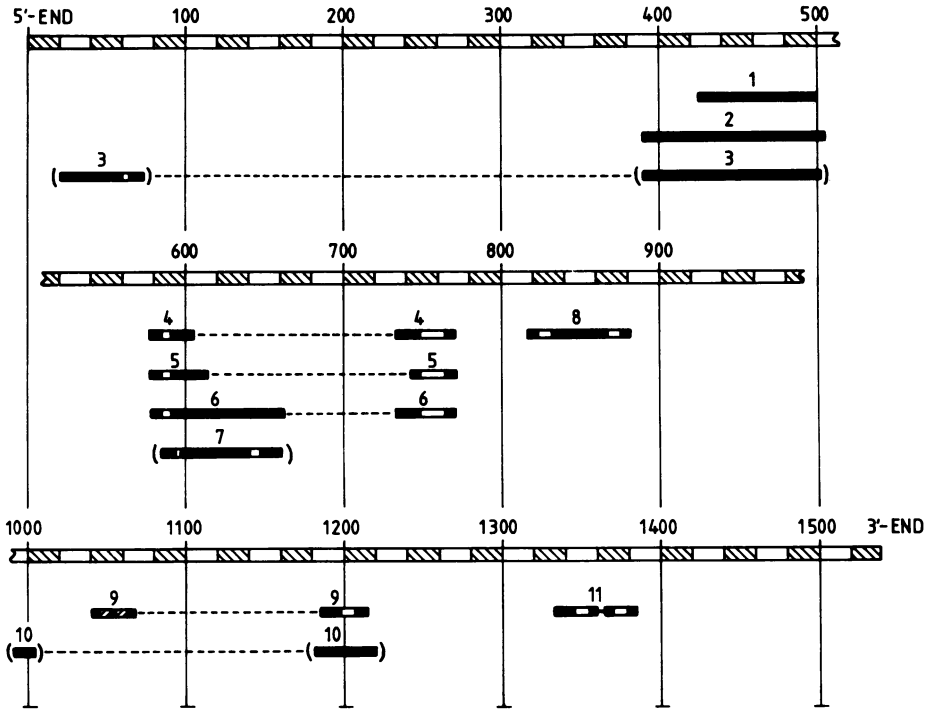



Figure 3: Location of the cross-linked complexes in 16S RNA (15, 16). The scale of nucleotides is numbered from the 5'-end, and each number (1 to 11) corresponds to a single cross-linked complex. The gaps in the blacked-out boxes denote oligonucleotides missing from the sequence analysis (the 5'-section of complex 9 showing two possibilities, see text), and the brackets denote tentative assignments.

the regions where oligonucleotides were "missing" from the sequences.

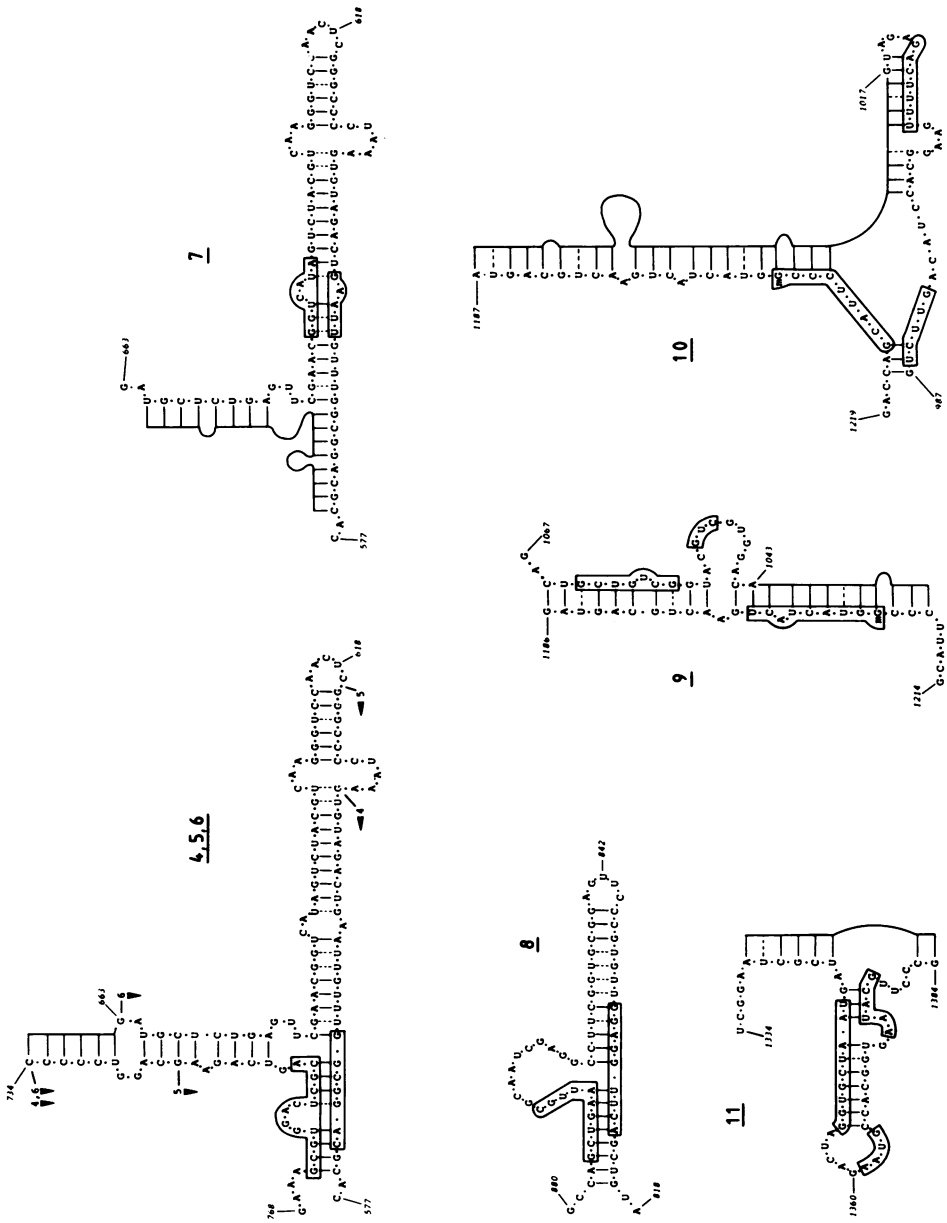
In the case of complexes 1 and 2, no missing regions could be positively identified, although $T_1$-oligonucleotides were found which gave anomalous products on digestion with ribonuclease A, suggesting that a cross-link was present within the sequence. On the other hand in complex 3, which arose from the same area of the 16S RNA (bases ca. 400 - 500), an additional sequence region was observed from the 5'-end of the RNA (Fig. 3), and in the latter region the C-A-A-G sequence (expected at positions 58 - 61 (15,16))was absent (cf. Fig. 2). This fragment complex also gave $T_1$-oligonucleotides which showed anomalous

ribonuclease A digestion products, but it is assigned as tentative in Fig. 3, since the complex was only clearly seen in one experiment.

The remaining complexes (numbers 4 to 11, Fig. 3) all lie within helical domains of the secondary structure which we have proposed for the 16S RNA (7), and the regions of this structure containing the various cross-linked complexes are illustrated in Fig. 4. Again, the "missing" ribonuclease $T_1$ oligonucleotides are indicated. An intra-RNA cross-link could be expected either to occur within a single domain of the RNA, or else to join two separate domains which lie close together in the tertiary structure. The fact that all of the complexes so far found correspond to the first of these two types (with the possible exception of complex 3, Fig. 3, cf. ref. 7) is very likely a consequence of our hydrolysis conditions. We have already observed (6,7) that our conditions tend to favour the appearance of fragments from certain regions of the RNA, most notably from bases 570 to 770 (7). It is quite possible therefore that many more ultraviolet-induced cross-links of both types are formed, which have so far escaped our attention.

Complexes 4 to 7 (Fig. 3) all lie within the same domain of the 16S RNA structure (Fig. 4, and ref. 7), although in complex 7 the cross-link is in a different position. The latter is assigned as tentative in Fig. 3, since it was not found in every experiment, but the data were nevertheless clear. The cross-link site is very well-defined in this case, comprising the missing U-U-A-A-G from one strand, and A-U-A-C-U-G from the other, both missing oligonucleotides being flanked on either side by sequences which were definitely present in the ribonuclease $T_1$ fingerprints (cf. Fig. 2). The boxed-in regions in Fig. 4 show the maximum number of nucleotides which could contribute to the cross-link site, and the G-residue at position 645 is therefore included in the boxed region for complex 7.

Complexes 4, 5 and 6 were all related. Each contained C-A-C-G (positions 577 - 580) and A-A-A-G (765 - 768), although the component fragments were of different lengths as indicated in Fig. 4. The missing oligonucleotide region was characterized by the absence of C-A-G (581 - 583) and C-U-C-A-G (755 - 759), and


4,5,6
7
8
9
10
11

the short non-characteristic oligonucleotides (G, C-G, U-G) flanking these missing sequences are included in the boxed-in region. A-C-G (756 - 758) is also tentatively included in the box, since A-C-G occurs twice in this RNA domain, but only one mol equivalent of the trinucleotide was found in the fingerprint analysis.

In complex 8, the 5' and 3'-ends of the sequence are defined by the presence of A-U-G and A-C-C-G, with A-C-U-U-G and U-U-A-A-G being absent (Fig. 4). As above, the small neighbouring oligonucleotides (G, A-G, C-G) are included in the boxes, and U-C-G (bases 874 - 876) is also included in the upper box. U-C-G occurs again in the lower part of the sequence (positions 821 - 823) and could contribute to the cross-link site instead, but since one mol of U-C-G was definitely present in the fingerprint analysis, we have only boxed-in one of these two U-C-G sequences.

Complex 9 was particularly reproducible and well-defined (cf. Figs. 1 and 2). Here the missing oligonucleotides comprise U-C-A-U-C-A-U-G from one strand, and one molecule of U-C-G or C-U-G from the other. Since these two trinucleotides cannot be distinguished from one another by ribonuclease A digestion and occur three times in this short sequence region, it is not possible to conclude which of the three is actually involved at the cross-link site. It is however worth noting that, if the structure of this RNA region is a continuous stack, then simple model-building shows the looped-out U-residue at position 1059 lying directly above the U-residue at position 1198. The displacement of the boxed-in regions from each other in this instance is therefore not incompatible with the structure drawn for this RNA domain (cf. the cross-link reported by Wagner and Garrett (2)). In this context it should also be noted that all the cross-linked

Figure 4: Sequences of the cross-linked complexes, in the secondary structure of ref. 7. Nucleotides are numbered from the 5'-end, and the "skeleton" base-pairings indicate RNA present in the secondary structure (7), but not in the complexes. Boxed-in regions show the "missing" oligonucleotides (see text). Complexes 4 to 6 all had bases 577 and 768 at their outer ends (cf. Fig. 3), the other ends of the fragment pairs being indicated by the arrowheads.

regions (Fig. 4) are associated with imperfectly base-paired parts of the structure, which allows a number of possibilities for the precise point of the cross-link.

Complex 10 arises from the same domain as complex 9. Here however only one component of the complex (positions 1187 - 1219) showed a clearly missing oligonucleotide. The other strand was characterized by the presence of the oligonucleotides A-C-A-U-C-C-A-C-G and A-A-G, but the cross-linked sequence could not be defined more precisely. For example, A-U-G was present in amounts of one mol, but this trinucleotide occurs both at positions 1015 - 1017 and 1187 - 1189, and it is not possible to know which of these was present. We conclude that either U-C-U-U-G or U-U-U-U-C-A-G was likely to have been involved in the cross-link site, but as a result of this uncertainty the complex is assigned as tentative (Fig. 3).

In the last example, complex 11, three oligonucleotides were missing, namely U-A-A-U-C-G, A-A-U-G and A-A-U-A-C-G. One mol of U-G was found in the fingerprint, although two are present in the sequence (1353 - 1354 and 1371 - 1372), and the former is arbitrarily boxed-in (cf. U-C-G in complex 8). It is not possible to say which two of the three missing regions are cross-linked, although it seems most likely that the A-A-U-G sequence is simply excised from the loop top. This excision was sometimes observed in analyses of non-cross-linked interacting fragments from this region (7).

It has already been mentioned above that the cross-linked oligonucleotides remained at the sample application point in the normal "mini-fingerprint" system (13). It can be seen from Fig. 4 that this is consistent with the contents of the boxed-in regions, even if the small non-characteristic oligonucleotides (G, C-G etc.) are not included; each cross-linked component must contain at least two G-residues, and several of the missing oligonucleotides contain more than one U-residue. Fig. 5 illustrates some examples of the ribonuclease A digests from the cross-linked oligonucleotides, after isolation with the help of the third chromatographic dimension (cf. Fig. 2). The cross-linked oligonucleotide from complexes 4 to 6 showed the most complex pattern, with three anomalous spots (Fig. 5a), which could re-

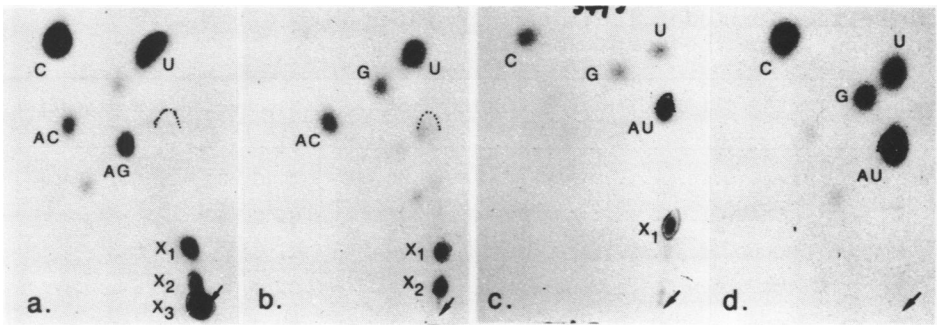



Figure 5: Ribonuclease A digestion products of the cross-linked oligonucleotides (cf. Fig. 2), in the double-digestion system of ref. 13. Direction of chromatography is from right to left, then from bottom to top. The arrow denotes the sample application point, and the dotted horseshoes the position of the xylene cyanol marker. The anomalous digestion products are marked "X". a. Digest of the cross-linked oligonucleotide from complex 6 (cf. Figs. 3 and 4). b. Corresponding digesst from complex 7. c. Corresponding digest from complex 9. d. Control digest of U-C-A-U-C-A-U-G (se text).

flect either an incomplete ribonuclease digestion near the cross-link site, or a heterogeneity within the cross-link site itself. The cross-linked oligonucleotide from complex 7 also showed two extra ribonuclease A digestion products (Fig. 5b), whereas that from complex 9 gave a single anomalous spot (Fig. 5c). In the latter instance the ribonuclease A digest from the corresponding non-cross-linked oligonucleotide U-C-A-U-C-A-U-G (cf. Fig. 4) is included for comparison (Fig. 5d). In each case the anomalous spots in Fig. 5 have mobilities consistent with those expected for short cross-linked oligonucleotides. This result should be contrasted with the peptide-containing products obtained from RNA-protein cross-linked complexes in our previous experiments (9,14), where very high mobilities in both dimensions of the chromatographic system were observed.

The ribonuclease A digestion alone does not allow a precise identification of the cross-link points. However, the ribonuclease A digestion products can be used to demonstrate that the composition of each cross-linked ribonuclease $T_1$ oligonucleotide (cf. Fig. 2) is consistent with that expected from the missing

Table 1: Ribonuclease A digestion products derived from the cross-linked oligonucleotides[a], compared with oligonucleotides "missing" from the complexes (Fig. 4).

| Complex number. | Oligonucleotides missing from the complexes[b]. | Ribonuclease A digestion products[c]. |
|---|---|---|
| 4,5,6 | CAG.(G.CG.G).....(ACG).CUCAG.(G.UG.CG) | C, G, U, AC, AG, (AAC)[d] |
| 7 | UUAAG.....AUACUG.(G) | G, U, AC |
| 8 | ACUUG.(G.AG.G)...(CG).UUAAG.(UCG) | C, G, U, AC, AAG |
| 9 | (G).CUG.UCG.....UCAUCAUG.(mG) | C, G, U, AU |
| 10 | UCUUG...UUUUCAG..(mG).CCCUUACG | C, G, U, AC, (AAC)[d] |
| 11 | UAAUCG.(UG.G).....AAUG....AAUACG | C, G, U, AC, AAU[e] |

a. Compare Fig. 2, where the cross-linked oligonucleotides are marked "X". b. The oligonucleotides from the boxed-in regions (Fig. 4) are given, with the short non-characteristic oligonucleotides (see text) included in brackets. c. Compare Fig. 5; in each case one or more anomalous slow-running spots were observed in addition to the oligonucleotides listed. d. A weak A-A-C spot was sometimes observed (cf. Fig. 5). e. A-A-U was present in some experiments, absent in others.

oligonucleotides corresponding to the boxed-in sequences in Fig. 4. The results of the ribonuclease A analyses for complexes 4 to 11 are given in Table 1, which shows on the one side these missing oligonucleotides, and on the other the products found in the ribonuclease A digests of the cross-linked oligonucleotides. It can be seen that, with the exception of the occasional appearance of small amounts of A-A-C in the analyses corresponding to complexes 4 - 6 (cf. Fig. 5a) and 10, there are no inconsistent products. Preliminary experiments have shown that further digestion with ribonuclease $T_2$ of the oligonucleotides resistant to ribonuclease A (Fig. 5) leads in some cases to the release of Ap, but in no case leads to complete degradation to mononucleotides.

## CONCLUSION

The results described here demonstrate that ultraviolet irradiation induces a number of covalent intra-RNA cross-links within the 30S subunit. The analyses provide the first detailed localisations of such cross-links within the 16S ribosomal RNA

molecule, thereby directly defining several neighbourhoods in the RNA structure to within a few nucleotides. These neighbourhoods are in precise agreement with the secondary structure which we have derived for the 16S RNA (7). Further experiments are in progress to pinpoint the nucleotides actually linked together in these complexes, as well as to apply the analytical systems to cross-links induced by bi-functional chemical reagents.

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