A PARTIAL LOCALISATION OF THE BINDING SITES OF THE 50 S SUBUNIT PROTEINS L1, L20 AND L23 ON 23 S RIBOSOMAL RNA OF *ESCHERICHIA COLI*

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1. Introduction

There are some 55 proteins in the *E. coli* ribosome and about one third of them interact directly with 16 S, 5 S or 23 S ribosomal RNA's (reviewed in [1]). Of these, 10 proteins have been identified that bind specifically to 23 S RNA [2–4].

A number of attempts have been made to isolate RNP fragments containing sections of RNA and the protein, after controlled ribonuclease digestion of reconstituted single protein–RNA complexes, especially from 16 S RNA (summarised in [5]) and 5 S RNA [6], for which the nucleotide sequences are largely known. Considerable progress has now been made in the sequencing of 23 S RNA [7] and it has been demonstrated that the RNA in an RNP fragment, obtained after nuclease digestion of a complex of protein L 24 and 23 S RNA, derives from the 5′-end of the 23 S RNA molecule [8].

In this work, we describe the isolation and the characterisation of specific RNP fragments isolated after controlled hydrolysis of reconstituted complexes L1, L20 and L23 with 23 S RNA. Fingerprints of T_1 ribonuclease digests obtained from the RNA of the RNP fragments, enabled us to locate their positions, approximately, within the central two thirds of the 23 S RNA.

2. Materials and methods

^{32}P-labelled and unlabelled 23 S RNA was prepared from ribosomes of *E. coli* (MRE 600 and A19) as described [8]. The 23 S RNA was deproteinised by three successive phenol extractions. It exhibited very low retention on Millipore nitrocellulose filters [9]. Proteins were purified by carboxymethylcellulose chromatography and Sephadex gel filtration [10] and identified electrophoretically [11]. Uncontaminated protein batches were used.

Protein-23 S [^{32}P] RNA complexes were prepared as described earlier [3,8] in TMK reconstitution buffer (0.03 M Tris–HCl, pH 7.4, 0.02 M $MgCl_2$, 0.35 M KCl and 6 mM 2-mercaptoethanol). The complex was digested at a concentration of about 1 mg/ml with T_1 ribonuclease (Sankyo, Japan). The RNP fragments were fractionated electrophoretically in 8% polyacrylamide gels containing Mg^{++} [8]. R_f values of the RNP's were measured relative to a Bromophenol Blue marker. The gel slices, containing the RNP fragments, were divided into two parts. The RNP of one part was eluted electrophoretically [12]. Some of this was completely digested with T_1 ribonuclease and fingerprinted [13,14]. The rest of the RNP was deproteinised by phenol treatment [8,9] and reconstituted with the corresponding protein, as described earlier [9], and the formation of a complex was established by the Millipore nitrocellulose filter technique [9].

Results

3.1. *Isolation and dissociation of RNP fragments*

RNP fragments containing L1, L20 and L23 were

isolated electrophoretically; they are considered separately below.

3.1.1. L1-RNP

The RNP fragment isolated from an L1-23 S RNA complex after T_1 ribonuclease treatment (enzyme: RNA ratio 1:7 w/w), migrated with an R_f value of 0.18 (fig.1a). 23 S RNA degraded under identical conditions produced no RNA in the corresponding gel region. The RNP fragment yield decreased rapidly up to an enzyme:RNA ratio of 1:2 (w/w) when no more RNP was detected. The RNP yield also decreased when the complex was hydrolysed at decreasing Mg^{++}

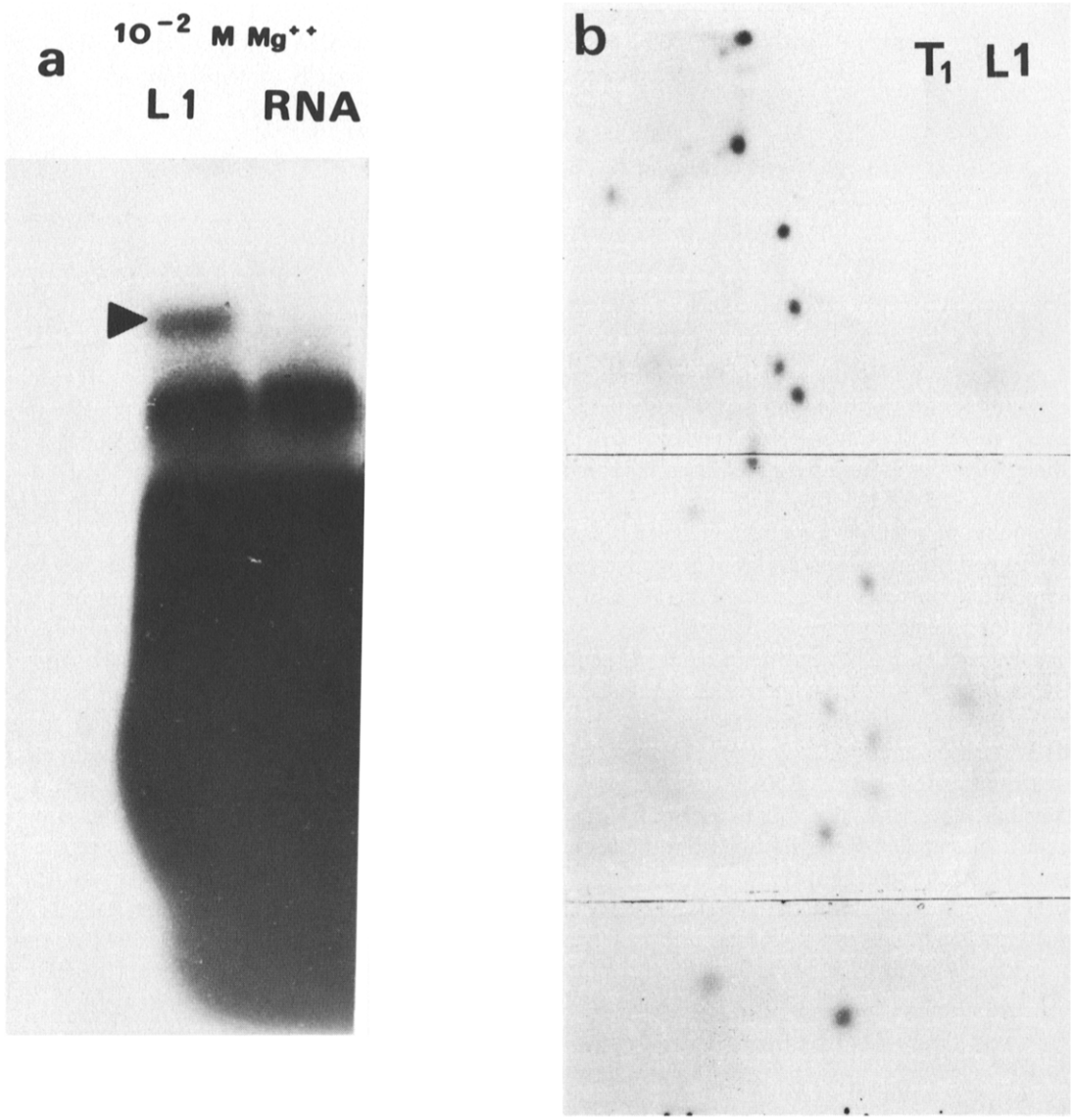


Fig.1. L1-RNP. a) Fractionation of a T_1 ribonuclease digest of an L1–23 S RNA complex (left side) and 23 S RNA alone (right side). The digestions were performed in TMK buffer at a T_1 ribonuclease:RNA ratio of 1:7 w/w, for 30 min at 0°C. The digest was electrophoresed in an 8% polyacrylamide gel containing 10^{-2} M Tris–acetate, pH 7.8, and 10^{-2} M Mg-acetate. The buffer was circulated. The RNP fragment, containing L1, is indicated by an arrow. b) A fingerprint of a T_1-digest of the RNA moiety of the L1-RNP fragment.

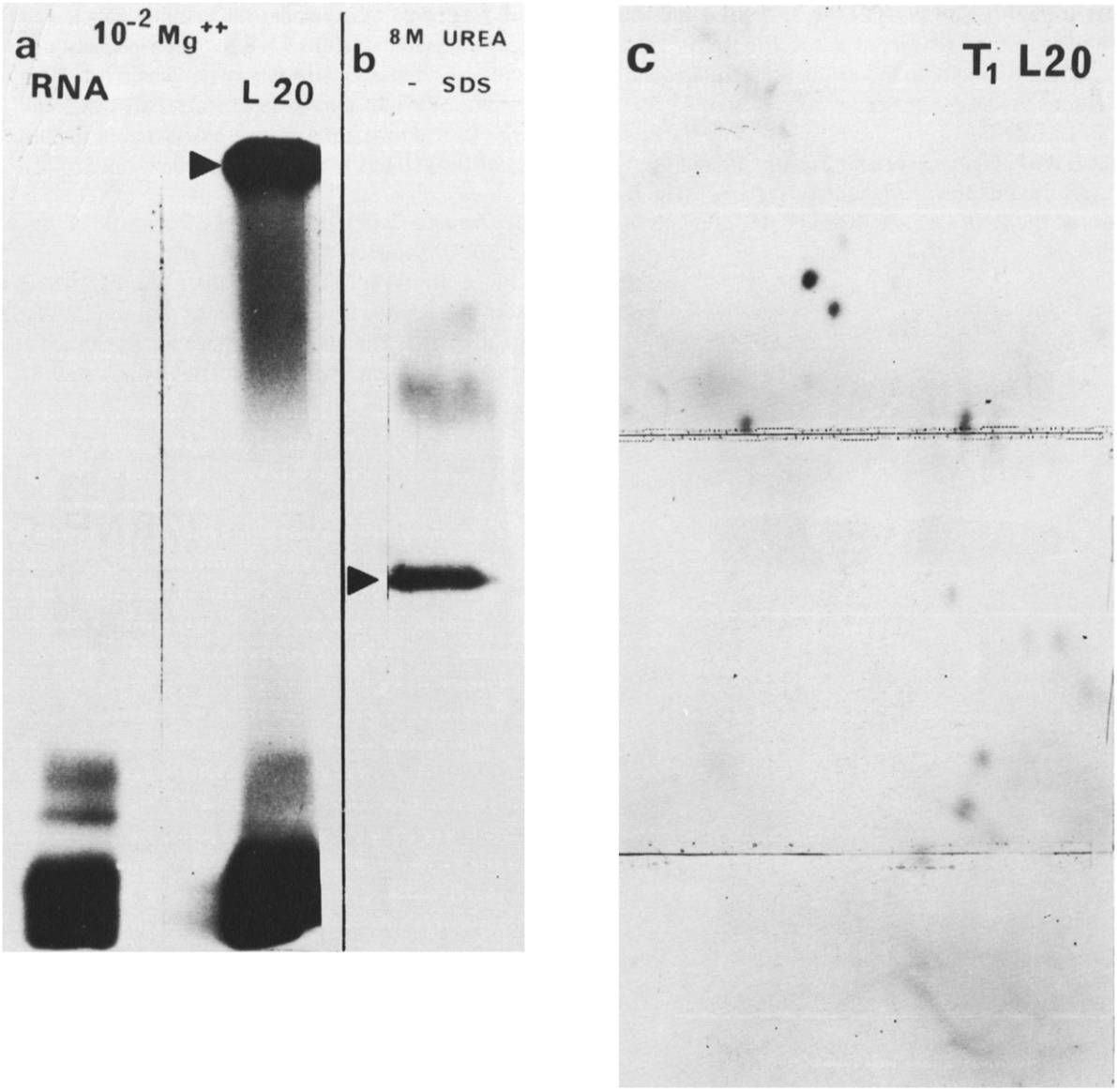


Fig.2. L20-RNP. a) Fractionation of the T_1 ribonuclease hydrolysate of 23 S RNA (left side) and of an L20–23 S RNA complex (right side) as described in the legend to fig.1a, but at an enzyme:RNA ratio of 1:10 (w/w). The RNP fragment, containing L20, remained near the origin (indicated by an arrow). b) The gel piece containing the L20-RNP was treated with 8 M urea and 0.1% SDS, to dissociate the protein, before electrophoresing in a 12% polyacrylamide slab gel containing 8 M urea [8]. c) A fingerprint of a T_1-digest of the main RNA fragment originating from the L20-RNP fragment.

concentrations. From 10^{-2} M to 10^{-4} M Mg^{++} the yield decreased by about 50%.

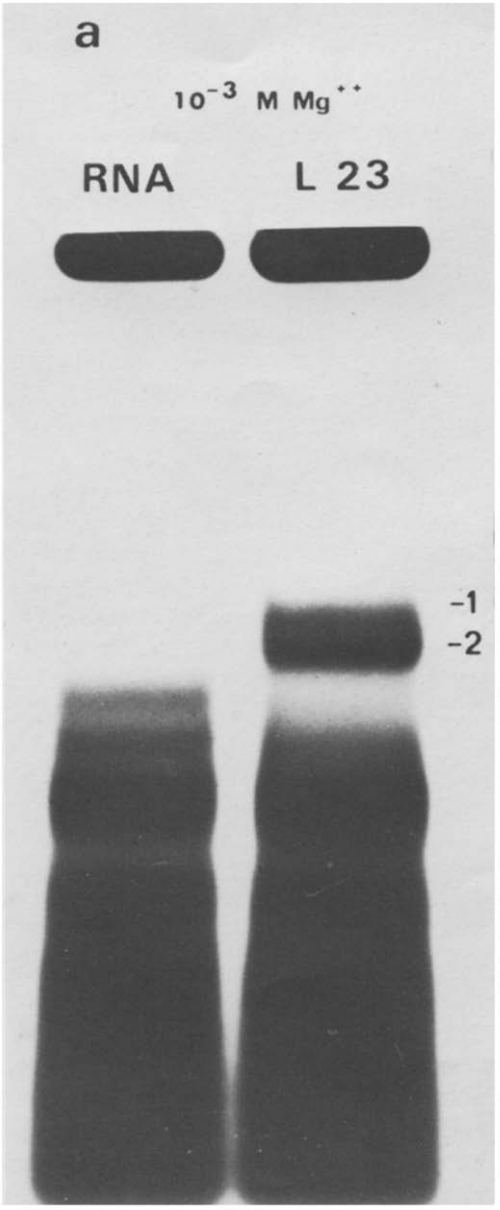
3.1.2. L20-RNP

The RNP fragment, containing L20, remained in an aggregated form below the origin of the polyacrylamide gel (fig.2a). It was stable over a wide range of enzyme: RNA ratio's and Mg^{++} concentrations. Under denaturing conditions it invariably yielded one strong discrete band (fig.2b) and a very low level of heterogeneous RNA fragment. The latter were shown by analysis, to

derive from different parts of the 23 S RNA and were attributed to the low level of unspecific RNA aggregation normally found at the origin, under the conditions used.

3.1.3. L23-RNP

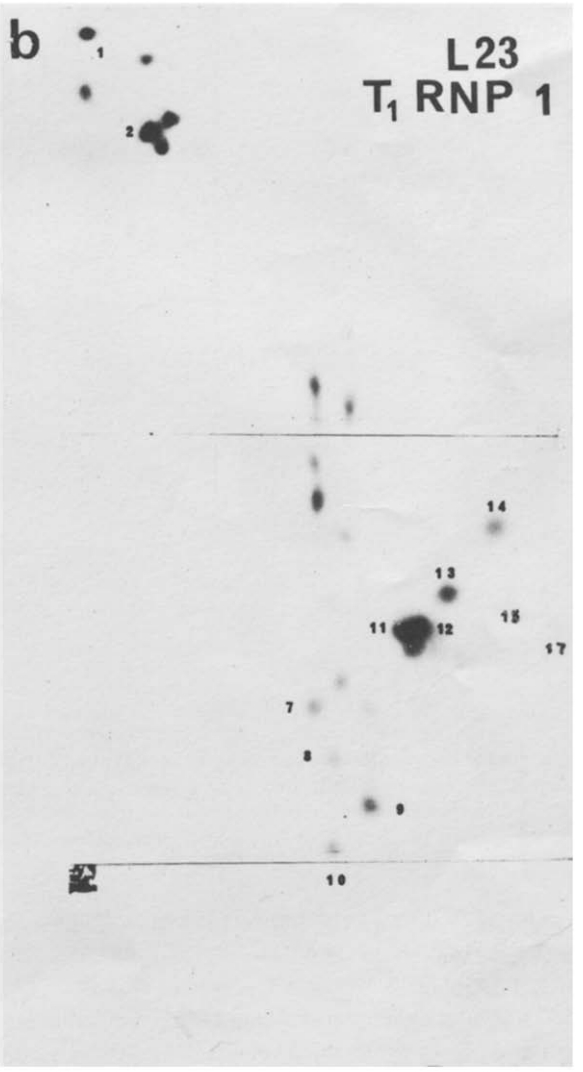
Two RNP fragments (1 and 2), containing L23, were resolved in polyacrylamide gels (fig.3a). The R_f values of the RNP's were 0.40 and 0.45, respectively.



Both fragments were stable over a similar enzyme and Mg^{++} range to that of the L1-RNP; the yields also decreased, similarly, at higher enzymic and lower Mg^{++} concentrations. In some experiments, two very weak RNP's (not shown in fig.3a) were detected in the upper part of the gel, but were not analysed.

3.2. *Characterisation of the RNA content of the RNP fragments*

Fingerprints of T_1-digests of the L1 and L23-RNP's, and of the RNA fragment obtained from the L20-RNP, were prepared. The oligonucleotides were analysed further for sequence with pancreatic ribonuclease, U_2



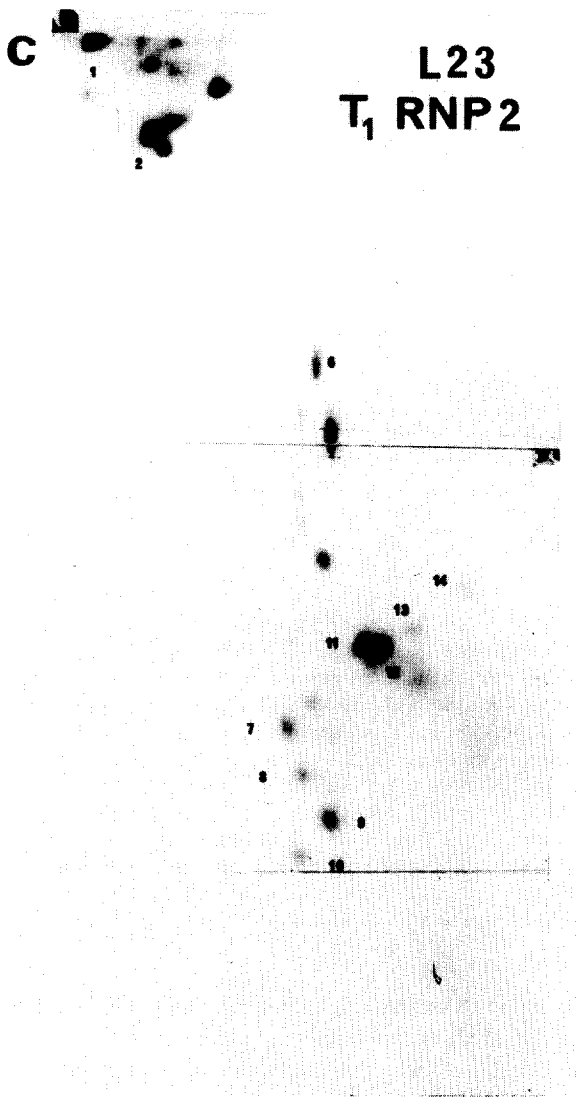


Fig.3. L23-RNP. a) Fractionation of the digestion products of 23 S RNA (left side) and of an L23–23 S RNA complex (right side) as described in the legend to fig.1a. Hydrolysis was at a T_1 ribonuclease:RNA ratio of 1:12 (w/w), for 10 min at 0°C. Two RNP fragments, 1 and 2, were resolved. b,c) Fingerprints of the T_1 digests of the RNA moities L23-RNP 1 and L23-RNP 2.

ribonuclease and venom phosphodiesterase. Full details of these analyses will be presented later. The results enabled us to compare the sequences with those of characterised fragments, produced by limited hydrolysis of 23 S RNA or 50 S subunits, that have been partially sequenced ([7] Branlant et al., in preparation) and are set out diagrammatically in fig.4.

3.2.1. L1-RNP

It was estimated, from the oligonucleotide analyses (fig.1b) that the RNA region contained 150-170 nucleotides. The region was present in fragment II but not in fragment B (fig.4) and could be placed between nucleotides 2000 and 2400.

3.2.2. L20-RNP

Fingerprint analyses of the main repurified L20–RNA fragment showed that it was a pure fragment of about 40–50 nucleotides (fig.2c). This fragment was found within fragment I but not in fragment A and must therefore lie between nucleotides 500 and 1300 (fig.4).

3.2.3. L23-RNP

Owing to an incomplete separation of the RNP's 1 and 2, there was slight cross-contamination of their RNA contents, as can be seen on the fingerprints of their T_1 digests (fig.3b, c). Nevertheless, oligonucleotides analyses indicated that the two RNP's 1 and 2 have a common region, about 80 nucleotides in length. This corresponds to about 70% of fragment 7c, that was obtained by mild digestion of 23 S RNA and analysed [7]. The numbered oligonucleotides in fig.3b, c correspond to those of fragment 7c [7]. RNP I contained an additional 30–40 nucleotides and RNP 2 contained about 20 extra nucleotides. In both RNP's some oligonucleotides corresponding to these additional nucleotides do not occur in fragment 7c. Since some oligonucleotides of fragment 7c are not present in RNP I or in RNP II, it can be concluded that at least one of the RNP's contains a discontinuous section of RNA. Both RNA regions contained in RNP I and in RNP 2 do not occur in fragment I and II and therefore lie between nucleotides 1300 and 2000 (fig.4).

3.3. *Specificity of the fragments*

Evidence for the specificity of the RNA binding sites derives from three sources. 1) Each of the three digested complexes containing unlabelled RNA was electrophoresed in polyacrylamide disc gels, in duplicate, as described earlier [9]. One gel was stained exclusively for protein with Coomassie Brilliant Blue and the other for RNA with pyronin G. Protein was shown only to occur on RNA bands that migrated with the same R_f value as those of the analysed L1, L20 and L23-RNP's

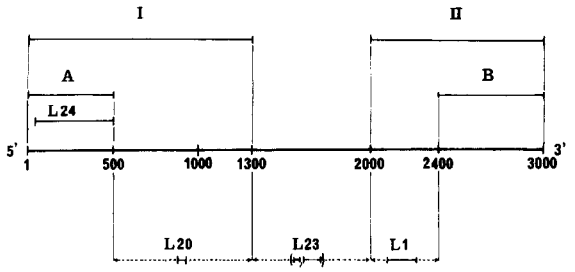


Fig.4. A scheme of the 23 S RNA showing the distribution of various RNA fragments. The RNA fragment A (which contains the L24 binding site) was obtained by mild digestion of the 23 S RNA and shown, by sequence analysis, to occur at the 5'-end of the molecule [7]. The fragments I, II and B, obtained by limited hydrolysis of 50 S subunits, derived from both ends of the 23 S RNA (C. Branlant et al., in preparation). A comparison of our oligonucleotide analysis with those of the fragments enabled us to localise, approximately, the binding sites of proteins L1, L20 and L23.

in figs.1a, 2a and 3a (E. Ungewickell helped with these experiments). 2) In digested 23 S RNA control experiments no RNA bands migrated in the position corresponding to the RNP fragments. In the experiment with the L20–RNA complex there was only a very small amount of RNA at the origin of the digested 23 S [^{32}P] RNA control (fig.2a). 3) Rebinding experiments were performed in which the RNP fragments were deproteinised and reconstituted with the isolated protein, as described earlier [9]. Complex formation was checked by the Millipore nitrocellulose filter technique [9]. The appropriate control experiments were performed. The results, in table 1, indicate that each of the RNA regions, isolated from the RNP's, reassociated exclusively with its corresponding protein.

4. Discussion

The results presented demonstrate that the RNA regions are the binding sites of the three proteins. It cannot be excluded, however, either that parts of the RNA binding sites have been excised, or that the protein binding site constitutes only a part of the isolated RNA region.

The properties of the RNP's differed from those of the L24-RNP, and from each other, in a number of respects. For example, the L1 and L23-RNP's were

Table 1
Binding of proteins to the RNA regions extracted from the RNP's under reconstitution conditions

RNA from	Protein added	% retention on filter
L1 - RNP	L1	50
	S4	4
	L23	6
L23 - RNP 1	L23	30
	S4	1
	L24	3
L23 - RNP 2	L23	26
	S4	2
	L24	2
L20 - RNA fragment	L20	24
	S4	2
	L23	3

[^{32}P] RNA regions (10 000 c.p.m.) were mixed with 0.7 A_{260} units unlabelled 23 S RNA in 0.3 ml TMK reconstitution buffer, in triplicate. A 4:1 molar excess of protein was added. The mixture was incubated at 42°C for 1 hr. The solution was chilled on ice, passed through a nitrocellulose filter (Millipore) under pressure, and washed with 3 ml cold TMK buffer. The filters were dried and counted in scintillation liquid and the three results for each RNA region were averaged. The retention of the RNA region alone was invariably below 3%; this value was subtracted from those given above. Incubated solutions of the proteins with [^{32}P] tRNA always gave less than 4% retention.

less stable to nuclease digestion than that of L24. Also, the L20-RNP, whilst being very stable to enzymic degradation showed a strong tendency to aggregate, probably due to protein-protein interactions, and remain near the origin of the polyacrylamide gel. The L23-RNP was exceptional in that although both RNP 1 and 2 contained a common section of RNA, they contained additional different parts. This could be due to a) heterogeneity of the RNA sequence or b) two different conformations of the RNA; it is being further investigated.

Attempts were made to isolate RNP fragments using other 23 S RNA binding proteins [2–4]. No success was obtained with proteins L6 and L16. Proteins L3 and L4 gave some protection and, although the RNA regions obtained were not completely reproducible, a part of the L1-RNA region was obtained

with L3. An RNP containing L2, L5, L18, L25, 5 S RNA and a section of 23 S RNA has also been isolated and is being further characterised.

The results are compatible with those of Spierer et al. [15] who bound proteins to the 13 S and 18 S RNA fragments derived from the 5′- and 3′-ends of 23 S RNA, respectively. L20 bound to the 13 S RNA fragment and L1 and L23 bound to the 18 S RNA fragment.

More detailed positioning of the protein binding sites will facilitate the determination of the RNA folding within the 50 S subunit, especially in co-ordination with immuno-electronmicroscopic localisation of 50 S subunit proteins (reviewed in [16]). The finding that proteins L1, L20 and L23 are all located in the ribosomal subunit interface region [17] is already significant since their binding sites extend over 1000 to 2000 nucleotides of RNA. This would suggest either an extended subunit interface region, or that the RNA is extensively folded so as to arrange parts of these proteins in the same surface region.

The specific RNP fragments that have been isolated all contain RNA within the approximate length range of 40 to 180 nucleotides. This is much smaller than for the previously isolated L24-RNP (about 500 nucleotides). They differ from the L24-RNP also in that the proteins in the RNP's are all rapidly digested by trypsin (C. Schulte and R. G., in preparation) whereas L24 is highly resistant [8]. L1, L20 and L23 are also much more accessible in the 50 S subunit to antibodies than L24 (reviewed in [16]). Therefore, a model such as that proposed for the L24-RNP, with the RNA tightly-wrapped around the protein, would be unsatisfactory for these RNP's. Probably, large parts of proteins L1, L20 and L23 are not interacting with the RNA and are accessible on the ribosomal surface.

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