 Nucleic Acids Research, 1999, Vol. 27, No. 13 **2745–2752**

Characterization of a *Leishmania donovani* gene encoding a protein that closely resembles a type IB topoisomerase

Sonia Broccoli, Jean-François Marquis[1], Barbara Papadopoulou[1], Martin Olivier[1] and Marc Drolet*

Département de Microbiologie et Immunologie, Université de Montréal, CP 6128, Succursale Centre-Ville, Montréal, Québec H3C 3J7, Canada and [1]Centre de Recherche en Infectiologie du CHUL, Ste-Foy, Québec G1V 4G2, Canada

Received February 26, 1999; Revised and Accepted May 7, 1999 DDBJ/EMBL/GenBank accession no. AF145121

ABSTRACT

In order to clone the gene encoding a type I DNA topoisomerase from *Leishmania donovani*, a PCR-amplified DNA fragment obtained with degenerate oligodeoxyribonucleotides was used to screen a genomic library from this parasite. An open reading frame of 1905 bases encoding a putative protein of 635 amino acid residues was isolated. A substantial part of the protein shares a significant degree of homology with the sequence of other known members of the IB topoisomerase family, in a highly conserved region of these enzymes termed the core domain. However, homology is completely lost after this conserved central core. Moreover, no conventional active tyrosine site could be identified. In fact, the protein expressed in *Escherichia coli* did not show any relaxation activity *in vitro* and was unable to complement a mutant deficient in topoisomerase I activity. The results of Southern blot experiments strongly suggested that the cloned gene was not a pseudogene. Northern analysis revealed that the gene was transcribed in its full length and also excluded the possibility that some form of splicing is necessary to produce a mature messenger. Furthermore, our results indicate that the gene is preferentially expressed in actively growing *L.donovani* promastigotes and that it is also expressed in other kinetoplastid parasites.

INTRODUCTION

DNA topoisomerases participate in nearly all cellular activities involving DNA (reviewed in 1). Various cellular processes such as replication, transcription and the movement of helicases along the DNA template generate supercoils that are subsequently removed by topoisomerases. Furthermore, these enzymes could also be involved in recombination and are known to play an important role in chromosome segregation, condensation and decondensation. DNA topoisomerases alter the topology of DNA via a reaction pathway that entails: (i) non-covalent binding of the enzyme to duplex DNA; (ii) cleavage of the DNA with concomitant formation of a covalent DNA–protein intermediate; (iii) strand passage; (iv) religation of the broken strand(s) (1). The DNA is nicked by the topoisomerase following a transesterification reaction during which the enzyme forms a covalent phosphotyrosyl bond with the DNA via the tyrosine active site (1,2). Topoisomerases can essentially be divided into two classes: type I and type II DNA topoisomerases. Type I topoisomerases are monomeric proteins that nick one strand of the DNA duplex and relax the double helix by rotating the non-covalently held segment about the phosphodiester bond in the unbroken strand opposite the protein-induced nick (1,2). These enzymes can be further divided into type IA (bacterial) and IB (eukaryotic) topoisomerases. Type II topoisomerases are homodimeric enzymes (one exception is DNA gyrase from *Escherichia coli*) that alter DNA topology by producing a transient nick in both strands of the double helix, forming a gate through which both strands of the DNA duplex can pass (1).

Topoisomerases have been shown to be the molecular targets of several molecules of natural and/or synthetic sources (reviewed in 3,4). Some of these drugs specifically interact with type I topoisomerases, whereas others recognize type II topoisomerases. Camptothecin, an extensively studied type IB topoisomerase poison, interferes with the breakage–reunion reaction of this enzyme, by trapping the key covalent intermediate, the cleavable complex (5). Cell death is believed to occur when the replication machinery collides with an open cleavable complex (6–8).

Parasites of the genus *Leishmania*, members of the trypanosomatidae family, are intracellular protozoans that infect the macrophages of mammals, including humans. Various pathologies can develop following infection by *Leishmania*, depending on the species involved, encompassing cutaneous, mucocutaneous and the deadly visceral leishmaniasis. The incidence of all three types of leishmaniasis is currently on the rise, mainly because of the growing number of travellers in endemic areas of the world, the lack of effective vaccines, the difficulty of vector control and the parasite's increasing

*To whom correspondence should be addressed. Tel: +1 514 343 5796; Fax: +1 514 343 5701; Email: marc.drolet@umontreal.ca

resistance to chemotherapy (9). Some classical anti-trypanosomal drugs were recently identified as topoisomerase poisons (10). These compounds do not induce DNA fragmentation in mammalian cells, but they stabilize cleavable complexes in kinetoplast DNA of trypanosomes (10,11). Other studies in trypanosomes have shown a direct correlation between cleavable complex accumulation (leading to double-strand breaks) and cytotoxicity of several type I and II topoisomerase inhibitors, including camptothecin (11–15). Taken together, the results of these studies strongly suggest that a different drug selectivity can be established between the human and trypanosomal topoisomerases. Topoisomerase I activity has been detected in several parasites of the trypanosomatidae family, including *Leishmania donovani* (11). As a first step in the development of chemotherapy against *L.donovani* infections based upon the use of DNA topoisomerase I as a molecular target, we have used degenerate oligonucleotides to isolate a type I topoisomerase gene from this parasite. We have cloned a topoisomerase I-like gene from *L.donovani* coding for a protein of 635 amino acids and presenting an extensive degree of homology with the central core. Surprisingly, the C-terminal region of the protein does not seem to present any resemblance to the highly conserved C-terminal domain of type IB topoisomerases which normally contains the tyrosine active site within a conserved SKXX**Y** motif (16–18). To our knowledge, this is the first report of a topoisomerase I-like gene from this parasite.

MATERIALS AND METHODS

Cloning of the *L.donovani TOP1*-like gene

The sequences for synthetic DNA primers were previously generated by reverse translation of peptide sequences in a region of extensive homology among eukaryotic type I topoisomerases (19). The sequences for the 5' and 3' oligonucleotides, consisting of 256 and 384 permutations, respectively, to reflect all possible codon degeneracies are: d[GGGGGG-AATTCTT(T/C)(C/A)G(T/C/G/A)AC(A/G/CIT)TA(T/C)AA-(T/C)GC] for the 5' primer (amino acid residues F589–A594 of human Topo I) and d[AAAAAAAGCTT(C/T)TG(A/G)TG-(A/G)TT(A/G)CA(A/G/C/T)A(A/G)(A/G/T)AT] for the 3' primer (residues Q633–I628 of human Topo I). Two micrograms of genomic DNA from *L.donovani* were used for the PCR reaction, which was carried out at 25°C for 2 min, followed by a 1 min extension at 72°C, for 60 cycles. The PCR products were routinely analyzed by electrophoresis on a 5% polyacrylamide gel. A 138 bp fragment was obtained and subcloned into pBluescript SK (Stratagene) using the *Eco*RI and *Hin*dIII sites that had been introduced into the 5' and 3' primers. A random labeled probe (Random Primed DNA Labeling Kit; Boehringer Mannheim) was prepared from this 138 bp PCR fragment and used to screen a genomic library of *L.donovani infantum* (strain LEM1317). The library was obtained as described (20). Essentially, partially digested *Sau*3A genomic DNA fragments were cloned into the *Bam*HI site of the cLHyg cosmid shuttle vector. Four cosmids probed positive: $B_7$65, $C_2$78, C_{10}29 and $D_2$67. Since a Southern blot experiment revealed that cosmid C_{10}29 shared more common DNA fragments with *L.donovani* genomic DNA than any of the other three cosmids, it was chosen for subcloning of the *TOP1*-like gene.

Hybridization studies

DNA from *L.donovani* Sudanese strain 1S (a generous gift from Dr Albert Descôteaux, Institut Armand-Frappier, Laval, Québec, Canada) was extracted as described (21). DNA fragments generated by various restriction enzymes were resolved on a 0.8% agarose gel and transferred to a Nytran Plus (Mandel) nylon membrane as described (22). DNA probes were generated by random labeling. To verify the presence of pseudogenes, DNA fragments of the cloned *TOP1*-like gene were used as probes. Total RNA from *Leishmania* cells was extracted using TRIzol (Gibco BRL). RNA was resolved on a 1% agarose gel, transferred to a Nytran Plus nylon membrane and probed with *Sac*I DNA fragments from the cloned *TOP1*-like gene. Hybridization, in the absence of formamide, and washings were performed as described (22). Hybridization signals were revealed by autoradiography using Fuji medical X-ray films. Membranes were re-used by stripping in 0.1 N NaOH (15 min) followed by two washes in 0.5 M Tris, 0.1% SDS for a period of 15 min at room temperature or by exposing them to high temperatures.

Sequencing of the *TOP1*-like gene from *L.donovani*

A 3.5 kb *Sal*I fragment from cosmid C_{10}29 that probed positive with the 138 bp PCR fragment and contained over half of the gene was subcloned into the M13mp19 phage vector. A 2.1 kb *Pst*I fragment from cosmid C_{10}29, with an overlapping region of 168 bp with the 3.5 kb *Sal*I fragment (see Fig. 1 for a restriction map), was also subcloned into the M13mp19 phage vector. This *Pst*I fragment also probed positive with the PCR fragment and contains the remaining part of the gene. The complete nucleotide sequence of the *L.donovani TOP1*-like gene was determined using an ALF automatic sequencer (Amersham Pharmacia Biotech) and a series of synthetic oligonucleotides as primers. The nucleotide sequence data was assembled and processed with the Oxford Molecular Group MacVector program, v.6.0. The multiple sequence alignment was established with the Clustal algorithm of MacVector.

Expression of the *L.donovani* Topo I-like protein in *Escherichia coli* and relaxation assays

In order to clone the *TopI* gene from *L.donovani* in a pET-21a expression vector (Novagen) in the appropriate reading frame, a PCR reaction was conducted on pSB129, a plasmid containing the *TopI* gene within a *Xho*I–*Pst*I fragment of ~3.5 kb in a pBluescript SK vector. Two primers, d(GATCGGATCCATG-AAGGTGGAGAATAG) and d(GATCGAGCTCCTCGTAC-TCGGGTGGA), were used in a PCR reaction with *Pfu* DNA polymerase (Stratagene) to obtain a 203 bp DNA fragment corresponding to the first 61 amino acids (183 bp) from the Topo I-like protein. The PCR product was cloned into pET-21a using the *Bam*HI and *Sac*I sites that had been introduced into the 5' and 3' primers. A unique *Ava*I site is located 156 bp downstream of the ATG of the *TOP1*-like gene. Following a double *Ava*I and partial *Sac*I digestion of pSB129 (the *Sac*I site is from pBluescript SK), the remaining portion of the gene was added to the first 156 nt (obtained by PCR). The resulting plasmid, pSB146, containing the entire *TOP1*-like gene under the control of an inducible T7 promoter and with a bacterial

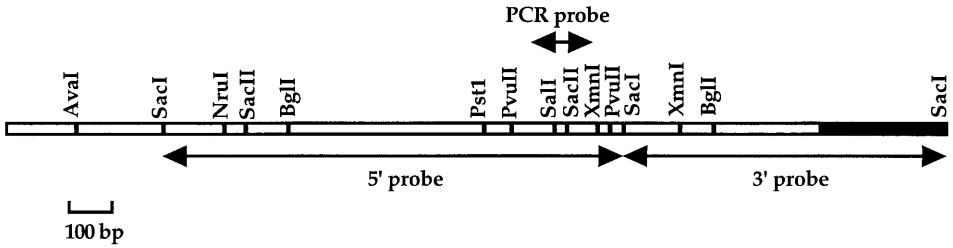

Figure 1. Restriction map of the *TOP1*-like DNA gene of *L.donovani*. The white area of the restriction map corresponds to the *TOP1*-like ORF, whereas the black area corresponds to the 285 bp long DNA that follows this ORF (the *Sac*I site is from the vector). The PCR probe is the 138 bp DNA fragment initially amplified from *L.donovani* genomic DNA that was used for genomic bank screening. 5' and 3' probes respectively correspond to the 5' (1069 bp) and 3' (750 bp) *Sac*I fragments that were used in the Southern blot experiments (Figs 5 and 6).

ribosome-binding site (Shine–Dalgarno sequence), was transformed into *E.coli* strain BL21(DE3) (Novagen). Protein expression and purification by chromatography on hydroxyapatite columns took place as previously described by Gatto *et al.* (23), except that induction was carried out for 2 h at 25°C. Induction was conducted at a lower temperature because we noted that large amounts of *L.donovani* Topo I-like protein formed inclusion bodies at 37°C.

Relaxation assays were carried out in total volumes of 25 µl containing ~200 ng of supercoiled DNA, 5 µl of 5× B cocktail (containing 200 mM Tris, pH 7.5, 500 mM KCl, 50 mM $MgCl_2$, 2.5 mM DTT, 2.5 mM EDTA, 150 µg/ml BSA) and 10 µl of several dilutions of either crude extracts containing the expressed proteins or several dilutions of a dialyzed fraction (0.6 M KP_i) of the expressed proteins. Where indicated, EDTA was added to the reactions in molar excess over Mg^{2+}. The assays were carried out at 37°C for 30 min and the reactions were arrested by phenol extraction. The products of the reactions were subsequently analyzed by electrophoresis in 0.8% agarose gels.

Parasite cell culture and growth induction

Promastigotes from *L.donovani* parasites were grown in supplemented SDM-79 medium at room temperature as described (24,25). *Leishmania* parasites were obtained from Dr Albert Descôteaux (Institut Armand-Frappier, Laval, Québec, Canada). For the growth induction experiments, 10 ml of a *L.donovani* promastigote culture at stationary phase (day 7) were transferred to a total of 50 ml of fresh SDM-79 medium for the given periods of time.

RESULTS AND DISCUSSION

Cloning of the *TOP1*-like gene from *L.donovani*

Degenerate oligonucleotides were constructed and used to generate a PCR fragment from *L.donovani* genomic DNA as described in Materials and Methods. A 138 bp fragment was obtained and its nucleotide sequence was determined. Over 80% of the nucleotides in the third positions of the codons were found to be guanine or cytosine residues (data not shown), which is a hallmark of *Leishmania* genes (26). An alignment of the predicted amino acid sequence with the corresponding region of known eukaryotic type I topoisomerases demonstrated that 35 of the 46 amino acids in that segment were conserved (data not shown). This suggested that we had indeed amplified the targeted region of the *L.donovani* topoisomerase I gene. A random labeled probe was prepared from the above 138 bp PCR fragment and used to screen a *L.donovani infantum* genomic library. Three of the four cosmids that probed positive with the fragment as well as genomic DNA from *L.donovani* were analyzed by Southern blot. What the Southern blot essentially revealed is that common fragments probed positive from both *L.donovani* genomic DNA and the tested cosmids (data not shown). The cosmid with a larger number of common fragments (C_{10}29) was selected for this study. The fragments that were common to both genomic and cosmid DNA were considered for subcloning.

Sequence analysis of the Topo I-like protein

Both strands of two fragments containing altogether the entire *TOP1*-like gene with an overlapping region of 168 bp were sequenced: a 3.5 kb *Sal*I fragment containing roughly the first half of the gene and a 2.1 kb *Pst*I fragment containing the remaining part of the gene (see Fig. 1 for a restriction map). Taking *L.donovani* codon usage into consideration, sequence data was assembled and processed. A single open reading frame (ORF) consisting of 1905 nt was found. The predicted amino acid sequence (635 residues) is presented in Figure 2 as well as its alignment with type I topoisomerases from several sources. It was found that the *L.donovani* cloned ORF and human Topo I share 38.4% identity and 54.4% similarity rates. Roughly, the first 472 amino acid residues of the ORF present an extensive degree of homology with known eukaryotic type I topoisomerases. Eukaryotic topoisomerases are monomeric in nature and can essentially be divided into four major domains (Fig. 2), two of which, the core and the C-terminal domains, are required for its catalytic activity (27–29). In the human counterpart, roughly the first 197 amino acid residues represent the N-terminal domain that is likely important for targeting the enzyme to the nucleus of the cell. This domain is poorly conserved and its length varies among representatives. For this reason, its sequence in the other known type I topoisomerases was not included in the alignment. The core domain in the human Topo I encompassing approximately residues K198–I651 is highly conserved and is important for preferential binding of the enzyme to supercoiled DNA. The C-terminal domain extends roughly from residue Q697 to F765 and contains the catalytically important active site tyrosine (Y723). The core and the C-terminal domains are connected by a poorly

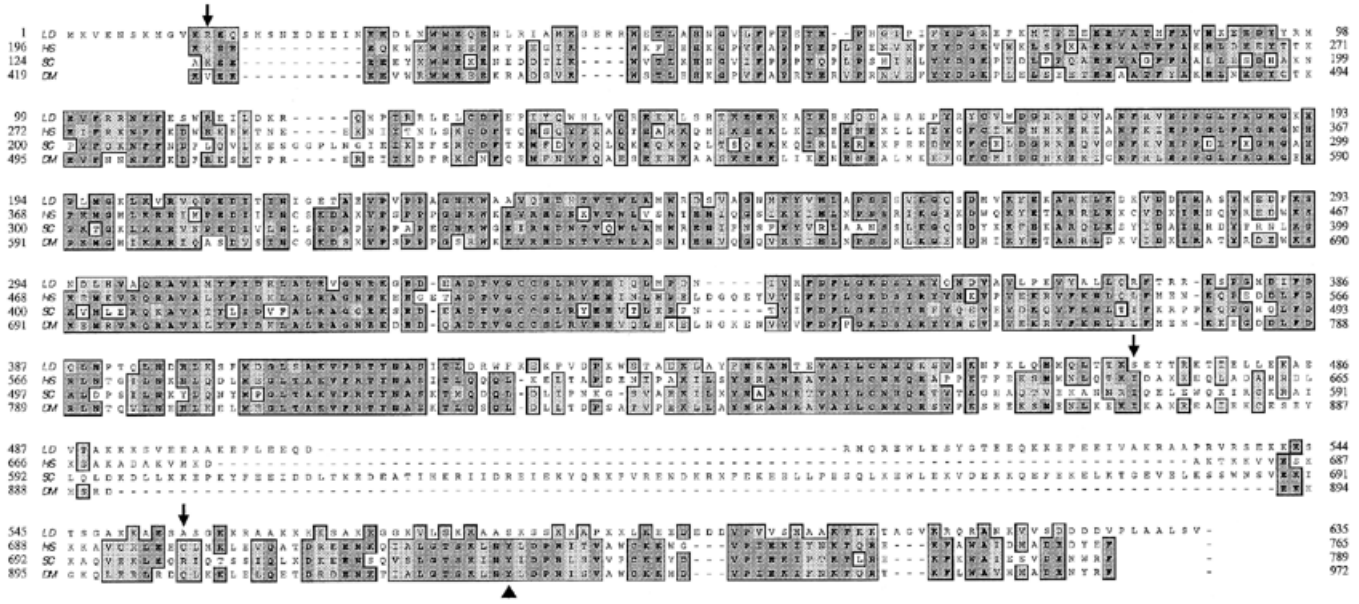

Figure 2. Sequence alignment of the Topo I-like protein from *L.donovani* (LD) with known Topo I from *Saccharomyces cerevisiae* (SC), *Drosophila melanogaster* (DM) and *Homo sapiens* (HS). Amino acid residues in light and dark gray boxes, respectively, represent conserved substitutions and identical residues. Sequences between the first two arrows include the human Topo I core domain, whereas sequences delimited by the second and third arrows correspond to the human Topo I linker domain. Sequences that follow the last arrow correspond to the human Topo I C-terminal domain that normally includes the active site tyrosine residue (indicated by an arrowhead) which is absent in the LD sequence. The *L.donovani* Topo I-like sequence, together with the corresponding nucleotide sequence, has been submitted to the GenBank database and issued the accession number AF145121.

conserved linker region (approximately residues D652–E696) (27,29). The ORF region of the *TOP1*-like gene from *L.donovani* that resembles a eukaryotic type I topoisomerase corresponds to the central core domain (Fig. 2). Interestingly, the N-terminal region of the *L.donovani* protein is relatively short. In fact, there are only 10 amino acid residues before the homology with the central core domain of topoisomerases I begins.

Alanine substitution mutation studies with vaccinia topoisomerase I showed that His265 is essential for the enzyme catalytic activity (30). Interestingly, this His residue is conserved among all known members of the IB topoisomerase family in a VAILCN**H** motif and is also present in the predicted amino acid sequence of the *L.donovani TOP1*-like gene (H453; Fig. 2). His265 is only nine amino acids away from the tyrosine active site in the vaccinia enzyme and the interval between the homologous histidine of cellular topoisomerases and their respective tyrosine active site is interrupted by a linker region of variable length. The linker region has no counterpart in the poxvirus topoisomerases and is poorly conserved within the cellular IB topoisomerase family. It is also dispensable for catalytic activity (27–29). The tyrosine active site of these enzymes usually lies within a SKXX**Y** motif within the C-terminal end of topoisomerase I (16–18). Amino acid sequence analysis of the *L.donovani* ORF after the first 472 amino acid residues seems to indicate a loss of homology starting approximately in the region where the linker domain would be expected to begin if such a domain were to exist. Moreover, no SKXX**Y** motif could be identified. However, following a region of substantial disparity among the sequences of the aligned topoisomerases, we found a stretch near the end of the ORF (in the conserved DVPIEK motif region) in which homology seems to be recovered (in amino acid residues D597–K609 of the protein; Fig. 2). At this point, we propose three explanations for the observed phenomenon: (i) one or several mutations had occurred within the cosmid, leading to the loss or replacement of the tyrosine active site; (ii) this is a new class of eukaryotic type I topoisomerase with an active site other than the conventional tyrosine; (iii) we had cloned a pseudogene.

Sequencing of the 3'-region of the gene from genomic DNA

It is a known fact that the overproduction of active topoisomerases in bacteria is problematic, if not lethal, for the cells (for example see 31). It is therefore possible that inactivated versions of the topoisomerase I gene are the only ones that could be maintained in the cell. In that case, only clones encoding a mutated inactive form of topoisomerase I could be recovered. To verify this hypothesis, we conducted a PCR reaction with genomic DNA from *L.donovani* using one 5' oligonucleotide corresponding to positions F409–S415 of the ORF (before the loss of homology) and another in the 3'-end of the gene (236 nt following the stop codon). The PCR product was subcloned and sequenced. The nucleotide sequence obtained was identical to that stemming from the cosmid DNA, excluding the possibility of mutations (data not shown). This result also clearly demonstrates that the cloned DNA sequence corresponds to one gene from *L.donovani* and, therefore, that it is not the result of multiple insertions of DNA sequences from disparate regions of the genome. This is also supported by the results of Southern blot experiments described in another section.

Protein expression and relaxation assays

Catalytic residues other than the tyrosine active site have never been identified in topoisomerases, although histidine and cysteine residues were suggested (1). Because they share common

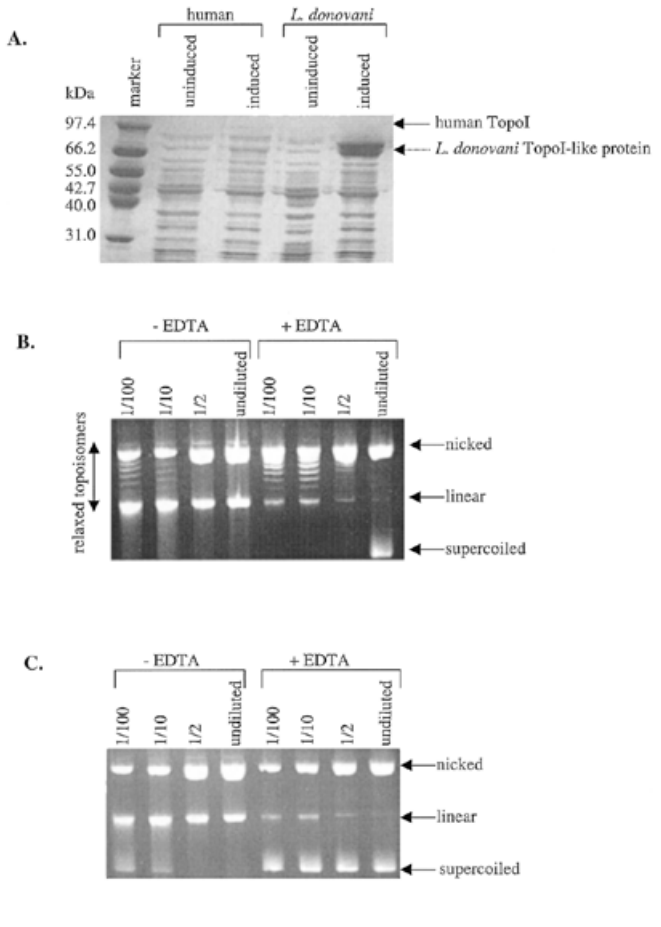

Figure 3. Relaxation assays with crude extracts of *E.coli* cells overproducing the Topo I-like protein of *L.donovani*. Crude extracts of induced (0.4 mM IPTG) or uninduced *E.coli* BL21(DE3) cells carrying either pET1B (human Topo I) or pSB146 (*L.donovani* Topo I-like protein) were obtained as described in Materials and Methods. (**A**) An aliquot of the various crude extracts as indicated was loaded on a polyacrylamide gel that was stained with Coomassie brilliant blue after electrophoresis. (**B** and **C**) Relaxation assays were performed as described in Materials and Methods with crude extracts from induced cells carrying pET1B and pSB146, respectively.

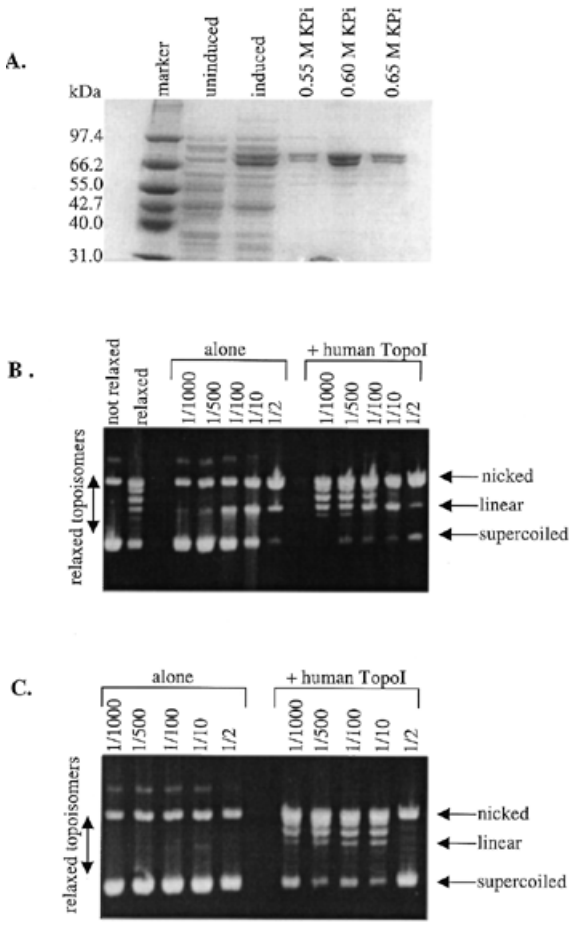

Figure 4. Relaxation assays with partially purified Topo I-like protein of *L.donovani*. The Topo I-like protein of *L.donovani* from crude extracts of induced *E.coli* BL21(DE3) cells carrying pSB146 was purified as described in Materials and Methods. (**A**) An aliquot of crude extracts from induced and uninduced cells and of different fractions eluted from the hydroxyapatite column were loaded on a polyacrylamide gel as indicated. After electrophoresis, the gel was stained with Coomassie brilliant blue. (**B** and **C**) Relaxation assays were performed as described in Materials and Methods with the 0.60 M KP_i fraction in the absence or the presence of EDTA, respectively. Ten units of purified human topoisomerase I were added (+ human Topo I) or not (alone) during the relaxation assays.

structural and mechanistic features, type IB DNA topoisomerases and site-specific recombinases are, more often than not, believed to derive from a common ancestral strand transferase (32). DNA strand breakage and joining by site-specific recombinases occurs by transesterification reactions. The integrase family of the conservative site-specific recombinases perform these reactions via a tyrosine residue, as is the case with topoisomerases, while the members of the resolvase–invertase family accomplish the same reactions by a serine active site (33). Interestingly, a serine (S580) of the *L.donovani* ORF is aligned with the tyrosine active sites of other known members of the topoisomerase IB family (Fig. 2). With this in mind, the *TOP1*-like gene was cloned into a pET-21a expression vector with its own start ATG codon, under the control of a bacterial Shine–Dalgarno sequence and a phage T7 RNA polymerase promoter (plasmid pSB146; Materials and Methods). We first tested for the ability of pSB146 to correct the growth defect of an *E.coli topA* null mutant (34). Whereas plasmid pET1B carrying the human topoisomerase I gene in a similar expression vector (23) was able to correct the growth defect of this mutant, we found that pSB146 was not (data not shown). The complementation of this *topA* null mutant by human topoisomerase I expression has been previously demonstrated (31). The fact that the *L.donovani TOP1*-like gene was not able to correct the growth defect of the same *topA* null mutant suggested that the Topo I-like protein was either not produced in *E.coli* or was catalytically inactive. To address this question, plasmids pSB146 and pET1B were both introduced into *E.coli* BL21(DE3) cells and protein overproduction was induced as described in Materials and Methods. As can be seen in Figure 3A, a protein with a molecular weight that matches the estimated 73.6 kDa according to the amino acid composition of the Topo I-like protein of *L.donovani* was detected in the crude extract of induced cells

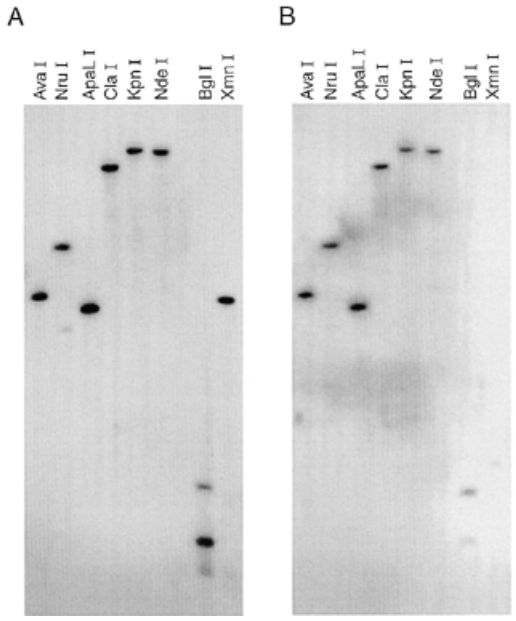

Figure 5. The first Southern blot analysis to screen for the presence of *TOP1*-like pseudogenes in *L.donovani*. Southern blot experiments were performed as described in Materials and Methods. The membranes were probed with the 1069 bp 5' *Sac*I DNA fragment (**A**), stripped and then reprobed with the 750 bp 3' *Sac*I DNA fragment (**B**).

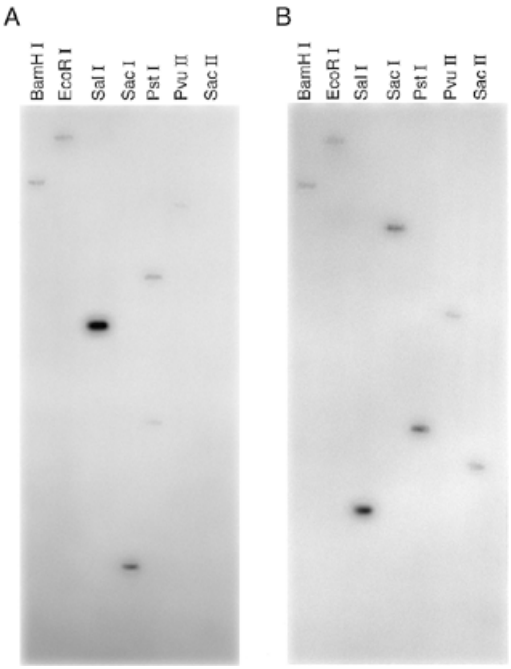

Figure 6. The second Southern blot analysis to screen for the presence of *TOP1*-like pseudogenes in *L.donovani.* Southern blot experiments were performed as described in Materials and Methods. The membranes were probed with the 1069 bp 5' *Sac*I DNA fragment (**A**), stripped and then reprobed with the 750 bp 3' *Sac*I DNA fragment (**B**).

carrying pSB146 (compare lane 4, uninduced, with lane 5, induced). Such a protein was not detected in a crude extract from induced cells carrying the vector alone (pET-21a; data not shown). A small amount of human topoisomerase I protein (91 kDa) was detected in the crude extract of induced cells carrying pET1B (compare lane 2, uninduced, with lane 3, induced). Despite the fact that human topoisomerase I was produced in much smaller amounts as compared to *L.donovani* Topo I-like protein, relaxation activity, at a dilution of at least up to 100-fold, was only detected in crude extracts of cells over-producing the human protein (compare Fig. 3B, human topoisomerase I, with Fig. 3C, *L.donovani* Topo I-like protein). Relaxation activity was detected despite the presence of a potent nuclease activity in the crude extract (Fig. 3B, –EDTA). Relaxation assays were also performed in the presence of excess EDTA to chelate divalent cations such as Mg^{2+}, in order to inactivate the nuclease activity. As expected, since divalent cations are not required for type IB topoisomerase I activity (1), human topoisomerase I activity was still detected (Fig. 3B, +EDTA). Adding EDTA did not, however, allow the detection of relaxation activity in crude extracts containing *L.donovani* Topo I-like protein (Fig. 3C, +EDTA). These results strongly suggested to us that this protein was catalytically inactive, at least in the crude extract. The Topo I-like protein was then partially purified by chromatography on a hydroxyapatite column. The proteins were eluted with a linear gradient of 0.2–0.9 M potassium phosphate as described in Materials and Methods and the protein of interest was eluted in fractions between 0.55 and 0.65 M KP_i inclusively, with a peak at 0.6 M KP_i (Fig. 4A). This is consistent with the elution pattern of human (23) and *Caenorhabditis elegans* topoisomerases I (35). Relaxation assays with dialyzed fractions under several experimental conditions, including substitution of Mg^{2+} for Mn^{2+}, proved unsuccessful (see, for example, Fig. 4B and C). In order to demonstrate that there were no inhibitors in the bacterial extracts that were masking the activity, human topoisomerase I, purified by the same protocol, was added to some reactions. As can be seen in Figure 4B (–EDTA) and C (+EDTA), human topoisomerase I activity was not inhibited. A nuclease activity was still present in the eluted fractions in the absence of EDTA (Fig. 4B). A similar amount of this nuclease activity was also detected in the 0.6 M KP_i fraction from induced cells carrying the vector alone (pET-21a), demonstrating that this activity is not linked to the *L.donovani* Topo I-like protein (data not shown). Altogether, these results strongly suggest that the *L.donovani* Topo I-like protein is not catalytically active.

Screening for other *TOP1*-like genes

In order to verify the possible existence of additional *TOP1*-alleles, genomic DNA from *L.donovani* was analyzed by Southern blot. DNA was digested with a total of 15 restriction enzymes. The results of the described Southern blots are presented in Figures 5 and 6. Genomic DNA from the parasite was digested with several enzymes whose restriction sites were absent in the *TOP1*-like gene: *Bam*HI, *Apa*LI, *Kpn*I, *Cla*I, *Eco*RI and *Nde*I (Figs 5 and 6; see Fig. 1 for a restriction map). The membranes were first probed with a 1069 bp *Sac*I fragment spanning the first part of the gene (Figs 5A and 6A), that which substantially resembles a *top1* gene. In all cases, a single

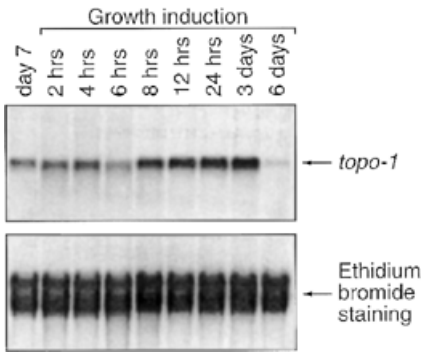

Figure 7. Expression of the *TOP1*-like gene in *L.donovani* as a function of growth. Parasite culture and northern blots were performed as described in Materials and Methods. The *TOP1*-like mRNA (*topo-1*) is 3.4 kb long according to molecular weight markers.

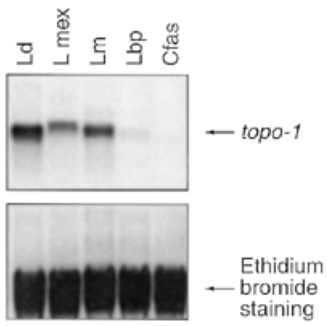

Figure 8. Detection of *TOP1*-like mRNA in various kinetoplastid parasites. RNA from various kinetoplastid parasites including *L.donovani* (Ld), *Leishmania mexicana* (L mex), *Leishmania major* (Lm), *Leishmania braziliensis* (Lbp) and *Crithidia fasciculata* (Cfas) was used for northern blot analysis with the 1069 bp 5' *Sac*I DNA fragment from the *TOP1*-like gene of *L.donovani* as a probe.

band probed positive with this fragment. Restriction enzymes with known sites in the gene were also used in the blots: *Sal*I, *Sac*I, *Pst*I, *Pvu*II, *Sac*II, *Ava*I, *Nru*I, *Bgl*I and *Xmn*I (Figs 5 and 6; see Fig. 1 for a restriction map). Again, the bands that probed positive with the 1069 bp *Sac*I fragment are consistent with what was predicted by the restriction map and no other bands appeared on the blot. Together, these results suggest that there are no additional *TOP1* alleles in the genome of *L.donovani* or that the used probes did not allow the detection of other *TOP1*-like genes.

The membranes were subsequently stripped as described and probed with a random labeled 750 bp long *Sac*I fragment (Figs 5B and 6B), corresponding to the 3'-end of the gene plus an extra 285 bp of DNA following the stop codon (Fig. 1). In the case of enzymes that do not cut in the *TOP1*-like gene, the bands that had probed positive with the 5' *Sac*I fragment also probed positive with the 3' fragment. All our results therefore indicate that the cloned gene really exists and that it is present as a single copy in the genome of the parasite.

Expression of the *TOP1*-like gene

A northern blot experiment was conducted to establish how the *TOP1*-like gene is expressed in the parasite. Total RNA was extracted from *L.donovani* promastigotes at different time intervals, as described in Materials and Methods. The RNA was subsequently probed with the 1069 bp *Sac*I fragment overlapping the region of the gene coding for a protein that resembles a type I topoisomerase. In each case, a single band of ~3.4 kb probed positive with the fragment. As can be seen in Figure 7, the intensity of the band increases as the parasite enters the growing phase and dramatically decreases when the stationary phase is reached. This suggests that the *TOP1*-like gene is preferentially expressed in actively growing *L.donovani*. Additionally, the fact that a single band probed positive with the *TOP1*-like fragment seems to suggest that no other forms of the messenger exists in the cell. Trypanosomatids have a distinctive way of storing and expressing their genetic information. No introns have been identified in the genes of *Leishmania*, so alternative splicing has not to date been described in these and related parasites (reviewed in 36). To further examine the question of whether the *TOP1*-like gene was fully transcribed, the membrane was stripped and reprobed with the *Sac*I fragment in the 3'-region of the gene. Again, a band of 3.4 kb probed positive with the fragment, suggesting that the gene is transcribed in its full length and confirming our belief that no form of alternative splicing occurs to yield a mature *TOP1* messenger (data not shown). Messengers of similar sizes were also detected in other kinetoplastid parasites when the 1069 bp *Sac*I fragment from the *TOP1*-like gene of *L.donovani* was used as a probe (Fig. 8).

In light of our results, we sought to explain how this gene could participate in conferring a topoisomerase I-like activity in *L.donovani.* Three possibilities are considered. First, it is conceivable that the gene does encode a fully active topoisomerase I but that no activity was detected under our *in vitro* and/or *in vivo* conditions. Despite the fact that a type IB topoisomerase with an active site other than the conventional tyrosine has never been described, the possibility cannot be ruled out, and it would account for the fact that the protein was inactive under the assay conditions that were used with other known eukaryotic type IB topoisomerases. Since not much is known on a molecular level of *L.donovani* and other related parasites, another possibility is that some unknown and yet undescribed form of post-transcriptional or post-translational modification(s) occurs in these parasites to yield a fully active topoisomerase I. The third hypothesis is that the active site of the *L.donovani* topoisomerase I is carried by a second subunit and therefore encoded in a different gene. It is now well established that human topoisomerase I activity can be reconstituted *in vitro* by mixing purified core domain together with C-terminal fragments of variable length (28). Recently, a two-hybrid system was used to identify proteins that interact with the central conserved domain of *Saccharomyces cerevisiae* DNA topoisomerase I (37). Several different C-terminal domain-containing fragments of topoisomerase I were identified as specific interacting polypeptides. None of these fragments overlapped with the core domain. Moreover, co-expression of the two domains in yeast partially complemented the growth problems of a mutant deficient in topoisomerase I activity and *in vitro* assays confirmed this restored activity (37). For the

moment, we can only speculate on what really occurs in the parasite and work is currently in progress to test the various hypotheses.

ACKNOWLEDGEMENTS

We thank Dr Albert Descôteaux (Institut Armand-Frappier) for the *L.donovani* strain and invaluable help in many aspects of this work. We also thank Dr Leroy F. Liu (UMDNJ, Robert Wood Johnson Medical School) for plasmid pET1B and members of its laboratory for advice on topoisomerase I assay and purification. M.D. and M.O. are recipients of a scholarship from les Fonds de la Recherche en Santé du Québec and B.P. is a recipient of a scholarship from the Medical Research Council of Canada. M.O. holds a Burroughs Wellcome Fund Award in Molecular Parasitology.

REFERENCES

1. Wang,J.C. (1996) *Annu. Rev. Biochem.*, **65**, 635–692.
2. Wang,J.C. (1994) *Adv. Pharmacol.*, **29A**, 1–19.
3. Drlica,K. and Franco,R.J. (1988) *Biochemistry*, **27**, 2253–2259.
4. Chen,A.Y. and Liu,L.F. (1994) *Annu. Rev. Pharmacol. Toxicol.*, **34**, 191–218.
5. Hsiang,Y.-H., Hertzberg,R., Hecht,S. and Liu,L.F. (1985) *J. Biol. Chem.*, **260**, 14873–14787.
6. Hsiang,Y.-H., Lihou,M.G. and Liu,L.F (1989) *Cancer Res.*, **49**, 5077–5082.
7. D'Arpa,P., Beardmore,C. and Liu,L.F. (1990) *Cancer Res.*, **50**, 6919–6924.
8. Covey,J.M., Jaxel,C., Kohn,K.W. and Pommier,Y. (1989) *Cancer Res.*, **49**, 5016–5022.
9. Ouellette,M. and Papadopoulou,B. (1993) *Parasitol. Today*, **9**, 150–153.
10. Shapiro,T.A. and Englund,P.T. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 950–954.
11. Burri,C., Bodley,A.L. and Shapiro,T.A. (1996) *Parasitol. Today*, **12**, 226–231.
12. Bodley,A.L. and Shapiro,T.A. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 3726–3730.
13. Bodley,A.L, Wani,M.C., Wall,M.E. and Shapiro,T.A. (1995) *Biochem. Pharmacol.*, **50**, 937–942.
14. Werbovetz,K.A., Lehnert,E.K., Macdonald,T.L. and Pearson,R.D. (1992) *Antimicrobial Agents Chemother.*, **36**, 495–497.
15. Werbovetz,K.A., Spoors,P.G., Pearson,R.D. and Macdonald,T.L. (1994) *Mol. Biochem. Parasitol.*, **65**, 1–10.
16. Eng,W., Pandit,S.D. and Sternglanz R. (1989) *J. Biol. Chem.*, **262**, 13373–13376.
17. Lynn,R.M., Bjornsti,M., Caron,P.R. and Wang,J.C. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 3559–3563.
18. Shuman,S., Kane,E.M. and Morham,S.G. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 9793–9797.
19. Hsieh,T.-S., Brown,S.D., Huang,P. and Fostel,J. (1992) *Nucleic Acids Res.*, **23**, 6177–6182.
20. Lamontagne,J. and Papadopoulou,B. (1999) *J. Biol. Chem.*, **274**, 6602–6609.
21. Medina-Acosta,E. and Cross,G.A.M. (1993) *Mol. Biochem. Parasitol.*, **59**, 327–330.
22. Shambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23. Gatto,B., Sanders,M.M., Yu,C., Wu,H.-Y., Makhey,D., LaVoie,E.J. and Liu,L.F. (1996) *Cancer Res.*, **56**, 2795–2800.
24. Olivier,M. and Tanner,C.E. (1987) *Infect. Immunol.*, **55**, 467–471.
25. White,T.C., Fase-Fowler,F., Van Luenen,H., Calafat,J. and Borst,P. (1988) *J. Biol. Chem.*, **263**, 16977–16983.
26. Langford,C.K., Ullman,B. and Landfear,S.M. (1992) *Exp. Parasitol.*, **74**, 360–361.
27. Stewart,L., Ireton,G.C. and Champoux,J.J. (1996) *J. Biol. Chem.*, **13**, 7602–7608.
28. Stewart,L., Ireton,G.C. and Champoux,J.J. (1997) *J. Mol. Biol.*, **269**, 355–372.
29. Champoux,J.J. (1998) *Prog. Nucleic Acid Res. Mol. Biol.*, **60**, 111–132.
30. Petersen,B.F. and Shuman,S. (1997) *J. Biol. Chem.*, **7**, 3891–3896.
31. Taylor,S.T. and Menzel R. (1995) *Gene*, **167**, 69–74.
32. Cheng,C., Kussie,P., Pavletic,N. and Shuman,S. (1998) *Cell*, **92**, 841–850.
33. Sadowski,P.D. (1993) *FASEB J.*, **7**, 760–767.
34. Drolet,M., Phoenix,P., Menzel,R., Massé,É., Liu,L.F. and Crouch,R.J. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 3526–3530.
35. Park,S.M. and Koo H.-S. (1994) *Biochim. Biophys. Acta*, **1219**, 47–54.
36. Vanhamme,L. and Pays,E. (1995) *Microbiol. Rev.*, **2**, 223–240.
37. Park,H. and Sternglanz,R. (1998) *Chromosoma*, **107**, 211–215.