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Studies on the binding of the ribosomal protein complex L7/12-L10 and protein L11 to the 5'-one third of 23S RNA: a functional centre of the 50S subunit

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ABSTRACT

The RNA binding sites of the protein complex of L7/12 dimers and L10, and of protein L11, occur within the 5'-one third of 23S RNA.

Binding of the L7/12-L10 protein complex to the 23S RNA is stimulated by protein L11 and vice-versa. This is the second example to be established of mutual stimulation of RNA binding by two ribosomal proteins or protein complexes, and suggests that this may be an important principle governing ribosomal protein-RNA assembly.

When the L7/12-L10 complex is bound to the RNA, L10 becomes strongly resistant to trypsin. Since the L7/12 dimer does not bind specifically to the 23S RNA, this suggests that L10 constitutes a major RNA binding site of the protein complex. Only one of the L7/12 dimers is bound strongly in the (L7/12-L10)-23S RNA complex; the other can dissociate with no concurrent loss of L10.

INTRODUCTION

The complex of protein L12, its N-serine acetylated form L7, and protein L10 was the first stable protein complex to be characterised from the 50S subunit of Escherichia coli (1). It consists of two L7/12 dimers and an L10 monomer (2), and constitutes a flattened, elongated structure (2). The proximity, and interdependence, of proteins L7/12, L10 and L11 in the ribosome has been demonstrated by a variety of methods including chemical cross-linking (3) and in vitro reconstitution (reviewed in 4). Indeed, this group of proteins constitutes an important functional centre on the ribosome: the L7/12 dimers have been implicated in the ribosomal binding sites of initiation factor IF-2, elongation factors EF-G and EF-Tu and the release factors RF-1 and RF-2 (4,5); protein L11 reacts strongly with photoaffinity analogues of GTP and GDP which are bound to the ribo-

some in the presence of EF-G (6,7), and there is also evidence for a direct involvement of L7/12 in EF-Tu-dependent GTPase hydrolysis (8).

Earlier, we demonstrated that protein L11 and the complex L7/12-L10 independently associate with 23S RNA (9,10), and proposed that the capacity of the latter protein complex to bind specifically to the 23S RNA was an important criterion for its nativity (9). In the present work, we have localized the protein binding sites on a fragment of 23S RNA, demonstrated the interdependence of the RNA-binding of L7/12-L10 and L11, and provided evidence that L10 constitutes a major RNA attachment site of the protein complex.

MATERIAL AND METHODS

Proteins, 16S and 23S RNAs, RNA fragments and protein-RNA complexes

The protein complex and protein L11 were prepared under non-denaturing conditions as described earlier (11). They were identified and characterised for purity by 2-D gel electrophoresis (12) and dodecylsulphate-slab gel electrophoresis (13). 16S and 23S RNA's were extracted with phenol and dodecylsulphate (9). 13S and 18S RNA fragments of 23S were isolated from 50S subunits by mild treatment with carrier-bound RNase A and the 11S RNA fragment was prepared in a sucrose gradient after stronger nuclease digestion (14). The RNA's and fragments were characterised electrophoretically for purity, homogeneity and size (14), and were shown to be protein-free by heating 3-5 A_{260} units of each RNA in 10% dodecylsulphate and then electrophoresing in a slab gel (13) and staining for protein.

Complexes were prepared by incubating the RNA or an RNA fragment with protein for 1 hr at 40°C, in 0.3 ml HMK buffer (10 mM Hepes, 20 mM $MgCl_2$, 350 mM KCl, and 6 mM 2-mercaptoethanol, pH 7.4) and cooling to 0°C. In some experiments, Hepes was replaced by Tris (TMK). Protein : RNA molar ratios of 5 : 1 were employed unless otherwise stated. The following molecular weights were taken: L7/12 - 12,200, L10 - 17,700, L11 - 14,800 (15), 16S RNA - 0.55 x 10^6, 23S RNA - 1.1 x 10^6, 13S RNA - 0.45 x

10^6, 18S RNA - 0.62 x 10^6 and 11S RNA - 0.34 x 10^6 (14). Protein concentrations were determined by a nitrogen assay (16) and by amino acid analysis.

Assay for protein-RNA complex formation

The incubation mixture was passed over a Sephacryl S-200 column (1 x 48 cm) at 4°C with a flow rate of 12 ml/hr. 1.0 ml fractions were collected and protein or RNA-bound protein was precipitated with 5% trichloroacetic acid and examined on slab gels (9). The protein was quantitated by measuring the intensity of Coomassie brilliant blue-stained bands and comparing with calibration curves (9).

Trypsin digestion experiments on the L7/12-L10 complex

Proteins L7/12-L10 and L7/12-L10+L11 were bound to 23S RNA and the protein-RNA complexes were purified by passing through a Sephacryl S-200 column. They were digested with trypsin (1/50th of the weight of protein, assuming a 1:1 molar complex) at 0°C for 1 h. The digestion was stopped by addition of a 3-fold weight excess of soya bean trypsin inhibitor. The free proteins were digested under the same conditions for comparison; the digestion was stopped by boiling the sample in a 1% dodecylsulphate solution.

RESULTS

The elution profile from a Sephacryl S-200 column of 23S RNA complexed with L7/12-L10 and protein L11 is shown in the upper part of Fig. 1. The 23S RNA-protein complex was resolved from the 5S RNA. In order to establish that the unbound protein was separated from the 23S RNA-protein complex a mixture of L7/12-L10 and L11, incubated in the absence of 23S RNA, was chromatographed under the same conditions. The lower part of Fig. 1 shows (a) that both the L7/12-L10 complex and L11 were completely separated from the RNA peak and (b) that no strong complex formation occurred between protein L11 and the L7/12-L10.

The binding capacities of the proteins to fragments of 23S RNA were examined with a view to localising the binding sites of

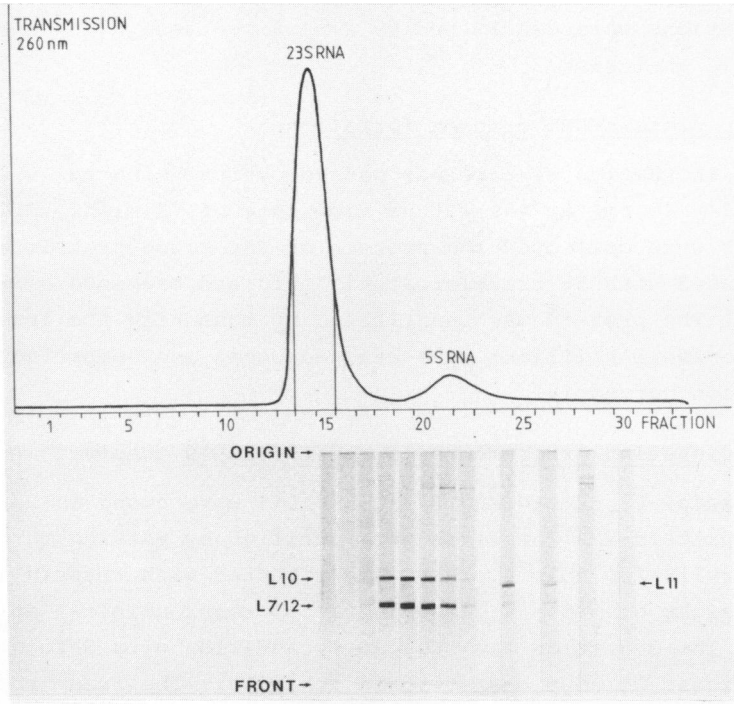

Figure 1: Elution profile of 250 µg 23S and 5S RNA from a Sephacryl S-200 column after incubating with a five-fold molar excess of L7/12-L10 and protein L11 in 0.3 ml HMK buffer. In a control experiment, L7/12-L10 and L11 were incubated in the absence of RNA and run on the same column; the gel strips which show the protein contents of this eluate are placed below the corresponding column fraction.

the protein complex and L11, within the 23S RNA sequence. 23S RNA, 16S RNA and the fragments 18S RNA (nucleotides ∿ 1,200 - 3,100), 11S RNA (∿ 2,000 - 3,100) and 13S RNA (∿ 1 - 1,200) were incubated under similar conditions with the protein complex and L11, and assayed for complex formation. The specificity of the binding was controlled by establishing (i) that no strong protein binding occurred to the 16S RNA and (ii) that molar protein:RNA ratios did not exceed about 1:1 for either the protein complex or L11. Qualitative results are shown in Fig. 2 and the quantitation is given in Table 1. For the L7/12-L10, the gels show that binding occurred only to the 23S RNA and the 13S RNA

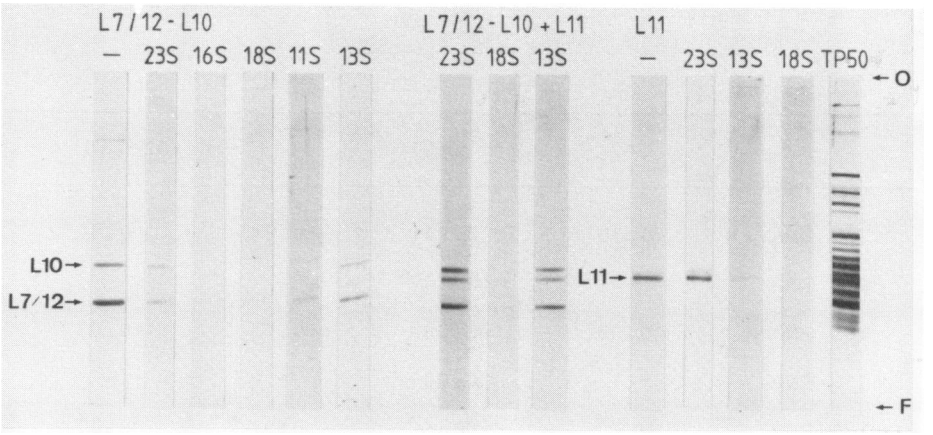

Figure 2: Binding of L7/12-L10 and protein L11 to 23S, 16S RNA and to 23S RNA fragments. The positions of the RNA-bound proteins are indicated by arrows. For most of the samples 50 to 65 pmoles RNA complex were applied to the gel. Higher loads were applied for (L7/12-L10)-11S RNA (100 pmoles), (L7/12-L10 + L11)-23S RNA (90 pmoles) and L11-23S RNA (170 pmoles). A low load was applied for the (L7/12-L10)-23S RNA complex (34 pmoles). (-) refers to unbound proteins which were co-electrophoresed for comparison; total 50S subunit proteins (TP 50) were also run as markers.

fragment. Very weak binding was observed to the 18S and 11S RNA fragments, and to 16S RNA in the control experiment. Protein:RNA molar ratios were estimated by densitometry of the gels and are listed in Table 1.

Protein L11 tends to saturate at less than a 1:1 molar ratio with the 23S RNA (10). The ratio in the L11-23S RNA-complex, eluted from the Sephacryl column at 12 ml/h, was lower (0.35:1) than that found before (10) with a faster Agarose column at 60 ml/h (0.5:1). When the L11-23S RNA complex was quickly separated from excess L11 using a very fast Sephadex G-50 column run at 100 ml/h a molar ratio of approximately 1:1 was found. Clearly, the L11-23S RNA-complex tends to dissociate under non-equilibrium conditions such that the time required for the isolation of the RNA-protein complex determines the molar ratio obtained. When the binding of L11 to the 13S RNA fragment was determined the slow running Sephacryl column was employed. A molar ratio of 0.23:1 was found for the 13S RNA and very low

Table 1: Molar binding ratios in reconstituted complexes of proteins and RNA or RNA fragments.

	L11	L7/12-L10	(L11 + L7/12-L10)	
23S RNA	0.35	0.94	0.84	0.90
16S RNA	-	<0.03	-	-
18S RNA	<0.03	<0.03	<0.03	<0.03
11S RNA	-	0.03	-	-
13S RNA	0.23	0.80	0.90	0.87

The binding assays and quantitation were performed as described in Methods. The molar ratios given for the L7/12-L10 complex are based on the amount of L10 present in the slab gels (see Text). An upper limit of 0.03 is given for the very low binding values; no attempt was made to quantitate further the very weak protein bands. Error limits for the high molar binding ratios were estimated at approximately ± 15%. (-) indicates that no binding experiment was performed.

binding occurred to 18S RNA (Table 1).

Possible cooperative binding effects between L11 and the L7/12-L10 complex were investigated. In the first experiment, a limited amount of L7/12-L10 (one-fold molar excess) was added in the presence and absence of a saturating amount of L11 (five-fold molar excess). In the second experiment, L11 was incubated with the 23S RNA at a 5:1 molar ratio at which 0.35 mole of L11 would normally complex with 1 mole of the RNA. The incubation was performed in the presence and absence of a 5:1 molar excess of L7/12-L10 which would normally produce approximately a 1:1 molar complex of the latter with 23S RNA (9). In both experiments the complexes were separated from unbound protein by gel filtration, as shown in Fig. 1, and the bound proteins were examined on slab gels, and their amounts quantitated.

The results of the two experiments are shown in Fig. 3. The quantitation is given in Table 2. In the first experiment, a large increase in the binding of L7/12-L10 occurred in the pres-

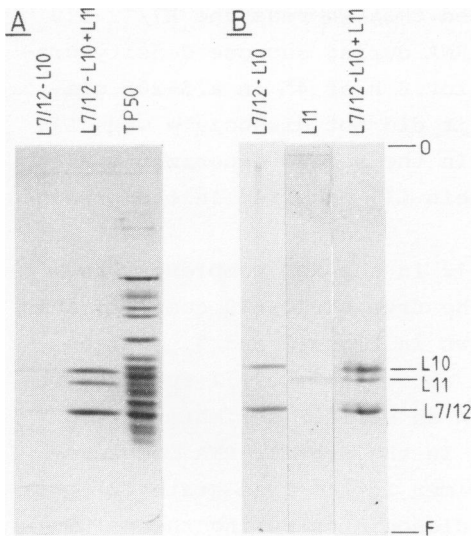

Figure 3: Cooperative protein-RNA binding effects for L7/12-L10 and L11 to 23S RNA. A: Stimulation of the binding of L7/12-L10 by L11 (L7/12-L10 in one-fold, L11 in five-fold molar excess). B: Stimulation of the binding of L11 by L7/12-L10 (both in a five-fold molar excess). 80-100 pmoles of each RNA-protein complex was loaded in each gel pocket. Total 50S subunit proteins (TP50) were co-migrated as markers.

Table 2: Mutual stimulation of RNA binding by L712-L10 and L11

A: Stimulation of L7/12-L10 by L11		
Proteins added	Proteins bound L7/12-L10	L11
L7/12-L10 alone (1:1)	0.10	
+ L11 (5:1)	0.83	1.1
B. Stimulation of L11 by L7/12-L10		
L11 alone (5:1)		0.35
L7/12-L10 (5:1)	0.94	0.84

Protein:RNA molar ratios in the incubation mixtures are given under the proteins. The protein:RNA molar ratios estimated for the RNA-bound L7/12-L10 complex are based on the amount of protein L10 that was bound.

ence of L11. The latter results were confirmed in an independent experiment when it was demonstrated that whereas the L7/12-L10 complex dissociated from the 23S RNA during sucrose density gradient centrifugation (25,000 rpm for 8 h at 4° in a 5-20% gradient in a Beckman SW 40 rotor), it did not dissociate when L11 was also bound (data not shown). In the second experiment, a large increase in binding of protein L11 occurred in the presence of excess L7/12-L10.

The stoichiometry of the L7/12 in the RNA complexes was always lower than that found in the free L7/12-L10 complex; this is clear from the gel results shown in Figs. 2 and 3. Whereas the L7/12:L10 ratio was estimated at 3.95 (± 0.3):1 for the free protein complex, in agreement with an earlier estimate (2), a ratio of 2.1 (± 0.15):1 was found in the protein-RNA complexes. Since protein L7/12 exists as a dimer in the free state (2) this strongly suggests that one dimer dissociates during the column fractionation of the (L7/12-L10)-23S RNA complex. The presence of L11 on the 23S RNA did not prevent this dissociation (Figs. 2 and 3).

The structural relationship between the L7/12 dimer and the L10 and between the L7/12-L10 complex and L11 were probed further by examining the sensitivity of the protein moieties to trypsin when complexed and uncomplexed with 23S RNA. For the unbound L7/12-L10 complex whereas the L7/12 was highly resistant to trypsin, the L10 was rapidly digested. When bound to the 23S RNA (Fig. 4A), however, both the L7/12 dimer and the L10 were strongly protected (Fig. 4B). A similar result was obtained with the (L7/12-L10+L11)-23S RNA complex. Whereas unbound L11 was rapidly digested to a resistant fragment no L11 digestion was observed in the protein-RNA complex. For both complexes, the protein moieties remained bound to the 23S RNA (Fig. 4B). When the L11-23S RNA complex was treated with trypsin, the L11 was rapidly digested; however this may only reflect the relatively high dissociation rate of L11 from the complex (see above).

DISCUSSION

The primary binding sites of L7/12-L10 and L11 lie within the 13S RNA fragment at the 5'-end of 23S RNA. Proteins L4, L20

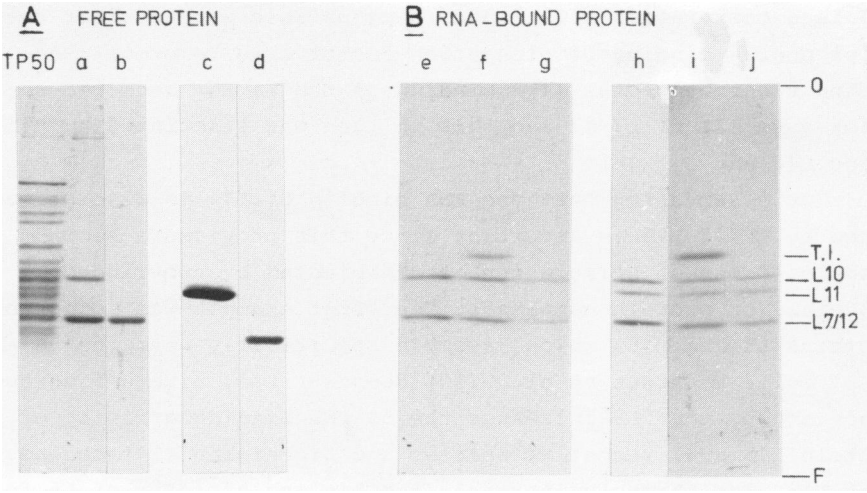

Figure 4: Trypsin treatment of L7/12-L10, L11, the (L7/12-L10)-23S RNA complex and the (L7/12-L10+L11)-23S RNA complex. A. Free proteins: (a) L7/12-L10 undigested; (b) L7/12-L10 incubated with trypsin for 1 h at 0°; (c) L11 undigested; (d) L11 treated with trypsin for 1 h at 0°. B. 23S RNA-bound proteins: (e) L7/12-L10 undigested; (f) digested as in (b); (g) the sample in (f) was re-isolated on a Sephacryl S-200 column; (h) L7/12-L10+L11 undigested; (i) digested as in (d); (j) the sample in (i) was reisolated on a Sephacryl column. T.I. indicates the position of the trypsin inhibitor which was added to stop digestion.

and L24 also bind to this RNA region (summarised in ref. 15) and proteins L13, L21 and L22 will assemble to it in the presence of the former proteins (14,17). There is also evidence to suggest that this whole protein-RNA region assembles early during in vitro 50S subunit reconstitution (18) and that it may constitute a major part of a stable core structure within the 50S subunit (19). An attempt was made to further localize the binding site of the L7/12-L10 complex within the 13S RNA fragment by T_1 ribonuclease digestion of complexes of L7/12-L10 and [^{32}P]-23S RNA. A large resistant ribonucleoprotein was obtained and subjected to oligonucleotide fingerprint analysis. Although the RNA moiety was primarily within the 13S RNA fragment region, the ribonucleoprotein was relatively unstable and difficult to prepare reproducibly in high yield.

The stimulation of the RNA binding of L7/12-L10 by protein L11 is a cooperative effect and is compatible with earlier results on the binding of elongation factor EF-G to protein-depleted ribosomal cores (4); binding of the factor required the prior assembly of L7/12 and this in turn was stimulated by proteins L10 and L11 (4).

Our observation that the RNA binding of L11 is also stimulated by L7/12-L10 is important since this provides a second example of mutual stimulation of RNA binding by two ribosomal proteins, or protein complexes. The first example was found for proteins L5 and L18, which assemble specifically with the 5S RNA of E. coli. Feunteun et al., (20) demonstrated, with a fluorescence study, that L5 increases the 5S RNA binding affinity of protein L18 and, recently, Spierer and Zimmermann (21) showed that L18 also strongly increases the binding affinity of L5 for 5S RNA. Although such mutual stimulatory effects may not necessarily operate during 50S subunit assembly, there would be obvious advantages for the accuracy of assembly if such dual mechanisms did operate. Both effects would probably arise as a result of the close proximity of the RNA binding sites of proteins and in the simplest, but by no means only, model the two protein moieties would be interacting when bound on the RNA.

The L7/12 dimer binds very weakly to the 23S RNA and this binding appears to be of an unspecific nature since it occurred to each of the RNA's tested. We also found no specific interaction of L10 with 23S RNA (10). The latter is supported by the findings of Spierer et al. (22), but not by those of Pettersson (23) who found that L10 can bind directly to 23S RNA; the reason for this disagreement is, at present, unclear although it may reflect the different modes of protein and/or RNA preparation. The trypsin digestion experiments, in Fig. 4, provide indirect evidence that L10 interacts with 23S RNA when bound to the L7/12 and this inference is supported by the protein modification studies of Pettersson (23). L10 may, therefore, constitute the main attachment site of the protein complex on the 23S RNA.

The binding results demonstrate that only one dimer of L7/12 remains bound to the (L7/12-L10)-23S RNA complex after

column fractionation. We infer that the second dimer is weakly bound and dissociates on the Sephacryl column (12 ml/h) because when the complex was rapidly fractionated on an Agarose column (60 ml/h), the L7/12:L10 molar ratio in the RNA complex was approximately 4:1 (9), the same as in the unbound protein complex. The dimer of L7/12 which remained in the (L7/12-L10)-23S RNA complex after Sephacryl gel filtration was stably bound since when the complex was rerun over the Sephacryl column there was no change in the L7/12:L10 molar ratio (see Fig. 4: gels (g) and (j)).

These results have important implications for the constitution of the protein complex and reveal that whereas one dimer binds strongly to L10, the other is more weakly associated with L10 and/or the first L7/12 dimer. This in turn provides evidence that the two dimers may be functionally differentiated on the ribosome, as has been suggested by others (C.G. Kurland, personal communication). These results also provide a rationale for the controversy over how many copies of L7/12 exist in the 50S subunit; estimates have varied between two and four (4). Our data suggest that since one pair can be readily dissociated, the number of copies of L7/12 per subunit is four.

Two main topographical models have been proposed for L7/12 on the 50S subunit which are based on immune electron microscopy. In one the proteins lie in the body of the subunit (24) whereas in the other they constitute a "stalk" structure which projects from the subunit (25). For the former model, there are various ways in which the L7/12-L10 might interact with the RNA, whilst for the latter, a model with L10 at the base of the "stalk", linking the L7/12 dimers to the 23S RNA seems feasible. Although antigenic determinants of both L10 and L11 were shown to be accessible on the surface of the 50S subunit for antibody binding (26), so far only those of L11 have been localized by immune electron microscopy (25); two widely separated determinants were positioned one of which is adjacent to L7/12.

In conclusion, we have shown that a group of proteins, which are known to be involved in an important functional site on the 50S subunit, assemble cooperatively to the 5'-end of the 23S RNA. This affords the exciting prospect of performing

functional studies on a "fragment" of the ribosome.

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