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Evidence for tertiary structural RNA-RNA interactions within the protein S4 binding site at the 5'-end of 16S ribosomal RNA of Escherichia coli.+

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ABSTRACT

Evidence is presented for tertiary structural interaction(s) (interaction(s) between two regions of an RNA molecule that are widely separated in the RNA sequence) within the 5'-one third of the 16S ribosomal RNA of Escherichia coli that constitutes the binding site of protein S4. The two main interacting RNA regions were separated by about 120 nucleotides (sections Q to M) of the 16S RNA sequence. A second, smaller gap, of 13 nucleotides, occurred within section C". The two main interacting regions contain about 150 nucleotides (sections H" to Q) and 160 nucleotides (sections M to C"). They are folded back on one another and, especially in the presence of protein S4, are strongly protected against ribonuclease digestion. The intermediate region (sections Q to M), however, is relatively accessible to ribonucleases in the S4-RNP.

By partial removal of subfragments from the RNA complex it was possible to localise the two main interacting sites within sections H" - H and sections I" - C". Three main criteria for the specificity of the RNA-RNA interactions were invoked and satisfied. The possibility of other tertiary structural RNA-RNA interactions occurring in other regions of the 16S RNA is discussed. Finally, all the structural information on the S4-RNP is summarised and a tentative model is proposed.

INTRODUCTION

Protein S4 is an important protein for the in vitro assembly of E.coli 30S subunits[1] and binds to, and protects against ribonuclease digestion, a large part of the 5'-one third of the 16S RNA[2-6]. The determination of most of the nucleotide sequence of the 16S RNA[7-10] has provided a basis for detailed studies on the structure of this RNA region.

Recently, we have characterised the precise parts of the 16S RNA that are protected against ribonuclease digestion by S4[4]. The S4-RNP was deproteinised and electrophoresed in polyacrylamide gels containing urea and it yielded, reproducibly,

a series of RNA subfragments[4]. These were analysed for sequence and their precise positions in the RNA sequence were determined. The amount of digestion at each enzyme cutting position and the identities of excised parts of the RNA sequence were also established[4].

About 120 nucleotides near the centre of the RNA region were either present in low yields or absent, and a second small gap in the sequence occurred near the 3'-end[4]. These observations led us to question, and to investigate, whether the two large RNA regions were complexed through the S4 protein or exclusively by RNA-RNA interactions.

The strategy of the present study was as follows. The S4-RNP was deproteinised by electrophoresing in polyacrylamide gels containing dodecylsulphate and magnesium. It was found that the two large non-contiguous regions, and the small one at the 3'-end, co-migrated in a complex (henceforth referred to as F1-RNA). Washing this RNA complex with 4 M urea selectively removed some of the smaller subfragments and led to a partial localisation of the interacting sites.

The RNA-RNA interaction(s) (that we reported briefly earlier[4]) were shown to be of a specific nature, and not simply unspecific aggregates, by applying the following three criteria. (1) That non-contiguous RNA regions were present in the complex in equimolar quantities. (2) That the RNA complex could be dissociated into subfragments and then reconstituted. (3) That not any RNA fragment was incorporated into the reconstituted RNA complex.

MATERIALS AND METHODS

Preparation of the S4-RNP

A complex of protein S4 and [^{32}P] 16S RNA was formed by incubating in TMK reconstitution buffer (30 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$, 0.35 M KCl and 6 mM 2-mercaptoethanol) as described earlier[4]. The complex was digested at a T_1 ribonuclease:RNA ratio of 300-450:1 Kunitz units per mg, for 30 min at 0° and immediately fractionated on an 8% polyacrylamide slab gel containing 7.5 mM Tris-acetate, pH 8, and 5 mM Mg acetate[4]. The faster migrating RNP 2, (henceforth referred to as S4-RNP), was

investigated.

Characterisation of the RNA subfragments

The [^{32}P] S4-RNP was excised from the gel, equilibrated in an 8 M urea solution in water for 3 hr at room temperature and then electrophoresed in a standard denaturing gel, namely, a polyacrylamide slab gel containing 7 M urea, 0.1% Na dodecylsulphate, 0.1 M Tris-borate, pH 8, and 2.5 mM EDTA. Full details of the procedure have been presented[4,11]. A series of subfragments were reproducibly fractionated. Their positions in the gel were determined autoradiographically as before[4]. Exposures varied between one hour and one week at 4°.

Each band was excised and electrophoresed from the gel onto DEAE paper and eluted[11]. Each sample was divided into two parts. One part was digested completely with T_1 ribonuclease and the other with pancreatic ribonuclease. Oligonucleotide analyses were performed by the methods of Sanger and co-workers[12,13]. These analyses sufficed to place a given subfragment within the known RNA sequence[9].

Deproteinisation of the S4-RNP

In most of the experiments the RNP was deproteinised by dodecylsulphate treatment. In a few experiments the S4 protein was removed either by phenol-dodecylsulphate extraction or by trypsin treatment. The procedures are described separately below.

Dodecylsulphate treatment. The gel slice, containing the [^{32}P] S4-RNP (10^6 counts/min) was soaked for 4-5 hr, at 20°, in 0.1% dodecylsulphate, 8 mM Tris-acetate, 1 mM Mg acetate, pH 8.0. It was then maintained at 20°, or 50°, for a further 10 min before cooling slowly. It was set in the top of a 10% polyacrylamide slab gel[4], containing the above buffer, by slowly polymerising a little acrylamide on either side of the gel slice. The temperature within the gel slice was checked with a thermistor and did not rise above 20°. Electrophoresis, at 18°, was continued for 16-18 hr at 150 V and 20 mA. The subfragments of each of the resolved RNA bands were fractionated, in a denaturing gel, as described above.

Phenol-dodecylsulphate extraction. [^{32}P] S4-RNP (10^6 counts/min)

was extracted from the gel by passing the gel-piece through a small syringe, stirring the crushed gel-piece overnight in 3 ml 0.1% dodecylsulphate, 0.2 M NaCl, 10 mM Mg acetate, at 4°, and centrifuging. The supernatant was shaken at 20° for 30 min with an equal volume of phenol saturated with the same buffer. After centrifuging, the phenol layer was washed twice with buffer and the combined supernatants (8 ml) were passed slowly through a 0.45 µ nitrocellulose filter (Millipore) to remove remaining RNP's. The RNA was precipitated with 2.5 volumes ethanol for 36 hr at -30°.

Trypsin treatment. [^{32}P] S4-RNP (20,000 counts/min) was eluted from the gel with 3 ml 8 mM Tris-acetate, pH 8, 5 mM Mg acetate and precipitated with 1.5 vol. ethanol for 48 hr. After dissolving in 0.1 ml of this buffer 1 µg carrier protein S4 and then 2.5 µg trypsin (Merck, Darmstadt) was added. The mixture was incubated for 2 hr at 10°.

It had been demonstrated, using radioactively-labelled S4, that the protein was completely removed from 16S RNA by both the phenol-dodecylsulphate and trypsin treatment[14] (R.G., E.U. and C.Schulte, unpublished work). The RNA prepared by the latter methods was electrophoresed in a 10% polyacrylamide slab gel containing 8 mM Tris-acetate, pH 8 and 5 mM Mg acetate, at 4°, for 16 hr at 30 mA and 200 V. The RNA bands were excised and fractionated into subfragments as described above.

Urea-wash of the deproteinised RNA complex (F1-RNA)

The slice from the dodecylsulphate gel containing the [^{32}P] F1-RNA band was soaked for 5 hr at 23° in 4 M urea, 8 mM Tris-acetate, pH 8 and 1 mM Mg acetate, and then polymerised into the top of a 10% polyacrylamide slab gel containing the above buffer. The sample was electrophoresed, at 4°, for 16 hr at 18 mA and 150 V. Each band was characterised for its subfragment content as described above.

Dissociation and reconstitution of the F1-RNA

The [^{32}P] F1-RNA was eluted from the gel piece by passing it twice through a small syringe and stirring vigorously overnight in 8 mM Tris-acetate, pH 8 and 1 mM Mg acetate. Gel pieces were removed by centrifuging and passing the supernatant through

a nitrocellulose filter (Millipore). In some experiments the RNA was extracted directly from the S4-RNP by phenol-dodecylsulphate treatment and the F1-RNA was fractionated in a dodecylsulphate-polyacrylamide gel (see above). One part of the F1-RNA solution was used for protein-rebinding experiments (see below). The remainder was dissociated into subfragments by heating in the presence of EDTA and urea and reconstituted by heating with TMK buffer according to the following procedure.

The F1-RNA solution (8 ml) was dialysed for 9 hr against 2.5 mM EDTA and 8 M urea, pH 5.9. It was then heated at 60°, for 5 min, in the dialysis bag to effect complete denaturation of base-pairing[15] and immediately dialysed for 15 hr against double-distilled water at 4°. It was concentrated to 2 ml with Ficoll 400 (Pharmacia). The latter had been deionised by extensive dialysis against double-distilled water and then lyophilised. The RNA solution was precipitated with 2.5 volumes ethanol for 48 hr. (In some experiments it was lyophilised at this stage with no difference in the results.) The precipitate was dried under a vacuum in a desiccator, dissolved in 0.05 ml water and heated to 60° for 5 min. An equal volume of double-concentration TMK reconstitution buffer was added that had been preheated to 60°. The RNA was maintained at 60° for a further 5 min before placing in a beaker containing warm water, and allowing it to cool slowly to room temperature thereby effecting optimum reconstitution[16]. The sample was then electrophoresed, at 4°, in a 10% polyacrylamide slab gel containing 10 mM Tris-acetate, 5 mM Mg acetate, pH 8, at 20 mA and 200 V.

Protein-rebinding experiments

Binding assays were performed with 0.45 μ nitrocellulose filters (Millipore), as described earlier[4]. The [^{32}P] F1-RNA was prepared by the dodecylsulphate gel method, extracted as described above, and dialysed against TMK reconstitution buffer for 16 hr to remove dodecylsulphate. The percentage of retention of the F1-RNA on the filter was determined so as to establish that it had been deproteinised. Binding experiments and suitable controls were performed with ribosomal proteins S4, S8, S20, L24 and a mixture of proteins S16 and S17 prepared as described earlier[17].

RESULTS

1. A description of the S4-RNP and of its RNA subfragments

The S4-RNP that was fractionated electrophoretically is shown in Fig.1. Degradation of 16S RNA alone produced no such RNA component. After treatment with urea and dodecylsulphate the S4-RNP was fractionated into 19 subfragment bands (Fig.1), three of which (8, 10 and 11) were mixtures of two subfragments. Each fractionated RNA band (except 6, 14 and 15) was isolated from at least four S4-RNP's, prepared over the T_1-ribonuclease range of 300-450 Kunitz units per mg RNA, and shown to be of reproducible sequence except for changes in the length of subfragment 11a at the 5'-end of the RNP. Subfragment bands 6, 14 and 15 were present in variable amounts but always in low molar yields. Subfragment 19 was sometimes resolved into two components. The sections of 16S RNA contained within the subfragments are given in Table 1. Their lengths, which ranged from about 180 to 15 nucleotides, are also listed.

The sequence of the whole RNA component of the S4-RNP is given in Fig.2 showing the order of the lettered sections and a tentative secondary structure. The large squares enclose the parent subfragments and the arrows mark enzyme cuts that produced the other subfragments.

In the present work the RNA subfragment fractionation pattern in Fig.1 has been used as a criterion for the presence of the RNA somponents of the S4-RNP snd it enabled us (1) to identify the subfragment content of an RNA complex (F1-RNA), (2) to establish which subfragments could be selectively removed from it, thereby partially localising the RNA-RNA interaction sites, and (3) to show that the RNA complex could be dissociated and then specifically reconstituted.

2. A description of the F1-RNA

The F1-RNA was prepared as follows. The S4-RNP was deproteinised by treatment with dodecylsulphate. The RNA was fractionated in a polyacrylamide slab gel, containing dodecylsulphate and magnesium. The result is shown in Fig.3a. Sample A, which was preincubated at 20° in dodecylsulphate and magnesium before applying to the gel, yielded one main band, the F1-RNA, which

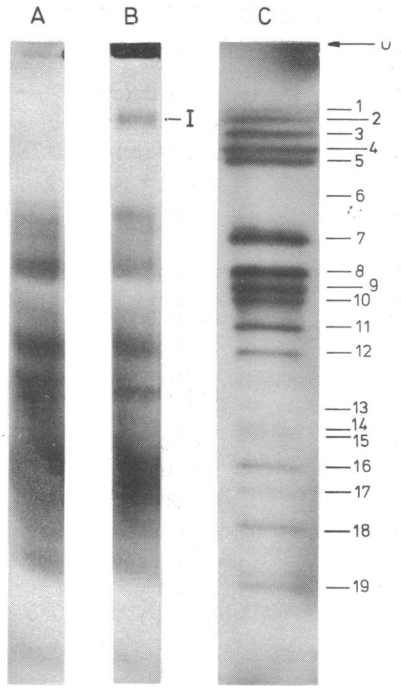

Figure 1. The electrophoretic fractionation of the [^{32}P]S4-RNP and its RNA subfragments.

Gel A. The 16S RNA was degraded and no significant protection of large RNA pieces was observed. Gel B. The S4-16S RNA complex was degraded and the S4-RNP resolved (indicated by I). Gel C. The S4-RNP was deproteinised, denatured and fractionated into RNA subfragments. They are numbered in a standard way and their identities are given in Table 1. Subfragments 6, 14 and 15 were present in variable but low yields. O denotes the origins.

migrated with a mobility similar to the S4-RNP. A similar result was obtained when the S4-RNP was deproteinised by phenol-dodecylsulphate or trypsin treatment and electrophoresed under similar conditions without dodecylsulphate. (The F1-RNA also had a similar subfragment composition (see below) except that small components tended to be lost during the phenol-dodecylsulphate extraction procedure.)

Sample B (Fig.3a) was incubated at 50°, before electrophoresis, and produced a more complex fractionation pattern. The F1-RNA band was absent, indicating that the RNA complex

Table 1. Identities and lengths of the RNA subfragments of the S4-RNP

Subfragments of S4-RNP	Sections of 16S RNA	Number of nucleotides
1	(M) B I' I I" (C")	~180
2	(M) B I' I I" (C")	~180
3	B I' I I" (C")	160
4	H" H' H Q' F (Q)	150
5	B I' I	132
6	-	-
7	(H) Q' F (Q)	88
8a	I	66
8b	H" H' (H)	67
9	H" H' (H)	66
10a	(I)	59
10b	(Q') F (Q)	59
11a	(I)	52
11b	F (Q)	52
12	B (I')	45
13	B + (H') H	41
14	-	
15	-	
16	(C")	27
17	(H) (Q')	27
18	(M)	22
19	(R) (G)	15

The subfragment numbers correspond to those shown in Fig.1. The section letters were defined during the 16S RNA sequence determination[7]. The brackets indicate that only part of an RNA section is present. The sequences of subfragments 6, 14 and 15 were not determined because they were normally present in very low yields. The nucleotide lengths were calculated from the sequence[9,10].

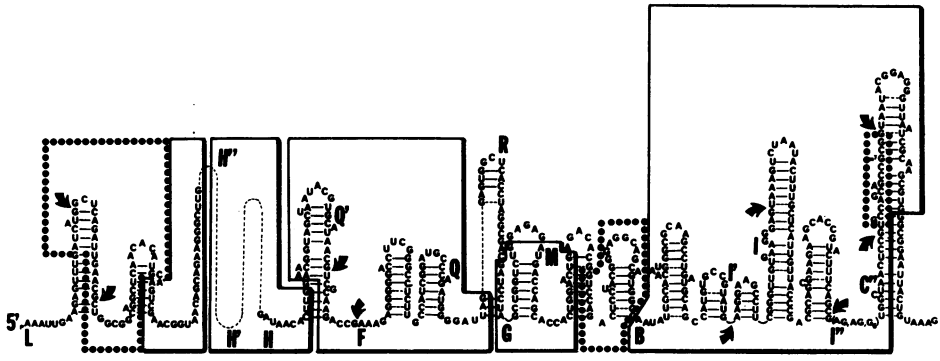

Figure 2. The sequence and possible secondary structure of the 5'-one third of the 16S RNA.

The squares drawn with full lines include the parent subfragments that are present in the S4-RNP. Arrows indicate the main enzyme cutting points within the parent subfragments that correspond to the ends of other RNA subfragments of the S4-RNP. The dotted squares indicate the regions that are present in very low yields. The dashed line in the sequence indicates that the order of the sequenced oligonucleotides is uncertain. Sequences that are not included within squares were excised by the ribonuclease and were absent from the S4-RNP.

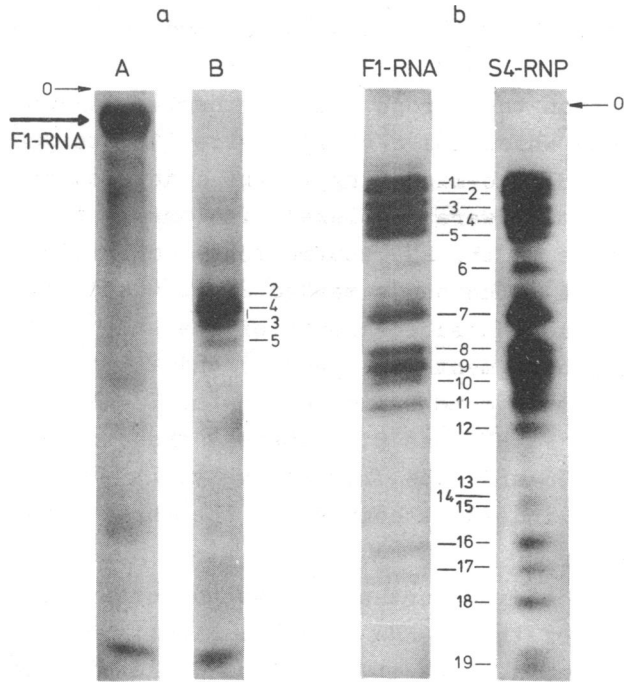

Figure 3. a. The S4-RNP was deproteinised in 0.1% dodecylsulphate and 1 mM Mg acetate. Sample A was incubated at 20° and sample B at 50°. They were fractionated in a 10% polyacrylamide gel containing the 0.1% dodecylsulphate and 1 mM Mg acetate at 18°. The F1-RNA was present in sample A but not in sample B. In sample B, bands 2, 3, 4 and 5 were identical to the RNA subfragments, of the same numbers, in Fig.3b (see also Table 1). b. The subfragments of the F1-RNA were fractionated in a denaturing gel. The parent S4-RNP was co-electrophoresed and the subfragments are shown for identification purposes. 0 denotes the origins.

had irreversibly dissociated during warming.

Each of the bands in the two gels in Fig.3a, including the F1-RNA, was further analysed in a denaturing gel for its RNA subfragment content. The subfragment content of the F1-RNA is shown in Fig.3b, together with the S4-RNP subfragments that were co-electrophoresed for comparison. Some RNA subfragments were absent from the F1-RNA. These included bands 18 and 19 corresponding to the central RNA region (covering sections (Q) to (M)) and subfragment 12 (sections B (I')). The short region in section C" was also completely absent. It was inferred,

therefore, that the F1-RNA band comprised three non-contiguous RNA regions.

The question then arose whether the two largest non-contiguous regions which had a similar size (150 to 160 nucleotides) were simply co-migrating in the dodecylsulphate gel (Fig. 3a) or whether they were complexed. The answer to this came from an analysis of the RNA subfragment composition of the bands in gel B (Fig.3a). The bands marked 2, 3, 4 and 5 were found to correspond to the RNA subfragments of the same numbers in Fig. 3b. The main subfragments 4 (5'-part) and 2 (3'-part) migrated separately and much more rapidly than the F1-RNA (Fig.3a). The R_F value of the F1-RNA was 0.05 whereas those of subfragments 4 and 2 were 0.36 and 0.34, respectively. It was concluded that the F1-RNA was, indeed, a complex of the three non-contiguous RNA regions.

Before proceeding further,it was necessary to establish two properties of the F1-RNA complex. First, that it was protein-free and second, that it constituted the binding site of protein S4 and, possibly, of other proteins that bind in this RNA region, namely S16/17 and S20[5]. First, the retention of the F1-RNA was measured on nitrocellulose filters. It exhibited very low retention (Table 2) and it was concluded that it was, indeed, protein-free. Second, it was found that a complex of F1-RNA with each of the proteins S4, S20 and S16/17 produced significant retention on the filters, whereas complexes with proteins S8 and L24 that do not bind specifically to this part of 16S RNA, produced low retention (Table 2).

To summarise the results on the F1-RNA, it had been established that it contained three non-contiguous RNA regions that were held together exclusively by RNA-RNA interactions, and that it was a specific complex because proteins S4, S20 and S16/17 were able to bind to it whereas other basic proteins were not.

3. Evidence for tertiary structural interaction(s)

The RNA region missing from the centre of the F1-RNA (sections (Q) to (M)) includes four "hairpin" structures (see Fig.2) and it can be concluded, therefore, that the two large RNA regions on either side of this gap are bound by a tertiary

Table 2. Protein binding to F1-RNA

F1-RNA sample	Protein added	Retention of [^{32}P]F1-RNA (%)
1	no protein	4
2	S4	22
3	S20	31
4	S16/17	29
5	S8	7
6	L24	8

Binding was determined by the degree of retention of [^{32}P] F1-RNA-protein complex on nitrocellulose filters. Triplicate samples of each protein were tested and the results were averaged. For a given protein the agreement between the results was within ± 10%.

structural interaction. Clearly, this conclusion is dependent on the secondary structural map, shown in Fig.2, being at least approximately correct.

Some experimental evidence supporting this theoretically derived structure was described earlier[8]. We undertook a further study in which the S4-RNP was heated in the presence of 0.1% dodecylsulphate and 1 mM Mg acetate. Progressively longer base-paired regions were irreversibly dissociated at increasing temperatures. Since the positions of the ribonuclease cuts in the RNA sequence were known, and they occurred mainly at unpaired-bases in double-helical regions, or in the loops at the end of "hairpins" (Fig.2), it was possible to correlate, quantitatively, the temperature at which a base-paired region was disrupted with that expected from the scheme in Fig.2. The results confirmed some of the "hairpin" structures in the S4-region including the relevant ones in the central region. These results will be published separately.

The second gap within the nucleotide sequence of the F1-RNA occurred within section C" (Fig.2). This was a small gap (13 nucleotides), but because the secondary structure proposed for section C" is the least certain of the whole F1-RNA region, it

is unclear whether subfragment 16 (3'-terminal) is maintained in the F1-RNA complex by secondary or tertiary structural RNA-RNA interactions.

4. Partial localisation of the RNA interaction sites

Some RNA subfragments, especially the smaller ones, could be further dissociated from the F1-RNA by treatment with increasing molarities of urea, in the presence of 1 mM Mg acetate. A range of urea concentrations was tested. Washing with 4 M urea resulted in the removal of several subfragments without completely disrupting the tertiary structural interaction. The effect of this treatment on the F1-RNA, after electrophoresis, is shown in Fig.4A. A series of bands were produced. Each was characterised for its RNA subfragment content. The tertiary structural interaction was maintained only in band I. This yielded the subfrag-

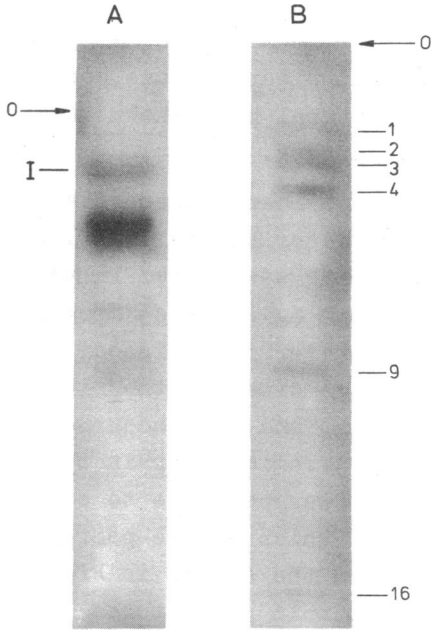

Figure 4. 4 M urea treatment of the F1-RNA

Gel A. The dissociation products of the F1-RNA resolved in a 10% polyacrylamide gel containing 4 M urea, 1 mM Mg acetate and 8 mM Tris-acetate, pH 8. Each band was analysed for its RNA subfragment content and only in band I was the tertiary structural interaction present. Gel B. Fractionation of the RNA subfragments of band I in a denaturing gel. They are numbered as in Fig.1.

ment pattern shown in Fig.4B. Only subfragments 4 and 9 were present from the 5'-region and 1 (in low yield), 2, 3 and 16 from the 3'-region. These subfragment contents of the 4 M urea-treated F1-RNA and F1-RNA are shown diagrammatically in Fig.5. The RNA composition of the S4-RNP is also shown for comparison.

The results for the 4 M urea-treated F1-RNA permitted a more precise localisation of the interacting sites. In the 5'-region the only small subfragment present in high molar yield was 9 (sections H" - H), therefore this must contain the main interacting site of the 5'-region. Subfragment 8b that differs from subfragment 9 only in sequence heterogeneity was removed. This suggests that the sequence difference in 8b produced a less stable interaction. (Such sequence heterogeneity effects were observed earlier during partial ribonuclease digestion of 16S RNA[7,8].)

In the 3'-region the larger subfragments 5, 8a, 10a (sections B-I, I and (I), respectively) were removed. Only those subfragments containing sections I" and (C") were retained in the complex. These observations, together with the fact that subfragment 12 (section B(I')) was not conserved in the F1-RNA, suggested that one or more interaction sites were located in sections I" to (C"). The absence of part of section C" in the F1-RNA may reflect the presence of two distinct interaction sites in sections I" and (C") or one site with a secondary structural link between sections I" and C" (Fig.2).

In summary, RNA interaction sites were located within sections H" - H and sections I" - (C").

5. Criteria for the specificity of the RNA-RNA interactions in the F1-RNA complex

Three main criteria were invoked for the specificity of the tertiary (and secondary) structural RNA-RNA interactions in the F1-RNA. They are considered separately below.

1) A specific RNA complex should contain equimolar amounts of the main interacting regions. This was tested by two methods. First, the autoradiogram of the subfragments was densitometered and the area under each peak was determined. Second, the bands were excised from the gel and the radioactivity estimated by Cerenkov counting.

Table 3. The molar proportions of the two large interacting RNA regions.

	5'-end subfragments								3'-end subfragments								Total 5'-end	Total 3'-end	Molar ratio 5'/3'-end
	4	7	8b	9	10b	11a/b	13b	17	1	2	3	5	8a	10a	12	13a	16			
S4-RNP	6.0	9.0	7.0	12	5.5	7.0	1.5	6.5	4.5	5.0	6.0	4.5	3.5	5.5	5.5	1.5	9.5	54.5	45.5	1.2
F1-RNA	4.5	9.5	7.5	13	5.0	13	-	5.0	4.0	6.5	8.5	2.5	3.5	5.0	1.0	-	11.5	57.5	42.5	1.3
4 M urea treated F1-RNA	21	-	-	27	-	-	3.0	-	5.0	11.5	14.5	-	-	-	-	3.0	15	51 (±2.5)	49 (±2.5)	1.0
Reconstituted F1-RNA	15.5	6.0	8.0	13.5	-	9.0	-	-	8.0	10	13	-	4.0	-	1.5	-	11.5	52 (±3.5)	48 (±7)	1.1

The central RNA region, containing subfragments 18 and 19, was present in the S4-RNP but is not included in the Table. The molar yield of each subfragment was calculated by dividing the area under the densitometer peak of subfragment by its nucleotide length (see Table 1). The sum of these values for each of the subfragments within a given RNA complex was then equated to 100%. The molar percentage of each subfragment was then calculated and is presented in the Table. For the subfragment mixtures, in particular 8a/b and 13a/b, the exact proportions of the two components were known only for the S4-RNP. The three F1-RNA complexes were assumed to contain the same ratios. For the urea-washed and reconstituted F1-RNA the error limits were calculated assuming that only 8a and 13a (3'-end) or only 8b and 13b (5'-end) were present. The error limits of measurement for the S4-RNP and untreated F1-RNA were relatively higher because they contained more subfragments.

Good agreement was obtained between the two methods. The results are presented in Table 3 for the former method. For each of the four RNA complexes, namely the S4-RNP, F1-RNA, 4 M urea-washed and the reconstituted F1-RNA (see criterion 2 below), approximately a 1:1 molar ratio of the two large interacting regions was obtained.

2) Under suitable solution conditions it should be possible to completely dissociate and reconstitute a specific RNA complex such that its electrophoretic properties remain about the same and that it satisfies criterion 1 (see above).

The F1-RNA was dissociated by heating in the presence of urea and then water, and reconstituted by heating in TMK buffer (see Materials and Methods for details). The reconstituted complex was electrophoresed in a polyacrylamide slab gel. About half of the reconstituted sample co-migrated with F1-RNA (see band I in Fig.6A) and yielded a similar subfragment pattern (Fig.6B) except that some smaller subfragments were not present or were present in very low yields. (These results are expressed diagrammatically in Fig.5.) Most of the remainder of the reconstituted RNA migrated in band II (Fig.6A). This yielded a less complex subfragment pattern (Fig.6C). From the mobility

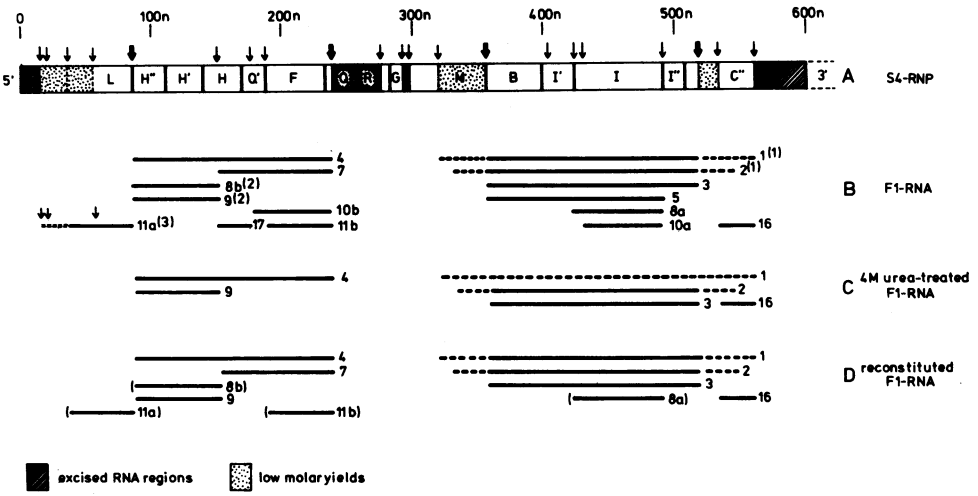

Figure 5. The RNA sections and subfragment content of the S4-RNP and F1-RNA's.

A. S4-RNP. The excised regions and those of low molar yields are indicated. The thickened lines mark the limits of the sections that were earlier defined during sequence determinations[9]. The larger and smaller arrows indicate positions of extensive and minor enzymic cutting, respectively. B. F1-RNA. The subfragments are numbered as in Fig.1 and Table 1. ——— shows regions present in high yield. ----- indicates those present in low yields. Subfragment 12 was sometimes present in very low yields. Footnotes: (1) - subfragments 1 and 2 are both mixtures of large subfragments. (2) - subfragments 8b and 9 differ in sequence heterogeneity. (3) - there is some variability in the length of subfragment 11a. C. 4 M urea-treated F1-RNA. Subfragment 13 was either absent or present in very low yield. D. Reconstituted F1-RNA. The brackets indicate that the yields of subfragment bands 8a/b and 11a/b are about 50%. It is unclear whether one, or both, of each pair of co-migrating subfragments is present.

of band II it was more likely that it contained RNA subfragments held together by secondary structural but not tertiary structural interactions.

For band I, criterion 1 was satisfied in that the molar ratio of the 5'/3'-regions was about 1:1 (Table 3). Addition of S4 protein during the reconstitution step did not affect the number or the amount of subfragments incorporated into the F1-RNA.

3) Not any RNA fragment should be incorporated into a specific

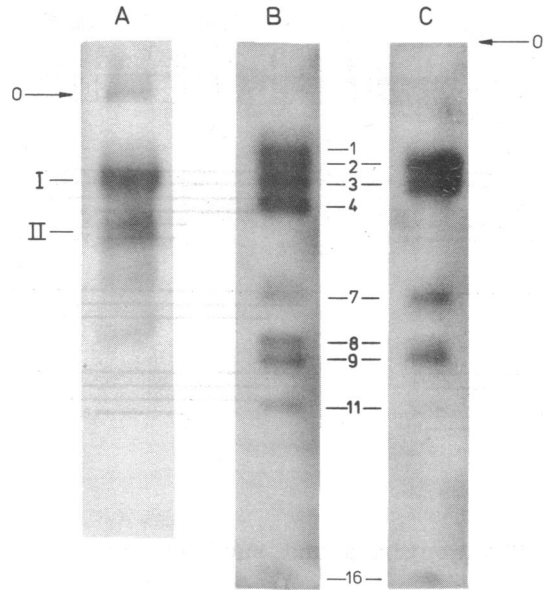

Figure 6. Reconstituted F1-RNA

Gel A. Fractionation of the reconstituted F1-RNA in a 10% polyacrylamide gel containing 5 mM Mg chloride and 10 mM Tris-acetate, pH 8. Gels B and C. Denaturing gels showing the RNA subfragments that derived from bands I and II, respectively, in gel A. O indicates the origins.

complex. In a specific complex, RNA-RNA interactions are likely to occur mainly through hydrogen-bonded bases, whereas unspecific binding is more likely to involve magnesium-bridging of phosphate groups. It follows that any RNA subfragments would bind in an unspecific RNA complex. By this criterion, therefore, the RNA-RNA interactions of the F1-RNA are specific since subfragments 5, 10a/b and 17 were not incorporated during reconstitution (see Fig.5). The reason for this is that these subfragments were only weakly base-paired in the F1-RNA complex (see Fig.2).

The results show that the F1-RNA complex satisfies all three criteria for specificity. Therefore, the tertiary structural RNA-RNA-interactions within the complex are of a specific nature.

DISCUSSION

Although the dimensions of the 30S subunit require that the 16S RNA has a folded structure within the subunit, this RNA tertiary structure could be maintained in its folded state by the proteins (as was suggested in earlier models[18,19]) or, as Cox has proposed[20], the RNA tertiary structure could be a helix, without additional intramolecular RNA-RNA interactions.

The possibility that isolated 16S RNA is extensively folded in the presence of magnesium and absence of protein was suggested by the following observations. (1) At increasing magnesium concentrations both the sedimentation coefficient[21,22] and the resistance to ribonuclease digestion of the RNA[23] increases markedly. (2) Proteins only bind specifically to the 16S RNA at magnesium concentrations above 1 mM[22,24].

In the present work we have demonstrated, conclusively, that such a folded structure does exist in free RNA and, moreover, that tertiary structural RNA-RNA interactions play a role in maintaining it, in the absence of protein, within the 5'-one third of the 16S RNA.

A partial localisation of the two main interacting sites within this region was made. One site occurs within sections H" - H, and the other lies within sections I" and the middle part of C". Although the chemical nature of the interaction cannot be inferred with certainty, the fact that it occurs in the absence of protein, suggests that hydrogen-bonding may be involved. The sequence of the H" - H region is, unfortunately, incomplete, and, therefore, it is not possible to investigate base-complementarity. However, an analysis of all the possible orders of the oligonucleotide sequences in this region demonstrates that no very stable "hairpin" structures can be formed (C.E. and P.S. unpublished results); a result in favour of it being free to base-pair with other parts of the 16S RNA.

The interaction(s) were judged to be specific by three main criteria. (1) The molar ratios of the subfragments of one interacting region were approximately equivalent to those of the other. (2) The F1-RNA complex could be dissociated and reconstituted, such that its electrophoretic properties were unchanged and criterion 1 was still satisfied. (3) Not any RNA

subfragment could be reconstituted into the F1-RNA complex, indicating that unspecific phosphate-phosphate interactions, through magnesium ions, were not predominant.

This concept for tertiary structural RNA-RNA interactions within ribosomal RNA molecules is in contrast to earlier hypotheses, the most recent of which, from Kurland[19,25], proposed that such interactions could only occur after proteins had bound to the RNA and then, exclusively, through the bridging of phosphate groups by magnesium ions.

Do other tertiary structural RNA-RNA interactions occur in 16S RNA?

There is circumstantial evidence to suggest that other tertiary structural interactions may occur in the 16S RNA and facilitate its folding. However, it should be stressed that no rigorous criteria for RNA-RNA specificity have been applied to these possible interactions. Although the first two (described below) exhibit similar characteristics to those of the S4-RNP, it cannot be excluded yet that proteins help to maintain the RNA complex. 1. In the S4-RNP 1 (an RNP, described earlier[4], that migrates more slowly than RNP 2), a part of sections D'O was identified (unpublished results). This occurs in the 16S RNA sequence some 200 nucleotides from the 3'-end of the S4-RNP[9]. 2. A complex of two widely separated regions of RNA was isolated in the absence of urea from partial ribonuclease digests of 16S RNA[9]. The two regions occurred in sections O' and A in the 3'-half of 16S RNA. These two regions occur at the RNA extremities of an RNP (containing proteins S7, S9, S10, S14, S19 and possibly S13)[26]. Since a gap occurs within the RNA sequence of the RNP[26], the observed RNA-RNA interaction could be a specific one maintaining a folded RNA structure. 3. An RNP (containing proteins S6, S8, S15 and S18) has an RNA component extending from section C" to O near the centre of the 16S RNA[5]. A gap in the RNA sequence occurs between sections C_2' and D'. Since the primary binding proteins (S8 and S15) attach on the 5'-side of this gap[4,5], the 3'-side may be maintained in the complex by RNA-RNA interactions.

A description of the S4-RNP

The results presented put a considerable restriction on the organisation of RNA within the S4-RNP and provides a basis for interpreting how protein S4 and this RNA region can interact.

We have the following information on the S4 protein. (1) It is a very elongated protein of molecular weight 22,500 and of approximate hydrated dimensions 130-170 x 20 x 20 Å.[27] Electron microscopic studies, using antibody markers[28], have demonstrated that this elongated structure is conserved in the 30S subunit. The protein must be very flexible, therefore, and able to interact intimately with the RNA structure. (2) A large continuous part of the protein (about 170 amino acids) is relatively inaccessible to trypsin molecules[13]. Also, several amino acids within this region can be cross-linked by ultraviolet irradiation to the 16S RNA[29]. Therefore, it probably constitutes the main binding region of the protein.

The information available on the RNA binding site can be summarised as follows. (1) Each of the five parent RNA subfragments (see Fig.2) shows significant binding to protein S4, as detected on nitrocellulose filters (unpublished work). (2) The central region of RNA, from sections (Q) to (M), is weakly protected by protein S4 against ribonuclease digestion[4] (although it is strongly protected by protein S20[4]). (3) The 3'-end of the S4-RNP comprising sections B to I" (see Figs. 2 and 5) is strongly protected against ribonuclease digestion by either the compact RNA structure, itself, or by protein S4. There are few enzymic cuts within this region. (4) The 5'-end of the S4-RNP, from sections (L) to (Q) is protected by both S4 and S20[4].

A model for the S4-RNP

We believe that the above data are compatible with a model which has the following characteristics.

(1) The whole RNA region is folded back on itself about sections Q to M to form an elongated structure.

(2) We have calculated, approximately, the maximum lengths of the two main regions that are protected by protein S4, and interact, namely sections H" to Q and B to I" (Figs. 2 and 5).

Using the parameters for an 11-fold RNA helix[30], namely helix diameter - 23 Å and base - separation (for single-strand stacked bases between "hairpin" structures) - 2.7 Å, then the maximum length of both regions (calculated from Fig.2) is 160-180 Å. The length of the whole RNA region, therefore, may well approach that of the estimated length of the protein (130-170 Å).[27,28]

(3) The protein lies along the RNA, possibly intertining with it, and interacting at several points along its length[14,29], and with at least one point on each parent RNA subfragment. Only the N-terminal region (40 - 50 amino acids) of the protein appears to be unimportant for the binding and may not interact with the RNA.[13,29]

(4) The 3'-part of the RNA region is less accessible to ribonuclease in the presence of S4 than the 5'-part (Figs. 2 and 5). The 5'-part is exclusively and strongly protected by protein S20[4]. Proteins S4 and S20 may lie, therefore, with their longer axes parallel to that of the RNA complex such that S4 interacts more with the 3'-part and S20 interacts mainly with the 5'-part.

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[+]Paper number 2 on protein binding sites in 16S RNA. The preceding paper was reference number 4.

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