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**RNA-protein cross-linking in *Escherichia coli* 30S ribosomal subunits; determination of sites on 16S RNA that are cross-linked to proteins S3, S4, S7, S9, S10, S11, S17, S18 and S21 by treatment with *bis*-(2-chloroethyl)-methylamine**

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SUMMARY

RNA-protein cross-links were introduced into *E. coli* 30S ribosomal subunits by treatment with *bis*-(2-chloroethyl)-methylamine. After partial nuclease digestion of the RNA moiety, a number of cross-linked RNA-protein complexes were isolated by a new three-step procedure. Protein and RNA analysis of the individual complexes gave the following results: proteins S4 and S9 are cross-linked to the 16S RNA at positions 413 and 954, respectively. Proteins S11 and S21 are both cross-linked to the RNA within an oligonucleotide encompassing positions 693-697, and proteins S17, S10, S3 and S7 are cross-linked within oligonucleotides encompassing positions 278-280, 1139-1144, 1155-1158, and 1531-1542, respectively. A cross-link to protein S18 was found by a process of elimination to lie between positions 845 and 851.

INTRODUCTION

Bifunctional alkylating agents of the nitrogen mustard type have been known for some years to introduce cross-links into ribosomes. In 1978 we reported the use of *bis*-(2-chloroethyl)-amine as an RNA-protein cross-linking reagent for *Escherichia coli* 30S and 50S ribosomal subunits (1), and more recently we have applied the rather more reactive derivative *bis*-(2-chloroethyl)-methylamine as an intra-RNA cross-linking agent (2,3). In the latter case we were able to identify a number of the intra-RNA cross-link sites on both the 16S (3) and 23S (2) RNA. *Bis*-(2-chloroethyl)-methylamine (which we shall refer to in this manuscript as "nitrogen mustard") has also been tested in our RNA-protein cross-linking studies, where it shows a reactivity approximately three to five times higher than its non-methylated counterpart (4). However, because of the technical difficulties involved in the isolation and analysis of RNA-protein cross-linked complexes (cf. ref. 5), we were not so far able to publish

any RNA-protein cross-link site localizations with this reagent.

These technical difficulties have now to a large extent been overcome, and in a parallel paper (6) we have described the application of a new procedure for the determination of RNA-protein cross-link sites, which enabled us to identify twelve cross-link sites on the *E. coli* 16S RNA, using the reagent methyl p-azidophenyl acetimidate ("APAI" (7)) as cross-linker. In this paper we report the identification of a corresponding set of nine RNA-protein cross-link sites on the 16S RNA, obtained from 30S ribosomal subunits cross-linked with nitrogen mustard. The new experimental procedure is described in detail in ref. 8, and involves a partial nuclease digestion of the cross-linked subunits, followed by removal of the non-cross-linked proteins and then removal of non-cross-linked RNA fragments. Finally, the individual RNA-protein cross-linked complexes are separated by two-dimensional gel electrophoresis and analysed for both their protein and RNA content. A further feature of the system is a new immunological procedure for the identification of the proteins in the complexes, described in ref. 9.

The cross-link data reported here, combined with the APAI cross-links just referred to (6) as well as with our previous data obtained using 2-iminothiolane (5,10), give a total of twenty-eight defined RNA-protein cross-link sites on the 16S RNA. These results have played a central role in the construction of a detailed model for the three-dimensional folding of the 16S RNA *in situ* in the 30S subunit (11).

### MATERIALS AND METHODS

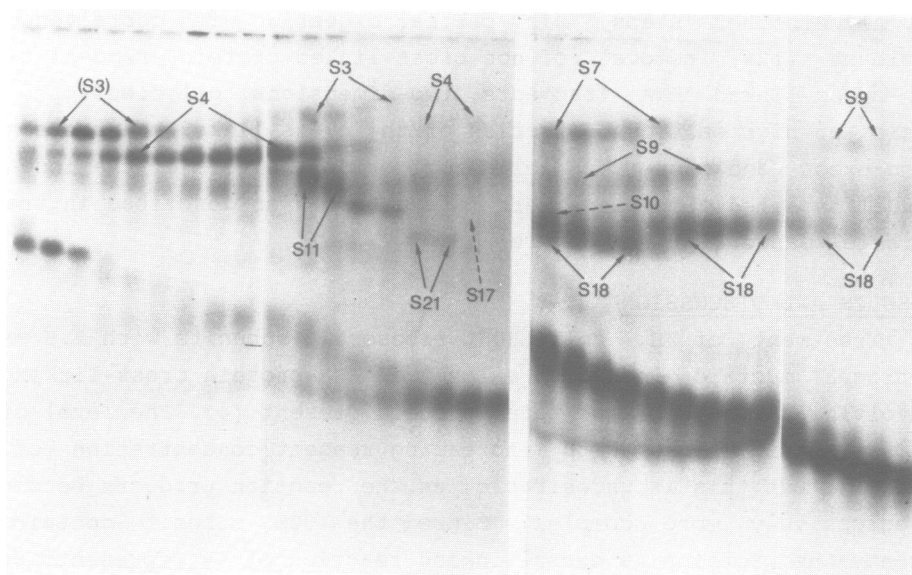
<sup>32</sup>P-labelled 30S subunits from *E. coli* strain MRE 600 were prepared by the method of Stiege et al (2). The subunits (ca. 5 A<sub>260</sub> units, 1.5 x 10<sup>9</sup> counts/min total) were dialysed into a buffer containing 50 mM KCl, 5 mM magnesium acetate, 25 mM sodium cacodylate pH 7.2. The subunit solution (ca. 0.6 ml) was made 2.5 mM in nitrogen mustard (EGA Chemie, Steinheim) by adding a suitable volume of a freshly prepared 100 mM solution of the reagent, and the mixture was incubated for 45 min at 37°. The reaction was stopped by adding cysteamine to a final concentration of 50 mM, and incubating for a further 15 min at 37°. The subunits were then precipitated with ethanol.

All further steps, *viz.* partial digestion with cobra venom nuclease (12), removal of non-cross-linked protein, removal of non-cross-linked RNA fragments, two-dimensional gel electrophoresis at high salt concentration of the cross-linked RNA-protein complexes, and protein and oligonucleotide analysis of the individual isolated complexes, were made precisely as described in the parallel manuscript (6) and ref. 8.

#### RESULTS AND DISCUSSION

Treatment of *E. coli* 30S ribosomal subunits with 2.5 mM nitrogen mustard leads to a level of RNA-protein cross-linking involving ca. 5% of the total protein present (4). The level of cross-linking rises with increasing reagent concentration (cf. ref. 1), but this is undesirable, as the reaction products become progressively more complex. Since the 30S subunit contains twenty-one proteins, a cross-linking reaction of 5% represents an average of roughly one RNA-protein cross-link per 16S RNA molecule. Under these conditions, as was the case with APAI-induced cross-linking (6), no 30S dimers could be detected after treatment with the reagent, as judged by sucrose gradient analysis (data not shown). This contrasts with other RNA-protein cross-linking procedures, where substantial dimer formation or aggregation have been reported (e.g. ref. 13). It should also be noted that at this low level of reaction, the concomitant intra-RNA cross-linking reaction (3) does not interfere with the analysis of the RNA-protein cross-links.

<sup>32</sup>P-labelled 30S subunits, cross-linked with nitrogen mustard, were partially digested with cobra venom nuclease as described in ref. 6. Non-cross-linked proteins were removed by electrophoresis through sucrose gradients in the presence of non-ionic detergent, and non-cross-linked RNA fragments were separated by glass-fibre filtration (cf. ref. 14), giving results that were virtually indistinguishable from those reported for APAI-induced cross-linking (Figs. 1a and 1b in ref. 6). The different specificity of the nitrogen mustard cross-linking reaction first becomes apparent in the subsequent two-dimensional gel electrophoretic separation of the individual cross-linked RNA-protein complexes, and examples of these gels are shown in Fig. 1 (cf. Fig. 2 of ref. 6).



**Figure 1:** Two-dimensional polyacrylamide gel separation at high salt concentration of RNA-protein cross-linked complexes. Direction of the first dimension is from left to right, and that of the second dimension from top to bottom. Each second dimension gel has 12 slots, containing the individual eluates from the first-dimension gel slices; four such gels from two different experiments (see text) are combined to give the total pattern. The rows of RNA-protein complexes lie above the "diagonal" of free RNA, and are marked with arrows giving the identity of the protein found in each row of spots. The dotted lines to proteins S17 and S10 indicate the approximate positions of complexes found in earlier experiments with a slightly different gel system.

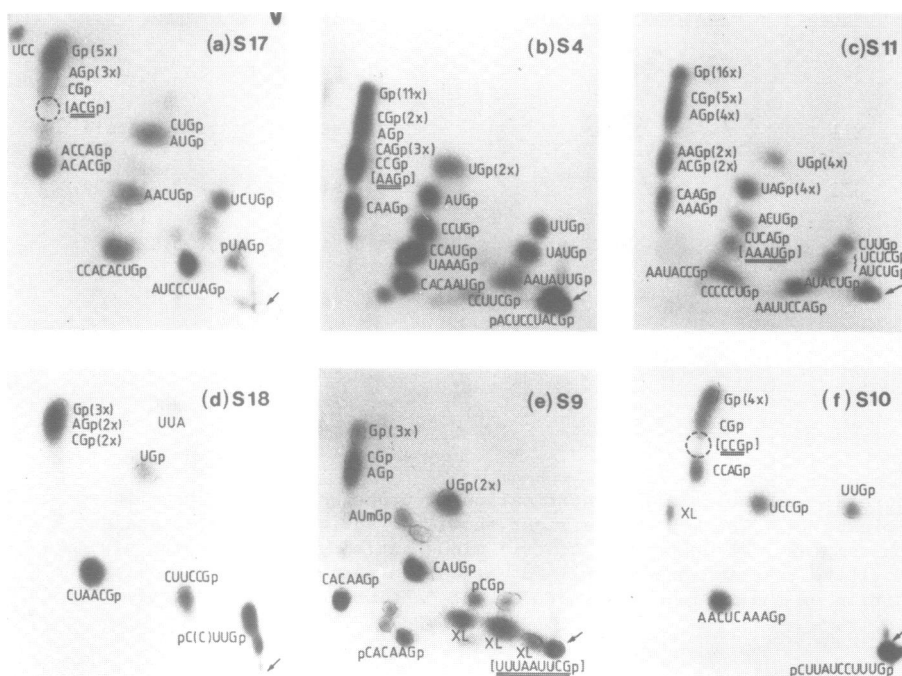
The patterns of nitrogen mustard cross-linked fragments were in general not quite so well-defined as those obtained with APAI (6), and parts of the two-dimensional gels tended to be diffuse. In order to facilitate the description of the results, the gels shown in Fig. 1 are combined from two experiments, where in the one case the larger complexes (left-hand half of Fig. 1) and in the other case the smaller complexes (right-hand half) were better resolved. As before (6), the gel patterns show a broad "diagonal" of free RNA fragments still remaining after the glass-fibre filtration step, running from upper left to lower right in the Figure. The RNA-protein complexes appear as rows of spots lying above this diagonal, and are designated according to the protein they were subsequently found to contain by the

COMPLEX	ANTIBODY TEST FILM	ANTIBODIES TESTED
S4		S3, <u>S4</u> , S5, S7,
S11		S6, S8, S9, <u>S11</u> , S12, S13, S14
S21		S10, S15, S16, S17, S18, S19, S20, <u>S21</u>
S18		S10, S17, <u>S18</u> , S19, S20, S21
S9		S6, S8, <u>S9</u> , S11, S12
S3		S2, <u>S3</u> , S4, 0
S7		S5, <u>S7</u> , S9

**Figure 2:** Immunological identification of proteins in the cross-linked complexes (9). The key on the right shows the antibodies tested against each complex, "0" denoting a control minus antibody. The proteins giving a positive reaction are underlined, as can be seen by comparison with the position of the radioactive spots on the "antibody test film" strip.

immunological test of ref. 9. The cross-links to proteins S10 and S17 were not resolved in the particular gels illustrated in Fig. 1, but the approximate positions where these complexes were found in other comparable gels are indicated by the dotted lines. The gels concerned were in fact earlier versions of the two-dimensional system, where the  $\text{Na}^+$  ion concentration was only 150 mM instead of 500 mM (cf. refs. 6,8). One complex involving protein S3 and a rather long RNA fragment (shown in brackets at the top left-hand corner of Fig. 1) was reproducibly found, but we were unable to pinpoint the cross-link site and this complex will not be discussed here.

Protein and oligonucleotide analyses of the individual cross-linked complexes isolated from the gels (Fig. 1) were made in an identical manner to that described for the APAI-induced cross-links (6), and here again the results given below represent a summary of a large number of determinations. Fig. 2 shows some examples of identifications of the proteins in the complexes, made with the help of antibodies to the 30S ribosomal proteins (cf. ref. 9). Fig. 3 gives examples of ribonuclease  $T_1$  fingerprints of the RNA moiety (after prior digestion with proteinase K



**Figure 3:** Examples of ribonuclease T<sub>1</sub> fingerprint analyses (16) of the cross-linked complexes. The first dimension runs from right to left, and the second dimension from bottom to top, the arrows indicating the sample application points. Identities and molarities of the oligonucleotides are shown. Underlined oligonucleotides in square brackets, together with the dotted circles, indicate the identities and expected positions of the missing (i.e. cross-linked) sequences. "XL" denotes an oligopeptide-oligonucleotide cross-linked species. (The oligonucleotide spots in some of the fingerprints are ringed, as a result of being photographed subsequent to the routine processing procedure for secondary digestion and analysis.)

(6,8)), and the locations of the RNA regions determined from the fingerprints in the sequence of 16S RNA (15) are illustrated in Fig. 4. Fig. 5 shows the details of the RNA sequences in the vicinity of each cross-link site.

The positions of the cross-link sites were inferred from the absence of characteristic oligonucleotides in the fingerprints (5,6,10). Nitrogen mustard is known to react preferentially with the N7 position of C-residues (2,3), and the positively-charged cross-linked product is resistant to ribonuclease T<sub>1</sub> digestion. In such cases the position of the cross-link site should be indi-

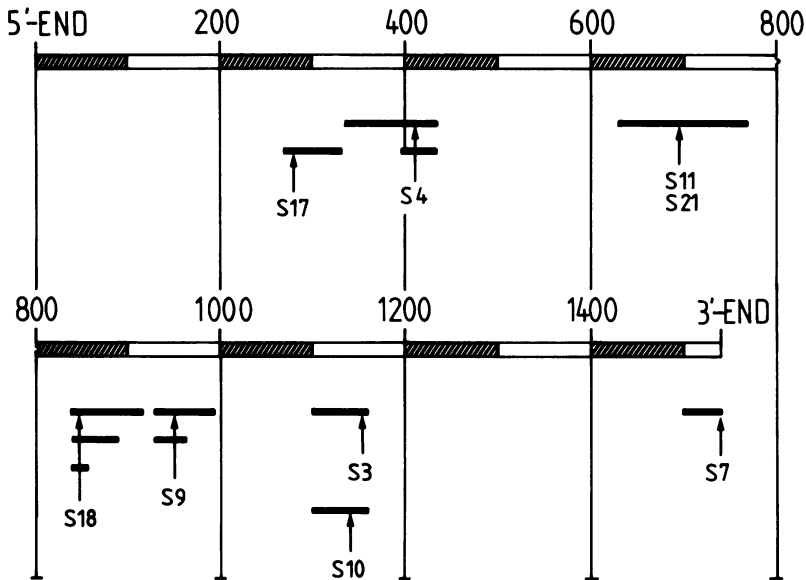


Figure 4: Locations of the cross-linked complexes in the 16S RNA sequence. The horizontal bars indicate the sequence positions of the RNA fragments in the complexes, with the arrow and protein number denoting the positions of the cross-link sites to the various proteins within these RNA fragments.

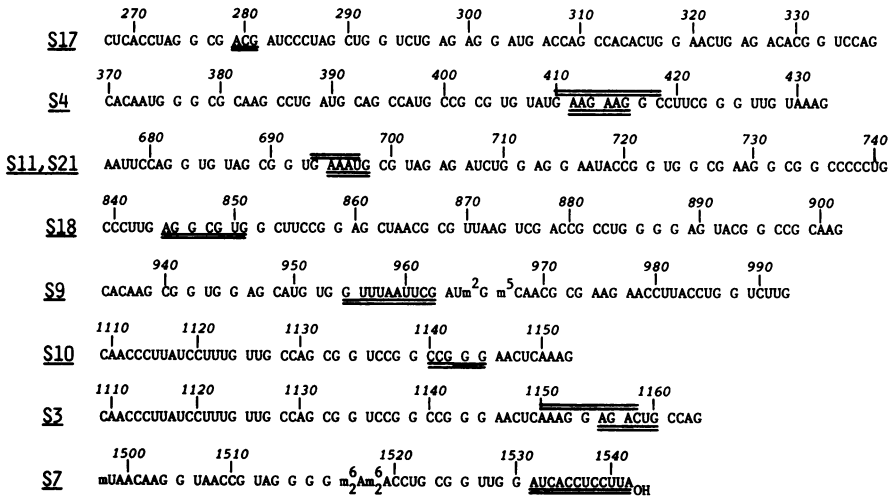


Figure 5: Details of the RNA sequence (15) in the vicinity of each cross-link site. The sequence is divided into ribonuclease T<sub>1</sub> oligonucleotides, to facilitate comparison with Fig. 3. Missing (i.e. cross-linked) T<sub>1</sub>-oligonucleotides are underlined, and the corresponding missing ribonuclease A oligonucleotides are marked by lines above the sequence concerned. The underlined sequence in the case of the S18 complex was deduced by a process of elimination (see text).

cated by the absence of two adjacent ribonuclease  $T_1$  oligonucleotides from their usual positions on the fingerprint. However, reaction with A-residues has also been observed (3), and reaction with U- or C-residues cannot be formally excluded, in which cases a single  $T_1$ -oligonucleotide should be missing at the cross-link site. Sometimes (but not always) an "extra" spot was observed on the fingerprint, corresponding to the cross-linked oligopeptide-oligonucleotide (cf. refs. 5,10), and data from ribonuclease A fingerprints were also used to clarify or confirm the positions of the cross-link sites. As before (6) the 5'- and 3'-ends of the RNA fragments in the complexes were sometimes difficult to define precisely, and the RNA sequences in such cases are discussed in terms of the first and last observed characteristic ribonuclease  $T_1$  oligonucleotide at each terminus. The deduction of each individual cross-link site is detailed in the following sections, the cross-links being described (both in the text and in the Figures) in the order in which they occur on the 16S RNA, in a 5'- to 3'-direction. The cross-linked complexes are named according to the protein which they contain.

Complex S17: As already mentioned, this complex was found in earlier versions of the two-dimensional gel system (cf. Fig. 1). At that stage the immunological test of Fig. 2 (9) had not been developed, and the protein identification was made instead by Ouchterlony double diffusion experiments (17), which gave a reproducibly positive reaction with anti-S17 (data not shown, but cf. the S17 analysis in ref. 5). The corresponding RNA analysis was also reproducible, and the ribonuclease  $T_1$  fingerprint is shown in Fig. 3a. In this instance both the 5'- and 3'-ends of the RNA fragment were clearly defined, as evidenced by the respective presence of pUAGp (positions 273-275) and UCC (positions 333-335) in the fingerprint. These termini correspond to known cleavage positions of cobra venom nuclease (18). The cross-link site was indicated by the absence from the fingerprint of ACGp (pos. 279-281, cf. Figs. 3a and 5). Since the 3'-adjacent oligonucleotide AUCCCUAGp (pos. 282-289) was present, the cross-link cannot be to G-281, and the presence of CGp (pos. 277-278) suggests that G-278 was also not involved, as CGp only occurs once in this sequence region. However, CGp is in general a very common



oligonucleotide, and its presence could be due to slight cross-contamination from other RNA regions. We therefore conclude that the cross-link site lies within the sequence GAC (pos. 278-280). Data from ribonuclease A fingerprints did not give any additional information.

Complex S4: Many different versions of this complex were observed with attached RNA fragments of different lengths, giving rise to the extended row of S4-containing spots in Fig. 1. A typical immunological test result can be seen in Fig. 2. The longest and shortest RNA sequence regions concerned are shown in Fig. 4, and an example of one of the ribonuclease  $T_1$  fingerprints, covering in this case the region from position 338 to ca. 433, is illustrated in Fig. 3b. Despite a slight cross-contamination with other RNA regions (Fig. 3b), the position of the cross-link site is clearly indicated by the total absence of AAGp. This oligonucleotide occurs twice in adjacent positions in the RNA (411-413 and 414-416, Fig. 5), and the reproducible absence of the trinucleotide pinpoints the cross-link site unambiguously to the G-residue at position 413. The corresponding oligonucleotide GAAGAAGGp (pos. 410-418) was also missing from its usual position in the ribonuclease A fingerprints.

Complexes S11 and S21: The complexes containing S11 and S21 appeared at distinct position on the two-dimensional gels (Fig. 1), and gave clear protein analyses (Fig. 2). The RNA regions and cross-link sites were identical in both cases. The ribonuclease  $T_1$  fingerprints (that from the S11 complex being shown in Fig. 3c) covered the RNA region from positions ca. 640 to 770, and the cross-link site was indicated by the absence of AAAUGp (pos. 694-698). GAAAUp (pos. 693-697) was missing from the corresponding ribonuclease A fingerprints, and the cross-link site therefore lies within the sequence GAAAU (pos. 693-697, Fig. 5). It should be noted that this same oligonucleotide was found to be involved in a high-yield intra-RNA cross-link (3), and thus appears to be a "hot spot" for reaction with nitrogen mustard in the 30S subunit.

Complex S18: As with the S4 complexes, S18 also appeared on the two-dimensional gels in an extended row of spots (Fig. 1), with attached RNA fragments of differing lengths. A typical

immunological analysis is shown in Fig. 2. The RNA fragments all had the same 5'-terminus, corresponding to the known cobra venom nuclease cleavage site at position 840-841 (18), which gives rise to the oligonucleotides pCCUUGp and pCUUGp (pos. 840 or 841 to 844, respectively, Fig. 5) on the ribonuclease T<sub>1</sub> fingerprint (Fig. 3d). The fragments extended to various positions in the 3'-direction (Fig. 4), the example in Fig. 3d terminating at position 872, as indicated by the presence of the oligonucleotide UUA. However no characteristic ribonuclease T<sub>1</sub> oligonucleotide was missing from the fingerprints. This led us to suspect that the cross-link site must lie among the short oligonucleotides (Gp, AGp, CGp, UGp) which are frequent near the 5'-end of this RNA region (Fig. 5). As already discussed in the case of the S17 complex, the molarities of these short oligonucleotides cannot be reliably determined from the fingerprints, as they are sensitive to slight cross-contaminations with other RNA fragments. Furthermore, the ribonuclease A fingerprint data do not help to resolve the problem, as there are two indistinguishable ribonuclease A oligonucleotides (GAGGCP, pos. 844-848, and GGAGCP, pos. 858-862) in the suspect regions. The shortest S18 complex found covered the region from positions 841 to 857, with a 3'-terminus indicated by the oligonucleotide CUUCC (pos. 853-857) on the T<sub>1</sub>-fingerprint. Thus, by a process of elimination, the cross-link site must lie between positions 845 and 851, as indicated in Fig. 5.

Complex S9: The S9 complex appeared in two distinct positions on the two-dimensional gel (Fig. 1), both of which corresponded to the same cross-link site. The protein analysis was clear (Fig. 2), and the two RNA fragments concerned are indicated in Fig. 4, the ribonuclease T<sub>1</sub> fingerprint of the shorter complex (covering positions ca. 934 to 966) being illustrated in Fig. 3e. The 5'-terminus of the fragment was heterogeneous, as evidenced by the presence of pCGp (pos. 932-933) and pCACAAGp (pos. 934-939), and this particular fingerprint showed a slight cross-contamination with another RNA fragment (arising from the 3'-terminus of 16S RNA, cf. the S7 complex below). The complex was unusual in that the T<sub>1</sub>-fingerprints contained a distinct row of spots corresponding to residual cross-linked oligonucleotide-oligopeptides (marked "XL" in

Fig. 3e). The oligonucleotide UUUAAUUCGp (pos. 955-963) was missing from its usual position at the origin of the fingerprint, and secondary digestion of each of the "XL" spots with ribonuclease A yielded AAUp, Cp, Gp and a large amount of Up. These spots are presumably heterogeneous in their peptide moiety. Since the modified oligonucleotide AUmGp (pos. 964-966) was present (Fig. 3e), the G-residue at position 963 cannot be involved in the cross-link, and thus the G-residue at position 954 (Fig. 5) must be the cross-linked nucleotide. This diagnosis was supported by the fact that GGUp was only weakly present in the corresponding ribonuclease A fingerprint, although this oligonucleotide should occur twice in the RNA region concerned (pos. 941-943 and 953-955).

Complex S10: As was the case with the S17 complex (see above), the S10 complex was found in earlier versions of the two-dimensional gel system (cf. Fig. 1), and the protein identification was made by the Ouchterlony method. The ribonuclease T<sub>1</sub> fingerprint (Fig. 3f) was reproducible and covered the region from position 1114 (as evidenced by the presence of the oligonucleotide pCUUAUCCUUUGp, pos. 1114-1124) to ca. position 1153. CCGp (pos. 1140-1142) was absent from the fingerprints, indicating that either G-1139 or G-1142 was the likely site of cross-linking. However, a weak spot corresponding to a cross-linked oligonucleotide-oligopeptide (marked "XL" in Fig. 3f) was also always observed, which yielded Up, Cp, AAAGp and an undigested residue upon secondary digestion with ribonuclease A. Since the oligonucleotide AACUCAAGp (pos. 1145-1153) was clearly present in the fingerprint, this result suggests that G-1144 or the 5'-end of the latter oligonucleotide was also involved in the cross-link but only in sub-molar amounts. This implies that the cross-link was heterogeneous, as we have observed in intra-RNA cross-linking studies with nitrogen mustard (3). We conclude that the crosslink lies predominantly within the sequence GCCG (pos. 1139-1142, Fig. 5), but sometimes extends to position 1144 (or possibly 1146). No ribonuclease A fingerprint data are available for this complex.

Complex S3: The immunological protein test for this complex is shown in Fig. 2, and the RNA region involved corresponds very

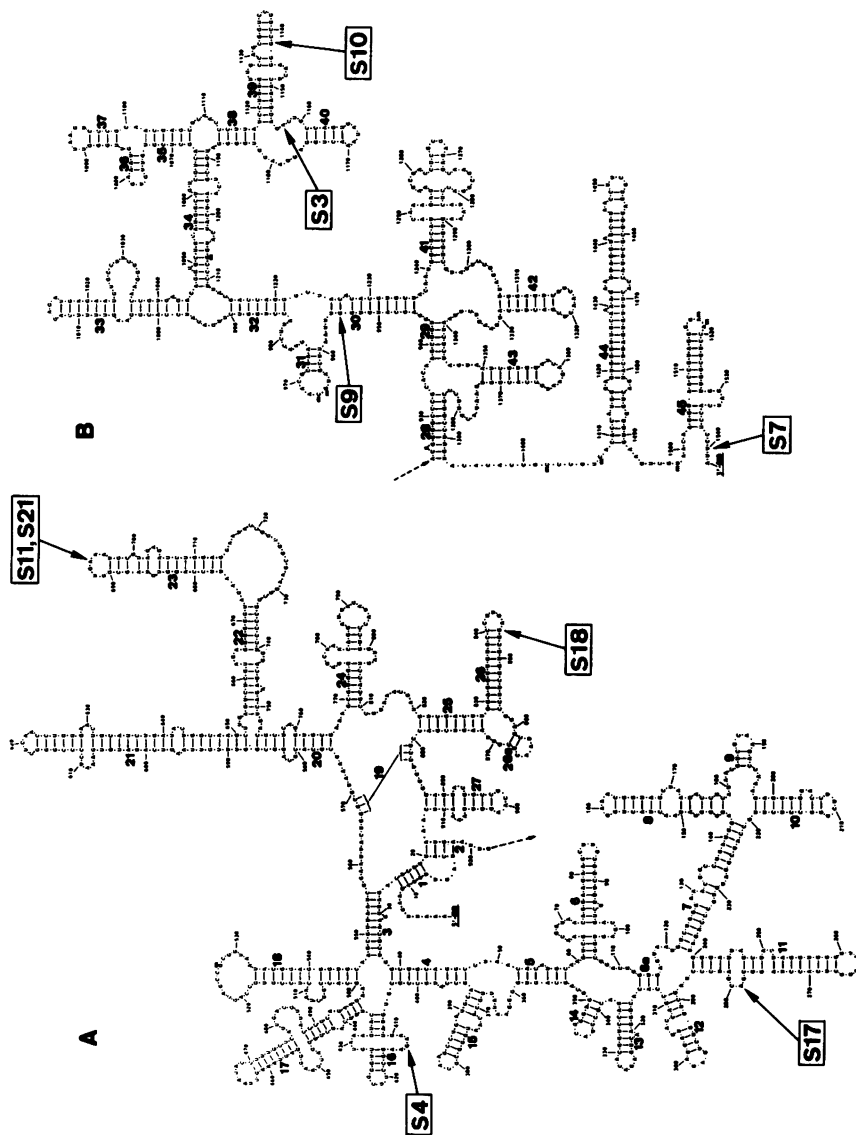


Figure 6: Locations of the nitrogen mustard induced cross-link sites in the secondary structure of 16S RNA, (cf. ref. 19).

closely to that just described for S10. The ribonuclease T<sub>1</sub> fingerprint was in fact identical to that shown in Fig. 3f, with the exceptions that CCGp was now present, and a stronger cross-linked spot in a somewhat lower position on the fingerprint was observed. This cross-linked oligopeptide-oligonucleotide yielded Up, Gp and an undigested residue upon secondary digestion with ribonuclease A. As in Fig. 3f, neither AGp (pos. 1155-1156) nor ACUGp (pos. 1157-1160) were present on the fingerprint, and there was only one molar equivalent of CCAGp (pos. 1128-1131 or 1161-1164). In the corresponding ribonuclease A fingerprint the oligonucleotide AAAGGAGACp (pos. 1150-1158) was not present, but instead a fast-running oligonucleotide-oligopeptide spot was observed, which liberated AAAGp and Gp on secondary digestion with ribonuclease T<sub>1</sub>. Taken together, these results indicate that the RNA fragment extended to position ca. 1160, and that the cross-link site lies at the 3'-end of the region within the sequence AGAC (pos. 1155-1158, Fig. 5), with G-1156 as the probable nucleotide involved.

Complex S7: The protein identification for this complex is shown in Fig. 2, and the RNA moiety arose from the 3'-terminal region of 16S RNA, covering positions ca. 1498 to the 3'-end. The T<sub>1</sub>-fingerprints were identical to those reported for the APAI-induced cross-link to protein S21 (Fig. 4i in ref. 6), and no example is therefore included in Fig. 3. As was the case with the latter cross-link, the site for protein S7 clearly lies within the 3'-terminal dodecanucleotide (Fig. 5), as evidenced by a cross-linked oligonucleotide-oligopeptide spot on the fingerprint which released AUp, ACp, Up and Cp upon secondary digestion with ribonuclease A.

### CONCLUSIONS

The locations of the nitrogen mustard cross-link sites described here in the secondary structure of the 16S RNA (slightly modified from the structure of ref. 19) are shown in Fig. 6. Taken together with the APAI cross-links described in the parallel manuscript (6), and those which we have already reported for 2-iminothiolane (5,10), this brings the total of known RNA-protein cross-link sites on the 16S RNA to two sites for protein

S3, two for S4, two for S5, three for S7, four for S8, two for S9, one for S10, two for S11, one or two for S13, two for S17, two for S18, one for S19 and three for S21.

Despite this progress, it is clear from the two-dimensional gel patterns (such as that of Fig. 1) that there are still many cross-links which we have not yet identified. This applies to all three of the cross-linking reagents just mentioned. The separation in the high-salt gel electrophoresis system (Fig. 1) is still by no means ideal, and cross-contamination of the individual complexes due to smearing on the gels is not uncommon (see some of the fingerprints in Fig. 3). It seems likely that this smearing is an inherent property of the denatured cross-linked RNA-protein complexes, and therefore that further significant improvement of the separation systems will prove very difficult. A more promising approach is to combine the existing system with affinity chromatography of the cross-linked complexes using appropriate antibodies. Thus, all the complexes corresponding to a single protein can be selected by affinity chromatography prior to the gel electrophoresis step, or alternatively the affinity chromatography can be applied as a final purification step for an individual isolated complex. Both possibilities have been shown to be feasible, and the results of these experiments will be reported elsewhere (20).

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