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RNA-protein cross-linking in *Escherichia coli* 50S ribosomal subunits; determination of sites on 23S RNA that are cross-linked to proteins L2, L4, L24 and L27 by treatment with 2-iminothiolane

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ABSTRACT

RNA-protein cross-links were introduced into E. coli 50S ribosomal subunits by treatment with 2-iminothiolane followed by mild ultraviolet irradiation. After partial digestion of the RNA, the cross-linked RNA-protein complexes were separated by our recently published three-step procedure. In cases where this separation was inadequate, a further purification step was introduced, involving affinity chromatography with antibodies to the ribosomal 50S proteins. Analysis of the isolated complexes enabled four new cross-link sites on the 23S RNA to be identified, as well as re-confirming several previously established sites. The new sites are as follows: Protein L2 is cross-linked within an oligonucleotide at positions 1818-1823 in the 23S RNA, protein L4 within positions 320-325, protein L24 within positions 99-107, and protein L27 within positions 2320-2323.

INTRODUCTION

Our laboratory has a long-standing interest in the application of RNA-protein cross-linking techniques to the study of ribosome structure in Escherichia coli (e.g. 1-4). The success of the approach depends on two factors, first the availability of suitable bifunctional reagents, and secondly the availability of efficient methodology for the analysis of the cross-linked products. We have concentrated our research to a large extent on the second of these two factors, and have recently developed a new three-step procedure for the analysis of RNA-protein cross-link sites on the ribosomal RNA (3,5). The use of the method, in conjunction with the cross-linking reagents methyl p-azidophenyl acetimidate (3) and bis-(2-chloroethyl)-methylamine (4), led in a short space of time to a quadrupling of the number of known cross-link sites to ribosomal 30S proteins on the 16S RNA. Combined with the older RNA-protein cross-link data obtained with

the reagent 2-iminothiolane (2,6), these cross-link sites have played a crucial role in establishing a three-dimensional model for the E. coli 16S RNA (7); the RNA-protein cross-links provide a positive link between the RNA structure and the known topographical arrangement of the ribosomal proteins, as determined by neutron scattering (8).

However, as we have already pointed out (4), the new methodology is still limited both by the complexity of the pattern of cross-linked products, and by the fact that the cross-linked RNA-protein complexes do not run very cleanly in the two-dimensional polyacrylamide gel system which is the final stage of the three-step procedure. Some of the cross-link data is thus lost, simply as a result of inadequate separations. This problem is clearly likely to be more serious in the case of the 50S ribosomal subunit, where the 23S RNA sequence is twice as long as that of the 16S RNA, and where there are nearly twice as many ribosomal proteins. With older methodology, we had previously established six RNA-protein cross-link sites on the 23S RNA induced by the reagent 2-iminothiolane (1), and in this paper we describe the application of the new analytical procedure (3,5) to the 50S subunit, using this same reagent. In order to overcome the problem of inadequate separation in the two-dimensional gels, we have introduced an affinity chromatography step, in which the cross-linked complexes isolated from the gels are purified where necessary by adsorption onto agarose or sepharose via antibodies to the individual ribosomal proteins. Eight RNA-protein cross-link sites are described, four of which are new, and four of which re-confirm our previous findings (1).

MATERIALS AND METHODS

Preparation, cross-linking and partial digestion of 50S ribosomal subunits: ^{32}P-labelled 50S ribosomal subunits (ca. 7 A_{260} units, 2 x 10^9 counts/min total) were prepared from E. coli strain MRE 600 by the method of Stiege et al (9). Cross-linking with 2-iminothiolane (Pierce Chemical Corp.) was carried out as described by Wower et al (1), with the exception that the ultraviolet irradiation treatment, which is the second step of the cross-linking reaction, was reduced from 3 min to 2 min. After

irradiation, the cross-linked subunits were incubated with 2-mercaptoethanol (1), then precipitated with ethanol. The precipitate was resuspended in buffer and subjected to digestion with cobra venom nuclease (10) exactly as described by Oβwald et al (3).

Separation and analysis of RNA-protein cross-linked complexes: The RNA-protein cross-linked complexes were isolated from the nuclease digest according to the three-step procedure of refs. 3, 5. Individual complexes were eluted from the two-dimensional gels (which represent the third stage of the latter procedure) in 10 mM Tris-HCl pH 7.8, 0.1% SDS, 1 mM EDTA, 500 mM sodium acetate, 6 mM 2-mercaptoethanol, and were either analysed for their protein and RNA content directly (without ethanol precipitation, cf. refs. 3, 5), or were subjected to further purification by affinity chromatography (see below). When this latter step was not incorporated, the complexes were analysed for their protein content by the immunological "spotting test" of ref. 11, and the cross-link sites on the RNA moiety were determined by total digestion with ribonuclease T_1 followed by "fingerprint" analysis on polyethyleneimine cellulose thin-layer plates (12), according to our standard procedures (9,13). The oligonucleotide data were fitted to the 23S RNA sequence of Brosius et al (14).

Final purification of cross-linked RNA-protein complexes by immuno affinity chromatography: Two alternative methods were used, one making use of protein A from Staphylococcus aureus (15,16) immobilized on sepharose in conjuction with antibodies to the individual 50S ribosomal proteins raised in rabbits, and the other making use of antigoat IgG (raised in rabbits) immobilized on agarose in conjunction with antibodies to the 50S ribosomal proteins raised in sheep. The antibodies were raised and characterized as outlined in ref. 11.

1. The "protein A-sepharose" method. Protein A-sepharose (Pharmacia) was suspended in 20 mM potassium phosphate buffer pH 7.7, 150 mM NaCl ("buffer A"). 60 µl aliquots of the suspended gel were mixed with 120 µl of buffer A and 120 µl of appropriate antiserum (raised in rabit) to one of the 50S proteins. The mixtures were shaken gently for 1 - 2 hr at 4°, and were then washed five times for 5 min in 1 ml of buffer A, with centrifuga-

tion between each wash. Vacant sites on the sepharose were saturated by shaking for 1 hr at 4° with a solution containing 1% bovine serum albumin (Sigma, nuclease free), 1% Triton X-100, 25 mM Tris-HCl pH 7.8, 500 mM NaCl, 5 mM EDTA. After centrifugation the surplus solution was decanted. ^{32}P-labelled RNA-protein complexes eluted from the two-dimensional gels (above) were warmed to 60° for 5 min in the elution buffer (1 ml), and were added to the antibody-sepharose preparations and shaken for 2 hr at 4°. After centrifugation the supernatant was removed, and the sepharose was washed four times for 5 min with 1 ml aliquots of 50 mM Tris-HCl pH 7.8, 500 mM NaCl, 1 mM EDTA, 2.5% Triton X-100, 250 mM sucrose and 0.5% SDS (17), followed by two further washes in the same buffer but without Triton X-100, sucrose or SDS. If required, the RNA-protein complexes remaining in the first supernatant were subjected to a second affinity chromatography step, using protein A-sepharose coupled to antibody to a different 50S protein.

The RNA-protein complex remaining bound to the sepharose at the end of this procedure was in each case eluted by suspending the sepharose in 200 µl of 10 mM Tris-HCl pH 7.8, 500 mM NaCl, 0.1% SDS together with 1 mg/ml of proteinase K, and incubating for 15 min at 37°. After separation of the sepharose by centrifugation, this elution step was repeated twice, and the combined eluates were warmed to 60° for 5 min. 20 µg of unlabelled carrier tRNA was added, and the solution was extracted with phenol for 1 hr at 4°. The RNA was isolated from the aqueous phase by ethanol precipitation, and subjected to fingerprint analysis, as described above.

2. The "agarose" method. 40 µl aliquots of agarose gel carrying immobilized antigoat IgG (Sigma chemical Corp., No. 6903) were washed twice for 5 min with 1 ml of buffer A (see above). 20 µl of appropriate antiserum to one of the 50S ribosomal proteins (raised in sheep) was added, together with 90 µl of buffer A containing 0.05% Tween 20 (Sigma, "buffer B"), and the mixtures were gently shaken for 2 hr at 4°. After centrifugation and removal of the supernatant, the agarose was washed six times for 5 min with 1 ml aliquots of buffer B. The ^{32}P-labelled RNA-protein complex (1 ml) was then added as in the

protein A-sepharose method, and the suspension was shaken for at least 3 hr at 4°. After centrifugation and removal of the supernatant, the agarose was again washed three times for 5 min with 1 ml of buffer B. The bound RNA was then eluted from the agarose by incubation with proteinase K as just described, and subjected to fingerprint analysis.

RESULTS AND DISCUSSION

The RNA-protein cross-linking reaction with 2-iminothiolane involves first a reaction with the ε-amino groups of lysine residues in the ribosomal proteins, and this is followed by a brief ultraviolet irradiation treatment, which causes cross-linking to the RNA, presumably by reaction between pyrimidine residues in the latter and the sulphur atom in the 2-iminothiolane (1,18). In previous experiments using this reagent with E. coli 50S ribosomal subunits (1), the cross-linked RNA was degraded by partial digestion with ribonuclease T_1. However, as we have since noted (e.g. 3,9) this digestion procedure is not very favourable, and much of the cross-link data was lost due both to selective over-digestion of certain regions of the RNA, as well as to selective aggregation of some of the cross-linked products under the separation conditions previously employed. For just this reason the patterns of RNA-protein cross-linked complexes observed on two-dimensional gels were deceptively simple in the older experiments (1,2).

In the new procedure (3,5), the cross-linked subunits are digested with cobra venom nuclease (10). Non-cross-linked proteins are removed by "sucrose gradient electrophoresis" in the presence of non-ionic detergent, and the RNA-protein complexes are then separated from fragments of free RNA by adsorption on glass-fibre filters (19). After elution from the filter, the individual cross-linked complexes are separated by two-dimensional gel electrophoresis at high salt concentration. Under these conditions (cf. 3,4) many more cross-linked complexes appear on the gels than were previously observed (1,2), and a typical gel pattern, obtained from ^{32}P-labelled 50S subunits cross-linked with 2-iminothiolane and digested with cobra venom nuclease, is illustrated in Fig. 1.

The gel of Fig. 1 shows the usual features (cf. refs. 3,4) of a weak "diagonal" of free RNA fragments still remaining after the glass-fibre filtration process, with the cross-linked RNA-protein complexes appearing as rows of spots lying above this diagonal. As noted in the Introduction, the resolving power of the gel is limited both by the complexity of the cross-linked products, as well as by a tendency for smearing in the second gel dimension, despite the high salt concentration which is an essential feature of the second dimension system (3,5). It is thus clear that a successful analysis of many of the cross-linked complexes (Fig. 1) requires a further purification step, and the immuno affinity chromatography procedures (see Material and Methods) were developed for this purpose.

The two immuno affinity methods described are both equally effective. In the one case, protein A from S. aureus (15,16) immobilized on sepharose is used to bind an antibody to the desired 50S ribosomal protein, and the immobilized protein

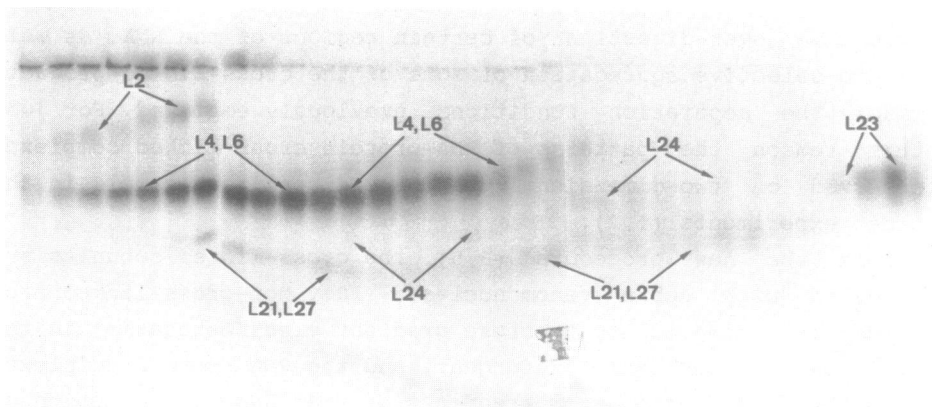

Figure 1: Two-dimensional gel electrophoresis (cf. refs. 3, 5) of RNA-protein cross-linked complexes. Direction of the first dimension is from left to right, and that of the second dimension from top to bottom. Each second dimension gel has twelve slots, containing the individual eluates from the first-dimension gel slices; three such gels are combined to give the total pattern. The rows of RNA-protein complexes are marked with arrows giving the identity of the proteins found in each row of spots.

A-antibody complex in turn binds the cross-linked RNA-protein complex. This "protein A-sepharose" method requires antibodies to ribosomal proteins which were raised in rabbits. In the alternative "agarose" method, antibodies raised in sheep are used, and in this case the immobilized agent is an antigoat IgG bound to agarose. Aliquots of the cross-linked RNA-protein complexes eluted from gels such as that shown in Fig. 1 were first subjected to the immunological "spotting test" of ref. 11, in order to find out which ribosomal proteins were present. In cases where only a single protein was found (cf. Fig. 2, below), the complex was subjected to oligonucleotide analysis directly. Otherwise, one or other of the affinity chromatography procedures was applied, and examples of both are given below. The amount of pure cross-linked complex which could be isolated by these procedures is obviously dependent on the proportion of the protein in question which was present in the mixture; typically, the corresponding gel bands from two or three adjacent slots of the second dimension gel (Fig. 1) were combined for elution, giving 20,000 - 100,000 counts/min of ^{32}P-radioactivity, of which 15 - 30% could be recovered from the agarose or sepharose as purified product by proteinase K digestion and phenol extraction. This

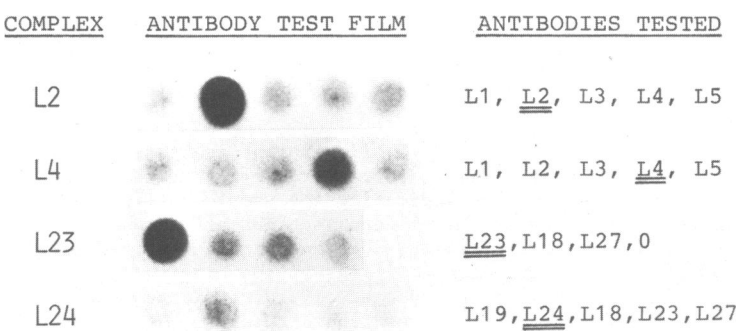

Figure 2: Immunological identification of proteins in the cross-linked complexes (11). The key on the right shows some of the antibodies tested against each complex, "0" denoting a control minus antibody. The proteins giving a positive reaction are underlined, as can be seen by comparison with the position of the radioactive spots on the "antibody test film" strip. The four strips are from the complexes whose respective RNA fingerprints are shown in Figs. 3a to d. (The L4 complex in the second row gave a negative reaction with anti-L6 (not shown, cf. text)).

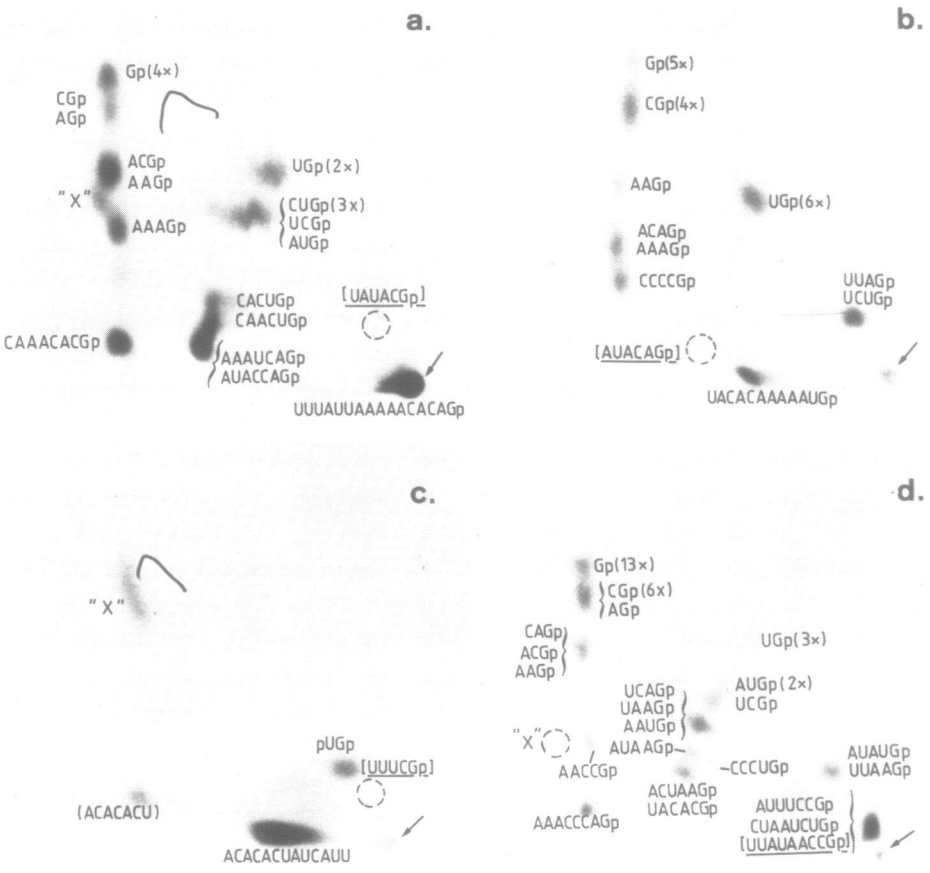

Figure 3: Examples of ribonuclease T_1 fingerprint analyses (12) of the cross-linked complexes. The first dimension ran from right to left, and the second from bottom to top, the arrows indicating the sample application points. Identities and molarities of the oligonucleotides are shown. Underlined oligonucleotides in square brackets, together with the dotted circles, indicate the identities and expected positions of the missing (i.e. cross-linked) sequences. "X", with or without a dotted circle, indicates the position of an oligopeptide-oligonucleotide cross-linked species. The fingerprints shown are of (a) the L2-RNA complex, (b) the L4-RNA complex (not purified by affinity chromatography), (c) the L23-RNA complex, and (d) the L24-RNA complex, purified by the agarose method. (The irregular "inverted U" markings in (a) and (c) indicate the position of a xylene cyanol dye marker on the thin-layer plates).

amount of radioactivity is amply sufficient for the subsequent oligonucleotide analysis. Furthermore, where significant amounts of two or more proteins were present in a single fraction eluted

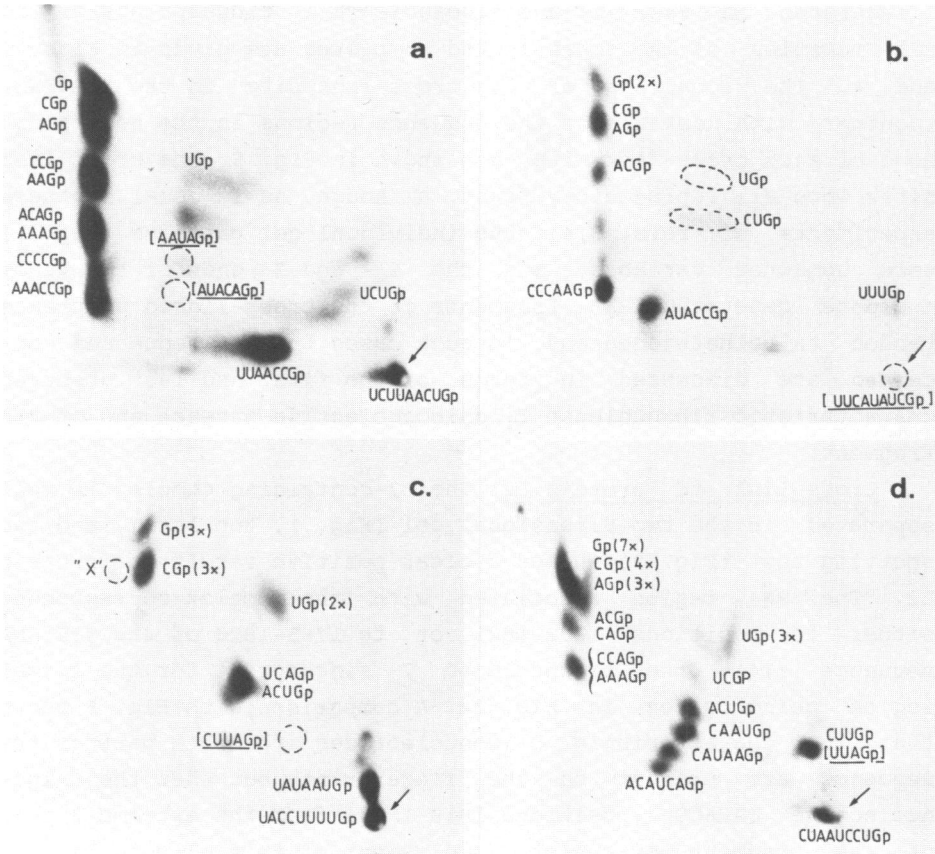

Figure 4: Examples of ribonuclease T_1 fingerprints of pairs of RNA-protein complexes isolated by immuno affinity chromatography. (a) and (b) are the fingerprints of complexes isolated by the protein A-sepharose method with anti-L4 and anti-L6, respectively. (c) and (d) are fingerprints of complexes isolated by the agarose method with anti-L21 and anti-L27, respectively. The fingerprints are marked as indicated in the legend to Fig. 3. (The UGp and CUGp oligonucleotide spots in (b) were smeared and faint, and are therefore ringed with dotted lines to indicate their presence).

from the two-dimensional gel, the cross-linked complexes corresponding to each protein could be recovered one by one by sequential applications of the affinity chromatography procedure using the appropriate antibodies.

Eight cross-link site analyes, making use of various combinations of the above procedures, are described in the following sections. Typical immunological spotting test results (11) are

illustrated in Fig. 2, and ribonuclease T_1 fingerprints of the RNA moieties of the cross-linked complexes are given in Figs. 3 and 4. The locations of the cross-link sites in the 23S RNA, together with details of the sequence regions in the neighbourhood of each cross-link site, are shown in Fig. 5. The cross-link sites were all reproducibly found, although, as is usual in these experiments (cf. refs. 3,4), the individual gel patterns (Fig. 1) were somewhat variable, and the 5'- and 3'-ends of the venom nuclease generated RNA fragments in the cross-linked complexes tended to be heterogeneous. In such cases the RNA sequences concerned are discussed in terms of the first and last observed characteristic ribonuclease T_1 oligonucleotide at each end of the fragment.

Cross-link to protein L2: The L2-containing complex is well separated in the two-dimensional gel (Fig. 1, top left), and the spotting test (Fig. 2) showed a clear positive result for protein L2. The RNA region associated with the complex corresponded either to positions 1732-1823 or to 1765-1823 of the 23S RNA sequence (14), the ribonuclease T_1 fingerprint for the former region being given in Fig. 3a. A comparison with Fig. 5 shows that the characteristic oligonucleotides for this part of the sequence are present on the fingerprint, but that the oligonucleotide UAUACGp (positions 1818-1823) from the extreme 3'-end of the fragment was absent. Involvement of this oligonucleotide at the cross-link site was however clearly indicated by the reproducible appearance of an anomalous spot on the fingerprint (marked "X" in Fig. 3a), which liberated ACp, Up and Gp together with an undigested residue upon secondary digestion with ribonuclease A. Since the cross-linked complexes were treated with proteinase K prior to ribonuclease T_1 digestion (cf. refs. 3,13), the spot "X" thus represents the residual cross-linked oligonucleotide-oligopeptide complex, and the secondary digestion products observed after ribonuclease A treatment suggest that the actual site of cross-linking was within the AU sequence at positions 1819-1820. (As we have previously noted (3), the residual oligonucleotide-oligopeptide complexes are often heterogeneous with respect to the peptide moiety and are sometimes rather insoluble, with the result that they tend to appear on the

fingerprints either in sub-molar amounts or as smears, and are semetimes not seen at all).

The cross-link to protein L2 is in excellent agreement with the work of K. Watanabe (20), who has identified a binding site on Bacillus caldolyticus 23S RNA for the protein corresponding to L2 in that organism. This binding site was at positions in the B. caldolyticus sequence which correspond to positions 1794-1825 in the E. coli 23S RNA (cf. Fig. 6 below).

Cross-link to protein L4: Complexes containing protein L4 were usually intermingled with L6-complexes on the two-dimensional gels (Fig. 1). However, in some instances (not visible in Fig. 1), the row of gel spots corresponding to these complexes was resolved into discrete groups, one of which contained only protein L4, as evidenced by the spotting test result (Fig. 2). The RNA region associated with this fragment arose from positions 284-350 of the 23S RNA sequence, and a comparison of the fingerprint (Fig. 3b) with the sequence region concerned (Fig. 5) shows that the cross-link site was within the oligonucleotide AUACAGp (positions 320-325), since this oligonucleotide was reproducibly absent from the fingerprint. No residual oligopeptide-oligonucleotide was observed in the case of this complex, so a further localization of the cross-link site within the AUACAG sequence was not possible.

The cross-link site to protein L4 comes from an entirely different region of the RNA as compared with the site previously found (positions 613-617, refs. 1,13). The latter site was however also found in this series of experiments when the affinity chromatography procedures were used, and sometimes both cross-link sites could be simultaneously observed (see below).

Cross-link to protein L23. A strong spot was always observed on the right-hand side of the two-dimensional gels (Fig. 1), which gave a positive reaction with L23 in the spotting test (Fig. 2). In our previous experiments, L23 was found to be cross-linked to the oligonucleotide UUUCGp (positions 137-141) within RNA fragments covering the sequence region between positions ca. 100 - 170 of the 23S RNA (1). In this case the fingerprint (Fig. 3c) shows that the RNA fragment here is very short, but it obviously corresponds to the same cross-link site

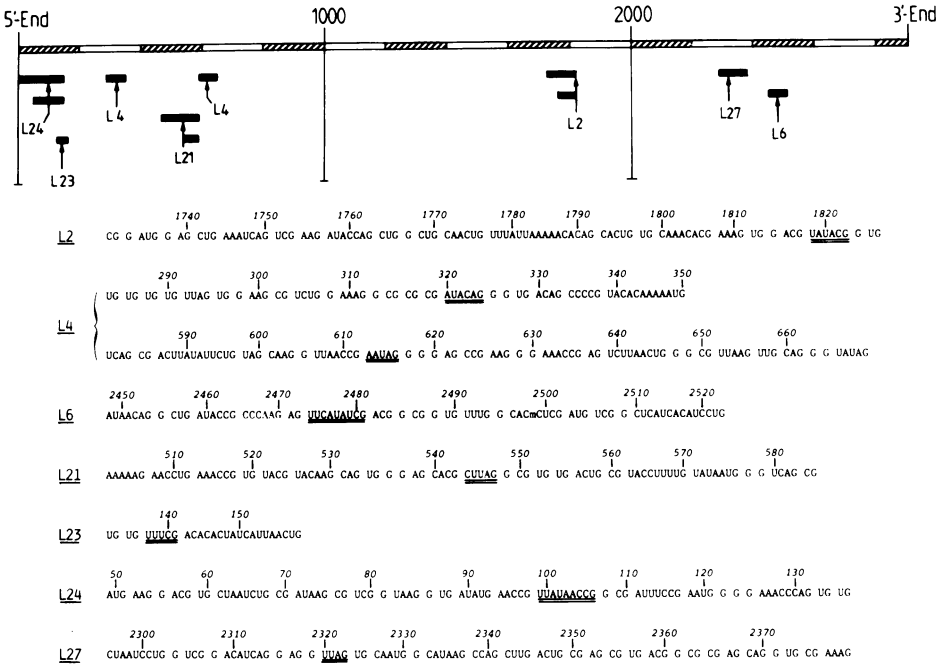

Figure 5: Locations of the cross-linked complexes in the 23S RNA sequence. The horizontal bars in the upper part of the Figure indicate the sequence positions of the most commonly observed RNA fragments in the complexes, with the arrow and protein number denoting the positions of the cross-link sites to the various proteins within the RNA fragments. The lower part of the Figure shows the details of the RNA sequence in the vicinity of each cross-link site. The sequence is divided into ribonuclease T_1 oligonucleotides to facilitate comparison with Figs. 3 and 4. Missing (i.e. cross-linked) oligonucleotides are underlined.

as before; the 5'-end of the fragment is represented by a venom nuclease cut at position 134/135, giving rise to the oligonucleotide pUGp on the fingerprint, the corresponding 3'-end being heterogeneous as evidenced by the appearance (cf. Fig. 5) of both ACACACU (positions 142-148) and ACACACUAUCAUU (positions 142-153). UUUCGp (positions 137-141) was absent, and instead an anomalous spot corresponding to the cross-linked oligopeptide-oligonucleotide was always seen (marked "X" in Fig. 3c), which gave rise to Up, Cp, Gp and an undigested residue upon treatment with ribonuclease A. The cross-link is thus to one of the U-residues at positions 137-139.

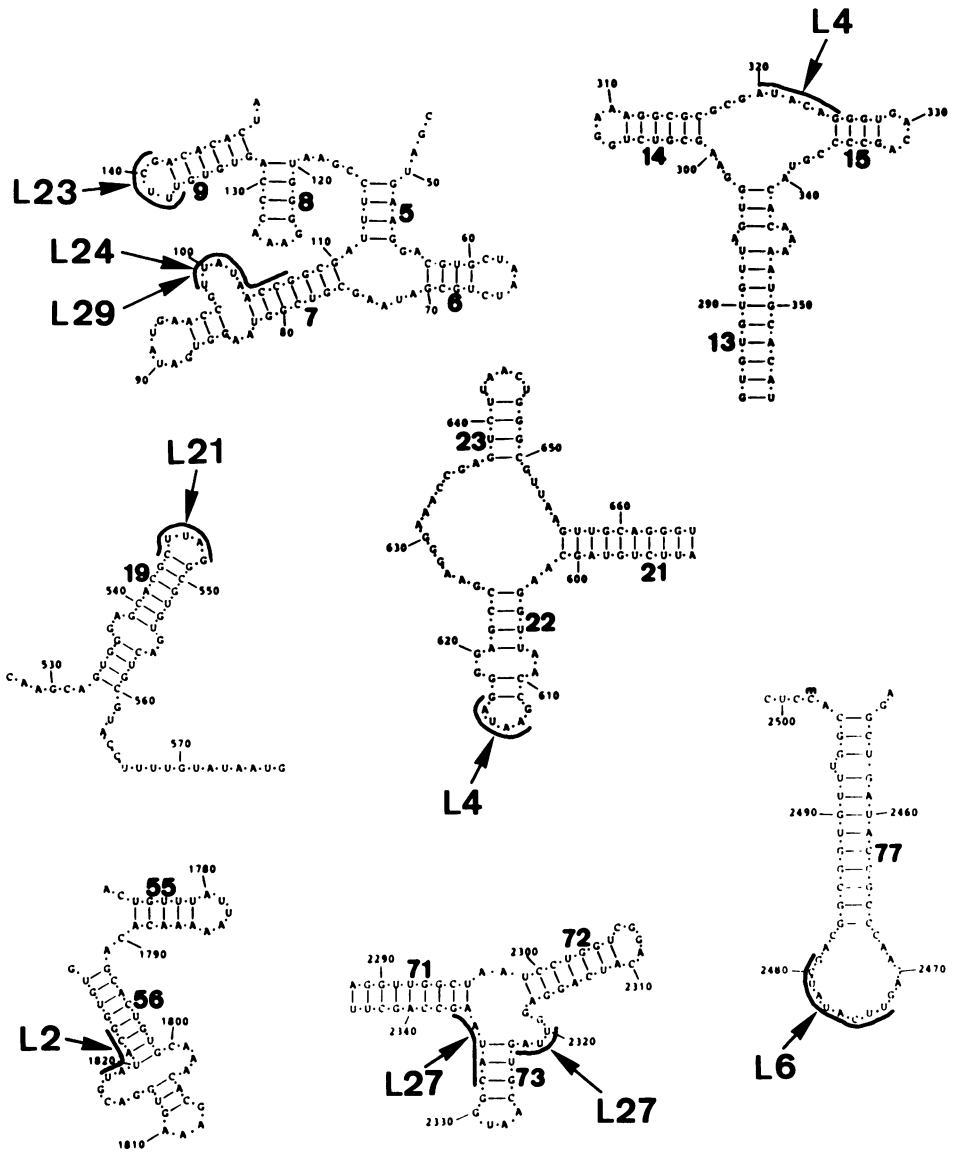

Figure 6: Location of the RNA-protein cross-link sites (including those from ref. 1) in the secondary structure of the 23S RNA (21). The solid lines indicate the oligonucleotides encompassing each cross-link (cf. Fig. 5). The structures are drawn in the same orientations as those of the secondary structure in ref. 21.

Cross-link to protein L24: Analysis by the spotting test of the faint row of spots on the two-dimensional gels (Fig. 1) below the "L4-L6" row showed a weak positive reaction for protein L24 (Fig. 2). On the basis of this result the affinity chromatography technique was applied, using anti-L24 and the "agarose" method (see Materials and Methods). This enabled L24-RNA complexes to be isolated which arose from the extreme 5'-end of the 23S RNA, the fragments covering positions 1-132, 50-132 or 80-132 of the sequence, according to the region of the two-dimensional gel (Fig. 1) from which the complex was taken. The fingerprint shown in Fig. 3d corresponds to the longest of these three fragments. The cross-link site lies within the oligonucleotide UUAUAACCGp (positions 99-107), as evidenced by the complete absence of AACp in secondary digests with ribonuclease A of the spot on the fingerprint which should have contained the latter oligonucleotide (Fig. 3d, the similar oligonucleotides AUUUCCGp and CUAAUCUGp being present). At the same time an anomalous spot "X" was often seen at the position indicated in Fig. 3d, which liberated AACp, AUp, Cp and Gp together with an undigested residue upon secondary digestion with ribonuclease A, indicating that the actual cross-link site was at U-99 or U-100. In one experiment, the oligonucleotide UUAUAACCGp was present, and the neighbouring oligonucleotide AUAUGp (positions 89-93) was absent instead (cf. Fig. 6).

Previously (1) we had found protein L29 to be cross-linked to the oligonucleotide at positions 99-107 in the 23S RNA, but although traces of L29 were found (in the expected position below the row of spots containing L21 and L27 (Fig. 1)), we were not able to isolate sufficient amounts of any L29 complexes for a fingerprint analysis in this series of experiments. A similar situation has been found in the 30S subunit (2,6), where proteins S8 and S17 were both cross-linked to the same oligonucleotide (positions 629-633) in the 16S sequence.

Cross-links to proteins L4 and L6: As already described above, the cross-link to protein L4 at positions 320-325 was occasionally found as a discrete spot on the two-dimensional gel. More usually, the situation visible in Fig. 1 was observed, in which L4 and L6 complexes were intermingled in a more or less

continuous row of spots on the gel. Here, the sequential application of the affinity chromatography step, using antibodies to L4 then L6 (or vice versa) in conjunction with either the protein A-sepharose or the agarose method (see Materials and Methods), enabled the complexes to be separated from one another and analysed. Figs. 4a and b show the results of a typical experiment, in this case with the protein A-sepharose method, Fig. 4a showing the fingerprint of the L4-containing component of the complex, and Fig. 4b the L6-containing component. It is immediately obvious that the two fingerprints are entirely different, although a minimal level of cross-contamination can be seen.

The RNA corresponding to protein L4 (Fig. 4a) shows oligonucleotides from two distinct regions of the 23S RNA sequence, covering positions 299-338, and 606-647 (cf. Fig. 5), as well as minor contamination with other sequences. In similar analyses from different regions of the gel (Fig. 1), longer sequences were observed, covering positions 284-350 and 586-647. An oligonucleotide was always missing from each of the two sequence regions, namely AUACAGp (positions 320-325) and AAUAGp (positions 613-617), respectively. The fingerprint in Fig. 4a thus represents a mixture of fragments containing the cross-link site to protein L4 described above, together with the site previously observed (1).

The RNA corresponding to protein L6 (Fig. 4b) covers the sequence from positions 2456-2494 (cf. Fig. 5), longer fragments (from positions 2405-2520) being observed in other analyses. In each case the oligonucleotide UUCAUAUCGp (positions 2473-2481) was absent, and the cross-link site is therefore the same as that previously determined for protein L6 (1). Figs. 4a and b thus demonstrate that, with the help of the affinity chromatography procedure, no less than three independent cross-link sites could be successfully analysed in a single eluate fraction from the two-dimensional gel (Fig. 1). It should be noted in this context that the stringent washing procedures described in Materials and Methods (cf. ref. 17) for the RNA-protein complexes bound to the sepharose or agarose are essential, in order to disrupt non-specific interactions or aggregation between the various different complexes present in the mixtures.

Cross-links to proteins L21 and L27: Cross-linked complexes containing proteins L21 and L27 were separated from one another in a similar manner to that just described for L4 and L6. Figs. 4c and d give examples of the fingerprints obtained, in this case after an affinity chromatography separation by the agarose method, using anti-L21 then anti-L27. In Fig. 4c the RNA corresponding to protein L21 is shown, and represents the sequence region covering positions 544-585. The cross-link site is indicated by the absence from the fingerprint of CUUAGp (positions 544-548) from the 5'-end of this region, and by the concomitant presence of an oligopeptide-oligonucleotide spot "X", which liberated AGp, Cp, Up and an undigested residue upon secondary digestion with ribonuclease A. The cross-link site is thus to one of the two U-residues at positions 545/546, and is the same as that previously observed for protein L21 (1). Longer fragments, covering positions 470-585 were found in other analyses, but the oligonucleotide CUUAGp was always absent.

The corresponding fingerprint for the L27 complex (Fig. 4d) shows an interesting variation on our previous data (1), where a cross-link site to this protein was localized in the oligonucleotide CAUAAGp at positions 2333-2337 in the 23S RNA. The fingerprint of Fig. 4d covers the same sequence region, from positions 2295-2379, but, instead of CAUAAGp, the nearby oligonucleotide UUAGp (positions 2320-2323) was absent (cf. Fig. 5). In some analyses (not visible in Fig. 4d) a cross-linked oligopeptide-oligonucleotide spot was visible, which liberated AGp on digestion with ribonuclease A. Again, longer fragments were also observed, but UUAGp was almost always absent, although in one or two experiments CAUAAGp was absent instead, as in our earlier studies (1). The two cross-link sites (positions 2320-2323 and 2333-2337) lie very close together on opposite sides of the same hairpin loop in the secondary structure of the 23S RNA (Fig. 6).

CONCLUSIONS

Figure 6 summarizes the RNA-protein cross-link data that we have so far reported, in this paper and in our previous work (1), using 2-iminothiolane as a cross-linking agent for 50S ribosomal subunits. The sites now number ten in all, and are shown in Fig.

6 in their respective positions in the secondary structure of the 23S RNA (21).

The results which we have described here demonstrate that the three-step procedure (3,5) for isolating RNA-protein cross-linked complexes does as expected lead to very complicated two-dimensional gel patterns in the case of the 50S subunit, but that application of the additional immuno affinity chromatography purification step enables the individual cross-linked complexes to be separated and analysed. This methodology is currently being applied to other cross-linking reagents, namely methyl p-azidophenyl acetimidate (3) and bis-(2-chloroethyl)-methylamine (4), with which we were unable to locate any cross-link sites at all in the case of the 50S subunit, using our older techniques (1,2). Preliminary localizations of a number of cross-link sites have now already been made using these reagents, and we are confident that in due course these experiments will lead to the derivation of a detailed three-dimensional model for the arrangement of the proteins and the 23S RNA in situ in the 50S subunit, at a similar level of resolution to that which has been achieved in the case of the 30S subunit (7).

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