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Transcription and processing of *ß*-tubulin messenger RNA in *Leishmania donovani* promastigotes

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Abstract Transcription of the multicopy *ß*-tubulin locus in *Leishmania donovani* promastigotes was examined by nucleic acid hybridization techniques. By northern analysis of promastigote RNA multiple *ß*-tubulin mRNAs were detected. The major species of 2·2 kb RNA is derived from the tandem repeat cluster of *ß*-tubulin genes, the other two (2·4 and 2·6 kb) are presumably derived from dispersed genomic loci. Combined S1-nuclease and primer extension mapping experiments demonstrated the presence of a single 5'-terminus with a 35 nucleotide spliced-leader sequence. The 3'-termini are heterogeneous. The development of a nuclear run-on system in *Leishmania* for studying transcription of individual genes is reported. Active but transient RNA polymerase II activity was observed in this system. Using specific DNA probes for labelled run-on RNA it was shown that *ß*-tubulin transcription occurs asymmetrically (*i.e.,* on one strand of the DNA template) in an *α*-amanitin sensitive manner. The significance of these results for the life cycle of the parasite is discussed.

Keywords. *Leishmania*; *ß*-tubulin mRNA; hybridization; nuclear transcription.

Introduction

Leishmania donovani is a kinetoplastid parasite protozoan that causes the disease known as kala-azar or visceral leishmaniasis in man. This vector-transmitted parasite has a dimorphic life cycle consisting of a free living flagellated promastigote stage within the gut of sandfly vectors, and a mammalian form, the amastigote, that resides within phagolysosomal vesicles of host macrophages (Zuckerman and Lainson, 1979).

Tubulins are major structural proteins of *Leishmania* promastigotes. During differentiation (transformation) of amastigotes to promastigotes, there is significant induction of tubulin biosynthesis in response to greater demands imposed by flagellar synthesis (Fong and Chang, 1981). The mechanism of this induction is not known. Both transcriptional (Landfear and Wirth, 1984) and post-transcriptional mechanisms (Wallach *et al.,* 1982; Fong *et al.*, 1984) have been implicated in other *Leishmania* species. Similarly, the promoter for tubulin mRNA transcription, and the biosynthesis of tubulin mRNA *via* RNA processing, are not well understood.

In this report, we characterize the *ß*-tubulin mRNAs of *L. donovani* promastigotes in terms of number, size and 5'- and 3'-termini. We also report for the first time the development of a nuclear run-on transcription system in *Leishmania* for studying features of *ß*-tubulin transcription.

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Materials and methods

Parasite cultivation

L. donovani strain UR6 (WHO nomendature-MHOM/IN/1978/UR6). Promastigotes were cultivated in modified Ray's (1932) agar medium containing 3·7% brain-heart infusion (Difco), 1% glucose, 1·5% agar, 10,000 units/ml penicillin, 10,000 μg/ml streptomycin and 1 % whole rabbit blood. Incubation was at 25°C for 48-72 h.

Preparation of parasite DNA and RNA

Total high molecular weight promastigote DNA was prepared by lysis with sodium dodecyl sulphate (SDS) and proteinase K, followed by phenol extraction, dialysis, RNase treatment and ethanol precipitation. Total RNA was prepared by the guanidium isothiocyanate-hot phenol method and mRNA selected by oligo-dT cellulose chromatography (Maniatis *et al.*, 1982).

DNA probes

Clone *β*T-1 contains a 3·3 kb *Hind*III-*Sal*I genomic fragment of the *L. donovani β*-tubulin tandem repeat (figure 1; Das and Adhya, 1990). Probe C is a 0·5 kb *Hind*III-*Xho*I fragment containing coding sequences of the *β*-tubulin gene, Probe UT is a *Pst*I fragment derived from the 3'-untranslated region of the gene. Probes were labelled by the random oligonucleotide priming method (Feinberg and Vogelstein 1983) using [α-^{32}P] TTP (3000 Ci/mmol, Bhabha Atomic Research Centre, Bombay). Probe A is a 240 bp *Pst*I fragment derived from the coding region of the gene (figure 1) cloned in both orientations in M13mp9 vector.

Northern blot hybridization

Total or polyadenylated RNA from *L. donovani* promastigotes was denatured as described by Thomas (1980) in 1 M deionized glyoxal, 50% dimethyl sulphoxide and 10 mM sodium phosphate, pH 7 at 50°C for 1 h. Denatured RNA was electrophoresed on a 1·2% agarose gel in 10 mM sodium phosphate, pH 7 at 70 V for 3–4h, then transferred by capillary diffusion to Zeta probe membrane (Bio Rad) using 10×SSC as transfer buffer. The filter was baked for 2h at 80°C under vacuum. Glyoxal adducts were removed from the RNA by pouring 20 mM Tris-HC1, pH 8, 1 mM EDTA at 95°-100°C on the filter and allowing to cool to room temperature. The membrane was pre-hybridized at 42°C for 18 h in 50% deionized formamide, 5 × SSC, 5 × Denhardt's solution (Maniatis *et al.*, 1982), 20 mM sodium phosphate, pH 7, 0·1% SDS and 200 μg/ml calf thymus or herring sperm DNA, Hybridization was performed in the same solution additionally containing denatured, oligo-labelled DNA probe (specific activity 10^7 cpm/μg, 10^5-10^6 cpm/ml) at 42°C for 48 h. The filter was then washed twice with 2× SSC, 0·1 % SDS, once with 0·5 × SSC, 0·1 % SDS and once with 0·1× SSC, 0·1 % SDS, each wash being for 15 min at room temperature with vigorous agitation. The filter was autoradiographed. To rehybridize the filter with a second probe, filter-bound [^{32}P] was stripped by

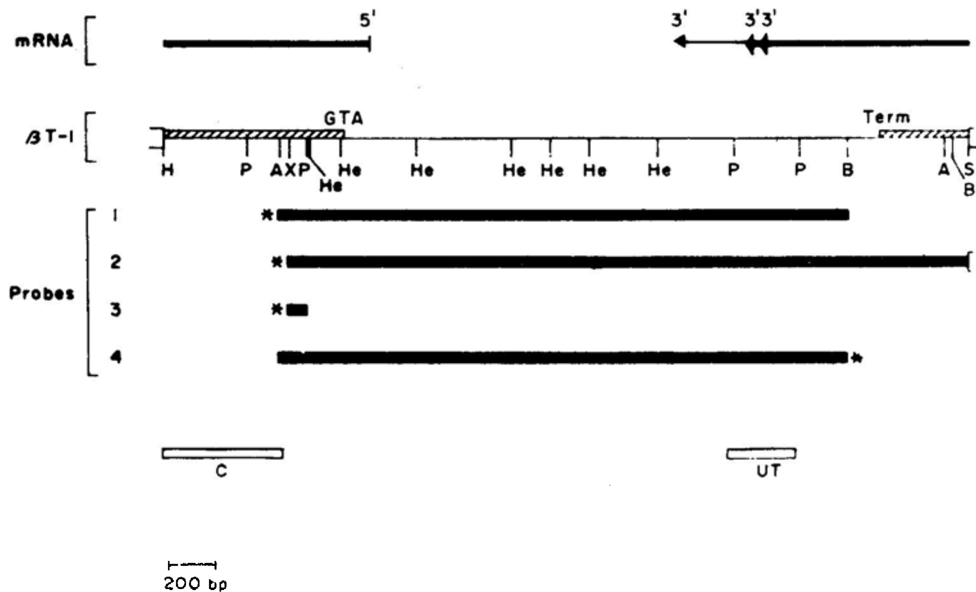

Figure 1. Restriction and transcription maps of the *β*-tubulin locus in *L. donovani* promastigotes. Locations of restriction sites on the genomic clone *β*T-1 are shown (H, *Hind*III; P, *Pst*I; A, *Aval*; X, *Xhol*; He, *Hae*III; B, *Bam*HI; S, *Sal*I). Square brackets flanking the restriction map represent pUC8 vector sequences. The mRNA map is shown above the restriction map; thick and thin lines represent major and minor RNA species, respectively, revealed by S1 mapping. DNA probes 1-4 used for S1 mapping and primer extension are depicted as filled bars, with the labelled ends marked with asterisks. Open bars represent fragments from the coding (C) and 3'-untranslated region (UT) of the gene used as hybridization probes.

immersion in a 95°C water bath for 10 min. Pre-hybridization, hybridization and washing were then carried out as before.

S1 mapping

DNA probes used for mapping are shown in figure 1. Restriction fragments were 5'-labelled using [γ-^{32}P] ATP and T4 polynucleotide kinase as described (Maniatis *et al.*, 1982). To label the 3'-terrninus of the *Bam*HI end of probe 4 (figure 1), an end-filling reaction with Klenow fragment and dGTP and [*α*-^{32}P] dATP was performed (Maniatis *et al.*, 1982). Uniquely labelled fragments were obtained by digestion with a second restriction enzyme followed by gel purification of the appropriate bands. Labelled probe (10-15,000 cpm) was hybridized with 1-2 *μ*g polyadenylated RNA in 10 *μ*l buffer containing 80% deionized formamide, 0·4 M NaCl, 40 mM PIPES, pH 6·4 and 1 mM EDTA under paraffin oil. After heating at 68°C for 10 min to denature the probe, reactions were transferred to the annealing temperature of 55°C for 6-15 h. Reactions were quenched with 0·2 ml Sl buffer (0·3M NaCl, 30 mM sodium acetate, pH 4·5, 1 mM $ZnSO_4$) containing 218 units S1 nuclease (P L Biochemicals). Digestion was carried out for 30 min at 37°C, S1-

resistant products were purified by phenol-extraction and ethanol precipitation and analyzed by electrophoresis on a 5% acrylamide-8 M urea gel at 200 V for 2 h.

Primer extension

The 5'-[^{32}P] labelled *Xho*I/*Pst*I fragment (probe 3, figure 1) was hybridized to 1·9 μg polyadenylated RNA as described above for the S1 mapping experiment. The reaction was chilled on ice and nucleic acids were ethanol precipitated. The hybridized primer was then extended with MMLV reverse transcriptase (BRL) in a l0 μl reaction containing 50 mM Tris-HCl, pH 7·5, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 1 mM each of the 4 deoxyribonucleoside triphosphates and 200 units of enzyme. After incubating at 37°C for 1 h, nucleic acids were ethanol precipitated and analysed by denaturing acrylamide electrophoresis as above.

Preparation of crude nuclei

All Operations were carried out at 0°–4°C. *L. donovani* promastigotes were harvested from blood-agar slants and washed twice with ice-cold phosphate buffered saline (150 mM NaCl, 20 mM sodium phosphate, pH 7·2). Approximately 1 ml packed cells were suspended in 5 ml buffer A (10 mM Tris-HCl, pH 8,10 mM NaCl, 1·5 mM $MgCl_2$, 1 mM EGTA, 5 mM DTT, 0·1 mM EDTA, 0·1 mM PMSF and 100 units/ml placental RNase inhibitor) containing 0·1 % Triton X-100. Cells were placed on ice for 5 min, then homogenized in a Dounce homogenizer (15-20 strokes). After microscopic examination for cell lysis, the homogenate was centrifuged at 3300 *g* for 10 min in a Sorvall SS34 rotor. The crude nuclear pellet was washed once with buffer A (lacking Triton) and suspended in 4 ml nuclei storage buffer (50 mM Tris-HCl, pH 8, 1·5 mM $MgCl_2$, 0·1 mM EDTA, 5mM DTT, 25% glycerol and 100 units/ml RNase inhibitor) at a concentration of 2×10^9 nuclei/ml. Nuclei were stored frozen in small aliquots at – 70°C.

Nuclear transcription

Reaction mixtures contained 12·5 mM Tris-HCl pH 8, 50 mM NaCl, 5 mM $MgCl_2$, 10 mM creatine phosphate, 1 mM each of GTP and CTP, 4 mM ATP, 10 μM UTP, 1 mCi/ml [α-^{32}P] UTP (Amersham, 3,000 Ci/mmol) and 10^9 nuclei/ml. Reactions were incubated for 10 min at 25°C. To assess the effect of inhibitors, nuclei were pre-incubated with α-amanitin (20 μg/ml) or actinomycin D (200 Mg/ml) for 15 min on ice before dilution with an equal volume of 2 × transcription buffer. Transcription was terminated by adding SDS (1%) and proteinase K (100 μg/ml) and incubation at 25°C for 15 min. RNA was phenol-extracted and ethanol precipitated, Contaminating DNA was removed by treatment with RNase-free DNase I (Boehringer-Mannheim). Unincorporated [^{32}P] triphosphates were separated by chromatography on a Sephadex G-100 column followed by ethanol precipitation of the excluded RNA peak, or by two ethanol precipitations in the presence of 2·5 M ammonium acetate. This RNA was used for hybridization. Incorporation of [^{32}P] nucleotide into RNA was measured by spotting aliquots of reaction mixtures on DE81 filters (Whatman), washing 5 times with 0·5 M NaH_2PO_4, twice with H_20, once with ethanol, followed by drying and liquid scintillation counting.

Hybridization analysis of labelled nuclear RNA

[^{32}P] labelled nascent nuclear RNA was hybridized to slot of cloned plasmids (1-5 μg) on nitrocellulose or Zeta probe membranes. Filters were pre-hybridized in 3 × SSC, 5 × Denhardt's solution, 10 mM sodium phosphate, pH 7, 0·1 % SD S and l00 μg/ml tRNA for 16-18 h at 65°C. Hybridization was for 48 h at 65°C in the same buffer containing [^{32}P] nascent RNA (10^5-10^6 cpm/ml). After hybridization, filters were washed twice with 3 × SSC, 0·1 % SDS for 15 min at 50°C, then twice with 0·3 × SSC, 0·1 % SDS for 15 min at 50°C, and autoradiographed.

Results

Northern blot analysis of β-tubulin mRNA

Total or polyadenylated RNA from *Leishmania* promastigotes was electrophoresed under denaturing conditions in an agarose gel, transferred to a nylon membrane and hybridized with [^{32}P] labelled probe C (figure 1) derived from the coding region of the β-tubulin gene. As shown in figure 2, three specific β-tubulin RNA bands were observed: a major species of 2·2 kb length and two minor species of 2·4

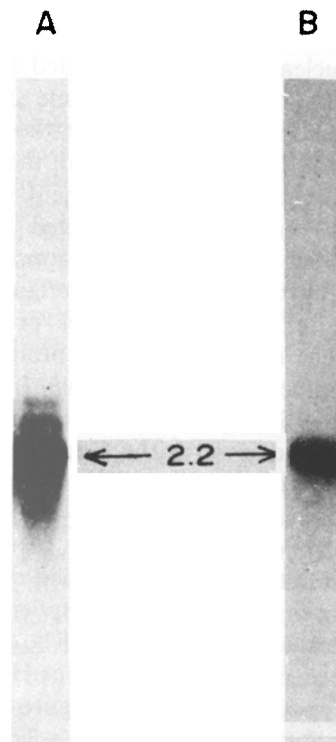

Figure 2. Northern blot hybridization of *L. donovani* promastigote RNA. Lane A, total RNA (10 μg) hybridized with coding probe C (figure 1). Lane B, the same filter was stripped of probe and re-hybridized with non-coding probe UT (figure 1). The major 2·2 kb species is indicated. The minor species are of 2·4 and 2·6 kb respectively.

and 2·6 kb respectively. All species are retained by an oligo-dT cellulose column, *i. e.*, they are poly A^+ RNAs (data not shown).

In a separate study (Das and Adhya, 1990) we have shown that there are at least 3 chromosomal loci for *β*-tubulin genes in *L. donovani.* Clone pLD *β*T-1 (figure 1) is derived from the major cluster of 12-15 genes. The observation of multiple *β*-tubulin mRNA species raises the question as to whether all are transcribed from the same chromosomal locus. To answer this question probe C was melted off the filter and filter-bound RNA re-hybridized with probe UT, which is derived from the 3'-untranslated region of the clone (figure 1). Only the major 2·2 kb species hybridized to this probe (figure 2). Since 3'-untranslated regions of the same genes at different chromosomal loci usually vary considerably in sequence, while coding regions are conserved, this result demonstrates that the 2·4 and 2·6 kb RNA species are probably transcribed from loci distinct from the major repeat cluster.

Mapping of steady state mRNA

To precisely delineate the 5’ and 3’ boundaries of *β*-tubulin mRNA on the genome, S 1 nuclease mapping and primer-extension experiments with reverse transcriptase were performed on poly A^+ promastigote RNA using end-labelled restriction fragments of *β*T-1 as probes or primers. When the 2·2 kb *Ava*I/*Bam*HI fragment, labelled at the *Ava*I-*end* (probe 1, figure 1) was hybridized to poly A^+ RNA and the hybrid digested with S1 nuclease, a single protected labelled DNA fragment 310 nucleotides (nt) long was obtained (figure 3A, lane 2). In the absence of added mRNA, there was no protection, confirming that the observed band was due to mRNA: probe hybrid and not due to reannealing of the probe (figure 3A, lane 1). A similar result was observed when the probe fragment was 5’-labelled at the *XhoI-end* (figure 1, probe 2); in this case the protected band was 270 nt in length (figure 3B, lane 1). From these observations it is apparent that (i) the direction of the transcription is from right to left as depicted in figure 1; and that (ii) a single 5'-terminus (or discontinuity, in case of splicing) is present in the mRNA at the position shown (figure 1). The nature of the discontinuity, if any, was investigated by a primer extension experiment using a 70 bp -*Xho*I/*Pst*I fragment, 5'-labelled at the same *Xho*I site as in the S 1 experiment (probe 3, figure 1), to hybridize with mRNA and subsequently extend towards the 5'-terminus of the RNA with reverse transcriptase. As shown in figure 3B, lane 2, a single primer extension product, 310 nt long was observed, which is larger than the corresponding S1-protected product by about 40 nt. It is therefore evident that an exon or leader sequence, not contiguously coded with the *β*-tubulin gene, is added on to the body of the mRNA by a transcriptional priming or RNA processing (splicing) event.

The 3'-termini of the steady state *β*-tubulin mRNA were mapped by using the 2·2 kb *Bam*HI/*Ava*I fragment, 3'-labelled at the *Bam*HI end (probe 4, figure 1), as probe for S1 nuclease mapping. As shown in figure 3C, lane 2, three protected species, 370, 440 and 740 nt long, were observed in a 3·6:9:1 ratio (as determined by densitometry of the bands). Since polyadenylated RNA was used in the experiment, these bands probably represent poly A-addition sites on the genome, mapping at the positions shown in figure 1. Thus multiple 3'-termini are present in promastigote *β*-tubulin mRNA.

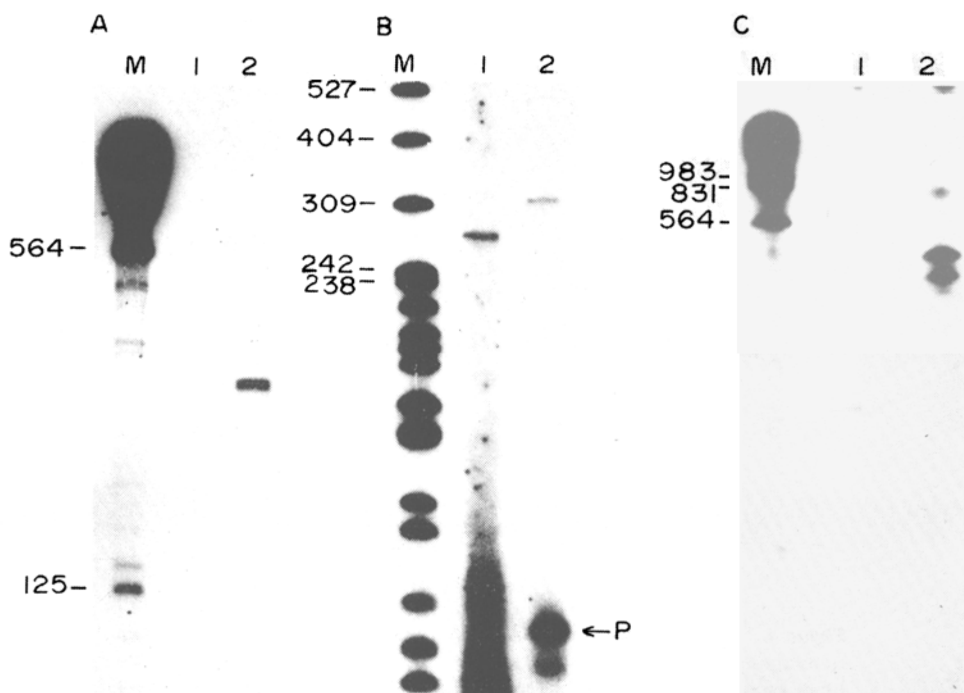

Figure 3. S1 nuclease and primer extension mapping of β-tubulin mRNA. Panel A: 5'-[^{32}P] labelled probe 1 (see figure 1) was hybridized to polyadenylated RNA (1·8 μg, lane 2) or no RNA (lane 1), then digested with S1 nuclease. Panel B: Lane 1, polyadenylated RNA (1·9 μg) was hybridized with 5'-[^{32}P] labelled probe 2 (see figure 1) and the hybrids were digested with S1 nuclease; lane 2, polyadenylated RNA (1·9 μg) was hybridized with 5'-[^{32}P] labelled probe 3 (see figure 1) and the primer was extended with reverse transcriptase. Panel C: 3'-[^{32}P] labelled probe 4 (see figure 1) was hybridized with polyadenylated RNA (1·8 μg, lane 2) or no RNA (lane 1) and the hybrids were digested with S1 nuclease. All reaction products were analyzed by 5% acrylamide-8 M urea gel electrophoresis. Lanes M, molecular weight markers, whose sizes (in nucleotides) are shown on the left of each panel.

Transcription of β-tubulin genes in isolated nuclei

The transcriptional status of specific chromosomal loci can be directly assessed by the nuclear run-on assay. This method is based on the fact that in isolated nuclei or chromatin, transcribing RNA polymerase remains bound to the DNA and to nascent RNA transcripts. Incubation of the nuclei or chromatin under appropriate conditions with radiolabelled ribonucleoside triphosphates results in the completion of the nascent chains (run-on) with incorporation of radioactivity. Labelled RNA can then be used as a probe to hybridize with specific cloned DNA fragments.

A crude preparation of promastigote nuclei was incubated with [^{32}P] labelled nucleoside triphosphates at 25°C. Rapid incorporation of [^{32}P] into RNA was observed (figure 4). After 10 min the reaction rate was reduced. If incorporation was stopped at 10 min by dilution of the label with an excess of unlabelled UTP, labelled RNA declined exponentially with a half life of about 39 min (figure 4). In

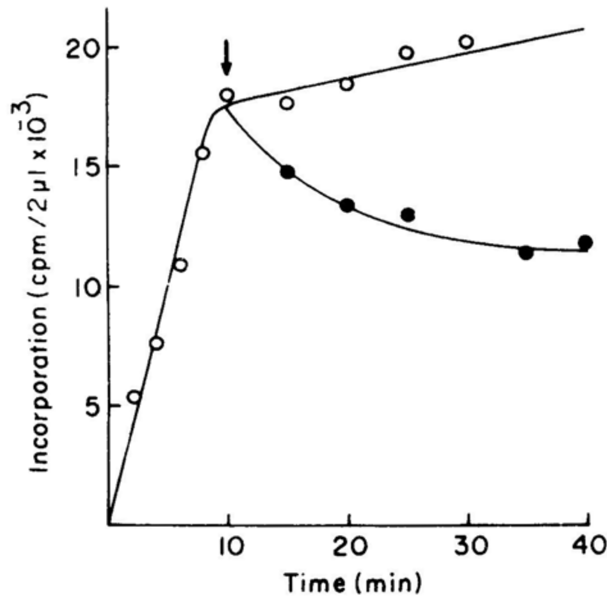

Figure 4. Kinetics of nascent RNA synthesis in isolated promastigote nuclei. A nuclear transcription (30 μl) containing 10 μM [α- ^{32}P] UTP was incubated at 25°C and at the times indicated 2 μl aliquots were withdrawn for incorporation assay by DE81 filter binding (O). To assess stability of the RNA synthesized, 1 mM unlabelled UTP was added to a parallel reaction at 10 min (arrow) and incorporation assessed at intervals thereafter (●).

other experiments (not shown) we have observed more rapid degradation of the RNA after a peak at 10 min. It is therefore essential to block cellular nucleases with appropriate amounts of RNase inhibitor during nuclear transcription. When the nuclei were pre-incubated with a low concentration (20 μg/ml) of α-amanitin, an inhibitor of RNA polymerase II, incorporation was reduced by more than 80% (table 1). Thus the majority of nascent RNA in this system is transcribed by RNA polymerase II.

Table 1.

Reaction	Time of incubation (min)	Incorporation cpm/μl	Net incorporation cpm	Inhibition (%)
Complete	0	2714	—	—
	5	12878	10,154	—
	10	16831	14,112	—
Complete + 10 μg/ml α-amanitin	0	1102	—	—
	5	2915	1813	82
	10	3073	1971	86

In the complete system, promastigote nuclei were incubated with rNTPs at 25°C in a vol of 20 μl. To assess effect of α-amanitin, nuclei were pre-incubated with 40 μg/ ml α-amanitin for 15 min at 0°C before addition of NTPs and incubation at 25 C. Aliquots (1 μl) were assayed for incorporation by DE81 filter binding.

Radiolabelled nuclear RNA was hybridized to specific DNA probes immobilized on nylon or nitrocellulose membranes. As shown in figure 5A, [^{32}P] nascent RNA hybridized specifically to *β*Tl-DNA (slot 1), and only background binding was observed with pUC8 (slot 2). Slots 3 and 4 contained complementary single strands of the 240 bp *Pst*I fragment from the N-terminal coding region of *β*T-l (figure 1) in M13. Hybridization occurred to only one strand (slot 3). Thus transcription at the *β*-tubulin locus is asymmetric. Specific transcription was sensitive to a low concentration (10 *μ*g/ml) of α-amanitin (figure 5B, slot 2) and to l00 *μ*g/ml actinomycin D (figure 5B, slot 3). This indicates that DNA dependent RNA polymerase II mediates transcription of *β*-tubulin genes in this system.

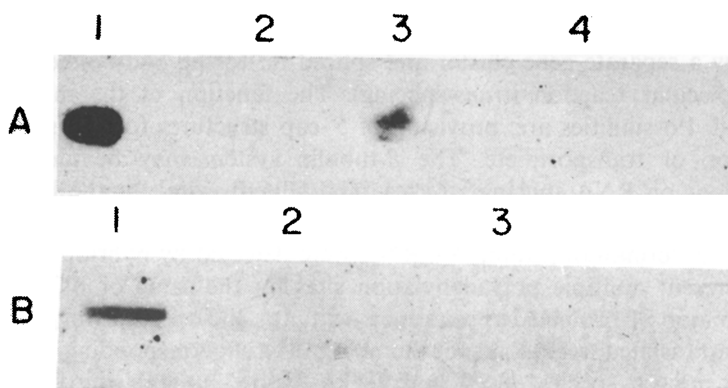

Figure 5. Hybridization analyses of nascent nuclear RNAs. [^{32}P] labelled RNA was obtained by transcription in isolated promastigote nuclei and hybridized to slots of cloned DNAs immobilized on Zeta probe (panel A) or nitrocellulose (panel B) membranes. Panel A: Specificity of *β*-tubulin transcription. Slot 1, *β*T-1 DNA (2·35 *μ*g); Slot 2, pUC8 DNA (2·1 *μ*g); Slot 3, M13 subclone A1 single-strand DNA (1·35 *μ*g); Slot 4, M13 subclone A2 single-strand DNA (1·35 *μg)*. Panel B: Effect of transcription inhibitors. Nuclear transcription was carried out in the absence of inhibitors (slot 1), or in the presence of 10 *μ*g/ml *α*-amanitin (slot 2) or 100*μ*g/ml actinomycin D (slot 3). The RNAs were separately hybridized to slots of *β*T-1 DNA (1 *μ*g each).

Discussion

In this report we have studied expression of *β*-tubulin mRNA in *Leishmania* promastigotes by nucleic acid hybridization techniques.

Recently we have cloned a genomic *β*-tubulin fragment from the parasite and studied chromosomal organization of *β*-tubulin genes using this cloned DNA as probe (Das and Adhya, 1990). These experiments showed that multiple copies of the *β*-tubulin gene are clustered as a tandem repeat on a single *Leishmania* chromosome. A few dispersed copies are also present. This pattern of major and minor genetic loci is reflected in the mRNA profile. Thus, one major (2·2 kb) and two minor (2·4 and 2·6 kb) species are observed. Hybridization with a gene-specific probe (figure 2) showed that major species is transcribed from the tandem repeat while the minor species may be derived from the dispersed genes. No information is yet available on the function of the minor mRNA species. They could

encode distinct *ß*-tubulin proteins. Alternatively, one or more of these mRNAs may be developmentally regulated during transformation of one form of the parasite to another.

The major 2·2 kb *ß* -tubulin mRNA has been mapped to the genomic DNA clone by S1 nuclease and primer extension experiments (figure 3). A unidirectional, bipartite structure is evident; the two halves are separated by a spacer of approximately 1 kb (figure 1). This structure confirms the tandemly repeated arrangement of *ß*-tubulin genes previously inferred from southern blot hybridization experiments (Das and Adhya, 1990).

SI nuclease mapping experiments showed that a single 5'-terminus of *ß*-tubulin mRNA is present. This includes a 35-nucleotide leader (or miniexon) sequence. Recent work with kinetoplastid protozoa (Van der Ploeg, 1986) has shown that all nuclear mRNAs from these organisms have such a 5'-leader. This sequence is encoded by a separate gene cluster and spliced on to the body of cellular mRNAs in a bimolecular reaction (*trans*-splicing). The function of the miniexon is not understood. Possibilities are: provision of 5'-cap structures for translation, mRNA stabilization or transport, etc. The *ß*-tubulin system may be ideal for further investigations on RNA splicing which is undoubtedly vital for the life cycle of the parasite.

Multiple 3'-termini of *ß*-tubulin mRNA were detected by hybridization (figure 3), These represent multiple polyadenylation sites on the same or different mRNAs. The two major SI-protected species map within a 300-bp *Pst* I fragment (figure 1). This 3'-untranslated region is not homologous to corresponding regions of the other two mRNA species of 2·4 and 2·6 kb (figure 2). Thus the two major, and probably also the minor, species are different polyadenylated versions of the same 2·2 kb mRNA. There is a relatively a long 3'-untranslated region of about 0·6 kb (figure 1). Such long noncoding 3'-sequences are a characteristic feature of *Leishmania* mRNAs. It remains to be seen whether such regions have any physiological function, such as mRNA stabilization and/or transport, translational regulation, etc. In summary our mapping results show that *ß*-tubulin mRNAs undergo post-translational processing at both 5'- and 3'-termini.

Although a great deal is known about the mechanism of RNA splicing in *Leishmania* and other kinetoplastid organisms, the mechanism of transcriptional initiation at specific promoters remains poorly understood. There are many reasons for this: (i) the initiation sites for mRNA precursors have not been previously mapped, and (ii) in *vitro* transcription of promoter-containing DNA has not been established.

Recently, two independent reports (Bellofatto and Cross, 1989; Laban and Wirth, 1989) have described transient expression of CAT genes under control of parasite DNA. This system will be most useful for studying *Leishmania* gene expression in future.

In order to define the transcription unit for *ß*-tubulin we have initiated nuclear run-on experiments with *Leishmania.* Incorporation studies (table 1) show that addition of radiolabeled ribonucleoside triphosphates to nuclear-kinetoplast preparations from promastigotes leads to a rapid rise in RNA synthesis followed by a plateau. Surprisingly, about 80% of the activity is ascribable to the *α*-amanitin sensitive RNA polymerase II. The reason for cessation of RNA polymerase II activity after about 10 min at 25°C is unknown, but may be due to inappropriate conditions of transcription. The remaining 20% of α-amanitin resistant activity may

be due to RNA polymerases I, III and the corresponding kinetoplast enzyme. By hybridization of nascent [^{32}P] labelled nuclear RNA to specific DNA probes (figure 4), it was demonstrated that β-tubulin genes are transcribed by RNA polymerase II in a strand-specific manner. Other polymerase II genes are also transcribed in this system (unpublished data). We are continuing such hybridization experiments to precisely define the structure of the β-tubulin pre-mRNA. This will be essential for definition of the β-tubulin promoter in *Leishmania.*

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