Analysis of the Binding of Xenopus Ribosomal Protein L5 to Oocyte 5 S rRNA

THE MAJOR DETERMINANTS OF RECOGNITION ARE LOCATED IN HELIX III-LOOP C*

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Xenopus ribosomal protein L5 was expressed in Escherichia coli and exhibits high affinity (K_d = 2 \times 10^{-6}) and specificity for oocyte 5 S rRNA. The pH dependence of the association constant for the complex reveals an ionization with a pK_a value of 10.1, indicating that tyrosine and/or lysine residues are important for specific binding of L5 to the RNA. Formation of the L5:5 S rRNA complex is remarkably insensitive to ionic strength, providing evidence that nonelectrostatic interactions make significant contributions to binding. Together, these results suggest that one or more tyrosine residues may form critical contacts through stacking interactions with bases in the RNA. In order to locate recognition elements within 5 S rRNA, we measured binding of L5 to a collection of site-specific mutants. Mutations in the RNA that affected the interaction are confined to the hairpin structure comprised of helix III and loop C. Earlier experiments with a rhodium structural probe had shown that the two-nucleotide bulge in helix III and the intrinsic structure of loop C create sites in the major groove that are opened and accessible to stacking interactions with the metal complex. In the present studies, we detect a correlation between the intercalative binding of the rhodium complex to mutants in the hairpin and binding of L5, supporting the proposal that binding of the protein is mediated, in some part, by stacking interactions. Furthermore, the results from mutagenesis establish that, despite overlapping binding sites on 5 S rRNA, L5 and transcription factor II A utilize distinct structural elements for recognition.

The metabolism of Xenopus 5 S ribosomal RNA during oogenesis provides the opportunity to study the interaction of several different proteins with the same nucleic acid. Moreover, these individual ribonucleoprotein complexes appear to determine the intracellular translocation of 5 S RNA. Initially, the primary transcripts are transiently associated with the La antigen in the nucleus (1, 2). However, the synthesis of 5 S rRNA and ribosome assembly are discontinuous in the early stages of oogenesis, so that much of the RNA is stored in the cytoplasm complexed either with transcription factor II A (TFIIIA) or with the protein p43 as part of a large multicomponent 42 S RNP complex (3, 4). Coincident with the expression of the ribosomal proteins during vitellogenesis, an increasing amount of cytoplasmic 5 S rRNA becomes associated with ribosomal protein L5 (5). This latter complex then moves to the nucleolus, where it becomes integrated into nascent ribosomes (1, 6). Mutant forms of 5 S rRNA that are unable to bind to TFIIIA or L5 are retained in the nucleus of the oocyte, indicating that the RNA can only be exported to the cytoplasm in the form of an RNP complex (2). Likewise, the ultimate return of cytoplasmic 5 S rRNA to the nucleolus depends on the formation of the complex with L5 (6, 7). It is not known how the formation of a particular RNP particle creates a signal for nucleocytoplasmic transport.

The interaction of TFIIIA with oocyte 5 S rRNA is the most thoroughly characterized of the four complexes. The factor makes multiple contacts over much of the nucleic acid through the nine zinc finger domains of the protein (8, 9). Recognition is mediated by the secondary structure of the RNA with the major determinants located in a central domain composed of helix II-loop A-helix V-loop E (10). The other protein involved in the storage of cytoplasmic 5 S rRNA, p43, also contains nine zinc finger domains; however, it shares only 33% amino acid identity with TFIIIA (11). Similar to the case with TFIIIA, the identity elements for p43 appear to be dispersed over a considerable amount of the 5 S RNA molecule including helices II, IV, and V as well as loop D (12). Contacts made by p43 to helical regions of the RNA appear to be more sequence-dependent compared with those made by TFIIIA (12). Notwithstanding the structural similarities between TFIIIA and p43 and their overlapping binding sites on 5 S rRNA, the determinants of high affinity binding are not identical for the two proteins.

Protection (13–16) and interference (17) experiments have been used to determine the binding site for rat ribosomal protein L5 or its yeast equivalent, YL3, on 5 S rRNA. The cumulative results are in good agreement and indicate that helices I, II, IV, and V, as well as loops D and E provide the major contact surface for the ribosomal protein. Despite having nearly congruent binding sites on 5 S RNA, L5 and TFIIIA share no similarity in the sequences of their amino acids (5, 18). Thus, two proteins have arisen independently to bind to the same RNA molecule and present a means for the comparative analysis of RNA recognition by two disparate proteins. In order to identify the important recognition elements for Xenopus L5, we have measured binding of the protein to a collection of site-specific mutants made in oocyte 5 S rRNA. Additionally, we have taken the occasion to compare these results with those from analogous experiments involving TFIIIA.

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The abbreviations used are: TFIIIA, transcription factor II A; RNP, ribonucleoprotein; DTT, dithiothreitol; MES, 2-(N-morpholino)ethanesulfonic acid; MBP, maltose binding protein; L5, the chimeric protein composed of maltose binding protein fused to ribosomal protein L5; Rh(phen)_2(phi)_3^2−, bis(phenanthroline)(phenanthrenequinone diimine)rhodium(III).

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EXPERIMENTAL PROCEDURES

Cloning of the Gene Encoding Ribosomal Protein L5 into the Expression Vector pMAL-c—A DNA clone (L5b) of the gene encoding ribosomal protein L5 from Xenopus laevis was kindly provided by Dr. W. M. Warnington (University of Virginia) and used as a template for polymerase chain reaction amplification (5). The 38-nucleotide 5' primer (5'-TAGGATCCCTCGAAGGCTAGTGGTCTAGAAGGTC-3') contained, in turn, a BamHI restriction site, the codons for Ile-Glu-Gly, which is the recognition sequence for Factor Xa, and 18 nucleotides complementary to the first six codons of L5. The 26-nucleotide 3' primer (5'-CAGGTACCTTATTAGCTGTCTGCCTT-3') contained, in turn from the 3'-end, the final six codons of L5, an additional stop codon, and a BamHI restriction site. Amplification by polymerase chain reaction was performed in a final volume of 100 μl containing 20 ng of the template plasmid; 100 pmol each of the 5'- and 3'-primers; 20 mM Tris-HCl, pH 8.8; 30 mM KCl; 10 mM (NH₄)₂SO₄; 2 mM MgSO₄; 0.1% Triton X-100; 0.2 mM dNTP; 2 μl of Vent DNA polymerase (New England Biolabs). Cycling was performed at 94°C (1 min), 50°C (2 min), 72°C (2 min) for 30 cycles. The final cycle had an extension time of 10 min. The amplified fragment (920 bp) was purified by electrophoresis on a 1% agarose gel and then digested with BamHI. The expression vector, pMAL-c (New England Biolabs) was digested with BamHI and treated with calf intestine alkaline phosphatase. The digested vector and polymerase chain reaction product were ligated, and the mixture was used to transform Escherichia coli DH5α. Individual colonies were screened by restriction mapping for recombinant pMAL-c plasmids with the L5 gene in the proper orientation. The DNA sequence of the L5 gene in the resulting recombinant plasmid, designated pMAL-cL5, was verified using the Sequenase kit for chain-termination sequencing (Amersham Corp.).

Expression and Purification of MBP-L5 Fusion Protein—E. coli (DH5α) transformed with pMAL-cL5 were grown at 37°C in Luria-Bertani medium containing 0.1% glucose until the absorbance of the culture at 600 nm reached approximately 0.4. Isopropyl-1-thio-β-D-galactopyranoside was then added to a final concentration of 60 μM. Cells were harvested 1 h later by centrifugation at 4°C and suspended in 20 ml of ice-cold affinity column buffer (10 mM Tris-HCl, pH 8.0; 200 mM NaCl; 5% glycerol; 1 mM EDTA; 1 mM DTT) plus protease inhibitors (2 μg/ml leupeptin; 16 μg/ml pepstatin; 0.8 mM phenylmethylsulfonyl fluoride; 50 μg/mg benzamidine). The solution was degassed prior to adding DTT and protease inhibitors. Cells were sonicated at all times to keep the cells chilled on ice. The cell suspension was lysed using a French press, and the cellular debris was removed by centrifugation for 20 min at 20,000 rpm in a Beckman Type 50.2 Ti rotor. The clarified cell extract was then subjected to affinity chromatography at 4°C through amylose (New England Biolabs) column equilibrated with the same buffer. The column was developed with 0.25–1.0 ml of column buffer was digested with 0.25–1.0 ml of Factor Xa (Promega) at 4°C and stored at −20°C. Analysis of whole cell extracts of uninduced and induced cells by SDS-polyacrylamide gel electrophoresis shows the appearance of a single, major protein of molecular mass of 75 kDa (Fig. 1, lane 3). Purification of L5—The fusion protein was expressed as a fusion protein, fL5, using the pMAL-c expression vector (25). The chimeric protein consists of the E. coli MBP as the amino-terminal domain connected to the ribosomal protein by a linker sequence containing the cleavage site for the protease Factor Xa. The molecular mass of fL5 predicted from the amino acid sequences of the MBP and linker (41,761 Da) plus L5 (34,075 Da) is 75,836 Da. The 5'-primer used for polymerase chain reaction amplification was designed so that cleavage of fL5 by Factor Xa releases the ribosomal protein without any additional amino acid residues at the amino terminus. E. coli (DH5α) carrying the plasmid pMAL-cL5 were grown to mid-log phase and induced to express fL5 by isopropyl-1-thio-β-D-galactopyranoside. Analysis of whole cell extracts of uninduced and induced cells by SDS-polyacrylamide gel electrophoresis shows the appearance of a single, additional protein after induction having an apparent molecular mass of 75 kDa (Fig. 1, lane 3). The fusion protein is prone to proteolytic degradation; therefore, cells collected by centrifugation were suspended in buffer containing a mixture of protease inhibitors. In addition, samples were continuously kept on ice during all steps of the purification. L5 appears to be easily oxidized, resulting in denatured, insoluble protein. For this reason, all buffers were degassed and contained 1 mM DTT. In experiments to optimize binding conditions, the standard binding buffer was used with the exception of the component being tested. Protein and RNA at the indicated concentrations were mixed gently, avoiding air bubbles as much as possible, and incubated at room temperature for 1 h. After addition of 0.5 μl of sample solution (0.25% bromophenol blue; 0.25% xylene cyanole; 60% sucrose) each reaction mixture was loaded onto the gel. Autoradiographs of mobility shift gels were scanned with a laserdensitometer, and this binding data was processed using the program EZ-fit (24). Binding assays were performed in duplicate with a minimum of two different preparations of RNA.

RESULTS

Purification of L5 Expressed in E. coli—X. laevis ribosomal protein L5 was expressed as a fusion protein, fL5, using the pMAL-c expression vector (25). The chimeric protein consists of the E. coli MBP as the amino-terminal domain connected to the ribosomal protein by a linker sequence containing the cleavage site for the protease Factor Xa. The molecular mass of fL5 predicted from the amino acid sequences of the MBP and linker (41,761 Da) plus L5 (34,075 Da) is 75,836 Da. The 5'-primer used for polymerase chain reaction amplification was designed so that cleavage of fL5 by Factor Xa releases the ribosomal protein without any additional amino acid residues at the amino terminus. E. coli (DH5α) carrying the plasmid pMAL-cL5 were grown to mid-log phase and induced to express fL5 by isopropyl-1-thio-β-D-galactopyranoside. Analysis of whole cell extracts of uninduced and induced cells by SDS-polyacrylamide gel electrophoresis shows the appearance of a single, additional protein after induction having an apparent molecular mass of 75 kDa (Fig. 1, lane 3). The fusion protein is prone to proteolytic degradation; therefore, cells collected by centrifugation were suspended in buffer containing a mixture of protease inhibitors. In addition, samples were continuously kept on ice during all steps of the purification. L5 appears to be easily oxidized, resulting in denatured, insoluble protein. For this reason, all buffers were degassed and contained 1 mM DTT. Cells were lysed using a French press rather than by sonication, since the latter method also appeared to promote oxidation of the protein. Despite these precautions, preparations of the protein, on average, were between 50 and 60% active as determined by Scatchard analysis of 5 S rRNA binding activity (results not shown). Protein that was below this level of activity or that yielded high molecular weight complexes in mobility shift gel assays was discarded.

After lysis and centrifugation to remove cellular debris, the crude cell extract was passed through an amylose affinity column, which was subsequently washed with buffer overnight. The retained fusion protein was eluted with buffer containing 10 mM maltose (Fig. 1, lane 4). Digestion of the purified fL5 with Factor Xa produces two polypeptide products having molecular masses of 42 and 34 kDa, which correspond to the MBP domain and L5, respectively (Fig. 1, lane 5). Generally, a small portion of fL5 was resistant to Xa cleavage, presumably due to

L55 S rRNA
denaturation and/or aggregation of the protein, which prevented access to the cleavage site. Intact L5 was purified after digestion of the fusion protein by chromatography over DEAE Sephacel as described in “Experimental Procedures.” No contaminating E. coli S 5 S rRNA could be detected in samples of affinity-purified fl5 upon staining SDS-polyacrylamide gels with silver.

Comparison of fl5 and L5 Binding to 5 S rRNA—The binding of the fusion protein to 5 S rRNA was compared with L5 purified from the former. In direct titrations of 5 S rRNA, equivalent molar concentrations of the individual proteins bound the same amount of the nucleic acid (Fig. 2A). Purified maltose binding polypeptide has no apparent affinity for 5 S rRNA (Fig. 2B, lanes 1 and 3) nor does it influence the binding of L5 (Fig. 2B, lanes 2 and 4). A stoichiometric mixture of the maltose binding polypeptide and L5, likewise, has the same RNA binding activity as an equivalent amount of the intact fusion protein (Fig. 2B, lane 2 compared with lane 5). The fusion protein, fl5, is more stable than purified L5. Moreover, the former produces a greater retardation of 5 S rRNA in mobility shift gels that decrease the time required for sufficient electrophoretic separation of free and bound RNA. Since the MBP domain has no effect on the binding of L5, we used fl5 in binding assays with mutant 5 S rRNA. However, in all cases where a mutation in 5 S rRNA affected binding of fl5, assays of these RNAs were repeated with L5. In no instance did we observe a quantitative difference between the binding of L5 and the fusion protein.

Characterization of Buffer Conditions for Binding Assays—We determined the activity of L5 as a function of pH and monovalent and divalent cation concentrations in order to optimize conditions for binding assays with mutants of 5 S rRNA. Standard binding conditions were buffered at pH 6.0 and 6.5 with 20 mM MES, at pH 6.8, 7.4, and 8.0 with 20 mM HEPES, and at pH 8.5, 9.0, and 9.5 with 20 mM Tris-HCl. Binding isotherms were used to determine the apparent association constant at each value of pH (Fig. 3A). A bell-shaped binding response was obtained with optimal binding occurring between pH 7.5 and 8.5; consequently, we performed standard binding assays at pH 8.0. The data points of the pH profile can be fit to an expression derived for pH effects on the apparent association constant. In this instance, the expression contained terms for two ionizations, and nonlinear regression analysis yielded pK_a values of 5.4 and 10.1. The data point at pH 9.5 was not included in the curve fitting, since it represents either the effect of an ionization associated with a third pK_a or the appearance of nonspecific binding in these alkaline conditions. It is difficult to attribute the first ionization to a specific amino acid or nucleotide residue, and this value may represent a complex function of both. The second pK_a, however, is equivalent to those for the ionization of tyrosine and lysine. Xenopus L5 contains 19 tyrosine residues and 36 lysine residues. We cannot discount that this pK_a represents deprotonation of a ring nitrogen of guanosine or uridine, although these latter possibilities seem much less likely. The marked dependence of the binding constant on pH is somewhat unusual for the association of two macromolecules, since oftentimes several functional groups with widely different pK_a values contribute to the interaction. For example, the binding of TFIIIA and p43 to 5 S rRNA exhibit little change as a function of pH with only modest decreases in affinity at values above 8.0 (12). The pH dependence of L5 affinity for 5 S rRNA may indicate that a small number of interactions mediate binding or that many of the contacts are chemically similar. The slope of the curve at pK_a 10.1 is approximately 2, which indicates that this number of ionizable residues contributes to this transition (26).

Magnesium moderately enhances binding of L5 to 5 S rRNA at low concentrations, but a decrease in affinity is detectable at concentrations greater than 2 mM (Fig. 3B). At 8 mM magnesium, the apparent K_a decreases by more than an factor of 10. Yeh et al. (27) have shown that high concentrations of magnesium promote dissociation of the yeast YL3-5 S RNA complex. Our standard binding assays contained 0.5 mM MgCl_2. Since the zinc finger domains of TFIIIA mediate binding of the factor to nucleic acids, micromolar concentrations of zinc are generally included in buffers for this protein. We found that binding of L5 to 5 S rRNA is severely inhibited by micromolar concentrations of zinc with no detectable binding above 30 mM ZnCl_2. Consequently, this divalent cation was not included in the standard binding buffer.

The interaction of L5 with 5 S rRNA is insensitive to ionic strength. The apparent K_a of the complex remains constant over a range of 0–0.4 mM KCl (data not shown). Only a 2-fold decrease in binding affinity was observed at a monovalent salt concentration of 0.6 M. These results indicate that an appreciable contribution to binding comes from nonelectrostatic interactions, which is surprising given that L5 is a highly basic protein with a net charge of +22 (5). However, this behavior has been observed for other RNA-binding proteins (28), including ribosomal proteins (29). The absence of an appreciable dependence on salt concentration is particularly meaningful in light of the pH profile that suggests the involvement of tyrosine and/or lysine residues in binding. These results indicate that it is the former amino acid that accounts for the pK_a of 10.1. Thus, contacts in which this aromatic residue is stacked upon the base moieties of a nucleotide may contribute significantly to the overall free energy of binding of the ribosomal protein to 5 S rRNA.

**Fig. 1. Expression and purification of fl5.** Protein samples were analyzed on a 10% SDS-polyacrylamide gel stained with Coomassie Blue dye. Lane 1, molecular mass standards of 69, 45, and 29 kDa; lanes 2 and 3, extract prepared from E. coli cells before and after induction of expression of fl5 with isopropyl-β-D-thiogalactopyranoside, respectively; lane 4, fl5 eluted from amylose affinity column; lane 5, digestion products of fl5 after treatment overnight at 0°C with factor Xa (weight ratio of fl5 to Xa was 100). The positions of the MBP polypeptide (a), L5 (b), and Xa (c) are indicated.

**Fig. 2. Binding of fl5 and L5 to 5 S rRNA.** A, internally radiolabeled 5 S rRNA (10 nM) was incubated with 5, 20, 50, or 100 nM L5 (lanes 2–5) purified from the deaved fusion protein by chromatography on DEAE-Sephalc or with the same concentrations of fl5 (lanes 6–9). Samples were analyzed by electrophoresis on 8% nondenaturing polyacrylamide gels followed by autoradiography. B, 5 S rRNA (10 nM) was incubated with 50 nM (lanes 1 and 2) or 20 nM (lanes 3 and 4) MBP in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of an equal concentration of L5; lane 5, binding assay containing 50 nM fl5.
A concentration of 0.1 M KCl was chosen for standard assays of L5, since this is comparable with the conditions that were used to measure binding of TFIIIA to these same mutants in 5 S rRNA. Moreover, higher salt concentrations appear to stabilize the nucleic acid binding activity of the protein.

Specificity of fL5 Binding to 5 S rRNA—The specificity of binding of fL5 to 5 S rRNA relative to tRNA was tested in a competition assay (Fig. 4). Internally radiolabeled wild-type 5 S rRNA and fL5 were incubated together with increasing concentrations of competing, unlabeled 5 S rRNA or E. coli tRNA. A 100-fold excess of tRNA relative to 5 S rRNA causes only a 40% decrease in binding of fL5 to the cognate RNA. From this experiment the dissociation constant for tRNA relative to that for 5 S rRNA is greater than 50. Thus, the protein discriminates well between 5 S rRNA and nonspecific RNA.

Analysis of the Binding of L5 to Site-specific Mutants of 5 S rRNA—Experiments to define the binding site for L5 (rat) or YL3 (yeast) on 5 S rRNA indicate that the contact surface for the protein is quite substantial (13–17). In fact, it has been proposed that the nucleic acid binding domains in the three eubacterial proteins that bind to prokaryotic 5 S rRNA have been brought together in the single eukaryotic protein (13), although this conservation is not evident from an analysis of amino acid sequence data (18). The binding site for rat L5 defined in footprinting experiments overlaps, but is not identical to, that for TFIIIA, explaining why binding of these two proteins to 5 S rRNA is mutually exclusive (16). TFIIIA protects most of 5 S rRNA from chemical or enzymatic degradation; however, the central domain of the nucleic acid (helix II-loop A-helix V-loop E) provides the major determinants for binding of the factor (9, 19, 30–32). In order to identify the location of the major identity elements for Xenopus L5, we measured binding of the protein to a collection of site-specific mutants of 5 S rRNA.

The binding affinities of fL5 for the mutant 5 S rRNAs were measured by titrating 1 nM internally radiolabeled RNA with increasing concentrations of protein. Free and bound 5 S rRNA were separated by electrophoresis on nondenaturing polyacrylamide gels (Fig. 5). Autoradiographs of these gels were scanned with a laser densitometer, and the integrated volumes of the individual bands were entered into the program EZ-Fit (24), which generates binding isotherms (Fig. 6) and a value for the $K_d$ of the complex. We used at least two different preparations of each RNA and performed each assay in duplicate, so there is a minimum of four assays for each mutant. In each series of experiments, the dissociation constants of the mutants were determined relative to that for wild-type 5 S rRNA measured in the same experiment in order to control for any differences in the activity of fL5 (or L5) from one series of assays to another. For those mutants that have a greatly reduced affinity for L5 (e.g. $K_{d}$50), it is not possible to saturate binding; aggregation of L5 in binding buffer at concentrations above 100 nM begins to interfere with the electrophoretic assay. Therefore, titrations did not exceed this concentration, and values for the dissociation constants of these mutants were calculated by the EZ-Fit program without complete binding of the RNA. The dissociation constant measured here for the L5$z$5 S rRNA complex in optimized conditions is 2 nM. The dissociation constants for each mutant relative to wild-type 5 S rRNA are presented in Table I. The binding of TFIIIA (isolated from Xenopus oocytes) to these mutant RNAs has also been measured using the same electrophoretic assay\(^2\); these data are included in Table I in

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Fig. 3. Dependence of the $K_a$ for the L55 S rRNA complex on pH and magnesium concentration. A, apparent $K_a$ values were determined from binding isotherms measured at appropriately buffered values of pH. The curve through the data points represents the best fit for a process containing two ionizations with $pK_a$ values of 5.4 and 10.1. The data point at pH 9.5, represented by an open circle, was not included in the curve fitting. B, apparent $K_a$ values were determined in standard binding buffer containing increasing concentrations of MgCl$_2$. Note that the abscissa of the plot is logarithmic scale.

Fig. 4. Specificity of fL5 binding to 5 S rRNA. fL5 (40 nM) and radiolabeled 5 S rRNA (5 nM) were incubated with either 5, 25, 67, 125 nM unlabeled, competitor 5 S rRNA (□), or 10, 50, 250, 500 nM unlabeled tRNA (○). The autoradiograph was scanned with a densitometer to quantitate the amount of bound radiolabeled 5 S rRNA.
order to compare the recognition elements in the RNA for the two proteins.

The mutations in 5 S rRNA that affect binding of L5 are located in the hairpin structure encompassing helix III-loop C. Deletion of the two bulged adenosines at positions 49 and 50 of the helix caused a very marked decrease in affinity as did a mutation in loop C at the stem-loop junction (T43A/C44G). Another mutation in loop C (A42C) has no impact on binding. The only mutation outside of this region that affects binding of L5 is the transversion T76G located in loop E. We have shown earlier that this substitution engenders an alternative conformation in the RNA that is in equilibrium with the wild-type structure.2 These two forms of 5 S rRNA are resolved on mobility shift gels (Fig. 5) with the alternative structure migrating faster than the wild-type conformation.

**DISCUSSION**

We have expressed the eukaryotic ribosomal protein L5 in E. coli and have shown that it has high affinity and specificity for 5 S rRNA. In the past, this protein has been difficult to characterize due to its notorious insolubility, especially when freed of its cognate RNA (see Ref. 16 and references therein). Consequently, most experiments to date have used the L5-5 S rRNA complex that is released from ribosomes upon treatment with high concentrations (25 mM) of EDTA. The expression of sufficient amounts of active protein enable us to avoid this procedure and to reconstitute the complex directly. Scatchard analysis has shown that preparations of fL5 never exceeded 60% binding activity, indicating that some solubility problems may still exist for the protein expressed in bacteria. Since the presence of the MBP domain in the chimeric protein appears to stabilize the 5 S rRNA binding activity, protein is stored in this form and only cut with Xa protease immediately before use in binding assays. Most important, we have never detected any effect of the MBP domain on the RNA binding activity or specificity of L5.

Characterization of the binding of L5 to the collective mutants of 5 S rRNA identifies the hairpin structure composed of helix III and loop C as the major determinant of recognition.

![Image](image-url)

**Fig. 5.** Mobility shift assays for binding of fL5 to mutant 5 S rRNAs. Autoradiographs for a selection of mutants are presented. In each assay 1.0 nM 5 S rRNA internally labeled with [32P]GTP was incubated with increasing concentrations of fL5, which ranged from 0 (first lane of each gel) to 160 nM for WILD TYPE, A42C, ΔA83) or 300 nM (for T43A/C44G, ΔA49/50, T76G). The two dots on the autoradiograph for T76G mark the two equilibrium conformations of this RNA; the upper form corresponds to the native conformation.

![Image](image-url)

**Fig. 6.** Binding isotherms derived from RNA mobility shift assays. Autoradiographs of the nondenaturing polyacrylamide gels were scanned with a laser densitometer to quantitate the intensity of the individual bands. Exposures were within the linear response range of the film. Curves were fit to the data by nonlinear regression analysis (24).

- **A**, wild-type (●); A42C (△); T43A/C44G (▲).
- **B**, wild-type (●); ΔA49/50 (△); ΔA49,50,83 (▲).
- **C**, wild-type (●); G70C/G71C (△); T76G (▲).

The large changes in $K_d$ values for these mutants indicate that much of the free energy of binding is derived from contacts made to this region. This is in contrast to TFIIIA, where point mutations, or even block mutations, seldom produce more than a 3-fold decrease in binding, suggesting that this latter interaction is directed by several weak contacts dispersed over a large amount of the RNA secondary structure (8, 10, 30).

The results of these binding experiments are unexpected, since much of the earlier work to define the site on 5 S rRNA for the cognate ribosomal protein all indicated that the arm of the RNA molecule composed of helix IV-loop E-helix V as well as helices I and II comprise the contact site (13–17, 33, 34). How-
ever, there is some evidence for contact between L5 and the helix III-loop C region (2, 14, 15), although in at least one case this interaction appeared to be weak and secondary to those in other regions of the RNA (14). α-Sarcin, a purine-specific ribonuclease, was used in footprinting experiments with the 7 S RNP complex released from rat ribosomes (16). In accord with earlier work, L5 protected a substantial region of the RNA that includes helices I, II, IV, and V and loop E. α-Sarcin only cuts at positions 37, 41, and 42 in the segment of 5 S RNA extending from nucleotide 28 to 46, leaving a significant amount of this region of the RNA untested by the nuclease. There is no detectable protection of these three nucleotides nor of the run of 5 purines from positions 47–51. Contacts between L5 and the stem-loop junction will not be detectable using α-sarcin. Since this is a blind spot with respect to the RNA, it is difficult to understand why protection from α-sarcin is not seen in helix III, unless all contacts with L5 are to the 5′-strand of the helix (i.e. nucleotides 27–32) leaving the 3′-strand exposed and susceptible to the nuclease. On the other hand, no mutation, excepting T76 → G, within the footprint for L5 has an effect on binding. Of particular interest is the quadruple mutant (G70 → C/G71 → C/G81 → C/G82 → C) that should destabilize both helices IV and V, and probably that entire arm of 5 S RNA. This mutation causes a 20-fold increase in the dissociation constant for TFIIIA. Remarkably, this mutant binds L5 with wild-type affinity.

In other studies, the 3′ terminus of 5 S RNA was chemically cross-linked to rat L5, suggesting that helix I is in close proximity to the protein (33). Fragments of 5 S RNA that remained associated with rat L5 after digestion of the 7 S RNP complex with ribonucleases included oligoribonucleotides encompassing positions 1–21, 77–102, and 106–120, which is in concordance with the experiments using α-sarcin (14). Interestingly, small amounts of fragments extending from nucleotide 41 to 52 or 56 were recovered in some experiments. Experiments with RNase T1 showed protection by L5 at nucleotides 37, 47, 48, and 89 (15), providing the only supporting evidence for an interaction between the helix III-loop C structure and the ribosomal protein. Comparable experiments with the yeast homologue, YL3, have yielded similar results. Fragments encompassing residues 1–12 and 79–121 remain bound to the protein after digestion with ribonuclease (13). Modification of interference experiments identified important contact sites in helices I, II, and IV (17). We note that we have not assessed helix I in our mutagenesis experiments.

Experiments that specifically address the Xenopus L5S S RNA complex, however, are in good agreement with the results presented here (2). RNPs complexes were immunoprecipitated from oocytes injected with mutant 5 S RNA genes that were predicted to delete or disrupt particular elements of secondary structure. The only variant RNA that could not be recovered by immunoprecipitation with anti-L5 antibodies was a mutant missing nucleotides 11–41; this deletion removes the domain of the RNA comprised of helix II-loop B-helix III-loop C. Mutants that disrupted the structure of the other arm of 5 S RNA had no detectable effect on binding. Microinjection of somatic and oocyte 5 S RNA into oocytes revealed preferential association of the somatic-type RNA with L5; five of the six nucleotides that differ between these two RNAs are located in helix III and loop B (7).

It is difficult to resolve the apparent discrepancies mentioned here. L5, like TFIIIA, may protect a substantial amount of 5 S RNA from digestion by ribonucleases; yet, unlike the transcription factor, it may contact only a small number of nucleotides within this protected region. This would account for the large impact particular mutants in the RNA have on the affinity of L5 compared with the much smaller effects seen with TFIIIA (Table I). Missing nucleoside experiments in combination with chemical footprinting should help to resolve this issue (9). It is worth noting that the earlier studies used samples of the L5S S RNA complex that is released from ribosomes treated with EDTA. It will be important to determine whether this procedure somehow alters the integrity of the native structure. It is unlikely that there are significant differences between the interactions of the rat and Xenopus proteins with 5 S RNA, since the two have 92% identity with respect to the sequences of their amino acids.

The higher order structure of Xenopus oocyte 5 S RNA has been examined using the metal complex Rh(phen)2(phen)(II)+ (35). This probe, which binds to nucleic acids on the basis of shape selection, promotes strand cleavage upon photocatalysis. The rhodium complex is sterically excluded from double-stranded regions of RNA because the major groove of an A conformation is too narrow, nor does the probe bind to unstructured regions of RNAs because the major groove is open and accessible to stacking interactions with the metal complex, including stem-loop junctions, noncanonical or mismatched base pairs, base triples, and bulges of more than one nucleotide (35, 36). Positions cleaved by Rh(phen)2(phen)(II)+ are indicated on the secondary structure of 5 S RNA in Fig. 7. Major sites of cleavage are clustered in loop E and along the helix III-loop C hairpin. The conformation of loop E has been determined by NMR spectroscopy and it approximates an A-form helix (37). However, a reverse Hoogsteen A:U pair, possibly involved in a base triple interaction with an adjacent bulged guanosine residue, and three consecutive mismatched appositions open the major groove, while base stacking maintains helical structure. These structural features of loop E account for the strong sites of cleavage by the transition metal complex. Loop E is an essential determinant for the binding of TFIIIA to 5 S RNA, presumably because of its unique geometry and because contacts

### Table I

| Mutation | IL5 | TFIIIA |
|----------|-----|--------|
| A12 → C | 1.26 ± 0.29 | 0.86 ± 0.01 |
| T62 → A | 17.1 ± 5.5 | 1.5 ± 0.29 |
| ΔA9/ΔA30 | 102 ± 4.2 | 1.2 ± 0.26 |
| ΔA99/ΔA90/ΔA30 | 103 ± 3.5 | 1.4 ± 0.30 |
| G30 → T | 1.19 ± 0.18 | 1.6 ± 0.13 |
| T30 → A | 1.44 ± 0.30 | 0.96 ± 0.11 |
| T50 → G | 1.18 ± 0.45 | 1.4 ± 0.05 |
| T60 → G/C79 → G | 0.84 ± 0.02 | ND |
| G70 → A | 1.19 ± 0.18 | 0.99 ± 0.14 |
| G70 → C | 1.08 ± 0.56 | 3.3 ± 0.20 |
| G70 → C/G71 → C/G81 → C/G82 → C | 0.75 ± 0.14 | 21.4 ± 0.13 |
| A12 → G | 1.05 ± 0.25 | 1.1 ± 0.20 |
| A12 → C | 1.24 ± 0.44 | 1.6 ± 0.39 |
| T70 → G | 82 ± 7.4 | 8.6 ± 1.9 |
| ΔA30 | 1.02 ± 0.14 | 1.2 ± 0.10 |
| G80 → A | 1.04 ± 0.38 | 0.73 ± 0.01 |
| T80 → A | 1.08 ± 0.24 | 0.89 ± 0.22 |
| T80 → G | 1.03 ± 0.17 | 0.84 ± 0.23 |
| A100 → C | 0.95 ± 0.12 | 4.7 ± 0.18 |
| A100 → G | 0.99 ± 0.22 | 2.3 ± 0.22 |
| A100 → T | 1.27 ± 0.20 | 1.1 ± 0.34 |
| A100 → C | 1.04 ± 0.21 | 1.1 ± 0.18 |
| C105 → T | 1.02 ± 0.16 | 0.71 ± 0.04 |
| T109 → C | 1.25 ± 0.32 | 2.1 ± 0.10 |

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a Binding is expressed as the ratio of the apparent dissociation constant of the mutant nucleic acid relative to the apparent dissociation constant for the wild-type nucleic acid. The numbers are the average of at least four independent determinations ± S.E.

b See Footnote 2.

c Not determined.
Furthermore, the complete absence of cleavage at C39 in this mutant provides evidence for a change in the structure of the putative tetraloop. This mutant also has a substantially reduced affinity for L5. Alternatively, the transversion A42 → C binds L5 with wild-type affinity. Although this latter mutation alters the cleavage pattern of Rh(phen)_2(phi)_3 in loop C and does diminish cleavage at C34, C39, C42, and C44, enhanced cleavage is measured at A32 and a new cleavage site is now observed at C35. Thus, the loop region of this mutant remains opened to stacking interactions with the metal complex, which is reflected in the high affinity binding of L5. It is important to note that these mutations change only the local structure of the RNA; no long-range effects were detected (35). There appears to be three sites in the hairpin that can accommodate binding of Rh(phen)_2(phi)_3; these are found at the bulged adenosines, at the stem-loop junction, and within the loop possibly stacked on the putative Hoogsteen A:U pair. This number can be compared with the two residues indicated by the slope of the pH curve at pK_a 10.1. The close parallel between intercalative binding of Rh(phen)_2(phi)_3 and binding of L5 to mutants of 5 S rRNA adds strong support to the suggestion that binding of the ribosomal protein through the major groove of the helix III-loop C hairpin structure is mediated, at least in part, by stacking interactions involving tyrosine residues. Experiments with the fluorescent probe biscoumophthalenesulfonic acid detected the presence of hydrophobic sites on the surface of yeast YL3 that became exposed upon disruption of the RNP complex (27). Increased turbidity upon dissociation of the complex also indicated exposure of hydrophobic regions on the ribosomal protein. Like Xenopus L5, the yeast protein is rich in tyrosine residues, despite there being only 45% homology between the two amino acid sequences.

L5 and TFIIIA both utilize sites on 5 S rRNA where major groove structures are opened and accessible (9, 35). However, in all other respects the interactions of these two proteins with the nucleic acid are different. The binding of TFIIIA depends on the overall secondary structure of 5 S rRNA with weak contacts dispersed over a large surface of the RNA (8, 45). For this reason, individual mutations in 5 S rRNA generally have small effects on the binding affinity of the transcription factor (8).

Disruption of the helix IV-loop E-helix V domain of 5 S rRNA by the quadruple mutant G70 → C/G74 → C/G81 → C/G82 → C has a substantial impact on TFIIIA because of the cumulative effect of disrupting several contacts that occur through this region of the RNA; yet, this same mutant has no effect on L5. Those mutations that do influence binding of L5 cause a considerable decrease in affinity and are confined to a relatively small region of 5 S rRNA. Of most significance, perhaps, are the differences in the physicochemical properties of the two complexes. Like many protein-nucleic acid interactions, the pH profile of TFIIIA binding to 5 S rRNA is rather featureless, exhibiting a modest decrease in affinity above pH 8.0 (19). An analysis of apparent binding constants as a function of ionic strength indicates that there are approximately five ion pairs formed between the transcription factor and 5 S rRNA, although there is an additional contribution from nonelectrostatic interactions to the free energy of binding (19). In marked contrast is the distinct pH profile for L5 and the exceptional insensitivity of binding to ionic strength that point to the importance of one or more tyrosine residues. These results indicate that stacking or intercalative, rather than electrostatic, interactions are the major determinants of the L5-S rRNA complex. Our earlier experiments with Rh(phen)_2(phi)_3 demonstrate that sites for such contacts are present in the helix III-loop C hairpin structure (35).

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