A TOPOGRAPHICAL STUDY OF THE 5'-REGION OF 16 S RNA OF *ESCHERICHIA COLI* IN THE PRESENCE AND ABSENCE OF PROTEIN S4

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Received 20 June 1977

1. Introduction

It was demonstrated, earlier, that the digestion of either 16 S ribosomal RNA, or a complex of protein S4 with the 16 S RNA of *Escherichia coli*, at very low levels of soluble pancreatic ribonuclease, yields a resistant RNA fragment that sediments at about 9 S [1,2]. Oligonucleotide fingerprint analyses of the RNA fragment revealed that it derives from the 5'-end of the 16 S RNA. This RNA region (S4–RNA) interacts exclusively with protein S4 [1,2]. More recently, a similar RNA region was isolated by degrading a protein S4–16 S RNA complex with T_1 ribonuclease and the identities of (a) the subfragments contained within the RNA region and (b) the enzyme cutting positions, were established [3]. These RNA subfragments lie within a discontinuous region of sequence, extending from section L to C'', and constitute a total of about 500 nucleotides.

Since protein S4 (mol. wt 22 500) is small compared with the S4–RNA site (maximum molecular weight about 165 000), the basis of the protein protection, or stabilization, of the RNA site was assumed to be partly due to the protein, rendering a few critical sequence regions inaccessible to the ribonuclease, and partly to the secondary and tertiary organisation of the RNA. The former explanation has been reinforced recently by the finding that cross-links induced in the S4 protein–16 S RNA complex by ultraviolet radiation occur primarily within a small RNA region [4].

In the present work, the structure of the RNA region has been investigated, using carrier-bound pancreatic ribonuclease as a probe, and evidence is presented for the following:

(1) The RNA region is highly-structured such that it is very resistant to digestion with carrier-bound ribonuclease at 21°C.

(2) The RNA structure is essentially the same in the presence and absence of protein S4, although minor conformational heterogeneities may occur in the absence of the protein.

(3) A few accessible sites occur on the RNA structure at which ribonuclease cuts, or excisions of sequence, are produced in high yield.

2. Materials and methods

400 μg 16 S [^{32}P]RNA was renatured by incubating for 1 h at 40°C in TMK reconstitution buffer (300 mM Tris–HCl (pH 7.4), 20 mM $MgCl_2$, 350 mM KCl, 6 mM 2-mercaptoethanol) and cooling slowly to 0°C. Either renatured 16 S RNA or the S4–16 S RNA complex

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Paper number 3 on protein binding sites on 16 S RNA. The preceding paper was reference number 6.

North-Holland Publishing Company – Amsterdam

(prepared as described earlier [3]) was then digested in 0.15 ml with carrier-bound pancreatic ribonuclease (Boehringer) at a nuclease : RNA ratio (w/w) of between 0.6 and 0.9:1, by rapid mixing for 5 h at 21°C. The solution was cooled quickly to 0°C before the enzyme was removed by centrifuging at 8000 × *g* for 5 min. The digest was electrophoresed in a 5% polyacrylamide slab gel containing 20 mM Tris-acetate (pH 8.0) and 5 mM Mg acetate, at 500 V for 15 h. The gel was run at 4°C and the buffer was circulated. The S4–RNA and S4–RNP were excised and the RNA subfragments were dissociated in the gel piece and resolved in a dodecylsulphate-EDTA-urea containing gel as described earlier [3]. The RNA dissociation products were excised, repurified electrophoretically, eluted and then fingerprinted [3].

3. Results and discussion

The isolation of the S4–RNA and S4–RNP, by polyacrylamide gel electrophoresis, is demonstrated in fig.1. The S4–RNP migrated more slowly than the S4–RNA; both were obtained in high yield. These

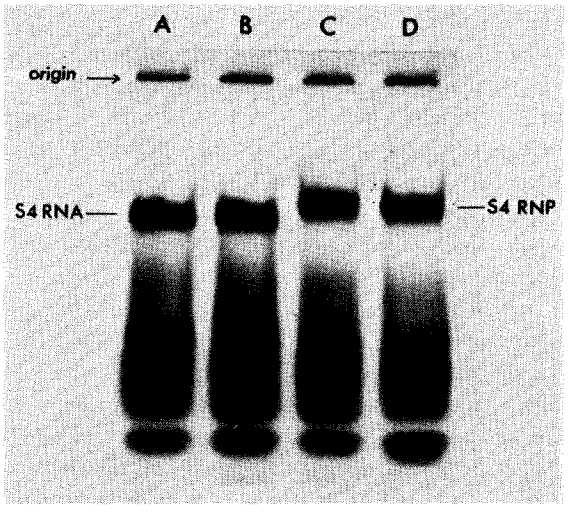

Fig.1. An autoradiogram showing the separation of the S4–RNA and S4–RNP in a polyacrylamide slab gel (see text for details). The following ratios of carrier-bound pancreatic ribonuclease : RNA (w/w) were used: for the renatured 16 S RNA A. 0.6 : 1, B. 0.9 : 1, and for the S4–16 S RNA complex C. 0.6 : 1, D. 0.75 : 1.

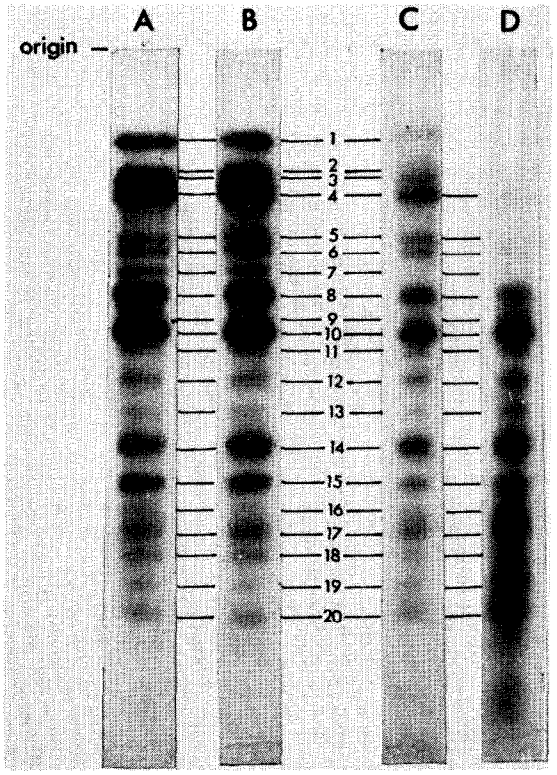

Fig.2. The RNA subfragment dissociation pattern of (A, B) the S4–RNA and (C, D) the S4–RNP, that are shown in fig.1. They were resolved in a 12–15% polyacrylamide gel containing 7 M urea. The subfragments are numbered.

two properties remain fairly constant over a wide range of enzyme hydrolysis conditions. The subfragment compositions of the two RNA moieties, after protein dissociation and denaturation, are presented in fig.2. The patterns are very similar. This suggests that the S4–RNA and the S4–RNP may contain the same subfragments and incur the same enzyme cuts. However, the following difference was observed in the relative yields of the subfragments: the RNA within the S4–RNP was slightly more susceptible to ribonuclease digestion than that in the S4–RNA, at the higher enzyme concentration, such that the larger subfragments from the S4–RNP tended to be more degraded into smaller ones.

Oligonucleotide fingerprint analyses, using T_1 ribonuclease, were made on the subfragments obtained from both the S4–RNA and the S4–RNP with the

following results. (1) No differences were found in the sequences of the corresponding subfragments. (2) At the lower enzyme digestion conditions, no differences in the yields of the subfragments were observed (fig.2). (3) Although the S4–RNP prepared at the higher enzyme concentration contained higher yields of the smaller subfragments, no new cutting positions were observed in the RNA sequence.

The sequence identitites of the subfragments shown in fig.2 are listed in table 1. The distribution of these subfragments along the RNA sequence and the approximate relative yields of the subfragments from samples A, B and C (fig.2) are summarised in fig.3A. The precise locations of the cutting positions produced by the carrier-bound pancreatic ribonuclease, are illustrated in fig.4.

Table 1

Subfragments from S4–RNA		Sections of 16 S RNA	Number of nucleotides
1		(R)GMBI′II″	232
2	(weak)	(M)BI′I + (B)I′II″(C″)	~150
3		(B)I′II″	137
4_1		(L)H″H′H(Q′)	129
4_2		(B)I′II″	116
5		H″H′H(Q′)	101
6		(I′)II″	92
7		(R)GM(B)	85
8_1		(H′)H(Q′)	69
8_2		(G)M(B)	71
9		(I)	59
10_1		(Q′)F(Q)	54
10_2		(I)	54
11		Mixture	
12_1		F(Q)	49
12_2		(G)(M)	45
13		Mixture	
14		(F)(Q)	32
15(a)		Mixture, very weak	
15(b)		(C″)	25
16 to 20		Mixture of very small subfragments	

The numbering of the subfragments corresponds to that given in fig.2. (a) and (b) represent subfragments that were resolved, subsequently, in a higher percentage gel. Subscripts 1 and 2 refer to two components in one band that were not resolved. The letters denoting the RNA sections were defined during the 16 S RNA sequence determination [7]. The bracketed letters indicate that only part of the RNA section is present. The sequences of subfragments 11, 13 and 15(a) were not determined because they contain several components (3 or more) in low molar yields. Fragments 16 to 20 contain mixtures of very small fragments (less than 20 nucleotides) and could not be analysed. The nucleotide lengths were calculated from the sequence (see fig.4).

Three important conclusions can be drawn from these results. They are considered separately below.

(1) The absence of any obvious differences in the major enzyme cutting positions in the S4–RNA and the S4–RNP indicates that the S4 protein binds to a part of the RNA structure that is inaccessible to the carrier-bound pancreatic ribonuclease but is accessible to the soluble pancreatic and T_1 ribonucleases (see below). Therefore, the protein must be partly buried in the RNA structure.

(2) The presence of the protein does not block any ribonuclease cleavage sites, nor does it produce any new ones. This indicates that the main secondary and tertiary organisation of the S4–RNA is essentially the same in the presence and absence of protein S4.

Although the S4–RNP was slightly more susceptible to ribonuclease digestion than the S4–RNA (see fig.2), the observed difference was not always as marked as shown in fig.2B,D. This variation was due to errors in pipetting small amounts of carrier-bound ribonuclease. Nevertheless, in a series of experiments the greater accessibility of the S4–RNP to ribonuclease was confirmed. This result may reflect some minor conformational heterogeneity in the S4–RNA. If, for example, the S4 protein binds preferentially to one of the more open conformations it could shift the equilibrium to this conformation thereby increasing the extent of cleavage in certain sites that were less accessible, on average, in the S4–RNA.

(3) The results obtained with carrier-bound pancreatic ribonuclease (see fig.3A) were compared with those obtained with soluble ribonucleases. This was not possible for the soluble pancreatic ribonuclease. In our experiments, the latter produced an S4–RNP that migrated similarly to that shown in fig.1, but it contained only RNA subfragments less than 30 nucleotides in length that were difficult to fractionate and analyse for sequence. The additional cuts that are produced by the soluble ribonuclease almost certainly reflect its greater penetrating power into the RNA structure. One S4–RNP prepared by a very mild digestion with pancreatic ribonuclease containing some larger RNA subfragments has been reported [5] and is considered below. T_1 ribonuclease, on the other hand, probably because of its more limited

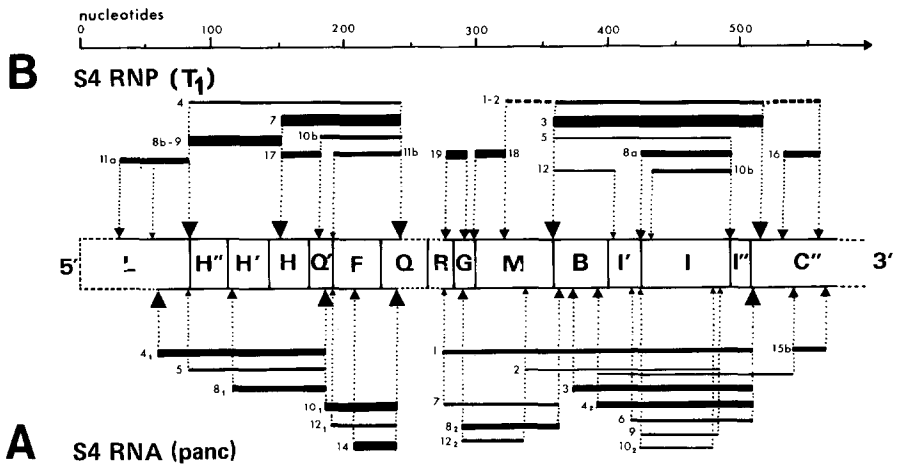

Fig.3. A plan showing the ordering of the RNA subfragments along the 5'-part of the 16 S RNA sequence. (A) Cutting positions in the S4–RNA, prepared with carrier-bound pancreatic ribonuclease, are indicated. Larger arrow-heads indicate major cutting positions. The thickness of the subfragment lines is proportional to their yield. (B) A similar plan showing the subfragments of the S4–RNP I produced by T_1 ribonuclease (as described in refs [3] and [6]). The amount of section L present was variable. The striated horizontal lines indicate that the amount of sequence present was variable and in low yield.

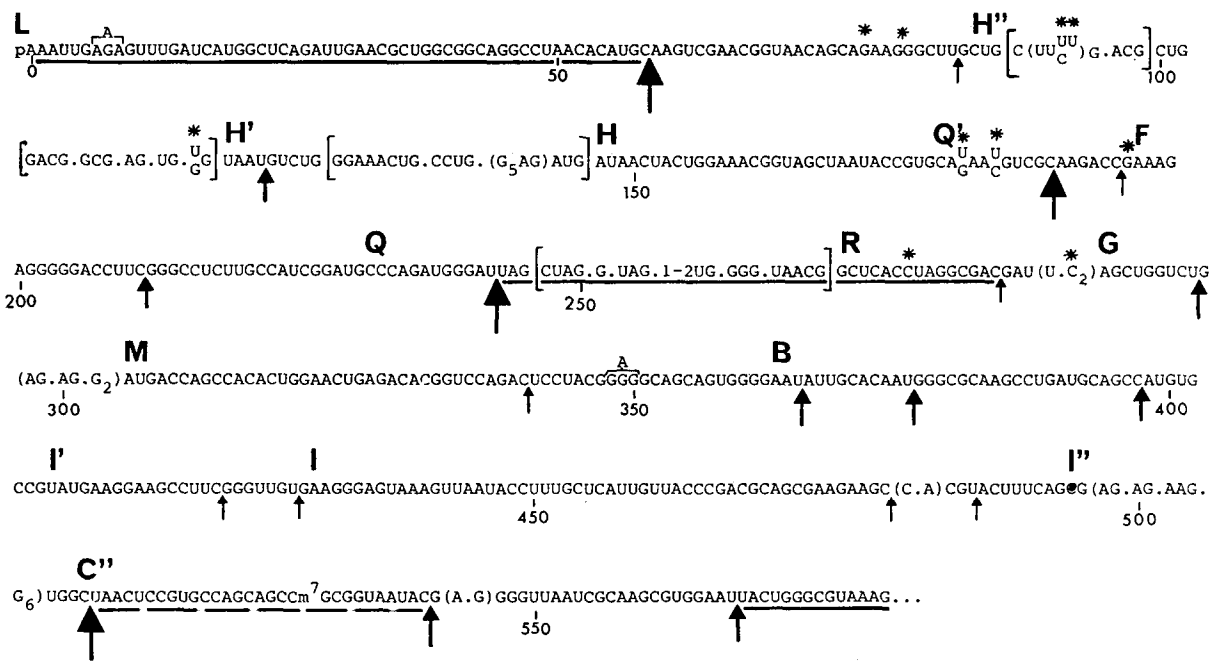

Fig.4. The sequence of the 5'-part of the 16 S RNA (according to refs. [7] and [8]) showing the precise positions of the cuts produced by the carrier-bound pancreatic ribonuclease. The larger arrow-heads indicate the cuts that occur in higher yields. (——) Denotes the sequence regions completely excised. (– –) Denotes the sequence region present in minor yield. (*) Denotes sequence heterogeneities.

specificity (cutting only at G-residues), yields an S4–RNP always containing relatively large RNA subfragments [3,6]. A map of its subfragments showing their relative yields, their positions along the RNA sequence and the enzyme cutting positions, is illustrated in fig.3B for comparison. There are the following striking similarities between the two maps:

(a) The extremities of the RNA region within the 16S RNA sequence are almost the same (section L to section C″).

(b) The sequence excisions in (i) section QR and (ii) section C″ occur in approximately the same positions. These excisions are compatible with the previously described interactions between widely separated sequence regions [6].

(c) Almost all the enzyme cuts that occur in the S4–RNA and S4–RNP, prepared with carrier-bound pancreatic ribonuclease, occur in neighbouring sequence positions in the S4–RNP prepared with T_1 ribonuclease. A few of these cutting positions have also been characterised, approximately, in an S4–RNP prepared after very mild treatment with soluble pancreatic ribonuclease [5] and our results are compatible with these.

A few differences are also apparent between the two RNA moieties shown in fig.3. Some of these can be attributed to the specificity differences of the enzymes. For example, the sequence AG·AG·G_2 in section G could only be excised by T_1 ribonuclease. Moreover, according to the tentative secondary structure model [3] only A and G residues are not base-paired in the part of section G that is excised by T_1 ribonuclease. Additional cuts that are only produced by T_1 ribonuclease, as found for example in section H, may also be due to the greater penetration of the soluble enzyme into the RNA structure.

In conclusion, this study has demonstrated (1) that the RNA region is highly structured, (2) that the RNA conformation is essentially the same in the presence and absence of S4 protein and (3) the identities of several points along the RNA sequence that are especially accessible to carrier-bound ribonuclease. The latter topographical information should prove particularly useful in model-building studies on this RNA region.

Acknowledgements

Dr J. P. Ebel is thanked for encouragement and support. The Délégation à la Recherche Scientifique et Technique, the Commissariat à l'Energie Atomique and the Deutsche Forschungsgemeinschaft provided financial support. EMBO travel grants were gratefully received by R.G.

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