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Mini-exon derived RNA gene of *Leishmania donovani*: structure, organization and expression

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Abstract. Mini-exon derived RNA is a small nuclear RNA of trypanosomatid protozoa such as *Leishmania* which donates its 5'-terminal 39 nucleotides to the 5'-ends of cellular messenger RNAs by trans-splicing. We have cloned a mini-exon derived RNA gene from *Leishmania donovani* and studied its organization and expression. About 200 copies of the gene per haploid genome are organized as a tandem repeat on a single chromosome. The gene is transcribed as a 95-nucleotide RNA. The first 39 nucleotides of mini-exon derived RNA is also found at the 5'-terminus of a cellular mRNA (β-tubulin), thus confirming its identity. Sequence analysis of the gene and its flanking regions showed that while classical RNA polymerase II promoter elements such as TATA and CAAT are absent from the 5'-upstream region, intragenic sequence motifs resembling RNA polymerase III promoter elements are present. The implications of this finding for mini-exon derived RNA expression are discussed.

Keywords. Mini-exon derived RNA; *Leishmania donovani*; cloning; organization; promoter elements.

1. Introduction

Mini-exon derived RNA (med RNA) is a small nuclear RNA molecule, complexed as a ribonucleoprotein particle, in protozoal parasites of the kinetoplastid family such as *Leishmania.* This RNA participates in *trans*-splicing reactions with cellular mRNA precursors, donating its 5'-terminal 39 nucleotides as a mini-exon (or spliced leader) to the 5'-termini of all mRNAs (reviewed by van der Ploeg 1986).

The precise function of the mini-exon sequence is unknown, but in view of its universal occurrence among kinetoplastids, it is obviously metabolically important. Med RNA genes have been cloned and sequenced from a number of kinetoplastid species (DeLange *et al* 1984; Milhausen *et al* 1984; Nelson *et al* 1984; Miller *et al* 1986; Muhich *et al* 1987) but the mechanism of transcription of the gene remains controversial. Evidence favouring RNA polymerase II mediated transcription has been presented (Laird *et al* 1985). However, on the basis of nuclear transcription experiments, it has been proposed that RNA polymerase III is involved (Grondal *et al* 1989).

To resolve these issues, we have isolated a med RNA gene from *Leishmania donovani,* studied the organization and expression of the gene, and carried out detailed sequence analyses of the gene and its flanking regions. Our results favour the polymerase III mechanism by virtue of the presence of polymerase III promoter-like elements in the med RNA intragenic region.

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2. Materials and methods

2.1 *Parasite cultivation*

L donovani strain UR6 (WHO nomenclature MHOM/IN/1978/UR6) promastigotes were cultured on blood-agar media (Ray 1932) containing 3·7% brain heart infusion (Difco), 1% glucose, 1·5% agar, 100 units/ml penicillin, 100 μg/ml streptomycin and 1% whole rabbit blood, for 48-72 h at 25°C.

2.2 *Preparation of parasite DNA and RNA*

Genomic DNA was isolated by SDS/proteinase K digestion, and total promastigote RNA by the guanidine isothiocyanate-hot phenol method as described (Maniatis *et al* 1982).

2.3 *Synthetic oligonucleotides*

Several oligonucleotides from the conserved region of the med RNA gene (figure 1) were synthesized in a Du Pont automated DNA synthesizer. Crude oligonucleotides were purified by 15% denaturing Polyacrylamide gel electrophoresis (PAGE), visualization of the major band by ultra-violet shadowing, followed by excision of the gel band, elution by the crush-and-soak method (Maxam and Gilbert 1977) and ethanol precipitation. Oligonucleotides were [^{32}P] labelled at 5'-termini using [γ -^{32} P] ATP and T4 polynucleotide kinase (Maniatis *et al* 1982).

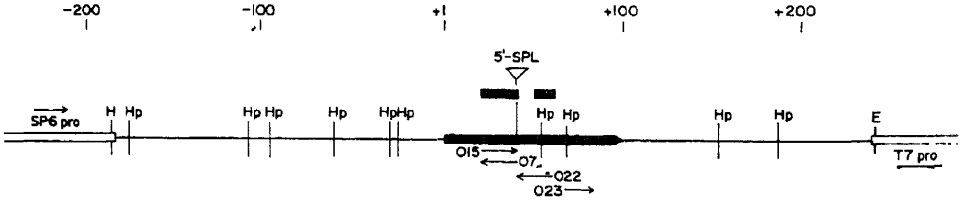

Figure 1. Physical map of med RNA clone pME1.

The 440 bp genomic insert containing the med RNA gene and 5'- and 3'-flanking sequences is shown. pGEM 3Zf(+) vector sequences flanking the insert are depicted as open boxes with orientation of SP6 and T7 RNA polymerase promoters as indicated. H=HindIII site, E = EcoRI site, Hp = HpaII site. The med RNA gene is depicted as a thick arrow. Under the gene the positions of various synthetic oligonucleotides used for DNA sequencing are shown. The hatched box above the gene represents conserved sequences (see text). The 5'-splice site for *trans*-splicing is designated as 5'-SPL. Numbers above the map indicate coordinates (in bp) with respect to the med RNA transcription initiation site (position + 1) .

2.4 *Uniform labelling of DNA probes*

The insert in plasmid pME1 was isolated by electrophoresis of HindIII- EcoRI double digest followed by electroelution and purification. This was labelled with [α $^{-32}$ P] TTP using the random priming method (Feinberg and Vogelstein 1983). Labelled DNA

(about 10^7-10^8 cpm/μg) was recovered by ethanol precipitation in the presence of 2·5 M ammonium acetate.

2.5 *Preparation of* [^{32} *P*] *labelled RNA probe*

Minus strand RNA probe was obtained by transcription of Hind III-linearized pME1 DNA with T7 RNA polymerase in the presence of [α-^{32} P] UTP. Reactions (10 μl) contained 40 mM Tris-HCl, pH 7·5, 6mM Mg Ac_2, lmM spermidine, 10 mM DTT, 0·5 mM ATP, 0·5 mM CTP, 0·5 mM GTP, 10 μM UTP including 10–20 μCi of [α-^{32} P] UTP (Bhabha Atomic Research Centre, Bombay, 3000 Ci/mmol), 20 units of placental RNase inhibitor, 0·5 μg DNA and 5 units T7 RNA polymerase. After incubation for 1 h at 37°C [^{32}P] labelled RNA was recovered by ethanol precipitation.

2.6 *Cloning of the med RNA gene*

Genomic DNA from *L. donovani* strain UR6 was digested with Haelll and the fragments were resolved by gel electrophoresis. Blunt-ended DNA fragments in the size range 400-500 bp were eluted from the gel and ligated to an equimolar mixture of [^{32}P]labelled Hind III and EcoRI linkers. Ligation products were digested to completion with EcoRI and HindIII enzymes and passed through a Sepharose CL-4B column (Pharmacia) to separate excess linkers. DNA was then ligated to EcoRI-Hindlll double-digested vector fragment of plasmid pUC 19 and used to transform *Escherichia coli* by the method of Hanahan (1983). All DNA manipulations were as described (Maniatis *et al* 1982). Clones were screened by colony hybridization using [^{32}P]labelled synthetic oligonucleotide O–7 (figure 1) spanning the universally conserved region of the med RNA gene. Hybridization was performed in 6X NET (IX NET: 0·15 M NaCl, 15 mM Tris-HCl, pH 8·0, 1 mM EDTA), 0·1% SDS, 5X Denhardt's solution, 20 mM sodium phosphate, pH 7·0, 200 μg/ml herring sperm DNA and $1{\cdot}6 \times 10^7$ cpm probe for 5 h at 42°C. Filters were washed 5 times with 6X SSC, 0·1% SDS (1X SSC: 0·15 M NaCl, 15 mM sodium citrate) at room temperature for 5 min, then with the same buffer at 42°C for 1–2 min before autoradiography. One positive clone was identified. The 440 bp insert from this clone was transferred to plasmid vector pGEM 3Z f(+) (Promega) to yield plasmid pME1.

2.7 *Southern blot analysis*

Blots of restriction digests of *L. donovani* genomic DNA on nylon membranes were prepared as previously described (Das and Adhya 1990). Filters were pre-hybridized overnight at 42°C in 50% deionized formamide, 5X SSC, 5X Denhardt solution, 20 mM sodium phosphate, pH 7·0, 0·1% SDS and 200 μg/ml herring sperm DNA. Hybridization was performed in the same solution additionally containing [^{32}P] labelled, denatured pME1 insert (figure 1) at 10^5-10^6 cpm/ml for 48 h at 42°C. Blots were washed employing a stringency of 0·lX SSC, 0·1% SDS at room temperature as described (Das and Adhya 1990) and autoradiographed.

2.8 *Copy number determination*

Quantitative dot blot hybridization of *L. donovani* genomic DNA with cloned med RNA gene probe was performed to determine gene copy number. Details of the procedure have been previously described (Das and Adhya 1990). Dots of 0·125 8µg *L donovani* (strain UR6) DNA and control dots of 0·81–52ng of plasmid pME1 were prepared on a Zeta probe (Bio Rad) membrane and hybridized with [^{32}P] labelled pME1 insert probe as above. After autoradiography, spots were densitometrically scanned to determine relative signal intensities. By interpolation of unknown points (genomic DNA) from the plasmid Standard curve, the copy number could be computed.

2.9 *Northern blotting*

Total RNA from *L. donovani* strain UR6 promastigotes was denatured in 1 M glyoxal as described (Adhya *et al* 1990) and electrophoresed on a 5% acrylamide–8 M urea gel at 200 V for 3 h. RNA was then electrophoretically transferred to a Zeta probe membrane in a Trans Blot apparatus (Bio Rad) under the following conditions: running buffer 0·25 M Tris-borate, 2·5 mM EDTA, at 350 mA for 4 h at 4°C. The filter was then rinsed with 0·1 M Tris borate, 1 mM EDTA dried and baked *in vacuo* at 80°C for 30 min. Hybridization of the filter with [^{32}P] labelled pME1 insert was as previously described (Adhya *et al* 1990).

2.10 *RNase mapping*

[^{32}P] labelled antisense pME1 probe RNA (10,000 cpm) was hybridized for 18 h at 45°C with total *L. donovani* promastigote RNA or control tRNA (10μg) in 30 μl containing 80% formamide, 40 mM PIPES, pH 6·4, 0·4 M NaCl, 1 mM EDTA, Reactions were diluted into 0·3 ml of 0·3 M NaCl, 20 mM Tris-HCl, pH 7·5, 1 mM EDTA containing 10μg RNase A and 100 units RNase T1 and incubated at room temperature for 1h. RNase-resistant hybrids were recovered by sequential proteinase K treatment, phenol extraction and alcohol precipitation. Protected RNA was suspended in 80% formamide, 1 mM EDTA, 0·1% xylene cyanol, 0·1 % bromophenol blue, heated at 65°C for 5 min and electrophoresed in a 5% acrylamide—8 M urea gel at 200 V for 3 h, followed by fixing, drying and autoradiography.

2.11 *Primer extension sequencing of mRNA*

A 5'-[^{32}P]labelled synthetic oligonucleotide primer (5 pmol) complementary to nucleotides –21 to –38 of the β-tubulin 5'-untranslated region (S Das and S Adhya, unpublished) was annealed to 10 μg poly A$^+$ mRNA from *L. donovani* promastigotes in 15 μl of 50 mM KCl, 50 mM Tris-HCl, pH 8·3, 5 mM $MgCl_2$ 10 mM DTT, by heating to 65°C for 3 min followed by room temperature incubation for 45-60 min. Annealed primer-template (3 μl) was distributed into separate reactions containing ddATP, ddGTP, ddCTP and ddTTP respectively (ratio of dideoxy to corresponding deoxytriphosphate = 40 μM: 200 μM) and

extended with 10 units AMV reverse transcriptase at 42°C for 1 h. Products were analysed by 8% acrylamide—8 M urea sequencing gel electrophoresis followed by autoradiography.

3. Results

3.1 *Copy number of the med RNA gene*

The number of copies of the med RNA gene per haploid genome of *L. donovani* was determined by a quantitative dot blot hybridization experiment (figure 2). Different

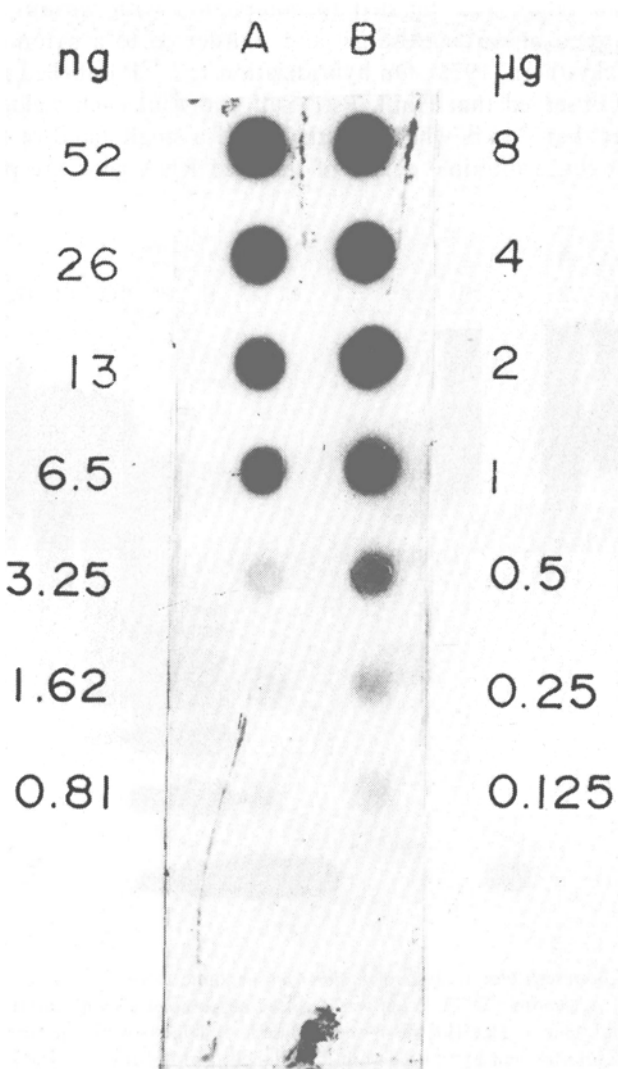

Figure 2. Copy number determination of med RNA gene.

Indicated amounts of plasmid pMEl DNA (**A**) or *L. donovani* genomic DNA (**B**) were applied as dots on a zeta probe membrane and hybridized with [^{32}P]labelled pME1 insert probe, as described in § 2.

known amounts of pME1 plasmid clone and parasite genomic DNA were applied as dots on a nylon membrane and hybridized with [^{32}P]labelled pMEl insert DNA probe. By densitometry, an equivalence of signal intensity was observed at 16·6 ng of pME1 DNA and 1 μg of genomic DNA (figure 2). Knowing the molecular weight of pME1 plasmid and the haploid genome content of *L. donovani* (Leon *et al* 1978), it was calculated that approximately 200 copies of the gene are present per haploid genome.

3.2 *Organization of med RNA genes*

Genomic *L· donovani* DNA was digested to completion with various restriction enzymes, electrophoresed on an agarose gel and transferred to a nylon membrane by the procedure of Southern (1975). On hybridization to [^{32}P]labelledinsert from clone pME1, it was observed that Hinfl, PstI, SalI and AluI each yielded a single large band of greater than 23 kb, whereas with HaeIII a single band of 450 bp was obtained (figure 3A). Since multiple copies of the med RNA gene are present (see

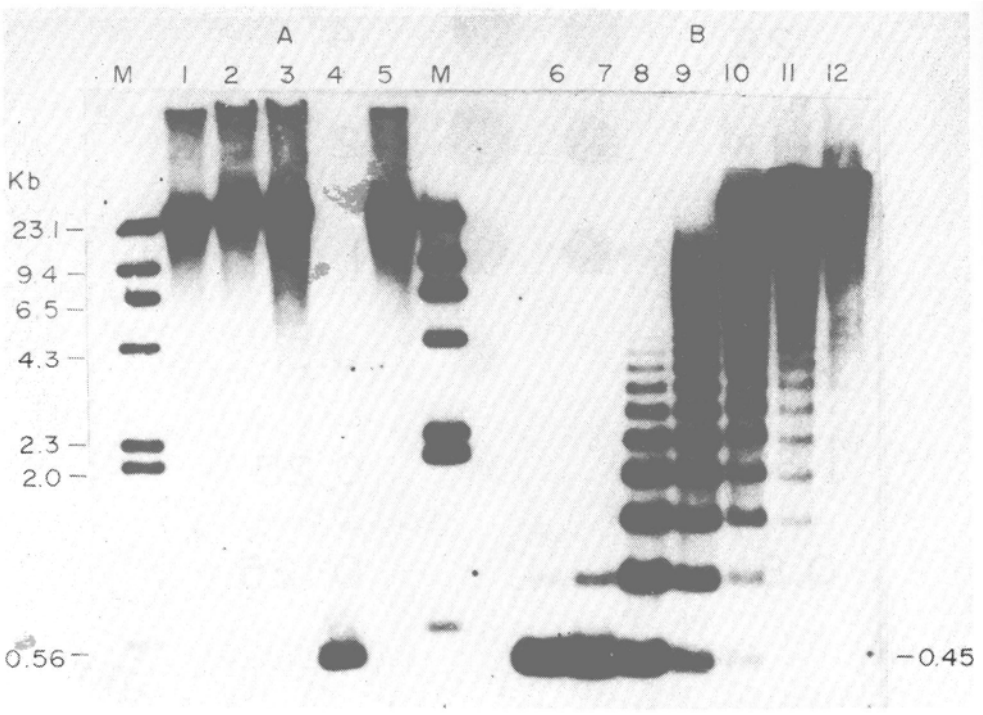

Figure 3. Southern blot analysis of *L. donovani* genomic DNA.

In panel A, genomic DNA (5μg) was digested to completion with Hinfl (lane 1), PstI (lane 2), AluI (lane 3), HaeIII (lane 4) and SalI (lane 5). Digests were electrophoresed on an agarose gel, blotted and hybridized with [^{32}P] labelled pME1 insert probe as described in §2. In panel B, genomic DNA (10μg) was partially digested for 1 h at 37°C with the following amounts of HaeIll: 10, 5, 2·5, 1·25, 0·625, 0·312 and 0·156 unit respectively (lanes 6–12). Electrophoresis and hybridization were carried out as in panel A. M, marker lanes. The sizes (in kb) of marker fragments are shown at the left. The position and size of the unit-length HaeIII repeat are depicted on the right.

above), this result indicates that the location of HaeIII sites in the vicinity of all the genes must be identical.

When genomic DNA was partially digested with varying amounts of HaeIII and probed with pMEI as before, a ladder of bands corresponding to multimers of 450 bp was observed (figure 3B). This indicates that multiple copies of the med RNA gene are arranged as a tandem repeat on a single *L. donovani* chromosome, since this arrangement would give rise to uniform spacing of any restriction site occurring once per repeat.

3.3 *Analysis of med RNA*

Expression of med RNA in *L. donovani* promastigotes was studied by Northern blot analysis. Total cellular RNA was electrophoresed on a Polyacrylamide gel, electrophoretically transferred to a nylon membrane and hybridized to [^{32}P] labelled pME1 insert probe. A single band about 100 n in size was observed (figure 4A).

To confirm this result, an RNase protection assay was performed. A negative-strand RNA probe was synthesized from HindIII-linearized pME1 (figure 1) by transcription with T7 RNA polymerase in the presence of [α-^{32}P] UTP. [^{32}P] labelled RNA probe was hybridized with total *L. donovani* RNA, and RNA:RNA hybrids were digested with a mixture of RNase A and RNase Tl. Protected RNA probe was analysed by gel electrophoresis. A major band of about 95 n was observed (figure 4B). This corresponds to the med RNA species observed by Northern blot analysis (figure 4A). A second band, about 76 n long was observed by RNase protection assay but not by Northern blot. This may be due to an artefact of the RNase assay arising from the presence of 4 U residues at position 76 of the med RNA (see figure 6), leading to "breathing" of the RNA: RNA duplex at this position with concomitant cleavage by the single-strand specific RNase. Alternative explanations such as unusual RNA structures revealed only by RNase protection analysis cannot be excluded at this point.

3.4 *Sequence of the 5'-mini-exon in a cellular mRNA*

The 5'-terminal 39 nucleotides of med RNA is transferred to all mRNAs of *Leishmania* and other kinetoplastid protozoa. We made use of this information to directly sequence the mini-exon (leader) region on a specific cellular mRNA. The gene for *L. donovani* β-tubulin was previously cloned and sequenced in this laboratory (Das and Adhya 1990). An oligonucleotide complementary to nucleotides +1 to +20 (relative to the ATG codon) was 5'-[^{32}P] labelled and annealed to poly A^+ mRNA from *L. donovani* promastigotes. Annealed primer was extended with reverse transcriptase in the presence of dideoxytriphosphates and the products analysed by sequencing gel electrophoresis (data not shown). It was observed that the mRNA sequence matched the β-tubulin DNA sequence up to – 70 (figure 5). Further upstream the mini-exon could be discerned, which aligned with part of the cloned med RNA gene (see below).

3.5 *DNA sequence analysis*

Plasmid pME1 was subjected to dideoxy chain termination sequencing using

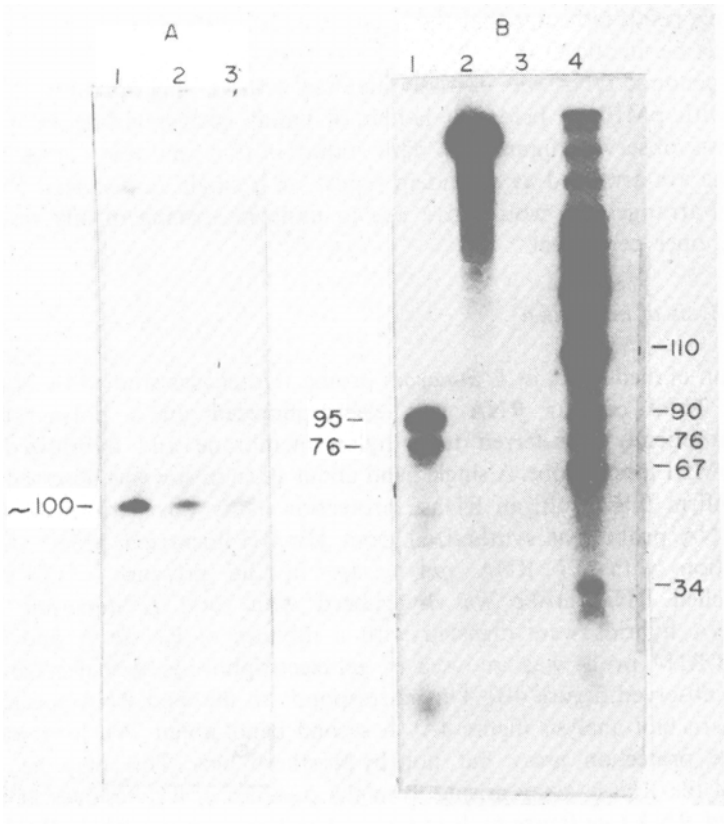

Figure4. Analysis of med RNA in *L. donovani* promastigotes.

Panel A, Northern blot hybridization. Total promastigote RNA was electrophoresed on a denaturing 5% acrylamide gel, electro-blotted on a nylon membrane and hybridized with [^{32}P]labelled pME1 insert. Lanes 1-3 contain 50, 25 and 10 μg of total RNA, respectively. The size of the med RNA transcript (in nucleotides) is shown at the left. Panel B, RNase protection assay. Total promastigote RNA (10 μg) was hybridized to [^{32}P] labelled minus-strand RNA probe, RNA: RNA hybrids were digested with RNases A and T1 and the protected fragments resolved by 5% acrylamide—7 M urea gel electrophoresis. Lane 1, 10 μg total promastigote RNA; lane 2, input probe RNA; lane 3, 10 μg tRNA in place of promastigote RNA; lane 4, [^{32}P] labelled single stranded DNA marker fragments. The sizes of the markers are indicated on the right, and those of the protected fragments on the left.

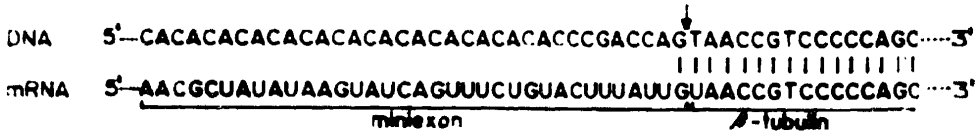

Figure 5. Sequence of *L donovani* β-tubulin mRNA obtained by primer extension with reverse transcriptase. The arrow shows position –70 at which the DNA and RNA sequences diverge. The 39-nucleotide mini-exon sequence (see figure 6) can be discerned further upstream on the mRNA but not on the DNA. The DNA and RNA sequences (plus strands) are aligned. Vertical lines show identity.

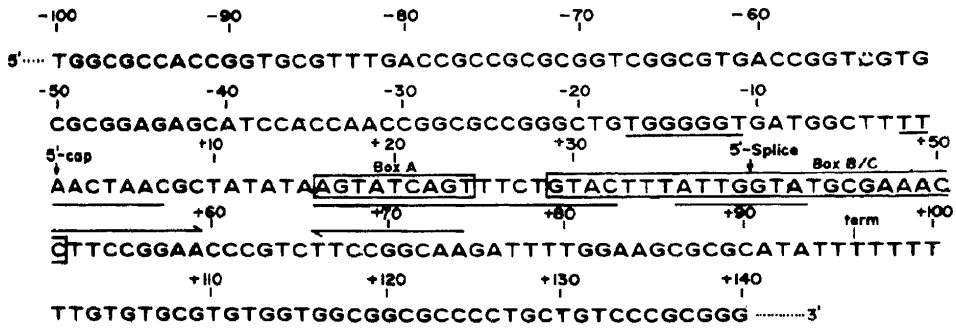

Figure 6. DNA sequence of the *L. donovani* med RNA gene and flanking regions.

The 96-nucleotide gene is located between the 5'-cap and 3'-termination sites, as indicated. Conserved sequence motifs among different kinetoplastid med RNA genes are underlined. Horizontal arrows show the position of a palindromic sequence. Putative RNA polymerase III promoter elements (box A and box B/C) are boxed. Box B is contained within the larger box C-like motif.

various oligonucleotide primers (shown in figure 1). The sequence of 240 bp including the gene and flanking sequences is depicted in figure 6. Assignment of the 5'-terminus at position +1 was made on the basis of (i) the RNA sequence of the mini-exon as determined by primer-extension sequencing of *β*-tubulin mRNA (see above) and (ii) the known sequence of the 5'-cap nucleotides in *Trypanosoma brucei* (Perry *et al* 1987). The 3'-terminus of the med RNA was assigned to position +96 on the basis of med RNA length (figure 4).

When the med RNA gene from *L. donovani* was compared to that from other trypanosomatid species (DeLange *et al* 1984; Milhausen *et al* 1984; Nelson *et al* 1984; Miller *et al* 1986; Muhich *et al* 1987) a number of conserved sequence blocks were noted (figure 6). The sequence TpyAACTAA is present at the transcription start site (positions -2 to +6). This is followed by a stretch of 7 A:T base pairs. A universally conserved block CAGTTTCTGTACT is present at positions +21 to + 33, followed 2 nucleotides later by the sequence ATTGGTA which includes the 5'-donor site for *trans-splicing*. A palindromic sequence between positions +51 and +75 was also observed. Palindromes were also found at similar positions on the med RNA genes from other kinetoplastid species (*Op. Cit*) however, the primary sequence is not conserved.

In contrast to the intragenic sequences, the 5'- and 3'-flanking regions from different kinetoplastid protozoa share little or no sequence homology. Known transcriptional control elements for RNA polymerase II, such as TATA box, CAAT box, etc., are absent from the appropriate upstream locations. The sequence PyG_5T between positions -11 and -17 (figure 6) is also present upstream of the *L. enriettii* (Miller *et al* 1986) and *Crithidia fasciculata* (Muhich *et al* 1987) med RNA genes but the significance of this sequence is unknown. The absence of specific RNA polymerase II promoter elements upstream of the med RNA gene led us to examine the intragenic regions for transcription control sequences. The only eukaryotic RNA polymerase known to utilize intragenic promoter elements (other than enhancers) is RNA polymerase III (Geiduschek and Tocchini-Valentini 1988). Genes for tRNA, 5S RNA and a number of viral RNAs contain sequence elements

Table 1. Comparison of kinetoplastid med RNA consensus sequence with A, B and C box consensus sequences for eukaryotic RNA polymerase III genes.

Gene	Box A	Box B	Box C
tRNA, VA RNA Consensus	RRYNNARYGG	GGTTCGANTCC	
5S RNA Consensus			GTACTTGGATGGGAGACC
L. donovani	AGTACAGTTT	GGTTCGANTCC	GTACTTGGATGCGAAACC
	^T^	^TG^	^TTTA^ ^T^
Kinetoplastid med RNA consensus	AGAACAGTTT	GGTAAGAARCT	GTACTTGGATGAGAARCT
	$\overline{T}$	^TG^	^TTTA^ ^T^ $\overline{C}$

Consensus box A, B and C sequences for RNA polymerase III genes from higher eukaryotes were taken from Geiduschek and Tocchini-Valentini 1988. The *L. donovani* and consensus med RNA sequences have been aligned for maximum homology (extra nucleotides are bracketed by carets). The kinetoplastid med RNA consensus was derived by comparison of published sequences of 6 different kinetoplastid species including *L. donovani* (see text for specific references).

known as box A, B and C (table 1). Box A is common to all genes; whereas box B is present in tRNA and viral RNA genes and box C is specific for 5S RNA genes. Examination of the *L. donovani* med RNA sequence revealed that a box A–like sequence is present at position + 16. Further downstream, starting at position + 29, a region of strong homology to the *Xenopus* 5S RNA internal control region (Sakonju *et al* 1980), including box C, was observed. Part of this region is also homologous to consensus box B of tRNA gene (table 1). These observations indicate that the med RNA gene promoter may be recognized by RNA polymerase III and accessory transcription factors. Box A and box B/C like elements were also found at similar positions on the med RNA genes from other kinetoplastid species.

4. Discussion

In this report we have examined the structure and organization of the med RNA gene in *L donovani* promastigotes in order to understand the mechanisms involved in transcription of the gene and involvement of the med RNA in *trans-splicing*.

About 200 copies of this gene are present as a tandem repeat (figures 2 and 3). The high copy number is presumably a reflection of the necessity of high level synthesis of med RNA which is required in stoichiometric amounts for *trans-splicing*. The genes are transcribed as a single RNA species about 96 nucleotides in length (figure 4) of which the 5'-terminal 39 nucleotides are donated as a leader to cellular mRNAs by *trans-splicing*.

Sequencing of the cloned gene and its neighbouring sequences (figure 6) revealed a number of intriguing features. The gene is embedded in G: C rich spacer DNA, initiates at a conserved octanucleotide sequence (AACTAACG) and terminates as a stretch of T residues. Among various kinetoplastid species the med RNA sequence is highly conserved but little-or no homology was observed in the 5'- and 3'-flanking regions. Promoter elements for RNA polymerase II such as TATA and CAAT boxes are noticeably absent from the 5'-flanking region. Thus it is likely that the control sequences are intragenic, a characteristic of RNA polymerase III genes (Geiduschek and Tocchini-Valentini 1988). Indeed, examination of the med RNA

gene sequence revealed elements closely resembling box A and box B/C of RNA polymerase III promoters (table 1) . The extended homology with the internal control region (including box C) of Xenopus 5S RNA genes is particularly striking. In higher eukaryotes, boxes A and B interact with RNA polymerase III transcription factor TF IIIC, whereas box C interacts with TF IIIA (Geiduschek and Tocchini-Valentini 1988). It remains to be seen whether similar factors from *Leishmania* bind to these sequence elements within the med RNA gene.

Other properties of the med RNA gene support the contention that it may be transcribed by RNA polymerase III. Like other RNA polymerase III transcripts such as tRNA, 5S RNA, U6 small nuclear RNA, med RNA is a small molecule encoded by multiple genes and, as part of a ribonucleoprotein particle, performs an essential housekeeping function in RNA metabolism, *i.e.*, *trans*-splicing. In common with other RNA polymerase III genes, the transcripts terminate within a stretch of T residues. Recent experiments in *T. brucei* (Grondal *et al* 1989) show that med RNA transcription in isolated nuclei resembles that of 5S RNA with respect to divalent cation requirement and sensitivity to inhibitors. It will be important to develop a cell-free transcription system for RNA polymerase III in *L. donovani* in order to test these possibilities and elucidate the role of the control elements in transcription of the med RNA gene.

Acknowledgements

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