Defining the RNA-Protein Interactions in the Trypanosome Preribosomal Complex

Lei Wang, Martin Ciganda, Noreen Williams

Department of Microbiology and Immunology and Witebsky Center for Microbial Pathogenesis and Immunology, University at Buffalo, Buffalo, New York, USA

In eukaryotes, 5S rRNA is transcribed in the nucleoplasm and requires the ribosomal protein L5 to deliver it to the nucleolus for ribosomal assembly. The trypanosome-specific proteins P34 and P37 form a novel preribosomal complex with the eukaryotic conserved L5-5S rRNA complex in the nucleoplasm. Previous results suggested that P34 acts together with L5 to bridge the interaction with 5S rRNA and thus to stabilize 5S rRNA, an important role in the early steps of ribosomal biogenesis. Here, we have delineated the domains of the two protein components, L5 and P34, and regions of the RNA partner, 5S rRNA, that are critical for protein-RNA interactions within the complex. We found that the L18 domain of L5 and the N terminus and RNA recognition motif of P34 bind 5S rRNA. We showed that Trypanosoma brucei L5 binds the β arm of 5S rRNA, while P34 binds loop A/stem V of 5S rRNA. We demonstrated that 5S rRNA is able to enhance the association between the protein components of the complex, L5 and P34. Both loop A/stem V and the β arm of 5S rRNA can separately enhance the protein-protein association, but their effects are neither additive nor synergistic. Domains in the two proteins for protein-protein and protein-RNA interactions overlap or are close to each other. This suggests that 5S rRNA binding might cause conformational changes in L5 and P34 and might also bridge the interactions, thus enhancing binding between the protein partners of this novel complex.
does not include loop C/stem III, as has been shown for rat and yeast L5 proteins (8, 17–20).

Here, we utilized filter binding assays to analyze which domains of P34 and L5 are involved in the binding of 5S rRNA and which regions of 5S rRNA are responsible for the associations with P34 and L5. Previous in vitro immune capture assays with L5 and P34 in the presence and absence of 5S rRNA showed that the addition of 5S rRNA to the reaction mixture increased the levels of P34 in the immune capture precipitate, suggesting that 5S rRNA might augment the bridging and stabilization of the interaction between L5 and P34 (11). Here, we utilized both fluorescence resonance energy transfer (FRET) and immune capture assays to determine the regions of 5S rRNA responsible for the enhancement of protein-protein interactions. These results increase our understanding of how L5, P34, and 5S rRNA associate and the mechanisms utilized by 5S rRNA to enhance the protein-protein associations in this trypanosome-specific preribosomal complex.

The extensive characterization of these associations that we have undertaken in this work will be exploited to target this essential process in these pathogenic organisms.

**MATERIALS AND METHODS**

**Recombinant proteins.** P34 (GenBank accession no. AF020695) was cloned into plasmid pQE-1 (Qiagen) and expressed as a polyhistidine fusion protein in Escherichia coli strain M15, as described previously (10, 11). L5 (GenBank accession no. XM_822569), cerulean-L5, enhanced yellow fluorescent protein (eYFP)-P34, the N and C termini and L18 domain of L5, and the N and C termini and RRDM domain of P34 were cloned into pTrcHis-TOPO (Invitrogen) for expression as polyhistidine fusion proteins in TOP10 One Shot cells (Invitrogen), as described previously (16). All of the recombinant protein expression plasmids were confirmed by DNA sequence analysis. The expression and purification of recombinant proteins were performed as described previously (10, 11). Fractions containing recombinant proteins, as visualized by SDS-PAGE with Coomassie blue staining and Western blot analysis using anti-P34/P37 (21), anti-L5 (11), anti-polyhistidine (H1029; Sigma), or anti-green fluorescent protein (GFP) (A10260; Life Technologies), were pooled, desalted using a PD-10 column (GE) with storage buffer (10 mM Tris [pH 7.6], 150 mM KCl, 0.1 mM dithiothreitol [DTT], 0.1% NP-40, 0.1 mM phenylmethylsulfonyl fluoride [PMSF]), and flash-frozen at −80°C in 400-μl aliquots.

**5S rRNA and 5S rRNA oligonucleotides.** T. brucei 5S rRNA (rDNA) (GenBank accession no. M14817.1) was amplified using primers 5STS5wd (5′-ATTTAACAATGAAGTGACCCATCTTGCCGCGG-3′) and 5ST3Rev (5′-AGATGAAACACCGCCGTTG-3′) to generate a template that was used for T3 polymerase-directed in vitro transcription (MAXiScript; Life Technologies). After in vitro transcription, 5S rRNA was purified (MEGAclear; Life Technologies). The 5S rRNA oligonucleotides 5′-GGCGGGGUGCAGAUCCAUACGGCCUCUGG-3′ (loop A/stem V), 5′-GGCCAGAUGCAUACGCGCGCCG-3′ (loop B/stem V), 5′-GCCGCAUGCGAAGCCGCGAUGAUGAAGUAGCAGCCGC-3′ (arm, including loops B and C and stems II and III), and 5′-GGCCUGUGAGUACGGCGCAGUGAUCAGCCGCGC-3′ (GG-3′ (γ arm, including loops D and E and stems IV and V) were purified from Integrated DNA Technologies.

**Absorption and fluorescence measurements.** Samples were measured in 96-well clear-bottom plates using a Synergy H1 hybrid reader (BioTek). Absorption spectra were recorded from 375 nm to 600 nm in 1-nm intervals. Emission spectra were collected from 450 nm to 600 nm (excitation, 400 nm; slit width, 1 nm; sensitivity, 100). Excitation at 400 nm improves the dynamic range, as the excitation of eYFP is at a minimal level (22). Excitation at 400 nm leads to emission of cerulean at 475 nm. When FRET occurs, excitation at 400 nm leads to the emission of eYFP at 530 nm (23, 24). Proteins and RNA were incubated in binding buffer (10 mM Tris [pH 7.6], 150 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, 0.1% NP-40) for 20 min at room temperature. All measurements were repeated at least three times with three preparations of proteins.

**Immune capture experiments.** Protein A magnetic beads (Dynabeads; Life Technologies) were cross-linked to anti-L5 antibody. No antibody was added to the Dynabeads as a negative control for nonspecific interactions with the Dynabeads. The Dynabeads were washed in phosphate-buffered saline (PBS) and blocked in PBS-bovine serum albumin (BSA) (1 mg/ml). Antibody was added to the beads, and the mixture was incubated at 4°C for 1 h. Dimethyl pimelimidate (Thermo Scientific Pierce) dissolved in 0.2 M triethanolamine (Sigma-Aldrich) in PBS was used to cross-link the antibody to the beads (three incubations of 30 min each at room temperature, separated by brief washes with 0.2 M triethanolamine). After cross-linking, the reaction was quenched with 50 mM ethanolamine (Sigma-Aldrich) in PBS (two incubations of 10 min each). Excess antibody was eluted in two washes with 1 M glycine (pH 3). The antibody-coated beads were washed with PBS–Tween 20 and then incubated with 200 ng of recombinant L5 and 200 ng of recombinant P34, in the absence or presence of 1 μM in vitro-transcribed 5S rRNA or 5S rRNA oligonucleotides, at 4°C for 1 h. Supernatants were collected, beads were washed three times with PBS–Tween 20, and the captured antigen was eluted with SDS–PAGE loading buffer at 70°C for 10 min. Immune capture fractions were analyzed by Western blot analysis using affinity-purified anti-P34 or anti-L5 antibody. The experiments were repeated three times with different preparations of recombinant proteins, 5S rRNA, and 5S rRNA oligonucleotides.

**Filter binding assay.** T. brucei 5S rRNA was transcribed in vitro in the presence of [α-32P]UTP as described previously (10, 11). The 5′ ends of 5S rRNA oligonucleotides were labeled with [α-32P]ATP (KinMax; Life Technologies). Radiolabeled 5S rRNA and 5S rRNA oligonucleotides were purified using NucAway spin columns (Life Technologies). A constant concentration of 5S rRNA (equivalent to 10,000 dpm and always lower than 0.5 nM) was used for all reactions. The 5S rRNA or 5S rRNA oligonucleotides and increasing concentrations of recombinant proteins were added in a total volume of 20 μl, in binding buffer (10 mM Tris [pH 7.4], 150 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, 0.1% NP-40, 10% glycerol, 100 μg/ml BSA). After 30 min of incubation at room temperature, the reaction mixtures were loaded onto prewetted nitrocellulose filters. The filters were washed once with binding buffer and then dried. A nylon filter underneath the nitrocellulose filters was used to capture unbound RNA. Radioactivity associated with the filters was measured using a PhosphorImager (Bio-Rad). All reactions were performed three times with different preparations of recombinant proteins and 5S rRNA and 5S rRNA oligonucleotides. The bound fraction for each data point was calculated as a ratio of the signal on the nitrocellulose filter to the total signal on both filters; 100% binding is the total input RNA, the theoretical maximal signal that could be obtained if all of the RNA in the reaction mixture were bound by protein. The dissociation constant was calculated using GraphPad Prism 5.

**RESULTS**

5S rRNA binds the N-terminal and the RRDM domain of P34 and the L18 domain of L5. Previous FRET and immune capture assays showed that the N-terminal APK-rich domain and the RRDM domain of P34 and the L18 domain of L5 are important for the association between P34 and L5 (16). Immune capture assays also showed that 5S rRNA enhances the protein-protein association (11). Therefore, we wanted to analyze whether 5S rRNA binds to the same or different domains compared with those required for the association between L5 and P34. These results might indicate whether the association of 5S rRNA directly causes conformational changes in the protein-protein binding domains of L5 and P34, which then enhance the protein-protein association. We expressed P34, L5, and their truncates (Fig. 1A and 2A) in E. coli and...
assessed their binding properties using in vitro filter binding assays with radiolabeled 5S rRNA.

Full-length P34 and the N terminus and RRM domain of P34 are capable of binding 5S rRNA (Fig. 1B). Their mode of binding corresponds to that of a bimolecular equilibrium with saturation. The $K_d$ (dissociation constant) value calculated for the full-length P34 and 5S rRNA binding reaction was 48.6 nM, as reported previously (10). The $K_d$ values calculated for the P34 truncates were 205.6 nM for the N terminus of P34 and 59.2 nM for the RRM domain. The C terminus of P34 did not bind 5S rRNA to a measurable extent (Fig. 1B).

Although there is good overall conservation between T. brucei L5 and the eukaryotic consensus sequence, some of the substitutions are located in the C terminus of T. brucei L5, which is one of the regions implicated previously in binding to 5S rRNA (25). Therefore, we next evaluated the domains of T. brucei L5 required for interaction with 5S rRNA. We found that both the full-length L5 and the L18 domain of L5 bind 5S rRNA. The association of full-length L5 with 5S rRNA showed a $K_d$ value of 11.9 nM, as reported previously (25). The $K_d$ value for the L18 domain of L5 with 5S rRNA was 17.6 nM, very close to the value for the full-length L5 protein. However, neither the N terminus nor the C terminus of L5 was capable of binding 5S rRNA (Fig. 2B). The filter binding data indicate that the binding sites of P34 and L5 that participate in the protein-protein and protein-5S rRNA interactions overlap or are close to each other.

P34 interacts with 5S rRNA through a high-affinity contact with loop A/stem V, and L5 interacts with 5S rRNA through a high-affinity contact with the $\beta$ arm. T. brucei 5S rRNA is composed of 119 nucleotides, and we showed previously that it possesses a typical secondary structure for eukaryotic 5S rRNA (10). The folded structure of 5S rRNA has 5 stems and 5 loops, with loops C and E highly accessible (Fig. 3). Previous data from enzymatic footprinting experiments showed that the binding site for Xenopus ribosomal protein L5 is loop C/stem III, which is located in the $\beta$ arm (7, 8) (Fig. 3A, blue oval). Results from competition experiments showed that P34 binds 5S rRNA through a high-affinity interaction with loop A/stem V (10) (Fig. 3A, red oval). Here, we utilized filter binding assays to delineate which regions of T. brucei 5S rRNA contribute to the binding of T. brucei P34 and L5. RNA folding predictions (with Mfold [26]) based on thermodynamics showed that loop A/stem V and the $\beta$ arm can fold the same as in the full-length 5S rRNA, but an oligonucleotide spanning the sequence of loop C/stem III alone cannot fold correctly. Thus, the $\beta$ arm containing loops B and C and stems II and III, instead of loop C/stem III, was used in this assay. Increasing concentrations of recombinant T. brucei P34 and L5 were incubated with radiolabeled 5S rRNA oligonucleotides, and protein-RNA complexes were analyzed by filter binding assays. As expected from our previous work, loop A/stem V of 5S rRNA was able to bind P34 with a $K_d$ value of 180 nM (Fig. 3B), and neither the $\beta$ arm nor the $\gamma$ arm of 5S rRNA was capable of binding P34.
This is consistent with our previous results showing that a high-affinity interaction with P34 occurred with only loop A/stem V (10). Those results also showed that, although P34 does not bind directly to loop C within the β arm, it protects this loop in RNase protection assays, either directly or by causing a conformational change in the RNA such that this loop is no longer accessible (10).

We next evaluated the interactions of L5 with the 5S rRNA oligonucleotides. The results showed that the β arm of 5S rRNA was capable of binding L5 with a $K_d$ value of 171.2 nM (Fig. 3C). However, loop A/stem V and the γ arm of 5S rRNA were not capable of binding L5. The filter binding assays showed that the T. brucei L5 binding site is consistent with that of Xenopus L5, which is located in loop C and stem III (7, 8), rather than those of rat and yeast L5 proteins, which encompass a much larger contact surface composed of stems I, II, IV, and V as well as loops D and E (8, 17–20). Taken together, these results show that T. brucei L5 and P34 bind to distinct regions of 5S rRNA.

Enhancement of the association between L5 and P34 by 5S rRNA can be demonstrated using FRET. Previous results from immunoprecipitation experiments showed that recombinant P34 associates directly with L5, and 5S rRNA is not required to maintain this association (11). However, the addition of 5S rRNA leads to more P34 being immunoprecipitated by anti-L5 antibody, indicating that 5S rRNA might modulate the protein association (11). Here, FRET was used to analyze the effect of 5S rRNA on the association between P34 and L5. When 1 μM cerulean-tagged L5 was incubated with 1 μM eYFP-tagged P34 and excited at 400 nm, it showed decreased cerulean emission around 475 nm as well as a sharply increased emission around 530 nm (Fig. 4, red trace). This increase is significantly larger than can be accounted for by the direct excitation of 1 μM cerulean-L5 (Fig. 4, brown trace), 1 μM eYFP-P34 (blue trace), or 1 μM cerulean-L5 plus 1 μM eYFP (black trace; nonspecific interactions), as shown previously (16). Therefore, these results indicate that L5 and P34 have associated and their fluorescent tags are within the range required for energy transfer to occur. In order to use FRET to analyze the effect of 5S rRNA on the association between L5 and P34, 1 μM (Fig. 4, purple trace) or 2 μM (Fig. 4, green trace) in vitro-transcribed and puri-
fied 5S rRNA was added to the 1 μM cerulean-L5 plus 1 μM eYFP-P34 solution. Figure 4 shows that the emission peak at 530 nm increased as 5S rRNA was added, indicating that 5S rRNA enhanced the association between cerulean-L5 and eYFP-P34. As a negative control, the polyanion polymer poly(dl-dc) was added to the 1 μM cerulean-L5 plus 1 μM eYFP-P34 solution, but no effect on the FRET signal was detected (data not shown). Therefore, these FRET results are consistent with previous results from immunoprecipitation experiments (11), which suggested that 5S rRNA enhances the interaction between L5 and P34.

Loop A/stem V and the β arm of 5S rRNA enhance the association between L5 and P34. Previous results from immune capture experiments (11) and the FRET data presented here show that the addition of 5S rRNA enhanced the protein-protein association. Our results also show that the domains of P34 and L5 for protein-protein and protein-RNA interactions overlap or are very close to one another, suggesting protein conformational changes upon RNA binding. We next wanted to determine which regions of 5S RNA are important in enhancing the protein-protein association. This would indicate whether the conformational change of one protein might cause the enhanced protein-protein interaction.

For this purpose, 5S RNA oligonucleotides were added to the solution containing 1 μM cerulean-L5 and 1 μM eYFP-P34 to determine which regions of 5S RNA were able to increase the emission at 530 nm. Figure 5A shows that, compared with the emission of the 1 μM cerulean-L5 plus 1 μM eYFP-P34 solution (red trace), the addition of 1 μM 5S RNA (brown trace) strongly increased the emission at 530 nm. The addition of 1 μM γ arm (Fig. 5A, blue trace) did not affect the FRET signal of the cerulean-L5–eYFP-P34 interaction. The addition of 1 μM loop A/stem V (Fig. 5A, purple trace) or the β arm (green trace) of 5S RNA also caused increases in the FRET signal, indicating that both loop A and the β arm can enhance the association between L5 and P34.

Although both loop A/stem V and the β arm of 5S RNA can increase the FRET signal, the increase is less than that observed with the addition of full-length 5S RNA. We showed previously that loop A/stem V 5S RNA binds to P34 and that the β arm of 5S RNA binds to L5. We wanted to test whether the effect of 5S RNA on binding is merely to provide a bridge between the proteins by binding them simultaneously or if binding of the rRNA changes the protein conformation in order to increase protein-protein affinity. To differentiate between these possibilities, both loop A/stem V and the β arm were added to the 1 μM cerulean-L5 plus 1 μM eYFP-P34 solution (Fig. 5A, orange trace). The results showed that loop A/stem V and the β arm together enhanced the protein-protein association. However, the effect of loop A/stem V together with the β arm was similar to that of loop A/stem V or the β arm alone, indicating that only full-length 5S RNA is able to bridge the protein-protein association efficiently. The obvious next step would be to design an RNA oligonucleotide containing both loop A/stem V and the β arm without the γ arm and to determine whether that would yield the same results as those for full-length 5S RNA. However, we were unable to design an RNA oligonucleotide lacking any significant number of γ arm residues that folded correctly.

Immune capture assays were also performed to confirm the regions of 5S RNA involved in enhancing the protein-protein interaction. Recombinant L5 and P34 were incubated in the absence or presence of full-length 5S RNA or 5S rRNA oligonucleotides. Fractions from the immune capture assays (Fig. 5B) using the anti-L5 antibody were subsequently analyzed using the anti-P34 antibody. In a negative control experiment, the beads alone did not interact nonspecifically with L5 (Fig. 5B, lane 4). Com-
pared with L5 and P34 alone (Fig. 5B, lane 7), the addition of full-length 5S rRNA (lane 10), loop A/stem V (lane 13), or the β arm (lane 16) to the reaction mixture increased the levels of P34 in the immune capture precipitate. The levels of P34 in the immune capture precipitate with the addition of loop A/stem V (Fig. 5B, lane 13) or the β arm (lane 16) were lower than those observed with the addition of full-length 5S rRNA (lane 10). No significant increase in P34 levels in the precipitate was detected with addition of the γ arm (Fig. 5B, lane 19).

Given that loop A/stem V and the β arm separately enhanced the levels of P34 in the precipitate, we wanted to determine whether adding the two oligonucleotides together would increase the association to the levels seen using full-length 5S rRNA. However, our results show that the levels of P34 in the immune capture precipitate with the addition of both loop A/stem V and the β arm (Fig. 5B, lane 22) were similar to those observed with the addition of either loop A/stem V (lane 13) or the β arm (lane 16).

Consistent with the FRET experiments, the immune capture assays showed that either loop A/stem V or the β arm can enhance the protein-protein association. The data also suggest that 5S rRNA interacts with both L5 and P34 and that the full-length 5S rRNA is required for full enhancement of the protein-protein association.

**DISCUSSION**

Unlike the conserved L5-5S rRNA RNP ribosomal precursor in other eukaryotes, trypanosome-specific protein P34 forms a pre-ribosomal complex with both L5 and 5S rRNA in the nucleoplasm of *T. brucei* (11). This novel preribosomal complex is essential for the stabilization of 5S rRNA and for ribosomal assembly (14). Although P34 is able to interact directly with L5 (11) and 5S rRNA (10), 5S rRNA can enhance the association between L5 and P34 (11). It has been shown that the N terminus and RRM domain of P34 and the L18 domain of L5 are important for the protein-protein interaction (16). To determine which domains of P34 and L5 are involved in the protein-RNA interactions, filter binding assays were performed; they showed that the N terminus and RRM domain of P34 (Fig. 1) and the L18 domain of L5 (Fig. 2) are also important for the protein-RNA interactions. These data indicate that the domains for protein-protein and protein-RNA interactions overlap or are close to each other.

The RRM domain, which forms a four-strand β sheet packed against two α helices, is the most abundant protein domain in eukaryotes. RRMs are involved not only in RNA recognition but also in protein-protein interactions (27). The RRM contains two highly conserved ribonucleoprotein motifs, RNP1 and RNP2, located in the central β strands. The aromatic amino acids of the RNP motifs are involved in base-stacking interactions with unpaired RNA bases (28). The N- and C-terminal regions, outside the RRM, are often able to enhance RNA binding affinity by enlarging the protein-RNA binding surface (27, 28). The N- and C-terminal extensions of the RRM of TcUBP1, an RRM-containing protein in the related kinetoplast *Trypanosoma cruzi*, have been shown to be involved in protein-protein interactions and to contribute to RNA binding by enlarging the RNA-protein interface (29). Our results show that both the N terminus and the RRM domain of P34 are able to bind 5S rRNA (Fig. 1), although the binding affinity for the RRM domain is significantly higher, which might indicate that both the N terminus and the RRM domain of P34 are required for full contact between proteins and RNA.

Several studies have been performed to characterize the specific domains of L5 that contribute to 5S rRNA binding activity. Previous RNA binding assays using deletion mutants showed that the N-terminal L18 domain of rat L5 is the critical 5S rRNA binding domain, while the C-terminal domain serves as the nucleolar targeting signal (5). Others found that both the N-terminal L18 domains and the C-terminal domains of human, rat, and *Xenopus* L5 proteins are required for 5S rRNA-specific binding activity (25, 30). Studies in yeasts suggested that both the N- and C-terminal domains of L5 are required for stable 5S rRNA interactions and that the basic amino acids on one face of the C-terminal α helix are involved in protein-5S rRNA interactions (31, 32). Mutations of these positively charged amino acids reduced 5S rRNA binding activities and growth rates (32). *T. brucei* L5 is a conserved ribosomal protein with 54% identity with *Xenopus* L5, and it consists of an L18 domain in the central region and a C-terminal domain which contains an α helix. In these studies, we found that the L18 domain of *T. brucei* L5 binds to 5S rRNA (Fig. 2), which is similar to the 5S rRNA binding of human, rat, and *Xenopus* L5 L18 domains (5, 25, 30). However, the C-terminal domain of *T. brucei* L5 does not bind 5S rRNA. We have found that a highly conserved arginine within the C terminus of *T. brucei* L5 is substituted for an uncharged alanine (yeast position 285). *T. brucei* L5 binds 5S rRNA with a *K*ₐ of 12 nM, 0.6 order of magnitude above the *K*ₐ value of *Xenopus* L5 (2 nM) (7). Mutating the alanine in the C terminus to a consensus arginine increases the binding affinity of *T. brucei* L5 for 5S rRNA (11). Therefore, we postulate that the lack of binding activity of the C terminus of L5 for 5S rRNA might be due to the lack of conservation in the C terminus of *T. brucei* L5, where critical positively charged amino acids are altered. Alternatively, the C terminus contributes to L5 binding affinity through conformational effects that do not occur in the absence of these conserved residues.

Results from filter binding assays, which are consistent with previous results from competition electrophoretic mobility shift assays (10), showed that P34 interacts specifically with 5S rRNA through loop A/stem V (Fig. 3B). In *Xenopus*, the zinc finger protein transcription factor IIIA (TFIIIA) binds 5S rRNA and translocates it from the nucleus to the cytoplasm for storage (33). No TFIIIA homologue has been identified in the genome of kinetoplasts (34). TFIIIA binds loop E, stem IV, loop A, and stem V of 5S rRNA, which contains the region to which P34 binds 5S rRNA (35). This might suggest a role for P34 in protecting 5S rRNA similar to the function of TFIIIA, although the two proteins show no sequence homology.

Filter binding assays also showed that *T. brucei* L5 interacts with only the β arm (including stems II and III and loops B and C) of 5S rRNA (Fig. 3C), which is the same binding region as for *Xenopus* L5 (7, 8). This is unlike the rat L5 binding region, which is composed of stems I, II, IV, and V, as well as loops D and E, and is therefore much larger (8, 17–20). Previous protection and interference experiments showed that a hairpin structure composed specifically of stem III and loop C is the contact site of *Xenopus* L5 (7). Since correctly folded loop C/stem III cannot be obtained using *in vitro* transcription (see above), we cannot use filter binding assays with this oligonucleotide to determine whether *T. brucei* L5 specifically binds stem III/loop C; it would not be biologically informative. However, it is clear that L5 and P34 utilize distinct structural elements for recognition.

FRET experiments and immune capture assays showed that
full-length 5S rRNA, loop A/stem V, and the β arm of 5S rRNA are capable of enhancing the association between L5 and P34. However, the enhancement of the protein-protein association with the addition of loop A/stem V or the β arm alone is less than that with the addition of full-length 5S rRNA, indicating that only full-length 5S rRNA is able to bridge the protein-protein association. Our data also show that the addition of both loop A/stem V and the β arm of 5S rRNA does not enhance the interaction to the same extent as does the addition of full-length 5S rRNA. Because the oligonucleotide containing both loop A/stem V and the β arm does not fold correctly according to the RNA folding prediction, we cannot directly investigate whether 5S rRNA without the γ arm can enhance the protein-protein association as well as the full-length 5S rRNA does.

A correlation between the ability of 5S rRNA oligonucleotides to enhance protein–protein interactions and their protein binding activity is evident. Formation of almost every RNA-protein complex involves conformational changes in the protein, the RNA, or both, and such conformational changes are a critical feature of RNP complex assembly (36, 37). Previous studies showed that conformational changes in loops B and C of 5S rRNA are induced by the binding of Xenopus L5 to loop C and stem III, creating contact sites for ribosomal protein L11 (7). The ribosomal proteins S6, S15, and S18 are the components of the small subunit of the ribosome. In Thermus thermophiles, S15 binds the 16S rRNA to stabilize the RNA tertiary structure that is required for S6 and S18 binding, and S6 can bind the ribosome only via heterodimerization with S18 (38). RNA binding to the RRM of the negative transcription elongation factor E subunit (NELF-E) induces the formation of a helix in the C terminus, which is often key for RNA recognition (39). Since the binding sites of P34 and L5 for protein-protein and protein-RNA interactions overlap or are close to each other, RNA binding might induce conformational changes in the protein binding domains of both P34 and L5 and increase the protein-protein association. Meanwhile, conformational changes in 5S rRNA might increase the protein-RNA interaction, thus bridging and stabilizing the protein-protein association.

The studies presented here not only identified the domains of L5, P34, and 5S rRNA for protein-RNA interactions but also show that 5S rRNA enhances the protein–protein interactions that are of potential functional significance for the stabilization and trafficking of 5S rRNA. These studies also suggest that 5S rRNA binding might induce conformational changes in L5 and P34 and promote their functions in this trypanosome-specific preribosomal complex. Although the ribosome is highly conserved, subtle differences enable the development of drugs targeting ribosomal assembly (40). Indeed, many existing drugs for the treatment of bacterial infections, such as macrolides, ketolides, lincosamides, and oxazolidinones, inhibit bacteria by binding specifically to the functionally relevant sites of bacterial ribosomes (41–43). Data from X-ray crystallography and mutagenesis studies provide effective tools for highlighting novel potential drug target sites (44). Experiments in which we are mutating the amino acids of L5 and P34 critical for protein-protein or protein-RNA interactions and analyzing the in vivo effects of these mutations are under way in our laboratory. Future studies will also focus on correlating these functional and biochemical data with structural data using X-ray crystallography of complexes containing L5 and P34 to identify docking sites for these trypanosome-specific interactions. This knowledge will be used to disrupt these interactions specifically with small-molecule inhibitors in future studies.

ACKNOWLEDGMENT

This work was supported by NIH grant GM092719 to N.W.

REFERENCES

1. Melse T, Xue Z. 1995. The nucleolus: an organelle formed by the act of building a ribosome. Curr. Opin. Cell Biol. 7:319–324.
2. Ciganda M, Williams N. 2011. Eukaryotic 5S rRNA biogenesis. Wiley Interdiscip. Rev. RNA 2:523–533.
3. Steitz JA, Berg C, Hendrick JP, La Branche-Chatot H, Metspalu A, Rinke J, Yario T. 1988. A 5S rRNA/L5 complex is a precursor to ribosome assembly in mammalian cells. J. Cell Biol. 106:545–556.
4. Deshmukh M, Tsay YF, Paulovich AG, Woolford JL, Jr. 1993. Yeast ribosomal protein L1 is required for the stability of newly synthesized 5S rRNA and the assembly of 60S ribosomal subunits. Mol. Cell. Biol. 13: 2835–2845.
5. Michael WM, Dreyfuss G. 1996. Distinct domains in ribosomal protein L1 mediate 5 S rRNA binding and nuclear localization. J. Biol. Chem. 271:11571–11574.
6. Luehrsens KR, Fox GE. 1981. Secondary structure of eukaryotic cytoplasmic 5S ribosomal RNA. Proc. Natl. Acad. Sci. U. S. A. 78:2150–2154.
7. Scripture JB, Huber PW. 2011. Binding site for Xenopus ribosomal protein L5 and accompanying structural changes in 5S rRNA. Biochemistry 50:3827–3839.
8. Scripture JB, Huber PW. 1995. Analysis of the binding of Xenopus ribosomal protein L5 to s5 S RNA: the major determinants of recognition are located in helix III-loop C. J. Biol. Chem. 270:27358–27365.
9. Woestenek EA, Gongadze GM, Shcherbakov DV, Rak AV, Garber MB, Hard T, Berglund H. 2002. The solution structure of ribosomal protein L18 from Thermus thermophilus reveals a conserved RNA-binding fold. Biochem. J. 363:553–561.
10. Ciganda M, Williams N. 2012. Characterization of a novel association between two trypanosome-specific proteins and 5S rRNA. PLoS One 7e30029. doi:10.1371/journal.pone.0030029.
11. Ciganda M, Prohaska K, Hellman K, Williams N. 2012. A novel association between two trypanosome-specific factors and the conserved L5-5S rRNA complex. PLoS One 7e41398. doi:10.1371/journal.pone.0041398.
12. Pitula JS, Park J, Parsons M, Ruyechan WT, Williams N. 2002. Two families of RNA binding proteins from Trypanosoma brucei associate in a direct protein–protein interaction. Mol. Biochem. Parasitol. 122:81–89.
13. Pitula J, Ruyechan WT, Williams N. 2002. Two novel RNA binding proteins from Trypanosoma brucei are associated with 5S rRNA. Biochem. Biophys. Res. Commun. 290:569–576.
14. Hellman K, Prohaska K, Williams N. 2007. Trypanosoma brucei RNA binding proteins p34 and p37 mediate NOPP44/46 cellular localization via the exportin 1 nuclear export pathway. Eukaryot. Cell 6:2206–2213.
15. Li J, Ruyechan WT, Williams N. 2003. Stage-specific translational efficiency and protein stability regulate the developmental expression of p37, an RNA binding protein from Trypanosoma brucei. Biochem. Biophys. Res. Commun. 306:918–923.
16. Wang L, Ciganda M, Williams N. 2013. Association of a novel preribosomal complex in Trypanosoma brucei determined by fluorescence resonance energy transfer. Eukaryot. Cell 12:322–329.
17. Gross B, Welfle H, Bielka H. 1985. Protein-RNA interaction in the rat liver 5S rRNA-protein L5 complex studied by digestion with ribonuclease. Nucleic Acids Res. 13:2325–2335.
18. Yaguchi M, Rollin CF, Roy C, Nazar RN. 1984. The 5S RNA binding protein from yeast (Saccharomyces cerevisiae) ribosomes: an RNA binding sequence in the carboxyl-terminal region. Eur. J. Biochem. 139:451–457.
19. Aoyama K, Tanaka T, Hidaka S, Ishikawa K. 1984. Binding sites of rat liver 5S rRNA to ribosomal protein L5. J. Biochem. 95:1179–1186.
20. Nazar RN, Wildeman AG. 1983. Three helical domains form a protein binding site in the 5S rRNA-protein complex from eukaryotic ribosomes. Nucleic Acids Res. 11:3155–3168.
21. Zhang J, Ruyechan W, Williams N. 1998. Developmental regulation of two nuclear RNA binding proteins, p34 and p37, from Trypanosoma brucei. Mol. Biochem. Parasitol. 92:79–88.
22. Martin SF, Tatham MH, Hay RT, Samuel ID. 2008. Quantitative analysis of multi-protein interactions using FRET: application to the SUMO pathway. Protein Sci. 17:777–784.
23. Markwardt ML, Kremers GJ, Kraft CA, Ray K, Cranfill PJ, Wilson KA, Day RN, Wachter RM, Davidson MW, Rizzo MA. 2011. An improved cerulean fluorescent protein with enhanced brightness and reduced reversible photoswitching. PLoS One 6:e17896. doi:10.1371/journal.pone.0017896.

24. Rizzo MA, Springer GH, Granada B, Piston DW. 2004. An improved cyan fluorescent protein variant useful for FRET. Nat. Biotechnol. 22:445–449.

25. Claussen M, Rudt F, Pieler T. 1999. Functional modules in ribosomal protein L5 for ribonucleoprotein complex formation and nucleocytoplasmic transport. J. Biol. Chem. 274:33951–33958.

26. Zuker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31:3406 –3415.

27. Maris C, Dominguez C, Allain FH. 2005. The RNA recognition motif, a plastic RNA-binding platform to regulate post-transcriptional gene expression. FEBS J. 272:2118 –2131.

28. Clery A, Blatter M, Allain FH. 2008. RNA recognition motifs: boring? Not quite. Curr. Opin. Struct. Biol. 18:290 –298.

29. Volpon L, D’Orso I, Young CR, Frasch AC, Gehring K. 2005. NMR structural study of TcUBP1, a single RRM domain protein from Trypanosoma cruzi. Biochemistry 44:3708 –3717.

30. Rosorius O, Fries B, Stauber RH, Hirschmann N, Bevec D, Hauber J. 2000. Human ribosomal protein L5 contains defined nuclear localization and export signals. J. Biol. Chem. 275:12061–12068.

31. Yeh LC, Lee JC. 1995. Contributions of multiple basic amino acids in the C-terminal region of yeast ribosomal protein L1 to 5 S rRNA binding and 60 S ribosome stability. J. Mol. Biol. 246:295–307.

32. Moradi H, Simoff I, Bartish G, Nygard O. 2008. Functional features of the C-terminal region of yeast ribosomal protein L5. Mol. Genet. Genomics 280:337–350.

33. Allison LA, Romaniuk PJ, Bakken AH. 1991. RNA-protein interactions of stored 5 S RNA with TFIIIA and ribosomal protein L5 during Xenopus oogenesis. Dev. Biol. 144:129–144.

34. Gongadze GM. 2011. 5 S rRNA and ribosome. Biochemistry (Mosc.) 76: 1450–1464.

35. Neely LS, Lee BM, Xu J, Wright PE, Gottesfeld JM. 1999. Identification of a minimal domain of 5 S ribosomal RNA sufficient for high affinity interactions with the RNA-specific zinc fingers of transcription factor IIA. J. Mol. Biol. 291:549–560.

36. Williamson JR. 2000. Induced fit in RNA-protein recognition. Nat. Struct. Biol. 7:834 – 837.

37. Draper DE, Reynaldo LP. 1999. RNA binding strategies of ribosomal proteins. Nucleic Acids Res. 27:381–388.

38. Agalarov SC, Sridhar Prasad G, Funke PM, Stout CD, Williamson JR. 2000. Structure of the S15,S6,S18-rRNA complex: assembly of the 30S ribosome central domain. Science 288:107–113.

39. Rao JN, Schweimer K, Wenzel S, Wohrl BM, Rosch P. 2008. NELF-E RRM undergoes major structural changes in flexible protein regions on target RNA binding. Biochemistry 47:3756–3761.

40. Hermann T. 2005. Drugs targeting the ribosome. Curr. Opin. Struct. Biol. 15:355–366.

41. Yonath A. 2005. Antibiotics targeting ribosomes: resistance, selectivity, synergism and cellular regulation. Annu. Rev. Biochem. 74:649–679.

42. Sutcliffe JA. 2005. Improving on nature: antibiotics that target the ribosome. Curr. Opin. Microbiol. 8:534 –542.

43. Poehlsagard J, Douthwaite S. 2005. The bacterial ribosome as a target for antibiotics. Nat. Rev. Microbiol. 3:870–881.

44. Bottger EC. 2006. The ribosome as a drug target. Trends Biotechnol. 24:145–147.