Nucleic Acids Research, 1997, Vol. 25, No. 24 **4977–4984**

The trypanosomatid *Leptomonas collosoma* 7SL RNA gene. Analysis of elements controlling its expression

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Received August 26, 1997 Revised and Accepted November 3, 1997

DDBJ/EMBL/GenBank accession no. AF006632

ABSTRACT

We have previously reported the co-purification of a tRNA-like molecule with the *Trypanosoma brucei* SRP complex [Béjà *et al.* (1993) *Mol. Biochem. Parasitol.* 57, 223–230]. To examine whether the trypanosome SRP has a unique composition compared with that of other eukaryotes, we analyzed the 7SL RNA and the SRP complex of the monogenetic trypanosomatid *Leptomonas collosoma.* The 7SL RNA from *L.collosoma* was cloned, and its gene was sequenced. In contrast to *T.brucei*, two 7SL RNA transcripts were detected in *L.collosoma* that originate from a single-copy gene. Using stable cell lines expressing tagged 7SL RNA, we demonstrate that the tRNA^{Arg} gene located 98 bp upstream to the 7SL RNA serves as part of the 7SL RNA extragenic promoter. The steady-state level of 7SL RNA was found to be tightly regulated, since the presence of the gene on the multi-copy plasmid repressed the synthesis of the chromosomal gene. Cell lines carrying truncated 7SL RNA genes were established and their expression indicated that domain I is essential for expressing the 7SL RNA. No constructs carrying portions of the 7SL RNA were expressed, except for a construct which lacked 23 nt from the 3′ end of the RNA. This suggests that 90% of the 7SL RNA molecule is important for its maintenance as a stable small RNA. We propose that the repression phenomenon may originate from a regulatory mechanism that coordinates the level of the 7SL RNA by its binding proteins.

INTRODUCTION

The signal recognition particle (SRP) in eukaryotes is a cytoplasmic ribonucleoprotein that targets presecretory proteins and membrane proteins to the endoplasmic reticulum (ER) membrane (1). SRP was shown *in vitro* to bind the signal sequence emerging from ribosomes and to trigger a transient pause in elongation. This blockage is relieved when SRP interacts with the ER SRP receptor, and normal protein synthesis is resumed, resulting in co-translational translocation of the protein into the ER lumen (1). The eukaryotic SRP carries a single RNA molecule, the 7SL RNA, that is composed of four stem–loop structures and six SRP proteins (2). Different functions were assigned to the different proteins. It was shown that SRP54 binds the signal peptide as it emerges from the ribosome, SRP 9/14 bind to domain I and function in elongation arrest, SRP68/72 mediate translocation into the ER, whereas SRP19 facilitates the binding of SRP54 to the RNA (3).

Extensive phylogenetic studies were performed on SRP RNAs and indicate that the different RNAs vary in size and in composition but all carry an invariant domain IV (4,5). It was proposed that the ancestral SRP molecule was reduced in size during bacterial evolution (4). However, it is currently unknown whether, in organisms carrying truncated forms of the SRP RNA, the missing RNA domains reside in other yet unidentified small RNAs. Extensive changes in the size and shape of domain I are found among eukaryotic 7SL RNAs. For example, domain I of higher eukaryotic 7SL RNAs can be folded as a tRNA-like molecule, but those of *Trypanosoma brucei* (6) and *Tetrahymena* lack one of the arms of the tRNA-like structure (7) and yeast have a significantly truncated form that bears no resemblance to the tRNA structure (8).

Structure–function aspects of the SRP were addressed *in vitro* in the canine system and *in vivo* in the fission yeast *Schizosaccharomyces pombe*. The results obtained from both systems indicate that the four domains (including domain III, that is missing from the bacterial SRPs) are critical for SRP function (9,10). It is currently unknown whether domain I of yeast also contributes to protein arrest, since the phenotype observed for domain I mutants may result from a failure to transcribe the gene (10).

Little is known about the regulation of 7SL RNA transcription. The only well characterized 7SL RNA promoter is the human promoter which is comprised of both upstream and internal elements (11,12). Homologies to A and B boxes of tRNA promoters can be found within the coding region of 7SL RNA. However, a CG dinucleotide at position +15 and +16 located outside this box was found to be essential for transcription whereas most of the mutations made within the A box had little effect (13). Analysis of the *T.brucei* 7SL RNA gene demonstrated the existence of extragenic elements located 97 bp upstream to the start site that controls the synthesis of the 7SL RNA. This element resides within a tRNA^{Lys} that is transcribed in the opposite direction (14).

Very little is known about protein translocation in trypanosomes. The finding that *T.brucei* has an SRP homologue was not surprising (6). However, the presence of a co-migrating tRNA-like molecule, that co-purifies with the *T.brucei* 7SL RNA (15), led us to

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hypothesize that the trypanosomatid SRP may differ from other SRPs, and is composed of two small RNP particles.

In this study, we have cloned, sequenced and expressed mutated and truncated versions of the monogenetic trypanosomatid *Leptomonas collosoma* 7SL RNA. This study is the first step towards understanding the structure–function relationship of the trypanosomatid SRP and the mechanism that tightly regulates its level of expression. The results indicate that *L.collosoma* 7SL RNA has a unique property as it is the only 7SL RNA described so far that is present in two stable RNA conformations. Studies performed with stable cell lines expressing truncated and mutated 7SL RNA demonstrate that the $tRNA^{Arg}$ located upstream to the 7SL RNA gene is part of the extragenic promoter element and that an intragenic element controlling the expression of the gene exists in domain I. This study also indicates that the steady-state level of 7SL RNA in trypanosomes is tightly regulated since the presence of mutated 7SL RNA on a multi-copy plasmid repressed the synthesis of the wild-type RNA. The repression was observed only when all 7SL RNA domains (known as the SRP protein binding sites) were present, suggesting a mechanism that coordinates the level of the 7SL RNA by its binding proteins.

MATERIALS AND METHODS

Oligonucleotides

5170, 5′-GTTAAAGTAGAGGAACTGGG-3′, sense to positions 159 to 177;
5303, 5′-GCAGAGCACCACGTCAACGC-3′, complementary to nt 82–101;
8606, 5′-TGCCGACATCAGTCCGTGTG-3′, antisense complementary to –88 to –105;
8721, 5′-CGGGATCCAGCCGGAGCCTTGCTC-3′, sense to positions 1 to 16 including a *Bam*HI site;
10021, *Sac*II linker carrying an *Eco*RI site 5′-GGACTGAATTCA-GTCCGC-3′;
10551, 5′-CAGGATTCGAACCTGCAACCC-3′, sense to –142 to –162 complementary to $tRNA^{Arg}$;
12615, 5′-CTGCTCCGTTCGGATCCTGCCGGCCTGA-3′, antisense to loop IV carrying insertion of a *Bam*HI site;
15137, 5′-CCGGATCCGGTGCGATGAAATGAGACGG-3′, complementary to nt 304–323 including a *Bam*HI site;
15366, 5′-GCTCTAGAGTCGACTTTGACCACCCATTAT-3′, sense oligo to nt –440 to –455 with *Xba*I and *Sal*I sites;
15367, 5′-GCGTCGACCAGCTGATAGGAAGTGCGGCAA-3′, antisense to –1 to –16, carrying sites for *Sal*I and *Pvu*II;
5804, 5′-CGGGATCCGCTTCACAGGATCGCCTGG-3′, antisense to 7SL RNA, complementary to positions 259 to 278 carrying a *Bam*HI site;
17208, 5′-TGCTCCGTTGCCGGCCT-3′, antisense to domain IV, complementary to nt 180–197;
17528, 5′-TCAGCTGGTGAAAGC-3′, antisense to domain I, complementary to nt 31–45;
18816, 5′-AGACGTGCGCGAGGTGG-3′, antisense to 200 to 216;
18662, 5′-CGCAGGTGATGACAGGCT-3′, antisense position 240 to 257;

RNA isolation and northern analysis

Total RNA was prepared with TRIzol reagent (GIBCO BRL). The RNA samples were fractionated on a 7 M urea/10% polyacrylamide gel and electroblotted onto a Nytran membrane. Hybridization with labelled oligonucleotides was performed at 37°C in 5× SSC (1× SSC consisted of 150 mM NaCl and 15 mM sodium citrate), 0.1% SDS, 5× Denhardt's solution and 100 µg/ml salmon sperm DNA. The *L.collosoma* 7SL RNA probe was obtained from excising the two 280 and 300 nt 7SL RNA molecules enriched in PRS from a preparative denaturing gel. The RNA was treated with alkaline phosphatase (16) to remove the phosphate termini and was 5′ end labeled with polynucleotide kinase (16).

Cloning of the gene encoding the 7SL RNA

Approximately 20 000 plaques of an *L.collosoma* λEMBL3 were screened with 5′ end-labeled 7SL RNA and four independent clones were isolated that also hybridized with antisense oligonucleotide complementary to nt 165–184 of the *T.brucei* 7SL RNA (6). The clones were digested with *Sal*I and a 2.8 kb fragment was subcloned into the pGEM-3 vector, and the *L.collosoma* 7SL 4/78 construct was generated. Two fragments, a 400 bp *Sau*3A fragment and a 1 kb *Taq*I fragment, were subcloned and sequenced using SP6 and T7 primers and internal gene primers.

Growth and extract preparation

Leptomonas collosoma cells were grown as previously described (17). Cells (5×10^{10}) were harvested, washed with PBS and resuspended in Buffer A containing 35 mM HEPES–KOH (pH 7.9), 10 mM $MgCl_2$, 50 mM KCl, 5 µg/ml leupeptin and 5 mM β-mercaptoethanol. The cell suspension was equilibrated in a nitrogen cavitation bomb (Parr Instruments Company) at 1000 psi for 10 min, and disrupted by release from the bomb. Post-ribosomal supernatant (PRS) preparation and DEAE-chromatography were essentially as described previously (18).

DNA transformation

About 4×10^7 cells were used for electroporation, conducted with two pulses of 500 µF and 2.5 kV/cm in a Bio-Rad gene pulser. Cells were transfected with 50–100 µg of the different DNA constructs. Transformants were selected in liquid medium in the presence of 20–50 µg/ml G418 (GIBCO). To elevate the copy number of the plasmid the transformants were grown in the presence of elevated G418 (500–1000 µg/ml).

Plasmid construction

To construct the 7SL RNA gene tagged in the *Sac*II site a linker (oligo 10021) was inserted into the unique *Sac*II site in *L.collosoma* 7SL RNA 4/78, located in position 239 within domain II of the 7SL RNA. The fragment carrying the mutation was subcloned into the pX expression vector (19). Two deletions of the upstream region were constructed using the 1460 bp *Acc*I–*Sal*I and the 1360 bp *Taq*I–*Sal*I fragments. To generate a pX plasmid containing the 7SL RNA gene transcription termination signals, (pX-7SL-t), a 1.2 kb *Hin*dIII–*Sma*I fragment, derived from the 4/78 plasmid, was cloned to the pX vector. The constructs described below are illustrated in Figure 5A. The construct (dI) carrying domain I was obtained by ligating to pX a *Sma*I–*Pvu*II containing a 1.3 kb upstream sequence and 41 nt of the 7SL RNA. The construct [p(dI)t] was made by ligating the same fragment as for [p(dI)] but to the pX-7SL-t. Constructs p(H)t and p(B)t were made by ligating the *Sma*I–*Hin*cII fragment carrying 86 nt of the 7SL RNA or *Sma*I–*Bss*HI carrying 147 nt of the 7SL RNA, respectively, to pX-7SL-t. Plasmid pdIX2 was

constructed by cloning a PCR product in to the *Bam*HI site obtained using oligos 8721 and 15137 into the pdI plasmid. Plasmid [p(-dII)t] was constructed by cloning a PCR product, obtained from oligos 18816 and 15366, to pX-7SL-t. The p(-3′)t plasmid was constructed by ligating the PCR product generated with oligos 18662 and 15366 to the *Sma*I site. The [p(-dI)t] construct was made by three point-ligation. The PCR product obtained using oligos 15367 and 15366 was digested with *Xba*I and *Pvu*II; the second PCR product, made with oligos 8721 and 15137, was digested with *Pvu*II and *Bam*HI. The two PCR products were ligated to pX digested with *Bam*HI and *Xba*I. The loop IV mutation was obtained by the Kunkel method (20). To generate the site-directed mutation, the 2.8 kb *Sal*I fragment was cloned into pBluescript KS(–). Oligonucleotide 12615 was used to generate the mutation, and the mutated fragment was cloned to the pX vector. The mutation was confirmed by sequencing. The sequence of the constructs generated by PCR and site-directed mutagenesis was verified. To synthesize an antisense 7SL RNA probe, a PCR product carrying the entire 7SL RNA gene (generated with oligos 8721 and 15137) was cloned into the *Bam*HI site of pGEM-3.

RNase protection assay

Antisense 7SL RNA was synthesized using SP6 polymerase. Total RNA (~10 µg) was mixed with 100 000 c.p.m. of *in vitro* transcribed anti-7SL RNA probe in buffer containing 80% formamide, 40 mM PIPES (pH 6.4), 0.4 M sodium acetate and 1 mM EDTA. After incubation at 85°C for 5 min, the RNA was hybridized for 12 h at 45°C. The unprotected RNA was digested with RNase One (Promega), and after deproteinization, the RNA was separated on a 6% denaturing gel.

RESULTS

Identification of the *L.collosma* 7SL RNA and sequence analysis of the gene and its flanking regions

As a first step towards elucidating the SRP complex of *L.collosoma* we identified its 7SL RNA. Whole cell extracts were prepared from *L.collosoma* and the 7SL RNA content was examined in these extracts after depletion of ribosomes (PRS). The RNA profile and the hybridization results with an antisense oligonucleotide, complementary to the *T.brucei* 7SL RNA (oligo EU-53) probe, are presented in Figure 1A, II. The results indicate the existence of two 7SL RNA molecules of 280 and 300 nt that hybridize to the 7SL RNA probe. These two molecules are present in a 1:1 ratio and are highly enriched in PRS. To examine whether the two molecules are distinct stable transcripts, the potential for interconversion of one species to the other was investigated. A fraction enriched with 7SL RNA was end labelled at the 5′ end and the labeled RNA (Fig. 1B, lane 1) was separated on a 10% denaturing gel. The separated 7SL I and 7SL II molecules were excised and analyzed. The results (Fig. 1B) suggest that there is no conversion *in vitro* between these species. Further separation of the 7SL RNA in different gel systems suggests that the 7SL RNA migrates as a single species only in the presence of 75% formamide and 7 M urea (Fig. 1C, panel 2), suggesting that only under severe denaturation conditions can the molecule be completely denatured. These results also suggest that the two distinct 7SL RNA conformations are stable and are generated *in vivo*.

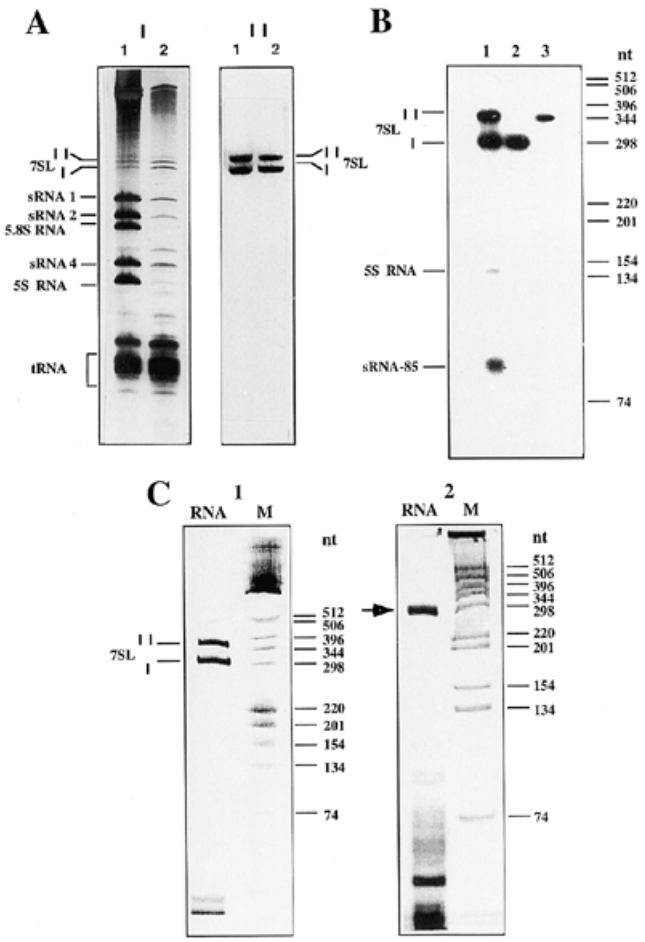

Figure 1. The *L.collosoma* 7SL RNA exits in two stable conformations. (**A**) I: Whole cell extract was prepared from 5×10^{10} cells subjected to centrifugation at 150 000 *g* (S150). Aliquots were deproteinized and the RNA was analyzed in a 6% denaturing gel and visualized by silver staining. Lane 1, whole cell extract; lane 2, S150. II: Equivalent RNA samples were subjected to northern analysis with antisense oligonucleotide EU-53, complementary to the *T.brucei* 7SL RNA. (**B**) Fractionation of 5′ end labeled 7SL RNA in a 10% denaturing gel. Fractions enriched for 7SL RNA were end-labeled at the 5′ end and the RNA was separated on a 10% denaturing gel. The 7SL I and II were excised from the gel, eluted and electrophoressed on a 10% denaturing gel. Lane 1, an aliquot of the 5′ end labeled RNA enriched for 7SL RNA; lane 2, pure 7SL I; lane 3, pure 7SL II. (**C**) Fractionation of 7SL RNA in different gel systems. Fractions enriched in 7SL RNA were separated next to a DNA marker; 1, RNA was separated in a 10% polyacrylamide gel containing 7 M urea; 2, 10% polyacrylamide containing 7 M urea and 75% formamide. The RNA was visualized by EtBr staining. DNA marker was the 1 kb ladder (BRL).

To gain more information on the structure of the *L.collosoma* 7SL RNA, the gene coding for the RNA was cloned and sequenced. An *L.collosoma* genomic library was screened with end-labeled 7SL RNA. Four independent λDNA clones carrying the 7SL RNA were isolated and sequenced. The sequence of the 7SL RNA gene and of the flanking regions is presented in Figure 2A and the proposed secondary structure is presented in Figure 2B. To examine the genomic organization of the 7SL RNA, *L.collosoma* DNA was digested with restriction enzymes of 6 and 4 bp recognition sites. The blot was hybridized with the

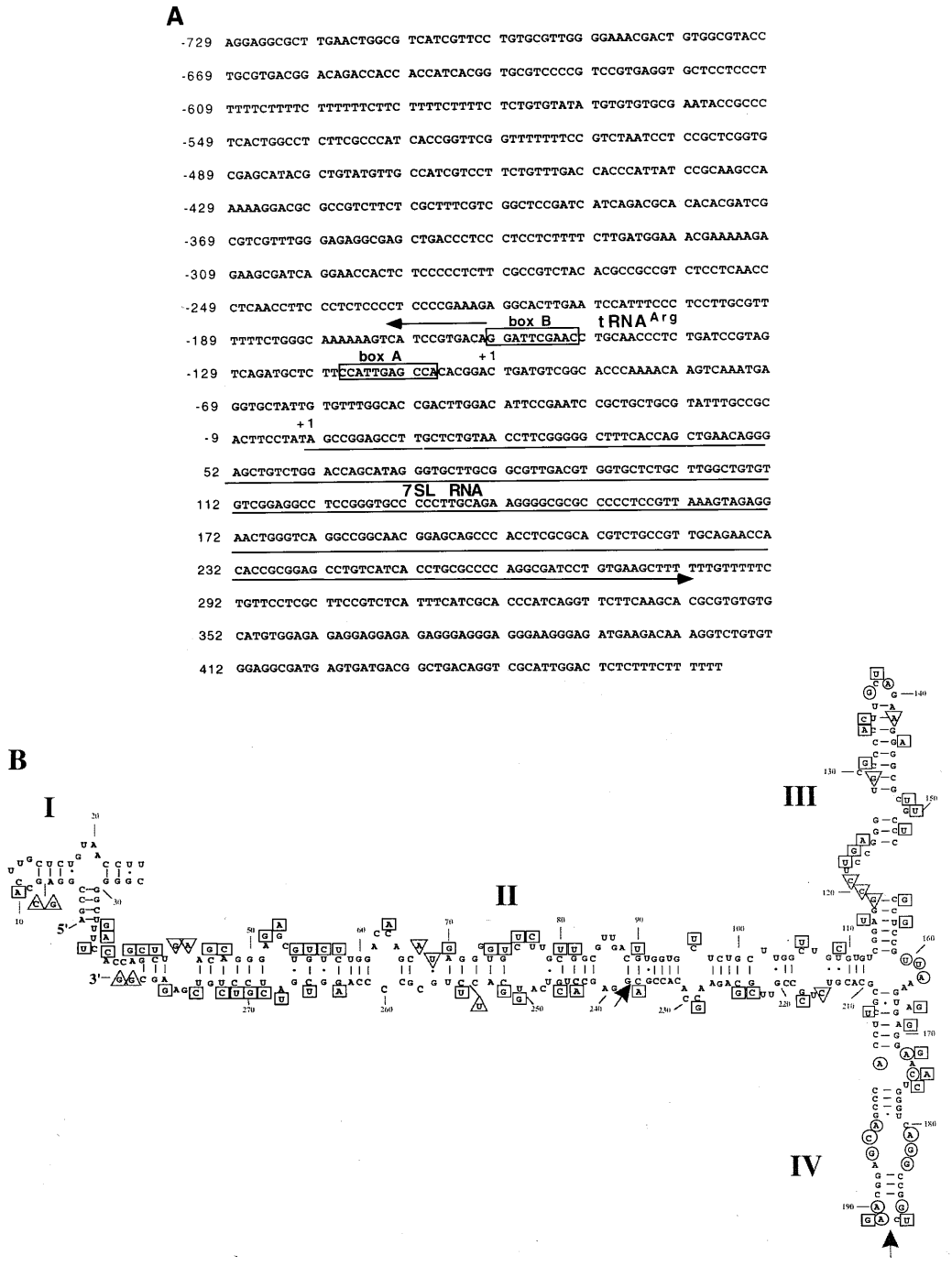

Figure 2. DNA sequence analysis of the 7SL RNA gene locus. (**A)** The coding regions of the 7SL RNA and the $tRNA^{Arg}$ are indicated by upper case lettering and the 7SL RNA sequence is underlined. The +1 position of the 7SL RNA and the $tRNA^{Arg}$ are indicated and the direction of transcription is marked with an arrow. The consensus promoter elements of the $tRNA^{Arg}$ (boxes A and B) are boxed. (**B**) The proposed secondary structure of the *L.collosoma* 7SL RNA. Differences between the *L.collosoma* and *T.brucei* 7SL RNAs are shown in boxes. Nucleotides that are missing in *L.collosoma* but exist in *T.brucei* are shown in triangles and nucleotides that exist in *L.collosoma* and missing from *T.brucei* are shown in inverted triangles. Nucleotides that are invariant in all 7SL RNA are circled. The position of inserting the tags is marked with arrows.

400 bp *Sau*3A 7SL RNA subclone, and the results (Fig. 3A) indicate that the 7SL RNA is a single-copy gene. To examine whether small size variations exist between alleles, genomic DNA and λDNA obtained from the different clones were subjected to PCR with oligos 8721 and 15137. The results presented in Figure 3B indicate that all the 7SL RNA genes that were isolated have an identical size, suggesting that there is no allelic size variation.

To determine the exact 5′ end of the 7SL RNA, primer extension was performed with oligonucleotide 5303. The results

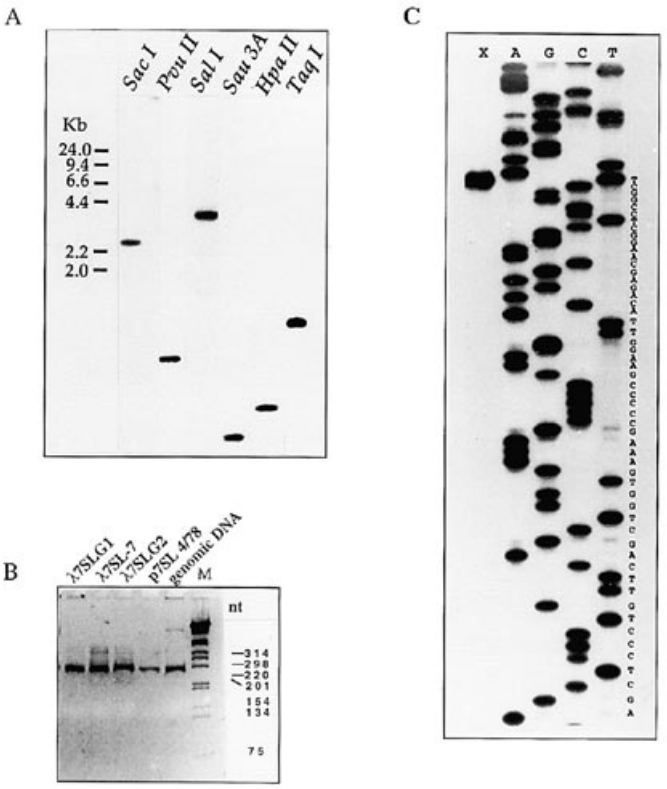

Figure 3. (**A**) Genomic organization of 7SL RNA. *Leptomonas collosoma* genomic DNA (10 µg) was digested with different restriction enzymes as indicated. After transfer, the membrane was hybridized with random-prime labeled 7SL RNA clone 7SL.7 *Sau*3A. Phage λ digested with *Hin*dIII was used as a marker, and the size of the fragments are indicated. (**B**) PCR analysis of λ phages and plasmids carrying the 7SL RNA gene and genomic DNA. DNA (~50 ng) was amplified with the oligonucleotides 5′-8721 and 3′-15137, and the PCR products were separated on a 6% native polyacrylamide gel. Marker was the 1 kb ladder (GIBCO BRL). The identity of the DNA is indicated. (**C**) Primer extension analysis was performed using an end-labeled oligonucleotide (5303). The products of sequencing reaction of 7SL.7 *Sau*3A using the same oligonucleotide were used as a reference. The sequence of the cDNA is indicated.

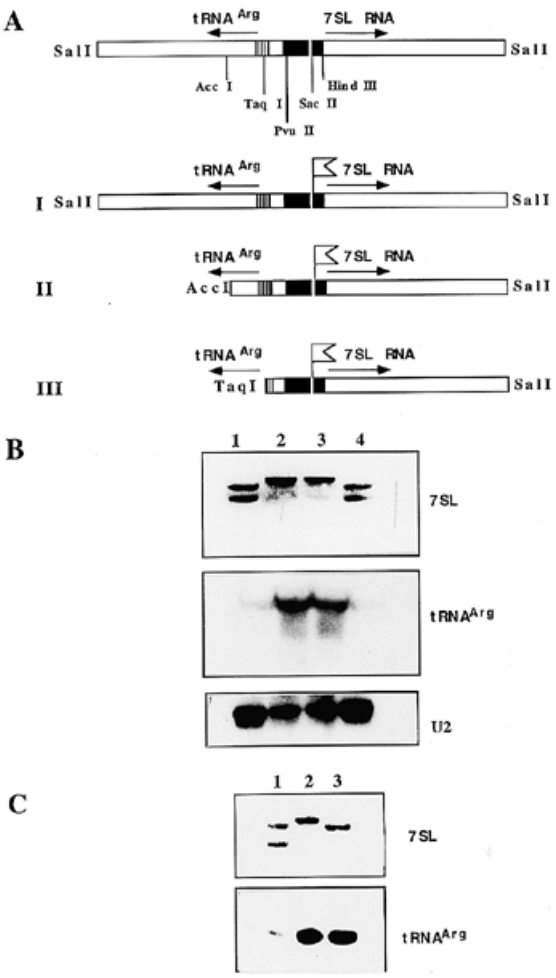

Figure 4. (**A**) Schematic presentation of constructs that vary in the 5′ flanks. The position of restriction enzymes that were utilized to create the constructs is indicated. The 7SL RNA coding region is marked by filled lines and the $tRNA^{Arg}$ by dashed lines. The arrows indicate the direction of transcription. (**B**) Northern analysis of RNA derived from wild-type and cell lines growing on 500 µg/ml G418 carrying the following constructs: lane 1,wild-type; lane 2, construct I; lane 3, construct II; lane 4, construct III. The RNA was probed with antisense oligonucleotides to 7SL RNA, $tRNA^{Arg}$ and U2 RNA. (**C**) Northern analysis of RNA derived from: lane 1, wild-type; lane 2, construct I; lane 3, mutation in loop IV. The northern blot was probed as indicated in (B).

presented in Figure 3C indicate that the +1 position is an A as indicated in Figure 2A and that the two 7SL RNA molecules do not differ at the 5′ end. The 280 nt *L.collosoma* 7SL RNA is 6 nt longer than the *T.brucei* 7SL RNA. The overall identity between the *T.brucei* and the 7SL RNA is only 69%; the differences between these two 7SL RNAs are shown in Figure 2B. The degree of identity varies between the domains, domain I and IV being the most conserved, 91% and 82%, respectively. Domain II can be further divided to two sub-domains on the basis of the micrococcal nuclease hypersensitive site of human 7SL RNA (21); the first part, spanning nt 40–83 and 241–280, exhibits only 51% identity with the *T.brucei* homologue, whereas the other part, covering nt 84–113 and nt 211–240, is highly conserved (85%). In the second part, the location of the bulges is also conserved. Several compensatory changes are found including the pairs 58–264 and 90–237. These pairs are GC in *L.collosoma* but AU in *T.brucei*. In addition, domain III differs in both the primary sequence (only 54% identity) and in the secondary structure. Whereas the *T.brucei* domain III is a long stem with a small loop, the *L.collosoma* sequence possesses two bulges of 2 and 5 nt each (Fig. 2B). Interestingly, the bulge of 5 nt is located in the 7SL RNA region homologous to the region that was shown to mediate the interaction of SRP with ribosomes (22).

A GenBank search with the sequences upstream to 7SL RNA revealed the presence of a tRNA gene located 98 bp upstream, which is 100% identical to $tRNA^{Arg}$ from *Leishmania tarentolae* (23). Interestingly, $tRNA^{Arg}$ was found upstream to the *Leishmania pifanoi* 7SL RNA gene (14). No other significant similarity was found to sequences located either upstream to the $tRNA^{Arg}$ or downstream of the 7SL RNA. Such a genome organization differs from that of *T.brucei*, since the U3 homologue with its accompanying $tRNA^{Arg}$ was found 90 bp upstream of the *T.brucei* 7SL RNA locus. The A and B boxes of the $tRNA^{Arg}$ are

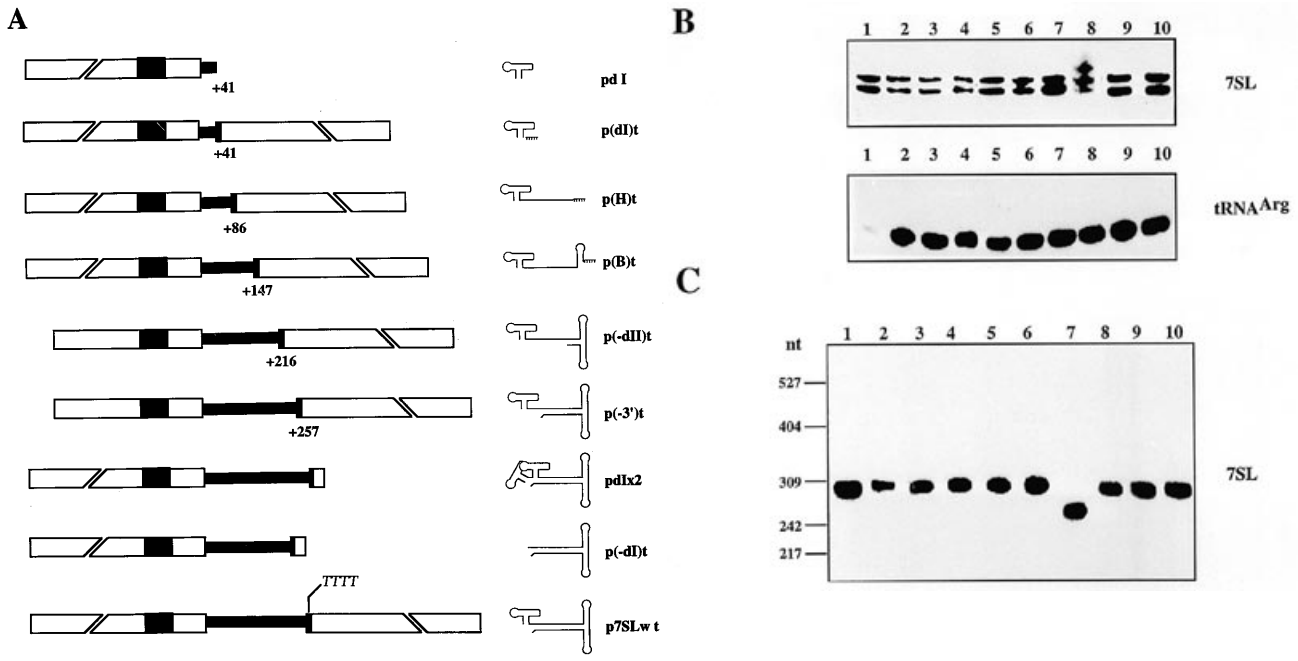

Figure 5. (**A**) Schematic presentation of constructs carrying truncated 7SL RNAs. The position of termination is indicated with runs of Ts. The $tRNA^{Arg}$ is boxed in black and the 7SL RNA coding region is indicated with a thick black bar. The potential secondary structure of the truncated 7SL RNA is indicated. (**B**) Northern analysis of RNAs from cell lines expressing the different constructs. All cell lines were grown on 500 µg/ml G418, and the blot was hybridized with antisense oligonucleotides to 7SL RNA, $tRNA^{Arg}$. Lanes 1, wild-type; lanes 2, pdI; lanes 3, p(dI)t; lanes 4, p(H)t; lanes 5, p(B)t; lanes 6, p(-dII)t; lanes 7, p(-3)t; lanes 8, pdIx2; lanes 9, p(-dI)t; lanes 10, p7SLwt. (**C**) RNAse protection assay. RNA was derived from the same cell lines as in (B). Size markers (pBR322 digested with *Hpa*II) are indicated.

indicated in Figure 2. Box A, 5′-TGGCTCAATGG-3′, deviates from the canonical sequence 5′-TRRYNNAGTGG-3′ by an A instead of a G in the 8th position, whereas the B box, 5′-GTTCGAATCC-3′, agrees well with the canonical sequence 5′-GTTCRANNCC-3′.

$tRNA^{Arg}$ is part of the 7SL RNA extragenic promoter

To study the structure–function of the 7SL RNA, we sought to overexpress mutated and truncated 7SL RNA genes. The 7SL RNA gene was marked by inserting a linker in the unique *Sac*II site (construct I in Fig. 5A) located at position 239 of the 7SL RNA (domain II), indicated in Figure 2B. A stable cell line expressing the mutated gene was obtained. The expression of the tagged RNA was examined by northern analysis, and the results indicate that the tagged 7SL RNA, which is larger than the wild-type RNA is efficiently expressed (Fig. 4B, lane 2). The expression of the tagged 7SL RNA repressed the synthesis of the wild-type RNA, since RNA transcripts, corresponding in size to wild-type 7SL RNA, were absent in cell lines carrying the tagged molecule (compare lanes 1 and 2). Since only a single RNA species was observed in lane 2, it may suggest that the tagged 7SL RNA does not undergo the conformational change proposed for the wild-type RNA.

To examine the extragenic sequences which regulate the synthesis of 7SL RNA, the expression of the tagged gene was examined in the presence of 274 and 154 bp of upstream sequence (constructs II and III, respectively). Construct III carries a truncated $tRNA^{Arg}$. RNA was prepared from cell lines and subjected to northern analysis with antisense 7SL, $tRNA^{Arg}$ and U2; the latter served as a control for the amount of RNA. The results are presented in Figure 4B. Lanes 3 and 4 indicate that the tagged gene was efficiently expressed in cell lines carrying only the 274 nt upstream sequence but not when the $tRNA^{Arg}$ was truncated, suggesting that the $tRNA^{Arg}$ is part of the extragenic elements that dictate the expression of the gene. Interestingly, despite the fact that the 7SL RNA was carried on a multi-copy plasmid, its cellular level was not elevated compared with wild-type cells, whereas the level of $tRNA^{Arg}$ increased ~10 times. This suggests that the level of the cellular 7SL RNA is tightly regulated. To examine whether or not the repression phenomenon is restricted to the mutation located in domain II, another mutation was introduced in loop IV. Stable cell lines were then established from a construct carrying this mutation (indicated in Fig. 2B), and the expression of the gene was examined by northern analysis (Fig. 4C). The results indicate that the 7SL gene mutated in loop IV also repressed the synthesis of the wild-type 7SL RNA and was found in a single conformation.

Expression of truncated and mutated 7SL RNAs

To characterize the sequences that regulate the expression of the 7SL RNA and to elucidate the mechanism that elicits the repression of the wild-type 7SL RNA by the gene carried on the multi-copy plasmid, several constructs were generated and are schematically presented in Figure 5A.

One possibility to explain the repression phenomenon is that the multi-copy plasmid titrates the 7SL RNA transcription factors. To examine this possibility the level of 7SL RNA was examined in cell lines carrying the upstream regulatory region and domain I, since this domain was shown in humans to carry

intragenic promoter elements (11). The results presented in Figure 5B (lanes 1, 2 and 3) indicate that the level of the cellular 7SL RNA was not changed in cells carrying constructs p(dI) and p(dI)t. A small transcript of 40 nt that could have been generated from domain I was not detected in total RNA preparation, even when the proper termination signals from the 7SL RNA gene were present [construct p(dI)t]. This data may suggest that the repression is not due to competition for transcription factors.

Hybridization of the same northern with the $tRNA^{Arg}$ probe (Fig. 5B) indicates that the level of $tRNA^{Arg}$ was elevated due to its presence on the multi-copy plasmid, suggesting that the $tRNA^{Arg}$ carried on these plasmids is highly expressed, irrespective of the expression of 7SL RNA.

To examine the factors that regulate the expression of the 7SL RNA, two constructs that differ only in domain I, i.e., deletion of domain I [p(-dI)t] or duplication of this domain (pdIx2) were constructed, and the expression of the 7SL RNA was examined. The results indicate that in the absence of domain I the 7SL RNA is not expressed, (Fig. 5B lane 9), suggesting that domain I may carry elements which are necessary for transcribing the gene, as in humans. Duplication in domain I did not interfere with the expression of the gene (lane 8), but this construct failed to repress the synthesis of the wild-type RNA.

To further explore the sequences essential for expressing the 7SL RNA, constructs harboring different lengths of the 7SL RNA were generated. Four constructs carrying split 7SL RNA genes harboring coding information for 86, 147, 216 and 257 nt were generated, and are schematically presented in Figure 5A. All these constructs carried the 7SL RNA transcription termination signals. The constructs were used to establish stable cell lines and the level of 7SL RNA was examined. The results, presented in Figure 5B, lanes 4–6, indicate that, despite the high level of expression of the accompanying $tRNA^{Arg}$, none of these truncated 7SL RNA were detectable in steady-state RNA preparation, suggesting that all four domains are essential for expressing stable 7SL RNA molecules.

To examine the level of 7SL RNA that may be present in minute amounts, a sensitive RNase protection assay was used. The results presented in Figure 5C indicate that, except for a construct lacking 23 nt from the 3′ end (lane 7), none of the truncated 7SL RNAs were expressed. Longer exposure of the gel did not reveal any additional bands that were not found in the wild-type control. However, the construct lacking only 23 nt from the 3′ end was efficiently expressed and repressed the synthesis of the wild-type RNA, as no fragment corresponding to protection with wild-type RNA was observed. The presence of two transcripts in Figure 5B, lane 7, suggests that this truncated 7SL RNA is capable of undergoing the conformational change, like the wild-type RNA.

The tight regulation on the 7SL RNA was also observed when the wild-type 7SL RNA locus was cloned into the pX plasmid. Despite the elevation in the copy number of the gene no increase in the cellular level of 7SL RNA was observed (Fig. 5B, lane 10).

DISCUSSION

In this study we have cloned and sequenced the 7SL RNA gene of the monogenetic trypanosomatid *L.collosoma* and examined the elements that regulate its expression. The results indicate that the level of 7SL RNA is tightly regulated, since synthesis of mutated 7SL RNA repressed the wild-type 7SL RNA. The repression took place only when the 7SL RNA, carried on the plasmid, was expressed. Apart from the 7SL RNA gene lacking only 23 nt from the 3′ end, none of the other split 7SL RNAs were expressed, suggesting that only when four 7SL RNA domains are present can the RNA be maintained as a stable small RNA. This study also demonstrates that the $tRNA^{Arg}$ is part of the extragenic elements that control the transcription of 7SL RNA and that domain I, like in humans, is essential for expressing the gene.

One of the most intriguing findings regarding the trypanosome 7SL RNA is its high degree of similarity to the human RNA (60%) compared with the yeast *S.pombe* RNA (45%) (6). This is also true for the *L.collosoma* RNA, that shares 52% identity with the human RNA and only 38% with the *S.pombe* RNA. This result is even more surprising considering the earlier divergence of trypanosomes compared with yeast from the eukaryotic lineage (24). Genetic transfer from the mammalian host to the parasite, is a mechanism that could have generated such relatedness. However, since *L.collosoma* lacks a mammalian host, the genetic transfer hypothesis should be ruled out.

Despite the sequence diversity between the yeast, human and trypanosome RNAs, yeast and trypanosome RNAs possess a truncated domain I compared with the tRNA-like domain of human RNA. The trypanosome domain I is an intermediate between the highly-truncated yeast domain and the tRNA-like structure of the human domain. In the mammalian domain I, a potential for base-pairing between the two hairpin loops exists. This potential does not exist in the trypanosome or *Tetrahymena* RNAs, since these RNAs carry a truncated second hairpin loop. Interestingly, however, these three domains carry the sequence homologous to the consensus sequence 5′-GCG-N3-5-CCUGUAAYCY-3′ that has been involved in binding the SRP9 and 14 (25). Based on the truncation of domain I in lower eukaryotes and the presence of a tRNA-like molecule that co-purifies with the trypanosome 7SL RNP (15) we propose that in those organisms which lack domain I, (like bacteria or eukaryotes that have a truncated domain I), a functional domain I may be carried by a separate RNP complex. We have previously reported the co-purification of a tRNA-like molecule with the *T.brucei* 7SL RNP (15) and recently identified the tRNA-like molecule that co-purifies with the *L.collosoma* 7SL RNP (our unpublished results).

The results suggest that the *L.collosoma* 7SL RNA is a single copy gene and that no size variation exists between the two 7SL RNA alleles. The data also indicate that the two 7SL RNA transcripts represent two stable conformers of the RNA. We observed that the ratio between 7SL I and II changes during growth, i.e., actively growing cells contain more 7SL I, and the ratio between the molecules reaches 1:1 when the culture ages. In addition, 7SL I is preferentially associated with ribosomes, and the *in vivo* conversion of 7SL I to II is inhibited in the presence of cycloheximide. These data suggest that the conformational change takes place during protein translocation (Ben-Shlomo *et al.*, unpublished data). The finding that 7SL RNA mutants located in domain II and IV exist in a single conformation may indicate that these mutated RNAs are not active in protein translocation and therefore do not undergo the conformational change.

The repression phenomenon observed in this study is similar to the observation made when human U1 RNA gene was expressed in mouse cells (26). It was found that despite the efficient expression of the human gene, the total amount of U1 RNA (both mouse and human) did not change, suggesting that multi-gene families encoding mammalian U1 RNA are subjected to dosage compensation. The finding of such a regulatory phenomenon

shared by two different small RNPs, may suggest that there is a common regulatory mechanism that co-regulates the level of the RNA with its RNP binding proteins. The repression was observed only when the 7SL RNA was efficiently transcribed and maintained as a stable RNA. We, therefore, favor the hypothesis that the SRP proteins regulate the level of 7SL RNA. Although we cannot rule out the possibility that the repression is due to the titration of a factor that binds to the coding region of the 7SL RNA, we consider this possibility most unlikely. From all the truncated 7SL RNA only the construct missing 23 nt from the 3′ end was expressed. This may suggest that the correct folding of the 7SL RNA into its protein binding domains is what dictates whether a truncated 7SL RNA will be maintained as a stable 7SL RNA. Support for a cellular mechanism that links and coordinates between the 7SL RNA and the SRP protein was obtained from the work in yeast (27) which indicated that cells impaired in the synthesis of SRP proteins showed a large reduction in the level of the SRP RNA.

Using transient transfection assays in *T.brucei*, it was previously demonstrated that the extragenic regulatory elements that control the synthesis of 7SL, U6 and U3 RNAs are found in the A and B boxes of the respective companion tRNA (14). The data presented in this study are consistent with this conclusion, since truncating the $tRNA^{Arg}$ abolished synthesis of the tagged 7SL RNA. The 10- to 20-fold amplification of the $tRNA^{Arg}$ compared to its level in wild-type cells supports the notion that no competition between factors that regulate the 7SL and tRNA gene occurs, as the same level of tRNA amplification was observed, regardless of how efficient the 7SL RNA gene was expressed. It is currently unknown how the companion tRNA elicits its regulatory effect on the 7SL RNA.

Several models were suggested to explain the transcriptional linkage between the small RNA and its companion tRNA gene. One model suggests that the tRNA locus might modify the chromatin structure at the 7SL RNA gene and render the region accessible for binding transcription factors. The second possibility is that the transcription factor TFIIB, that normally binds to the region located 45–50 nt upstream to the tRNA gene, may interact with a 7SL RNA transcription factor that is placed over the transcription start site of the 7SL RNA gene. Alternatively, the A and B boxes of the companion tRNA may have a dual function, they may serve as promoter elements for the tRNA itself while controlling the expression of 7SL RNA. However, it is difficult to rationalize this possibility, because the binding of transcription factors to tRNA genes occurs in an orientation-dependent manner, whereas the tRNA and the 7SL RNA are transcribed divergently (28). Our finding that only the level of the $tRNA^{Arg}$ is greatly amplified in cell lines carrying the two genes on a multi-copy plasmid may suggest that the transcription rate in the two directions is different, making the latter possibility unlikely. However, we cannot rule out the possibility that the cellular level of 7SL RNA is mostly regulated post-transcriptionally.

The repression phenomenon observed in this study can be further utilized to examine the structure–function relationship of the trypanosomatid 7SL RNA. Since the level of wild-type 7SL RNA was undetectable in cells carrying 7SL RNA mutated in domains II and IV, it may suggest that trypanosomes, like yeast (29), utilize an alternative protein translocation pathway.

ACKNOWLEDGEMENTS

This work was supported by research grant from the Cemach and Anna Oiserman Research fund and from the Leo and Julia Forchheimer Center for Molecular Genetics of the Weizmann Institute. We wish to thank Ora Asher for excellent technical assistance.

REFERENCES

1 Walter,P. and Johnson,A.E. (1994) *Annu. Rev. Cell. Biol.*, **10**, 87–119.
2 Walter,P. and Blobel,G. (1982) *Nature*, **299**, 691–698.
3 Siegel,V. and Walter,P. (1988) *Trends Biochem. Sci.*, **13**, 314–316.
4 Althoff,S., Selinger,D. and Wise,J.A. (1994) *Nucleic Acids Res.*, **22**, 1933–1947.
5 Poritz,M.A., Strub,K. and Walter,P. (1988) *Cell*, **55**, 4–6.
6 Michaeli,S., Podell,D., Agabian,N. and Ullu,E. (1992) *Mol. Biochem. Parasitol.*, **51**, 55–64.
7 Brennwald,P.J., Siegel,V., Walter,P. and Wise,J.A. (1991) *Nucleic Acids Res.*, **19**, 1942.
8 Poritz,M.A., Siegel,V., Hansen,W. and Walter,P. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4315–4319.
9 Siegel,V. and Walter,P. (1988) *Cell*, **52**, 39–49.
10 Liao,X., Selinger,D., Athoff,S., Chiang,A., Hamilton,D., Ma,M. and Wise,J.A. (1992) *Nucleic Acids Res.*, **20**, 1607–1615.
11 Ullu,E. and Weiner,A.M. (1985) *Nature*, **318**, 371–374.
12 Kleinert,H., Gladen,A., Geisler,M. and Benecke,B.J. (1988) *J. Biol. Chem.*, **263**, 11511–11515.
13 Bredow,S., Kleinert,H. and Benecke,B.J. (1990) *Gene*, **86**, 217–225.
14 Nakaar,V., Dare,A.O., Hong.D., Ullu,E. and Tschudi,C. (1994) *Mol. Cell. Biol.*, **14**, 6736–6742.
15 Béjà,O., Ullu,E. and Michaeli,S. (1993) *Mol. Biochem. Parasitol.*, **57**, 223–230.
16 Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
17 Goldring,A., Karchi,M. and Michaeli,S. (1995) *Exp. Parasitol.*, **80**, 333–338.
18 Michaeli,S., Roberts,T.G., Watkins,K.P. and Agabian,N. (1990) *J. Biol. Chem.*, **265**, 10582–10588.
19 Goldring,A., Zimmer,Y., Ben-Yehuda,E., Goncharov,I. and Michaeli,S. (1996) *Exp. Parasitol.*, **84**, 28–41.
20 Kunkel,T.A. (1985) *Proc. Natl. Acad. Sci USA*, **82**, 488–492.
21 Larsen,N. and Zwieb,C. (1990) *Nucleic Acids Res.*, **19**, 209–215.
22 Andreazzoli,M. and Gerbi,S.A. (1991) *EMBO J.*, **10**, 767–777.
23 Shi,X., Tom-Chen,D.H. and Suyama,Y. (1994) *Mol. Biochem. Parasitol.*, **65**, 23–37.
24 Sogin,M.L., Elwood,H.J. and Gunderson,J.H. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 1383–1387.
25 Strub,K. and Walter,P. (1990) *Mol. Cell. Biol.*, **10**, 777–784.
26 Mangin,M., Ares,M.J. and Weiner,A.M. (1985) *Science*, **229**, 272–275.
27 Brown,J.D., Hann,B.C., Medzihradszky,K.F., Niwa,M., Burlingame,A.L. and Walter,P. (1994). *EMBO J.*, **13**, 4390–4400.
28 Nakaar,V., Ullu,E. and Tschudi,C. (1994) *Parasitology Today*, **11**, 225–228.
29 Hann,B.C. and Walter,P. (1991) *Cell*, **67**, 131–144.