

Physical mapping across the dihydrofolate reductase–thymidylate synthase chromosome of *Leishmania major*

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SUMMARY

We have used a chromosome-specific approach to generate a 300 kb long ‘contig’ across *Leishmania major* 500 kb chromosome. Clones from a 13-hit genomic library served as templates to generate end-specific probes that were used in hybridization to a high density array of the library. The ‘contig’ generated contained 12 markers uniformly spaced. Three restriction endonucleases were mapped within the map extending its resolution. Map extension indicated a peculiar feature of sequence organization in subtelomeric regions where chromosome-specificity of mapping is lost. End-probes generated from clones mapping to the extremes of a 300 kb ‘contig’ rescued a high percentage of 2 types of clones from the genomic library, 1 of which showed positive hybridization to the hexameric telomere repeat. Fine mapping at these regions revealed that these 2 clones contained elements common to all chromosomes of the parasite. The physical map generated constitutes ready-to-use data for the study of many aspects of genome organization. Being cloned in a shuttle vector, the genomic sequences reordered in the map can be used to generate genetic information by transfection into the parasite.

Key words: *Leishmania*, physical map, telomeric region, DHFR–TS, genome organization.

INTRODUCTION

Trypanosomatid parasites of the genus *Leishmania* are the causative agent of a heterogeneous spectrum of diseases affecting 12 million people all over the globe. Many features of this parasite’s biology reflect a peculiar genome organization determining particular mechanisms of gene expression regulation, which may include gene amplification (Beverley, 1991), bent DNA (Marini *et al.* 1982), polycistronic transcription and subsequent mRNA processing (LeBowitz *et al.* 1993), and the absence of promoter sequences or centromeric regions that have not been identified yet.

Genome research is being continuously improved since the development of methodologies that permit the manipulation of large genomes and the establishment of genome mapping efforts on many model organisms, such as human (Cohen, Chumakov & Weissenbach, 1993), *Drosophila melanogaster* (Ajioka *et al.* 1991), *Caenorhabditis elegans* (Sulston *et al.* 1992), *Saccharomyces cerevisiae* (Riles *et al.* 1993; Dujon, 1996), *Escherichia coli* (Kohara, Akiyama & Isono, 1987). A global vision of genome

structure and function, introduced by genome projects, has led to a better understanding of gene expression and evolution in some organisms (Hartl *et al.* 1994; Zorio *et al.* 1994).

The integration of molecular and genetic data constitutes a major goal in genome research, since it can greatly improve our knowledge of any organism’s biology. Genetic mapping in *Leishmania* has been restrained for many years due to the lack of experimental crosses between genetically marked strains (Panton *et al.* 1991). However, the development of DNA transfection vectors (Kapler, Coburn & Beverley, 1990; Laban *et al.* 1990) generated powerful tools to be used in different approaches from gene knockouts (Cruz & Beverley, 1990) to functional genetic complementation (Ryan *et al.* 1993*b*). These molecular tools may also play an important role in genome research. Having a dual function, such vectors can be used in both physical and genetic mappings.

Physical mapping constitutes an important step in genome mapping. It consists of ordering genomic DNA fragments on their chromosomes using several methods. The reconstruction of the original genomic order into ‘contigs’ can be achieved by using fingerprinting (Coulson *et al.* 1986), fragment size matching (Olson *et al.* 1986), multi-origin chromosome walking and STS-content mapping (Mizukami *et al.* 1993). When physically ordered fragments are

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cloned in a transfection vector, such as cLHYG (Ryan, Dasgupta & Beverley, 1993a), the positioning of genetic loci relative to one another across the genome can also be revealed.

In our effort to physically map the *Leishmania major* genome in a chromosome-specific fashion we have successfully combined chromosome walking and non-random STS-content mapping strategies. The advantage of using such strategy lies in the fact that each step on map assembly confirms the previous one and corroborates the positioning of a given clone. A single contig covering the DHFR-TS chromosome constitutes ready-to-use data for the study of many aspects of chromosome structure and organization. Among those are chromosome polymorphism, genetic mapping and genic density, elements of transcriptional regulation, presence of repetitive elements and many other puzzling topics regarding the way genetic information is stored and expressed in these parasites.

MATERIALS AND METHODS

High-density array of a genomic library

The *Leishmania major* (LV39, clone 5-Rho/SU/59/P) library used was constructed by cloning sheared genomic DNA into the cosmid shuttle vector cLHYG (Ryan *et al.* 1993a). Independent colonies (11520) were transferred into LB medium in 96-well arrays and grown overnight at 37 °C. Glycerol was added (15%, v/v) and microtitre plates were stored at -70 °C. The library was robotically printed on GeneScreen Plus nylon membranes (DuPont) in a high-density array (at Sanger Centre, Hinxton, UK). Colonies were grown overnight at 37 °C on LB/50 µg/ml ampicillin plates. The colony filters were submitted to denaturation (0.5 M NaOH-8 min) and neutralization (0.5 M Tris, pH 8; 1.5 M NaCl-8 min). Dried membranes were baked for 2 h at 80 °C. The entire library was printed on 4 filters (3072 clones/filter). A set of 4 filters can be probed at least 8 times before loss of signal.

Single primer PCR amplification

End-specific probes were generated by single primer PCR amplification; the conditions for all PCRs were 30-sec denaturation at 94 °C, annealing for 1 min at 41 °C, and extension for 1 min at 72 °C. Reactions were continued for 30 cycles. Single primer reactions were conducted either with T7 (5'-AATACGACT-CACTATAG-3') or 13K (5'-GTCAACGAGAAA-CACC-3') primers.

Screening the library

Probes generated by PCR amplification or gel-extracted DNA fragments were radio-isotope

labelled by random priming as described (Feinberg & Vogelstein, 1983). Following pre-hybridization for 4 h at 67 °C in 25 ml of 5 × Denhardt's reagent (0.1% Ficoll; 0.1% polyvinylpyrrolidone; 0.1% bovine serum albumin) (Sambrook, Fritsch & Maniatis, 1989), hybridization was conducted at the same temperature for 15–40 h in 8 ml of the same solution. Filter washes were 2 × SSPE/0.5% SDS for 10 min at room temperature with 1 change of solution, and 2 × SSPE/0.5% SDS for 30 min at 65 °C with one change of solution. Filters were exposed to X-ray films for 8–48 h. Hybridization washing procedures for Southern blots and PFGE conditions have been described elsewhere (Cruz & Beverley, 1990).

DNA manipulations

Cosmids positive for a given probe had their cosmid DNA extracted by the alkaline/SDS method (Sambrook *et al.* 1989). Following digestion with *Xho*I, *Bam*HI or *Sst*II, and agarose gel electrophoresis, comparison of restriction profiles identified overlapping clones to be used in another round of probe generation, screening and map extension. The restriction endonucleases *Bam*HI, *Bgl*II, *Hind*III, *Eco*RI and *Xho*I were used to align and overlap clones precisely. *Bal*31 assays were done as described (Sambrook, *et al.* 1989).

Contig assembly

A contig assembler software was used to confirm contig organization. The software Sam (V2.2.1) is still under development at the Sanger Centre (by C. Soderlund) and produces maps containing ordered markers along with the alignment of clones, rather than a map of real physical distances. Those clones positive for more than one marker defined the assembly of the map.

RESULTS

The screening strategy

End-specific probes were prepared by amplifying insert ends using single-primer PCR. We used cLHYG vector as template in control reactions to confirm the position of primers flanking the *Bam*HI cloning site and found that the primers are 360 bp apart. Using primers separately in single primer reactions, product sizes were about 1 kb and 0.9 kb for T7 and 13K primers respectively. The amplified insert ends were used as probes in hybridization to high-density filters and allowed the detection of overlapping clones. The PCR products generated by either primer were always long enough to guarantee probe specificity. Single copy probes consistently

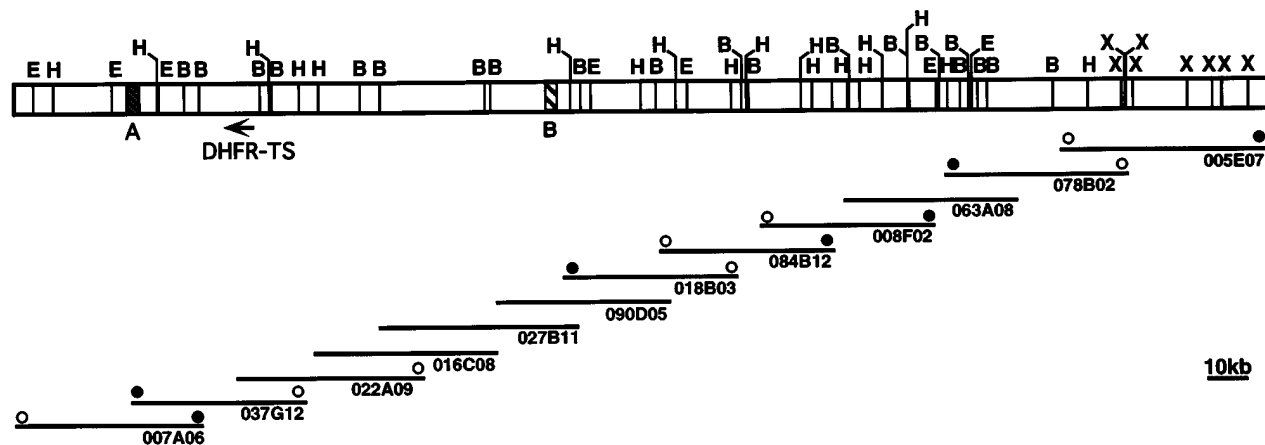


Fig. 1. The DHFR-TS chromosome map of *Leishmania major*. Representation of the 300 kb contig of DHFR-TS chromosome. Restriction sites of *Bam*HI (B), *Eco*RI (E), *Hind*III (H) and *Xho*I (X) are presented. Chromosome-specific markers used for map construction are hatched boxes placed on the chromosome representation (A and B). Position of DHFR-TS is shown and the black arrow indicates direction of transcription. Recombinant clones from which the restriction map was generated are represented below. Open and closed circles, placed close to each cosmid, represent T7 and 13K end-specific probes employed for map construction.

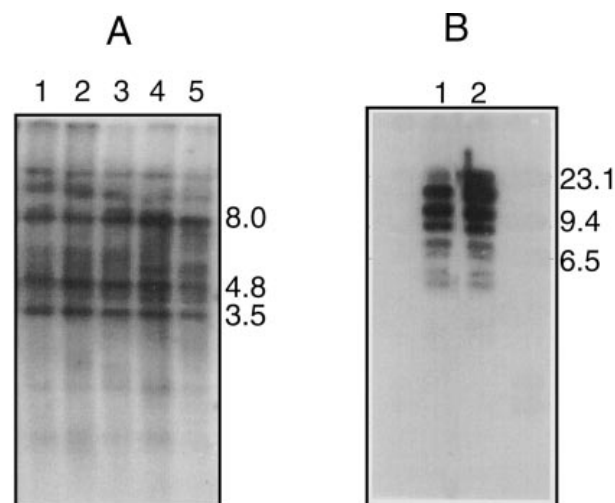


Fig. 2. T clones are at the chromosome ends. (A and B) Southern blots of digested genomic DNA (*Hind*III) previously submitted to increasing time incubations with *Bal*31. (A) Hybridization with intact clone T; lanes 1–5 represent *Bal*31 incubation times ranging from 8 to 14 min. (B) Hybridization with the 4.5 kb *Bam*HI/*Hind*III fragment from clone T (depicted as a bar in Fig. 5); 1 and 2 represent *Bal*31 incubation times of 0 and 30 min respectively.

rescued an average of 18 clones from the high-density filters. Insert ends running through repetitive elements were clearly identified at this stage by the higher number of positive clones (up to 10-fold increase) when compared to single copy probes.

Restriction profiles of hybridizing recombinants were compared to the parental clone. Those showing a clear overlap were selected and used as template for probe generation and extension of the contig.

The DHFR-TS chromosome contig

Using the strategy above we were able to reconstruct a 300 kb contig (Fig. 1). The seed for contig generation was the DHFR-TS gene, known to be localized in this chromosomal band (Beverley, Ellenberger & Cordingley, 1986). Two overlapping clones running through DHFR-TS gene are represented in the map (037G12 and 022A09); positioning of both clones was also confirmed by Southern blot hybridization with the same DHFR-TS probe. Both ends of the two-clone initial contig were used in the first steps of chromosome walking. The contig was extended about 30 kb to the left, while at the right end a span of about 210 kb was achieved in consecutive 10 steps. Overlaps at every point of map extension were confirmed by restriction profile analysis. Extension at one overlapping point (between 090D05 and 018B03) was carried out by using a gel purified fragment from 090D05 clone. Two other markers, named here A and B, previously shown to be in this chromosome (Beverley and co-workers, unpublished results), constituted important anchors during the contig building process. The chromosome walking generated a set of 15 evenly distributed markers across the 300 kb contig. Such markers were never separated by more than 30 kb (Fig. 1).

A set of 12 clones was chosen to minimally represent the contig (Fig. 1); these clones were analysed by detailed restriction mapping, which confirmed the original alignment (Fig. 1). Some of the cosmids along the contig were used as probes in blots containing PFGE-separated chromosomes and ensured the DHFR-TS chromosome localization.

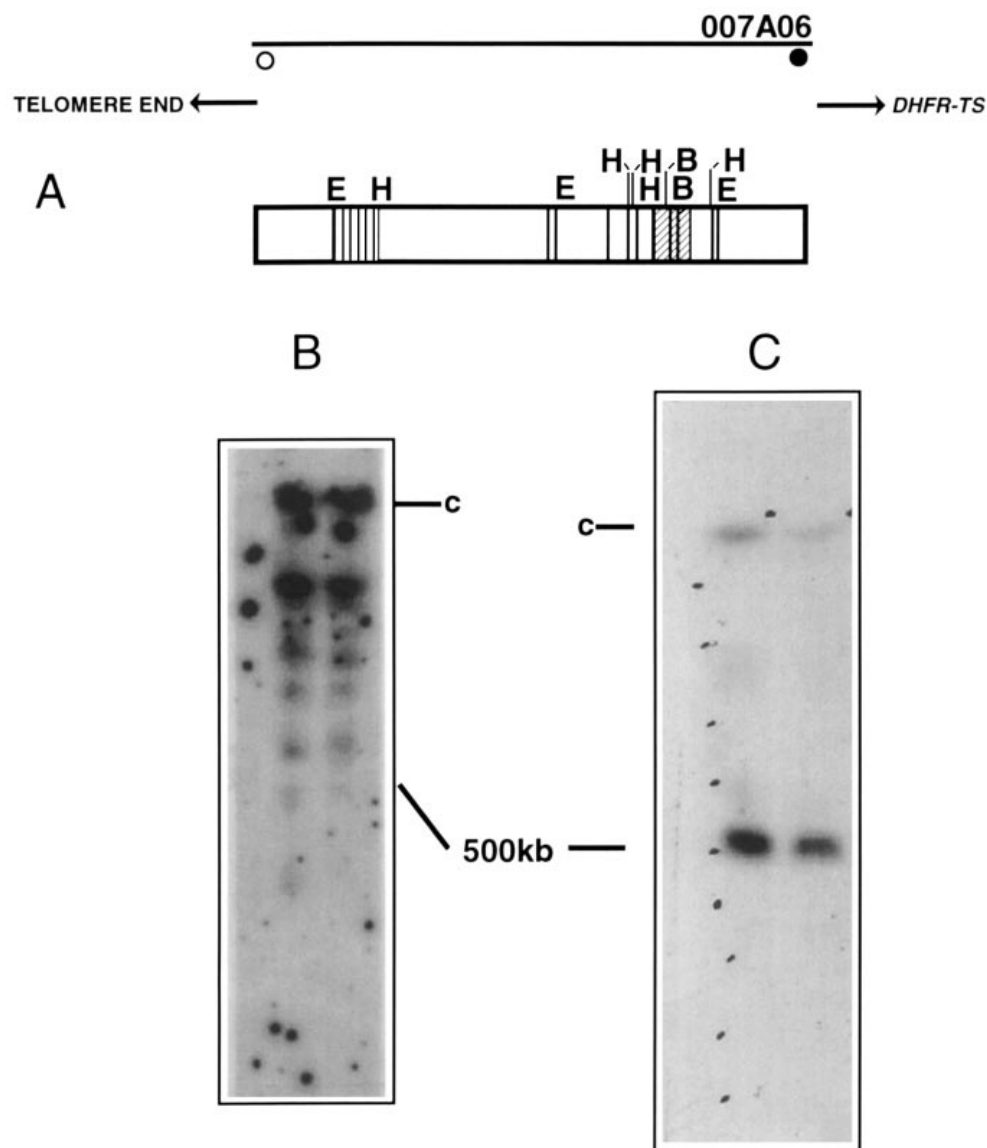


Fig. 3. Chromosome 'left end side': loss of chromosome specificity. (A) Schematic representation of clone 007A06 restriction map: *EcoRI* (E), *BamHI* (B), and *HindIII* (H). Hatched box corresponds to marker A, shown in Fig. 1. Upper horizontal line represents clone 007A06, positioning T7 and 13K primers as open and closed circles respectively. (B and C) Southern blots of PFGE-separated chromosomes of (1) *Leishmania major*, LV39 and (2) *L. major*, LT252 (PFGE-electrophoresis proceeded in a CHEF-DR II apparatus, with switch times varying from 35.4 to 73.6 sec in a linear ramp over 35 h). Hatched boxes indicate DNA fragments used as probes in each hybridization. Arrows indicate position of the clone relative to the chromosome.

These clones are: 007A06, 037G12, 027B11, 018B03 and 005E07 (data not shown). The markers used and their respective positive clones were analysed by Sam V2.2. The contig produced by Sam confirmed the positioning of clones and the order of markers along the map.

The contig ends

Probes generated from the extreme clones of the contig identified a set of recombinants that indicated that contig extension had reached a peculiar feature of chromosomal structure. Irrespective of the probe

used to extend the contig (T7 probe for 007A06 and 13K probe for 005E07), we observed that (i) none of the selected clones showed noticeable overlaps, as determined by restriction analysis, to the parental clones, (ii) restriction fragment analysis also revealed that a higher percentage of the clones were of 2 types, named T and S, (iii) *XhoI* digestion suggested the presence of an overlap, of about 6 kb, between clones T and S, (iv) virtually all chromosomal bands showed positive hybridization to intact clones T and S, (v) clone T, but not clone S, showed positive hybridization to the cloned telomeric repeat of *Trypanosoma brucei* (Van Der Ploeg, Liu & Borst, 1984).

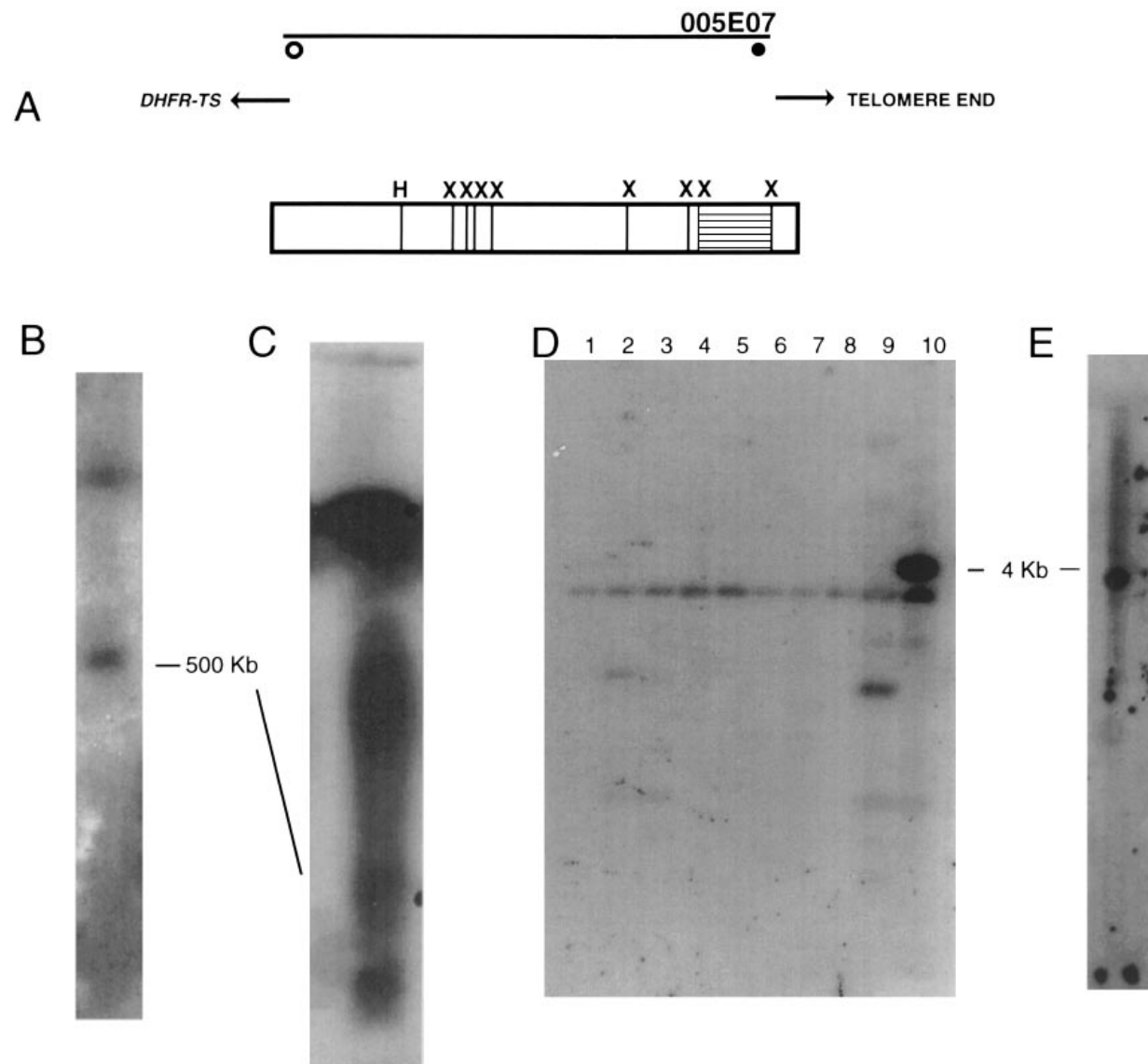


Fig. 4. Chromosome 'right end side': loss of chromosome specificity. (A) Schematic representation of clone 005E07 restriction map: *Hind*III (H) and *Xho*I (X). (B) Southern blot of PFGE-separated chromosomes of *Leishmania major* LV39 hybridized with intact clone 005E07. Hybridization of a 4 kb *Xho*I fragment from clone 005E07 to: (C) Southern blot of PFGE-separated chromosomal DNA of *L. major*, LV39; (D) Southern blot of the agarose gel with all clones from the contig: (1) 007A06, (2) 037G12, (3) 022A09, (4) 090D05, (5) 018B03, (6) 084B12, (7) 008F02, (8) 063A08, (9) 078B02, (10) 005E07 (E) Southern blot of an agarose gel containing the parasite genomic DNA digested with *Xho*I. PFGE conditions used are similar to that presented in Fig. 3. Arrows indicate position of the clone relative to the chromosome.

Different fragments derived from clone T were hybridized to digested genomic DNA. The entire clone T and isolated fragments tested in *Bal*31 assays confirm the position of clone T at the ends of chromosomes (Fig. 2). The fragments tested (Fig. 2B, and data not shown) suggested, in addition, the polymorphic nature of the region. As shown on Fig. 2B, one of the fragments identified a set of 7 major discrete bands decreasing in size in *Bal*31 assays.

Considering that this chromosome is about 500 kb, clones T and S would contain sequence elements present in the region between the telomeric ends and clones at both ends of the contig (007A06 and 005E07). However, restriction analysis did not reveal

an overlap between central contig and clones T and S. From this result it appears that probes derived from the termini of the contig (clones 007A06 and 005E07) are not specific for the DHFR-TS chromosome. In fact, clone 007A06 contains both a DHFR-TS chromosome-specific marker and non-specific sequences, as shown by hybridization to PFGE-separated chromosomes (Fig. 3). To gain information on the organization of the region, a 4 kb *Xho*I fragment from clone 005E07 was gel purified and used as a probe in Southern analysis (Fig. 4). The fragment hybridized to all bands in PFGE-separated chromosomes (Fig. 4C). Nevertheless, the probe hybridized to a unique 4 kb band in *Xho*I

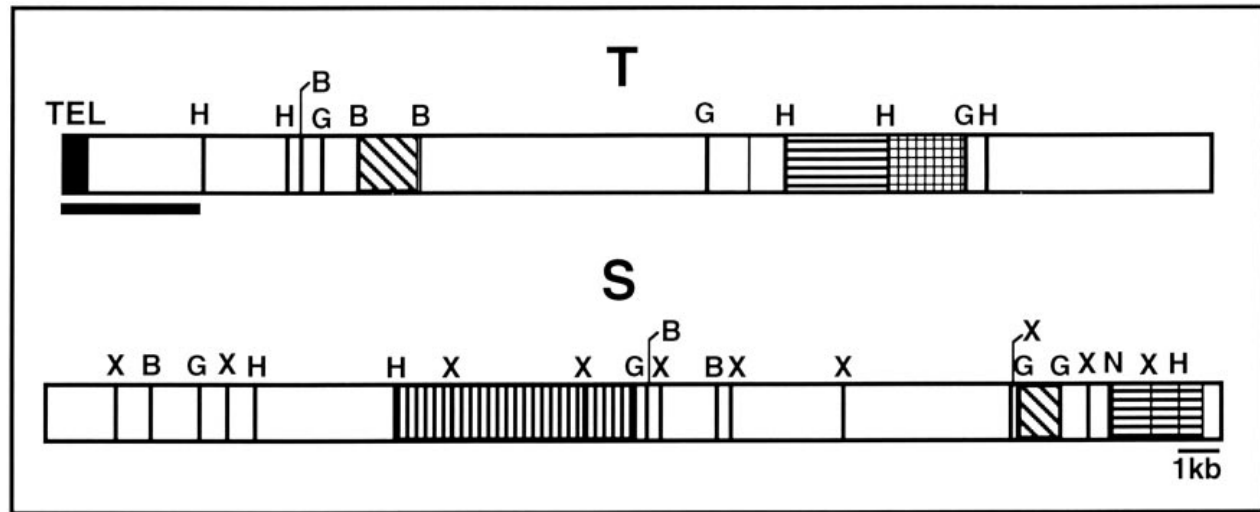


Fig. 5. Features of clones T and S. (A) Physical map of clones T and S with some restriction enzymes: *Bam*HI (B), *Bgl*II (G), *Hind*III (H) and *Eco*RI, (R). Known fragments containing sequences shared by T and S are depicted by hatched boxes with different pattern. The 4.5 kb *Bam*HI/*Hind*III fragment, used as probe, is indicated below T restriction map as a bar. (B) Southern blot of a PFGE of (1) *Leishmania major*, LT252 (2) *L. major*, LV39, (3) *L. braziliensis*, (4) *L. donovani infantum*, (5) *L. amazonensis panamanensis*, hybridized to the 4.5 kb *Bam*HI/*Hind*III fragment.

digested genomic DNA (Fig. 4E), suggesting that, in spite of being spread over the genome, such sequence is present in a conserved context. The fact that this fragment did not hybridize to clones within the map (Fig. 4D) may indicate that this sequence is widespread among the chromosomes at subtelomeric regions. Similar results were obtained when using a 4 kb *Xho*I fragment purified from clone 007A06, which is at the left end of the 300 kb central contig (data not shown).

Further analyses of clones T and S have shown that they are clearly non-overlapping regions as can be deduced from their restriction map with 3 different endonucleases (Fig. 5A). Hybridization experiments of clone T fragments to chromosomes of some *Leishmania* species (Fig. 5B), and data not

presented have shown that (i) none of the fragments tested was chromosome specific; with variable intensity virtually all chromosomal bands were positive (Fig. 5B), (ii) some degree of sequence homology is kept among the species tested (Fig. 5B), (iii) clones T and S carry common elements in a diverse organization.

DISCUSSION

In this paper we report the construction of an ordered contig map of the DHFR-TS chromosome of *Leishmania major*. We have successfully combined non-random STS and fragment-size matching methods in the construction of a contig across this chromosome. Twelve markers are uniformly spaced

within approximately 300 kb, and sites for 3 restriction endonucleases have been determined across the whole contig.

The use of end-specific probes presents advantages when compared to other mapping strategies. Overlaps of a few hundred nucleotides are detectable since probes are ~ 1 kb long (Evans, 1991). The efficiency of end-specific probe utilization has been demonstrated both theoretically (Palazzolo *et al.* 1991) and experimentally (Mizukami *et al.* 1993). Nonetheless, this strategy has always been associated to whole genome mapping efforts (Evans & Lewis, 1989; Palazzolo *et al.* 1991). We otherwise employed end-specific probes in a chromosome specific mapping approach. The use of probes generated from both ends of a given clone, enabled the rapid self-confirming construction of a complete map at a high resolution. Further restriction analysis allowed unequivocal positioning of overlapping clones. The combination of strategies constitutes an uncomplicated method in the sense that the map is built without the need of massive hybridization data collection and complex computer analyses.

The *L. major* genome size is estimated to be 36.5 Mb (Wincker *et al.* 1996). The 11520 arrayed clones would represent a 13-hit library. The use of single copy probes, such as DHFR-TS and most of the single-primer amplified DNAs, consistently rescued 15–24 recombinants from high density filters. A somewhat higher number of clones selected may indicate a cloning bias against particular features of the genome, such as those found at telomeric and subtelomeric regions. In fact, the analysis of about 900 clones by restriction digestion showed that only 4% of these clones are positive to the hexameric telomere probe, which is slightly lower than the fraction of telomeric clones expected.

Map completeness depends on the quality of the library. Our data indicate that the use of sheared DNA for library construction ensured the representation of the telomeric region. Mapping at chromosome ends would not be possible otherwise. Besides, a sheared genomic library will not be deprived of genomic regions where restriction sites are present at either a higher or lower frequency than each 40 Kb.

Map extension is hampered at the ends of the 300 kb contig. Sequences present in cosmids at the extremes of the map are common to all chromosomal bands indicating that chromosome specificity of probes is lost in both directions. Repetitive sequence elements at subtelomeric regions have been reported in human genome (Brown *et al.* 1990) and in many other eukaryotic organisms. The presence of subtelomeric repeats has been suggested as an important feature in determining genome plasticity in protozoa. Studies on structural organization at telomeric regions from *Plasmodium falciparum* chromosomes demonstrated that such regions contain arrays of

repetitive sequence elements. It has been suggested that these elements mediate chromosome pairing and meiotic recombination leading to the extensive chromosome polymorphism (de Bruin, Lanzer & Ravetch, 1994; Lanzer, Fischer & Le Blancq, 1995).

Chromosome size variation has long been demonstrated in *Leishmania* species (Giannini *et al.* 1986). Minisatellite DNA sequence has been recently described in *Leishmania infantum* genome (Ravel *et al.* 1995) and shown to be located in size-variable subtelomeric regions of at least 2 chromosomes of this parasite. Named LiSTIR1, such sequence was shown not to be present in *L. major* genome, being specific for the *Leishmania donovani/Leishmania infantum* group. However, the organization of this sequence is believed to be related to the subtelomeric repeat elements found in the human genome. Our results suggest that a similar pattern of chromosome organization might be possible for *L. major* chromosomes.

The fact that a chromosome specific 300 kb contig approaches a non-specific stretch closer to telomeric ends indicates that recombinants T and S might encompass a typical set of elements common to all chromosomes of *L. major*. However, unlike LiSTIR1, fragments dispersed along clone T are virtually present in all chromosomes of both Old and New World *Leishmania* species. Although our data suggested that this region is characteristically polymorphic, further analyses of these clones are necessary to define the extension of homology between different chromosome tips and organization of common elements.

The strategy designed permitted the reconstruction of an unusual 300 kb long contig across a 500 kb chromosome. Since the reconstructed sequence is cloned in a shuttle vector, recombinant cosmids assembled in the map can be functionally tested by transfection into *Leishmania*. Considering this property, new strategies for generating genetic information can be conceived and tested. Consequently, any physical map generated by the strategy described here constitutes the basis for genetic mapping, overcoming the lack of classic genetics as experimental tools for these parasites.

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