Precise Localization of the Site of Cross-Linking between Protein L4 and 23S Ribonucleic Acid Induced by Mild Ultraviolet Irradiation of *Escherichia coli* 50S Ribosomal Subunits†

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ABSTRACT: Mild ultraviolet irradiation of *Escherichia coli* 50S ribosomal subunits causes a cross-linking reaction between protein and RNA, whose primary target is protein L4 [Möller, K., & Brimacombe, R. (1975) *Mol. Gen. Genet. 141*, 343]. Here we have determined the site of this cross-link both on L4 and on 23S RNA. For the site on the protein, a cross-linked protein–oligonucleotide complex was isolated and subjected to successive digestions with various proteases. In each case the peptide–oligonucleotide complexes formed were analyzed. It could clearly be shown that the cross-link site was contained within a characteristic peptide 16–20 amino acids long and that the amino acid concerned was the tyrosine residue at position 35 in the recently completed L4 sequence (M. Kimura and B. Wittmann-Liebold, personal communication). For the site on the RNA, a cross-linked L4–23S RNA complex was subjected to mild nuclease digestion, producing a range of L4–RNA fragments which were isolated with the help of a new two-dimensional gel electrophoresis system. Oligonucleotide analyses of these fragments, combined with successive nuclease digestions of the residual oligonucleotide attached to protein L4, established that the site of cross-linking was homogeneous, involving a uridine residue at position 615 in the recently determined 23S RNA sequence [Brosius, J., Dull, T. J., & Noller, H. F. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 201].

A thorough understanding of ribosome structure requires that the spatial arrangement of the individual ribosomal components be examined at the amino acid/nucleotide level. However, despite the vast amount of topographical information which has been accumulated for the *Escherichia coli* ribosome [see, e.g., Brimacombe et al. (1978) for a review], very few studies have thus far been pursued to this level of detail. In the case of the ribosomal RNA molecules, the accessibility of specific guanosine residues on the surface of the ribosomal subunits has been analyzed by Noller and his co-workers [e.g., Herr et al. (1979) and earlier papers], with the help of the single strand specific reagent kethoxal. On the protein side, a pair of amino acid residues which are adjacent to each other within the 30S subunit has recently been identified by the analysis of a chemically cross-linked complex formed between proteins S5 and S8 (Allen et al., 1979). Our own studies in this field have been concerned with the determination of neighborhoods between ribosomal proteins and RNA, using RNA–protein cross-linking as a tool, and we have established the precise location of a cross-link site between protein S7 and 16S RNA induced by mild ultraviolet irradiation of 30S subunits (Möller et al., 1978; Zwieb & Brimacombe, 1979).

Special methods had to be developed in order to make possible this type of analysis of an RNA–protein cross-link site, and the mild irradiation system was chosen for this purpose since S7 was the only protein which was cross-linked to RNA to any significant extent under the conditions used. However, in more usual cross-linking situations, such as those involving the use of bifunctional chemical reagents [e.g., Ulmer et al. (1978), Bäumert et al. (1978), and Rinke et al. (1980)], several proteins are simultaneously cross-linked to the RNA within the subunit. This poses a considerable number of additional problems which need to be solved for the separation and analysis of the cross-linked products. A situation of intermediate complexity is obtained by using irradiated *E. coli* 50S subunits. Here, the primary product of cross-linking to 23S RNA under mild conditions is protein L4, but L2 is also cross-linked to a significant extent, and the background cross-linking of other proteins is notably higher than in the case of irradiated 30S subunits (Möller & Brimacombe, 1975).

In this paper, we present some new techniques for the isolation of individual RNA–protein complexes from mixtures of cross-linked products and describe the application of these methods to the analysis of the cross-link site between protein L4 and 23S RNA, induced by mild irradiation of 50S subunits.

Materials and Methods

Preparation and Irradiation of Ribosomal Subunits. Ammonium chloride washed 50S subunits from *E. coli* strain MRE 600, either unlabeled or labeled with [^{32}P]orthophosphate, were prepared by the usual procedure (Morgan & Brimacombe, 1972; Yuki & Brimacombe, 1975). The subunits were activated by dialysis as described (Zwieb & Brimacombe, 1979) and were irradiated with ultraviolet light for 12 min at a concentration of 5 A_{260} units/mL in 50 mM KCl, 5 mM magnesium acetate, 6 mM 2-mercaptoethanol, and 10 mM Tris-HCl, pH 7.8, exactly as described (Möller & Brimacombe, 1975; Möller et al., 1978). Activity of the subunits was tested in the poly(uridylic acid) [poly(U)] system of Dohme & Nierhaus (1976).

Determination of the Cross-Link Site in Protein L4. (*a*) *Isolation of the L4–Oligonucleotide Complex.* A total of 20 000 A_{260} units of unlabeled 50S subunits was irradiated as above, precipitated with ethanol, and taken up in irradiation buffer at a concentration of 180 A_{260} units/mL. A parallel sample containing 100 A_{260} units of ^{32}P-labeled irradiated subunits [(5–10) × 10^8 counts/min total radioactivity] was also prepared. In each case, the non-cross-linked protein was removed by the acetic acid–urea method of Hochkeppel et al. (1976), with the following minor modifications. Sometimes the RNA plus cross-linked protein did not precipitate immediately after the addition of acetic acid, in which case water

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0006-2960/80/0419-4179$01.00/0

was added until the solution began to become cloudy. After the first precipitation and centrifugation, the pelleted material was washed with 400 mM KCl, 20 mM magnesium acetate, and 30 mM triethanolamine hydrochloride, pH 9, and then dissolved overnight in 20 mM triethanolamine hydrochloride, pH 8, to a concentration of 200 A_{260} units/mL. After the second acetic acid precipitation, the pellets were washed twice with the high-salt buffer just mentioned and then once with 20 mM magnesium acetate and 30 mM triethanolamine hydrochloride, pH 9, and were finally dissolved in 5 mM triethanolamine hydrochloride, pH 8, and 1 mM EDTA, again to a concentration of 200 A_{260} units/mL. In the case of the unlabeled reaction mixture, solution was aided by sonication of the pellet, together with rapid stirring and warming to 37 °C.

The RNA–protein complexes thus obtained were digested with ribonucleases A and T_1 for 2 h at 37 °C, at final enzyme concentrations of 2 μg/mL and 200 units/mL, respectively. During this time, a precipitate of the L4–oligonucleotide complex appeared. The reaction mixture derived from the unlabeled subunits (100 mL) was treated with solid urea (3.6 g) and was then evaporated to 10 mL. This caused the precipitated complex to redissolve, and the solution was added at this stage to the small ^{32}P-labeled reaction mixture (0.5 mL). The clear solution was applied to a Sephadex G-75 (superfine) column (200 cm long, 2 cm in diameter) equilibrated in 6 M urea, 6 mM 2-mercaptoethanol, and 10 mM sodium acetate, pH 5.6. The column was eluted at 6.6 mL/h and 3.3-mL fractions were collected. ^{32}P radioactivity in each fraction was measured, and aliquots (15 μL) were analyzed for protein content on 15% polyacrylamide slab gels in the system of Laemmli & Favre (1973). After the aliquots run, the gels were stained with Coomassie blue and were also subjected to autoradiography. Column fractions containing the L4–oligonucleotide complex were pooled, dialyzed against 2% acetic acid (by using Spectrapor 3 dialysis tubing), and lyophilized.

(*b*) *Protease Digestions and Peptide Analyses.* The L4–oligonucleotide complex [ca. 2–4 mg, (2–3) × 10^5 counts/min] was dissolved in 1 mL of 0.05 M HCl and was treated with an aqueous solution of porcine pepsin to give an enzyme/substrate ratio of 1:30. After incubation for 1 h at 37 °C, the sample was made 10% in acetic acid and applied to a Sephadex G-25 (superfine) column (125 cm long, 1 cm in diameter). The column was eluted with 10% acetic acid at 4 mL/h, and 0.6-mL fractions were collected. Radioactivity in each fraction was measured, and aliquots (100 μL) were tested by chromatography on Polygram CEL 300 thin-layer plates (Macherey and Nagel, Germany) in pyridine/butanol/acetic acid/water (10:15:3:12 v/v) [cf. Hitz et al. (1977)]. The plates were stained with 0.004% fluorescamine (Vandekerckhove & Van Montagu, 1974) and were subjected to autoradiography. Column fractions found to contain the two ^{32}P-labeled peptides (see text) were then pooled and separated in the same thin-layer system. After the chromatography, the ^{32}P-labeled peptides were eluted with 50% acetic acid and lyophilized. One-third of each sample was submitted to amino acid and N-terminal sequence analysis (see below).

The remaining two-thirds of each peptide was dissolved in 0.5% morpholine–formate buffer, pH 8, and was further digested by incubating for 5 h at 37 °C with trypsin (from Worthington Biochemicals, relatively ribonuclease free, final concentration 2 μg/mL). The digest was lyophilized and then "fingerprinted" on CEL 300 thin-layer plates by using the two-dimensional electrophoresis/chromatography system of Hitz et al. (1977). After the sample was run, the plates were stained with fluorescamine and autoradiographed, and the peptides eluted from the plates as described above. Again, aliquots were taken for amino acid and sequence analysis.

An aliquot (~5 nmol) of the ^{32}P-containing peptide isolated after trypsin digestion was submitted to a final digestion in 0.5% morpholine–formate buffer, pH 8 (0.1 mL), with 2 μg of aminopeptidase M for 2 h at 37 °C, followed by a 30-min incubation with 2 μg of proteinase K. The hydrolysate was lyophilized and fingerprinted as above.

In addition, some of the early fractions from the G-25 column (above, see text) were treated with thermolysin in 0.5% morpholine–formate buffer, pH 8, containing 2 M urea for 4 h at 52 °C, at an enzyme/substrate ratio of ca. 1:100. The digest was made 5% in acetic acid and reapplied to the Sephadex G-25 column (above). The column fractions were analyzed on thin-layer plates as described above, and the ^{32}P-labeled peptides were extracted and submitted to amino acid analysis.

Amino acid compositions were determined in a Durrum 500 amino acid analyzer, according to the procedure of Hitz et al. (1977). N-Terminal sequence analysis was made by using the procedure of Chang et al. (1978).

Determination of Cross-Link Site in 23S RNA. (*a*) *Isolation of L4–RNA Complex.* Six to eight A_{260} units of ^{32}P-labeled 50S subunits [(5–8) × 10^8 counts/min total] were irradiated as above, concentrated by ethanol precipitation, and applied to a sucrose gradient containing dodecyl sulfate, as described previously (Möller et al., 1978). Gradient fractions containing 23S RNA and RNA–protein cross-linked complexes were pooled, ethanol precipitated, and dissolved in 0.2 mL of 0.2% Triton X-100, 2.5 mM EDTA, and 10 mM Tris-HCl, pH 7.8. Aliquots (50 μL) of this solution were incubated for 15 min at 37 °C with different amounts of ribonuclease A (0.5–5 ng of enzyme per A_{260} unit of RNA) and were then loaded into 1-cm slots of a 6% polyacrylamide slab gel, 40 cm long and 1 mm thick. This gel contained Triton X-100, LiCl, urea, EDTA, and Tris-HCl buffer, pH 7.8, as described (Zwieb & Brimacombe, 1978) but differed from the previous system in that the concentration of Tris–citric acid in the reservoir buffer was increased to 100 mM and that the sample slots were formed by pouring a short spacer gel 0.5 cm long. This spacer was of 5% polyacrylamide and contained no LiCl, and Tris–citric acid, pH 8.8, was substituted for Tris-HCl, pH 7.8. Other components were identical with those in the main separation gel. The gel was run until a xylene cyanol marker had travelled 15–20 cm and was then autoradiographed.

Gel strips 1 cm wide containing the samples were cut out and rinsed in 0.1% dodecyl sulfate, 0.05 M Tris, and 0.38 M glycine for 5 min. The upper 14 cm of each strip was then polymerized onto the top of a 13% slab gel (20 cm long and 1 mm thick) in the dodecyl sulfate system of Laemmli & Favre (1973). No spacer gel was used. This gel was run until a bromophenol blue dye marker had travelled 18 cm and was then autoradiographed. Parts of the gel containing cross-linked RNA–protein complexes (see text) were loaded onto a third gel, also in the system of Laemmli & Favre (1973) as above, but in this case the gel was of 17% polyacrylamide and contained 7 M urea in addition to the usual components. This gel was run for a similar distance and was also autoradiographed.

The ^{32}P-labeled RNA–protein complexes were extracted from the gel into dodecyl sulfate buffer, as described (Zwieb & Brimacombe, 1978), and were precipitated with 3.5 volumes of ethanol in the presence of 10–20 μg of unlabeled carrier

RNA. Each fraction was then taken up in 40 μL of 1% dodecyl sulfate and diluted to 0.2 mL with 1 mM EDTA and 10 mM Tris-HCl, pH 7.8. The recovered radioactivity was 5000–20000 counts/min in each case. To test for the presence of cross-linked protein L4, we mixed an aliquot of ~1000 counts/min from each sample with 5 A_{260} units of unlabeled 50S subunits and treated it with ribonucleases A and T_1 in Sarkosyl buffer, followed by several ethanol precipitations to remove free oligonucleotides, exactly as described (Ulmer et al., 1978). The samples were analyzed on the two-dimensional gel system of Mets & Bogorad (1974), again as described (Ulmer et al., 1978). The gels were stained for protein and autoradiographed.

(*b*) *Oligonucleotide Analysis of L4–RNA Complexes.* After the removal of aliquots for protein analysis, the remainder of each cross-linked RNA–protein sample was incubated with three 4-μL portions of proteinase K (5 mg/mL), each incubation being for 30 min at 37 °C. The samples were then extracted with an equal volume of phenol for 2 h at 4 °C, and the aqueous phase was made 100 mM in sodium acetate and treated with 50 μg of carrier RNA and 3.5 volumes of ethanol. The precipitated RNA was centrifuged off, washed with 80% ethanol, and dissolved in 10 μL of 0.01% dodecyl sulfate. The samples were hydrolyzed for 30 min at 37 °C with 5 μg of ribonuclease T_1, lyophilized, and dissolved in 5 μL of water. The oligonucleotides released were fingerprinted on poly(ethylenimine) thin-layer plates by using the "mini-fingerprint" system described by Volckaert & Fiers (1977), with the exception that the plates were washed with 70% ethanol instead of water to remove urea. After autoradiography, the oligonucleotide spots were eluted from the plates and subjected to a secondary digestion with ribonuclease A as described (Möller et al., 1978), the digestion products also being analyzed on poly(ethylenimine) plates in the "double-digestion" system of Volckaert & Fiers (1977).

(*c*) *Determination of the Nucleotide Cross-Linked to L4.* Cross-linked RNA–protein complexes isolated on the two-dimensional Triton–dodecyl sulfate gel system (above), but not purified on the dodecyl sulfate–urea system, were extracted from the gel, precipitated with ethanol, and redissolved as above. The samples were then digested with ribonuclease T_1 alone (conditions as above) and were applied to a standard 15% Laemmli–Favre (1973) gel. The band corresponding to the T_1–oligonucleotide–L4 complex was located by autoradiography and the complex extracted from the gel, again as described above. This complex was treated with ribonuclease A, ribonuclease T_2, or proteinase K, as previously described (Zwieb & Brimacombe, 1979), with the exception that resin-bound instead of free proteinase K was used in this instance. The digestion products were separated on poly(ethylenimine) plates, either in the two-dimensional double-digestion system of Volckaert & Fiers (1977) or in the one-dimensional system of Bernardi (1974), as before (Zwieb & Brimacombe, 1979). Where necessary, products were eluted from the plates by using 80% formic acid (for L4–oligonucleotide complexes), 5% formic acid (for peptide–oligonucleotide complexes), or 30% triethylammonium carbonate, pH 10 (for oligonucleotides). The eluted samples were lyophilized prior to the next digestion or separation. The sequence of digestions and separations involved in the experiment is described in the text.

Results and Discussion

The 50S subunits from *E. coli* MRE 600 ribosomes were subjected to mild ultraviolet irradiation as described under Materials and Methods. The identity of protein L4 as the primary target of cross-linking to 23S RNA under these conditions has already been established (Möller & Brimacombe, 1975), both by gel electrophoresis in two different systems and by Ouchterlony tests with protein-specific antisera. In the experiments to be described here, a shorter irradiation time was used [12 min as opposed to 30 min (Möller & Brimacombe, 1975)], since a kinetic analysis of the cross-linking reaction had shown that 40–50% of the protein L4 content of the 50S subunit was already linked to the RNA during this shorter time (Zwieb et al., 1978). The activity of the 50S subunits in the poly(U) system was reduced by ~75% as a result of this irradiation treatment, as compared with 50% after 3 min of irradiation (Rinke et al., 1980); the inactivation kinetics does not correspond to the kinetics of L4 cross-linking [cf. Zwieb et al. (1978)].

In order to identify the site(s) of cross-linking to L4 on both protein and RNA, we isolated cross-linked RNA–protein complexes from ^{32}P-labeled irradiated 50S subunits and subjected them to a series of nuclease and protease digestions, with a view to isolating, on one hand, an identifiable L4 peptide attached to a ^{32}P-labeled oligonucleotide and, on the other hand, an identifiable 23S RNA fragment attached to protein L4. In both cases, further digestions were carried out to localize the amino acid and nucleotide at the cross-link point. The two procedures, i.e., identification of the cross-linked peptide/amino acid and identification of the cross-linked RNA fragment/nucleotide, are experimentally unrelated, and they will therefore be described in two separate sections.

(*A*) *Analysis of the Cross-Link Site in Protein L4.* In a similar previous series of experiments (Möller et al., 1978) designed to identify the peptide in protein S7, which is cross-linked to 16S RNA, by ultraviolet irradiation of 30S subunits, the S7–16S RNA complex was freed from non-cross-linked protein by zonal centrifugation on sucrose gradients containing dodecyl sulfate. This complex was digested with nuclease, and the S7–oligonucleotide complex was isolated by chromatography on CM-cellulose. The cross-linked peptide was identified by tryptic digestion, followed by analysis of the tryptic peptides.

None of these procedures was found to be well-suited to the case of cross-linked L4. In the first place, the limited capacity of the zonal rotor necessitated an inconveniently large number of successive runs in order to collect sufficient material for the peptide analyses of L4. Secondly, when a nuclease digest of the L4–23S RNA complex was applied to CM-cellulose, at least 75% of the cross-linked material always became irreversibly bound to the column. Thirdly, the isolated L4–oligonucleotide complex proved to be very insoluble under the conditions required for tryptic digestion.

Accordingly, a different experimental system was devised for each stage. After irradiation of the 50S subunits, the RNA–protein cross-linked complex was isolated by a slight modification of the acetic acid–urea extraction procedure of Hochkeppel et al. (1976). Preliminary experiments showed that the two acetic acid extractions effectively removed all of the non-cross-linked protein and that the RNA was quantitatively precipitated despite the presence of the cross-linked protein. Accordingly, the protein–RNA complex was isolated on a preparative scale, as described under Materials and Methods. After the acetic acid extractions, the precipitated RNA fractions were taken up in dilute buffer and subjected to a total digestion with ribonucleases A and T_1 and then applied to a Sephadex G-75 column in order to separate the L4–oligonucleotide complex. The ^{32}P radioactivity in the column fractions was measured, and the proteins present in each fraction were analyzed by gel electrophoresis.

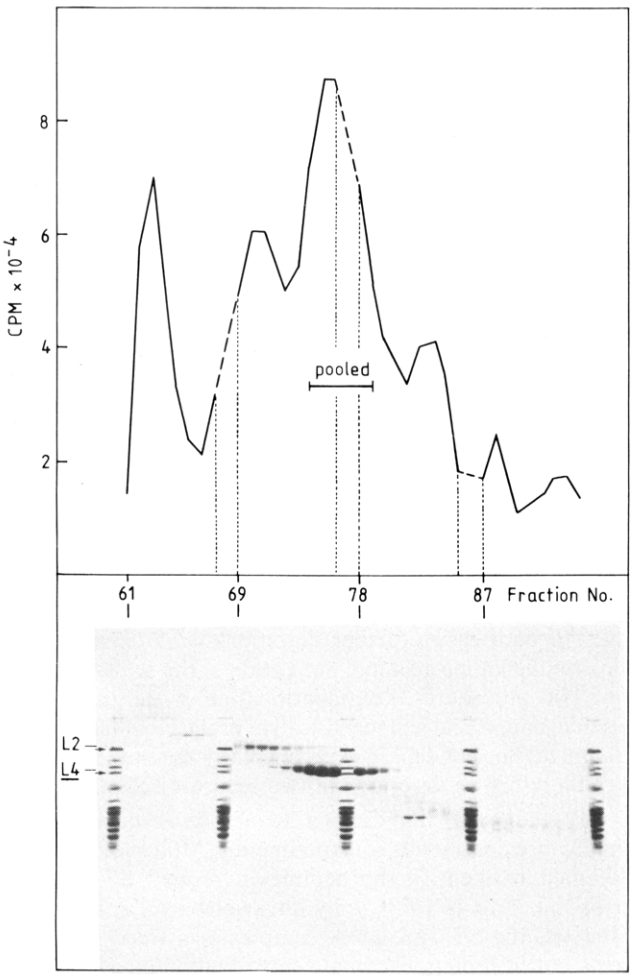

FIGURE 1: Separation of ^{32}P-labeled oligonucleotide–protein cross-linked complexes on Sephadex G-75. The upper half of the figure shows the relevant part of the column elution profile, and the lower half is the dodecyl sulfate gel obtained from aliquots of the corresponding column fractions, interspersed with five control samples of total 50S protein. The direction of electrophoresis is from top to bottom. The interruptions in the elution profile are merely to bring the upper and lower halves of the figure into exact correspondence. The positions of L4 (and L2) in the gel are marked, and the fractions pooled for further analysis of the L4 complex are indicated.

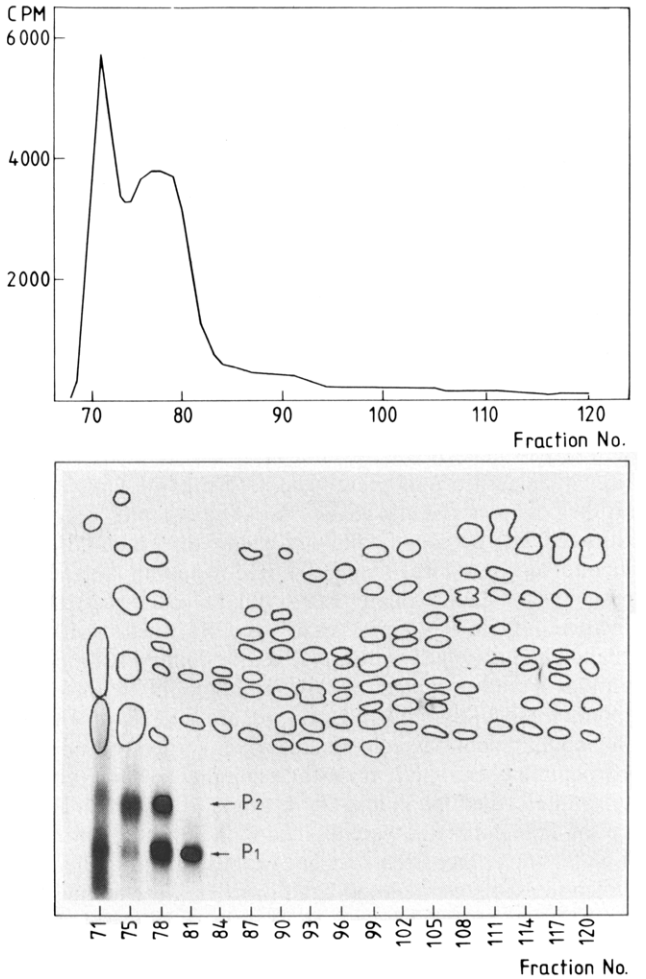

FIGURE 2: Separation of ^{32}P-labeled peptic peptides derived from L4 on Sephadex G-25. The upper half of the figure shows the relevant part of the column elution profile, and the lower half is the autoradiogram from the corresponding thin-layer cellulose chromatogram obtained from aliquots of every third column fraction. The direction of chromatography was from bottom to top. The thin-layer plate was also stained with fluorescamine, and the positions of the stained spots are traced onto the autoradiogram. The radioactive peptides P1 and P2 are indicated.

Figure 1 shows the relevant part of a typical elution profile from such a column, together with the corresponding gel. As expected, the L4–oligonucleotide complex was the major product, but significant amounts of other complexes were also observed, corresponding to L2 and a number of smaller proteins. Autoradiography of the gel showed that ^{32}P radioactivity was associated with the bands corresponding to the L4 complex [which runs slightly slower than protein L4 itself (Möller & Brimacombe, 1975)], thus confirming the presence of the oligonucleotide. Further elution of the column led to the appearance of a large peak of free oligonucleotide material (not shown in Figure 1).

Fractions containing the L4–oligonucleotide complex were pooled as indicated in Figure 1, dialyzed, and lyophilized. The yield of complex at this stage was ~3 mg, representing a yield of ~25% of the total initial amount of L4. The complex was then digested with pepsin, and the digestion products were separated on a Sephadex G-25 column. The column fractions were tested by thin-layer chromatography as described under Materials and Methods, and the thin-layer plates were both autoradiographed and stained with fluorescamine. Figure 2 shows a typical ^{32}P elution profile from this column, together with the corresponding thin-layer plate. It can be seen that the radioactivity is eluted rapidly from the column in two peaks (whose relative intensity varied) and that this radioactivity is associated almost entirely with two peptide products of low R_f value in the thin-layer system, which were weakly stained by fluorescamine. A third very slow-running radioactive product is seen in fraction 71 (Figure 2), corresponding to partially digested or undigested material in the first column peak (see later). The bulk of the peptide products, not attached to oligonucleotides, appeared in the later column fractions, as indicated by the fluorescamine-stained spots. Further elution of the column (Figure 2) did not release any more ^{32}P radioactivity.

The fractions from the G-25 column which contained the two major radioactive products were pooled, and these products, denoted P1 and P2 as indicated in Figure 2, were isolated by preparative thin-layer chromatography. The two oligopeptide–oligonucleotide complexes were extracted from the plates, and aliquots were subjected to further proteolytic digestions as outlined below, the details being given under Materials and Methods. At appropriate stages, the various fractions were subjected to amino acid analysis or to N-terminal sequence analysis by the double-coupling method of Chang et al. (1978). The amino acid analysis data obtained are summarized in Table I, and the partial sequences deduced

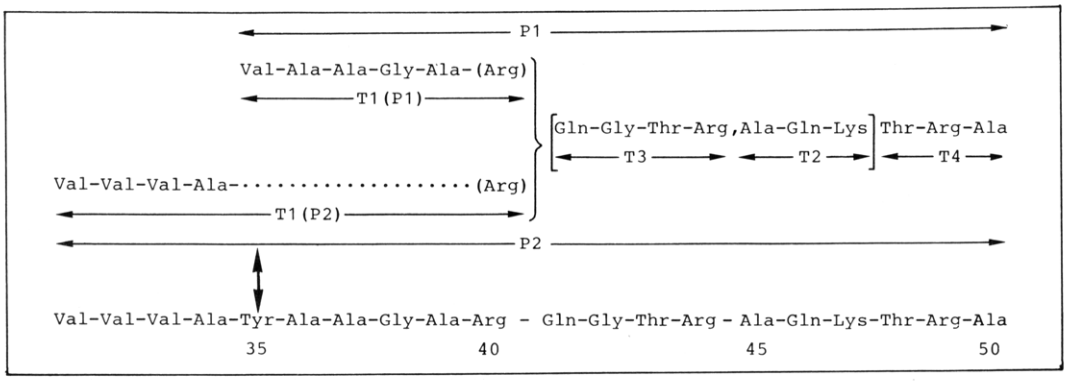

FIGURE 3: Sequence deduced for the cross-link region in protein L4 (peptides P1 and P2). The sequence is divided according to the tryptic digestion products of P1 (above) and P2 (below), with the dots indicating "missing" amino acids (see text for details). The corresponding region of the newly completed L4 sequence is included at the bottom of the diagram (M. Kimura and B. Wittmann-Liebold, personal communication). The valine residue at the beginning of peptide P1 is presumed to be an artifact (see text). The cross-linked tyrosine residue at position 35 is shown by the arrow.

Table I: Amino Acid Compositions[a]

peptide	amino acid composition							
	Thr	Glx	Gly	Ala	Val[b]	Ile*[c]	Lys	Arg
P1	1.8	1.9	3.6[d]	5.3	0.5	1.1	1.2	3.1
P2	2.0	2.3	3.6[d]	5.4	2.6		1.5	2.9
T1 (P1)			2.4[d]	3.0	0.5	0.6		1.0
T1 (P2)			3.0[d]	4.0	1.8			1.0
T2 (P1) / T2 (P2)		1.1		1.0			1.0	
T3 (P1) / T3 (P2)	1.0	1.0	1.1					0.9
T4 (P1) / T4 (P2)	1.0			1.1				1.1
MK (P1)			1.1[d]	1.0		0.6		
MK (P2)			1.9[d]	0.9	2.1			

[a] Values are given in relative moles for each amino acid present; absolute values were of the order of 0.8–1.5 nmol in each case. P1 and P2 are the ^{32}P-containing peptides (Figure 2). T1(P1) etc. are the tryptic peptides isolated from P1 and P2 (Figure 4), T1 containing the radioactivity. MK(P1) and MK(P2) are the ^{32}P-containing peptides isolated by digestion with aminopeptidase M and proteinase K. See text for detail. [b] Values found for Val tend to be low (see text). [c] Ile* is the unknown product found near to Ile in the amino acid analysis. [d] Ca. 1.5 mol of glycine arises from the oligonucleotide moiety (see text).

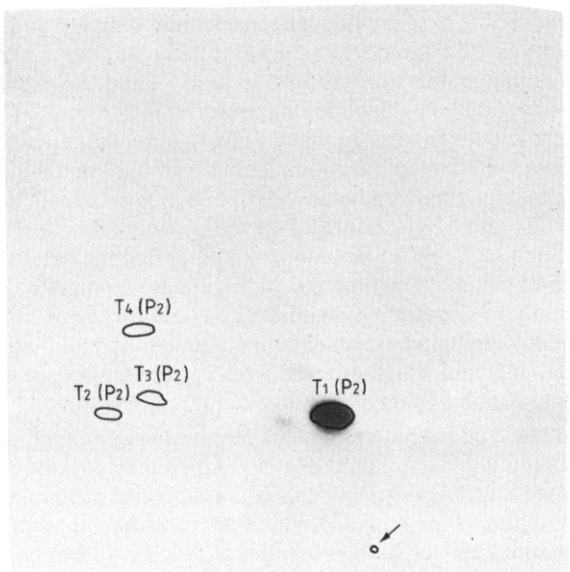

FIGURE 4: Two-dimensional peptide map obtained after tryptic digestion of peptide P2. The first dimension is electrophoresis (cathode left and anode right), and the second dimension is chromatography (bottom to top). The sample application point is indicated by the arrow. The figure is an autoradiogram of the thin-layer plate, with the positions of the fluorescamine-stained spots traced onto it. The positions of peptides T1–T4(P2) are indicated.

for peptides P1 and P2 are given in Figure 3. Shortly before this paper was accepted for publication, the amino acid sequence of protein L4 was completed, and the relevant region of this sequence is included in Figure 3 (M. Kimura and B. Wittmann-Liebold, personal communication). In the following section we present our results as they were found and interpret them in the light of these new sequence data.

First, the amino acid analyses of the undigested peptides P1 and P2 showed that they were closely related. The peptides were present in approximately equimolar amounts, and each consisted of 16–20 amino acids, the compositions differing only in that P2 had a higher valine content whereas P1 contained the amino acid denoted as "Ile*" (Table I). The latter appeared reproducibly in the amino acid analysis profile as a rather broad peak slightly displaced from the isoleucine peak in the direction of leucine, and its identity is not known. Such a peak could arise from an unhydrolyzed Ile–Ile dimer, but this can be ruled out in this instance, since there is no Ile–Ile in the relevant part of the L4 sequence. N-Terminal sequence analysis of the two peptides gave Val-Ala-Ala-Gly-Ala- for P1 and Val-Val-Val-Ala- for P2 (Figure 3). In both cases the stepwise degradation process broke down reproducibly at these points (see the discussion below). We were not able to make a satisfactory correlation of the loss of ^{32}P radioactivity with the individual amino acids released during the sequencing process, possibly as a result of the vigorous chemical reactions involved.

Next, aliquots of P1 and P2 were subjected to trypsin digestion and the products separated by the standard two-dimensional peptide mapping procedure (Hitz et al., 1977) on cellulose thin-layer plates. The plates were stained with fluorescamine and also autoradiographed. The two peptides gave almost identical tryptic maps, and a typical example (from P2) is illustrated in Figure 4. Only one of the tryptic digestion products contained ^{32}P radioactivity in each case, whereas three nonradioactive peptides were found. These are denoted T1(P1 or P2) etc., as indicated in Figure 4. Amino acid and sequence analyses showed that the three nonradioactive peptides derived from P1 and P2 were identical (Table I and Figure 3). Peptide T2 had the sequence Ala-Gln-Lys, T3 was Gln-Gly-Thr-Arg, and T4 was Thr-Arg-Ala. It is clear that our tryptic peptide T4 must lie at the C terminus of the peptic peptides P1 and P2, since it has alanine and not

lysine or arginine as its C-terminal amino acid, and comparison with the relevant section of the L4 sequence (Figure 3) shows that the order of these peptides is unambiguously T3, T2, T4.

The amino acid analyses of the radioactive tryptic peptides T1(P1) and T1(P2) differed from each other in precisely the same way as those of the parent peptides P1 and P2, namely, with respect to their valine and Ile* content. The values found for valine in the case of peptides derived from P2 were sometimes rather low (Table I), but this is most probably a result of the well-known resistance of valine–valine linkages to acidic hydrolysis. N-Terminal sequence analysis of the two radioactive peptides T1(P1 and P2) also gave the same results as the corresponding analyses of P1 and P2, again with a breakdown of the sequencing process at precisely the same points (see below). Clearly, these radioactive tryptic peptides T1(P1 and P2) must contain the cross-link site(s) and must lie at the N termini of P1 and P2 (Figure 3).

Two final experiments were made in order to obtain even shorter cross-linked peptide–oligonucleotide complexes. First, aliquots of T1(P1 and P2) were subjected to a total digestion with aminopeptidase M and proteinase K, and the products were separated on a peptide fingerprint as in Figure 4. These two enzymes were chosen for this purpose instead of Pronase, because they are free from nucleases and phosphatases. In each case, a single radioactive peptide was found, denoted MK(P1) and MK(P2), which gave the amino acid analyses indicated in Table I. Second, the undigested material from the G-25 column (fractions 69–74, Figure 2) was digested with thermolysin and analyzed similarly. Here, three radioactive peptides were found, which gave analyses corresponding to that of MK(P2) and which differed from each other only in their glycine content (data not shown).

It should be noted here that, as observed in our experiments with protein S7 (Möller et al., 1978), glycine can arise from hydrolysis of oligonucleotides. A more precise measurement showed that 1 mol of adenylic acid (and no other mononucleotide) gives rise to ~0.7 mol of glycine under the conditions of the amino acid analysis (data not shown), and, since the oligonucleotide attached to protein L4 after total digestion with ribonucleases A and T_1 is A-A-U (see next section), it follows that ~1.5 mol of glycine in the amino acid analyses of all the radioactive peptides arises from the nucleotide moiety.

If these data are compared with the appropriate region of the L4 sequence (Figure 3), it can be seen immediately that all the results obtained with peptide P2 or its derivatives [T1(P2) and MK(P2)] are consistent with a cross-link involving the tyrosine at position 35. The N-terminal sequencing procedure breaks down at the alanine residue preceding this tyrosine, and no tyrosine was found in any of the amino acid analyses from these peptides. In experiments with pUp cross-linked to ribonuclease by ultraviolet irradiation (Havron & Sperling, 1977), serine, isoleucine, and threonine were found to be cross-linked to the nucleotide, and, here also, the specific residues concerned "disappeared" in the amino acid analysis. Similarly, in the case of protein S7, a methionine residue was identified at the cross-link site (Möller et al., 1978), since the expected methionine in the sequence (Reinbolt et al., 1978) was never found in amino acid analyses of the cross-linked peptide.

In the case of the peptides derived from P1, the breakdown of the N-terminal sequencing procedure after the alanine residue (position 39) can be explained on the grounds that the next amino acid is arginine, which is difficult to identify by the procedure of Chang et al. (1978). However, the N-terminal amino acid of P1 and T1 (P1) was identified as valine, although tyrosine would have been expected at this position (Figure 3), and further the amino acid analyses showed the anomalous amino acid Ile* (Table I). In the shortest peptide, MK(P1), only alanine and Ile* were found, apart from the glycine arising from the oligonucleotide moiety. The simplest explanation for these findings is that in P1 and its derivatives the chemistry of the cross-link is different from that in P2, resulting in Ile* as a breakdown product of the cross-linked tyrosine in the amino acid analysis and also allowing the N-terminal sequencing procedure to carry on past the cross-link point.

The finding of valine at the N terminus of P1 and T1(P1) is not consistent, but it is possible that this valine residue was an artifact, analogous to Ile*, arising from the breakdown of the cross-linked tyrosine. It is noteworthy that the derivatives of valine and isoleucine have similar running positions in the system of Chang et al. (1978). Whatever the correct explanation for this discrepancy may be, the clear absence of the expected tyrosine from the analyses of peptides derived from P1 as well as from P2 indicates that tyrosine (position 35) is the cross-linked amino acid in both instances.

(*B*) *Analysis of the Cross-Link Site in 23S RNA.* In the corresponding series of experiments to identify the nucleotide in 16S RNA which is cross-linked to protein S7 by irradiation of 30S subunits, a number of stepwise degradations of the cross-linked RNA–protein complex were made, which involved the use of three separate systems. First, a preliminary localization of the cross-link site was made with the help of a nonionic detergent gel system to separate large cross-linked complexes from fragments of free RNA (Zwieb & Brimacombe, 1978). Next, RNA–protein complexes of intermediate length were isolated on dodecyl sulfate gels, for precise determination of the ribonuclease T1 oligonucleotide containing the cross-link site (Zwieb & Brimacombe, 1979), and, finally, a series of digestion procedures was employed to localize the cross-linked nucleotide itself (Möller et al., 1978; Zwieb & Brimacombe, 1979). In the experiments to be described here, these systems are combined into a single, coherent experimental procedure, centered on a two-dimensional gel electrophoresis step for the separation of heterogeneous mixtures of cross-linked RNA–protein complexes.

Highly radioactive ^{32}P-labeled 50S subunits were irradiated and were then freed from non-cross-linked protein on a dodecyl sulfate–sucrose gradient, as described under Materials and Methods. The isolated RNA–protein cross-linked complexes were taken up into a buffer containing the nonionic detergent Triton X-100, subjected to a mild digestion with ribonuclease A, and immediately applied to the two-dimensional polyacrylamide gel system. The first dimension of this system was a slightly modified version of the Triton X-100 gel system previously published (Zwieb & Brimacombe, 1978), and, in addition, long thin slab gels were used in order to improve the resolution. After electrophoresis, the gels were autoradiographed, and the upper one-third of the gel strips containing the samples were polymerized onto the top of further slab gels for the second dimension, which was a 13% gel containing dodecyl sulfate as described by Laemmli & Favre (1973). After this second electrophoresis step, the gels were again autoradiographed. Details of the procedure are given under Materials and Methods, and the principle of the separation is illustrated in Figure 5a.

In both gel dimensions, free RNA fragments run toward the anode and are separated on a size basis alone. These fragments therefore give rise to a "diagonal" in the two-di-

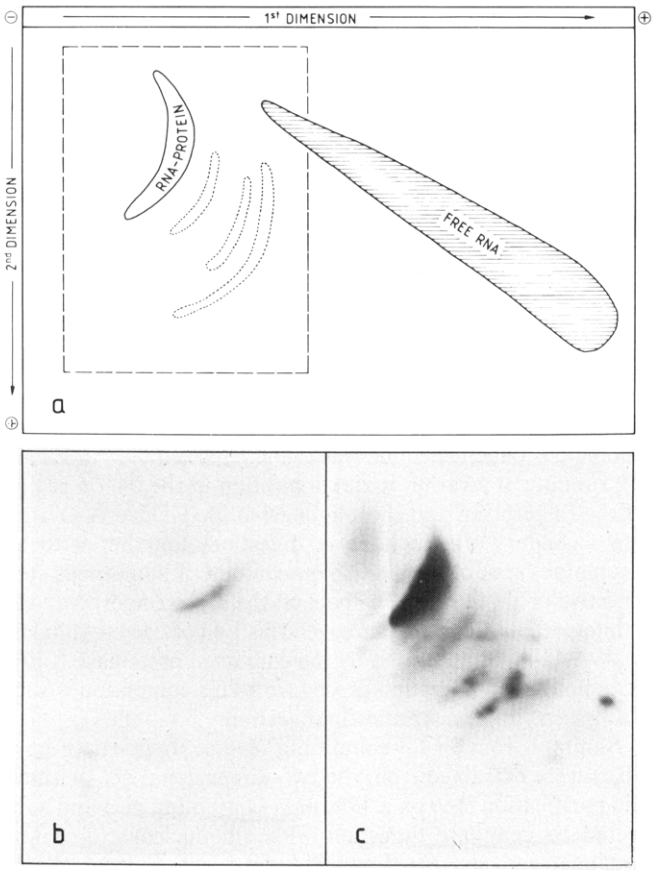

FIGURE 5: Two-dimensional gel separation of cross-linked RNA–protein complexes after partial digestion with ribonuclease A. The first dimension (in Triton X-100) is from left to right; the second (in dodecyl sulfate) is from top to bottom. (a) Sketch showing the principle of the separation, with free RNA forming a diagonal and the RNA–protein complexes forming reverse diagonals (see text). (b) Autoradiogram showing a reverse diagonal containing protein L4; the photograph corresponds to the boxed-in region of (a). (c) As for (b), in this case from a slightly milder nuclease digestion and with a longer autoradiographic exposure to show additional reverse diagonals corresponding to proteins other than L4.

mensional system, which contains the bulk of the radioactive material (Figure 5a). Cross-linked RNA–protein complexes, however, behave differently. In the first dimension, the separation in the presence of the nonionic detergent is on a charge basis, and the cross-linked proteins require an attached RNA fragment of a certain minimum length before they will move into the gel at all. As the RNA fragment becomes larger, so the complex moves farther into the gel. This system was shown previously to be capable of causing complete deproteinization of non-cross-linked ribonucleoprotein particles (Zwieb & Brimacombe, 1978), and it follows that the formation of spurious RNA–protein interactions during the electrophoresis can be ruled out. In the second dimension, the proteins in the cross-linked complexes become negatively charged with dodecyl sulfate, and the separation is based simply on size, so that the complexes containing the longer RNA fragments now move more slowly. The result is that cross-linked RNA–protein complexes appear on the two-dimensional gel as curved "reverse diagonals", running roughly at right angles to the "normal" diagonal formed by the free RNA fragments (Figure 5a).

Parts b and c of Figure 5 give two examples of the experimental results obtained in the case of RNA–protein complexes from irradiated 50S subunits. Figure 5b shows a lightly exposed autoradiogram in which a single "reverse diagonal" can be seen, which will be shown below to contain the cross-linked

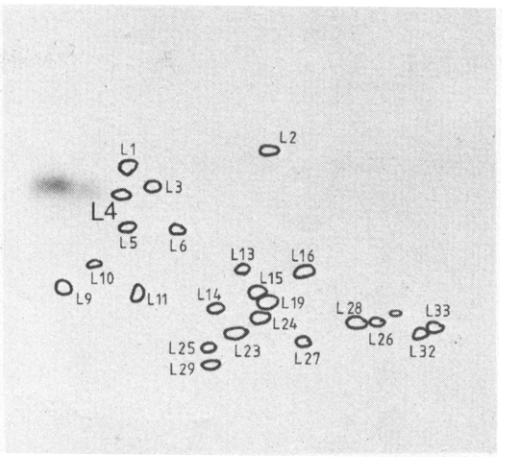

FIGURE 6: Two-dimensional protein gel analysis of an L4–oligonucleotide complex. The gel system is that of Mets & Bogorad (1974), with the direction of electrophoresis from left to right (first dimension) and top to bottom (second dimension). The sample consisted of an L4–RNA ^{32}P-labeled complex, digested with ribonuclease A and T_1 and applied to the gel together with unlabeled 50S protein. The figure is the autoradiogram from the gel, with the positions of the stained proteins [numbered according to Kyriakopoulos & Subramanian (1977)] traced onto it.

L4. In Figure 5c the corresponding autoradiogram was deliberately overexposed, so that, in addition to the strong reverse diagonal due to protein L4, a number of other well-separated reverse diagonals can be seen, arising from cross-linked proteins which were minor products of the irradiation process. In this case the extent of the ribonuclease digestion was somewhat less than in the example of Figure 5b, and the RNA fragments attached to L4 are noticeably longer. Ribonuclease A was found to give more reproducible results than ribonuclease T_1 in this system, but, nevertheless, in each experiment two or three samples were usually run with different concentrations of ribonuclease A, in order to guarantee that at least one sample would yield a reverse diagonal containing a spectrum of L4–RNA complexes of suitable size for the subsequent analyses. In control gels of ribonuclease-treated but nonirradiated samples, no reverse diagonals were seen.

The reverse diagonal suspected of containing protein L4 was cut out from the gel and divided into three or four pieces along its length. As a further purification step, these gel pieces were loaded directly onto a 17% slab gel, containing dodecyl sulfate and urea. After electrophoresis the gel was autoradiographed, and the presumed L4–RNA complexes were excised; the impurities appeared in each case as a faint smear running in front of the relatively sharp protein–RNA band [data not shown; cf. Zwieb & Brimacombe (1979)]. The protein–RNA complexes were extracted from the gel slices and each sample was split into two, one aliquot being used to demonstrate the presence of L4 and the other to analyze the RNA sequence. Details of the procedures are given under Materials and Methods. The aliquots for L4 analysis were mixed with unlabeled 50S subunits, digested with ribonucleases A and T_1, and applied to two-dimensional gels which were subsequently stained for protein and autoradiographed. A typical example is illustrated in Figure 6, which clearly shows a single radioactive spot running in the expected position, just to the left of protein L4. This spot is the fully digested L4–oligonucleotide complex [cf. Möller & Brimacombe (1975)] and was found as the sole radioactive product in all the samples from the reverse diagonals corresponding to L4 (Figure 5b,c).

The sample aliquots for RNA sequence analysis were treated with proteinase K and then extracted with phenol. The

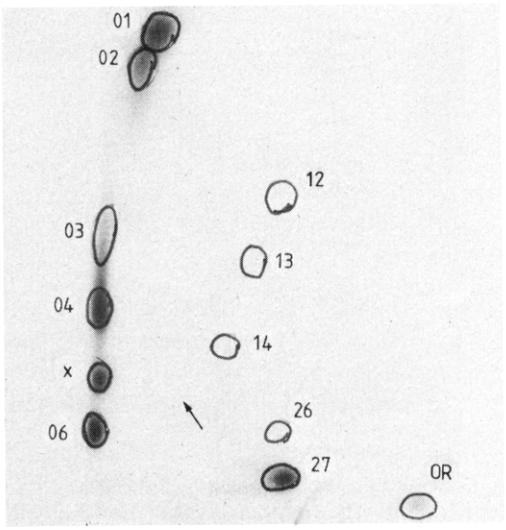

FIGURE 7: Fingerprint of an L4–RNA cross-linked complex. The two-dimensional thin-layer poly(ethylenimine) plate was run in the mini-fingerprint system of Volckaert & Fiers (1977), with the first dimension from right to left and the second from bottom to top. The sample was a proteinase K/ribonuclease T_1 digest of a ^{32}P-labeled L4–RNA complex, isolated as described in the text. The spots on the autoradiogram are numbered by using the system of Uchida et al. (1974), the principle components corresponding to G (01), A-C-A-G (04), A-A-A-C-C-G (06), U-U-A-A-C-C-G (27), and the oligonucleotide–peptide complex (X, see text). The minor components (12, 13, 14, 26, and the spot OR at the origin) did not contribute significant amounts of radioactivity in the analysis. The arrow shows the position where the missing oligonucleotide A-A-U-A-G would be expected to appear.

aqueous phase, which still contained all the ^{32}P radioactivity, was digested with ribonuclease T_1, and the oligonucleotides released were fingerprinted, a typical example being shown in Figure 7. Each oligonucleotide from the fingerprint was subjected to a secondary digestion with ribonuclease A, the products being separated in the double-digestion system of Volckaert & Fiers (1977). The combination of these two systems is ideal for the analysis of T_1–oligonucleotides from relatively short RNA fragments, particularly when the level of radioactivity is rather low.

The fingerprint analyses for the various segments of each reverse diagonal (Figure 5b,c) and from all the different experiments were combined and compared with the sequence of 23S RNA, which has recently become available (Brosius et al., 1980). The results are summarized in Figure 8. All the fragments arose from the same region of 23S RNA, and all contained the characteristic oligonucleotides U-U-A-A-C-C-G (27) and A-A-A-C-C-G (06). The 5′ and 3′ ends of the fragments could not usually be determined very precisely, since by the nature of the experiment each fragment sample taken for fingerprinting was heterogeneous, as a result of the initial ribonuclease A treatment of the L4–23S RNA complex. For this reason, the ends of each fragment in Figure 8 are only given to the nearest T_1–oligonucleotide which was definitely present. However, the important feature of the analyses is that, despite the presence of oligonucleotides 27 and 06, the intervening characteristic oligonucleotide A-A-U-A-G (15) was never found; an arrow in Figure 7 indicates the position where this oligonucleotide would be expected to appear. Instead, an anomalous oligonucleotide was found (marked "X" in Figure 7), running at a rather variable position in the 04–06 region of the fingerprint, and this oligonucleotide yielded A-G after the secondary ribonuclease A digestion, together with an anomalous spot or spots. Oligonucleotide X sometimes split into two or three smeared spots on the same fingerprint and is interpreted as being a heterogeneous L4 peptide cross-linked to A-A-U-A-G, generated by the combined proteinase K and ribonuclease T_1 digestion procedure. This contention is substantiated in the next and final section.

Samples from an L4-containing reverse diagonal (Figure 5b,c) were extracted from the two-dimensional gel (without the purification step on a 17% urea-containing gel) and subjected to complete digestion with ribonuclease T_1. The products were separated on a standard 15% dodecyl sulfate gel (Laemmli & Favre, 1973). This gel was autoradiographed and a sample of total 50S protein from an adjacent slot in the same gel was stained for protein. The result (Figure 9) showed a strong band of ^{32}P radioactivity corresponding to the T_1–oligonucleotide–L4 complex, together with a fast-running band of free oligonucleotides. The L4–oligonucleotide was extracted from the gel and subjected to ribonuclease A digestion, either with or without a subsequent proteinase K digestion [see Materials and Methods and Zwieb & Brimacombe (1979)]. The digestion products were analyzed on poly(ethylenimine) plates, giving the results shown in Figure 10a,b. In Figure 10a, it can be seen that the sample (not treated with proteinase K) gives A-G, together with a spot remaining at the origin. In Figure 10b, the sample (with proteinase treatment) also yields A-G, but the origin spot is replaced by a series of

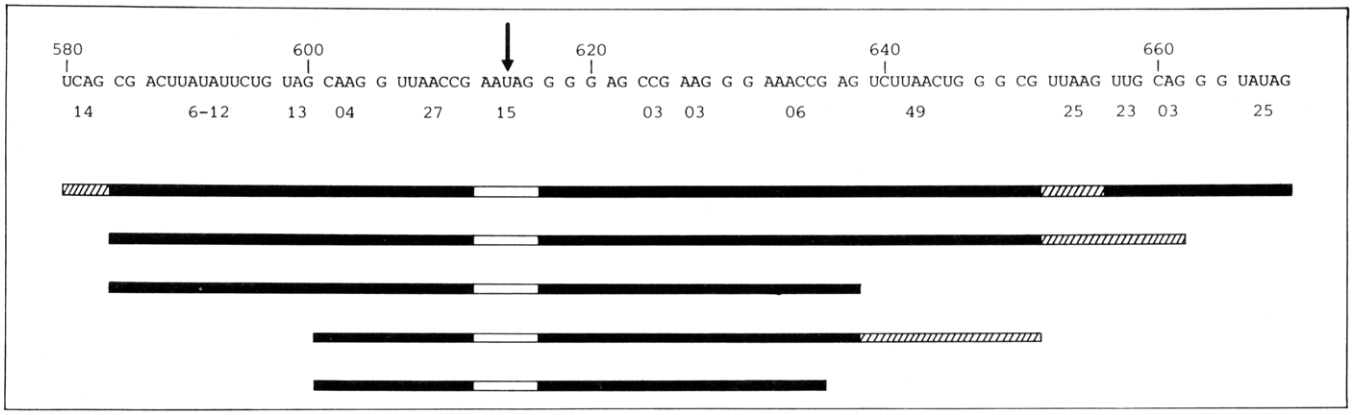

FIGURE 8: Part of the sequence of 23S RNA encompassing the cross-link site to protein L4. The sequence (Brosius et al., 1980) is divided into T_1–oligonucleotides, numbered as in Figure 7 (Uchida et al., 1974). The horizontal bars indicate the results of various fragment analyses, the solid areas indicating oligonucleotides present in molar amounts and the crosshatched areas those present in submolar amounts. A-A-U-A-G was not found in any analysis, and the arrow indicates the site of cross-linking to L4 (see text). The shortest fragment (positions 602–636) is the one whose fingerprint is shown in Figure 7. The large oligonucleotides 6–12 and 49 did not move from the origin of the fingerprints (cf. Figure 7) but could be readily distinguished by the subsequent ribonuclease A digestion. C-A-A-G (positions 602–605) did not appear in our analyses; instead, A-C-A-G was always found.

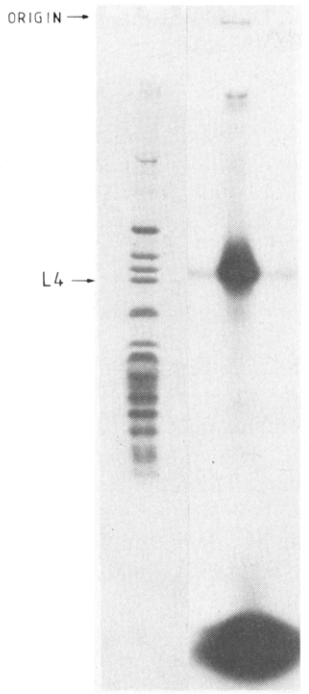

FIGURE 9: Isolation of the L4–oligonucleotide complex on a dodecyl sulfate gel. A ^{32}P-labeled L4–RNA complex was fully digested with ribonuclease T_1 and applied to the gel. The figure shows (left) a stained slot from the same gel containing total 50S protein and (right) the autoradiogram of the ribonuclease T_1 digest. The position of protein L4 is marked.

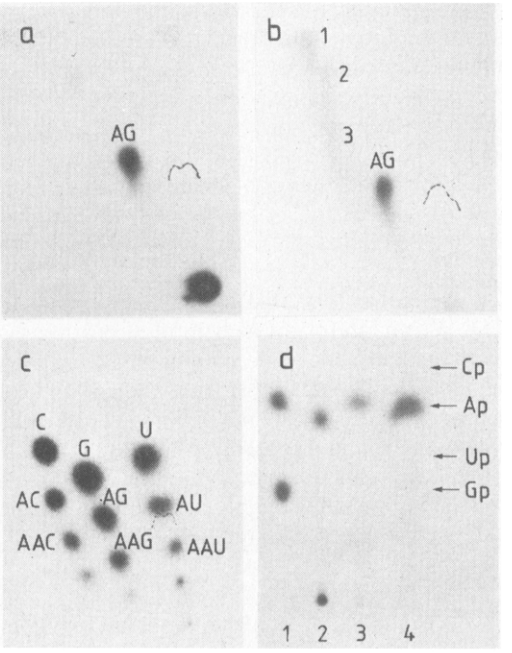

FIGURE 10: Secondary and tertiary digestions of the L4–oligonucleotide complex from Figure 9. (a), (b), and (c) are two-dimensional separations on poly(ethylenimine) plates, in the double-digestion system of Volckaert & Fiers (1977). The first dimension is from right to left and second dimension from bottom to top. The position of the dye marker is indicated (dotted line). (a) Complex digested with ribonuclease A. (b) Complex digested with ribonuclease A and then with proteinase K. (c) Control digest indicating the positions of the ribonuclease A products. (d) One-dimensional separation of ribonuclease T_2 digests in the system of Bernardi (1974). Positions of the four mononucleotides are indicated. (Sample 1) Digest of the A-G spot from (a). (Sample 2) Digest of the origin spot from (a). (Sample 3) Same as for sample 2 but with a proteinase K digestion in addition. (Sample 4) Digest of spot 3 from (b). The smeared spot due to the peptide–nucleotide complex in samples 3 and 4 is off the top of the diagram.

fast-running smeared spots; this was precisely the pattern obtained from ribonuclease A digests of the anomalous oligonucleotide X (Figure 7). It follows that the oligonucleotide remaining attached to the protein or peptide at this stage should be A-A-U.

All the spots were extracted from the thin-layer plates (Figure 10a,b) and were subjected to a final digestion with ribonuclease T_2. In addition, an aliquot of the spot from the origin in Figure 10a was treated with proteinase K as well as with ribonuclease T_2. The mononucleotides released were separated on the one-dimensional system of Bernardi (1974), some examples being shown in Figure 10d. It was confirmed that the A-G spot from Figure 10a,b does indeed liberate Ap and Gp (e.g., sample 1, Figure 10d), and, since this A-G was released by ribonuclease A, it must correspond to the A-G at positions 616 and 617 in the sequence (Figure 8) and not to the A-G at positions 621 and 622. (The latter might have been suspected if ribonuclease T_1 digestion near the cross-link site was not complete.) All the other spots from Figure 10a,b release Ap in the final ribonuclease T_2 digestion (see examples in Figure 10d), either together with faint fast-running smeared spots corresponding to peptide-containing material (not shown in Figure 10d) or together with a spot at the origin corresponding to intact protein (sample 2, Figure 10b) in the case where no proteinase treatment had been made. No trace of Up or any mononucleotide other than Ap was found in these digests. In each case, the radioactivity in Ap and the radioactivity in the peptide- or protein-containing spots were measured, giving ratios which lay between 1.5:1 and 2.5:1 (Ap/protein- or peptide-containing material), in the various experiments. These data establish unambiguously that the T_1–oligonucleotide–L4 complex contains the missing A-A-U-A-G (Figure 8) which liberates A-G and 2A by successive digestions with ribonuclease A and T_2. The site of cross-linking to protein L4 is therefore the uridine residue in this oligonucleotide A-A-U-A-G, at position 615 in the 23S RNA sequence.

Conclusions

The experiments described here provide the first direct analysis of an RNA–protein contact point within the 50S subunit. The cross-link site on protein L4 involves a tyrosine residue, whereas methionine was the amino acid concerned in the corresponding cross-link between protein S7 and 16S RNA in the 30S subunit (Möller et al., 1978; Zwieb & Brimacombe, 1979). No obvious similarity between the amino acid sequences in the vicinity of the cross-link can be observed in the two cases, but, on the other hand, the RNA sequences concerned are very similar. In both instances, a uridine residue is involved, contained in the sequence A-A-U-G-G in 16S RNA and in the sequence A-A-U-A-G in 23S RNA. In the 16S RNA case, experiments on the secondary structure of this region of the RNA [cf. Ross & Brimacombe (1979)] indicate that at least the A-A-U sequence is not involved in base pairing (C. Glotz and R. Brimacombe, unpublished experiments). It seems reasonable that an RNA sequence would be the determining factor in the ultraviolet-induced cross-linking reaction, at the wavelength (254 nm) used, although intercalation of the cross-linked L4 tyrosine residue may also be an important factor.

From the methodological standpoint, we believe that the systems described here provide a practical solution to the problem of analysis of multiple cross-linking sites induced by bifunctional chemical cross-linkers. As stated in the introduction, a number of such bifunctional RNA–protein reagents

are already available for the ribosome, and we are in the process of applying our methodology to the analysis of the cross-linking sites concerned. Preliminary localizations have so far been made for some sites in 30S ribosomal proteins cross-linked to RNA with bis(2-chloroethyl)amine (Ulmer et al., 1978), as well as for sites on 16S RNA cross-linked to protein with the imido ester of *p*-azidophenylacetic acid (Rinke et al., 1980).

Acknowledgments

We are grateful to Drs. M. Kimura, B. Wittmann-Liebold, and H. G. Wittmann for much helpful discussion as well as for making their sequence data on protein L4 available to us. Similarly, we thank Drs. J. Brosius and H. F. Noller for making their sequence of 23S RNA available. We also thank V. Nowotny for running the poly(U) assays, Dr. L. Mende and M. Montero for their help with the amino acid sequencing, and M. Meinke for her valuable assistance with the RNA analyses.

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