Chromosome profile of *Leishmania donovani*: Interstrain and interspecific variations

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The genome of *Leishmania donovani* AG83, a virulent strain causing kala-azar, was resolved into 29 chromosomal bands by pulsed field gel electrophoresis (PFGE) under standardized conditions. Comparison of the karyotype with those of other strains and species revealed variations. By Southern hybridization, specific genes were localized to individual chromosomes. Twenty-two copies of β-tubulin genes are located on band 27 (1·63 Mb); minor copies are present in band 16 (850 kb) and band 9 (650 kb). A β-tubulin related nontranscribed locus was isolated from a genomic library and shown to contain repetitive sequences hybridizing throughout the genome. Single chromosomes contain multicopy clusters of gp63 and mini-exon-derived RNA genes, but interspecific variations were observed in each case. The results emphasize the importance of using a standard reference strain of *Leishmania donovani* for coordinated genome mapping of this clinically important organism.

1. Introduction

The digenetic life cycle of the protozoan parasite *Leishmania donovani*, the causative agent of kala-azar, is characterized by the lack of a recognizable sexual stage and an unusual cell cycle in which mitosis proceeds without chromosome condensation or nuclear membrane dissolution. These factors preclude the application of classical genetic and karyotype analyses to the organism. The situation is aggravated by the lack of a sufficient number of mutants and genetic polymorphism (also termed 'genome plasticity') leading to variations within and between *Leishmania* species. Yet, in view of the obvious clinical importance of this human pathogen, a genetic approach to the problems of virulence, transmission, drug resistance and other phenomena, is highly desirable.

Over the last decade, progress has been made towards molecular karyotyping of *Leishmania* species through resolution of chromosomes by pulsed-field gel electrophoresis (PFGE; van der Pleog *et al* 1984; Pages *et al* 1989; Spithill and Samaras 1987; Das and Adhya 1990). Most of the work has been carried out on one particular strain of *L. major*, an Old World species that causes cutaneous leishmaniasis, and a WHO-sponsored genome mapping project on this organism is underway in Europe (Ivens and Blackwell 1996). PFGE-mapping has also been reported for *L. infantum*, a viscerotropic species which causes infantile kala-azar in the Mediterranean (Wincker *et al* 1996). Although it has been argued that the genome map of one strain would be applicable to another (Ivens and Blackwell 1996), there is little evidence to substantiate this claim. Indeed, it is known that differences in gene copy number and organization differ between *L. donovani*, *L. major* and other species. Moreover, it is difficult to reconcile the great differences in clinical symptoms caused by the different species with an identical genotype. For these reasons, it is necessary to develop a genome map of a well-characterized virulent Indian strain of *L. donovani* which will be useful to identify the genetic determinants for the unique features of Indian kala-azar.

We report here the molecular karyotype of an Indian strain of *L. donovani* which is being extensively used for genetic, biochemical and immunological studies. By

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hybridization with specific probes, several multicopy nuclear genes were localized to individual chromosomal bands. Comparisons were also made with other species and strains, confirming the existence of genetic polymorphism.

2. Materials and methods

2. *Cultivation of parasites*

The *Leishmania* strains used in the present study are shown in table 1. Promastigotes were revived from liquid nitrogen storage by cultivation on biphasic blood-agar slants (Das and Adhya 1990) in 5 ml screw-cap vials containing 0·2–0·5 ml overlay of Schneider's *Drosophila* medium supplemented with 20% fetal calf serum at 22°C for 3–4 h. The strains were serially adapted on Schneider's and M199 media containing 10% fetal calf serum. Parasite cultures were routinely maintained in M199 medium.

2.2 *Preparation of agarose blocks*

To prepare agarose blocks of parasites, about 100 ml of log-phase promastigote culture (10^7 cells/ml) was centrifuged at 500 *g* for 10 min to pellet down the cells. The pellet was washed with ice-cold phosphate-buffered saline (pH 7·0) 3–4 times, then suspended in 250 µl SE buffer (75 mM NaCl, 25 mM EDTA, pH 8·0) at a concentration of 10^8 cells/ml. To the cell suspension, 250 µl of 1% low-melting agarose at 45° was added, mixed quickly and thoroughly and poured into 5 wells in a block-former (Pharmacia) so that the number of parasites was about 2×10^7 per block. The block-former was placed on ice for 1 h to solidify the agarose. Then the immobilized cells were lysed *in situ* by incubating each block in 500 µl of ES buffer (0·5 M EDTA, pH 9·5, 1% sarcosyl) containing 0·5 mg proteinase K at 50° for 48 h. Finally, the DNA blocks were stored at 4° in 0·5X TBE (45 mM Tris-borate, 1 mM EDTA) buffer containing 10 mM phenyl methyl sulphonyl fluoride (PMSF) after extensive washing with 0·5X TBE.

Table 1. *Leishmania* strains.

Species	Strain	Year	Place	Source
L. donovani	DD8	1980	Bihar, India	1
L. donovani	AG83	1983	Bihar, India	2
L. donovani	GE1	1989	Bihar, India	2
L. donovani	GE2	1989	Bihar, India	2
L. donovani	GE6	1989	Bihar, India	2
L. donovani	LV9	1967	Ethiopia	3
L. tropica	UR6	1976	Bihar, India	2
L. infantum	Spanish isolate	not known	Spain	3

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2.3 *In situ restriction of chromosomal DNA*

For *in situ* *Eco*RI digestion, a half portion of a DNA block was pre-equilibrated with *Eco*RI buffer (50 mM Tris-HCl, pH 7·5, 10 mM $MgCl_2$, 0·1 M NaCl, 1 mM dithiothreitol) for 20 min on ice. The buffer was replaced by fresh *Eco*RI buffer (200 µl) containing 100 µg/ml acetylated BSA and 10–20 units of *Eco*RI enzyme were added. The blocks were incubated at 37°C overnight, washed thoroughly with 0·5X TBE and stored at 4° in the presence of 0·5X TBE containing 10 mM PMSF.

2.4 *Run conditions for pulsed field gel electrophoresis*

Prior to PFGE, a half portion of a DNA block was embedded inside each well of a 1% agarose gel. The gel was run in a Pulsaphor apparatus (LKB) in 0·5X TBE buffer at 15°. The buffer of the gel chamber was circulated continuously and the temperature was compensated by lowering the set temperature of the circulator to 8°. For resolution of a wide range of chromosomal sizes, pulse times, voltage settings and run times were adjusted as shown in table 2.

2.5 *DNA probes*

DNA probes were prepared by restriction digestion of the respective plasmid DNAs and gel elution of the insert fragments. Probes were labelled by the random priming method (Feinberg and Vogelstein 1983) with [α-^{32}P] TTP. A 0·5 kb β-tubulin coding probe [βT (5′)] was obtained by double digestion of the genomic clone pβT-1 (Das and Adhya 1990) with *Xho*I and *Hind*III. A 0·45 kb βT (3′) probe from the β-tubulin 3′-untranslated region was obtained by *Bam*HI digestion of clone pβT1 (Das and Adhya 1990). A 0·5 kb mini-exon derived RNA (med RNA) gene probe was obtained by double digestion of clone pME1 (Hassan *et al* 1992) with *Eco*RI and *Hind*III. A 0·39 kb gp63 coding probe was obtained by *Bam*HI digestion of a PCR clone spanning positions

Table 2. Run conditions for PFGE separation of *L. donovani* chromosomes.

Pulse time (s)	Voltage	Run time (h)	Range of separation (kb)
80	150	48	300–700
100	150	48	300–1000
300	100	72	900–2000
900	50	164	1000–> 2000

527–919 of the gp63 gene (Webb *et al* 1991). Two additional probes, a 2·9 kb *Eco*RI and a 0·8 kb *Hind*III/*Xho*I double digested fragment, were obtained from a genomic Lambda Dash II clone, λc4, containing β-tubulin cross-hybridizing sequences (see below).

2.6 *Southern blotting*

The digested DNA fragments (5 µg/lane) were electrophoresed on an 0·8% agarose gel for 3–4 h at 70 volts. The gel was UV-irradiated for 5 min after ethidium bromide staining and then the high molecular weight DNA fragments were depurinated by soaking the gel in 0·25 N HCl for 10–15 min. Then the DNA fragments were denatured in 0·5 N NaOH, 1·5 M NaCl for 30 min and the gel was neutralized with 0·5 M Tris-HCl (pH 7·5), 1·5 M NaCl for 30 min at room temperature. The DNA was transferred to a nylon membrane (Hybond N, Amersham) by capillary diffusion (Southern 1975) in the presence of 10X SSC (saline sodium citrate) for 48 h. The filter was rinsed in 2X SSC, dried and UV-irradiated for 3–4 min for cross-linking. Finally the filter was baked at 80°C in vacuum for 30 min.

2.7 *Northern blotting*

RNA samples (5 µg/lane) were heated in 1 M glyoxal, 50% dimethyl sulphoxide, 10 mM sodium phosphate (pH 7·0) at 50°C for 1 h (total volume 20 µl). The samples were loaded on a 1% agarose gel cast in 10 mM sodium phosphate (pH 7·0) in a horizontal gel apparatus (Bio Rad). The buffer was continuously circulated by a peristaltic pump. The gel was electrophoresed at 70 volts for 3–4 h and transferred to a nylon membrane (Hybond N, Amersham) by capillary diffusion in the presence of 10X SSC for 48 h. The filter was rinsed in 2X SSC, dried and baked at 80°C in vacuum for 30 min.

2.8 *Hybridization conditions*

Pre-hybridization was carried out in a solution containing 5X SSC, 5X Denhardt's solution, 20 mM sodium phosphate buffer, pH 7·0, 0·1% SDS, 200 µg/ml calf thymus DNA at 42°C for 4–6 h. The blot was then incubated with fresh pre-hybridization solution containing [α-^{32}P] labelled DNA probes (1×10^6 cpm/ml). Hybridization was performed in aqueous phase at 55°C overnight, or in the presence of 50% formamide at 42°C, for 48 h. The blot was successively washed with 2X SSC, 0·1% SDS; 1X SSC, 0·1% SDS and 0·1X SSC, 0·1% SDS for 15 min each at room temperature. Finally, high stringency wash was performed (0·1X SSC, 0·1% SDS) for 15 min at 65°C.

2.9 *Construction and screening of genomic library*

A genomic library of *L. donovani* strain AG83 DNA was constructed in Lambda Dash II vector (Stratagene). This vector has the advantage of spi$^-$ selection of recombinants on *Escherichia coli* strain MRA(P2). Vector DNA was double digested with *Bam*HI and *Xho*I restriction enzymes and the polylinker was removed by alcohol precipitation of the digested arms at low salt concentration. The vector arms were then ligated with gel eluted 9–23 kb Sau3A I genomic DNA fragments of *L. donovani* strain AG83. The ligated material was packaged with Gigapack II packaging extract (Stratagene) and recombinants were selected by plating on *E. coli* strain MRA(P2). A positive Lambda clone (λc4) of 13·1 kb was identified by plaque hybridization with a β-tubulin coding probe at low stringency. Hybridization was in 5X SSC at 50°C, with the final wash in 0·1X SSC, at room temperature. Subsequently, 2·9 kb *Eco*RI and 0·8 kb *Hind*III/*Xho*I fragments from this clone were subcloned into pBluescript SK(+) plasmid vector.

3. Results

3. *The molecular karyotype of L. donovani*

In order to unambiguously assign chromosomal addresses to individual genes of *L. donovani*, it was first necessary to standardize PFGE conditions for maximum resolution of chromosomal bands. No single condition resolved all the bands; rather, up to four different gel runs (figure 1 and table 3) were necessary. Forty eight h runs at a pulse frequency of 100 s gave good resolution up to 1 Mb, as well as maximum interstrain discrimination (figure 1B). For chromosomes between 1 and 2 Mb, pulsing at 300 s for 72 h was found to be optimal (figure 1C). More prolonged runs at higher pulse frequencies are tedious and do not dramatically improve resolution (figure 1D). Three other parameters were additionally found to be critical: (i) cell numbers embedded in agarose should not exceed 2×10^7 per plug and not more than half of a plug should be loaded in one lane to avoid overloading and smearing of the bands; (ii) the temperature of the circulating buffer should not exceed 15°C; the set temperature should be adjusted accordingly (about 8°C); (iii) at higher pulse times, lower voltages should be used.

A maximum of 29 chromosomal bands, ranging in size from 300 kb to greater than 2 Mb, of *Leishmania* strain AG83 were resolved (figure 1, table 3). Some of the bands were less or more intensely stained than others, suggesting the presence of multiple chromosomes, or less than the diploid number. The band sizes add up to 26·7 Mb, which is less than estimates of the genome size of 36 Mb for *L. infantum* (Wincker *et al* 1996) and

45–50 Mb for *L. donovani* (Comean *et al* 1986). This discrepancy is probably due to the presence of multiple chromosomes in individual bands.

The band profiles of different Indian strains of *L. donovani* are almost, but not quite, identical (figure 1). A number of interstrain variations were observed. (i) The 825 kb band 15, and 800 kb band 14 of strain AG83 are missing in strain DD8; conversely, strain DD8 contains a 790 kb band absent in strain AG83 (figure 1B). (ii) The 1·42 Mb band 25 of strain AG83 is present in strain DD8, but absent in strain GE2 and GE6 (figure 1C). (iii) Strains GE2 and GE6 contain additional bands in the 800–900 kb region that are absent in the other two strains (figure 1B). (iv) Differences were also observed between the Indian strains and the African strain LV9 of *L. donovani* (figure 1C) and between the *L. donovani* strains and the viscerotropic *L. tropica* strain UR6 (figure 1B).

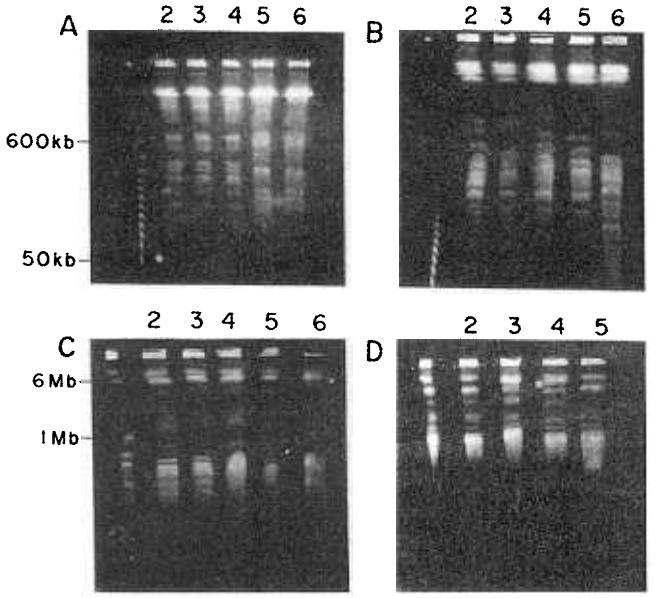

Figure 1. Separation of *Leishmania* chromosomes under different pulse conditions. Ethidium bromide stained gels are shown at different run times. (**A**) 80 s pulse, 48 h. Lane 1, marker λ-ladder (multiples of 50 kb), lanes 2,3, *L. donovani* strain AG83; lane 3, *L. donovani* strain DD8; lane 4, *L. infantum* (Spanish isolate); lane 5, *L. tropica* strain UR6. (**B**) 100 s pulse, 48 h. Lanel, marker λ-ladder (the smallest band is of 50 kb); lane 2, *L. donovani* strain AG83; lane 3, *L. donovani* strain DD8; lane 4, *L. donovani* strain LV9; lane 5, *L. infantum* (Spanish isolate); lane 6, *L. tropica* strain UR6. (**C**) 300 s pulse, 72 h. Lane 1, *S. cerevisiae* markers (220, 280, 360, 440, 550, 600, 680, 750, 790, 820, 910, 950, 1080, 1100, 1640 and 1900 kb); lane 2, *L. donovani* strain AG83; lane 3, *L. donovani* strain DD8; lane 4, *L. donovani* strain GE6; lane 5, *L. donovani* strain GE2, lane 6, *L. donovani* strain LV9. (**D**) 900 s pulse, 164 h. Lane 1, *L. donovani* strain AG83; lane 2, *L. donovani* strain DD8; lane 3, *L. donovani* strain LV9; lane 4, *L. infantum* (Spanish isolate); lane 5, *L. tropica* strain UR6. The positions of some markers are indicated at the left.

3.2 *Chromosomal address of β-tubulin genes*

Multiple β-tubulin genes are organized as tandem repeat clusters and dispersed copies in many *Leishmania* species (Landfear *et al* 1983; Huang *et al* 1984; Das and Adhya 1990). The same general organization was apparent in Indian isolates of *L. donovani* (figure 2A, B). Three chromosomal loci were observed by Southern hybridization with a cloned β-tubulin probe; for strain AG83, these correspond to band 27 (1·63 Mb), band 16 (850 kb) and band 9 (650 kb). The sizes of the corresponding bands in other species may be slightly different. The major locus, containing most of the gene copies as a tandem repeat is in band 27. To determine the total size of the β-tubulin locus in these chromosomes, the band was excised from the gel, the DNA digested *in situ* with *Eco*RI, re-electrophoresed at low pulse frequency, blotted and hybridized with β-tubulin probe. A single band of 80 kb was observed (figure 1C). Since *Eco*RI does not cut within the repeat (Das and Adhya 1990), this corresponds to the maximum size of the β-tubulin cluster.

Table 3. Molecular karyotype of *L. donovani* strain AG83.

Chromosome band number	Chromosome size (bp)	Chromosome localization	Gene function
	300		
2	375		
3	400		
4	450		
5	475		
6	500		
7	525		
8	575		
9	650	β-tubulin	Metabolic
10	675	gp63	Virulence
11	725		
12	750		
13	775		
14	800		
15	825		
16	850	β-tubulin	Metabolic
17	900	med RNA	Metabolic
18	950		
19	975		
20	1000		
21	1090	β-tubulin related	Pseudogene
22	1240		
23	1280		
24	1340		
25	1420		
26	1540		
27	1635	β-tubulin	Metabolic
28	1670		
29	> 2000		

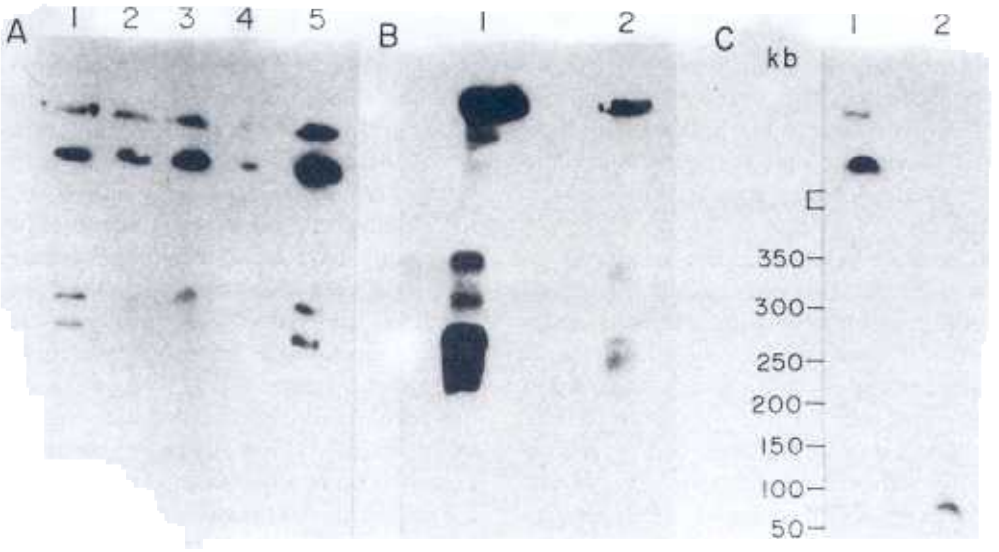

Figure 2. Chromosomal location of β-tubulin genes. (**A**) A Southern blot of chromosomes resolved at 300 s pulse was hybridized with βT(5′) coding probe. Lane 1, *L. donovani* strain AG83; lane 2, *L. donovani* strain DD8; lane 3, *L. donovani* strain GE6; lane 4, *L. donovani* strain GE2; lane 5, *L. donovani* strain LV9. (**B**) Southern blot strips of chromosomes of *L. donovani* strain AG83 resolved at 900 s pulse were hybridized with oligolabelled total promastigote DNA (lane 1), or with βT(5′) coding probe (lane 2). (**C**) release of the major β-tubulin cluster on band 27 by *Eco*RI. Band 27 of *L. donovani* strain AG83 (the major band in panel A) was excised from a gel run at 100 s pulse, incubated without (lane 1), or with (lane 2) *Eco*RI, and rerun at 20 s pulse for 16 h. After blot transfer, the DNA was hybridized with βT(5′) coding probe. Marker sizes are indicated at the left. Radioactivity at the top of each lane in this and subsequent figures represents unresolved nuclear DNA stuck at the origin.

3.3 *A β-tubulin related (β Tub R) locus: non-transcribed repetitive sequences linked to a putative pseudogene*

By screening a λ genomic library with a β-tubulin probe at low stringency (see § 2), a recombinant phage (λc4) with an insert of 13·1 kb was isolated (figure 3A). At least 2 β-tubulin related genes are present in the 8·3 kb region from the right end of the insert as determined by Southern hybridization of the clone with a β-tubulin probe (data not shown). The rightmost 2·9 kb *Eco*RI fragment A, containing one or more of these genes, hybridized at high stringency (see § 2) to band 21 (1·09 Mb) of strain AG83, distinct from the 1·6 Mb-band 25 which contains the major β-tubulin locus (figure 3B).The absence of hybridization to the major β-tubulin chromosomal band at high stringency indicates divergence from the β-tubulin sequence. The adjacent 0·8 kb-*Hind*III/*Xho*I fragment (figure 3A) hybridized weakly to multiple chromosomes, including two large bands between 1 and 1·6 Mb, and several smaller bands in the 650 kb region (figure 3B), indicating the presence of repetitive sequences. Neither region is transcribed to form stable RNAs (figure 3C). The β-tubulin related sequences could thus represent one or more non-functional pseudogenes.

3.4 *Localization of the gp63 locus*

gp63 is a major surface glycoprotein implicated in parasite virulence (Chang *et al* 1990). The multicopy gp63 locus has been reported to be present in a single 600–700 kb chromosome in different *Leishmania* species (Webb *et al* 1991). This is true for the Indian *L. donovani* strains AG83 and DD8, in which band 10 (650 kb) contains all the gp63 genes (figure 4). However, in the *L. infantum* Spanish isolate and the Ethiopian *L. donovani* strain LV9, two bands of 650 and 600 kb hybridize to gp63 probe (figure 4) with about equal intensity.

3.5 *Med RNA genes on a large chromosome*

Miniexon-derived RNA (med RNA) is a short RNA molecule which contributes its 5′ 39 nt to the ends of cellular mRNA by *trans*-splicing (Hassan *et al* 1992). By hybridization, med RNA genes were localized to a 900 kb chromosome in all strains tested (figure 5). The viscerotropic *L. tropica* strain UR6 contains a significantly higher copy number than the other strains, and also an additional chromosome of size 350 kb containing a few copies of the med RNA gene (figure 5).

4. Discussion

This report represents the first step towards a comprehensive genetic analysis of Indian strains of *L. donovani*. The molecular karyotype established here will form the basis of physical mapping, gene targeting and other studies aimed at understanding the genetic basis of parasite pathogenesis.

Significant differences in chromosome band patterns were detected within and between species (figure 1). This is undoubtedly a result of genome plasticity. It has been hypothesized that chromosome ends (telomeres) of *Leishmania* undergo rapid expansion and contraction, while the internal regions are relatively stable (Iovannisci and Beverley 1989). Gene conversions and/or unequal crossing over by homologous recombination should also lead to polymorphism within repeated gene clusters. Indeed, we have detected intraspecific restriction fragment length polymorphism at all the loci mapped in this study (unpublished data). Regardless of the mechanism, the presence of chromosome length variations emphasizes the importance of using a single, well-characterized strain of *L. donovani* for valid comparisons of mapping results obtained by different laboratories participating in the genome project. We suggest the adoption of strain AG83 as the reference mapping strain, for the following reasons: (i) virulent and avirulent (passaged) variants of this strain are available in different Indian laboratories as well as in the *Leishmania* cryobank of IICB; (ii) methodologies for cloning this strain by limiting dilution as well as by plating have been recently established (unpublished data); (iii) well-characterized animal models (mice and hamsters) for visceral leishmaniasis are available; (iv) *in vitro* macrophage infections with this strain have been extensively studied; (v) there is a large volume of literature on drug efficacy and drug targeting studies using strain AG83 as the model organism; (vi) similarly, extensive immunological investigations are being carried out in the mouse model of strain AG83 infection.

The chromosomal addresses of various genes are generally conserved within Indian strains of *L. donovani* (figures 2, 5). Interestingly, gp63 genes, which have been reported to be located on a single 600–700 kb band in other species (Webb *et al* 1991), was mapped to two

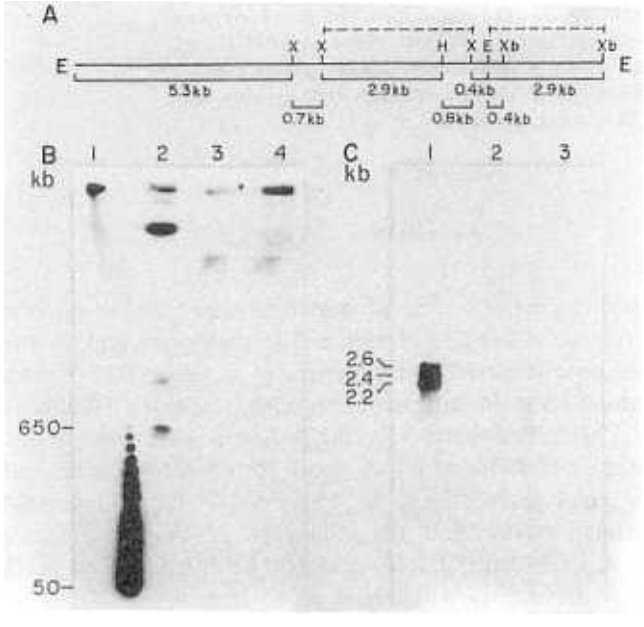

Figure 3. Organization and transcription of the β-tubulin related locus. (**A**) Restriction map of clone λc4. Fragments hybridizing with βT(5′) coding probe are shown by dotted lines. (**B**) Chromosomal location of the β-tubulin related region. Filter strips of *L. donovani* strain AG83 chromosomes resolved at 100 s pulse were hybridized separately with βT(5′) coding probe (lane 2), the 2·9 kb *Eco*RI fragment of clone λc4 (lane 3) and the 0·8 kb *Hind*III/*Xho*I fragment of clone λc4 (lane 4). Lane 1, λ-chromosomal ladder markers, hybridized with λDNA. (**C**) A Northern blot of poly A^+ RNA of *L. donovani* starin AG83 was hybridized with the βT(5′) coding probe (lane 1), the 2·9 kb *Eco*RI fragment of λc4 (lane 2) and the 0·8 kb *Hind*III/*Xho*I fragment of λc4 (lane 3). Sizes of the β-tubulin mRNAs (kb) are shown at the left.

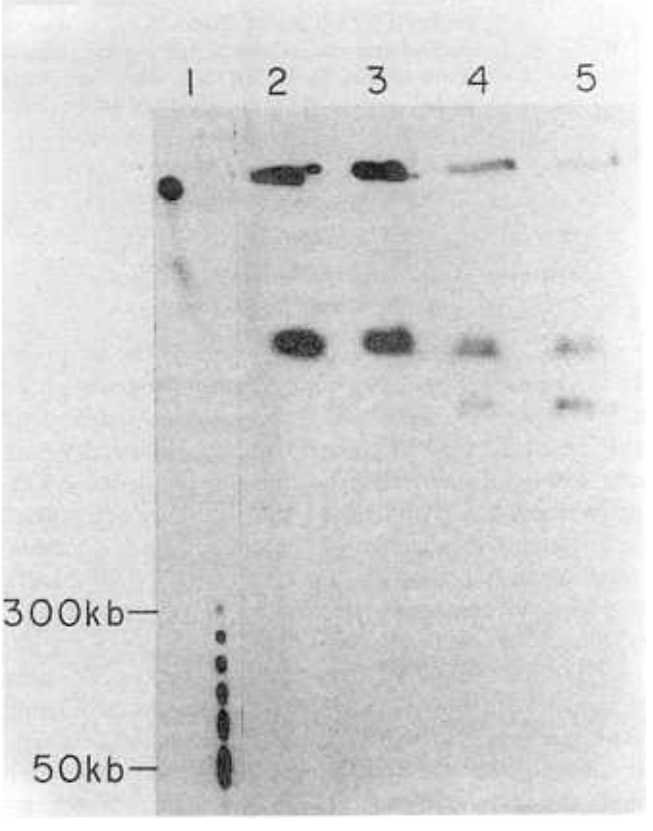

Figure 4. Chromosomal location of gp63 genes. Southern blots of chromosomes resolved at 80 s pulse were hybridized with a 0·39 kb gp63 coding probe. Lane 1, λ-chromosomal ladder markers hybridized with λDNA (sizes are shown at the left). Lane 2, *L. donovani* strain AG83; lane 3, *L. donovani* strain DD8; lane 4, *L. infantum* (Spanish isolate); lane 5, *L. donovani* strain LV9.

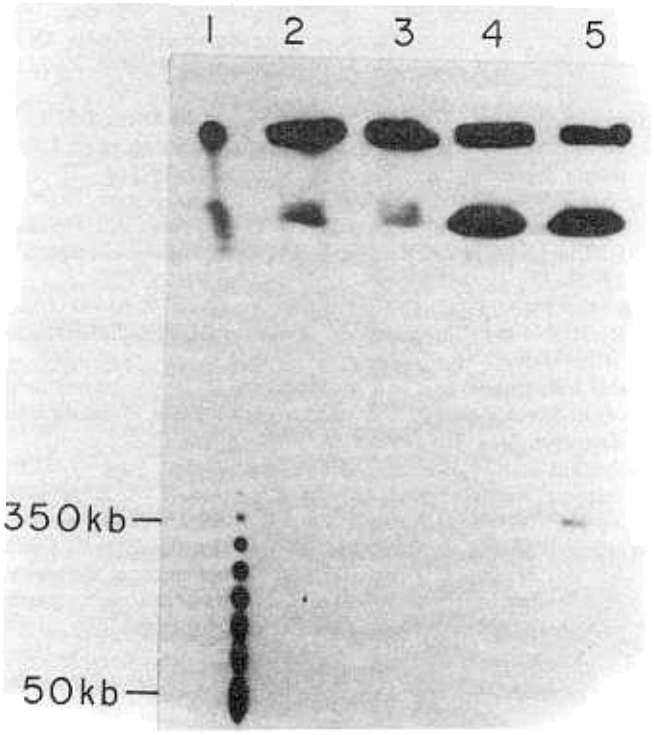

Figure 5. Chromosomal location of med RNA gene. Southern blots of chromosomes resolved at 80 s pulse were hybridized with a 0·5 kb med RNA coding probe. Lane 1, *L. donovani* strain AG83; lane 2, *L. donovani* strain DD8; lane 3, *L. infantum* (Spanish isolate); lane 4, *L. tropica* strain UR6. Marker sizes are shown at the left.

bands in the Spanish *L. infantum* strain, as well as the Ethiopian *L. donovani* strain (figure 4). One interpretation of this result is that the two bands in *L. infantum* and *L. donovani* strain LV9 represent alleles of the diploid gp63 locus, but the presence of homologous sequences on different chromosomal bands cannot be excluded at this time. Ploidy of *Leishmania* has often been a contentious issue in view of the presence of aneuploids and polyploids in parasite populations (Cruz *et al* 1993), but recently, heterozygotes have been generated at a number of loci by gene disruption, confirming that most genes are present in a diploid configuration (Cruz and Beverlay 1990; Curotto de Lafaille and Wirth 1992; Cruz *et al* 1993; Souza *et al* 1994; Webb and McMaster 1994; Hwang *et al* 1996; Mottram *et al* 1996).

It has been reported previously that medRNA genes are located on a single 350 kb chromosome in a particular strain of *L. infantum* (Wincker *et al* 1996). Our results, with a different *L. infantum* strain (Spanish isolate) as well as with *L. donovani* strains, are quite different: in each case a 900 kb chromosome contains most or all of the genes. Only in the case of *L. tropica* UR6 we could detect a few minor copies in a 350 kb band (figure 5). The latter strain also contains a significantly higher copy number of medRNA genes compared to the others. It is not known whether this is related to the ability of strain UR6 to grow rapidly on solid medium, and thus maintain a high rate of RNA metabolism.

We report here the identification of a previously undetected gene(s) homologous to, but distinct from, β-tubulin (figure 3). This gene is linked to repetitive sequences, is not transcribed, and adjacent regions are generally devoid of restriction sites (figure 3). These characteristics are compatible with a centromeric or telomeric location of this sequence, a possible pseudogene. Pseudogenes have not been previously reported in *Leishmania*. DNA sequence analysis will reveal the extent of homology of this region with β-tubulin genes.

The methodology described in this report will be generally applicable to other genes for which clones or sequence data are available. Furthermore, linkage maps may be constructed by using anonymous probes such as sequence-tagged sites (STS) or expressed sequence tags (EST). This developments will be crucial for construction of low and high-resolution maps of the *L. donovani* genome.

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