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Organization and chromosomal localization of ß-tubulin genes in *Leishmania donovani*

SAUMITRA DAS and SAMIT ADHYA*
Leishmania Group, Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Calcutta 700 032, India

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Abstract. The genomic organization and chromosomal location of the *ß*-tubulin isogenes in *Leishmania donovani* promastigotes has been studied by nucleic acid hybridization techniques using a cloned *ß*-tubulin gene. We have cloned a *ß*-tubulin gene fragment, 3·3 kbp long, from genomic DNA of *Leishmania donovani* using a heterologous *ß*-tubulin DNA as probe. Restriction maps of this clone have been prepared. It has been estimated that there are approximately 11-15 copies of the *ß*-tubulin genes per haploid genome. The majority of these isogenes are arranged in a tandem repeat with a length of 3·5 kbp on a single chromosome. In addition a few dispersed gene copies at different chromosomal loci were detected by pulse field gradient gel electrophoresis. Part of the internal coding region of the gene has been sequenced to confirm the identity of the *ß*-tubulin clone and is found to be nearly identical to that of *Leishmania mexicana amazonensis.*

Keywords. *Leishmania donovani; ß*-tubulin; gene organization; chromosome location; DNA sequence.

Introduction

Leishmania donovani is a pathogenic protozoan parasite of the order kinetoplastida with a dimorphic life cycle including a flagellated promastigote form in sandfly vectors and a non-flagellated amastigote form in the macrophages of infected mammals. This transformation of one form to other might be a reflection of altered expression of different stage specific cellular genes.

In *Leishmania,* tubulins are developmentally regulated proteins (Fong and Chang, 1981). Biosynthesis of tubulin increases rapidly during amastigote to promastigote transformation, and decreases in the reverse direction. Thus the study of organization and expression of the tubulins at the genetic level of this organism is of particular interest. Tubulin is the major constituent of the microtubular structures in *Leishmania* comprising cytoskeletal network, mitotic spindle and motility generating flagella. There are two types of tubulin, α and *ß.* Monomers of approximately 55,000 molecular weight form *αß* hetero dimers that are polymerized to form macromolecular filaments called microtubules. The nucleotide coding sequences of *α*- and *ß*-tubulin genes are highly conserved across phylogenetic boundaries, but the organization and copy number vary widely. In chicken and *Drosophila* (Cleveland *et al.*, 1981) there are 4 *α*- and 4 *ß*-tubulin genes. Chlamydomonas species have two *α*- and two *ß*-tubulin genes (Silflow and Rosenbaum, 1981) while the rat and human genome contain 10-20 unlinked genes for each subunit (Lemischka and Sharp, 1982; Wilde *et al.,* 1982). In *Leishmania*

*To whom all the correspondence should be addressed.
Abbreviations used: PBS, Phosphate buffer saline; SDS, sodium dodecyl sulphate; PFGE, pulsed field gradient gel electrophoresis.

species such as *L. enrietti* (Landfear *et al.,* 1983), L. mexicana (Fong *et al.,* 1984), *L. tripica* (Huang *et al.,* 1984),and *L. Major* (Spithill and Samaras, 1987), multiple copies of the *ß*-tubulin genes have been reported to be organized as tandem head to tail repeats The α- and *ß*-tubulin genes in *Trypanosoma brucei* have been found to be physically linked in tandemly repeated units of *αß* (Thomashow *et al*.,1983) In addition to the tandem repeat cluster, in some cases a few dispersed loci of *ß*-tubulin genes have also been identified. Existence of these scattered copies raises the possibility of the existence of functionally different tubulin proteins (Fulton and Simpson, 1976; Kowit and Fulton, 1974). Heterogeneity within the sequence of tubulin genes or proteins has been observed in some organisms, *viz.*, Chlamydomonas (Witman *et al.*, 1972), sea urchin (Bibring *et al.*, 1976), etc. This structural heterogeneity reflects a functional specialization among tubulin proteins. In *Leishmania,* whether all these tubulin genes are functional and if so, whether different genes have specialized functions, are not known. Hence it is important to correlate the tubulin gene number, chromosomal location and function to have an idea about the utilization of different tubulin proteins by the parasite.

We have studied the organization and chromosomal location of the *ß*-tubulin genes in *L. donovani* using a homologous genomic clone of *ß*-tubulin. By dot blot and Southern hybridization analysis the number and relative location of *ß*-tubulin genes have been determined. Major and minor gene copies have been mapped to different chromosome-sized DNA molecules by pulsed field gradient electrophoresis. To detect micro-heterogeneity within the gene the *ß*-tubulin clone has been partially sequenced. Results are discussed in terms of the prevailing ideas on tubulin polymorphism as determinants of cellular function.

Materials and methods

Parasite culture

L donovani strain UR6 (WHO nomenclature-MHOM/IN/1978/UR6) promastigotes were grown at 25°C for 48-72 h in modified Ray's (1932) agar medium containing 3·7% brain-heart infusion, 1% glucose, 1·5% agar, 10,000 U/ml penicillin, 10,000 μg/ml streptomycin and 1% rabbit blood. Two other strains of *L. donovani* AG 83 and WHO reference strain DD8 were grown at 25°C in medium 199 (Gibco laboratories) supplemented with 10–20% fetal calf serum.

Isolation of parasite genomic DNA

For isolation of genomic DNA *L. donovani* promastigotes were harvested with cold phosphate-buffered saline (PBS), washed 3–4 times with it and finally suspended in lysis buffer containing sodium dodecyl sulphate (SDS) and proteinase K followed by incubation at 37°C overnight. The lysate was then subjected to phenol extraction, dialysis, RNase treatment and ethanol precipitation DNA was estimated by its absorbance at 260 nm (1 A_{260} unit of absorbance = 50 μg double-stranded DNA) (Maniatis, *et al.*, 1982).

Cloning of β-tubulin gene

Total *L donovani* DNA was digested to completion with a combination of *Hind*III

and *Sal*I and 3–4 kbp fragments isolated by agarose gel electrophoresis and electroelution. These fragments were cloned into pUC8 vector which had been digested with same two enzymes. Transformants were screened with pLE*β*3, a *β*-tubulin genomic clone from *L. enrietti* (kindly provided by Dr. Scott Landfear of Harvard School of Tropical Public Health, MA, USA). Out of 200 clones screened, 3 positive, apparently identical *β*-tubulin clones were detected. One of these designated pLD*β*Tl has been used. The cloning procedure was according to Maniatis *et al.* (1982).

Copy number determination

Total *L. donovani* promastigote DNA (0·25–4 *μ*g) and the cloned *β*-tubulin gene pLD*β*T1 (0·125–4 ng) were denatured in 0·3 N NaOH and applied in a dot configuration on Zetaprobe (Bio-Rad) membrane using a dot blot apparatus (Bio-dot; Bio-Rad). After application the membrane was soaked in 1 × SSC for 2 min at room temperature then baked at 80°C for 1 h. The filter was then subjected to hybridization as detailed below with nick-translated [^{32}P] labelled pLD*β*Tl (herein abbreviated as *β*T1) DNA probe, followed by autoradiography. Quantitation of the band intensities were performed in two ways: (i) scanning of the autoradiogram at different exposure times in an Ultroscan densitometer (LKB) followed by peak weight determination or peak area estimation from the integration trace and (ii) excision of the slots for liquid scintillation counting. A standard curve of hybridization signal versus number of molecules of plasmid was plotted and corresponding mol number for genomic DNA obtained by interpolation. Knowing the cellular DNA content of *Leishmania* (Leon *et al.,* 1978) the copy number per cell (or haploid genome) could be computed.

Southern blot analysis

Total promastigote DNA was digested with restriction enzymes as indicated, electrophoresed in 1% agarose gels and transferred to nitrocellulose or Zetaprobe membrane by the method of Southern (1975), followed by hybridization with [^{32}P] labelled probe. Different probes were used which were [^{32}P] labelled either by nick-translation (Rigby *et al.*, 1977) or oligolabelling (Feinberg and Vogelstein, 1983) method. Southern blots on Zetaprobe membrane filters were prehybridized at 42°C for 24 h in 50% deionized formamide, 5 × SSC, 5 × Denhardt solution, 20 mM sodium phosphate, pH 7, 0·1% SDS and 200 *μ*g/ml calf thymus DNA or herring sperm DNA. Hybridization was performed by adding [^{32}P] labelled denatured probe (3×10^5 cpm/ml) to the prehybridization solution and incubating at 42°C for 24 h. The filters were 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 shaking, and then subjected to autoradiography. For the Southern blots on nitrocellulose membrane, prehybridization performed at 65°C for 4–6 h and hybridization was carried out at the same temperature for 24 h in aqueous medium using 3×10^5 cpm/ml probe and the filters were washed once with 3× SSC, 0·1% SDS, thrice with 1 ×SSC, 0·1% SDS, each wash for 30 min at 65°C, followed by autoradiography (24–48 h). Specific activity of the probe was 1-2 × 10^7 cpm/*μ*g DNA.

Pulse field gradient gel electrophoresis

Sample preparation: Promastigotes (1·25–2·5 × 10^7 cells) were washed with cold PBS and suspended in 100 μl of SE buffer (75 mM NaCl, 25 mM EDTA, pH 8) and 100 μl of 1% low melting agarose to form agarose blocks. Cells were then lysed *in situ* by incubating the blocks in 500 μl of ES buffer (0·5 M EDTA, pH 9·5; 1% sarkosyl) and 2·5 mg/ml proteinase K at 50°C for 48 h. The blocks were then washed thrice with 0·5 × TBE and stored at 4°C until use.

Run condition: Pulse field gradient gel electrophoresis (PFGE) of chromosome-size DNA of promastigotes of 3 different strains of *L. donovani* (UR6, DD8 and AG83) were performed by placing these agarose blocks inside the lanes of a 1% agarose gel and applying 150 V at different pulse times (60, 80 and 100 s) for 40 h in 0·5 × TBE gel running buffer at 15°C in a Pulsaphor apparatus (LKB). DNA was visualized by staining with ethidium bromide. For transfer chromosome-size DNA was depurinated by soaking the gel in 0·25 N HCl for 30 min and then blotted on to Zetaprobe membranes by the method of Southern followed by hybridization with [^{32}P] labelled βTl probe.

DNA sequencing

Part of the internal coding region and C-terminal coding portion of the cloned β-tubulin gene pLD βT1 were sequenced by enzymatic chain termination method (Hattari and Sakaki, 1986). Double stranded DNA sequencing was performed with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of dideoxy nucleotides and [α^{32}P] dTTP. Both universal forward and reverse primer (Promega) were used. The sequences were compared with the homologous region of the published β-tubulin gene sequence of *L. mexicana amazonensis.*

Results

Copy number determination

To determine the copy number of the β-tubulin gene a quantitative Southern blot hybridization was performed by using increasing amounts of *L. donovani* promastigote genomic DNA and βT1 plasmid DNA. DNA samples were denatured, applied on a nylon membrane and hybridized with nick-translated [^{32}P] labelled βT1 DNA probe (figure 2A). By comparing the intensity of hybridization of the β-tubulinprobe with the genomic DNA, to the amount which hybridizes to a known amount of the cloned β-tubulin plasmid, the copy number was determined. On the basis of densitometric analysis (see 'materials and methods') the graphical equivalence shows the amount of radiolabeled DNA in 1 μg genomic DNA corresponds to that for 1·4 ng of the βTl plasmid Standard which is equivalent to 35·3 × 10^{-5} pmol. The graphical equivalence obtained from liquid scintillation counting shows that the amount of radioactivity bound to 1 μg genomic DNA corresponds to that due to 1·75 ng of βT1 plasmid. This is equivalent to 44·1 × 10^{-5} pmol of the 6 kbp plasmid βT1. Knowing the *L. donovani* haploid

genomic content 5×10^7 bp (Leon *et al.*, 1978) the copy number was estimated to be in the range of 11-15 copies per haploid genome.

Tandem arrangement of the β-tubulin genes

The organization of the multiple *β*-tubulin gene was studied by probing Southern blots of restriction enzyme digested genomic DNA of *L. donovani* promastigotes with [^{32}P] labelled *β*Tl DNA. The restriction map of the cloned gene (figure 1)

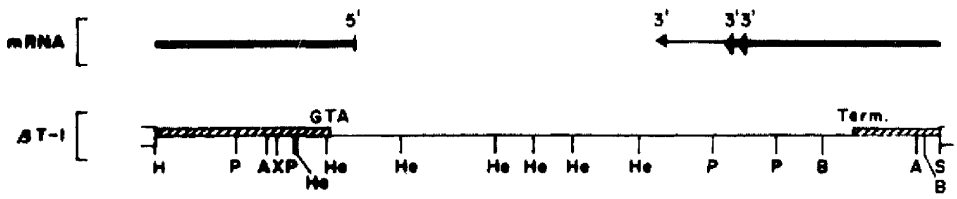

Figure 1. Restriction and transcription maps of the major *β*-tubulin locus in *L. donovani.* Locations of restriction sites on the genomic clone*β*T1 are shown. H, *Hind* III; P, *Pst*I; A, *Ava*I; X, *Xhol*; He, *Hae*III; B, Bam HI; S, *Sal*I. Square brackets flanking the restriction map represent pUC8 vector sequences. The mRNA map (Adhya *et al.*, 1990) is shown above the restriction map; thick and thin lines represent major and minor mRNA species respectively. Protein coding regions (determined by DNA sequencing) are designated by hatched bars (scale 0·8 cm = 200 bp).

showed that the gene contained single sites for *Hind*III, *Sal*I and *Xhol* enzymes, two sites for *Bam*HI and none for EcoRI. Digestion of genomic DNA with *Eco*RI yields a high molecular weight band (larger than 23 kbp) while with *Hind*III, *Sal*I and *Xhol* a single major band, 3·5 kbp in size was observed (figure 2B). Double digestion with *Hind* III and *Sal*I cause a reduction in the size of this band from 3·5 to 3·3 kbp. *Bam*HI digestion results in two bands of length 2·9 and 0·6 kbp (the lower band is not visible in the figure). *Hind*III and *Eco*RI double digestion yielded 3·5 kbp major band apart from a few minor bands (data not shown). From these results it was apparent that majority of the *β*-tubulin genes are organized as a precisely repeated structure in which the distance between adjacent sites for a given restriction enzyme is conserved, thus giving rise to a single major band. *Eco*RI enzyme has no recognition site within the gene and hence generates a single high molecular weight fragment that contains the entire repeat.

To confirm this repeated arrangement genomic DNA was partially digested with varying amounts of *Xho*I and subjected to Southern blot hybridization with [^{32}P] labelled *β*T1 probe. At intermediate concentrations of the enzyme *Xhol* a ladder of bands was observed of lengths 3·5, 7 and 10·5 kbp respectively (figure 2C). These lengths correspond to monomeric, dimeric and trimeric fragments from the cluster of tandemly repeated *β*-tubulin genes. Similar results were obtained using the enzymes *Hind*III and *Bam*HI (data not shown).

Recently these experiments have been repeated under conditions favouring detection of single copy fragments using probes derived from the N-terminal or C-terminal region of the gene (data not shown). Several minor bands corresponding to 5' or 3'-flanking sequences derived from the major cluster and from minor dispersed copies (see below) were observed.

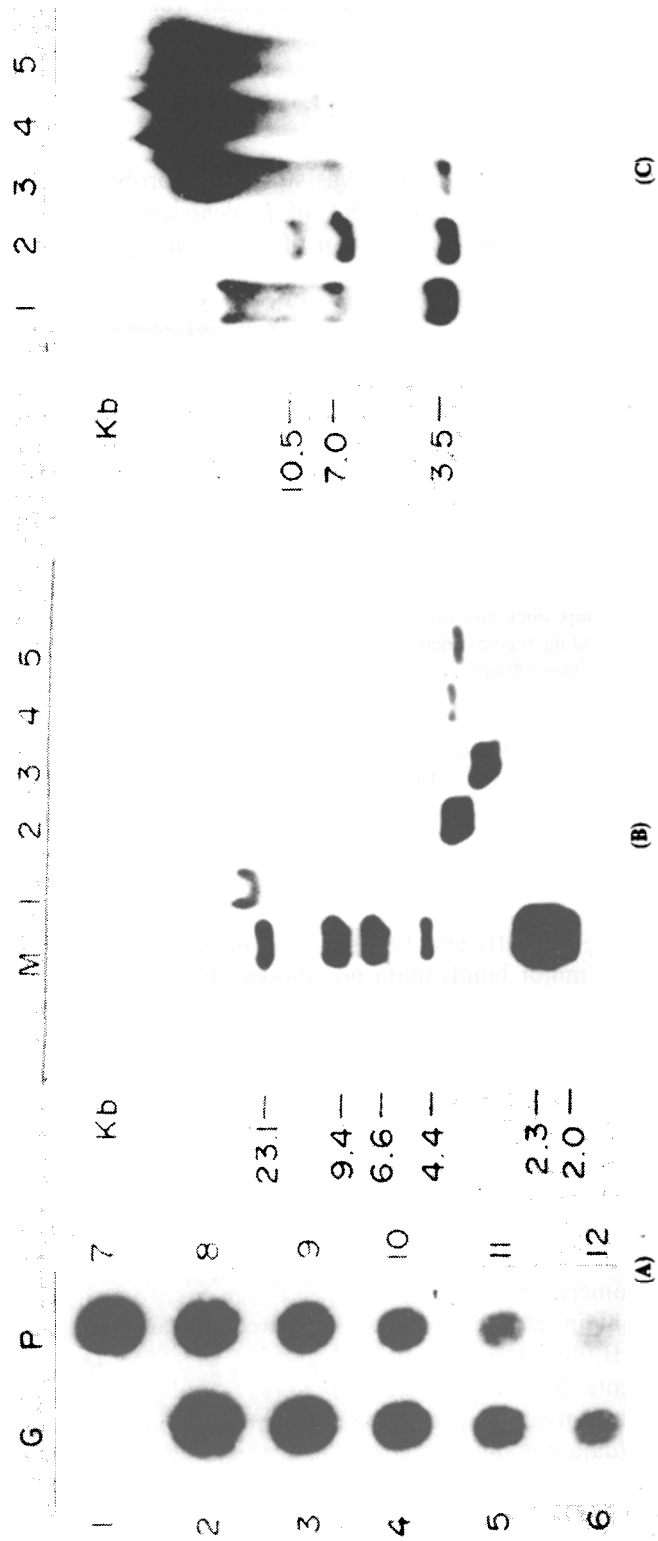

Figure 2. Hybridization analyses of β-tubulin genomic DNA with [^{32}P] labelled βT1 probe. (**A**) Copy number determination by dot blot hybridization. Lane G, dot 1, λDNA (4 μg); dots 2–6, *L. donovani* genomic DNA (4, 2, 1, 0·5, 0·25 μg respectively). Lane P, dots 7–12, pLDβT1 DNA (4, 2, 1, 0·5, 0·25 and 0·125 ng respectively). The filter was hybridized with oligo-labelled [^{32}P] βT1 insert DNA. (**B**) Southern blot hybridization of genomic DNA digested with different restriction enzymes. Lane M, λ-*Hind*III markers; lanes 1–5, DNA 5–10 μg) digested with *Eco*RI, *Hind*III *Bam*HI, *Sal*I and *Sal*I + *Hind*III respectively. Numbers to the left represent sizes (in kbp) of λ-*Hind*II molecular weight markers. (**C**) Southern blot hybridization of genomic DNA partially digested with *Xho*I. Lanes 1–5, DNA (10 μg) digested with 10, 2·5, 1, 0·5 and 0·25 units of *Xho*I, respectively, for 1 h at 37°. Numbers to the left show the sizes (in kbp) of the partial digest bands. In (**B**) and (**C**) filter-bound DNA was hybridized with [^{32}P] labelled pLDβT1 probe, washed and autoradiographed.

Chromosomal localization of β-tubulin isogenes

To map the chromosomal location of the *β* -tubulin isogenes and to determine whether this chromosomal location is conserved among different Indian isolates of viscerotropic *Leishmania,* PFGE followed by Southern blot hybridization with *β*Tl probe was performed. This electrophoresis system can separate wide range of chromosome size DNA molecules (Carle and Olson, 1985; Spithill and Samaras 1985). For organisms such as *Leishmania* which do not undergo chromosome condensation during mitosis, this is the only way to perform karyotype analysis and gene mapping. Chromosomes of 3 Indian *L. donovani* strains (UR6, DD8 and AG83) were resolved into about 20 bands (figure 3A). The band profile is similar in strain UR6 and the WHO reference strain DD8, but slightly different in AG83, which emphasizes the inherent plasticity of the *Leishmania* chromosomes. Southern blot hybridization of the chromosomes with *β*T 1 probe demonstrated 4 different loci for *β*-tubulin genes in *L. donovani* UR6 (figure 3B). The location of the major repeat cluster was assigned to band 20 (or even higher band, not resolved in the present gel) in all 3 isolates. The hybridization profile for the minor dispersed copies varies between the strains. Only two minor bands were detected for strain AG83 in contrast to 3 minor bands each for UR6 and DD8. As judged by its relative intensity (see below) the lower AG83 band may contain two copies on the same chromosome or one copy on each of two different unresolved chromosomes. Thus it is evident that only the chromosomal location of the major repeat is conserved, not that of the dispersed loci.

Densitometric scanning of the autoradiograms showed that the ratio of the dispersed copies to that in major repeat locus in UR6 as 1·6:1:17·7, in DD8 1·4:1:18·4 and in AG83 2·6:1:15·8.

Partial sequencing

The identity of the clone *β*T1 that has been used for studying organization of the *β*-tubulin genes, was confirmed by partial sequencing of the cloned gene from both ends using universal forward and reverse primer (Promega). The sequences were compared (figure 4A) with the published *β* -tubulin gene sequence of *L. mexicana* (Fong and Lee, 1988). The sequence at the internal coding region (from *Hind*III site onwards 117 bp, figure 1) shows 3 conservative base changes, one nonconservative base change (Val in *L. donovani,* Leu in *L. mexicana*) and one amino acid (histidine; position marked in the figure 4A) deletion. The C-terminal coding region (from *Sal*I site onwards 204 bp, figure 1) shows only one conservative change at the third base out of 204 nucleotides sequenced. The sequence data therefore demonstrates the high degree conservation of the *β*-tubulin gene sequences amongst different species of *Leishmania.*

Discussion

In this report we have shown that there are approximately 11-15 copies of *β*-tubulin genes per haploid genome of the pathogenic protozoan parasite *L. donovani.* The majority of these copies are arranged in a tandem repeat on a single chromosome (band 20). Additionally, a few copies are scattered on different

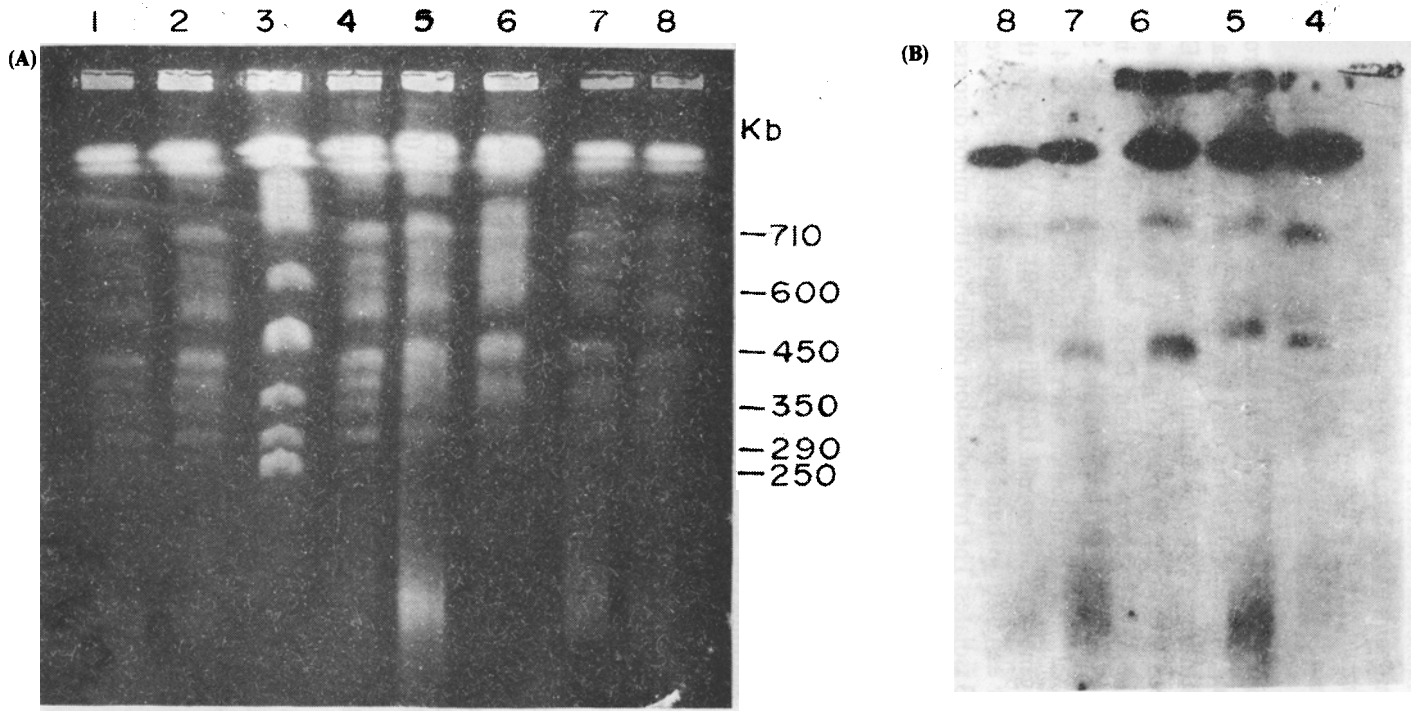

Figure 3. PFGE and Southern blot analysis of chromosomes from *L. donovani*. **(A)** Ethidium bromide-stained gel showing fractionation of chromosomes of *L. donovani* strains by pulse field gradient gel electrophoresis at a pulse time 80 s. Lane 1, 2 and 4, *L. donovani* strain UR6; lane 5 and 8, strain DD8; and lane 6 and 7, strain AG83. Lane 3, *S. cerevisiae* chromosomes run as molecular weight markers, whose sizes (kbp) are shown at the right. **(B)** Southern blot of part of the PFG gel shown in **(A)** hybridized with oligo-labelled βT1 probe for chromosomal localization of β-tubulin isogenes. Lane numberings are the same as in **(A)**.

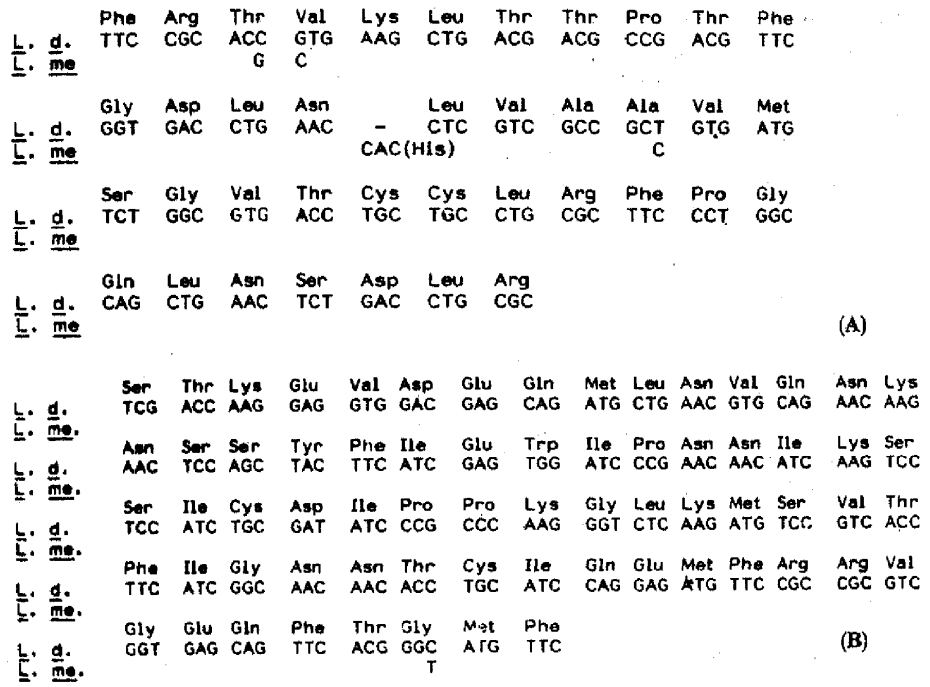

Figure 4. Partial nucleotide sequence of the *β*-tubulin gene from clone pLD*β*Tl. The sequence is compared with homologous region of the published *β*-tubulin gene sequence of *L. mexicana amazonensis* (Fong and Lee, 1988). Nucleotide differences between the two sequences are indicated. **(A)** DNA sequence of internal coding region (from *Hind*III end of the cloned gene). **(B)** C-terminal coding region (from *Sal*I end).

chromosomal loci. The organization of the *β*-tubulin repeat is very similar to that observed in other *Leishmania* species. With respect to repeat length (3·5 kbp) and restriction map, particularly, the structure most closely resembles that of *L. tropica* (Huang *et al.*, 1984). Existence of multiple copies of the tubulin genes may be a mechanism to allow high level of transcription of *β*-tubulin mRNA required for promastigote growth and division (Huang *et al.*, 1984).

In genomic Southern blots, apart from the major repeat band one or two dispersed copies were observed which are apparently single copy genes. The PFGE Southern blot hybridization experiment confirmed the dispersed copy genes. The ratio obtained by scanning of the autoradiogram, clearly shows the lower abundance of the dispersed tubulin genes in contrast to the major repeat. The function, if any, of these dispersed genes is unknown. They may encode functionally different variants; alternatively some or all of these genes may be non-functional pseudogenes. A transposon-interrupted k-tubulin pseudogene has been recently reported in *Trypanosoma brucei* (Affolter *et al.*, 1989). Multiple *β*-tubulin mRNAs have been observed in *L. donovani* (Adhya *et al.*, 1990) but only one of these is derived from the major repeat. A similar conclusion was derived in *L. major* (Spithill and Samaras, 1987). Therefore some or all of the dispersed copies are transcriptionally active. Cloning and sequencing of the dispersed genes should provide more information on their function.

The chromosomal location of the major repeat was found to be conserved in all 3 Indian isolates of *L. donovani,* but the chromosomal loci for the dispersed copies vary somewhat between the strains. Variation in the chromosome size and number of the dispersed loci of the *ß*-tubulin isogenes between the strains is an indicator of the genomic plasticity of these organisms. It should be possible to extend this method of direct chromosomal localization to other genes of *L. donovani* for which DNA probes are available.

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