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The tertiary folding of *Escherichia coli* 16S RNA, as studied by *in situ* intra-RNA cross-linking of 30S ribosomal subunits with bis-(2-chloroethyl)-methylamine

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SUMMARY

Intra-RNA cross-links were introduced into E. coli 30S ribosomal subunits by treatment with bis-(2-chloroethyl)methylamine. The subunits were partially digested with cobra venom nuclease, and the cross-linked complexes were separated by two-dimensional electrophoresis and analysed according to our published procedures. Tertiary structural cross-links in the 16S RNA were identified between nucleotides 31 and 306, and between the tetranucleotide 693-696 and nucleotides 794 or 799. Secondary structural cross-links, lying at the ends of double-helical regions, were found between nucleotides 46 and the trinucleotide 362-364, and between the dinucleotide 148-149 and nucleotide 174. Cross-links within double-helical elements were identified between the tetranucleotide 128-131 and nucleotide 232, between nucleotide 250 and the dinucleotide 274-275, and between nucleotides 1413 and 1486. Adenine as well as guanine residues were involved in the cross-links.

INTRODUCTION

During the last few years we have published several cross-linking studies on the ribosomal RNA from Escherichia coli (1-5). In all these experiments, 30S or 50S ribosomal subunits were used as the substrates for the cross-linking reactions, with a view to obtaining direct information on the three-dimensional folding of the RNA molecules in situ in the ribosome. The approach has led to the identification of an increasing number of intra-RNA contacts or neighbourhoods in both 16S and 23S RNA. Whereas the earlier experiments (1,2) only yielded cross-links lying within the secondary structures (6) of the molecules, the more recent results have included both secondary and tertiary structural cross-links (3-5), "tertiary" cross-links being those between RNA regions that are remote from one another in both the primary and secondary structure. The tertiary structural cross-links so

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far reported were induced by direct ultraviolet irradiation of 50S (4) or 30S (5) subunits, or by treatment of 50S subunits (3) with bis-(2-chloroethyl)-methylamine, "nitrogen mustard".

In this paper we describe a further series of cross-links, generated by nitrogen mustard in 30S subunits. As before (3-5), the procedure for identifying the sites of cross-linking involved (a) subjecting the cross-linked subunits to a partial digestion with cobra venom nuclease (7), (b) two-dimensional gel electrophoresis to separate the intra-RNA cross-linked complexes, and (c) fingerprint analysis of the individual isolated complexes. The cross-links found include five secondary structural and two tertiary structural sites, which, taken together with the information already obtained from the ultraviolet-induced cross-linking experiments (5), impose a number of rigid constraints on the folding of the 16S RNA in the 30S subunit. In contrast to the assumption previously made in evaluating the cross-linking data (3), it is clear from this series of experiments that nitrogen mustard can cross-link adenine residues in the RNA, in addition to guanines.

MATERIALS AND METHODS

^{32}P-labelled 30S ribosomal subunits from *E. coli* strain MRE 600 were prepared as described by Stiege et al (3), with the exception that 50 ml instead of 100 ml cultures were used. After isolation and activation dialysis, the 30S subunits (ca. 3 A_{260} units, 1.5 x 10^9 counts/min total) were dialysed into a buffer containing 5 mM $MgCl_2$, 50 mM KCl and 25 mM sodium cacodylate, pH 7.2. The cross-linking reaction was carried out in this buffer (ca. 0.5 ml) with 2 mM nitrogen mustard for 60 min at 37°, and excess reagent was then destroyed by incubation with 40 mM cysteamine hydrochloride for a further 15 min at 37°. The cross-linked subunits were precipitated with ethanol, and taken up in 60 µl of 1 mM $MgCl_2$, 20 mM NH_4Cl, 10 mM Tris-HCl, pH 7.8. A suitable quantity of cobra venom nuclease (cf. 3-5) was added, and the solution incubated for 60 min at 37°. Next, this solution was made 6 mM in EDTA and incubated for 10 min at 37° with 50 µg of proteinase K, then SDS was added to a concentration of 0.3% and incubation continued for a further 10 min. The digested

sample was applied directly (without ethanol precipitation (cf. 3)) to the two-dimensional gel electrophoresis system for separation of the cross-linked products.

Electrophoresis was carried out either exactly as previously described (3), or with a homogeneous 4% gel in the first dimension in place of the 3 - 15% gradient gel (3); in such cases the second dimension gels were of 7% polyacrylamide, instead of 10% and 20% (3). As before, each first dimension gel strip was divided into an upper and lower portion for the second dimension electrophoresis. Cross-linked RNA complexes were located on the gels by autoradiography, and were extracted and subjected to oligonucleotide analysis by total digestion with ribonuclease T_1 or A, according to our usual procedures (3-5). In some cases the extracted complexes were further purified by an additional electrophoresis step on 10% or 20% gels, prior to oligonucleotide analysis. The oligonucleotide data were fitted to the 16S RNA sequence of Brosius et al (8).

RESULTS

A two-dimensional gel separation of cross-linked 30S subunits after digestion with cobra venom nuclease is shown in Fig. 1. In contrast to our previous methodology (3-5), the venom nuclease digestion was carried out at low salt concentration, and the sample was applied to the gel without ethanol precipitation (see Materials and Methods). This procedure gives a better resolution of the cross-linked RNA complexes, which - as has already been demonstrated (3-5) - run in this gel system as discrete spots above the "diagonal" of free RNA fragments. The gel patterns obtained were similar but not identical in the different experiments. Taken together, however, the results led to reproducible identifications of seven different cross-link sites in the RNA, which are designated A to G according to their position in the 16S sequence (cf. Fig. 3, below). In the example shown in Fig. 1, spots corresponding to cross-links A, B and F are marked. Each of these cross-links was found in two spots in this gel, representing complexes containing RNA fragments of different lengths as revealed by the subsequent oligonucleotide analysis (cf. refs 3-5). A number of other spots corresponding

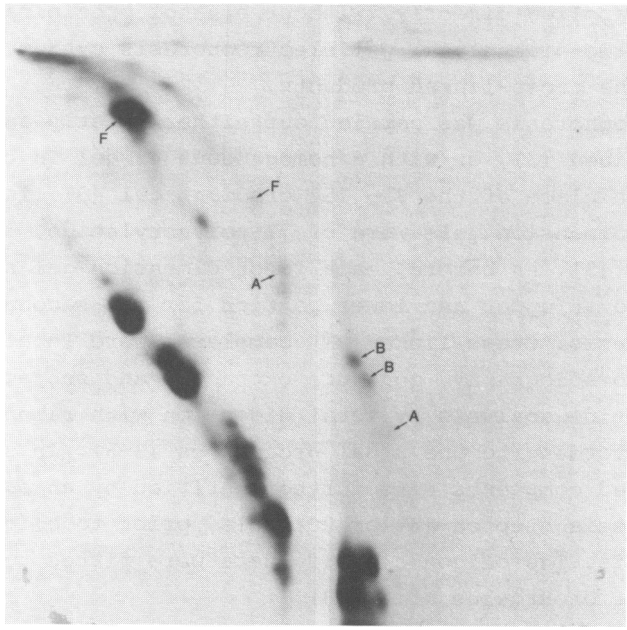

Figure 1: Separation of ^{32}P-labelled cross-linked RNA complexes on two-dimensional gels. The left-hand gel corresponds to the upper region of the first dimension gel strip (cf. ref. 3), the right-hand gel to the lower region. The first dimension gel was of 4% polyacrylamide, and the second dimension gels both 7% (see Materials and Methods). Direction of electrophoresis was from left to right (first dimension), and from top to bottom (second dimension). The lettered spots are the cross-linked complexes described in the text.

to cross-linked complexes can also be seen in Fig. 1, which in this particular gel did not give unequivocal analyses, but which in further similar gels led to the identification of the remaining cross-links C, D, E and G.

In the following section, oligonucleotide analyses of RNA complexes corresponding to one example of each of the cross-links A, B, C, D, E, F and G are described. Examples of the ribonuclease T_1 fingerprints obtained from the complexes are shown in Fig. 2, and the locations of the cross-links in the 16S RNA sequence (8) are indicated in Fig. 3.

Complex A: The ribonuclease T_1 fingerprint for complex A given in Fig. 2 is that of the faster-running of the two complexes marked "A" in Fig. 1. The RNA in the complex arises from two distinct regions of the 16S sequence, namely residues ca.

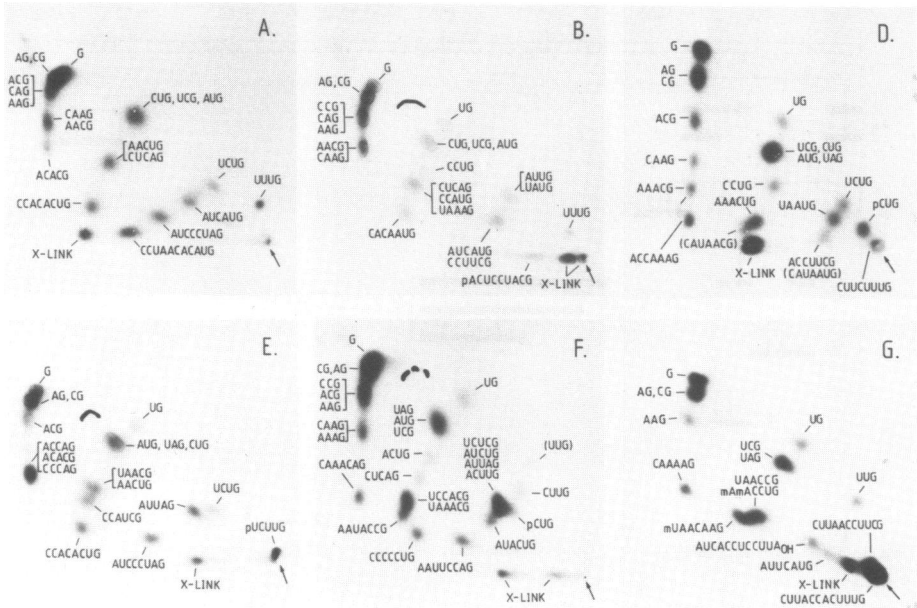

Figure 2: Ribonuclease T_1 fingerprints of cross-linked complexes A, B, D, E, F and G, on polyethyleneimine cellulose plates (9). The first dimension is from right to left, the second from bottom to top, with arrows denoting the sample application point. The identities of the oligonucleotides are given, "X-link" denoting the cross-linked oligonucleotide complex in each case. The fingerprints (with the exception of those from complexes D and G) were run in the more concentrated buffer system of ref. 5, which gives better resolution of oligonucleotides near to the origin, but poorer resolution of the very small oligonucleotides. The spots in some of the fingerprints are circled, as a result of being photographed subsequent to the routine processing for extraction and secondary digestion, the "horseshoe" indicating the position of a xylene cyanol marker dye.

7 - 72 and ca. 275 - 336. Characteristic oligonucleotides from each of these regions can be seen in the fingerprint, such as UUUG (positions 12-15), AUCAUG (16-21) or CCUAACACAUG (47-57) from the 5'-region, and AUCCCUAG (282-289), CCACACUG (311-318), or ACACG (327-331) from the 3'-region. The 5'- and 3'-termini of both regions correspond to established cobra venom nuclease cutting sites in the RNA (10), with the exception of the 5'-terminus at position ca. 7; the latter cut was sometimes observed, sometimes not, as evidenced by the absence or presence, respectively, of the 5'-terminal oligonucleotide pAAAUUG in the fingerprints of complex A.

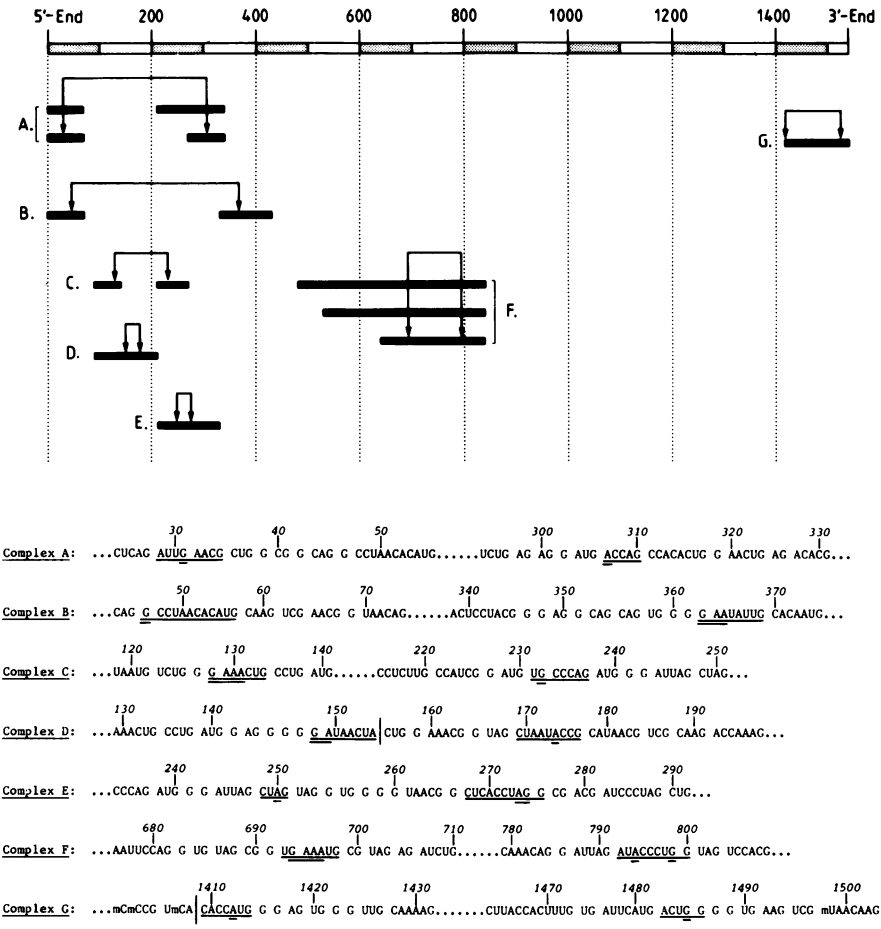

Figure 3: Locations of the RNA regions containing the cross-linked complexes in 16S RNA. The sequence is numbered from the 5'-end. In the upper part of the diagram the thick bars indicate the sequence regions contained in the most frequently observed versions of complexes A to G, the thin lines with arrow-heads denoting the cross-link in each case. The lower part of the Figure shows the details of the 16S sequence in the vicinity of the cross-link sites, the sequence being divided into ribonuclease T_1 oligonucleotides, to facilitate comparison with Fig. 2. The cross-linked T_1-oligonucleotides are underlined, with double underlining indicating the precise site of cross-linking within the T_1-oligonucleotides (see text). The vertical lines in the sequences for complexes D and G (at positions 155/156 and 1408/1409, respectively) indicate cobra venom nuclease cuts (cf. ref. 10).

The 5'-component of the cross-link site was identified by the complete absence from the fingerprint of AUUG (positions 28-31), as well as by the fact that AACG, which should occur twice

in the RNA region concerned (at positions 32-35, and 65-68, cf. Fig. 3), was only present in unimolar amounts. Since reaction with nitrogen mustard makes G-residues resistant to ribonuclease T_1 hydrolysis (3), this indicates that the site of cross-linking is to G-31. The 3'-component of the cross-link site was identified by the complete absence of ACCAG (306-310), but in this case both of the flanking ribonuclease T_1 oligonucleotides (AUG at positions 303-305, and CCACACUG at 311-318 (Fig. 3)) were present, which indicates that a residue other than guanine must have been involved in this component of the cross-link. In place of the missing oligonucleotides, an anomalous extra spot was reproducibly observed on the fingerprints of complex A (Fig. 2), and this liberated AU, U (weak), G, C, AG and an undigested residue upon secondary digestion with ribonuclease A. These products are consistent with the predicted composition of the cross-linked oligonucleotide, namely AUUGAACG (28-35) linked to ACCAG (306-310). The presence of C and AG, and the absence of AC in the secondary digest shows that the actual cross-link is from G-31 to A-306, with the ribonuclease A-resistant residue being GAAC (31-34) linked to AC (306-307).

Occasionally, other variants of cross-link A were found. In one instance AUG (positions 303-305) was absent as well as ACCAG (306-310), suggesting a cross-link to G-305 instead of A-306. More interestingly, the 5'-component of the cross-link site was once found at G-46 instead of G-31, and the 3'-component in another case at G-293 or G-297 instead of A-306. The significance of these variants will be elaborated below in the Discussion section.

Complex B: As with complex A, the RNA in complex B also arose from two distinct regions of the 16S molecule, the 5'-component being the same as that of complex A, namely residues ca. 7-72. The 3'-component comprised residues 338 to ca. 433, the 5'-terminus of this component being defined by the presence in the fingerprint of the oligonucleotide pACUCCUACG (338-346) (Figs. 2 and 3); this corresponds to an established cobra venom nuclease site (10). The cross-link site was delineated by the absence of the ribonuclease T_1 oligonucleotide CCUAACACAUG (47-57) from the 5'-component of the RNA and of AAUAUUG (363-369)

from the 3'-component. Each of these oligonucleotides is preceded in the 5'-direction by a single G-residue (positions 46 and 362, respectively) and thus the cross-link site could be between G-46 and G-362, or between positions within the two missing oligonucleotides; the analysis of complex A above shows clearly that, as already noted in the Introduction (cf. ref. 3), adenine as well as guanine can be cross-linked by nitrogen mustard. The 3'-terminal G-residues of the missing oligonucleotides (57 and 369) are however excluded as candidates for the cross-link site, since in each case the next oligonucleotide in the 3'-direction (CAAG, positions 58-61, and CACAAUG, positions 370-376) was present in the fingerprint. The cross-linked oligonucleotide appeared as a smeared spot at the origin of the fingerprint (Fig. 2), and liberated C (weak), AAC, AC, AU (strong), U and G upon digestion with ribonuclease A, together with a resistant residue. This is consistent with a cross-link between GCCUAACACAUG (46-57) and GAAUAUUG (362-369), with the actual cross-link being from G-46 to either G-362 or one of the A-residues at positions 363-364 (Fig. 3), leaving GC (46-47) cross-linked to GAAU (362-365) or AAU (363-365) as the ribonuclease A-resistant residue.

Complex C: The fingerprint of complex C (not given in Fig. 2) showed it to contain RNA covering the regions from residues ca. 95-138 and 217-265. The missing oligonucleotides, representing the cross-link site, were AAACUG (129-134) from the 5'-component of the RNA, and CCCAG (233-237) from the 3'-component. AAACUG is flanked in the 5'-direction by a single G-residue (Fig. 3), whereas the next ribonuclease T_1 oligonucleotide in the 3'-direction (CCUG, positions 135-138) was present. This points to G-128 or one of the three A-residues (129-131) as the 5'-component of the cross-link site. The missing oligonucleotide from the 3'-region, CCCAG (233-237), is flanked by UG and AUG (Fig. 3), both of these ribonuclease T_1 oligonucleotides occurring several times in this part of the RNA. The cross-linked oligonucleotide from the fingerprint released U, C, G and AG upon digestion with ribonuclease A, together with an undigested residue. The presence of AG in the digest excludes the possibility of G-237 being the cross-linked nucleotide, and the result is consistent with a cross-link between the oligonucleotides GAAACUG (128-134) and

UGCCCAG (231-237), with the precise site being between one of the positions 128-131 as just mentioned, and G-232. This would leave GAAAC (128-132) or AAAC (129-132) linked to GC (232-233) as the ribonuclease A-resistant residue.

Complex D: Complex D arose from a single RNA region, covering residues ca. 87 to 204. The 5'- and 3'-ends correspond to established cobra venom nuclease sites (10), but the analysis of the complex was made somewhat more complicated by the presence of a further cobra venom nuclease cut in the centre of the region between residues 155 and 156 (Fig. 3). This gave rise to an oligonucleotide corresponding to pCUG (156-158) (Fig. 2) on the ribonuclease T_1 fingerprint, but the expected 5'-adjacent oligonucleotide $AUAACUA_{OH}$ (149-155) (Fig. 3) was not observed, suggesting that this oligonucleotide or the next G-residue in the 5'-direction (position 148) was involved as the 5'-component of the cross-link site. The 3'-component of the cross-link was defined by the absence from the fingerprint of the oligonucleotide CUAAUACCG (169-177). Since both flanking oligonucleotides were present, namely UAG (166-168) and the heterogeneous CAUAACG or CAUAAUG (178-184) (see Fig. 2 and ref. 11), it follows that the cross-link site must be to an internal residue in the missing oligonucleotide between positions 169 and 176. The cross-linked oligonucleotide from the fingerprint (Fig. 2) liberated AAC, U, C, AAU and G on digestion with ribonuclease A, which is consistent with a cross-link between $GAUAACUA_{OH}$ (148-155) and CUAAUACCG (169-177). Since neither AU nor AC were observed in the ribonuclease A digest, it follows that the actual site of cross-linking is from G-148 or A-149 to A-174, leaving GAU (148-150) or AU (149-150) linked to AC (174-175) as the ribonuclease A-resistant residue.

Complex E: Complex E also arose from a single RNA region, covering residues 216 to 335, the latter position corresponding to a published cobra venom nuclease site (10). The 5'-end was defined by the presence of an oligonucleotide corresponding to pUCUUG (216-220) (Fig. 2), and the cross-link site was delineated by the absence of both CUAG (248-251) and CUCACCUAG (267-275). The first of these two oligonucleotides is flanked by AUUAG (243-247) and UAG (252-254), both of which were present in the ribo-

nuclease T_1 fingerprint, indicating that A-250 is the 5'-component of the cross-link site. The second missing oligonucleotide (267-275) is flanked on both sides by single G-residues (Fig. 3); however, ribonuclease A digestion of the cross-linked oligonucleotide from the fingerprint (Fig. 2) liberated only C, U and AC, but no AG. This pinpoints the 3'-site of the cross-link to A-274 or G-275, with AG (250-251) linked to AG (274-275) or AGG(274-276) being left as the ribonuclease A-resistant residue from the cross-linked oligonucleotide.

Complex F: The slower-running version of complex F (Fig. 1) is by far the most abundant nitrogen mustard cross-link which we have observed in the 30S subunit. This version of the complex covers the RNA region between residues ca. 475 and 836, the 5'- and 3'-termini corresponding to established cobra venom nuclease sites (10). The most clearcut analyses, however, were obtained from the smaller faster-running versions of the complex (cf. Fig. 1), and the fingerprint from one such complex is shown in Fig. 2. In this case the RNA region covered is from residues 637 to ca. 836, the 5'-terminal-oligonucleotide being pCUG (637-639). The cross-link was defined by the clear absence from the ribonuclease T_1 fingerprint of AAAUG (694-698) and AUACCCUG (792-799). The first of these oligonucleotides is flanked by the small uncharacteristic oligonucleotides UG and CG (Fig. 3), whereas the second is flanked at its 3'-end by G, and at its 5'-end by AUUAG (787-791). The latter was present in the ribonuclease T_1 fingerprints. The cross-linked oligonucleotide on the fingerprint ran reproducibly as two separate spots (Fig. 2), suggestive of an internal heterogeneity. Ribonuclease A digestion of these spots gave C, G, U and AU in the case of the faster-moving spot, and C, G, U, AC and AU in the case of the slower-moving spot (Fig. 2), together with undigested residues. The absence of AAAU (694-697) from these digests indicates that the 5'-component of the cross-link site is either G-693 or one of the three A-residues (694-696). The 3'-component of the cross-link is correspondingly either A-794 in the faster-moving cross-linked oligonucleotide or G-799 in the slower-moving oligonucleotide. This leaves GAAAU (693-697) or AAAU (694-697) cross-linked to AC (794-795) or to GG (799-800), respectively, as the ribo-

nuclease A resistant residue in the two cases.

Complex G: In contrast to cross-links A to F, which all lie in the 5'-proximal half of the 16S RNA, complex G arose from the extreme 3'-end of the molecule, covering residues 1409-1542. The position of the 5'-terminus is crucial to the analysis of this cross-link, since (as was the case with complex D) the 5'-component of the cross-link site turned out to be within an oligonucleotide generated by a venom nuclease cut. Such a cut has been established between positions 1408-1409 (10), but no oligonucleotide corresponding to pCACCAUG (1409-1415) (Fig. 3) was observed on the ribonuclease T_1 fingerprint (Fig. 2), although mCmCCG (1402-1405) was clearly absent and CAAAAG (1427-1432) clearly present. Instead of pCACCAUG, an anomalous spot was observed, which appeared to represent the cross-linked oligonucleotide, suggesting that the 5'-terminal oligonucleotide was also the 5'-component of the cross-link site. The 3'-component of the cross-link site was defined by the absence of ACUG (1483-1486) from the fingerprint. The 5'-flanking oligonucleotide AUUCAUG (1476-1482) was present in the ribonuclease T_1 fingerprints, whereas the oligonucleotide GGGGU (1486-1490) was missing from a corresponding ribonuclease A fingerprint (not shown). Thus, the 3'-component of the cross-link site is G-1486. The cross-linked oligonucleotide from the ribonuclease T_1 fingerprint liberated AC (strong), U, G, C and a spot corresponding to pCp upon digestion with ribonuclease A, together with an undigested residue. This is consistent with a cross-link from pCACCAUG (1409-1415) to ACUGG (1483-1487), with the precise site of the cross-link being from A-1413 to G-1486. The ribonuclease A-resistant residue is thus AU (1413-1414) linked to GG (1486-1487).

DISCUSSION

The positions of cross-links A to F in the 5'-proximal half of the 16S RNA secondary structure (6) are shown in Fig. 4. Cross-links C and E represent secondary structural cross-links, in which the nitrogen mustard has bridged a double-helical section of the RNA at a point where the base-pairing is interrupted. Cross-link G at the 3'-terminus of the RNA (not shown in Fig. 1) also belongs to this class, and links the A-residue (1413) of a

mis-matched A-G pair in helix 44 (6) of the RNA to a G-residue (1486) on the opposite strand adjacent to this A-G pair. Cross-links B and D are also secondary structural cross-links, in this case across RNA regions where three or more helices come together. Cross-link D is however in a region where the secondary structure of the RNA is in dispute, and it should be noted that in the secondary structure model of Noller (12), this cross-link would be across a G-A mis-matched pair within a double-helical element, like cross-links C and G.

The tertiary structural cross-links A and F are without doubt the most important with regard to deriving the three-dimensional structure of the RNA. Cross-link A, from G-31 to A-306, is particularly interesting for several reasons. First, it involves the same residue (G-31) that was found cross-linked to C-48 in our previous study (5), in which the cross-links were induced by ultraviolet irradiation of 30S subunits. Secondly, as described above in the Results section, several heterogeneous variants of cross-link A were occasionally observed. One of these, in which G-46 istead of G-31 was linked to A-306, is clearly very reasonable in view of the proximity of G-31 and C-48 (5) just mentioned. The other observed heterogeneities were at the 3'-site of the cross-link, and involved G-305, and G-293 or G-297 instead of A-306. Inspection of the secondary structure (Fig. 4) shows that all these positions are clustered together at the end of helix 12, and here (as with cross-link D above) it should again be noted that the Noller secondary structure model (12) proposes a slightly different base-pairing within this helix, in which residues 304-306 are looped out. Thirdly, cross-link A provides one of the most clearcut examples of a nitrogen mustard cross-link involving a G-residue linked to an A-residue. The other tertiary structural cross-link, F, which is the highest yield nitrogen mustard cross-link so far found, also shows heterogeneity in the 3'-component of the cross-link site, and here again both A- and G-residues are involved. This type of heterogeneity could reflect either the flexibility of the cross-linker or of the RNA structure itself, or both.

The finding that adenine as well as guanine residues can be cross-linked by nitrogen mustard necessitates a re-appraisal of

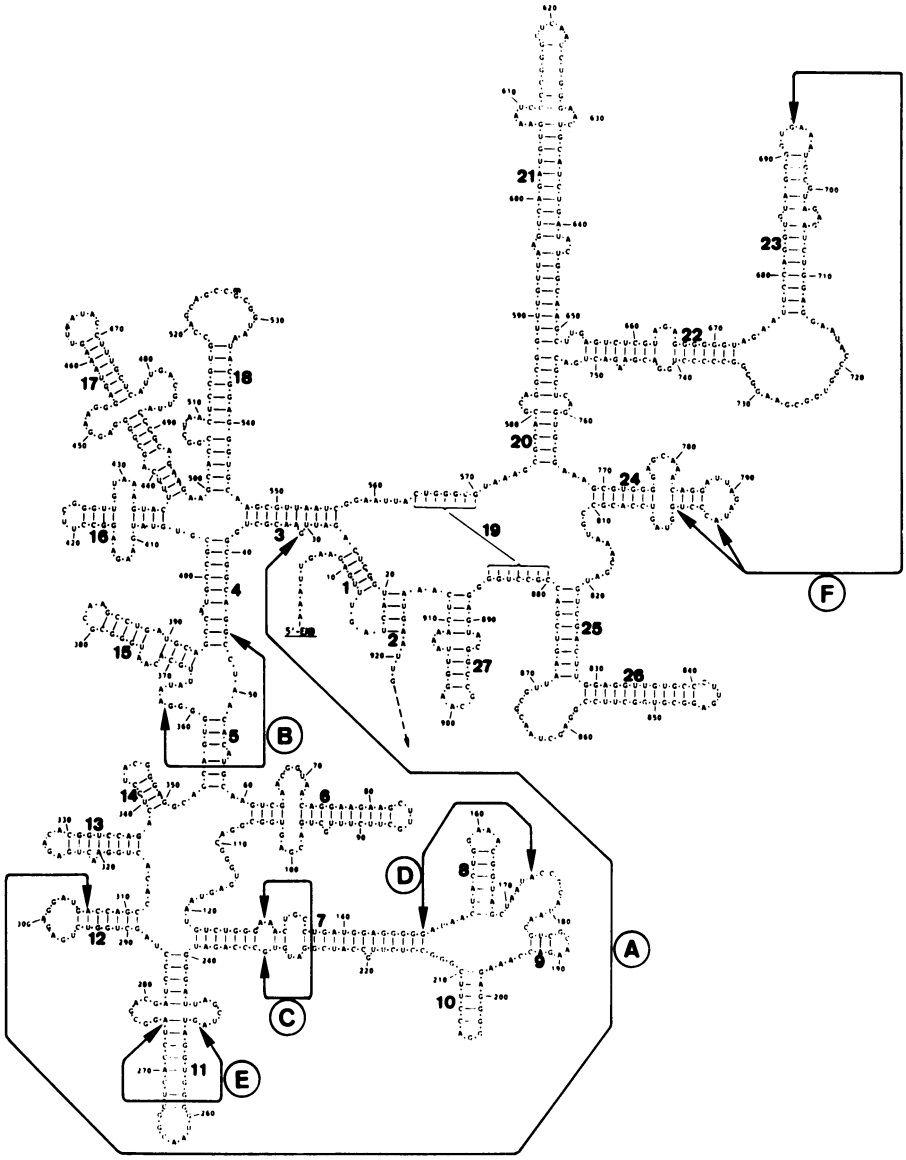

Figure 4: Location of cross-links A to F in the 5'-proximal half of the secondary structure (6) of 16S RNA.

our previous findings with 23S RNA (3), in which we assumed that only G-residues were involved. In fact, the possibility of reaction with A-residues makes essentially no difference to the

cross-links reported (3), with the exception of the cross-link between residues 1482 and 1501 of the 23S RNA. Here it now seems more likely that one of the four A-residues (positions 1502-1505) adjacent to G-1501 was involved as the 3'-component of the cross-link site, thus making this cross-link precisely analogous to the secondary structural cross-links C and G reported here. We cannot of course entirely exclude the possibility that pyrimidine residues can also take part in some nitrogen mustard cross-links, but in no case would this alter the deduced cross-link site positions by more than one or two nucleotides. A further series of experiments with the 50S subunit has shown that many more nitrogen mustard cross-links remain to be elucidated in the 23S RNA, but the problem of heterogeneity, as observed here in cross-links A and F, renders the 23S RNA data very difficult to evaluate, and so far only one new cross-link (between residues 2808 and 2890) has been unequivocally identified (our unpublished data).

The ultraviolet induced intra-RNA cross-links which we previously reported in the 30S subunit (5) included two tertiary structural cross-links in the 3'-proximal domain of the 16S RNA. Taken together with the tertiary cross-links reported here in the 5'-proximal domains (Fig. 4), the data impose an increasing series of rigid constraints on the folding of the 16S RNA secondary structure into three dimensions, in situ in the active 30S subunit. So far there appears to be little or no correspondence between our tertiary structural cross-links and those reported by other authors (13-16), using isolated RNA or inactivated 30S subunits as the substrate for cross-linking.

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