STUDIES OF THE BINDING OF *E. COLI* RIBOSOMAL PROTEIN S4 TO 16S rRNA AFTER UV IRRADIATION OF THE S4–16S rRNA COMPLEX

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1. Introduction

In the *E. Coli* ribosome several proteins directly bind to the 16S rRNA [1–5]. Conversion of these specific non covalent interactions to covalent cross-links could be of great help for studying the binding sites between a particular protein and the 16S rRNA.

It is now well known that upon u.v. irradiation covalent bonds are formed between polynucleotides and proteins. This technique was used to link covalently together DNA and DNA polymerase [6], synthetic $d(A\ T)_n$ and RNA polymerase [7], aminoacyl-tRNA synthetase and tRNA [8,9], lac operon and lac repressor [10], cyclic AMP and high affinity macromolecular receptors [11], polynucleotide and protein in virions [12,13]. We began this study with ribosomal protein S4 and 16S rRNA for different and obvious reasons. The primary structure of 16S rRNA is almost completely determined [14]. Protein S4 plays an essential role in the Nomura's assembly map [15] and its primary sequence is known [16]; it has been shown that in the specific 16S rRNA–S4 complex, S4 protects as much as one third of the RNA molecule against mild ribonuclease T_1 digestion [17–20] and this enhances the possibility of covalent cross-links between RNA and protein after u.v. irradiation; the contacts between protein S4 and 16S rRNA have been studied [5,17–22]. Recently the lysine residues of protein S4 involved in the binding with 16S rRNA were identified [23] and the C-terminus part of protein S4 was found to be involved in this binding site [24].

The results reported here show the specificity of this cross-linked 16S rRNA–S4 complex after u.v. irradiation; the tryptic peptides of S4 corresponding to the contacts between the protein and the I6SRNA have been characterized.

2. Materials and methods

Unlabelled 16S rRNA and labelled 16S-[^{32}P] rRNA from *Escherichia coli* MRE 600 were isolated as described previously [4,25]. Protein S4 was purified and identified as described earlier [26,27] and the complex 16S rRNA-protein S4 was prepared by the technique of Garrett et al. [4]. We used 0.1 nmol 16S [^{32}P] rRNA for labelled complexes and 2 nmol 16S rRNA for unlabelled complexes; protein S4 was added at a 3 to 5 molar excess over 16S rRNA. Under these conditions Garrett et al. [4] obtained a molar ratio protein : RNA of approximately 1:1.

Irradiation procedure : the 16S rRNA–S4 complex was placed on a glass plate covered wtih parafilm, on ice, irradiated for varying times at a distance of about 7 cm from two parallel 15 W u.v. tubes (ref. C15H18).

The labelled protein–RNA complex could be retained by filtration on nitrocellulose filters (Sartorius), while free RNA and protein passed through the filters. For isolation of the 16S rRNA–S4 complex and its separation from free RNA and S4, cesium chloride gradients were used. The formed complex, irradiated or not, was laid out onto 4.5 ml of a solution of CsCl (density 1.50) in TM4 buffer (Tris–HCl pH 7.5, 10^{-2} M, Mg acetate 10^{-4} M) or in TM2 buffer (Tris–HCl pH 7.5, 10^{-2} M, Mg acetate 10^{-2} M). The gradient was established by a 16 h centrifugation with a SW 50 rotor in a Spinco L75 at 4°C at 46000 rev/min. Fractions were collected and the radioactivity or the absorbance were measured. The 16S rRNA–S4 com-

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plex was collected, dialysed 48 h against high volumes of water, precipitated with 2.5 vol of ethanol, dissolved in water and then submitted to total tryptic digestion. The tryptic digestion was performed under rather drastic conditions to facilitate the attack of S4 in the complex by trypsin. A high molar ratio between the enzyme and the substrate was employed : 1 to 30. The reaction was performed in 0.1M *N*-methyl-morpholine buffer pH 8.0 for 3 h. The tryptic peptides were separated by a fingerprinting technique on cellulose thin layer plates. (Polygram Gel 400, Macherey-Nagel, Germany). The first dimension was an electrophoresis (400 V 1.5 h) and the second dimension was a chromatography in pyridin-*n*-butanol–acetic acid–water solvent (50:15:75:60, vol/vol). The peptides were stained by ninhydrin reagent.

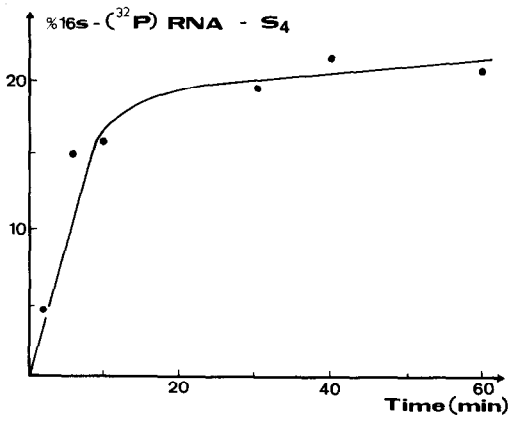

Fig.1. Percentage of 16S [^{32}P] rRNA cross-linked to protein S4 for various times of u.v. irradiations, measured by filtration on nitrocellulose filters, under dissociating conditions (urea 8 M – SDS 0.1%).

$$\%\ \text{u.v. cross-linked complex} = \frac{(\text{u.v. complex} - \text{non-u.v. complex})^{\text{dissociating condition}}}{\text{non-u.v. complex}}$$

3. Results and discussion

The formation of a stable specific complex between the ribosomal protein S4 and 16S rRNA by u.v. irradiation was studied by different approaches and several experiments were carried out to determine the specificity of the cross-linking reaction.

Our first assays were made by filtration on nitrocellulose filters. With this technique we were able to retain the labelled 16S[^{32}P] rRNA–S4 specific complex, while the free RNA and protein S4 passed through the filter. This kind of experiment was useful to optimise the conditions of u.v. irradiation, but the precision was not very high because the background of radioactivity retained on the filter was important. However the results were significant enough to establish the formation and specificity of the cross-linking reaction.

When a non covalent specific complex, formed between 16S rRNA and protein S4, was placed under dissociating conditions (urea 8 M–SDS 0.1%), the 16S [^{32}P] rRNA was eliminated by filtration on nitrocellulose filters. When the formed complex was irradiated by u.v. light, the radioactivity retained by the filter corresponded to the cross-linked complex formed; under the same conditions the radioactivity kept by the filter after filtration of the non-irradiated complex corresponded to the blank.

Fig.1 gives the percentage of 16S [^{32}P] rRNA cross-linked to protein S4 with respect to the radioactivity retained by the filter after filtration of the non irradiated complex, filtered under non dissociating conditions, for various times of irradiation. The yield of cross-linked complex reaches about 20% after 15 min of irradiation.

To determine the specificity of the formation of u.v. cross-links between 16S rRNA and protein S4, several controls were carried out. Under conditions suitable for complex formation when the 16S [^{32}P] rRNA was placed in the presence of non specific protein such as bovine serum albumine or protein L24 (a protein from 50S ribosomal subunit), and then irradiated, no radioactivity was retained on the filter. The same result was found when the 16S [^{32}P] rRNA and protein S4 were placed together in the same solution but under conditions which did not allow the formation of a complex, and then irradiated. These results seem to show that covalent specific u.v. cross-links are possible only with a specific protein and only when the specific complex is formed before the u.v. irradiation.

To study the u.v. cross-linked complex, it was necessary to separate the covalent complex from the non covalent one and to eliminate the free 16S rRNA and protein S4. The use of cesium chloride gradients allowed the isolation of the irradiated complex (fig.2).

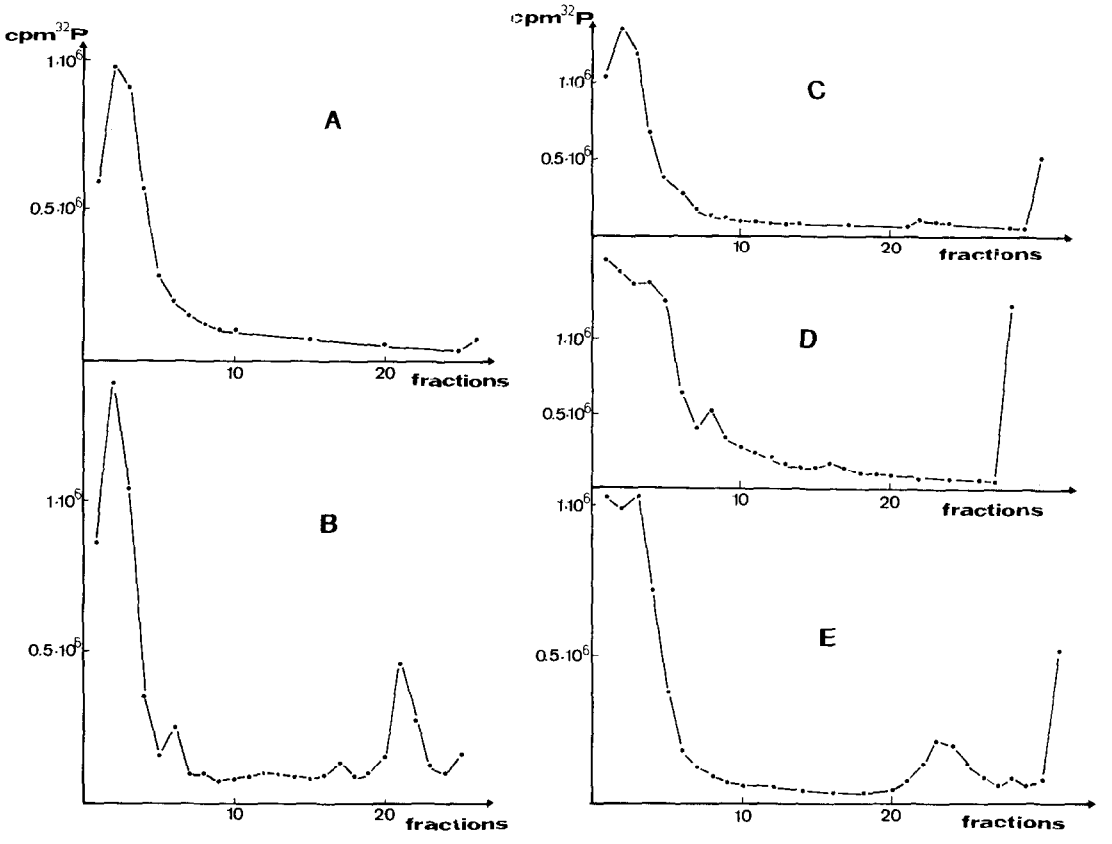

Fig.2. Cesium chloride gradients of: (A) 16S [^{32}P]rRNA (CsCl in TM2 buffer); (B) 16S [^{32}P]rRNA–S4 complex (CsCl in TM2 buffer); (C) 16S [^{32}P]rRNA–S4 complex (CsCl in TM4 buffer); (D) u.v. irradiated 16S [^{32}P]rRNA (CsCl in TM4 buffer); (E) u.v. irradiated 16S [^{32}P]rRNA–S4 complex (CsCl in TM4 buffer).

Figs.2A and 2B give respectively the position of free 16S rRNA and of the 16S rRNA–S4 complex under non-dissociating conditions (TM2 buffer) on the cesium chloride gradients. Fig.2C shows that under dissociating conditions (CsCl in TM4 buffer) the complex was dissociated during centrifugation. The position of the irradiated 16S rRNA is indicated on fig.2D. On fig.2E the specific 16S rRNA–S4 complex was irradiated 30 min then centrifuged under dissociating conditions: no dissociation was observed with the irradiated complex. The peak corresponds to the cross-linked complex, free of non-bound 16S rRNA and protein S4 which is found on the top of the gradient as can be seen from absorbance measurements.

The cross-linked complex was collected, precipitated, dissolved and submitted to total tryptic digestion as described in materials and methods. To study the binding sites of protein S4 with 16S rRNA unlabelled RNA was used. The tryptic peptides from cross-linked and non cross-linked S4 were separated by the fingerprint technique described in materials and methods. These fingerprints are illustrated on figs.3 and 4. The position of the tryptic peptides was assigned from the fingerprint on paper described by Schiltz and Reinbolt [28] and from some information kindly provided by Dr Yaguchi, Berlin. Comparison of the peptide maps revealed differences in some ninhydrin-stained spots. Thus peptides T_{13}, T_{14}, T_{14} + Lys, T_{20}, T_{20} + Lys stained very weak with ninhydrin on the fingerprint of cross-linked S4. These peptides correspond to the position 144–151 in the sequence proposed by Reinbolt and Schiltz [16] and would

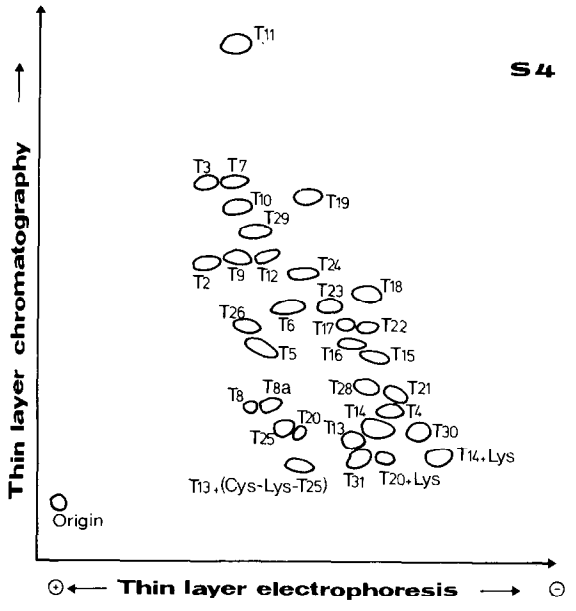

Fig.3. Tryptic fingerprint of protein S4.

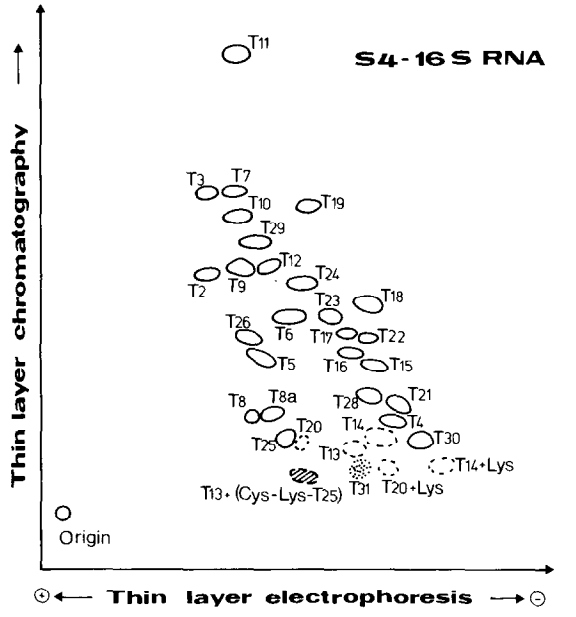

Fig.4. Tryptic fingerprint of protein S4 in the UV irradiated 16S rRNA–S4 complex; ( ) Peptides T13, T14, T14 + Lys, T20, T20 + Lys stained very weakly with ninhydrin; ( ) Peptide T31 stained weakly with ninhydrin; ( ) Peptide Cys–Lys-T25 did not stain well with ninhydrin.

be retained on the 16S rRNA after tryptic digestion of protein S4 cross-linked covalently to 16S rRNA by u.v. irradiation. This part of the sequence has a very high proportion of basic aminoacids and essentially lysine residues:

144 147 151
Glu–Lys–Ala–Lys–Lys–Glu–Ser–Arg

These results allow us to conclude that some of these aminoacids are involved in interactions with the 16S rRNA. It is interesting to observe that Amons et al. [23] found after reductive methylation of lysine residues of protein S4, that Lys-147 could be a part of 16S rRNA binding sites. This agrees very well with out results.

Peptide T13 also is located in position 56–59 of S4:

56 59
Glu–Lys–Gln–Lys.

Therefore this region of the sequence which presents basic properties, could also be involved in interactions with 16S rRNA.

Another peptide:

181 185
T31 : Arg–Lys–Pro–Glu–Arg

did not stain well with ninhydrin after u.v. irradiation of the 16S rRNA–S4 complex. It seems that this peptide, with several basic residues, can interact with 16S rRNA. The ninhydrin stained spot of the peptide Cys–Lys + T25, position 31–43, also seemed decreased and the peptide could be involved in interaction with the 16S rRNA.

Two peptides, T1 and T32, could not be characterized on a paper fingerprint. They do not move from the origin. On a t.l.c. fingerprint they could not be seen either. For this reason a comparison of the peptide maps of non cross-linked and cross-linked S4 did not allow us to determine whether the peptides, Tl and T32, are involved in contacts with 16S rRNA. Besides, it would be interesting to investigate whether the C-terminus part of protein S4 (T32) interacts with 16S rRNA, as shown by Daya-Grosjean et al. [24].

In conclusion it appears that a minimum of 2 or 3 clusters of basic regions of protein S4, position 144–151, 181–185 and perhaps 56–59 can form covalent bonds with 16S rRNA after u.v. irradiation of the 16S rRNA–S4 specific complex. Another region of S4 : position 31–43 could be involved in the S4–16S rRNA contacts. These regions are underlined in the sequence of S4 given in fig.5.

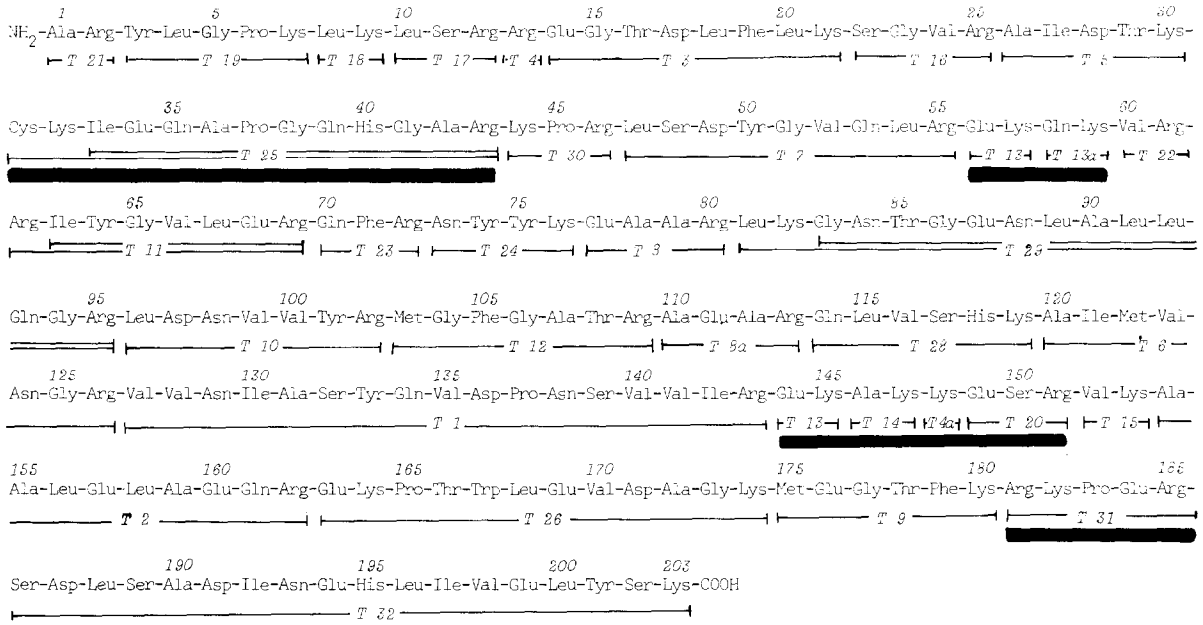

Fig.5. Sequence of protein S4. The tryptic peptides which are cross-linked to the 16S rRNA in the complex are underlined.

In the future we hope to determine more precisely these regions of the sequence of S4 that are involved in interactions with 16S rRNA. It can be easily foreseen that the u.v. irradiation method would also help to locate in the sequence of 16S rRNA the regions involved in the binding sites with protein S4. Such studies are under way.

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