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RNA-protein cross-linking in Escherichia coli 30S ribosomal subunits: precise localisation of the nucleotide in 16S RNA which is coupled to protein S7 by ultraviolet irradiation

Christian Zwieb and Richard Brimacombe

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

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ABSTRACT

It is well established that when E. coli 30S ribosomal subunits are irradiated with ultraviolet light under mild conditions a specific cross-link is formed between protein S7 and the 16S RNA. Methodology is presented for the analysis of the single nucleotide residue concerned in this cross-link. Firstly, the identity of the ribonuclease T_1 octanucleotide attached to S7 is confirmed by a new method, which involves isolation and analysis of S7-polynucleotide complexes containing 30 - 40 nucleotides. Secondly, the isolated S7-octanucleotide complex is digested successively with ribonuclease A, proteinase K and ribonuclease T_2, and the nucleotides liberated are identified. The results show unambiguously that uridine residue number 1239 in the 16S RNA sequence is cross-linked to protein S7.

INTRODUCTION

A number of RNA-protein cross-linking studies (1-6) have demonstrated that most if not all of the E. coli ribosomal proteins are capable of being linked to their respective ribosomal RNA molecules within the subunits. It follows that some regions of all the proteins concerned are either in close neighbourhood to, or in direct contact with, specific regions of the ribosomal RNA. However, as we have stressed in recent publications (4,7,8), these cross-linking studies have little ultimate value in gaining an understanding of the topography of protein and RNA within the ribosomal particles, unless the sites of cross-linking on protein and RNA are determined. Up to now the precise site of cross-linking on the RNA has only been inferred in one special case, namely the cross-linking of proteins to the 3'-terminus of 16S RNA via periodate oxidation of the 3'-terminal ribose, followed by Schiff's base formation with protein amino groups and subsequent stabilisation by borohydride reduction (2).

Serious doubt has however recently been cast on the validity of this method of cross-linking (9), largely because it has not been demonstrated that, under the experimental conditions used, the cross-linking takes place exclusively at the 3'-terminus of the RNA.

Our work has been concerned both with the development of general methods of RNA-protein cross-linking (3,4,6), and with the development of methodology for the analysis of sites of cross-linking on both protein and RNA (7,8). For the latter purpose we have made use of a simple cross-linking system, involving direct irradiation of the 30S subunit with ultraviolet light; under suitably mild conditions, only a single protein, S7, is cross-linked to the 16S RNA to a significant extent by this treatment (1). We have published a method for the direct analysis of the regions of RNA concerned in the cross-link (7), in which free RNA was separated from RNA linked to protein on a gel system containing nonionic detergent. From these experiments, the site of cross-linkage to S7 could be tentatively identified as lying within a specific ribonuclease T_1 octanucleotide in 16S RNA, and in a subsequent publication evidence was produced in support of this contention, by analysing the nucleotides which could be released from the S7-octanucleotide cross-linked complex by treatment with ribonuclease A (8). In the same paper, the site of cross-linking on S7 was determined as lying within a short peptide Ser-Met-Ala-Leu-Arg (positions 113 - 117 in the protein sequence), with methionine as the probable amino acid concerned.

In this paper, we describe the final precise localisation of the nucleotide in 16S RNA which is cross-linked to protein S7. The analysis has been considerably helped by the publication of an unambiguous sequence for the 16S RNA (10,11), and the experiments are divided into two parts. Firstly, the identity of the octanucleotide containing the cross-link site was positively proven, by isolating and analysing S7-polynucleotide complexes containing 30 to 40 nucleotides of RNA. Secondly the identity of the nucleotide at the cross-link site was determined by successive digestion of the S7-octanucleotide complex with ribonuclease A, proteinase K and ribonuclease T_2. The results in-

dicate clearly that a single uridine residue (at position 1239 in the 16S sequence) is cross-linked to protein S7, under the experimental conditions used.

MATERIALS AND METHODS

Preparation of 16S RNA from irradiated 30S subunits

Ammonium chloride-washed 30S subunits from E. coli strain MRE600, either unlabelled or labelled with ^{32}P in the 16S RNA moiety, were prepared as previously described (12,13). In some experiments the subunits were subjected to an activation dialysis overnight against 400 mM ammonium chloride, 20 mM magnesium acetate, 6 mM 2-mercaptoethanol, 20 mM Tris-HCl pH 7.8 (14); this dialysis alone (without heat treatment) is capable of restoring full activity to the subunits (G. Sieber and K. H. Nierhaus, personal communication). It was followed by a further dialysis against several changes of irradiation buffer, viz. 50 mM potassium chloride, 5 mM magnesium acetate, 10 mM Tris-HCl pH 7.8.

In order to generate the S7-16S RNA cross-link, the subunits were irradiated for 15 min with ultraviolet light exactly as previously described (1,8), and were then applied to 7.5 - 30% sucrose gradients containing dodecyl sulphate in an SW 25.1 rotor to separate non-cross-linked protein, again as described (8). Gradient fractions containing 16S RNA together with the S7-16S RNA cross-linked complex were pooled, precipitated with 2 vol. ethanol at -20°C in the presence of 100 mM sodium acetate, and dissolved in 0.05% dodecyl sulphate, 1 mM EDTA, 6 mM 2-mercaptoethanol, 10 mM Tris-HCl pH 7.8.

Partial digestion of 16S RNA containing cross-linked S7

In preliminary experiments, 6 - 7 A_{260} unit aliquots of unlabelled 16S RNA (from irradiated or unirradiated 30S subunits) in 50 µl of dodecyl sulphate buffer (see above) were digested at 37°C for 30 min with various amounts of ribonuclease T_1 (0.16 - 80 enzyme units per A_{260} unit of RNA). At the end of the incubation 10 µl of 55% trichloracetic acid was added, and the precipitate of RNA-protein complex together with large oligo- or polynucleotides was separated by centrifugation. The pellets were dissolved in dodecyl sulphate buffer, and applied to 15%

polyacrylamide slab gels (35 cm long) using the dodecyl sulphate system of Laemmli and Favre (15). 30S proteins were run in adjacent slots of the same gel, and, after electrophoresis, the bands were visualised by staining with amido black. Having found suitable digestion conditions in this manner, the experiment was repeated using ^{32}P-labelled 16S RNA from irradiated and unirradiated subunits, the bands on the gel being visualised in this case by autoradiography, and also by staining of a 30S protein control sample run on the same gel.

Bands suspected to contain S7-RNA cross-linked complexes were excised from the gel and were purified by loading the gel slices directly onto a 12% polyacrylamide gel containing dodecyl sulphate and urea, in the buffer system described in ref. 16. The bands were localised by autoradiography, and the RNA or RNA-protein complexes were extracted into dodecyl sulphate buffer as described (7). One third of each sample was analysed for the presence of S7 by incubating for 30 min at 37°C with excess ribonuclease T_1 (750 units enzyme per A_{260} unit in the original sample (8)), and applying the digested sample to a 15% Laemmli-Favre gel (15) as above, together with total 30S protein. After electrophoresis, the gel was stained, then cut into 5 mm slices and analysed for ^{32}P-radioactivity. The S7-oligonucleotide complex appears as a clean ^{32}P-labelled band, running slightly slower than protein S7 in the gel (1,7). The remaining two-thirds of the samples thus shown to contain cross-linked S7 were digested with ribonuclease T_1 and subjected to oligonucleotide analysis using the method of Sanger et al. (17), as described (13). Secondary digests of the ribonuclease T_1 oligonucleotides isolated from the fingerprints were made with ribonuclease A as described (7), the products being analysed on the two-dimensional thin-layer system of Volckaert and Fiers (18).

Analysis of cross-linked S7-oligonucleotide complexes

^{32}P-labelled 16S RNA containing cross-linked protein S7 was fully digested with ribonuclease T_1, and the S7-octanucleotide complex was isolated and purified by two-dimensional chromatography on polyethyleneimine thin-layer plates (18), exactly as described (8) with the exception that the complex was trans-

ferred onto the plates in the presence of 0.05% dodecyl sulphate. The complex was eluted from the plates with 80% formic acid and lyophilized, and was then subjected to further digestion with either ribonuclease A, ribonuclease T_2 or proteinase K as follows. All digestions were made in 50 µl buffer, in the presence of 5 µg of carrier RNA. Ribonuclease A digestions were made with 5 µg of enzyme in 0.05% dodecyl sulphate, 1 mM EDTA, 6 mM 2-mercaptoethanol, 10 mM Tris-HCl pH 7.8 for 30 min at 37°C. Ribonuclease T_2 digestions were made similarly, using 1 unit of ribonuclease T_2 (from Sigma), but with 50 mM ammonium acetate pH 4.5 in place of Tris buffer. Proteinase K digestions were made in 0.25% dodecyl sulphate, 10 mM magnesium acetate, 20 mM NaCl, 10 mM Tris-HCl pH 7.8 for 1.5 hr at 37°C, with 3 x 2 µl aliquots of enzyme suspension (5 mg/ml, obtained from Merck, Darmstadt) being added at 0.5 hr intervals. After the incubation the enzyme was inactivated by heating briefly to 95°C, and the suspension was cleared by centrifugation. The proteinase K did not show any significant ribonuclease activity.

The various digests were separated either by two-dimensional chromatography on polyethyleneimine plates using the system of Volckaert and Fiers (18) as above, or by one-dimensional chromatography on the same polyethyleneimine plates, developed in 10% acetic acid 1.4% pyridine pH 3.7, as published by Bernardi (19). The sequence of digestions and separations involved in the various experiments is described in the text.

RESULTS AND DISCUSSION

(a) Isolation and analysis of specific S7-polynucleotide cross-linked complexes.

16S RNA containing cross-linked S7 was isolated from irradiated unlabelled 30S subunits and was subjected to partial digestion with various amounts of ribonuclease T_1, as described in Materials and Methods. The reaction products were examined on dodecyl sulphate gels (15), together with total 30S protein as marker, and the result of a typical series of digestions is shown in Fig. 1A. The gel was stained with amido black, which shows little or no reaction with RNA, and, since protein S7 has been shown to be the only 30S protein to be cross-linked to a signi-

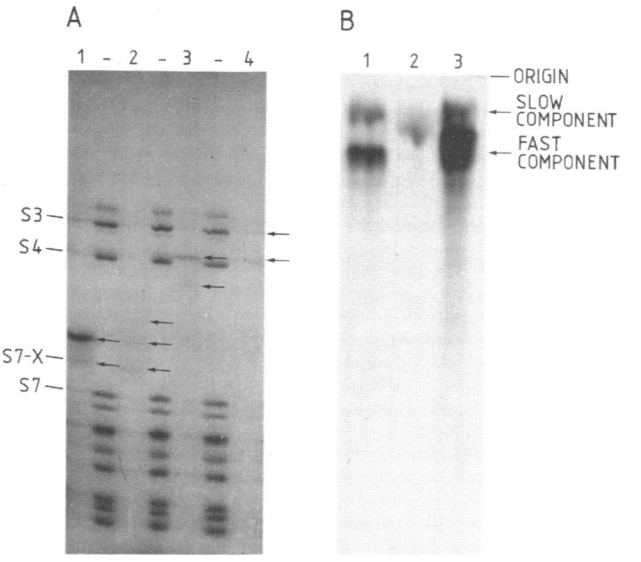

Figure 1: Isolation of cross-linked S7-RNA complexes.

A. Partial digestions with ribonuclease T1 of unlabelled 16S RNA containing cross-linked S7, separated on a dodecyl sulphate gel (15) and stained with amido black. Every alternate slot contained total 30S protein, and the positions of S3, S4 and S7, as well as the fully-digested S7-oligonucleotide complex (S7-X), are indicated on the left side of the diagram. Arrows on the right side of each slot indicate the positions of the principle stained complexes. Slot 1: 80 units enzyme per A_{260} unit RNA. Slot 2: 1.6 units. Slot 3: 0.8 units. Slot 4: 0.5 units. (Slot 1 is noticably contaminated with total protein from the adjacent control slot.)
B. Purification of ^{32}P-labelled complexes on a 12% gel containing dodecyl sulphate and urea. Slices from a gel such as that depicted in Fig. 1A were loaded directly onto the gel, and the bands located by autoradiography. Slot 1: S7-RNA complex II. Slot 2: Fully-digested S7-oligonucleotide complex. Slot 3: S7-RNA complex I. Arrows indicate the fast- and slow-running components (see text).

ficant extent to 16S RNA under the irradiation conditions used (1), it follows that the observed weak but nevertheless clearly-defined stained bands correspond very probably to S7-RNA complexes. Control samples of digested 16S RNA from unirradiated 30S subunits showed no bands after staining with amido black.

Fig. 1A shows that, as the amount of ribonuclease T_1 is re-

duced, progressively larger complexes are obtained, which move more slowly in the gel. In particular, in slot 3 a strong band can be seen moving at the position of protein S4. From the known molecular weights of S4 and S7 (23,100 and 17,100 respectively (20,21)), this suggests that the complex would in this case contain of the order of 20 nucleotides of RNA, if it is indeed an S7-RNA cross-linked digestion product. An attached RNA fragment of this length should be ideal for a positive and unambiguous identification of the RNA sequence attached to the protein. In fact, as will be seen later, the RNA fragments attached to protein S7 turned out to be rather longer than this crude estimate suggests.

The experiment of Fig. 1A was repeated, using 30S subunits labelled with ^{32}P, and digestion conditions similar to those of slot 3 (Fig. 1A). The gel was analysed both by autoradiography and staining (see Materials and Methods), and the results showed a complex array of ^{32}P-RNA fragments in the interesting region of the gel (between proteins S3 and S7, cf. Fig. 1A). By comparing with the results of Fig. 1A, a number of bands likely to contain S7-RNA complexes were excised from the gel in the region between proteins S3 and S4. The analysis of two of these putative complexes will be described in detail in the following section; they will be referred to as complexes I and II, and they ran approximately with proteins S3 and S4, respectively, in the gel (cf. Fig. 1A). As a first step, the two complexes were purified by a second electrophoresis in the presence of urea, as described in Materials and Methods, and the separation obtained is shown in Fig. 1B. A control sample of S7-16S RNA which had been subjected to a total digestion with ribonuclease T_1 (cf. ref. 8) was also included in this second gel.

As a result of the direct loading of gel slices from one system onto another, the radioactive complexes do not run very sharply in the second gel, but nevertheless Fig. 1B shows that both of the putative S7-RNA complexes under consideration separated into a slow- and a fast-running component, whereas the control sample (from the fully-digested S7-16S RNA) ran between. All the principle bands were extracted from this gel, and aliquots were analysed for the presence of cross-linked S7 by di-

gesting them fully with ribonuclease T_1, and running the products onto a dodecyl sulphate gel similar to that of Fig. 1A (see Materials and Methods, and cf. ref. 8). The gel was both stained and analysed for ^{32}P-radioactivity, a typical result being illustrated in Fig. 2 for the control sample and the two components of complex I, extracted from the gel of Fig. 1B. It can be seen that the control sample and the slow-moving component of complex I both show a single peak of radioactivity running at the expected position for the S7-RNA ribonuclease T_1 end-product, i.e. rather slower than free protein S7 (cf. Fig. 1A and ref. 8). In contrast the fast-moving component of

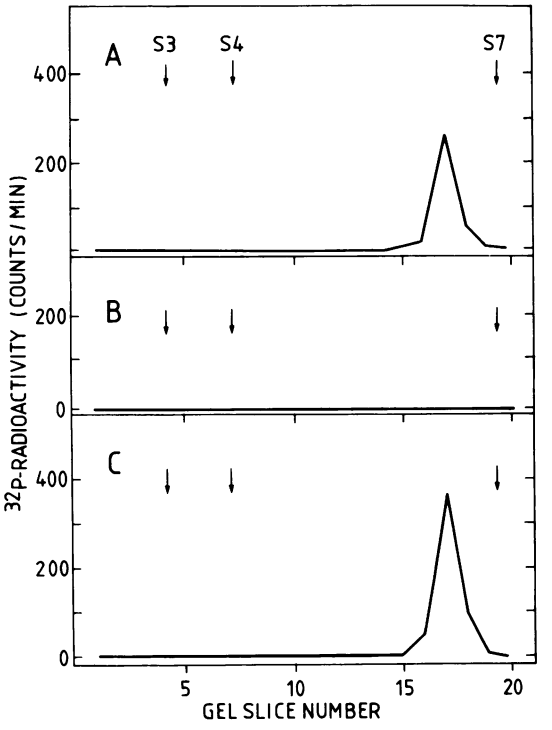

Figure 2: Determination of cross-linked S7 content.

^{32}P-labelled complexes extracted from the gel of Fig. 1B were fully digested with ribonuclease T1 and re-applied to a gel such as that of Fig. 1A (15), as described in Materials and Methods. A: Radioactive profile from complex I (slow-moving component, Fig. 1B). B: Profile from complex I (fast-moving component). C: Profile from control sample (slot 2, Fig. 1B).

complex I showed no radioactivity in this region of the gel, indicating that this component contained only RNA. A similar result was found for the two components of complex II (Fig. 1B).

The slow-moving components of complexes I and II (Fig. 1B), shown in this manner to contain cross-linked protein S7, were fully digested with ribonuclease T_1, and the oligonucleotides released were "fingerprinted" as described in Materials and Methods. The fingerprint obtained from complex I (shown in Fig. 3) contained eight well-defined spots, together with a faint background of contaminating oligonucleotides. The fingerprint of complex II was similar, with the exception that three spots were

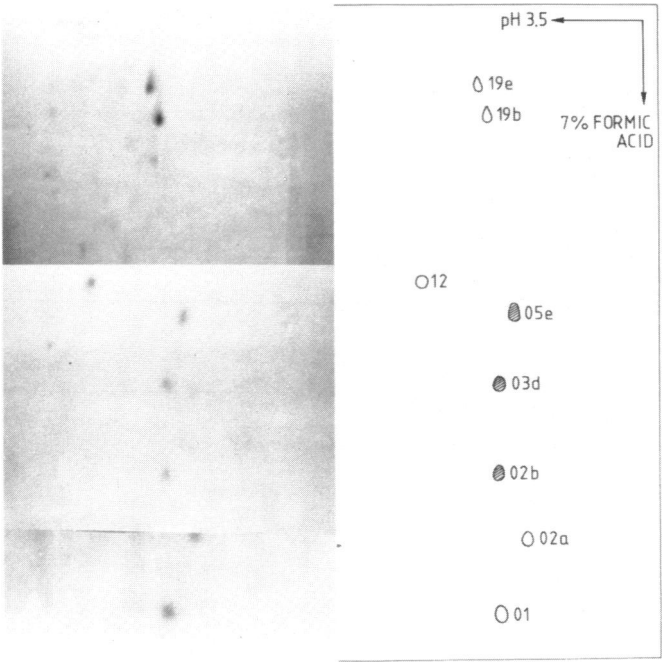

Figure 3: Fingerprint of S7-RNA complex I.

The fingerprint was obtained from the slow-moving component of complex I (Fig. 1B), the first dimension of electrophoresis being from right to left, the second from top to bottom. The oligonucleotide spots are numbered (right side of diagram) according to the system of Uchida et al (22), with the spots which were absent in the corresponding fingerprint from complex II indicated by shading.

missing, namely oligonucleotides 02b, 03d and 05e (denoted by shading on the key to Fig. 3). In contrast, the fingerprints of the fast-moving components of complexes I and II (Fig. 1B) indicated a totally non-specific RNA content, covering most of the 16S RNA sequence.

All the principle radioactive spots were cut out from the fingerprints of the S7-containing components of complexes I and II, and were subjected to a secondary digestion with ribonuclease A (see Materials and Methods). The results, together with the molarities measured for each oligonucleotide, are summarized in Table 1. Allowing for the fact that the molarities of the mono- and di-nucleotides are a little high (as a result of the faint background contamination of the fingerprints with other RNA sequences; Fig. 3), these results enable the RNA fragments to be placed precisely and unambiguously within the now completed sequence of 16S RNA (10,11), as indicated in Fig. 4. In both cases, oligonucleotide 28d, the presumed site of cross-linking

Table 1: Oligonucleotides released by ribonuclase T_1 digestion of S7-RNA complexes.

Spot Number[a]	Oligonucleotide Sequence[b]	Molarity[c] Complex I	Complex II
01	G	4.2	2.0
02a	C.G	1.4	1.2
02b	AG	1.0	n.d.
03d	AAG	0.6	n.d.
05e	AC.C.AG	0.6	n.d.
12	U.G	1.4	1.0
19b	C.U.AC.AC.AC.G	0.7	0.8
19e	C.AU.AC.AAAG	0.7	0.7

a. The spots (cf. Fig. 3) are numbered according to ref. 22.
b. The stops indicate the cutting points of ribonuclease A. Spots 05e and 19e can be uniquely placed in the 16S sequence (10,11) on the basis of the ribonuclease A digestion alone.
c. "n.d." denotes not detected. The molarity of the S7-oligonucleotide complex (remaining at the origin of the first dimension of the fingerprint) was 0.5 and 0.7 in complexes I and II respectively, assuming a chain length of eight nucleotides (see text).

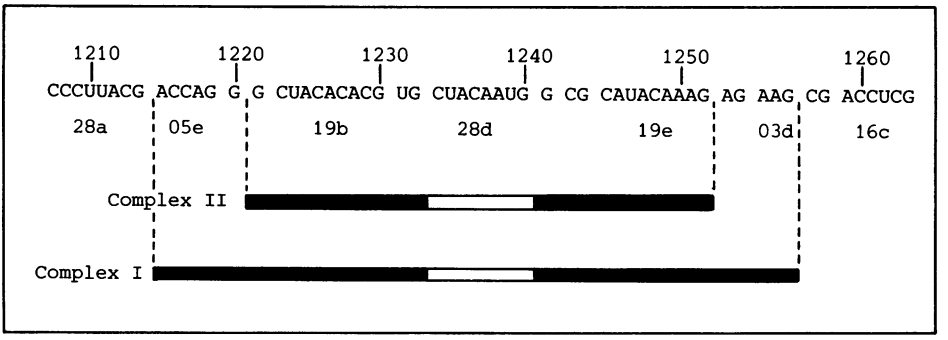

Figure 4: Location of complexes I and II in the 16S RNA.

Oligonucleotides present in the two fingerprints (see Table 1) are indicated on the appropriate part of the sequence (10,11) by black bars. Nucleotides are numbered from the 5'-end of the molecule. Spot 28d was absent from both fingerprints.

to S7 (7,8) was entirely missing from the fingerprint, although it occupies a central position in the sequence. A sharply-defined radioactive spot, whose ^{32}P-content matched that expected for the S7-28d complex, was always found remaining at the origin of the cellulose acetate first dimension in fingerprints of the S7-containing complexes, (see footnote to Table 1).

This result unambiguously confirms our previous conclusion (7,8) that oligonucleotide 28d is involved in the cross-linking to S7. It cannot however be decided at this stage whether G-residue 1241 (Fig. 4) has remained attached to the S7-octanucleotide complex, or whether it is liberated by ribonuclease T_1 in the oligonucleotide analysis. On the other hand it is clear that both U-G and C-G are present on the fingerprints of both complexes (Table 1), and therefore correspond to residues 1231-32 and 1242-43 respectively. It follows that neither of these dinucleotides has remained attached to the S7-octanucleotide complex.

(b) Analysis of the nucleotide attached to S7.

The results described above demonstrate that the cross-linking site to protein S7 lies within the octanucleotide C-U-A-C-A-A-U-G, possibly with a second G-residue (at position 1241, see Fig. 4) also being involved. In order to pursue the analysis

further, the ^{32}P-labelled oligonucleotide-S7 complex resulting from complete digestion with ribonuclease T_1 (Fig. 1B) was prepared and purified as previously described (8). This complex was digested variously with ribonuclease A, proteinase K or ribonuclease T_2 under the conditions described in Materials and Methods, and the results of some of the different digestions are illustrated in Fig. 5.

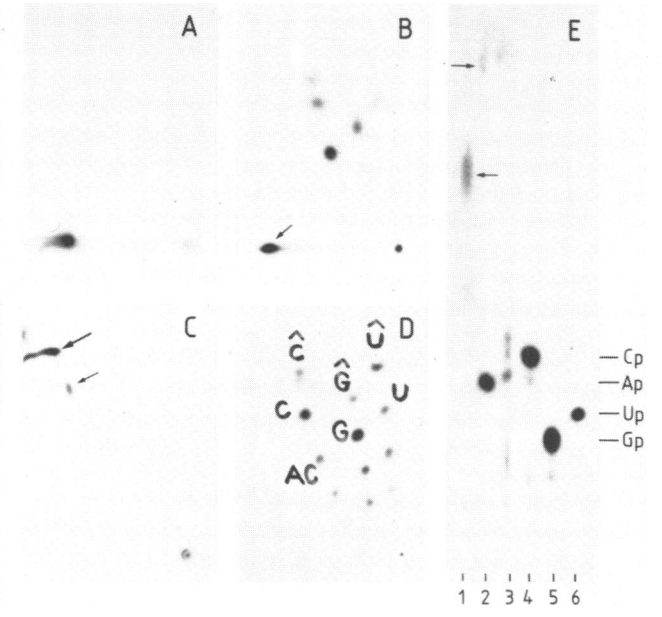

Figure 5: Digestions of the S7-octanucleotide complex.

A to D. Separations on poyethyleneimine plates in the two-dimensional system of ref. 18. The first dimension is from right to left, the second from bottom to top. A: Purified 32P-labelled S7-octanucleotide complex. B: S7-octanucleotide complex after digestion with ribonuclease A. The S7-trinucleotide complex is arrowed. C: S7-trinucleotide complex (from Fig. 5B), after extraction and digestion with proteinase K. The major and minor components are arrowed. D: Control digest showing positions of oligonucleotides. "^" denotes cyclic phosphate.
E. One-dimensional separations in the system of ref. 19. Spots corresponding to oligopeptide-containing material are arrowed. Slot 1: Main component from Fig. 5C. Slot 2: The same, after digestion with ribonuclease T2. Slot 3: Minor component from Fig. 5C, after ribonuclease T2 digestion. Slots 4 - 6: Cp, Gp and Up markers, extracted from the plate of Fig. 5B. See text for details.

Fig. 5A shows a two-dimentional chromatogram of the purified S7-oligonucleotide complex run on polyethyleneimine thin-layer plates in the system of Volckaert and Fiers (18). In contrast to our previous experiments (8), the complex was applied to the plate in 0.05% dodecyl sulphate, which gave a quantitative transference onto the plate, the complex running as a single ^{32}P-labelled spot on the chromatogram. If this complex was digested with ribonuclease A, the result shown in Fig. 5B was found. As has already been reported (8), this digest led to the release of C, A-C, U and G in near equimolar amounts together with C-cyclic phosphate (compare the control digest of Fig. 5D), and this result is included to demonstrate that no spot which could correspond to GpGp was found in the digest. It therefore follows that the oligonucleotide attached to S7 after T_1 digestion is the octanucleotide C-U-A-C-A-A-U-G and not the nonanucleotide C-U-A-C-A-A-U-G-G, and that the shorter oligonucleotide remaining after the ribonuclease A treatment must be the trinucleotide A-A-U. This was not clear in our previous studies (8), due to ambiguities in this region of the published sequence of 16S RNA, as available at that time (23).

The S7-A-A-U complex (arrowed in Fig. 5B) was eluted from the thin-layer plate, and was digested with proteinase K, and re-applied to the same separation system giving the result of Fig. 5C. This shows that the complex has been digested to an oligopeptide-oligonucleotide, the major component of the radioactivity running at the top left-hand corner of the plate, with a weak second spot moving just behind it (Fig. 5C). These complexes were eluted once more, and half of the major component and the whole of the minor component were subjected to a final digestion with ribonuclease T_2. The digestion products, together with the other (undigested) half of the major complex component, were run on a further polyethyleneimine plate in one dimension (see Materials and Methods). This system separates all four mononucleotides (19), and Cp, Up, and Gp eluted from the plate of Fig. 5B were used as markers. The result is presented in Fig. 5E.

It should be noted first that the Cp marker spot eluted from the plate of Fig. 5B contains small but significant amounts

of Ap. This was always observed, and is due to the known tendency of high concentrations of ribonuclease A to attack A-residues (24). This slight overdigestion could arise from any of the three A-residues in the S7-octanucleotide complex. The oligonucleotide-oligopeptide complex runs as a complex smear in this separation system, but Fig. 5E shows that, after digestion with ribonuclease T_2, the complex is split into a single fast-running component together with a strong spot corresponding to Ap. (The fast-running components are arrowed in Fig. 5E). The ratio of radioactive intensities of these two spots was approximately 2:1 (Ap:complex), and no trace of Up was found in any experiment. The same release of Ap by ribonuclease T_2 was observed in experiments where the ribonuclease A and T_1 treatments were made simultaneously as opposed to consecutively, and a comparable result was obtained even if the proteinase K treatment was omitted. This indicates that the ribonuclease T_2 has full access to the oligonucleotide despite the presence of the whole bulk of protein S7. The minor radioactive spot running behind the principle spot on Fig. 5C showed a release of Ap, Cp and Gp after digestion with ribonuclease T_2 (Fig. 5E), suggesting that this minor component represents an incomplete digestion by the ribonuclease A (Fig. 5B). Again however no trace of Up was seen.

These results demonstrate that the end-product of this series of digestions is an oligopeptide-mononucleotide complex, containing the uridine residue number 1239 in the sequence (Fig. 4). This residue is therefore the point of cross-linking to protein S7. The result was not affected when the 30S subunits were subjected to an activation dialysis prior to irradiation, as described in Materials and Methods, indicating that this particular RNA-protein neighbourhood in the subunit is not involved in a conformational change during activation.

The experiments reported here, taken together with those already published (7,8), have resulted in the development of three systems for analysing cross-linking sites on ribosomal RNA. These are firstly the non-ionic detergent gel system (7) for preliminary localisation of the cross-link site, secondly the isolation of RNA-protein complexes of intermediate length for precise localisation of the T_1-oligonucleotide concerned,

and finally the series of digestion procedures to identify the cross-linked nucleotide itself. Combinations of these systems are currently being used in our laboratory to identify the sites of cross-linking induced by treatment of the ribosomal subunits with bifunctional chemical reagents (4,6).

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