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# Interaction of small ribosomal and transfer RNAs with a protein from *Leishmania donovani*

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## ABSTRACT

**Using synthetic antisense RNA from the 5′-untranslated region of the β-tubulin gene as probe in gel retardation assays, a heat stable RNA-binding factor was identified in promastigotes of the kinetoplastid protozoan *Leishmania donovani*. The same or similar factors interact with several small ribosomal RNA (srRNA) species and, more weakly, with tRNA, as shown by binding and competition experiments. Deletion analysis indicated involvement of repeated purine-rich motifs on the antisense RNA, in the reaction. Related, conserved motifs occur on at least two of the srRNAs. By a modified Western blot assay, the RNA-binding species was identified as a single, small polypeptide. The activity is apparently specific for the promastigote stage of the parasite, being undetectable in amastigotes. The properties of this RNA-binding factor suggest that it is a novel, previously uncharacterised protein.**

## INTRODUCTION

The kinetoplastid protozoan *Leishmania* is characterised by a dimorphic life-cycle consisting of an intracellular stage (amastigotes) in the mammalian host and a flagellated stage (promastigotes) in the gut of sandfly vectors (1). During differentiation of one stage to the other, the synthesis of a number of proteins is regulated. For example, tubulin synthesis increases dramatically within a few hours of amastigote differentiation *in vitro* (2,3). Induction of β-tubulin synthesis in *L.mexicana* (4,5) and *L.donovani* (3) was previously shown to be regulated at the post-transcriptional level. Superimposed upon this specific genetic regulation are a number of global changes in amastigote metabolism, e.g., cellular and mitochondrial enlargement (6–8), increased respiration rate (9), and enhanced overall rates of protein synthesis (3). Regulation of translation may occur through the interaction of protein or nucleoprotein factors with the translation machinery, but whether the effect is specific or global would depend on the nature of the target (specific mRNAs, all mRNAs, ribosomes, translation factors, etc.) for the controlling factor.

*Leishmania* and other kinetoplastid organisms have a number of unique features in their translation machinery which set them apart from other eukaryotes. Cytoplasmic mRNAs contain a universal 5′-spliced leader sequence (reviewed in ref. 10) with a hypermodified cap structure (11). Secondly, the 28S rRNA is split into a number of fragments by processing; two large species, 28Sα and 28Sβ, of 1.9 and 1.8 kb, and several small ribosomal RNAs (srRNAs) including 5.8S RNA, and 220 nt, 180 nt, 140 nt, and 70 nt species (12–15). The srRNAs are apparently associated with polysomes (12), and the 180 nt and 140 nt species have predicted secondary structure resembling parts of domain VII of 28S rRNA from other eukaryotes (16). The reason for this elaborate 'ribosome-in-parts' is unknown. A third remarkable property is the apparent lack of mitochondrially-encoded tRNA genes (17) and the sharing of tRNAs between the mitochondrial and cytoplasmic compartments (17,18), leading to the hypothesis that mitochondrial tRNAs are imported from the cytoplasm. It is clear that studies of these small RNAs in *Leishmania* will lead to a better understanding of the unique aspects of translation regulation.

In this report we describe the identification and partial characterization of a factor in *Leishmania* promastigotes which binds with fidelity to a number of srRNAs as well as tRNA. The activity is detectable in the promastigote but not in the amastigote stage of the parasite.

## MATERIALS AND METHODS

### Leishmania strains

Promastigotes of *L.donovani* strains UR6 and AG83 were cultured on blood-agar media (19) and medium 199 (GIBCO) with 10% fetal calf serum, respectively. Strain AG83 was also passaged in BALB/c mice; amastigotes were isolated from infected spleens or livers by Percoll gradient centrifugation (3).

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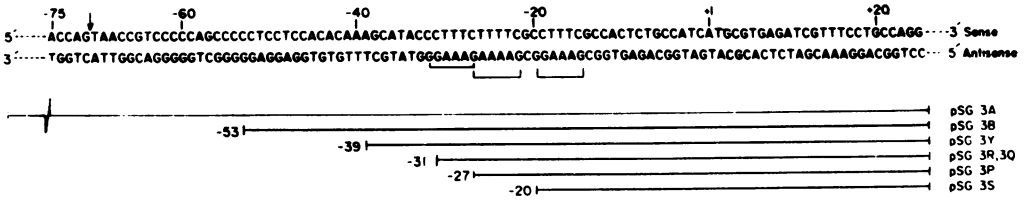


**Figure 1.** Nucleotide sequence of the 5'-upstream region of the *L.donovani* β-tubulin gene (EMBL data Library accession number X 51821). The N-terminal ATG starts at position +1. The arrow between nucleotides −70 and −71 designates the 3'-acceptor site for trans-splicing of the universal mini-exon (30). Below the sequence, the leftward end-points of various deletion clones are indicated (except pSG3A, which maps to −104); all inserts terminate at +25. Bracketed sequences on the antisense strand denote hexapurine repeats apparently involved in factor-binding (see text).

## DNA clones

All DNA clones were derived from subclone βT1-J (S.D and S.A., unpublished) containing the first 25 bp of the *L.donovani* β-tubulin gene and about 240 bp of 5'-upstream sequence. Unidirectional deletions from the 5'-upstream end were constructed using exonuclease III or nuclease BAL31, followed by linker-ligation and subcloning into polylinker regions of the dual transcriptional vectors pGEM3Zf(+) (Promega) and pSPT19 (Boehringer−Mannheim), according to standard procedures (20). DNA sequences of the inserts were determined by the dideoxy chain termination method (21).

## Preparation of RNA probes

$^{32}$P-labelled sense or antisense probes were synthesized from the deletion clones as template using the appropriate phage RNA polymerase. EcoRI- or HindIII-linearized DNA (0.5 μg) was incubated in 10 μl of 40 mM Tris−HCl, pH 7.5, 6 mM $MgAc_2$, 10 mM DTT, 1 mM spermidine, 0.5 mM each of ATP, GTP and CTP, 10 μM UTP including $10^4-10^5$ cpm/pmol of [α-$^{32}$P] UTP (Amersham or BARC, Bombay), 100 units/ml pancreatic RNase inhibitor and 500 units/ml of RNA polymerase, for 1 h at 37°. RNA was recovered by ethanol precipitation from 2.5 M $NH_4Ac$ at −20°. For competition assays, low-specific activity RNA was prepared identically except that input radioactivity was reduced 10-fold and UTP concentration raised to 0.5 mM.

Labelling of tRNA and small ribosomal RNAs was performed by dephosphorylation of total or poly A$^-$ RNA from promastigotes with calf intestinal phosphatase, followed by T4 polynucleotide kinase reaction with [γ-$^{32}$P] ATP (BARC), as described (20). Individual RNA species were separated on a denaturing gel for subsequent elution and ethanol precipitation.

Radiolabelled nuclear run-on RNA was prepared using crude promastigote nuclei as previously described (3).

## Preparation of soluble extracts

*Leishmania* promastigotes or Percoll-purified amastigotes were washed with phosphate-buffered saline at 4° and lysed with 5 cell volumes of 50 mM Tris−HCl, pH 7.5, 0.3 M KCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 20% glycerol and 0.1% Triton X-100 for 5 min on ice. Nuclei, kinetoplasts and cell-debris were removed by low-speed centrifugation to yeild the cytosol fraction, which was further centrifuged at 100,000×g for 1 h. The post-ribosomal supernatant (S-100) was dialysed against 20 mM Tris−HCl, pH 7.5, 0.1 M KCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT and 0.5 mM PMSF for 4−6 h at 4° and stored in aliquots at −70°.

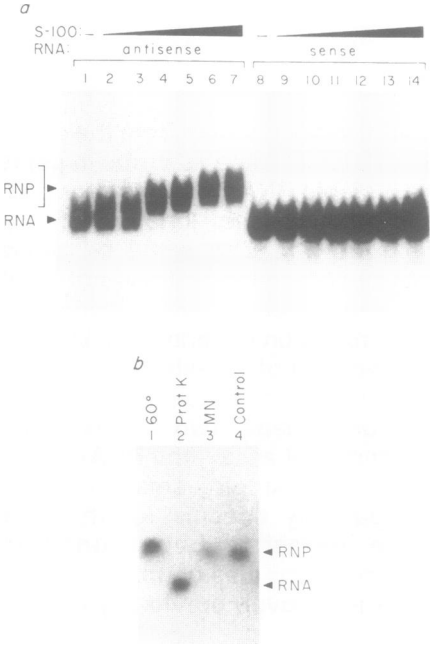


**Figure 2.** Binding of a heat-stable protein factor(s) to the *Leishmania* β-tubulin antisense RNA. **a,** $^{32}$P-labelled clone pSG3B sense or antisense RNA (2 fmol) was incubated with the following amounts (as total protein) of a *L.donovani* strain UR6 promastigote S-100 extract: lanes 1,8, none; lanes 2,9, 0.017 μg; lanes 3,10, 0.035 μg; lanes 4,11, 0.175 μg; lanes 5,12, 0.35 μg; lanes 6,13, 1.75 μg; lanes 7,14, 3.5 μg. **b,** $^{32}$P-labelled pSG3B antisense RNA was incubated with an S-100 extract (1.75 μg protein) that has been pre-treated as follows: lane 1, heat-treated (60°, 5 min) followed by centrifugal clarification; lane 2, proteinase K (2.5 mg/ml); lane 3, micrococcal nuclease (100 units/ml, 15 min at room temperature) plus $Ca^{2+}$ (1 mM), followed by EGTA (2 mM); lane 4, EGTA added before $Ca^{2+}$ and micrococcal nuclease. Heparin-resistant complexes were assayed by gel retardation as described in Materials and Methods.

## Gel retardation assay

Binding assays (10 μl) contained binding buffer (10 mM Tris−HCl, pH 7.5, 5 mM $MgAc_2$, 2 mM DTT), $^{32}$P-labelled RNA and cytosol or S-100 extract as indicated. After incubation for 30 min at 0°, heparin was added to 5 mg/ml and incubation continued for a further 10 min. Samples were electrophoresed on a native 5% polyacrylamide gel (acrylamide:bis-acrylamide = 60:1) in 50 mM Tris−borate, 1 mM EDTA, pH 8.3 at 8 V/cm for 2−3 h before autoradiography.

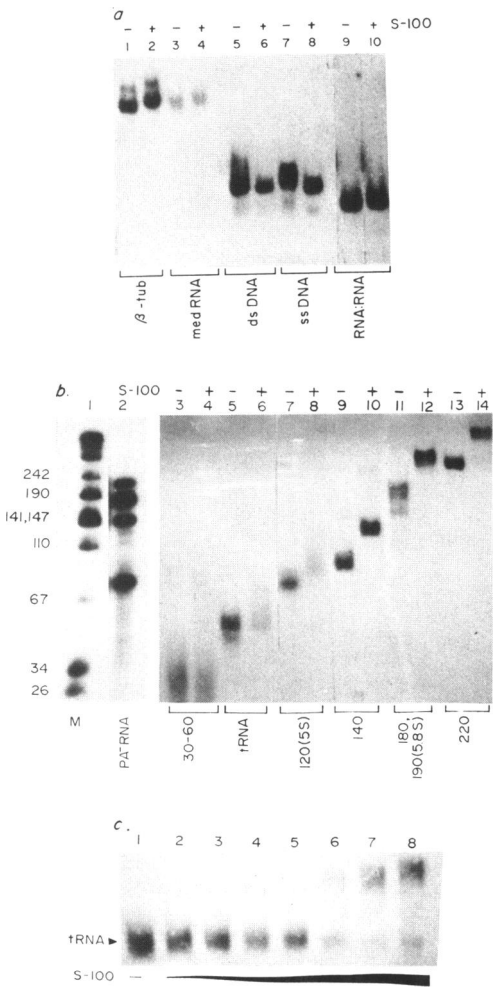


**Figure 3.** Binding of *Leishmania* factor(s) to ribosomal RNAs and tRNA. **a,** the following RNAs and DNAs were incubated without (−) or with (+) a promastigote S-100 extract: lanes 1,2, sense-strand RNA from clone βT-H (S.D. and S.A., unpublished) containing C-terminal and 3′-downstream regions of the *L.donovani* β-tubulin gene; lanes 3,4, sense-strand RNA from a *L.donovani* med RNA clone (30); lanes 5,6, linear double-stranded pSG3B DNA, 3′-end labelled on the antisense strand; lanes 7,8, the same DNA, heat-denatured; lanes 9,10, pSG3B sense−antisense RNA hybrid. **b,** poly A⁻ RNA from promastigotes was 5′-$^{32}$P labelled and electrophoresed on a denaturing 6% polyacrylamide gel (lane 2) alongside molecular weight markers (lane 1); individual bands/regions were excised, the RNAs eluted and 7000 cpm were incubated with (+) or without (−) a 60°-treated S-100 extract (3 μg). Lanes 3,4, 30−60 nt RNA; lanes 5,6, tRNA; lanes 7,8, 120 nt (5S) RNA; lanes 9,10, 140 nt srRNA; lanes 11,12, a mixture of 180 nt srRNA and 190 nt (5.8S) srRNA; and lanes 13,14, 220 nt srRNA. **c,** $^{32}$P-labelled tRNA (8000 cpm) was incubated with increasing amounts of a 60°-treated S-100 extract: lane 1, none; lanes 2−8, 0.1, 0.2, 0.4, 0.6, 1, 2 and 4 μg of extract.

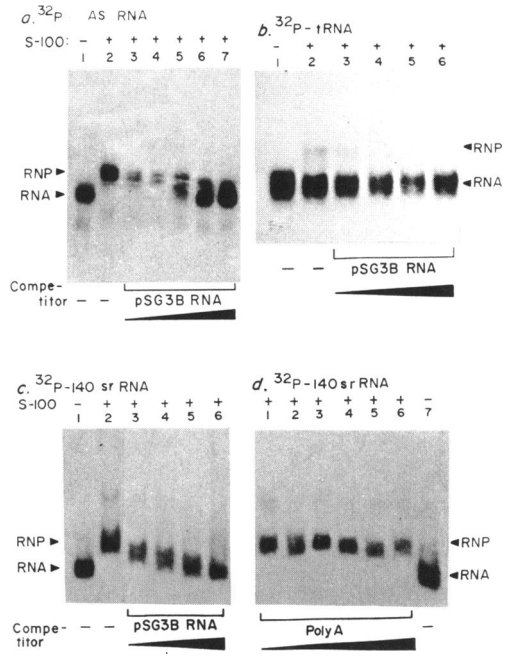


**Figure 4.** Competition assays. **a,** $^{32}$P-labelled pSG3B antisense RNA (2 fmol) was incubated with 0.175 μg of heated S-100 extract in presence of 0, 20, 20, 60, 100 and 200 fmol (lanes 2−7) of low-specific activity pSG3B antisense RNA; **b,** $^{32}$P-tRNA (8000 cpm) incubated with 0.75 μg of heat-treated S-100 extract in presence of 3, 15, 30, and 150 ng (lanes 3−6) of pSG3B antisense RNA; **c, d,** $^{32}$P-140 nt srRNA (8000 cpm) was incubated with 1.5 μg S-100 extract in presence of 10, 20, 50, and 100 ng of pSG3B antisense RNA (lanes 3−6 of panel **c**) or 10, 20, 50, 100 and 500 ng of poly A (lanes 1−6, panel **d**). Activity was assayed by gel retardation as before.

### Northwestern blotting

Total proten of cytosolic or S-100 extracts was electrophoresed on an SDS-12.5% polyacrylamide gel (22) and electro-blotted on nitrocellulose (23) for 18 h at room temperature. The filter was washed 4 times with 0.1 M Tris−HCl, pH 7.5, 0.1% NP40, each for 30 min at 4° with gentle agitation, then blocked with binding buffer (see previous section) containing 5% BSA and 0.01% Triton X-100 for 5 min. The blocked filter was incubated with $10^5-10^6$ cpm/ml of $^{32}$P-labelled RNA probe in binding buffer containing 0.01% Triton X-100 for 18 h at 4° with gentle agitation. The filter was washed 3 times for 5 min each with binding buffer, then autoradiographed.

## RESULTS

### Binding of synthetic β-tubulin antisense RNA with a protein factor(s) in *Leishmania* extracts

Post-ribosomal supernatants from detergent-lysed *Leishmania* promastigotes were incubated with $^{32}$P-labelled transcripts of the *Leishmania donovani* β-tubulin 5′-upstream region (Fig. 1), and then assayed by the gel retardation technique. No RNA−protein complexes with the plus strand were observed under the conditions of the assay (Fig. 2A). However, in a control experiment with the corresponding antisense strand, stable, specific heparin-resistant RNA−protein complexes were detected (Fig. 2a). Titration of extract resulted in progressive retardation of the complex, to a saturation level (Fig 2a). This 'step-profile' is characteristic of binding of other RNA ligands (see below), and indicates interaction of multiple molecules of factor with each molecule of RNA.

Figure 2b shows that (1) the factor is heat-stable (up to about 60°), (2) activity is sensitive to protease, and (3) there is apparently no micrococcal nuclease-sensitive component. Thus the active component is a heat-stable protein. Re-extraction and electrophoresis of the complexed RNA demonstrated that gel-retardation was not due to an irreversible covalent modification

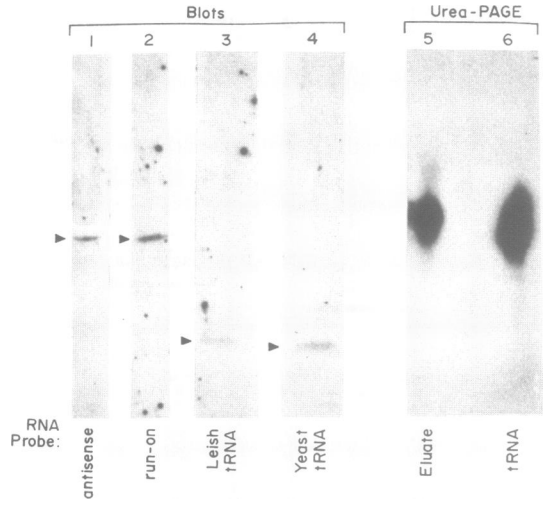


**Figure 5.** Northwestern blot analysis of RNA-binding factor. Western blots of heated S-100 extracts (60 μg protein) were incubated with the following $^{32}$P-labelled RNA probes, as described in Materials and Methods: lane 1, pSG3B antisense RNA; lane 2, nuclear RNA; lane 3, *L.donovani* tRNA; lane 4, yeast tRNA. Arrowheads show the 15-kDa band (lanes 1, 2 and 5, 6 were from different gels (12% and 10% polyacrylamide, respectively; thus the band has migrated differently). RNA in the band in lane 2 was eluted, electrophoresed on a denaturing 5% acrylamide gel and autoradiographed; lane 5, eluted RNA; lane 6, yeast tRNA marker.

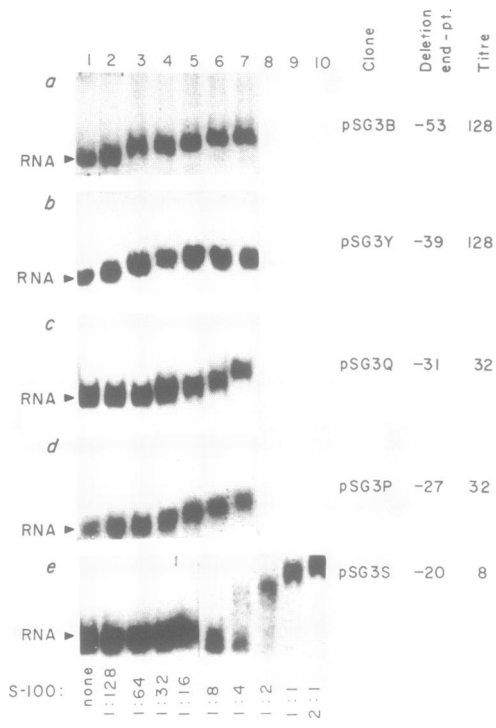


**Figure 6.** Factor-binding activity of antisense RNA deletion clones: Gel retardation assay. $^{32}$P-labelled antisense RNA (2 fmol) from clones pSG3B (panel **a**), pSG3Y (panel **b**), pSG3Q (panel **c**), pSG3P (panel **d**) and pSG3S (panel **e**) was incubated with the following dilutions of a 60°-treated promastigote extract (2 mg/ml original protein concentration; same batch of extract used in all cases): none, 1 μl each of 128, 64, 32, 16, 8, 4, and 2-fold dilutions, and 1 and 2 μl of undiluted extract (lanes 1–10 respectively). For each clone, the deletion-endpoint and titre, i.e., the lowest dilution at which no band-retardation was observed, are indicated on the right.

mediated by a protein factor. Complex formation occured in presence of $K^+$ concentrations of up to 1 M, and was not dependent on $Mg^{2+}$ (data not shown).

## Specific binding of tRNA and small ribosomal RNAs

To check sequence-specificity of binding, a number of labelled RNAs and DNAs were incubated with a heated S-100 extract. Synthetic RNAs from the β-tubulin C-terminal region and a cloned med RNA gene did not form detectable complexes (Fig.3a). Double-stranded or denatured DNA, or sense–antisense RNA hybrids, were similarly unaffected (Fig.3a). Thus the binding factor is specific for single-stranded RNA.

Next, total or poly $A^-$ RNA from *Leishmania* promastigotes was 5′-$^{32}$P-labelled with polynucleotide kinase and the small labelled RNA species isolated after polyacrylamide gel electrophoresis. Several abundant small RNA species were labelled by this protocol (Fig. 3b), including tRNA, 5S RNA and the 220, 180, 140, and 70 nt small ribosomal RNAs (srRNAs) previously identified in kinetoplastid organisms (12–16). When incubated with S-100 extracts, all RNAs, with the exception of RNA in the 30–60 nt region, showed complex formation (Fig. 3b). With tRNA or 5S RNA, a smear rather than a specific band was observed at this extract concentration. Extract titration experiments with labelled tRNA (Fig. 3c) and the 140 nt srRNA (not shown) revealed the same 'step-profile' seen with antisense RNA (Fig. 2). Yeast tRNA showed similar binding activity (data not shown). It is to be noted, however, that detection of these complexes requires higher extract concentrations than the antisense RNA reaction (compare Figs. 2 and 3) and that while all batches of extract exhibit antisense binding activity, some do not demonstrably bind tRNA. This argues for either (1) a lower affinity of the same factor for srRNAs compared to antisense RNA, or (2) different factors binding to the different RNAs.

## Competition assays to determine binding specificity of different RNAs

To determine whether the same, similar or different factors binds to antisense RNA and other RNAs, competition assays were performed. Binding to labelled antisense RNA, tRNA and the 140 nt srRNA were effectively competed by excess low-specific activity antisense RNA (Fig. 4a, b and c), but not by poly A (Fig. 4d), poly U, poly C or an antisense DNA oligonucleotide spanning positions −22 to −38 (see Fig. 1) of the β-tubulin 5′-upstream region (data not shown). These results indicate that the same or similar factors interacts with all three types of RNA, and that the binding is sequence-specific (otherwise the synthetic polynucleotide would compete). However, tRNA was a poor competitor of antisense RNA binding (data not shown), probably reflecting the much lower affinity of tRNA–protein interaction.

## Binding of labelled RNAs to factors immobilised on nitrocellulose: the 'Northwestern' blot

To obtain information on the subunit composition of the RNA-binding factor, an S-100 extract from *Leishmania* promastigotes was electrophoresed on an SDS-polyacrylamide gel, and

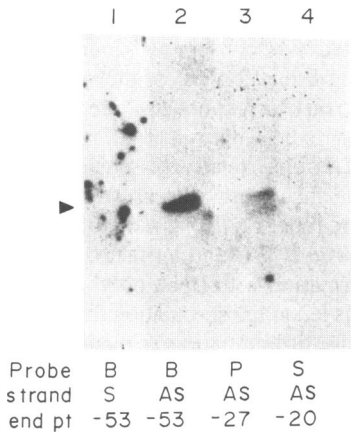


**Figure 7.** Deletion analysis of antisense RNA: Northwestern blot. Western blot strips of S-100 extract (50 μg) were incubated with $10^6$ cpm/ml of the following $^{32}$P-labelled RNAs: lane 1, clone pSG3B sense(S); lane 2, clone pSG3B antisense (AS); lane 3, clone pSG3P antisense; lane 4, clone pSG3S antisense. The arrowhead shows the 15.1-kDa band. The apparent doublet in lane 3 is a gel artifact.

'Western' blotted to nitrocellulose. After sequential renaturing and blocking steps, the filter was incubated with $^{32}$P-labelled RNA probe. Following washing and autoradiography, a single band of molecular weight approximately 15 kDa was observed with the β-tubulin antisense RNA probe (Fig. 5). In control experiments, (1) no band was observed with the corresponding sense strand as probe (Fig. 7), and (2) the binding activity was resistant to prior heat-treatment of the extract. Labelled tRNA was bound to an identical-sized band (Fig. 5). These results are consistent with the hypothesis that β-tubulin antisense RNA, and tRNA (as well as, possibly, the srRNAs) recognize similar or identical protein species.

One application of the Northwestern technique is the selection of specific RNA ligands from a heterogenous RNA probe. We searched for a physiologically relevant RNA ligand by using $^{32}$P-labelled promastigote nuclear run-on RNA to probe a Western blot. Again, the 15-kDa band was observed (Fig. 5). RNA bound to the filter was eluted, concentrated and run on a denaturing polyacrylamide gel; a broad band was observed which roughly comigrated with tRNA (Fig. 5).

Extracts from rat liver and *E.coli*, when similarly blotted, did not react with β-tubulin antisense RNA (data not shown). The activity therefore appears to be cell-specific or stage-specific (see below).

### Deletion analysis of the factor-binding site in the β-tubulin 5′-upstream antisense region

Unidirectional deletions were constructed within the β-tubulin 5′-upstream region (Fig.1). Titration of extract showed that all the deletions mapping between −53 and −20 bound factor, but apparently with different affinities (Fig. 6). A clone mapping to about +1 was inactive (data not shown). To compare activities of different clones, serial dilutions of extract were incubated with RNA and the end-point or titre, i.e., lowest dilution at which no complex is observed, determined. It was reasoned that the higher the end-point for a particular clone, the greater would be the affinity of RNA−protein complex formation.

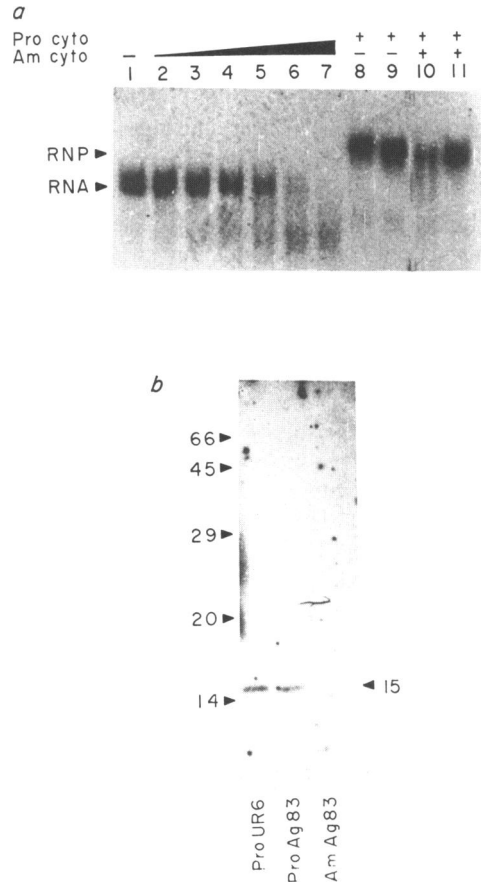


**Figure 8.** RNA binding activity in different stages of the *L.donovani* life-cycle. **a,** Gel retardation assay. $^{32}$P-labelled pSG3A antisense RNA (2 fmol) was incubated with heat-treated cytosolic extract as follows: lane 1, none; lanes 2−7, 0.034, 0.068, 0.136, 0.272, 0.544 and 1.09 μg of protein, respectively, of amastigote extract; lanes 8, 9, 0.5 and 0.125 μg of promastigote extract; lane 10, 0.25 μg of promastigote plus 0.27 μg of amastigote extract; lane 11, 0.25 μg of promastigote plus 0.068 μg of amastigote extract. **b,** Northwestern blot. Total protein (65 μg) of an S-100 extract from *L.donovani* strain UR6 promastigotes (lane 1), cytosol from *L.donovani* strain AG83 promastigotes (lane 2) and cytosol from *L.donovani* strain AG83 amastigotes (lane 3) was Western blotted and probed with pSG3B antisense RNA probe ($10^6$ cpm/ml). Sizes (in kDa) of protein standards are indicated on the left.

Figure 6 shows that, as nucleotides were progressively deleted from −53 to −20, there was a stepwise loss of activity reflected by lower titres. Deletion of 26 nucleotides (between −53 to −27) reduced the titre about 4-fold. Between −27 and −20, there was a further 4−8 fold drop in titre (Fig. 6).

The order of affinities pSG3B (mapping to −53) > pSG3P (−27) > pSG3S (−20) was confirmed by two other experiments. 1) Cross-competition assays showed that pSG3B RNA was more effective in competing with labelled pSG3P RNA than with itself (data not shown). 2) Western blot strips of S-100 extracts were incubated with equivalent amounts of pSG3B, pSG3P and pSG3S RNA. A strong 15.1-kDa band was observed with pSG3B, a weaker band with pSG3P and no band with pSG3S (Fig. 7).

Examination of the antisense sequence in this region revealed the presence of repeated purine-rich motifs (Fig. 1). Between −13 and −27 there are two overlapping repeats of GGCGAAA A/G G. A part of this motif, GAAAGG, is repeated a third time

between −27 to −32. Clones mapping at −53, −31 and −20 contain, respectively, 3, 2 and 1 intact copies of the hexapurine motif, roughly correlating with their relative activities.

### RNA binding activity at different stages of the *Leishmania* life cycle

The above experiments were performed with extracts from the flagellated, promastigote form of the parasite. To assess the role, if any, of the factor in differentiation, cytosolic extracts from purified amastigotes (the intracellular form present in mammalian hosts) were assayed for RNA binding activity by gel retardation. Titration of amastigote extract failed to show activity (Fig. 8a). Mixing experiments with active promastigote extracts showed that this lack of activity was not due to presence of proteases or inhibitors in the amastigote extracts (Fig. 8a).

One problem with the solution assay is the presence of nuclease activity in the amastigote preparations (leading to partial probe degradation; Fig. 8a). This problem could be circumvented by the blot assay, since SDS-PAGE would presumably separate nucleases from the factor. As shown in Fig. 8b, the 15-kDa band was observed using promastigote but not amastigote extracts.

## DISCUSSION

A combination of gel retardation assays, competition experiments and blotting techniques was used to demonstrate a heat-stable factor(s) in *Leishmania* promastigotes that specifically interacts with a population of functionally related small RNAs: the srRNAs and tRNA. Specificity was judged by a number of criteria: (1) formation of discrete complexes resistant to heparin, DNA, synthetic polynucleotides and high salt concentrations; (2) absence of detectable binding to single- or double-stranded DNA, double-stranded RNA or other single-stranded RNAs. Therefore in terms of specificity the factor lies somewhere between non-specific histones, single-strand binding proteins, etc., and the highly specific translational regulators which interact with single mRNAs.

The role of specific regions of the β-tubulin antisense RNA in the binding reaction was investigated by deletion mutagenesis. The apparent affinity of binding was reduced as the purine-rich motifs CGAAAGG, CGAAAAG and AAAGGG were progressively deleted (Figs. 1, 6 and 7). Thus, these and similar motifs may constitute part or whole of the binding site. The proximity of these motifs to each other (Fig. 1) suggests cooperativity in the antisense RNA-factor interaction.

Competition experiments indicated that the antisense RNA, srRNAs and tRNA share recognition motifs for the *Leishmania* factor (Fig. 4). Hence, a computer-aided search of the highly conserved 180 nt, 140 nt and 70 nt srRNA sequences of trypanosomes (16) and crithidia (24) for the above purine-rich motifs were conducted. Two observations were made: (1) The homologous sequences GAAGGG, CGAGAGG and CGAAGGG occur at positions 11, 53 and 96 respectively of the 180 nt srRNA. The 140 nt srRNA contains the motif CGAGAG at position 18. The 70 nt srRNA does not contain a similar motif. (2) All of these motifs lie within hairpin-loop regions of the predicted secondary structures (16). The CGAGAGG motif on the 180 nt srRNA is part of a highly conserved sequence on 28S rRNA in other eukaryotes that acts as a cleavage site for α-sarcin (25) and constitutes part of the ribosomal A-site. The sequence of *Leishmania* 5S RNA (which also binds factor, Fig.3) has not been published, but a survey of 5S RNA sequences from other sources revealed the conserved sequence YGGGAA at the junction of helix E and loop d′ of the secondary structure (26). The binding site on tRNA is not easily recognizable at the primary sequence level, since only a few residues are universally conserved (27). The relatively purine-rich D-loop is one possibility. The factor may discern a conserved secondary or tertiary structure motif on tRNA rather than a primary sequence.

The *Leishmania* RNA binding factor was originally detected by complex formation with RNA polymerase-generated *in vitro* transcripts. This led to the speculation that regulation of β-tubulin synthesis during differentiation is mediated by antisense RNA and its cognate factor. Such a possibility was rendered unlikely by three observations: (1) No antisense RNA derived from the β-tubulin 5′-untranslated region was detectable by RNase mapping of total cellular RNA (M.Bhaumik and S.A., unpublished data). (2) Transcription of tubulin genes in *Leishmania* (28) and *Crithidia* (29) is strand-asymmetric. (3) During amastigote-to-promastigote differentiation tubulin synthesis is induced earlier (at 1−4 h; ref.3) than the RNA binding activity (at about 24 h; data not shown). It may be argued, therefore, that the antisense RNA fortuitously possesses sequences that act as strong binding sites for the factor, and that the true physiological ligand is some other cellular RNA, such as tRNA or srRNA. Binding-selection of nuclear RNA showed that the RNA ligand(s) is indeed present *in vivo* (Fig.5). The identity of this RNA, which migrates in the tRNA region of the gel, remains unknown pending sequence analysis.

Although β-tubulin antisense RNA is a non-physiological ligand, it targets a single protein species in *Leishmania* (Fig.5), and is capable of competing with srRNAs and other ligands for binding to the factor (Fig.4). These facts may be exploited to determine the location and function of the RNA binding protein by *in vivo* and *in vitro* targetting experiments.

## ACKNOWLEDGEMENTS

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