CHARACTERIZATION OF THE REGIONS FROM *E. COLI* 16 S RNA COVALENTLY LINKED TO RIBOSOMAL PROTEINS S4 AND S20 AFTER ULTRAVIOLET IRRADIATION

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Received 18 April 1977

1. Introduction

Protected RNA regions containing the binding sites of four 30 S ribosomal proteins (S4, S8, S15 and S20) have recently been prepared from reconstituted single protein 16 S RNA complexes by mild enzymatic digestion of the non-protected RNA regions [1]. The area protected by protein S4 encompasses about 500 nucleotides in discontinuous regions extending from sections L to C″, in the 5′-region of the 16 S RNA molecule. The S20-protected region lies within the S4-protected region, stretching from section H″ to section M and comprises about 300 nucleotides. Although S4 is known to be an elongated protein [2], it seems unlikely that it could interact with such a large RNA region and it is probable that these proteins bind to a number of sites within the depicted RNA regions that must be maintained in a stereochemical configuration due to RNA–protein and RNA–RNA interactions. Such RNA–RNA interactions have recently been shown to exist within the protein S4-RNA binding site of 16 S RNA [3], and two main interacting sites have been located between sections H″–H and I″–C″.

It was necessary to discriminate between the respective contribution of these RNA–RNA and protein–RNA interactions. The use of ultraviolet irradiation to form photochemical covalent bonds between the 16 S RNA and a ribosomal protein is a reliable method to check RNA regions which are interacting with the protein. This technique was successfully used to covalently link RNA or DNA and specific proteins in several cases (for references, see [4]). In the case of the ribosome, it has been shown that the irradiation of 30 S [5] and 50 S [6] subunits using high doses of ultraviolet light allowed the covalent binding of almost all the ribosomal proteins to the 16 S or 23 S RNAs. Using mild conditions, only proteins S7 and L4 could be covalently linked to the 16 S and 23 S RNAs, respectively [7] and the 16 S RNA region linked to protein S7 has now been characterized [8].

In a previous paper, the specificity of the photoreaction was demonstrated [9] and the tryptic peptides from proteins S4 [9] and S7 [10], photochemically linked to the 16 S RNA complexes, were identified.

In this paper, we report the sequences of the RNA regions which can be photochemically linked to proteins S4 and S20 after ultraviolet irradiation of the specific S4–16 S RNA and 20 S–16 S RNA complexes.

2. Materials and methods

The ^{32}P-labeled 16 S RNA was prepared as described previously [11] by phenol extraction from isolated 30 S subunits in the presence of 0.01 M Tris–HCl, pH 7.5; 0.001 M $MgCl_2$; 0.1 M KCl; 0.006 M mercaptoethanol.

Protein–16 S RNA complexes were prepared according to Garrett et al. [12] by incubating the protein and RNA for 1 h at 42°C in reconstitution buffer (0.03 M Tris–HCl, pH 7.4; 0.02 $MgCl_2$; 0.35 M KCl) at a final concentration of 1 mg RNA/ml. Proteins were added at a 3–5 : 1 molar excess over 16 S RNA. Purified S4 and S20 proteins from *E. coli* were kindly given by Professor Wittmann (Berlin) and Professor Daune (Strasbourg) respectively.

The irradiation procedure has been described in a

preceding paper [9]. The irradiation time used was 30 min and under these conditions, about 20–30% of the RNA becomes crosslinked to the proteins.

The complexes, irradiated or non-irradiated, were digested with T_1 RNAase for 30 min at 4°C, at a T_1 : RNA ratio of 333–1666 units/mg RNA. The resultant ribonucleoprotein particles (RNPs) were then isolated as described earlier [1] on 8% polyacrylamide gel slabs containing 0.01 M Tris acetate, pH 8.0, 0.01 M Mg acetate, at 4°C, for 16 h at 500 V and 40 mA. After autoradiography, the specifically protected RNP was excised and dissociated using the procedure described in ref. [1], involving the dialysis of the complex against 8 M urea followed by fractionation on composite polyacrylamide gel slabs containing 10–15% acrylamide, 8 M urea, 0.09 M Tris borate, pH 8.3, 0.0025 M EDTA with a 2 cm layer on top of the gel containing 0.1% sodium dodecyl sulfate. During this treatment, the non-covalently bound RNA fragments become dissociated from the protein whereas the linked RNA fragments remain attached.

After autoradiography, the subfragments were excised and eluted electrophoretically [13] and subsequently digested with T_1 RNAase and phosphatase (and in several cases pancreatic RNAase) for fingerprinting [14]. The nomenclature used for the oligonucleotides and fragments is that described by Ehresmann et al. [15].

3. Results and discussion

In a preceding paper, it has been shown that the photochemical reaction involving the 16 S RNA is specific for the 30 S proteins [9], proteins such as serum albumin or 50 S ribosomal proteins not being linked. Furthermore, in order to form a covalent bond between the protein and RNA, a stable complex must be present before the irradiation step.

It has been verified that under these experimental conditions, the irradiation does not alter the mobility of the 16 S RNA during polyacrylamide gel electrophoresis with the exception of a small amount of RNA which remains aggregated on the top of the gel, suggesting that RNA–RNA crosslinks occur to a very low extent. Controls were also made to show that the irradiation does not alter the ability of the RNA to form S20–16 S RNA and S4–16 S RNA complexes (results not shown).

For both proteins S4 and S20, the protein–16 S RNA complexes were divided in two parts, of which only one was irradiated, the other being used as a control. During the first step, the RNP particles were isolated on polyacrylamide gels containing Mg^{2+} and no difference of mobility was detected between irradiated and non-irradiated samples. After the urea–SDS treatment, the non-covalently bound fragments were removed from the protein and differences between the dissociation patterns of the irradiated and non-irradiated samples were observed (see figs 1 and 2). In the case of the non-irradiated complex, the isolated RNP particle contained the protein and RNA fragments which are maintained together as a result of RNA–protein and RNA–RNA interactions. In the case of the RNP particle from the irradiated complex, some, if not all, of the RNA–protein interactions have been converted into covalent bonds. The protein-linked RNA fragments should then be expected to migrate more slowly, due to the presence of the linked protein. This new electrophoretic mobility depends directly on two different factors:

(i) The size of the RNA fragments in relation to the size of the protein (the smaller the RNA fragment, the greater the effect of the protein on the migration delay).

(ii) The presence of one or several RNA fragments bound to the same protein molecule. Consequently, a decrease of the yield of the corresponding RNA fragments may be detected by comparison with the control pattern.

In the case of the S4–16 S RNA complex, experiments were performed using increasing T_1 RNAase hydrolysis conditions (333–1666 units/mg RNA). The dissociation products of the non-irradiated material were identical to those already described by Ungewickell et al. [1], using the same conditions. At low T_1 : RNA ratios, a large fragment, identified as section BI′I was strongly and reproducibly reduced in intensity, as well as subfragments containing section I; small fragments corresponding to part of section C″ and in several cases to section M were also reduced, but in a more variable manner. Additional material observed near the origin, which could not be analysed because of the low ^{32}P-activity, is thought to correspond to the linked protein–RNA material.

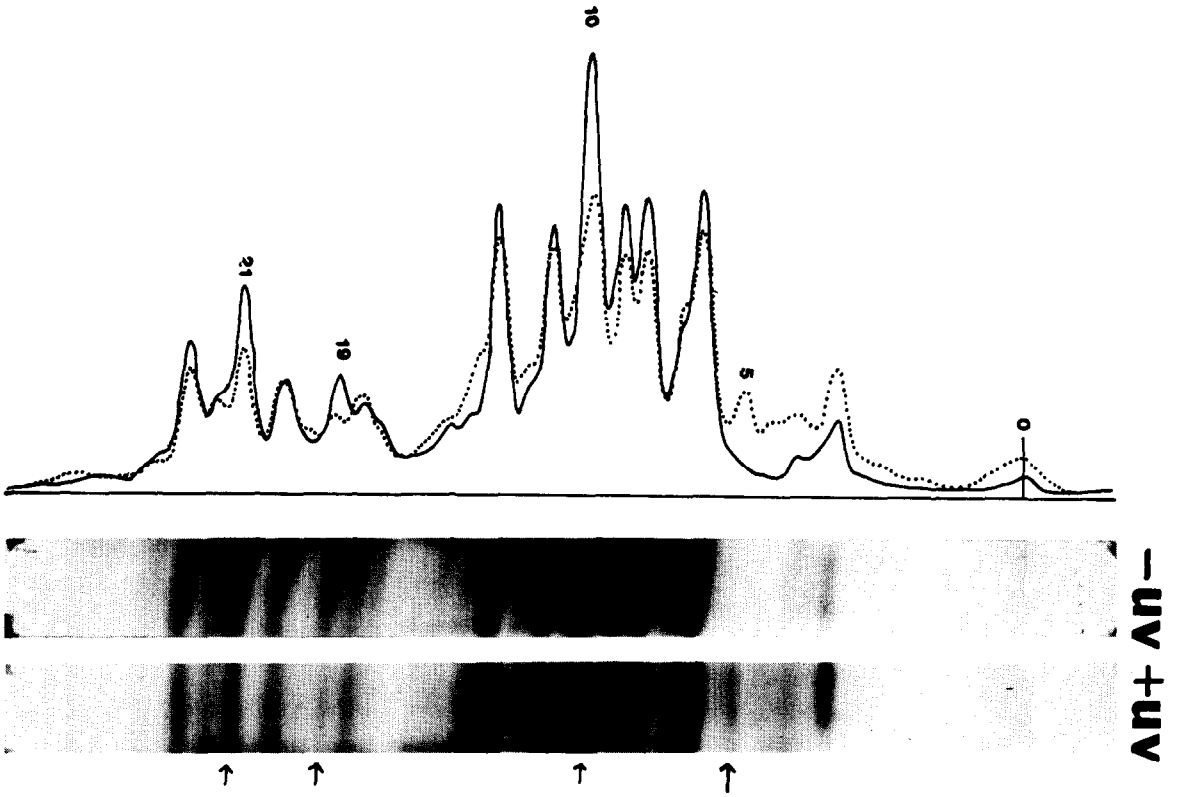

Fig.1. An autoradiogram of the dissociation products from the RNPs obtained after T_1 RNAase hydrolysis of the S4-RNA complexes, both irradiated and non-irradiated. The hydrolysis conditions used were 833 T_1 units/mg RNA. The fractionation was carried out by electrophoresis through a 12–15% polyacrylamide gel in the presence of urea. Densitograms from the irradiated (- - - -) and non-irradiated (——) samples are shown.

By increasing the extent of hydrolysis, smaller subfragments were obtained and a fragment corresponding to section I (band 10, fig.1) was strongly and reproducibly decreased in intensity. Moreover, a new band was clearly present (band 5, fig.1). This new product was eluted and analysed and its RNA content found to be identical to that of fragment 10, i.e., section I. Although the presence of the S4 protein could not be directly shown, due to the very low amount of material, only the presence of the linked protein can explain the alteration of the migration of this RNA component. In addition, the yields of several small fragments were decreased (for example bands 19 and 21, fig.1), corresponding to a part of section C'' and in several cases, to subfragments from sections I or M. Occasionally, subfragments from section L showed a decreased intensity, but such results should be integrated with care, because section L behaves in a very variable manner and the variation observed cannot be related with certainty to the irradiation process.

The results obtained from the small fragments are not so clear as those obtained in the case of section I because the decrease in yield is variable and new bands containing the linked protein could not be identified. Two main reasons can be proposed:

(i) the different photochemical linkages do not occur at the same rate for every binding site and section I seems to be a preferential site, explaining the variability observed in the case of these small fragments and the difficulties of detection of the linked material.

(ii) the photochemical linkages can occur on different RNA molecules; on a single 16 S RNA molecule, any number of covalent attachment points

can be formed, leading to a distribution of the RNA-protein linked material into a range of RNP particles migrating to different positions on the gel. In fact, several bands, insufficiently labelled for analysis, can be observed within the dissociation pattern of the irradiated sample.

In the case of the S20–16 S RNA complex, only one fragment was found to be strongly decreased in intensity (fragment 8, fig.2). This fragment was identified as an RNA region comprising (F) QR (G) (see fig.3). The other subfragments present were analysed and found to correspond to those RNA regions already described by Ungewickell et al. [1]. An additional product was evident (band 6, fig.2), its RNA content was analysed and found to be identical to band 8, i.e., (F) QR (G). In this case also, the retarded migration can only be explained by the presence of the linked protein.

The results are summarized in fig.3. Protein S4 was found to be linked mainly to an RNA fragment encompassing 74 nucleotides, namely section I and, to a weaker and more variable extent, to part of sections C″ and M (and perhaps to a part of section L). It is of interest that these attachment sites (with the possible exception of section L) are all located within the 3′-half of the protected RNA region. Because it has been shown that RNA–RNA interactions exist between the two halves [3], namely between H″H′H and I″ (C″), it can be suggested that protein S4 interacts directly with several parts of the 16 S RNA (in section I, C″, M) and that the 5′-region (L)–(Q) is maintained within the complex by RNA–RNA inter-

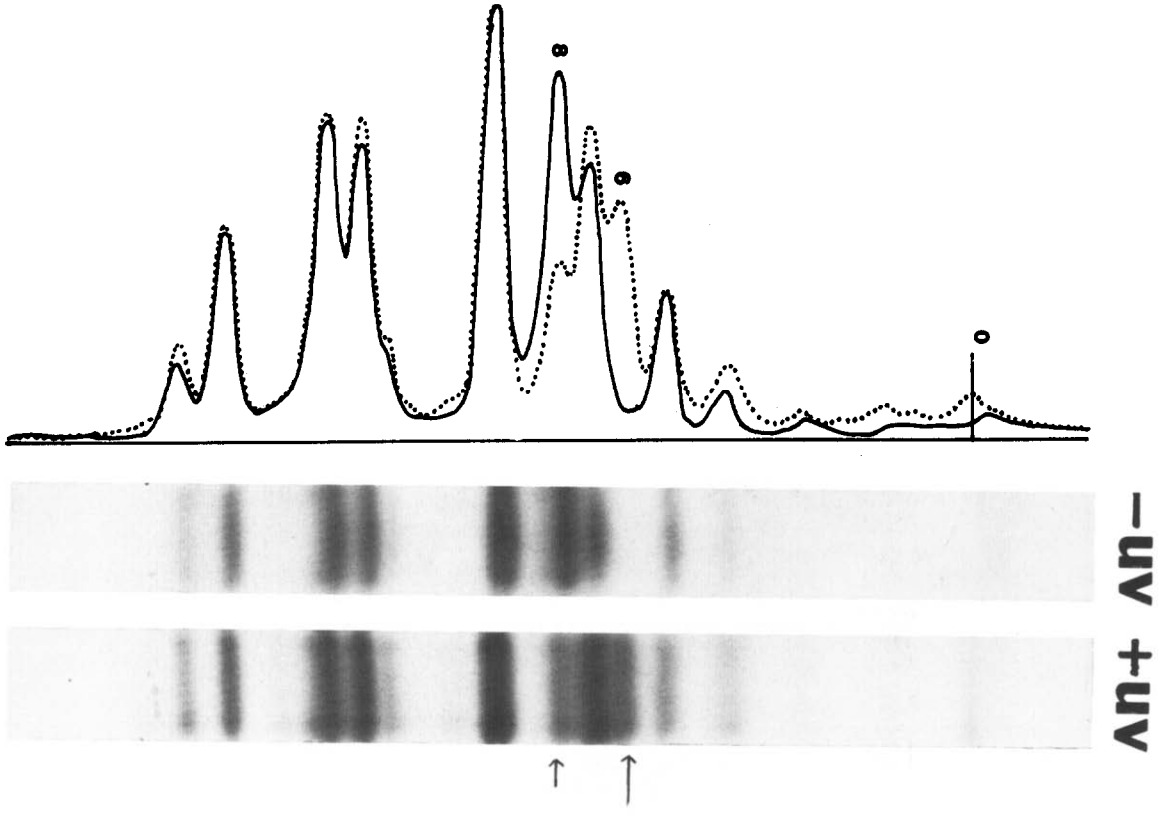

Fig.2. An autoradiogram of the dissociation products from the RNPs obtained after T_1 RNAase hydrolysis of the S20-RNA complexes, both irradiated and non-irradiated. The hydrolysis conditions used were 666 T_1 units/mg RNA. The fractionation was carried out by electrophoresis through a 12–15% polyacrylamide gel in the presence of urea. Densitograms from the irradiated (- - - -) and non-irradiated (——) samples are shown.

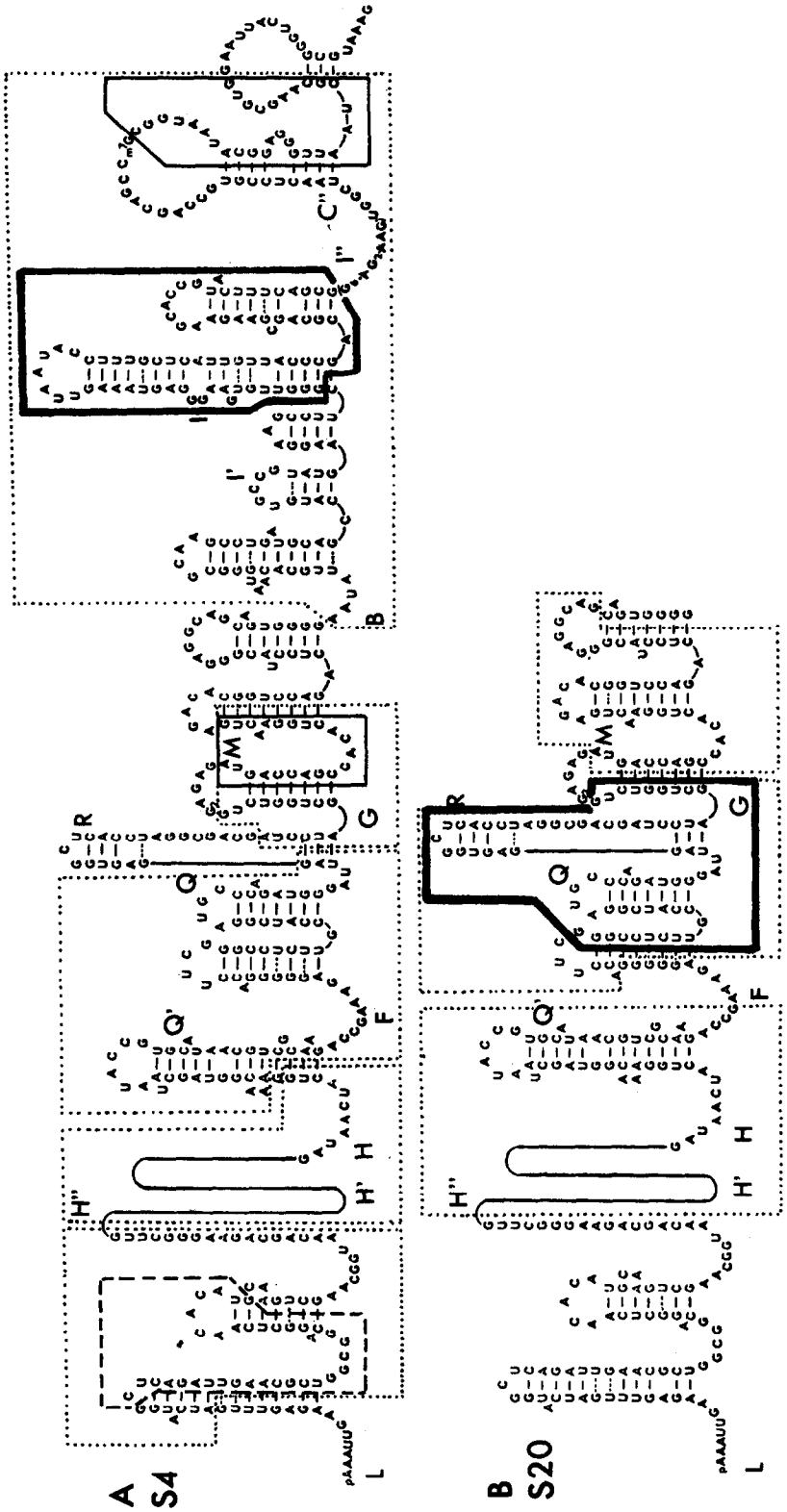

Fig.3. The nucleotide sequence and proposed secondary structure of RNA regions covalently linked to proteins S4 (A) and S20 (B). The sequences enclosed in the square (. . . .) indicate the RNA regions protected by proteins S4 and S20 according to ref [1]. The sequences enclosed in the square (——) indicate the RNA fragments strongly linked to either protein S4 or S20. The sequences enclosed in the square (——) indicate the RNA fragments linked to protein S4 to a weaker and more variable extent. The sequences enclosed in the square (- - - -) indicate the RNA fragment which cannot be shown with certainty to be linked to protein S4. The secondary structure model proposed is slightly different from that proposed in ref [1]. This model has been corrected in accordance with recent sequence improvement, especially in section C'' (Ehresman, C., Stiegler, P. and Ebel, J. P., unpublished results).

actions. These RNA–RNA interactions may have a possible role in the stabilisation of the complex. In the case of protein S20, only one fragment could be linked, encompassing 80 nucleotides and corresponding to section (F) QR (G), arising from the middle part of the S20-RNA protected region (fig.3). It is important to note that this fragment contains the part of the RNA which is excluded from the S4-protected region (Q) (R). The remaining part of this fragment: (F) (Q) is present within the protected S4-RNA region but cannot be linked to protein S4. This suggests that, although the protein-protected areas have been found to be overlapping, the sites of interaction between the 16 S RNA and these two proteins must be distinct.

Nevertheless, the presence of other binding sites cannot be completely excluded and it may be possible that a particular kind of protein–RNA interaction, required for the formation of such photochemical bonds, is selected for under these conditions.

Work is now being carried out in order to localise more precisely the nucleotides involved in the photochemical bonds, in relation to those peptides which can be demonstrated as cross-reacting with the RNA. It should be noted that 3 or perhaps 4 different regions within protein S4 have been shown to form covalent bonds with the 16 S RNA after ultraviolet irradiation [9], but as yet is too early to draw any definitive relationship between them and the 3 or 4 RNA regions which have been discussed here.

Acknowledgements

We thank Professor H. G. Wittmann (Berlin) and Professor M. Daune (Strasbourg) for their gift of proteins S4 and S20 used in this work. This work was supported by the 'Délégation à la Recherche Scientifique' and the 'Commissariat à l'Energie Atomique'.

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