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The role of the basic N-terminal region of protein L18 in 5S RNA - 23S RNA complex formation

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ABSTRACT

Of the three proteins, L5, L18 and L25, which bind to 5S RNA, the former two effect the interaction of 5S RNA with 23S RNA. We have used trypsin as a probe to investigate the roles of the proteins in this RNA-RNA assembly, with the following results:

(1) In complexes with 5S RNA, the highly basic N-terminal region of L18 is accessible to trypsin. This accessibility is unaffected by L25. However, its presence is essential for stimulating L5 binding. (2) In 5S RNA-protein-23S RNA complexes proteins L5 and L18 are both strongly resistant to proteolysis. (3) No 5S RNA-23S RNA complex formation occurs in the presence of L5 and the C-terminal L18 fragment. Two possible models for the mechanism of RNA-RNA assembly are proposed.

INTRODUCTION

In the presence of proteins L5, L18 and L25, 5S RNA associates stoichiometrically with 23S RNA (1 - 4); the presence of proteins is essential for this RNA-RNA assembly. At present the mechanism of this interaction is not understood although it has been proposed either that the proteins form a link between the RNA's (2, 5), or that intermolecular RNA-RNA interactions occur (6) possibly as a consequence of protein-induced conformational changes in the 5S RNA which are known to occur (7 - 9).

L18 is essential for 5S RNA - 23S RNA interaction (2, 4) and we have shown, in a trypsin digestion study of an L18 - 5S RNA complex, that whereas the C-terminal part of L18 (residues 18 to 117) contains the primary 5S RNA binding site, the highly basic N-terminal region is accessible (5). We proposed, therefore, that this region of L18 may play a role, direct or indirect, in the interaction with 23S RNA. In the present work, we have extended these studies on the role of the proteins in the 5S RNA-23S RNA interaction and have shown that the N-terminal region of L18 is essential for stimulating L5 binding and for effecting, in conjunction with L5, the RNA-RNA interaction.

MATERIALS AND METHODS

Preparation of 5S RNA, 23S RNA, proteins L5, L18 and L25, and the C-terminal fragment of L18.

5S RNA was isolated from 50S subunits of *E. coli* MRE 600 as described by Monier and Feunteun (10). 23S RNA was isolated from 50S subunits by the phenol-dodecylsulphate procedure (2) and was separated from 5S RNA by gel filtration on an Agarose A 0.5 column (100 x 2 cm) containing 30 mM Tris-HCl, pH 7.4 and 1 mM Mg chloride.

Proteins L5, L18 and L25 were fractionated either by the method of Hindennach *et al.* (11), or by first preparing the 5S RNA-protein complex (12) and then fractionating the proteins on a phosphocellulose column (13). Batches of protein L5 were generally cross-contaminated with small amounts of L3, L6, L30 and L32. Some batches of L18 contained up to about 10% by weight of L15. The C-terminal fragment of protein L18 ($L18_F$) was prepared as described by Newberry *et al.* (5). Protein concentrations were determined by amino acid analysis in a Durrum analyser with 5 to 10 ug protein.

Preparation of protein-RNA complex

5S RNA-protein complexes were prepared by incubating 5S RNA with a 3-fold molar excess of proteins L5, L18, $L18_F$ or L25 in TMK reconstitution buffer (30 mM Tris-HCl, pH 7.6, 20 mM $MgCl_2$, 350 mM KCl and 6 mM 2-mercaptoethanol) at 33° for 45 min. The 5S RNA was first renatured by incubating at 55° for 5 min in the TMK buffer and slowly cooling (14). The protein-RNA complexes were cooled slowly and separated from unbound protein on a Sephadex G-100 column. The 5S RNA-protein complex was precipitated with 1.7 volumes of ethanol for 16 h at -30° and dissolved in the TMK buffer.

Complexes with 23S RNA were prepared by incubating 5S RNA-protein complexes with 23S RNA at 1.5:1 molar RNA:RNA ratio in the TMK buffer at 33° for 45 min. The 23S RNA-protein-5S RNA complex was then separated from the 5S RNA-protein complex by Agarose A 0.5 gel filtration (150 x 1,5 cm column) in 30 mM Tris-HCl, pH 7.6, 20 mM $MgCl_2$ and 250 mM KCl, or by sucrose density gradient centrifugation. In the latter, the complexes containing [^{32}P]-5S RNA were layered on gradients containing 5-20% sucrose in 30 mM Hepes, pH 7.8, 20 mM $MgCl_2$, 30 mM KCl and 6 mM 2-mercaptoethanol and centrifuged for about 9 hr at 28,000 rev/min in an SW 40 rotor. 0.5 ml fractions were collected, the optical absorbance was measured at 260 nm. [^{32}P]-5S RNA was measured by Cerenkov counting. Proteins and RNA were precipitated with 5% tri-

chloracetic acid and the proteins bound in the 5S RNA-23S RNA complex were identified by electrophoresis in 15% polyacrylamide slab gels containing dodecylsulphate (5).

Trypsin digestion of proteins and protein-RNA complexes

Proteins were dissolved in TMK buffer, pH 7.4, at a concentration of 0.2 mg/ml. Trypsin was added at a ratio of 1:500 to 1:10 (w/w) trypsin to protein or 1:50 to 1:1 for the protein-RNA complexes, and incubated on ice for 1 hr. The reaction was stopped by boiling the samples for 5 min in 1% dodecylsulphate before electrophoresing them in 15% polyacrylamide slab gels containing dodecylsulphate.

RESULTS

Binding of proteins L5, L18 and L25 to 5S RNA

Each of the three proteins binds invidivually to 5S RNA. Saturation of binding of the proteins L18 and L25 at about 0.8:1 and 0.4:1, respectively, was demonstrated earlier (5, 15). The relatively low saturation value of the latter probably reflects that some dissociation of L25 occurred under the non-equilibrium conditions of the binding assay, since L25 has a markedly lower binding affinity for 5S RNA than L18 (3, 9, 16); this view has recently been substantiated by experiments in which a fragment of 5S RNA is protected against ribonuclease A digestion by the same L25 batch as that used here; yields above 80% were generally produced (17; S. Douthwaite, unpublished data). L5 binds very weakly to 5S RNA (3, 18-20) and we generally observed protein:RNA molar ratios of about 0.15:1, for a five-fold molar excess of protein input; this increased to 0.25 to 0.3:1 when L18 was present in the reaction mixture (see also 15). Other cooperative RNA binding effects have been observed between the proteins; in particular L5 and L25 have both been shown to increase the binding affinity of L18 to 5S RNA (3, 9).

Binding of 5S RNA to 23S RNA

Gray *et al.*, (2) used partially purified proteins and a nitrocellulose membrane assay for detecting 5S RNA-23S RNA complex formation. They demonstrated that 5S RNA-23S RNA complex formation requires the presence of protein L18 and either L5 (later corrected to L6) (3) or L25. We have investigated these effects in the following experiment, using more direct methods of gel filtration and electrophoresis.

A mixture of 5S RNA and 23S RNA was incubated with the following combi-

nations of proteins: (L5 + L18), (L5 + L25), (L18 + L25) and (L5 + L18 + L25). The complexes containing 23S RNA were separated from those containing only 5S RNA on an Agarose column. The 23S RNA-bound proteins were analysed in a dodecylsulphate-containing gel. Strong binding was observed with protein combinations (L5 + L18) and (L5 + L18 + L25) but no binding was observed with (L18 + L25) nor with the individual proteins (see Fig. 1). It was concluded, therefore, that proteins L5 + L18 are essential for the 5S RNA-23S RNA interaction. Protein L25 appears to be unimportant except, possibly, in that it tends to stabilize L18 binding to 5S RNA (3); it tended to dissociate from the 5S RNA-L5 + L18 + L25-23S RNA complex during purification (Fig. 1). These conclusions are in agreement with the results recently reported by Spierer *et al.*(4).

Trypsin digestion characteristics of the proteins and the single protein-5S RNA complexes.

The trypsin digestion characteristics of protein L18 and the L18-5S RNA complex were presented earlier (5). Whereas the complex yields a resistant fragment extending from residues 18 to 117, the free protein yielded a group of three fragments, including the latter which rebound to the RNA and two slightly smaller fragments which did not rebind.

Digestion of L25 produced a homogeneous resistant fragment which is shown in Fig. 2. It was deficient in its N-terminal sequence; the fragment se-

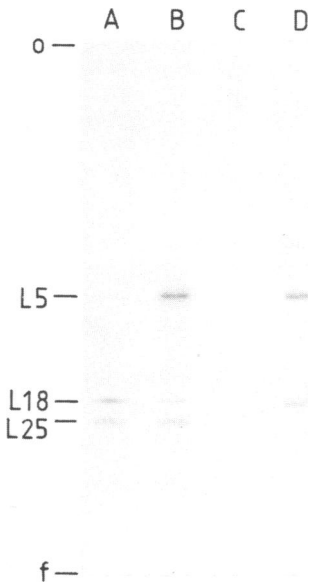

Figure 1: A: 5S RNA-(L18 + L25) and B: 5S RNA-(L5 + L18 + L25). C, D: the complexes in A and B, respectively, after incubating with 23S RNA and purifying the 23S RNA complex on an Agarose column. The proteins were dissociated from the RNA and resolved in a dodecylsulphate polyacrylamide slab gel as described in Methods. O denotes the origin and f- the dye marker.

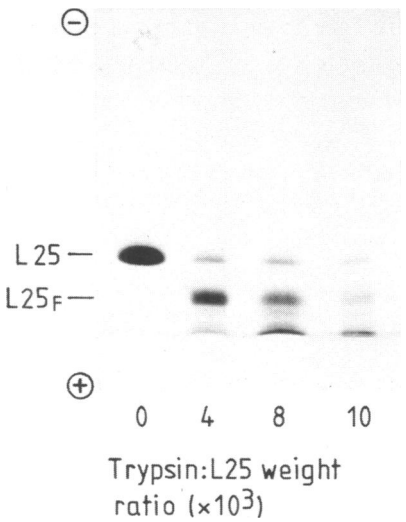

Figure 2: Trypsin treatment of protein L25 for 1 hr at 0°C and separation in a dodecylsulphate slab gel.

quence extends from residue 22 to the C-terminus (residue 94) (21; data not shown). L25 resembles L18, therefore, in that the C-terminal region is the most highly structured. It differs, however, in that the N-terminal region of L25 appears to be involved in 5S RNA binding. The C-terminal fragment does not bind to 5S RNA (data not shown), and the N-terminal region is highly resistant to trypsin digestion in the L25-5S RNA fragment even at trypsin:L25 ratios of 1:1 (w/w) (Fig. 3; gel B).

Protein L5 was the only protein that was readily digested without yielding a resistant fragment; the 5S RNA-bound protein was also susceptible to the trypsin and was slowly digested (see Fig. 4).

The accessibility of the N-terminal region of protein L18.

A: In 5S RNA-protein complexes.

Cooperative binding effects which occur between 5S RNA and proteins L5, L18 and L25 (3, 9, 20) suggest that protein-protein interactions may occur. In order to establish whether the basic N-terminal region of protein L18 is involved in these putative interactions and, therefore, less accessible to trypsin in the presence of the other proteins, 5S RNA complexes were prepared containing L25, L18 and (L18 + L25) and their susceptibilities to trypsin were examined. The results are shown in Fig. 3. For the 5S RNA-L18 and 5S RNA-(L18 + L25) complexes the $L18_F$ was produced in high yield and it was concluded that L25 did not reduce the accessibility of the N-terminal region of L18.

The results for the L5-5S RNA complex were less conclusive because

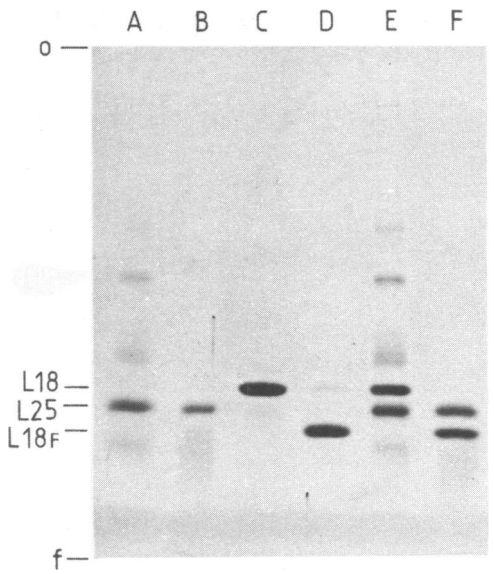

Figure 3: Digestion of 5S RNA-protein complexes at a trypsin:protein ratio of 1:200 w/w for 1 hr at 0°C. The digested proteins were dissociated and resolved in a dodecylsulphate-polyacrylamide slab gel. A, B: L25-5S RNA before and after trypsin treatment, respectively. C, D: L18-5S RNA before and after trypsin treatment, respectively. E, F: (L18 + L25)-5S RNA before and after trypsin treatment, respectively. The L25 batch contained small amounts of unidentified contaminating proteins.

first, the L5:5S RNA stoichiometry was low and second, L5 itself was slightly susceptible to trypsin under the conditions required to produce the L18 fragment. The results which are presented in Fig. 4 provide no evidence for any mutual protection of the proteins against trypsin digestion.

B: In the 5S RNA-L5 + L18 + L25-23S RNA complexes

Complexes were formed between 5S RNA and 23S RNA by incubating the RNA's with proteins L5, L18 and L25, separating the 23S RNA-containing com-

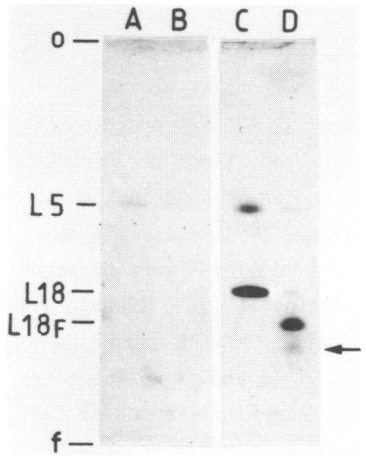

Figure 4: A, B: 5S RNA - L5 complex before and after digestion. C, D: 5S RNA -(L5 + L18) complex before and after digestion with trypsin, respectively. The arrow may show a fragment deriving from L5. All samples were digested at a trypsin:protein ratio of 1:200 for 1 hr at 0° and resolved in a dodecylsulphate slab gel.

plexes by gel filtration and precipitating with ethanol. L25 tended to dissociate from the 23S RNA complex during the purification. The resulting complex was treated with trypsin. The results are shown in Fig. 5 and demonstrate that no degradation occurred. A control sample containing 5S RNA-(L5 + L18 + L25) which was incubated with trypsin under the same conditions, produced the L18 fragment in high yield (Fig. 5). Therefore, both proteins L5 and L18 which were totally and partially degraded, respectively,when complexes with 5S RNA (Figs. 3 and 4) were strongly protected in the presence of the 23S RNA. The significance of this result is considered later.

The importance of the N-terminal region of L18 for the 5S RNA-23S RNA interaction.

23S RNA was incubated with 5S RNA in the presence of proteins L5 + L18 + L25, L5 + L18, and L5 + $L18_F$. Control samples containing only L5 or L18 or no protein were also run. The 23S RNA-protein-[^{32}P]-5S RNA complexes were separated from unbound [^{32}P]-5S RNA-protein complexes by sucrose density gradient centrifugation. The results are presented in Fig. 6. About 50% of the (^{32}P)-5S RNA was bound to the 23S RNA in the presence of proteins L5 and L18. The presence of L25 produced no detectable increase in the yield of 23S RNA-5S RNA complex formation. Only a very small amount of complex formation occurred in the samples containing L5, L18 and no protein; the same result was obtained for the (L5 + $L18_F$)-5S RNA complex.

The 5S RNA-containing peaks in Figure 6 were precipitated with trichlor-

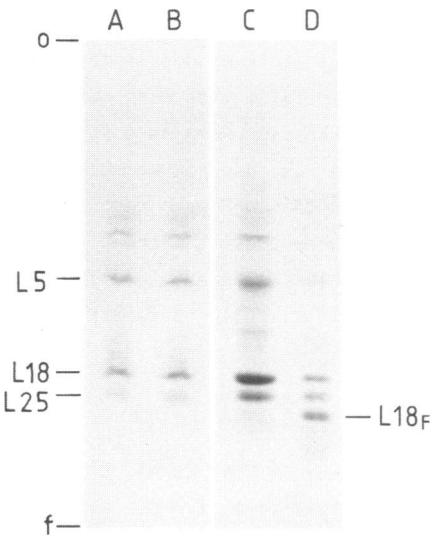

Figure 5: A, B: 5S RNA - (L5 + L18 + L25)-23S RNA complex before and after digestion with trypsin. C, D: control samples with the 5S RNA-(L5 + L18 + L25) complex before and after digestion under the same conditions; namely at a trypsin:protein ratio of 1:50 (w/w) for 1 hr at 0°C. The L5 batch contained identified contaminating proteins (see Materials and Methods).

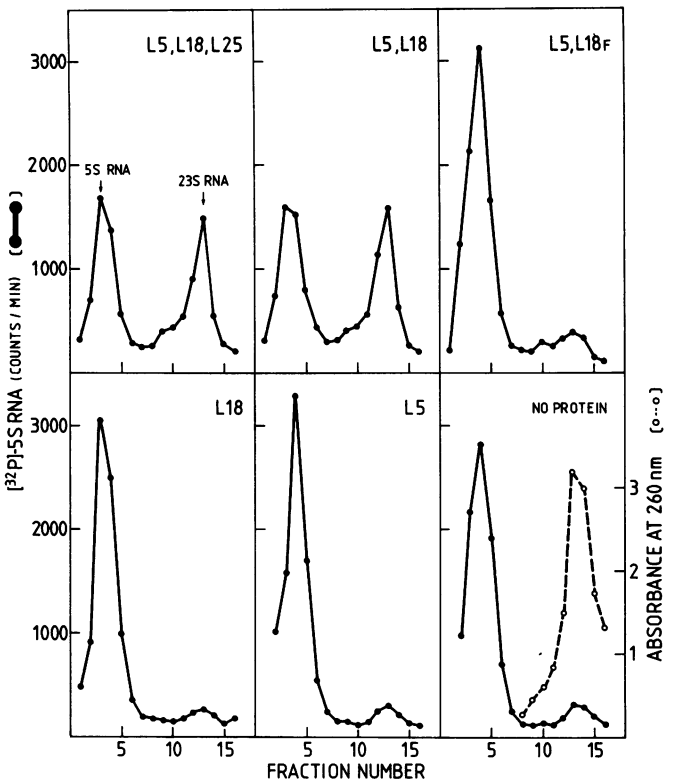

Figure 6: Sucrose gradient centrifugation of 5S RNA-protein - 23S RNA complexes (see Methods for details). The proteins which were added are indicated.

acetic acid and the bound proteins were examined in dodecylsulphate-slab gels. The molar protein:RNA ratios were estimated approximately by densitometry of the gels. No L5 was detected for samples A and B implying that when both L5 and L18 were bound the 5S RNA complexed with 23S RNA. For samples C and E weak L5 binding was observed (0.15-0.2:1). $L18_F$, unlike L18, did not stimulate the L5 binding. The protein contents of the 23S RNA-containing peaks were also examined. For samples A and B the input proteins were present whereas for C-F, including the sample with $L18_F$ + L5, no proteins were detected. The results indicate that the N-terminal region of L18 is essential for 5S RNA-23S RNA complex formation.

DISCUSSION

The present results extend the earlier studies of Gray *et al.* (1, 2) and Spierer *et al.* (4) in demonstrating the essential role of proteins in the assembly of 5S RNA with 23S RNA; in particular they establish the importance

of the unusually basic N-terminal region of L18 (nine out of seventeen amino acids are arginines or lysines - see ref. 22 for comparison with other ribosomal proteins). The possibility of a direct RNA-RNA interaction between residues 82 to 89 of 5S RNA and 143 to 154 of 23S RNA, as proposed by Herr and Noller (6), although supported by both the strong conservation of this 5S RNA sequence during evolution (23) and by the accessibility of the adjacent guanine-141 on the 50S subunit to kethoxal (6) it is not supported by the present studies, nor by topographical studies on the 5S RNA-protein complexes (15).

Earlier Gray *et al.*(2), using a nitrocellulose filter assay, inferred that either L18 + L5 (corrected from L6 (3)) or L18 + L25 could effect the 5S RNA - 23S RNA interaction. In the present experiments we see no effect with L18 + L25. A similar result was recently attained by Spierer *et al.* (4). There are two possible reasons for this discrepancy which we cannot distinguish between at present. One is that weak complex formation occurs which is stable in the filter assay but breaks down during gel filtration or in sucrose gradients. The other is that the L25 was cross-contaminated with small amounts of L5 in the earlier experiments (2); this would be compatible with our recent observation on dodecylsulphate slab gels, that when ribosomal proteins are fractionated on carboxymethylcellulose columns in 6 M urea L5 tends to smear through the salt/pH gradient, presumably due to aggregation, thereby producing cross-contamination; previously cross-contamination of L6 with L5 led to the incorrect inference that the former protein was involved in the 5S RNA-23S RNA complex formation (2, 3).

This strong tendency for protein L5 to aggregate, even in urea, provides a serious hindrance to the further investigation of 5S RNA-23S RNA assembly. For example, it is difficult to isolate L5 in either a pure state or high yield. Also, the aggregation may influence the L5-5S RNA binding properties; the low binding which we, and others (18 - 20), observe may result from a tendency for the protein to aggregate before it can bind to 5S RNA. In this context, the observed stimulation of L5-5S RNA binding in the presence of L18 could result from a weak protein-protein interaction between L5 and L18, occurring off the RNA, which prevents L5 from aggregating and allows it to bind to the RNA with a higher stoichiometry.

The interdependence of L5 and L18, during 5S RNA-23S RNA assembly, does correlate with other results. For example, they bind cooperatively and stabilize each other on 5S RNA (3, 4) and, unlike L25, they can be displaced together by removal of bivalent metal ions (24). L18 and L5 have been chemical-

ly cross-linked in the 50S subunit; L18 and L25 have not (25). Proteins equivalent to L18 and L5, but not L25, are generally found on 5S RNA-protein complexes isolated directly from halophilic and thermophilic bacteria (18, A. Matheson, personal comm.). Moreover, protein sequencing studies indicate that a large 5S RNA-bound protein from yeast may have evolved by fusion of the genes corresponding to L5 and L18 (26). Protein L25, on the other hand, appear to assemble relatively independently of L18; it has a separate binding sites on the 5S RNA (15, 17) and, apparently, induces independent changes in the 5S RNA structure (7, 15, 17).

The trypsin digestion studies presented here tell us the following about the 5S RNA-23S RNA interaction. The basic N-terminal region of protein L18, which can be readily excised from the L18-5S RNA complex (5), is essential for cooperative stimulation of L5 binding to the 5S RNA. The results indicate an interaction of the N-terminal region of L18, direct or indirect, with L5 (but not with L25). The binding results show that the 5S RNA-(L5 + $L18_F$) complex does not associate with the 23S RNA; this result indicates that the N-terminal region is essential for the 5S RNA-23S RNA interaction. This inference is reinforced by the observation that both L5 and the N-terminal region of L18 are strongly protected against trypsin in the 23S RNA complex.

This leaves us with essentially two models for the interaction which are depicted in Fig. 7. In model A, the basic N-terminal region provides a link between L5 and 23S RNA possibly with the L5 maintaining the correct RNA binding conformation of the N-terminus of L18. In model B, the basic N-terminal region of L18 interacts with L5, possibly with the very acidic region in the centre of the sequence (27) and thereby effects a conformational change in the L5 such that it can bind to 23S RNA. Both of these models imply that a protein-protein interaction occurs prior to interaction with 23S RNA; and they require that a conformational change occurs in either L18 (model A)

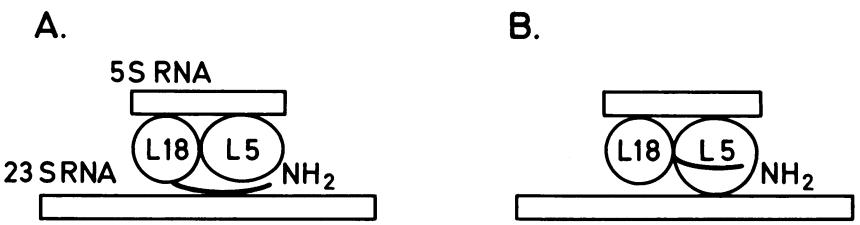

Figure 7: Two alternative hypothetical models for the role of the basic N-terminal region of L18 in the 5S RNA-23S RNA assembly.

or L5 (model B) as a result of that protein-protein interaction.

Whilst much more complex models are conceivable. For example, with the 5S RNA-(L5 + L18) lying in a pocket in the 23S RNA tertiary structure with complex interactions occurring between all three components and the 23S RNA, we feel that the features presented in one of the two models will play an essential role in the interaction of 5S RNA and 23S RNA.

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