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Protein-RNA crosslinking in *Escherichia coli* 30S ribosomal subunits. Identification of a 16S rRNA fragment crosslinked to protein S12 by the use of the chemical crosslinking reagent 1-ethyl-3-dimethyl-aminopropylcarbodiimide

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ABSTRACT

1-ethyl-3-dimethyl aminopropylcarbodiimide (EDC) was used to crosslink 30S ribosomal proteins to 16S rRNA within the E.coli 30S ribosomal subunit. Covalently linked complexes containing 30S proteins and 16S rRNA, isolated by sedimentation of dissociated crosslinked 30S subunits through SDS containing sucrose gradients, were digested with RNase T1, and the resulting oligonucleotide-protein complexes were fractionated on SDS containing polyacrylamide gels. Eluted complexes containing 30S proteins S9 and S12 linked to oligonucleotides were obtained in pure form. Oligonucleotide 5'terminal labelling was successful in the case of S12 containing but not of the S9 containing complex and led to identification of the S12 bound oligonucleotide as CAACUCG which is located at positions 1316-1322 in the 16S rRNA sequence. Protein S12 is crosslinked to the terminal G of this heptanucleotide.

INTRODUCTION

Understanding ribosomal functions during the complex process of protein biosynthesis requires detailed knowledge of the structure of the ribosome and of the spatial relationships between its constituent molecules. A great deal of information in this regard has been accumulated, especially concerning protein-protein neighbourhoods, by crosslinking (1), neutron scattering (2) and immune electron microscopy (3). In contrast, protein-RNA proximity relationships and interactions have been studied primarily by partial nuclease digestion of reconstituted 16S RNA-protein complexes or gently unfolded 30S subunits (reviewed in 4). This technique has generally led to the identification of relatively large regions of 16S rRNA to which proteins remain bound, and only in the case of proteins S8 and S15 has a reasonably small binding site been well characterized (5,6).

30S ribosomal proteins have also been crosslinked to 16S RNA by

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UV-irradiation (7-9) and by a wide range of bifunctional chemical reagents (10-19). The sites in 16S rRNA to which proteins S4,S7, and S20 are crosslinked have been identified following UV irradiation of native 30S subunits or reconstituted protein-16S rRNA complexes (7,8,20) and a region of close proximity of protein S1 and 16S rRNA has been located using a photoactivable crosslinking reagent and reconstituted 30S particles (21). We have previously shown (22) that under suitable conditions 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC) can be used to activate side chain carboxyl groups in ribosomal proteins in the intact ribosome and provoke formation of amide bonds between the activated glutamyl and aspartyl residues of these proteins and amino groups in ribosomal RNA. Here we identify the heptanucleotide CAACUCG situated at positions 1316-1322 in 16S rRNA as the site to which protein S12 is crosslinked by treatment of 30S subunits with EDC. Its identifications, greatly facilitated by knowledge of the sequence of 16S rRNA (23), was achieved by analysis of peptide-oligonucleotide-complexes using the rapid sequencing technique of Donis-Keller et al (24). We further show that the nucleoside involved in the crosslink with protein S12 is the guanosine residue.

MATERIALS AND METHODS

Preparation of 30S ribosomal subunits :

30S ribosomal subunits were prepared from E.coli strain MRE 600 as previously described (25) and stored before use at -70°C in 10 mM triethanolamine (TEA)-HCl pH 7.5, 10 mM Mg acetate, 50 mM KCl, 10 mM 2-mercaptoethanol.

Preparation of carrier total 30S proteins (TP30S)

30S proteins were extracted by the acetic acid procedure (26). To eliminate contaminating RNA, crude protein preparations were resuspended in 10 mM Na-acetate pH 4.5, 6 M urea, 0.1% SDS, 1 mM dithiothreitol ; 5 N NaOH was added to a final concentration of 0.4N and the suspension was incubated at 37°C for 2 hours. Proteins were then precipitated by addition of 5 vol. of acetone, dissolved in 8 M urea and stored at -20°C at a concentration of 20 mg/ml.

Crosslinking reaction

A suspension of 30S subunits (2000 $A_{260\ nm}$) was dialyzed at 4°C, against 500 vol. of crosslinking reaction buffer (1 mM Na-cacodylate, 0.5 mM magnesium chloride, 50 mM KCl, adjusted to pH 6.5),

for 20 hours with a change of buffer after 5 hours of dialysis. The concentration of 30S subunits was then adjusted to 10 A_{260nm} ml by addition of crosslinking reaction buffer, EDC was added to a final concentration of 50 mM, and the pH was immediately readjusted to 6.5 with 0.1 N HCl. Reaction was allowed to proceed at 25°C for 25 min. with stirring, and then stopped by addition of 1/10 vol. of quenching buffer (1 mM Na-cacodylate, 0.22 M Mg acetate, 1.74 M ammonium acetate, 1.56 M ammonium chloride, adjusted to pH 6.5) and incubation of the resulting mixture at 25°C for 1 hour.

Purification of 30S crosslinked subunits : elimination of oligomers and aggregates

EDC treated 30S subunits were precipitated with 0.7 vol. of cold ethanol at -20°C for 30 min., recovered by centrifugation, resuspended in 10 mM TEA-HCl pH 7.5, 400 mM NaCl, 10 mM Mg acetate,10 mM 2-mercaptoethanol at a concentration of 125 $A_{260\ nm}$/ml and purified by centrifugation (20 000 rpm, 17 hours, 4°C, Spinco SW27 rotor) through 5-20% sucrose gradients made in the same buffer and loaded with 1 ml samples. 30S subunit peaks were pooled, taking care to avoid any contamination by dimers and 30S monomers were precipitated with 0.7 vol. of cold ethanol at -20°C for one hour and recovered by centrifugation.

Isolation of 30S protein-16S RNA complexes

Purified EDC treated 30S subunits resuspended in dissociation buffer (10 mM TEA-HCl, pH 7.5, 0.1% SDS, 100 mM LiCl, 1 mM dithiothreitol) were heated at 40°C for 10 min. 16S rRNA either free or bound covalently to 30S proteins was separated from free 30S proteins and protein-protein complexes by centrifugation (26 000 rpm, 19 hours, 10°C, Spinco SW 27 rotor) through 5-20% sucrose gradients made in dissociation buffer and loaded with 100 $A_{260\ nm}$ of dissociation products. 16S peak fractions were pooled and free RNA and protein-RNA complexes were precipitated with 2 vol. of cold ethanol at -20°C for one hour, after addition of NaCl to a concentration of 100mM,recovered by centrifugation, dissolved in water, and stored at -20°C.

Iodination of the crosslinked proteins

30 $A_{260\ nm}$of 30S protein-16S rRNA complexes (about 0.3 mg of protein) were iodinated with 3.7 MBq of ^{125}I (Amersham, 5.55×10^{2}MBq/

µgI) according to the chloramine T procedure (27). Complexes were separated from unreacted iodine by filtration through a sephadex G-25 column (130x5 mm) preequilibrated with 10 mM TEA-HCl pH 7.5, 100 mM NaCl, were precipitated with 2 vol. of cold ethanol and recovered by centrifugation.

RNase digestion of 16S RNA-30S protein complexes and isolation of oligonucleotide-protein products

170 $A_{260\ nm}$ of unlabelled complexes and 30 $A_{260\ nm}$ of (^{125}I) complexes were mixed with 2000 units of RNase T1 (Sankyo) in 1.2 ml of digestion buffer (10 mM TEA-HCl pH 7.5, 1.5% N-lauroyl-sarcosine , 5 mM Na-EDTA, 10 mM 2-mercaptoethanol) and the resulting solution was incubated at 37°C for 1 hour. After digestion, 30S protein-oligonucleotide complexes, precipitated by addition of 5 vol. of acetone and collected by centrifugation, were resuspended in 50 mM Tris-HCl pH 6.8, 1% SDS, 10 % glycerol, 0.01% bromophenol blue and fractionated by electrophoresis at 500 V on 350x 160x1 mm SDS containing 15% polyacrylamide gel slabs for 40 hours at 4°C (28). 30S protein-oligonucleotide complexes were visualized either by autoradiography (products to be eluted for oligonucleotide sequence analysis) or by staining with Coomassie Blue (products to be eluted for protein identification) and bands corresponding to these complexes were excised, loaded directly onto a fresh gel slab and a second electrophoretic separation was carried out under the same conditions.

Identification of the crosslinked proteins

A small part of each complex isolated after the first stage of gel purification was used to identify the crosslinked proteins. Samples of excised gel bands were ground in heat-sealed 1 ml Eppendorf pipette tips plugged with siliconized glass wool.Nine vol. of elution buffer (10 mM Tris-HCl, pH 7.9, 7 M urea, 1 mM Na-EDTA, 0.1 % SDS, 150 mM NaCl containing 0.3 mg/ml of TP30S)were added and elution was carried out at 37°C overnight with shaking. The sealed tips were then cut off the pipettes and the eluates were expelled through the glass wool plugs by applying pressure with a syringe. TP30S and complexes were precipitated from the eluates by addition of 5 vol. of acetone, dried in vacuo,and dissolved in 50 µl of 10 mM Na-acetate pH 4.5,6 M urea,0.1% SDS, 1 mM dithiothreitol. The oligonucleotide moieties of the complexes

were hydrolyzed with alkali as described above, and the resulting mixtures of TP30S and crosslinked proteins were precipitated several times with 5 vol. of acetone, dried in vacuo, dissolved in 8 M urea and analyzed by two-dimensional polacrylamide gel electrophoresis (29). Gels were stained with coomassie brilliant blue and autoradiographed.

5'terminal labelling of crosslinked oligonucleotides

Protein-oligonucleotide complexes, recovered from purification gels as described above, were dissolved in 40 µl of 10 mM Tris-HCl pH 8.0, 10 mM Mg acetate, 15 mM 2-mercaptoethanol and added to 7.4 MBq of ^{32}P ATP (Amersham, 111 TBq/mmol). 5'terminal labelling was carried out with 2 units of T4 polynucleotide kinase(NEN) at 37°C for 60 min.

One half of the labelled products were digested for 60 min. at 37°C with proteinase K (Merck) using an initial enzyme/TP30S weight ratio of 0.5 which was increased to 1 after 30 minutes by addition of a second amount of proteinase K.

The two samples (± proteinase K treatment) were dried in vacuo, dissolved in 100 mM Tris-borate pH 8.3,2.5 mM Na-EDTA, 8 M urea, 0.05% bromophenol blue, 0.05% xylene cyanol and fractionated by electrophoresis at 2000 V for 2.5 hours on a 20 % polyacrylamide gel slab (400x300x0.5 mm) prepared in 100 mM Tris-borate pH 8.3, 2.5 mM NaEDTA containing 8 M urea, and preelectrophoresed at 1000 V for 90 min. before use. Electrophoresis buffer was 100 mM Tris-borate pH 8.3, 2.5 mM NaEDTA. Fractionated products were visualized by autoradiography.

Elution and purification of ^{32}P labelled fragments

Interesting 5'labelled fragments (figure 4) were eluted according to Maxam et Gilbert (30), precipitated with 10 µg of carrier tRNA, purified by electrophoresis on 17% polyacrylamide 8 M urea gels made in 100 mM Tris borate pH 8.3, 2.5 mM NaEDTA, visualized by autoradiography, and eluted as above.

Sequence analysis of crosslinked RNA fragments

Purified 5'labelled fragments were subjected to partial enzymatic digestions by the following enzymes : RNase T1 (Sankyo) (24) ; RNase U_2 (Sankyo) (24) ; RNase CL_3 from chicken liver (Bethesda Research Laboratories) (31) ; RNase A (Worthington) (23) ; RNase BC from Bacillus cereus (PL-Biochemicals) (32) and RNase Phy M

(PL-Biochemicals) (33). Reaction mixtures (5 µl) contained 1 µg of carrier tRNA and were incubated under the following conditions: 30 min. at 55°C in 20 mM Na-citrate, pH 5.0, 7 M urea, 1 mM Na-EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol, at enzyme/carrier tRNA ratios of 0.025 and 0.0025 (RNase A), 0.1 unit/µg (RNase T1), 0.5 unit/µg (RNase U_2) and 10 units/µg (RNase Phy M); 30 min. at 55°C in the same buffer without urea at 10 units/µg (RNase Bc) ; and 60 min. at 37°C in 100 mM KH_2PO_4, pH 6.5 at 1 unit/µg (RNase CL_3). Non specific cleavage was performed by partial alkaline hydrolysis (24). Digestion products were fractionated by electrophoresis at 2000 V for 3 hours on 400x300x0.5 mm 25 % polyacrylamide gels prepared in 100mM Tris-borate pH 8.3 2.5 mM Na-EDTA, 8 M urea and prerun at 1500 V for 1.5 hours before use. Separated products were visualized by autoradiography at -80°C, using an Ilford $(CaWo)_4X$-ray intensifying screen (34).

Identification of the 5'terminal nucleotide

Mixtures of 5'labelled oligonucleotide-peptide complexes and carrier tRNA were totally digested with RNase P1 (PL-Biochemicals) in 50 mM NH_4 acetate pH 5.3 at 37°C for 1 hour with an enzyme/carrier tRNA ratio of 0.02 (w/w). Released 5'nucleotides were analyzed by thin layer cellulose chromatography with concentrated HCl/isopropanol/H_2O (17.6/68.0/14.4) as solvent (35). 5'labelled nucleotides were visualized by autoradiography and marker 5'nucleotides by inspection under UV light at 254 nm.

RESULTS AND DISCUSSION

EDC is a water soluble carbodiimide which, like other compounds of this class, activates the carboxyl group (36). In our conditions it catalyses amide bond formation between carboxyl and amino groups and this reaction has been used to activate aspartyl and glutamyl residues in proteins in order to convert them to asparaginyl and glutaminyl residues (37) and to form protein-protein bridges between the subunits of a hormone (38). However, to our knowledge, its ability to introduce amide bonds between carboxyl and aminogroups has not been used to explore the structure of nucleoproteins such as the ribosome. We have previously shown (22) that EDC crosslinks 30S proteins efficiently to 16S rRNA in the E.coli 30S ribosomal subunit and simultaneously introduces

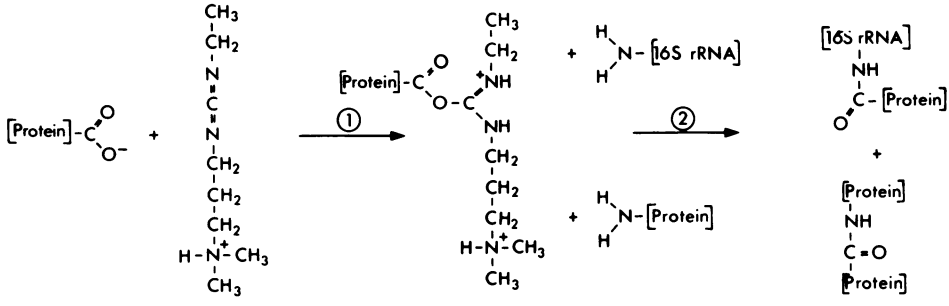


Figure 1 - Scheme of the crosslinking reaction mechanism
1) In the experimental conditions used, EDC activates a carboxyl group leading to the formation of an O-acylisourea
2) This highly reactive intermediate condenses with a nucleotide or protein aminogroup creating a protein-nucleic acid or protein-protein amide linkage.

protein-protein crosslinks. The mechanism of these reactions is schematized in Figure 1.

Fractionation and analysis of 30S protein-16S rRNA complexes ; identification of crosslinked proteins

Mixtures of free 16S rRNA and crosslinked 30S protein-16S rRNA complexes, isolated from EDC treated 30S subunits and separated from free 30S proteins and free 30S protein-protein complexes by sedimentation of dissociated crosslinked subunits on SDS containing sucrose gradients, were digested with T1 RNase and the digestion products were fractionated on SDS containing 15% polyacrylamide gels along with control TP30S. The results of a typical fractionation gel (Fig.2) show that the digestion products of the crosslinked 30S protein-16S rRNA complexes contain several components which migrate in the same gel region as the indicated 30S proteins and could therefore be oligonucleotide-protein complexes. Material migrating in the S2-S4 region was found to be very heterogeneous (oligonucleotide-protein-protein, and protein-oligonucleotide-protein complexes etc.. results not shown) and was not further studied. Stained products with electrophoretic mobilities similar to those of 30S proteins S7-S14 were divided into four fractions Xa,Xb,Xc, Xd for subsequent analysis. Figure 3 shows the results of experiments carried out to identify the proteins present in the complexes eluted from zones Xa,Xb,Xc and Xd. Fig. 3a and 3c identify protein S9 in zone Xa and protein S12 in

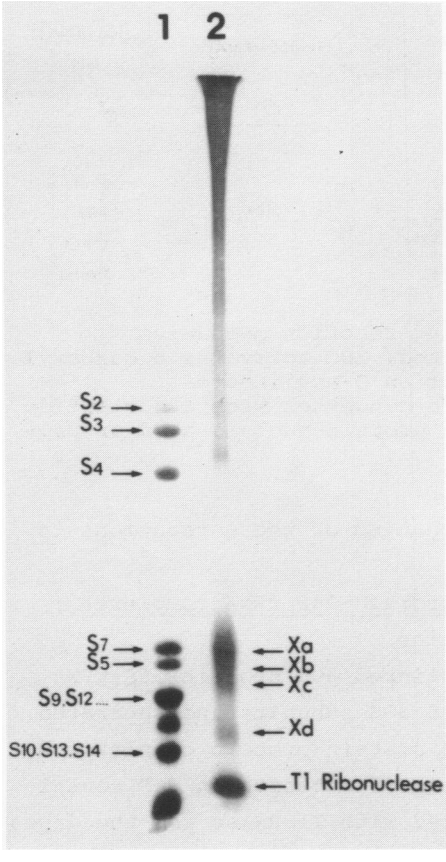


Figure 2 - Fractionation of 30S protein-oligonucleotide complexes by electrophoresis on a 15% polyacrylamide gel in 0.375 M Tris-HCl, pH 8.8, 0.1% SDS as described (28). (1) control TP30S, (2) 30S protein-oligonucleotide complexes. Proteins were visualized by staining with coomassie brilliant blue. Material found to be heterogenous and to consist mainly of protein-protein-RNA complexes. It was not migrating at and above S4 was further analyzed.

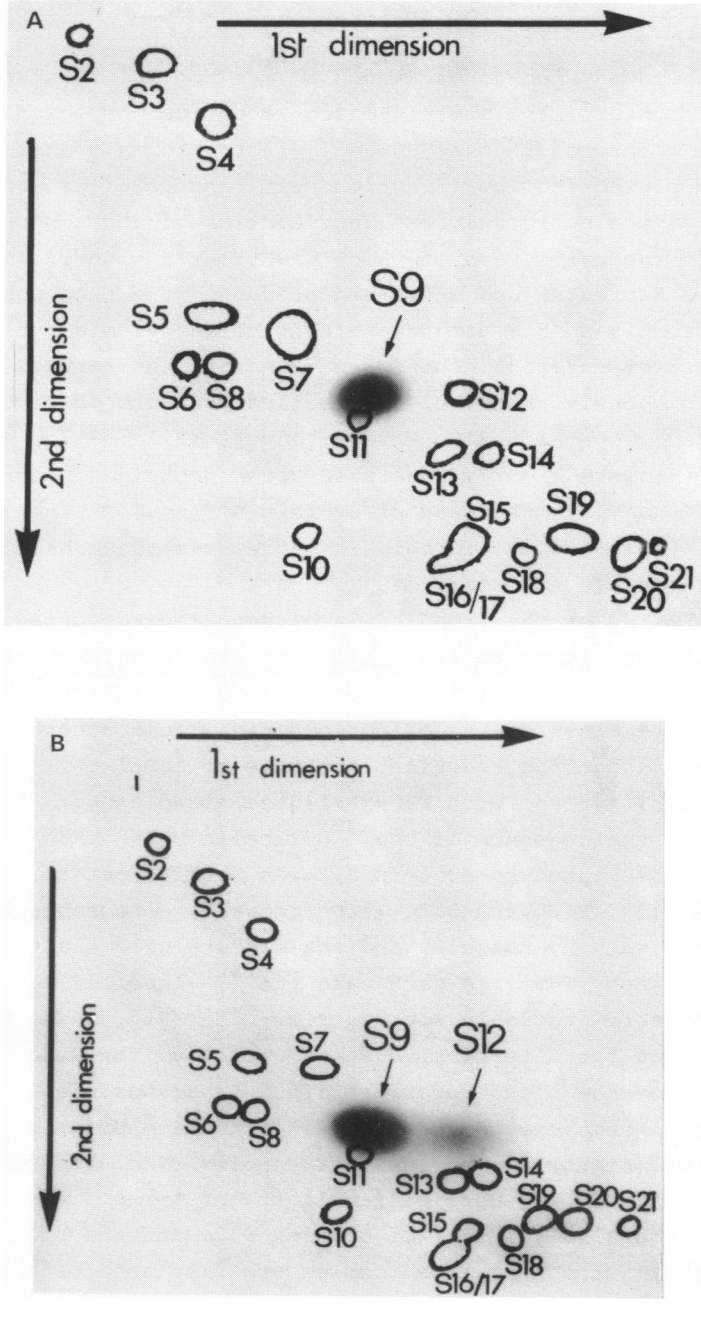
zone Xc, and show unambiguously that these two proteins have been crosslinked to 16S rRNA since, after alkaline hydrolysis to remove oligonucleotides, the corresponding radioactive spots are located at the positions of control proteins S9 and S12, whereas, before alkaline hydrolysis, zones Xa and Xc are slightly displaced with respect to these proteins (see figure 2). Figure 3b shows that zone Xb contains a mixture of proteins S9 and S12 suggesting the presence of a mixture of the components found in zones Xa and Xc. This was confirmed by electrophoresis analysis of material eluted from zone Xb (results not shown) which revealed the presence of two well-separated bands with the same mobilities as bands Xa and Xc in Figure 2. Figure 3d shows that band Xd contains proteins S10 and S13/14. For this reason, material from this band was not further analysed.

Sequence of oligonucleotides crosslinked to 30S proteins

In order to verify that the presence of oligonucleotides in the materials eluted from zones Xa and Xc was not due to unspecific adsorption to protein, protein-oligonucleotide complexes were submitted to a second stage of SDS-gel electrophoresis (Materials and Methods), eluted from purification gels, and labelled with ^{32}P ATP and polynucleotide kinase. The reaction products were then divided into two parts, one of which was treated with proteinase K. Proteinase K treated and untreated products were then analyzed by electrophroesis on 8 M urea containing polyacrylamide gels (Fig.4). In the case of material eluted from zone Xc, the results obtained show unambiguously that the ^{32}P labelled products observed were crosslinked to protein S12. Three products (S12a, S12b, and S12c in Figure 4) were clearly generated after proteinase K treatment. On the contrary, in the case of protein S9, no ^{32}P labelled fragments were released by proteolysis suggesting that the post-labelling reaction had not occurred.

Inaccessibility of the 5'end of an oligonucleotide crosslinked to protein S9 due either to its small size, to an unfavourable protein conformation, or to location of the crosslinking point at or near the 5'terminus of the oligonucleotide could account for failure of the labelling reaction. Attempts to label this oligonucleotide at its 3'end with T4 RNA ligase are planned.

Analysis of the sequence of the ^{32}P labelled 16S rRNA oligonucleotide crosslinked to protein S12 was carried out as follows : Complexes S12a, S12b,and S12c (see figure 4) were subjected to total hydrolysis by RNase P_1 and the digests were analyzed by homochromatography (35). In each case the ^{32}P-labelled mononucleotides comigrate precisely with carrier CMP (Fig.5). We therefore conclude that the 5'terminal base is cytosine. The second and third residues from the 5'end were identified as adenosines by partial digestion of complexes S12a and S12b with RNase U_2(A) and RNase Phy M (A+U) (Fig.6). In the case of complex S12c, the results of sequence analysis are more difficult to interpret. As seen in Figure 6, the alkali ladder of complex S12c and the spot present in the -enzyme track of this complex are displaced with respect to corresponding patterns obtained with the heptanucleotide present in complexes S12a and S12b. This suggests that the prepara-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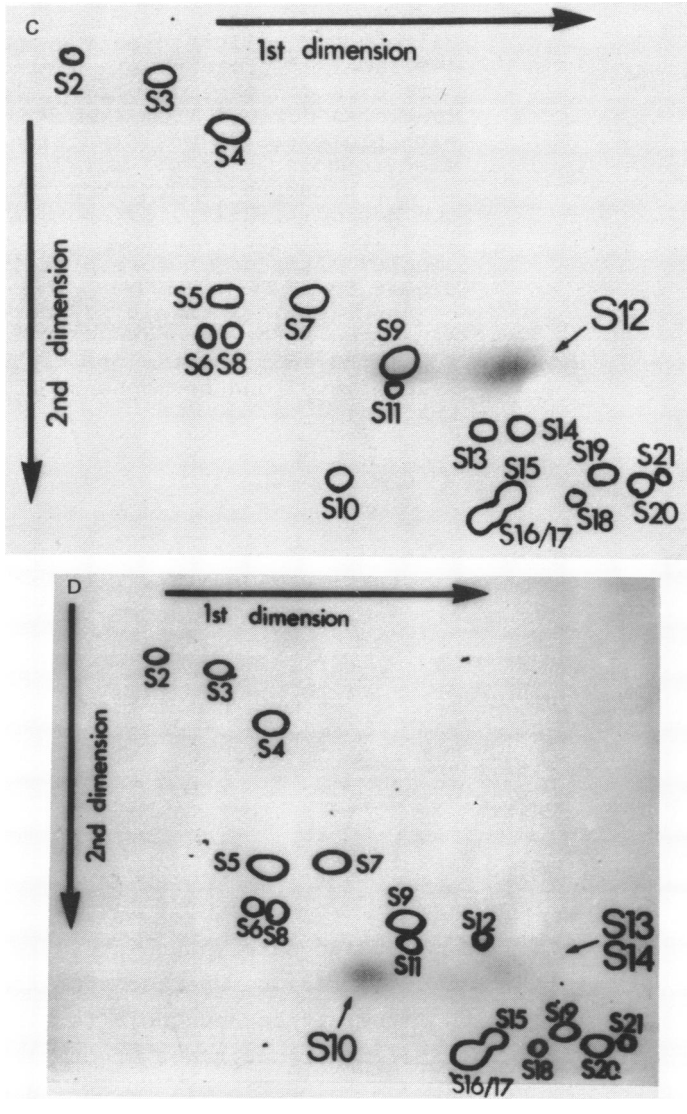

Figure 3 - Identification of crosslinked proteins
The products of alkaline hydrolysis of ^{125}I labelled crosslinked 30S protein-oligonucleotide complexes mixed with unlabelled TP30S were analyzed by two-dimensional gel electrophoresis as described (29)
Labelled crosslinked proteins revealed by autoradiography were identified by reference to the positions of stained unlabelled 30S proteins
a) proteins from zone Xa (figure 2)
b) " " " Xb (")
c) " " " Xc (")
d) " " " Xd (")

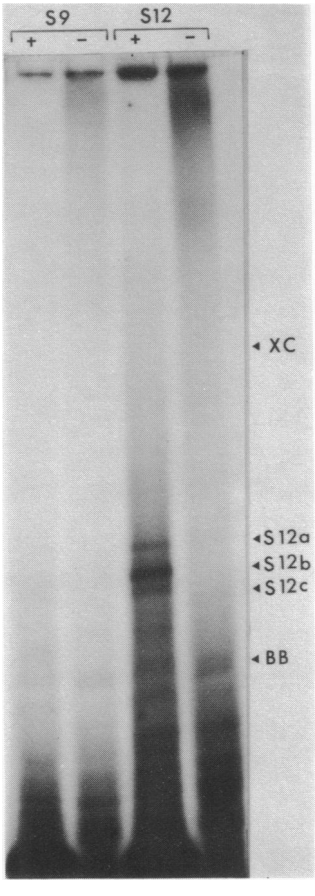

Figure 4 - Fractionation of crosslinked oligopeptide-oligonucleotide complexes. ^{32}P labelled protein-oligonucleotide complexes and peptide-oligonucleotide complexes derived from them by digestion with proteinase K were fractionated by electrophoresis on polyacrylamide gels in the presence of 8 M urea (Materials and Methods). S9(+), S9(-) ; ^{32}P labelled oligonucleotide-protein S9 complex after and before proteinase K digestion S12(+), S12(-) ; ^{32}P labelled oligonucleotide-protein S12 complex after and before proteinase K digestion Xc and BB indicate the positions of xylenecyanol and bromophenol blue tracking dyes.

tion of complex S12c used to obtain data shown in Figure 6 contained mainly an oligonucleotide one residue shorter than those present in complexes S12a and S12b ; it lacked the 5'terminal cytidine referred to above (different preparations of complex S12c were used in the experiments described in Figures 5 and 6).However a small amount of the entire heptanucleotide was also present since a faint spot corresponding to the first position of the heptanucleotide is visible in the RNase A $(C+U)^1$ track of complex S12c and spots corresponding to the third position can be seen in the RNase U_2(A) and RNase Phy M(A+U) tracks. We therefore conclude that the pyrimidine present at the fourth position in the oligonucleotide of complex S12c corresponds in fact to the fifth residue of the heptanucleotide present in complexes S12a and S12b.

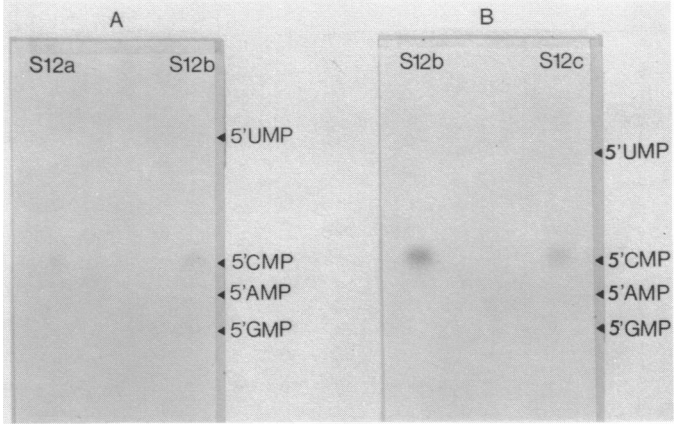

Figure 5 - Identification of the 5'terminal residue of oligonucleotides crosslinked to protein S12. The oligonucleotide moieties of crosslinked complexes S12a, S12b and S12c (see figure 4) were totally digested with RNase P1 and the resulting products were separated by homochromatography (35). (a) analysis of complexes S12a and S12b, (b) analysis of complexes S12b and S12c. The positions of marker nucleotides are indicated at the right of each figure. The direction of migration is from bottom to top.

The latter therefore has the sequence CAA"X"pyr"X""X". The hexanucleotide observed in complex S12c probably arises by cleavage between the first and second residues of the heptanucleotide. The bond between these two residues seems to be fragile since a spot corresponding to 5'terminal cytidine is visible in the lower part of the enzyme track of the analysis of the complex S12a. It is noteworthy(Figure 6) that the sensitivity of complexes S12a,S12b and S12c to RNase A is very different. Under identical conditions (enzyme/carrier tRNA = 0.025), complexes S12a and S12b were completely digested whereas complex S12c was only partially digested producing a clear band corresponding to the fourth position of its hexanucleotide (fifth position of the heptanucleotide)showing that the oligonucleotide-peptide complexes differ in their susceptibility to enzymatic attack.
In two separate experiments, the fifth residue in the 3'direction of the heptanucleotide was also identified as a pyrimidine in the case of complex S12b. In the first, partial digestion of complex S12b by RNase Bc from B.cereus (C+U) gave rise to a band at the fifth position from the 5'end (Fig. 7). In the second experiment

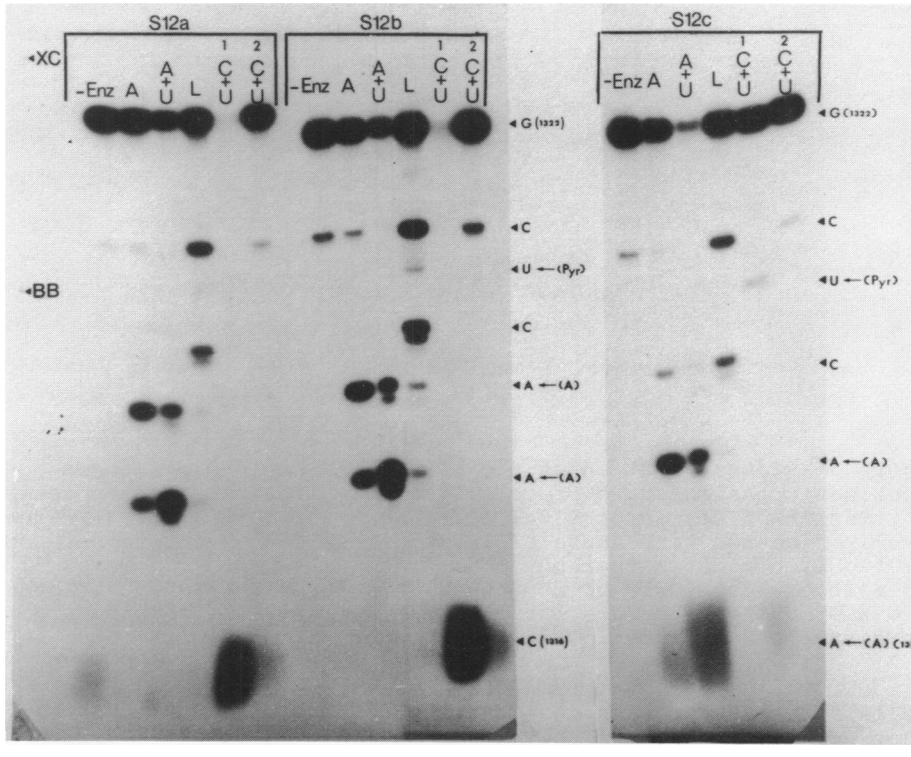

Figure 6 - Sequencing gel of 5'-labelled oligonucleotide-peptide complexes S12a, S12b, and S12c (see figure 4). Partial digestions were performed with RNase U_2(A), RNase PhyM (A+U), pancreatic RNase A (C+U) and by limited alkaline hydrolysis (L). Digestion products were fractionated on a 25% polyacrylamide gel as described in the legend to figure 4. Enzyme/carrier tRNA ratios of 0.025 (1) and 0.0025 (2) (wt/wt) were used for hydrolyses by RNase A (C+U). Arrows indicate the nucleotides the identities of which (in parentheses) were deduced from this figure.

a hexanucleotide fragment of complex S12b (see Fig.6, only band in -Enz track) was similarly analyzed by partial enzymatic cleavage (Fig.8). Following treatment with RNase Bc (C+U) a band at the position of the fifth nucleotide confirms the identity of this base as a pyrimidine. A faint band at the same position after digestion with RNase Phy M (A+U) further suggests that the fifth nucleotide may be a uridine. The sixth residue could not be identified in this way, since cleavage occurred at this position even in the absence of enzymes (Fig. 6,7).

The presence of a gap of variable size between the sixth and se-

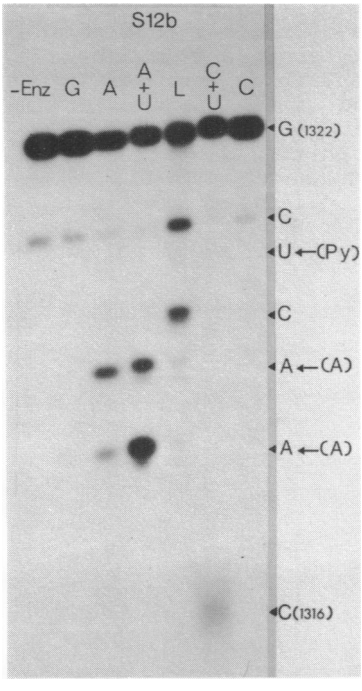

Figure 7 - Sequencing gel of the 5'-labelled oligonucleotide-peptide complex S12b (see figure 4). Partial digestions were carried out with RNase T1 (G), RNase U_2 (A), RNase Phy M (A+U), RNase Bc (C+U), RNase CL_3 (C) and by limited alkaline hydrolysis (L). Digestion products were analysed on a 25% polyacrylamide gel as described in the legend to Figure 4. Arrows indicate the nucleotides the identities of which (in parentheses) were deduced from this figure.

venth nucleotides from the 5'end was evident in limited alkaline hydrolyses of each complex (Fig. 6,7). The size of this gap was strictly correlated with the order of migration of the complexes in Figure 4. We suggest that this gap is due to the presence of peptide moieties of different sizes covalently bound to the seventh nucleotide. Knowing that the first five residues in the heptanucleotide were CAA-"X"-pyrimidine and that the point of crosslinking was located on the 3'terminal residue, we searched for RNase T1-oligonucleotides from 16S rRNA which contained seven or more residues and which began with CAA"X"Py. Only three such fragments occur : CAAACAG (778-784), CAACUCG (1316-1322), and CAACCCUUAUCCUUUG (1108-1123). In spite of its length, the third could not be eliminated a priori, since cleavage after the seventh phosphodiester bond might have occurred as a result of the crosslinking reaction. However we can eliminate this oligonucleotide since its seventh residue is uridine, which does not contain an amino group and threfore cannot participiate in amide bond formation. Moreover, its fifth nucleotide, a cytidine, is not consistent with the band observed at the corresponding position follo-

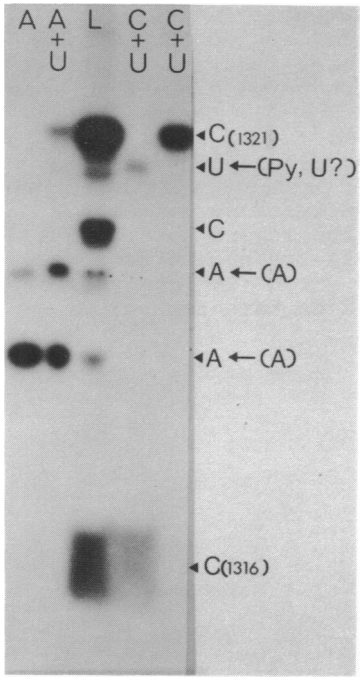

Figure 8 - Identification of the fifth residue of the oligonucleotide crosslinked to protein S12. A hexanucleotide containing residues 1-6 from the 5'end of the heptanucleotide crosslinked to protein S12 was subjected to partial digestions with RNase U_2(A), RNase Phy M(A+U), RNase Bc (C+U) and to limited alkaline hydrolysis (L). Digestion products were fractionated on a 25% polyacrylamide gel.

wing limited hydrolysis of the crosslinked fragment with RNase Phy M (A+U) and RNase BC (C+U) (Fig.8). For the same reason, we can eliminate the oligonucleotide CAAACAG (778-784). Furthermore this oligonucleotide should yield a spot corresponding to the fourth residue (A) which is not seen in Figure 8. We therefore conclude that the only RNase T1-oligonucleotide from 16S rRNA whose identity is consistent with our experimental results is CAACUCG (1316-1322).

Although the cleavage patterns of the crosslinked oligonucleotides were at times difficult to interpret, we feel that such abnormalities constitute further evidence that these RNA fragments contained covalently bound peptides. In particular, the relative inaccessibilities of the fourth and fifth residues to enzymatic cleavage may have been due to the attached peptide moiety inhibiting proper-substrate recognition by the nucleolytic enzymes. Systematic cleavage at the sixth residue (see figures 6,7) is of particular interest since it permits relatively efficient elimination of the crosslinked nucleotide. For example, after incubation in 5% acetic acid for 1 hour, 40% of the complex S12b was converted

to a hexanucleotide fragment (data not shown). Such cleavage in the enzymatic digestion experiments described above (Fig.6,7) was possibly due to the fact that samples had been adjusted to pH 5.0 with Na-citrate. Introduction of a covalent crosslink of this type therefore seems to destabilize the polynucleotide to the 5' side of the crosslinked nucleotide. If this is a general phenomenon, incubation at an acid pH may be a useful technique in analyzing oligonucleotides which contain residues that are protected against ribonuclease attack by crosslinked peptides.

Structural and functional implications of an S12-G1322 crosslink

It is apparent that under our experimental conditions, pH 6.5 and 0.5 mM Mg^{++}, at least part of the peptide chain of protein S12 is situated very close to the 16S oligonucleotide CAACUCG (1316-1322). The integrity of 30S subunits after the crosslinking reaction was established by subsequent isolation on sucrose gradients.Although an unchanged sedimentation coefficient cannot be considered as a sufficient proof of an unchanged conformation in crosslinked 30S subunits we consider that the S12-16S rRNA crosslink which we observe probably reflects the spatial relationship between these two ribosomal components within structurally intact 30S subunits. Several indirect arguments support this conclusion.

Protein S12 has been shown to be located in the immediate neighbourhood of protein S4 by chemical crosslinking (1) and neutron scattering (39). It is also known that streptomycin dependence caused by mutation of S12 can be reversed by a mutation in S4(40). Since S4 has been shown to associate with the 5'terminal region of 16S RNA (4) or domain I as defined in (41),and since the fragment crosslinked to protein S12 is found near the 3'end of 16S RNA, i.e. in domain III (41),these results suggest two possibilities. Either protein S12 is situated between widely separated domains I and III or these two domains approach each other closely within the 30S subunit.

According to the model of Lake (42), the position of S12 lies near the cleft of the 30S subunit, but on the surface facing away from the 50S subunit. Crosslinking data suggests that at least part of protein S12 must extend near the interface since it has been crosslinked to proteins L2,L16, L21 and L32 (43). Initiation factor 3 has also been crosslinked to S12(44,45) and its site of

attachement to 30S subunits has been localized near the cleft as well (42). Consequently, protein S12 appears to be situated near the region of the 30S subunit which is actively involved in initiation of protein synthesis.

Furthermore, UV irradiation of 70S-$tRNA^{val}$complexes containing $tRNA^{val}$ with 5 carboxymethyluridine in the wobble position bound to the P site leads to formation of a covalent bond between this residue and C1400 of 16S rRNA (46 and Ehresmann, C., personal communication). Since the distance between C1400 of 16S rRNA and the CAACUCG fragment (1316-1322) is relatively small, proximity of protein S12 and this fragment is consistent with the possible role of this protein in the decoding process and with its position in the 30S subunit.

Crosslinking of protein S12 to G_{1322} does not imply that sequence CAACUCG is the only region of 16S RNA which is close to or interacts with S12. Three general conditions are required for the formation of a protein-RNA crosslink :

(1) RNA and protein must be associated in a temporally and spatially stable relationship,(2) regions of close approach of the associated molecules must be accessible to the crosslinking reagent and (3) these regions must contain the appropriate reactive groups close to one another. Regions of close approach which do not fulfill these conditions may exist.

Finally we note that the heptanucleotide CAACUCG forms part of a hairpin loop, according to secondary structural models which are supported by phylogenetic comparisons (41,47,48). This region varies considerably in primary sequence, but displays a remarkably constant size and secondary structure over a wide phylogenetic range (49). These features may thus be of fundamental importance with respect to the structure of the 30S subunit and may be related to the functional importance of its neighbour, protein S12.

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