FURTHER CHARACTERISATION OF THE RNA STRUCTURE IN THE BINDING REGION OF PROTEIN S4 ON 16 S RIBOSOMAL RNA OF *ESCHERICHIA COLI*

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1. Introduction

Protein S4 of the *Escherichia coli* ribosome assembles with a large region of RNA at the 5′-end of the 16 S RNA (reviewed in [1]). This RNA region (S4–RNA) is exceptional in that it can be organised into a compact structure, thereby creating a binding site for protein S4 (see [2]). Protein S4, itself, contains two distinct domains, namely the C-terminal three-quarters of the protein and the N-terminal quarter [3–5]. The former domain contains the primary RNA binding region of the protein, and three peptides have been identified in this region that cross-link towards the 3′-end of the RNA region [6–7]. The N-terminal domain is probably primarily involved in interacting with other proteins [4].

Earlier, a complex of the RNA region and protein S4 (S4–RNP II) was characterised for (a) the identities of the nucleotide sequences that it contains, (b) the positions of the ribonuclease cuts which yield important topographical information about the RNA structure, and (c) the approximate localisation of those interacting regions that are distantly separated in the RNA sequence and facilitate the folding of the 16 S RNA (e.g. 8–11). In the present work, we report analyses of a larger RNA region (S4–RNP I) that is found associated with protein S4 after mild T_1 ribonuclease digestion. The enzyme cutting positions occur in essentially the same positions as for the smaller S4–RNP II [2,9]. However, the larger RNA region contains additional sequences at the 5′- and 3′-ends and, also, three fragments that derive from the centre of the 16 S RNA and are separated by about 200 nucleotides of primary sequence from the S4–RNA region. Evidence is presented that these three fragments interact with the S4–RNA within the 16 S RNA structure, in the absence of protein S4, and it is proposed that this is a second interaction that facilitates the tertiary folding of the free 16 S RNA.

2. Material and methods

44 μg S4–16 S RNA complex containing about 10^7 cpm [^{32}P]RNA in 0.1 ml TMK reconstitution buffer (30 mM Tris–HCl, pH 7.4, 20 mM $MgCl_2$, 350 mM KCl, 6 mM 2-mercaptoethanol) was digested with T_1 ribonuclease (Sankyo, Japan) over the range 7 to 35 units for 20 min at 0°C. In a control experiment 44 μg protein-free 16 S RNA was digested over the range 7 to 20 units. In some experiments a mixture of 16 S [^{32}P]RNA + 23 S RNA was used with no difference in the results. The S4–RNP's were then

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isolated in an 8% polyacrylamide gel containing 20 mM Tris-acetate (pH 8.0), 5 mM Mg acetate [9]. Enzyme conditions were selected to give the maximum yield of the larger S4–RNP and it was prepared for RNA analysis starting with about 10^8 cpm 16 S [^{32}P]RNA. The RNA subfragments from the S4–RNP region were resolved in a dodecylsulphate-EDTA-urea containing gel [9]. Their yields were estimated by microddensitometry of autoradiograms. The subfragment bands were all excised, repurified on 15% or 20% gels, eluted and fingerprinted as described before [9].

3. Results

The kinetics of digestion of renatured 16 S RNA and of the protein S4–16 S RNA complex, are shown in fig.1. In the 16 S RNA samples, ribonuclease-resistant material was clearly discernible only at the lowest enzyme concentration (A_1) where two main bands occurred, namely RNA I and RNA II. In the S4–16 S RNA complexes, however, slower migrating bands were present over the whole nuclease range, although the larger S4–RNP I was only present over the lower part of the range.

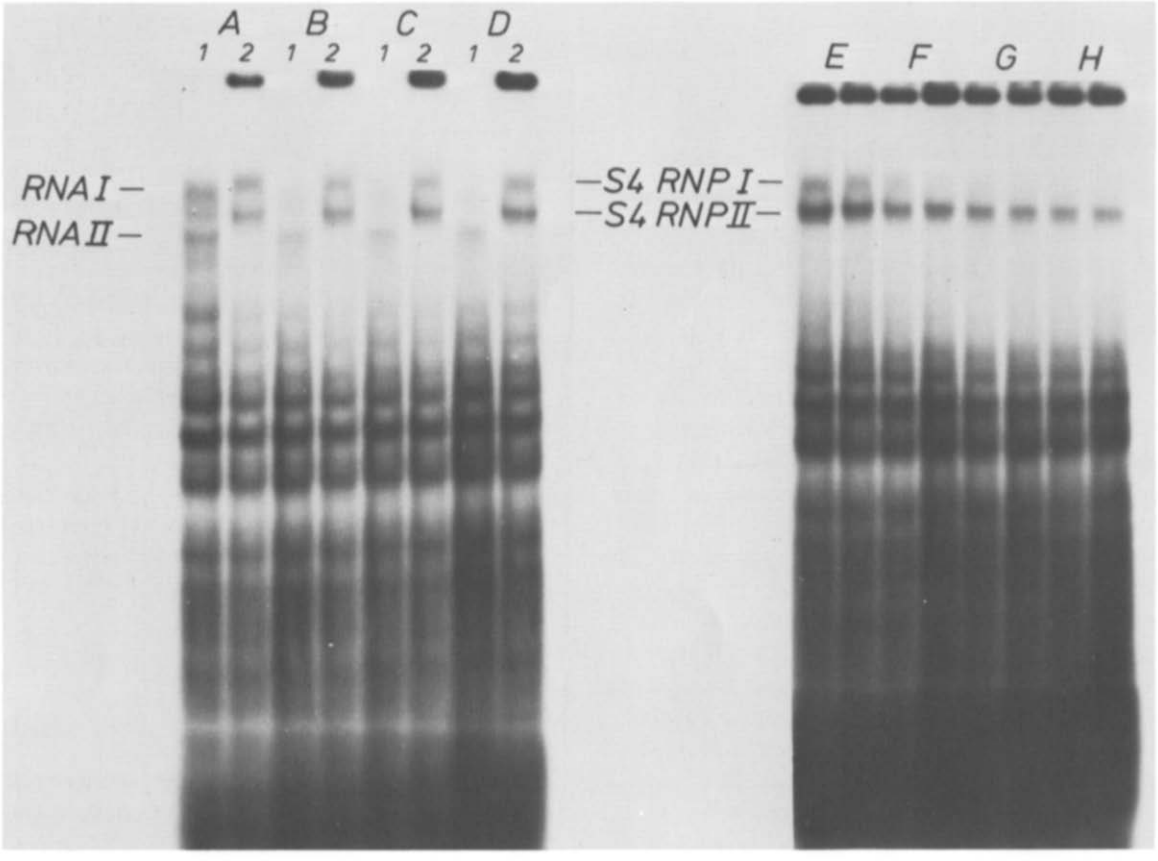

Fig.1. An autoradiogram showing the fractionated digests of 16 S (+ 23 S) RNA and the S4–16 S RNA complex (+ 23 S RNA) at increasing T_1 ribonuclease concentrations. In samples A_1, B_1, C_1 and D_1, 44 μg RNA was digested in the absence of protein with 7, 12.5, 15 and 20 units T_1 ribonuclease, respectively. In the remaining samples, namely A_2 to D_2 and E-H, 44 μg S4–RNA complex was incubated with 7, 12.5, 15, 20, 22.5, 25, 30 and 35 units T_1 ribonuclease (samples E to H were run in duplicate). Digestion was continued for 20 min at 0°C. The samples were electrophoresed in an 8% polyacrylamide slab gel containing 20 mM Tris-acetate, pH 8.0 and 5 mM Mg acetate at 500 V for 15 h. The resistant RNA I and II in sample A_1 and the S4–RNP I and II are indicated.

The S4–RNP I was prepared at the lowest enzyme concentration where no ribonuclease-resistant fragment was present in the control gel (as in fig.1B_2). It was fractionated into its subfragments in a denaturing gel. The result is compared in fig.2, with that obtained from the S4–RNP II that was prepared in the same gel. These two patterns of subfragments were reproducible at the given enzyme concentration and the sequence identities of the subfragments are given in table 1.

Most of the corresponding subfragments from the S4–RNP I and S4–RNP II were identical in nucleotide sequences. Some additional sequences were found in the S4–RNP I, however, and they are considered below.

Fig.2. An autoradiogram of the RNA subfragments from S4–RNP I and II. They were resolved in a 12–15% compound polyacrylamide gel containing 7 M urea (see text for details). Bands 7, 11, 13, 15 and 17 are exclusively present in the S4–RNP I.

Table 1

Subfragments from S4–RNP I	Sections of 16 S RNA	Number of nucleotides
1	Very weak	
2	BI′II″(C″)	200
3	H″H′HQ′F(Q)	158
4	BI′II″	148
5	BI′I(I″)	~140
6	(H)Q′F(Q)	83
+7	(L)	81
8(a)	II″	75
+8(b)	(L)	72
9	H″H′(H)	68
10	I	66
11(a)	B(I′)	49–53
+11(b)	(C″) (Very weak)	~50
12	B(I′)	46–49
+13	(C″)	45
14	Mixture	
+15(a)	(0)	33
+15(b)	(D′)	26
16	(M)	22
+17	Mixture	
18	(R)(G)	18
19_1	(R)(G)	14
+19_2	(0)	14

The subfragment numbers correspond to those shown in fig.2. a and b represent subfragments that were resolved in a higher percentage gel. Subscripts 1 and 2 refer to two components in a band that were not resolved. The section letters were defined during the 16 S RNA sequence determination [10]. The brackets indicate that only part of an RNA section is present. The sequences of subfragments 1, 14 and 17 were not determined since they were present either in very low yield or they contained three or more components. The nucleotide lengths were calculated from the sequence [10]. (+) Denotes the subfragments that occur in the S4–RNP I but not in S4–RNP II.

(1) Bands 7, 11, 13, 15 and 17 were only present in the S4–RNP I.

(2) Bands 7 and 8(b) contain additional sequences in section L, close to the 5′-end of the 16 S RNA; variability in the protection of section L in the S4–RNP II was reported earlier [9]; nevertheless, the sequence extension towards the 5′-end is significantly increased in the S4–RNP I.

(3) Bands 11(b) and 13 contain additional sequences in section C″ such that the region is extended slightly at its 3′-end.

(4) Two co-migrating subfragments occur in band 15 that both derive from sections D'0 close to the centre of the 16 S RNA molecule. These two subfragments contain about 60 additional nucleotides and, together with the extended sequences in sections L and C'', they probably account for the mobility difference of the S4–RNP I compared with S4–RNP II.

(5) Band 19 contained part of section 0 that was contiguous in sequence with section D in band 15(b).

A T_1 ribonuclease fingerprint of the total RNA region from the S4–RNP I is shown in fig.3A. The spots are numbered according to ref. [10]. The new spots, that were not observed in the S4–RNP II prepared under the same conditions, are indicated by

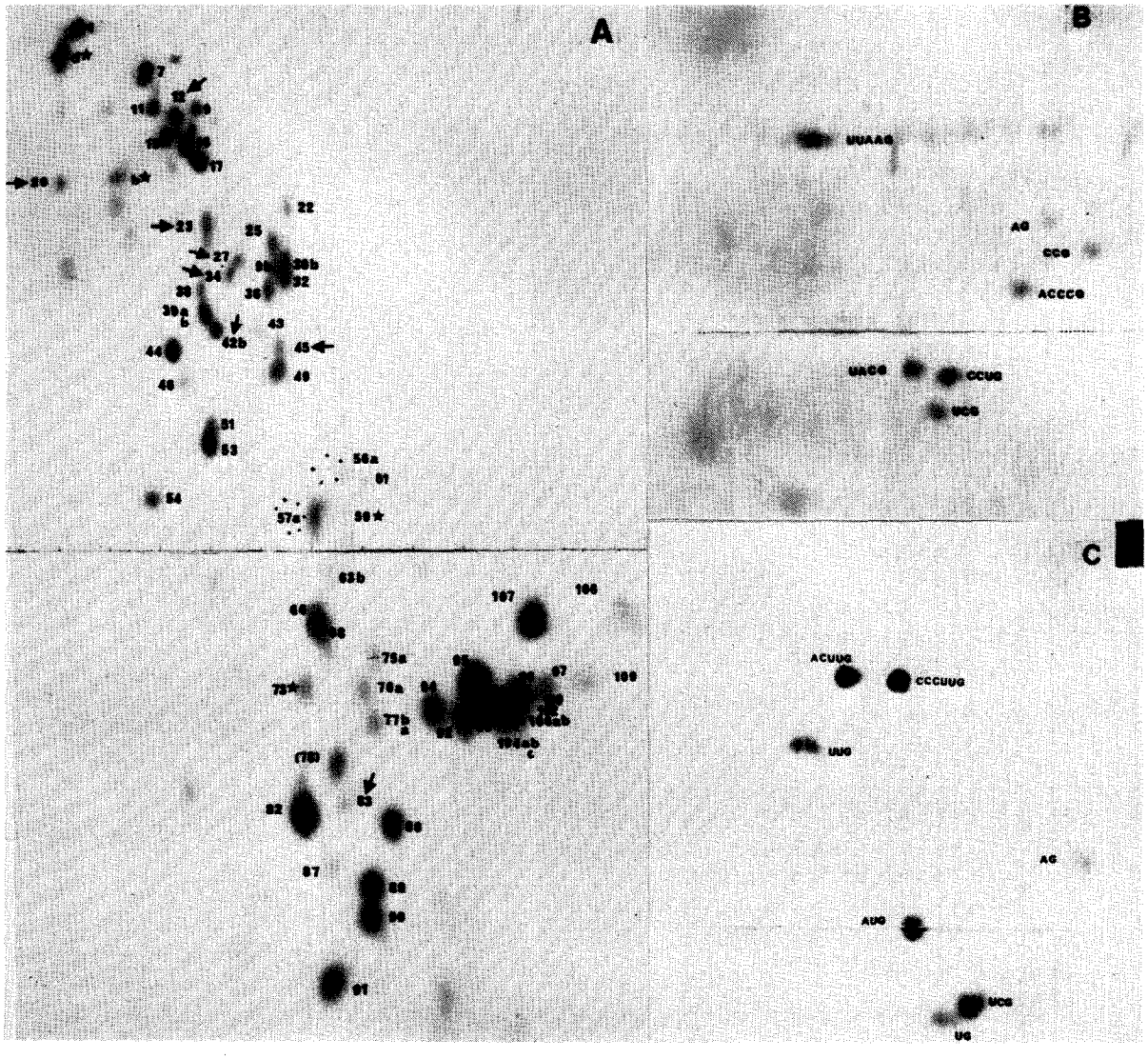

Fig.3. T_1 ribonuclease oligonucleotide fingerprints of (A) the whole RNA region contained in the S4–RNP I, (B) and (C) the two components 15(a) and 15(b), respectively, that were purified from band 15 (see fig.2). 15a derives from section 0 and 15(b) from section D'. The standard numbering system is used for the oligonucleotide spots [10]. In (A) arrows mark the spots that occur exclusively in S4–RNP I and not in S4–RNP II. (*) indicates sequence heterogeneity.

arrows. (i) spots 20, 23, 34 and 44 derive from section L, (ii) spots 12, 27 and a second copy of spot 66 are from section C″, (iii) spots 42(b) and 45 are from section D′, and (iv) spot 83 derives from section 0. Fingerprints of the two subfragments 15(a) and 15(b) that contain the sequence near the centre of the 16 S RNA are shown in fig.3B,C.

The resistant RNA regions, RNA I and II, that were prepared from the free 16 S RNA (fig.1), were examined to establish whether they were related in structure to the S4–RNP I and II. The patterns of the subfragments from the RNA I and II were almost identical to those of the S4–RNP I and II, respectively (fig.2). The only difference was that generally the yields of subfragments 1, 2 and 3 were slightly lower in RNA I and II than in the S4–RNPs. In order to confirm this structural analogy, the RNA's I and II were fingerprinted with T_1 ribonuclease. By visual inspection the fingerprint of the RNA I was identical to that in fig.3A. Moreover, the fingerprint of the RNA II was identical to that obtained earlier for the S4–RNP II. We concluded, therefore, that the nucleotide sequences of RNA I and II were identical to those of S4–RNP I and II, respectively.

Finally, a map is given in fig.4, which shows the yields and the alignment of the RNA subfragments along the 16 S RNA sequence. It also demonstrates the large separation of the three fragments in section D′0 from the S4–RNA.

4. Discussion

The RNA binding site of protein S4, as was clearly demonstrated in the preceding paper, and confirmed in the present one, is a stable compact structure that can be isolated from 16 S RNA in the absence of protein S4. Earlier [11] we provided evidence that an interaction occurs, within the RNA site, between two widely separated regions of sequence that occur in sections H″H′ and I″C″. This was the first experimental localisation of an RNA–RNA interaction that occurs in protein-free 16 S RNA between widely separated regions of sequence, and three criteria were invoked to establish its specificity. It was concluded that this interaction was important for stabilising the tertiary structure of the RNA site in the absence of protein S4.

In the present work we provide evidence for the occurrence of another such interaction between sections D′0 near the centre of the molecule, and the S4–RNA region. The finding that this interaction was present after degrading protein-free 16 S RNA, established that the presence of protein S4 was not required. The only criterion of specificity that we have, for this interaction, is that the additional sequence in section D′0 (and also in sections L and C″) occurs in approximately one copy per S4–RNA as indicated by the intensities of the spots in the total fingerprint (fig.3A) and from the intensities of the RNA subfragments in the denaturing gel. We consider it very unlikely, however, given their high G-contents, that the three fragments from D′0 could be released from the S4–16 S RNA complex, or free 16 S RNA, during degradation, in the presence of T_1 ribonuclease, and subsequently become strongly and unspecifically associated with the S4–RNA during

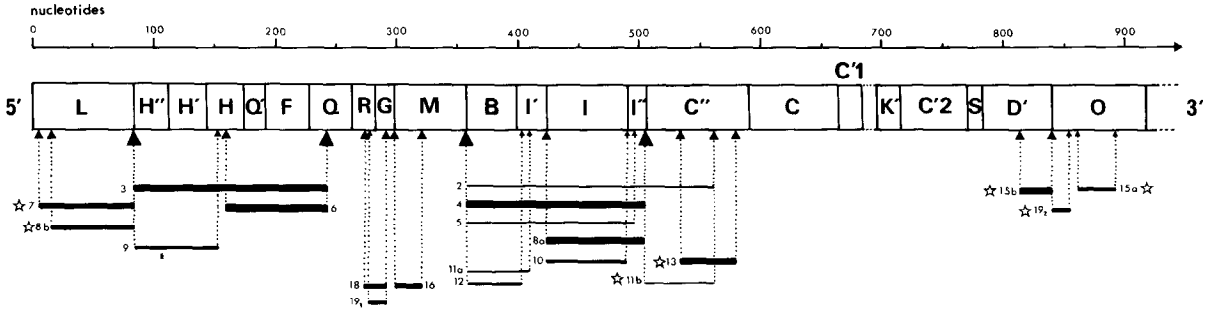

Fig.4. Diagram showing the ordering of the RNA subfragments from the S4–RNP I along the 5′-region of the 16 S RNA sequence. The standard nomenclature for the section letters is used. The thickness of each line is proportional to the approximate yield of each subfragment. The arrows indicate the cutting positions of T_1 ribonuclease and the larger arrow-heads indicate the cuts that occur in higher yield. (☆) marks the subfragments that were not present in the S4–RNP II.

its preparation.

The conversion of S4–RNP I to S4–RNP II results in the loss of part of section L, section C'' and the three fragments in region D'0. Although they could be simultaneously degraded, in the presence of protein S4, the observation that no intermediate products are formed, suggests that the dissociation may be cooperative, in which case these three regions may be interacting.

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