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Topography of the E. coli 5S RNA- protein complex as determined by crosslinking with dimethyl suberimidate and dimethyl-3, 3'-dithiobispropionimidate

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ABSTRACT

5S RNA-protein complexes were prepared in vitro using partially purified E. coli 5S RNA and total E. coli 70S ribosomal proteins. The complexes were isolated from sucrose gradients and shown to contain proteins L5, L18, L25 and a fourth protein not heretofore characterized and designated L31'. The complexes were treated with the crosslinking reagents dimethyl suberimidate and dimethyl-3,3'-dithiobispropionimidate. Both reagents gave identical patterns of crosslinked proteins when analyzed by one-dimensional polyacrylamide/dodecylsulfate gel electrophoresis. Dimers of L5-L31', L5-L18 and L18-L18 and a trimer containing L5, L18 and L31' were identified by diagonal polyacrylamide/dodecylsulfate gel electrophoresis of the proteins crosslinked with dimethyl-3,3'-dithiobispropionimidate. No crosslinking was detected between L25 and the other three proteins.

INTRODUCTION

The importance of the 5S RNA molecule in ribosome structure and function is well established. E. coli ribosomes lacking the 5S RNA show reduced activities for polypeptide synthesis, elongation factor T-dependent aminoacyl-tRNA binding to the A-site, initiation factor-dependent aminoacyl-tRNA binding to the P-site and peptidyl-transferase activity (8). However, a precise functional role for the 5S RNA is yet to be established.

Three ribosomal proteins, L5, L18 and L25, form stable complexes with the 5S RNA (35). Photoaffinity labeling experiments using a guanosine-5'-diphosphate affinity label under elongation factor G-dependent binding conditions results in the labeling of L5 and L18 (24). Also, a tRNA fragment (T-Ψ-C-G), which binds to the 5S RNA-protein complex, inhibits enzymatic binding of aminoacyl-tRNA to the ribosome (31). These two results suggest that the 5S RNA and its binding proteins are at, or near, the ribosomal A-site (for a review of the evidence see Reference 10).

A variety of techniques have been used to probe the nature of the 5S RNA-

protein complex. The stoichoimetry and optimal binding conditions have been determined for each of the three binding proteins (33). Circular dichroism (1,12) and small-angle X-ray scattering (28) have been used to study conformational changes in the 5S RNA upon protein binding. Regions of the 5S RNA involved in protein binding have been examined by RNase digestion (11,14), and by chemical modification of the RNA (13). In addition, the nucleic acid binding site of L18 to the 5S RNA has been investigated using proteolytic digestion (27) and chemical modification (Fanning and Traut, Biochim. Biophys. Acta, in press).

This report describes the protein-protein neighbors of the isolated 5S RNA-protein complex as determined by the use of crosslinking reagents. In addition to the three well established ribosomal proteins (L5, L18 and L25), a fourth protein has been found in the 5S RNA-protein complexes made in this laboratory. This protein has been designated L31' because of its structural similarity to ribosomal protein L31 (Eistetter, Fanning, Butler and Traut, manuscript in preparation).

MATERIALS AND METHODS

Reagents. Dimethyl suberimidate dihydrochloride and dimethyl-3,3'-dithiobispropionimidate dihydrochloride were from Pierce Chemical Co.; RNase free sucrose and "ultra pure" urea were obtained from Schwartz-Mann; glycylglycine, iodoacetamide and chloramine T were from Sigma Chemical Co.; acrylamide, bisacrylamide and TEMED were from Eastman Chemical Co; ^{125}I was from New England Nuclear. All other chemicals were reagent grade.

Preparation of 5S RNA and ribosomal proteins. Ribosomes were prepared from *E. coli* MRE 600 as described previously (16). Ribosomal RNA was prepared from approximately 40 mg of 70S ribosomes in 10 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, 10 mM $Mg(OAc)_2$, 10 mM 2-mercaptoethanol, 0.5 mM EDTA (TNM buffer). The solution was made 0.1% in sodium dodecylsulfate and an equal volume of buffer saturated phenol was added. The mixture was vortexed for 30 min in the cold and the aqueous phase was collected after centrifugation. The phenol extraction was repeated without dodecylsulfate and the aqueous phase was dialyzed against 20 mM Tris-HCl, pH 7.5, 20 mM $Mg(OAc)_2$. The dialyzed RNA solution was adjusted to 1.0 M NaCl and kept at 0° C for 12-16 hr. In 1.0 M NaCl most of the high molecular weight RNA precipitated leaving the 5S RNA in solution (7). After centrifugation to remove the precipitated RNA, a portion of the supernatant fraction was analyzed by polyacrylamide gel electrophoresis (17). On a molar basis the 5S RNA constituted about 90% of the RNA remaining in solution.

The partially purified 5S RNA preparation was dialyzed against 20 mM Tris-HCl, pH 7.5, 20 mM $Mg(OAc)_2$ 20 mM 2-mercaptoethanol and stored at 4°C. Ribosomal proteins were prepared from approximately 80 mg of 70S ribosomes in TNM buffer. The proteins were extracted in the presence of 4 M urea, 3 M LiCl (22). After centrifugation to remove the RNA, the proteins were dialyzed against 20 mM Tris-HCl, pH 7.5, 1.0 M K(OAc), 20 mM $Mg(OAc)_2$, 20 mM 2-mercaptoethanol. The dialyzed proteins, including a small amount of precipitated material, were stored at 4°C.

Preparation of 5S RNA-protein complexes: The crude 5S RNA preparation (8 ml) was preincubated for 15 min at 30°C to allow any denatured 5S RNA to assume its native conformation (30). The dialyzed protein solution (4 ml) was vortexed to suspend the slight precipitate and the entire protein mixture was added dropwise to the preincubated 5S RNA solution with vortexing. The resulting reconstitution mixture had the following composition: 20 mM Tris-HCl, pH 7.5, 300 mM K(OAc), 20 mM $Mg(OAc)_2$, 20 mM 2-mercaptoethanol (RB buffer). The reconstitution mixture was incubated at 30°C for 30 min, cooled on ice, and insoluble material removed by centrifugation at 12,000 g for 10 min. The 5S RNA-protein complexes were isolated by centrifugation in 5-20% sucrose gradients made up in RB buffer. After centrifugation at 4°C for 24-36 hr at 33,000 rpm in the SW 41 rotor the gradients were fractionated and fractions containing the 5S RNA-protein complexes were pooled and stored at 4°C (Fig. 1).

Crosslinking with dimethyl suberimidate (DMS). A freshly prepared 1.0 M stock solution of DMS was made by dissolving DMS in 0.5 M triethanolamine-HCl, pH 8.0, containing 2.0 M NaOH. The pH of the stock solution was about 8.0. Triethanolamine-HCl, pH 8.0, was added to aliquots of the 5S RNA-protein complexes to give a final concentration of 50 mM and the complexes were incubated with either 5 mM or 10 mM DMS for 4.5-5 hr at 0°C. The reactions were stopped by adding glycylglycine to 100 mM and the ribosomal proteins were extracted in the presence of 4 M urea, 3 M LiCl (22). The extracted proteins were dialyzed against 7.5% acetec acid, freeze-dried and analyzed by one-dimensional polyacrylamide/dodecylsulfate gel electrophoresis (26).

Crosslinking with dimethyl-3,3'-dithiobispropionimidate(DTBP). An aliquot of the 5S RNA-protein complexes was dialyzed for 4-6 hr against 20 mM triethanolamine-HCl, pH 8.0, 300 mM K(OAc), 20 mM $Mg(OAc)_2$ to remove 2-mercaptoethanol. After dialysis, triethanolamine-HCl, pH 8.0, was added to a final concentration of 50 mM. DTBP was dissolved in 0.5 M triethanolamine-HCl, pH 8.0, containing 2.0 M NaOH to give a 1.0 M solution and was added to aliquots

of the dialyzed complexes to give final concentrations of either 5 mM or 10 mM. The reactions were allowed to proceed for 4.5-5 hr at 0°C and stopped by the addition of glycylglycine to 100 mM. Iodoacetamide was added to 40 mM (21) and the proteins were extracted in the presence of 4 M urea, 3 M LiCl. After dialysis against 7.5% acetic acid and freeze-drying the proteins were analyzed either by one-dimensional polyacrylamide/dodecylsulfate gel electrophoresis or by two-dimensional diagonal polyacrylamide/dodecylsulfate gel electrophoresis (32). In some instances the freeze-dried proteins were labeled with ^{125}I prior to gel electrophoresis.

Identification of crosslinked proteins. Freeze-dried samples of DTBP crosslinked proteins (10-15 µg) were dissolved in 8 M urea, 20 mM triethanolamine-HCl, pH 8.0, 1% dodecylsulfate. NaI and ^{125}I were added to final concentrations of 2-4 mM and 2-6 µCi respectively. Chloramine T was then added to give a slight molar excess over iodide. After 5-15 min at room temperature the iodinated proteins were dialyzed against 6 M urea to remove excess ^{125}I and analyzed by one-dimensional polyacrylamide/dodecylsulfate gel electrophoresis, or by two-dimensional diagonal polyacrylamide/dodecylsulfate gel electrophoresis.

Crosslinked iodinated proteins were identified essentially as described previously (19) In brief, stained bands corresponding to crosslinked proteins were cut from one-dimensional polyacrylamide/ dodecylsulfate gels and the proteins were eluted with 0.5 M Tris-HCl, pH 7.5, 1% dodecylsulfate, 2% 2-mercaptoethanol. The protein solutions were made 8 M in urea, mixed with 300 µg of nonradioactive 70S ribosomal proteins and passed through a column of Dowex 1-X8 to remove stain and dodecylsulfate. After dialysis, the proteins were freeze-dried, dissolved in 8 M urea and analyzed by two-dimensional polyacrylamide/urea gel electrophoresis. The gels were stained, dried and then autoradiographed with Kodak No-Screen X-Ray film.

RESULTS

In-Vitro reconstitution of 5S RNA-protein complexes. A partially purified preparation of 5S RNA was mixed with total ribosomal proteins under conditions known to promote the formation of 5S RNA-protein complexes (14). The complexes were then sedimented into sucrose gradients to remove contaminating 16S and 23S RNA and unbound proteins. Fig. 1A shows a typical sucrose gradient profile. The broad peak at the top of the gradient was composed primarily of 2-mercaptoethanol and unbound proteins. Two-dimensional polyacrylamide/urea gel electrophoresis of this material demonstrated the presence of all of the

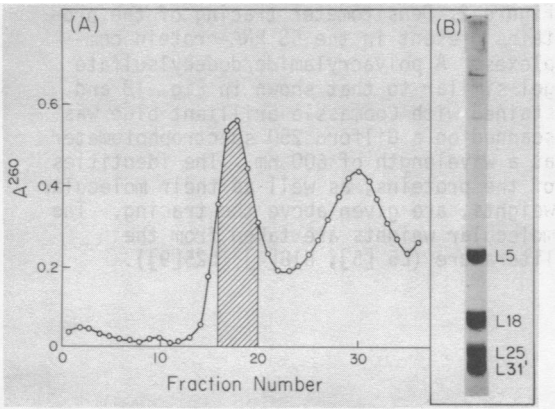

Figure 1. Isolation and protein composition of 5S RNA-protein complexes. (A) Sucrose gradient profile of the complexes prepared as described in Materials and Methods. (B) Polyacrylamide/dodecylsulfate gel of the proteins extracted from the pooled 5S RNA-protein complexes (fractions 16-20 in panel A).

ribosomal proteins including the 5S RNA binding proteins L5, L18, L25 and L31'. This was consistent with the molar excess of proteins over 5S RNA used in the reconstitution mixture.

Pooled material from the peak, at fractions 16-20 (Fig. 1A), was analyzed by one-dimensional polyacrylamide/dodecylsulfate gel electrophoresis. Four protein components were detected (Fig. 1B). The identity of the four proteins, isolated from one-dimensional polyacrylamide/dodecylsulfate gels, was established by iodination with ^{125}I and analysis by two-dimensional polyacrylamide/urea gel electrophoresis in the presence of nonradioactive marker ribosomal proteins. The four proteins present in the complexes were identified as L5, L18, L25 and a fourth component, henceforth referred to as L31' (data not shown).

Protein L31' was present at 0.6-1.0 copies, relative to L5, per 5S RNA-protein complex based on densitometer tracings of gels stained with Coomassie brilliant blue (Fig. 2). This suggested that L31' was a genuine 5S RNA binding protein. Based upon the known molecular weights of the other three 5S RNA binding proteins the molecular weight of L31' was estimated to be approximately 8,700 daltons (Fig. 2).

5S RNA-protein complexes were disrupted with dodecylsulfate and the RNA examined by polyacrylamide gel electrophoresis (17). 5S RNA, greater than 90% in its native form, was the only RNA species detected in the gels (data not shown).

Crosslinking with dimethyl suberimidate (DMS). 5S RNA-protein complexes were treated with 5 mM and 10 mM DMS and analyzed by one-dimensional polyacrylamide/dodecylsulfate gel electrophoresis. Extensive crosslinking was

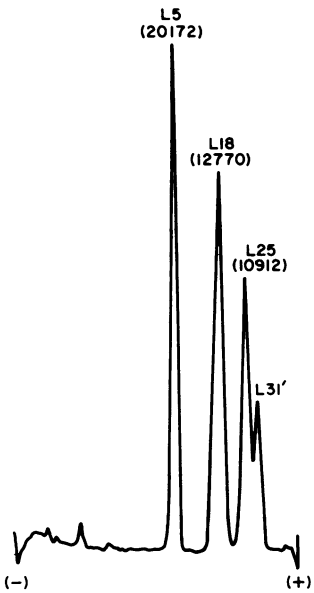

Figure 2. Densitometer tracing of the proteins present in the 5S RNA-protein complexes. A polyacrylamide/dodecylsulfate gel similar to that shown in Fig. 1B and stained with Coomassie brilliant blue was scanned on a Gilford 250 spectrophotometer at a wavelength of 600 nm. The identities of the proteins, as well as their molecular weights, are given above the tracing. The molecular weights are taken from the literature (L5 [5]; L18[4]; L25[9]).

observed with new electrophoretic products at positions corresponding to approximately 26,000, 29,000, 33,000 and 45,000 daltons (Fig. 3). Control experiments in which the complexes were disrupted by dialysis against 8 M urea prior to DMS treatment showed no high molecular weight bands.

The identity of the DMS-crosslinked products could not be established unambiguously on the basis of molecular weight alone. The 29,000 dalton product had a molecular weight nearly identical to that expected for an L5-L31' dimer. However, the influence of possible intramolecular crosslinks on the mobility of the proteins in the gels could not be predicted. Thus, to distinguish an L5-L31' dimer (ca. 29,000 daltons) from an L5-L25 dimer (ca. 31,000 daltons) with confidence was virtually impossible.

Crosslinking with dimethyl-3,3'-dithiobispropionimidate(DTBP). A second reagent, DTBP, was used in order to overcome the difficulties encountered in identifying the DMS promoted crosslinks. DTBP, which has a somewhat shorter crosslinking radius than DMS (9 Å vs 11 Å), has the advantage that it is cleavable by disulfide reducing agents such as 2-mercaptoethanol.

Treatment of the 5S RNA-protein complexes with DTBP gave a pattern of crosslinks virtually identical to that found with DMS when analyzed by one-dimensional polyacrylamide gel electrophoresis (Fig. 4). The major difference between the two reagents was in the efficiency of promoting crosslinks. DMS,

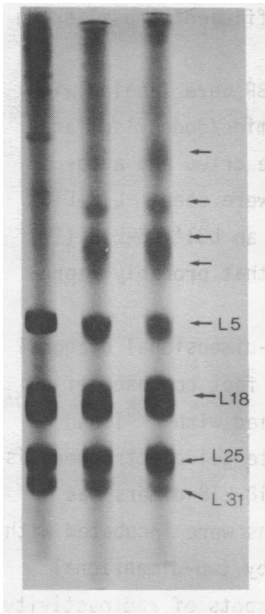

Figure 3. Crosslinking of proteins in the 5S RNA-protein complexes induced by treatment with dimethyl suberimidate (DMS). 5S RNA-protein complexes were treated with DMS and the proteins were analyzed by polyacrylamide/dodecylsulfate gel electrophoresis. Gel 1, no DMS; gel 2, 5mM DMS; gel 3, 10mM DMS. The positions of the crosslinked proteins are indicated by the arrows.

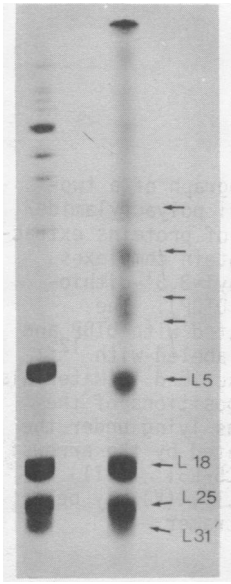

Figure 4. Crosslinking of proteins in the 5S RNA-protein complexes induced by treatment with dimethyl-3,3'-dithio-bispropionimidate (DTBP). 5S RNA-protein complexes were treated with DTBP and the proteins were analyzed by polyacrylamide/dodecylsulfate gel electrophoresis. Gel 1, no DTBP, gel 2, 10 mM DTBP. The positions of the cross-linked proteins are indicated by the arrows.

possibly because of its longer length, was a much more efficient crosslinking reagent than DTBP (compare Fig. 3 to Fig. 4).

Samples of 5S RNA-protein complexes treated with DTBP were labeled with ^{125}I and analyzed by two-dimensional diagonal polyacrylamide/dodecylsulfate gel electrophoresis. After electrophoresis the gels were dried and autoradiographed (Fig. 5). The following crosslinked pairs were seen: L18-L18 (I), L5-L31' (II), and L5-L18(III). Also detectable was an L31'-L5-L18 (IV) trimer as well as a higher molecular weight product (V) that probably represents an L31'-L5-L18-L18 tetramer.

To confirm that the L5-L31' dimer identified by two-dimensional diagonal polyacrylamide/dodecylsulfate gel electrophoresis was in fact composed of L5 and L31', a sample of DTBP crosslinked proteins was labeled with ^{125}I and subjected to one-dimensional polyacrylamide/dodecylsulfate gel electrophoresis. The region of the gel corresponding to the L5-L31' and L18-L18 dimers was excised and the proteins were eluted. The eluted proteins were incubated with 2-mercaptoethanol to reduce the crosslinks and analyzed by two-dimensional polyacrylamide/urea gel electrophoresis (Fig. 6). Four spots of radioactivity could be seen, three of which correspond to proteins L5, L18 and L31'. The fourth spot did not correspond to any ribosomal protein in the stained gel. This material probably represents non-reduced dimers, but could also represent L5 that has undergone some form of chemical modification during the crosslinking and/or labeling process.

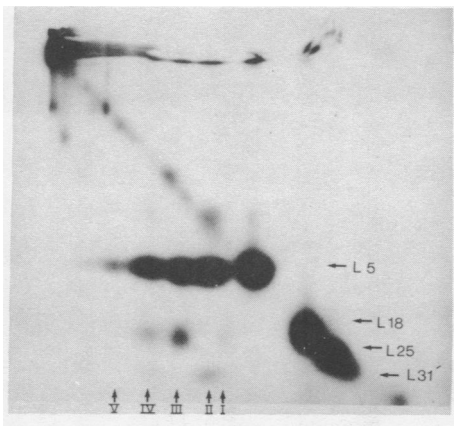

Figure 5. Autoradiograph of a two-dimensional diagonal polyacrylamide/dodecylsulfate gel of proteins extracted from 5S RNA-protein complexes treated with dimethyl-3,3'-dithiobispropionimidate (DTBP). The complexes were treated with DTBP and the proteins were labeled with ^{125}I and analyzed as described in Materials and Methods. The positions of the crosslinked proteins lying under the diagonal are indicated by the arrows: (I) L18-L18, (II) L5-L31', (III) L5-L18, (IV) L31'-L5-L18, (V) may be an L31'-L5-L18-L18 tetramer.

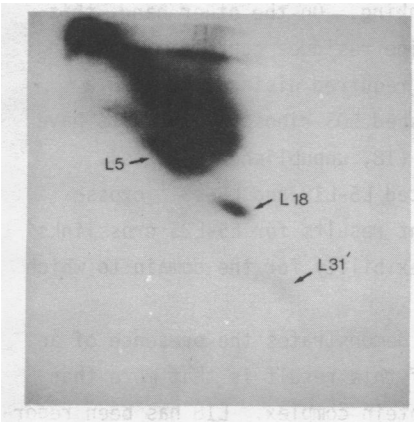

Figure 6. Identification of the L5-L31' dimer. Proteins extracted from dimethyl-3, 3'-dithiobispropionimidate-treated 5S RNA-protein complexes were labeled with ^{125}I and subjected to one-dimensional polyacrylamide/dodecylsulfate gel electrophoresis. The crosslinked proteins migrating in the 26,000-30,000 dalton range were extracted from the gel, incubated with 2-mercaptoethanol, and analyzed by two-dimensional polyacrylamide/urea gel electrophoresis in the presence of nonradioactive marker ribosomal proteins.

DISCUSSION

The 5S RNA-protein complexes described here contain a protein component, L31', not previously described in the literature. L31' is similar to, but not identical with, a sequenced (3) ribosomal protein identified as L31. The failure of others to detect L31' in isolated 5S RNA-protein complexes is probably related to the fact that the amount of L31' associated with the 50S ribosomal subunit is very sensitive to the salt concentration used in ribosome purification: several cycles of centrifugation through 0.5 M NH_4Cl is sufficient to remove nearly all of the L31' from the ribosomes of a number of *E. coli* strains, e.g., Q 13, B and D 10 (Eistetter, Fanning, Butler and Traut, manuscript in preparation).

The present report demonstrates the close proximity of proteins L5 and L31' and L5 and L18 in the 5S RNA-protein complex. The proximity of L5 to L18 is consistent with the results of others (33) who showed that the presence of L18 enhances the binding of L5 to the 5S RNA. This enhancement presumably occurs by a mechanism involving either protein-protein interaction or an L18 induced conformational change in the 5S RNA (33). The data presented here do not distinguish between these two possibilities, but are consistent with a protein-protein interaction between L5 and L18. Protein-protein interaction may be involved in L31' binding to the 5S RNA-protein complex. Attempts to establish the binding constant and stoichiometry of L31'-5S RNA complex formation have, to date, proven unsuccessful since L31', purified by phosphocellulose/urea chromatography, fails to bind 5S RNA (T. Fanning, unpublished).

The absence of crosslinks involving L25 suggest that L25 and the other three proteins occupy separate domains on the complex sufficiently distant as

to be unavailable for protein-protein crosslinking. On the other hand, this result may be due to the fact that no L25 lysine residues capable of reacting with the crosslinking reagents are within the required distance to form a crosslink. Crosslinking studies with salt washed 50S ribosomal subunits have yielded L5-L18, L5-L31' and L5-L25 crosslinks (18, unpublished results). Similar studies on the 70S ribosome demonstrated L5-L18 and L5-L31' crosslinks, but no L5-L25 crosslinks. The different results for L5-L25 crosslinks are suggestive of a certain conformational flexibility for the domain to which L25 is bound.

The crosslinking pattern shown in Fig. 5 demonstrates the presence of an L18-L18 dimer. The simplest interpretation of this result is that more than one molecule of L18 can bind to the 5S RNA-protein complex. L18 has been reported to be present at two copies in the 5S RNA-protein complex (14,25) and in the 50S ribosome (34). Others, however, have found only a single copy (6,15). The stoichiometry of L18 in the 5S RNA-protein complexes used in the present study could not be accurately assessed. Densitometer tracings of Coomassie brilliant blue R-250 stained gels suggested two copies of L18 relative to the other proteins in the complex. However, when the concentration of a sample of purified L18 was determined either with Coomassie G-250 (2) or with the Folin-Ciocalteau reagent (23) the value obtained by the Coomassie method was twice as high as by the Lowry method (T. Fanning, unpublished). The anomalous staining behavior of certain proteins with Coomassie has been noted by others (20,29). Therefore, it seems unlikely that L18 is present at appreciably more than one copy in the 5S RNA-protein complexes. Very likely the L18-L18 dimer arises from the crosslinking of a small fraction of 5S RNA-protein complexes which contain two L18 molecules (28). (Note: the intensities of the spots in Fig. 5 are probably due to the number of tyrosine residues/protein, ie. L5 contains six tyrosines, L18 and L25 contain three tyrosines each and L31' contains a single tyrosine). The results presented here clearly demonstrate that the binding site of the second L18 molecule is near enough to that of the first to allow a crosslink to form.

In summary, the results presented here indicate that in addition to the three well established 5S RNA binding proteins, L5, L18 and L25, a fourth protein, L31', can be detected in 5S RNA-protein complexes. Crosslinking experiments indicate the proximity in the 5S RNA-protein complex between proteins L5 and L31', and L5 and L18. No crosslinks involving L25 were detected and no L18-L31' dimers were seen. In addition to the L5-L31' and L5-L18 dimers, some L18-L18 dimers were detected.

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FOOTNOTE

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