

Mitochondrial and Nuclear Localization of Topoisomerase II in the Flagellate *Bodo saltans* (Kinetoplastida), a Species with Non-catenated Kinetoplast DNA*

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Ivana Gažiová‡ and Julius Lukeš§

From the Institute of Parasitology, Czech Academy of Sciences and Faculty of Biology, University of South Bohemia, 37005 České Budějovice, Czech Republic

We have studied topoisomerase II (topo II) in the cells of *Bodo saltans*, a free-living bodonid (Kinetoplastida). Phylogenetic analysis based on the sequence of the entire topo II gene, which is a single-copy gene, confirmed that *B. saltans* is a predecessor of parasitic trypanosomatids. Antibodies generated against either an overexpressed unique C-terminal region of topo II or a synthetic oligopeptide derived from the same region did not cross-react with cell lysates of related trypanosomatids, while they recognized a single specific band in the *B. saltans* lysate. Immunolocalization experiments using both antibodies showed that topo II is evenly dispersed throughout the kinetoplast. This is in striking difference from the localization of topo II in other flagellates, where it occurs in two antipodal centers flanking the kinetoplast disk. Moreover, the same topo II has a distinct localization in multiple loci at the periphery of the nucleus of *B. saltans*. With a minicircle probe derived from the conserved region we have shown that all relaxed non-catenated minicircles are confined to the globular kinetoplast DNA bundle. Therefore, in the mitochondrion of this primitive eukaryote topo II does not catenate relaxed DNA circles into a network *in vivo*, while a decatenating activity is present in partially purified cell lysates.

Topoisomerase II (topo II)¹ is a ubiquitous enzyme that catalyzes strand passing of double-stranded DNA in an ATP-dependent manner. It is involved in a number of processes, including replication and transcription of nuclear and mitochondrial DNA, chromosome segregation, and chromatin organization (1).

Topo II is an important component of the kinetoplast-mitochondrion organelle of the kinetoplastid protozoa. These flagellates represent one of the most primitive groups of eukaryotes, equipped with a number of unique features. Their mitochon-

drial DNA, termed kinetoplast (k) DNA, contains up to 40% of total cellular DNA and is composed of thousands of minicircles and dozens of maxicircles. Maxicircles encode mitochondrial genes, the transcripts of which undergo extensive editing of the uridine insertion/deletion type. Minicircles bear guide RNA genes, which provide information for the editing process (for recent review see Refs. 2 and 3).

The order Kinetoplastida is divided into the suborder Bodonina, which comprises free-living commensalic or parasitic biflagellated species, and the suborder Trypanosomatina, members of which are equipped with a single flagellum and are obligatory parasitic. In the mitochondrion of Trypanosomatina, the DNA molecules are present as relaxed circles catenated into a single giant network (for recent review see Refs. 4 and 5). So far, topo II was studied in detail in the model trypanosomatids *Crithidia fasciculata* and *Trypanosoma brucei*, where it is involved in decatenation, replication, and recatenation of the kDNA minicircles (6–8). The enzyme has also a very characteristic localization in two opposing antipodal protein centers (8). Topoisomerases are considered to be a prime target for the antitrypanosomal (9, 10) and antileishmanial (11) therapy.

In primitive kinetoplastids that belong to the Bodonina, the kDNA is composed of either large circles (~200 kb) bearing tandemly arranged minicircle-like sequences, as is the case in *Trypanoplasma borreli* (12), or small circles (1.4–10.0-kb-long minicircles). In *Cryptobia helicis*, these minicircles are supercoiled and non-catenated (13), while they are relaxed and non-catenated in *Bodo saltans* and *Dimastigella* spp. (14, 15). *B. saltans* is a free-living omnipresent flagellate that represents a significant component of the biological community of the sewage cleaning technologies. As an evolutionary predecessor of parasitic trypanosomatids (14, 16, 17), it qualifies as an important model organism for the studies of RNA editing and kDNA structure (18). Its minicircles encode classical guide RNA genes (14), which serve for editing of the maxicircle-located genes that have a novel order and editing pattern (19). Due to their function, proper replication and segregation of minicircles is therefore critical for the survival of daughter cells.

Insight into the role of topo II in this primitive bodonid is important for our understanding of evolution of catenation, replication, and other exciting features of the kinetoplast. Herein, we present characterization of the topo II gene of *B. saltans* and novel localization of the protein in its mitochondrion and nucleus. Surprisingly, topo II is dispersed throughout the kinetoplast bundle, despite the absence of a catenated kDNA network in this species. This suggests a significant functional difference between the *B. saltans* and trypanosomatid topoisomerases.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY083347.

‡ Present address: Institute of Entomology, Czech Academy of Sciences, 37005 České Budějovice, Czech Republic.

§ To whom correspondence should be addressed: Institute of Parasitology, Czech Academy of Sciences, Branišovská 31, 37005 České Budějovice, Czech Republic. Tel.: 00420-38-7775416; Fax: 00420-38-5310388; E-mail: jula@paru.cas.cz.

¹ The abbreviations used are: topo II, topoisomerase II; kDNA, kinetoplast DNA; RT, reverse transcriptase; PBS, phosphate-buffered saline; DAPI, 4',6'-diamidino-2-phenylindole; BSA, bovine serum albumin.

EXPERIMENTAL PROCEDURES

Cell Culture—Cultivation of *B. saltans*, strain K1 isolated from Lake Konstanz, Germany, was up-scaled to obtain sufficient amount of cells (10^8 – 10^9) and performed essentially as described (19). Feeder bacteria *Alcaligenes xylosoxidans denitrificans* were carefully removed by repeated overnight sedimentation in columns at 4 °C followed by multiple differential sedimentation of bacteria and flagellates during centrifugations. *C. fasciculata*, *Leishmania tarentolae*, and *T. brucei* were cultivated according to standard protocols.

Construction and Screening of the Genomic Library—Total DNA of *B. saltans* was isolated as described elsewhere (14, 19). The 1.5-kb-long ATP-binding domain of the topo II gene of *B. saltans* (BstopoII) was PCR-amplified with degenerate oligonucleotides 12C6 (CATGT(A/C)CT(C/G)(A/C)T(A/G)(C/A)(G/A)CC(G/A)GAGA(T/C)GTAC) and 12C-7 (CC(A/G)TC(T/G)GC(A/G)TCCTG(A/C)TC(T/G)GTAC(G/A/A) using the following program: 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 2 min (30 cycles). The amplicon was cloned into the pBluescript SK⁻ vector (Stratagene) and sequenced using the Prism DyeDeoxy Terminator Cycle Sequencing Kit (PerkinElmer Life Sciences). A genomic library was constructed using partially *Sau3AI*-digested DNA that was, after phenol-chloroform extraction and ethanol precipitation, ligated into the *Bam*HI-digested λ GEM11 vector arms and subsequently packaged using the Packagene extract (Promega) and amplified in the MB406 *Escherichia coli* cells. The resulting library contained $\sim 1.25 \times 10^5$ plaque-forming units, and half of it was screened with a fragment containing the ATP-binding domain of the BstopoII gene radioactively labeled by nick translation. Screening was performed in the hybridization solution (3 \times SSC, 5 \times Denhardt's solution (0.1% Ficol, 0.1% BSA, 0.1% polyvinyl pyrrolidone), 0.1% SDS, 25 μ g/ml sheared herring sperm DNA, and 10% dextran sulfate) at 65 °C overnight. The membranes were washed twice in 3 \times SSC, 0.1% SDS, twice in 1 \times SSC, 0.1% SDS, twice in 0.3 \times SSC, 0.1% SDS, and twice in 0.1 \times SSC, 0.1% SDS, each wash at 65 °C for 20 min. Three positive plaques were purified by two rounds of plating and hybridization. The recombinant phage DNA from two different selected clones was analyzed by Southern hybridization after the digestions with *Eco*RI, *Bam*HI, *Hind*III, *Sac*I, and *Sal*I, resolved on 0.75% ethidium bromide-stained agarose gel, transferred to a Hybond N⁺ membrane (Amersham Biosciences), and hybridized with the same probe. Hybridizing fragments were cloned into appropriately predigested pGEM11f or pGEM7f vectors (Promega) and sequenced.

Phylogenetic Analysis—Additional topo II gene sequences used in this work were retrieved from GenBankTM and include: *Leishmania infantum* (AY004225), *Leishmania donovani* (AF150876), *Leishmania chagasi* (O61078), *Crithidia fasciculata* (P27570), *T. brucei* (P12531), *Trypanosoma cruzi* (P30190), *Dictyostelium discoideum* (P90520), *Plasmodium falciparum* (P41001), *Saccharomyces cerevisiae* (P06786), *Schizosaccharomyces pombe* (AL031174), *Encephalitozoon cuniculi* (AL590444), *Drosophila melanogaster* (P15348), *Caenorhabditis elegans* (Z49069), *Sus scrofa* (O46374), and *Homo sapiens* α (P11388) and β (Q02880) subunits. Multiple alignment of the amino acid sequences was performed using the CLUSTAL W package (www.ddbj.nig.ac.jp/searches/e.html) and is available upon request. Maximum likelihood and maximum parsimony analyses were performed using PUZZLE-TREE (20) and PAUP* (21), respectively, with *P. falciparum* as an outgroup. The Dayhoff amino acid substitution model and the model of rate heterogeneity with γ -distribution in 10 rate categories, as implemented in PUZZLE-TREE, were applied. The corresponding Quartet-puzzling and bootstrap analyses included 1000 replicates.

Southern Hybridization and RT-PCR—10- μ g aliquots of total genomic DNA of *B. saltans* were digested with *Bam*HI, *Eco*RI, *Sal*I, *Sma*I, and *Xho*I, separated on 0.75% ethidium bromide-stained agarose gel, and blotted. A C-terminal region of BstopoII used for expression (see below) was labeled with DIG/dUTP (Roche Molecular Biochemicals) by PCR and used as a probe. Total cellular mRNA was extracted from 1×10^8 exponentially growing cells using the QuickPrep micro mRNA kit according to manufacturer's instructions (Amersham Biosciences). The first cDNA strand was synthesized from 300 ng of poly(A) using the SuperScript Preamplification system (Invitrogen). Control was performed without the addition of reverse transcriptase. Two primer pairs were used: IK2-GCAAGGAGCTCTGTGATCTC (positions 1481–1500 within the coding region) and IK6-CTTCTTGAGTTGCGT-GTTTCAG (positions 3091–3111), and IK20 and IK21 (positions 2896–2916 and 3572–3591; for sequences see below). The PCR program consisted of 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 90 s with a final extension 72 °C for 10 min.

Expression and Purification of Recombinant Protein—A 700-bp-long

fragment covering most of the C-terminal variable region of BstopoII was PCR-amplified from the *Eco*RI genomic clone with oligonucleotide IK20-GCCATATGCATCATCATCATCATGCAGATTCCTCCGCA-AGCCCC (containing the *Nde*I site that creates the initiation codon and six histidine codons (underlined)) and IK21-CCGGATCCTCAACGCA-CCACCATCTCCCCAG (containing the *Bam*HI site and the stop codon (underlined)). The amplicon digested with *Nde*I and *Bam*HI was gel-purified and cloned into the pRSET A expression vector (Invitrogen) digested with the same restriction enzymes. The resulting expression plasmid encoding the His₆-3'-topo II end was transformed into the *E. coli* strain BL21(DE3) (Novagen). 1 ml of overnight culture was added to 0.5 liter of medium and incubated at 25 °C until A₆₀₀ reached 1.0. Isopropyl-1-thio- β -D-galactopyranoside was then added to a final concentration 0.05 mM, and the culture was grown for another hour at 25 °C. The cells were collected by centrifugation, resuspended in the 1 \times binding buffer (Novagen), and lysed by the addition of 100 μ g/ml lysozyme and three cycles of freezing and thawing followed by sonication for 30 s. The suspension was centrifuged, and the His-tagged overexpressed protein was purified from the supernatant by the metal-chelate affinity chromatography on a Ni²⁺ chelate resin under native conditions as specified by the manufacturer (Novagen).

Preparation of Antibodies and Western Blotting—Polyclonal antibody BstopoII-1 was prepared by immunizing rabbit at 2-week intervals with four subcutaneous injections of 0.5 mg of purified recombinant topo II protein emulsified with complete (first injection) and incomplete (following injections) Freund's adjuvant. Another rabbit antibody (BstopoII-2) was raised against a synthetic oligopeptide corresponding to the 1163–1177-amino acid region (QRSNEKWKFFRRKCC) from the unique C terminus of the *B. saltans* topo II gene. The last cysteine was added to enhance efficient conjugation of the synthetic peptide to ovalbumin. Conjugation was performed as recommended by the manufacturer (Pierce). Each injection, containing 0.3 mg of a conjugate of the synthetic BstopoII peptide and ovalbumin, was injected into a rabbit following the protocol described above. Serum was collected 7–10 days after the third and fourth injections and was tested by Western blotting. Both the His-topo II fusion protein and the synthetic peptide were immobilized on the AminoLink Plus Coupling Gel (Pierce) and used for affinity purification of both polyclonal antisera. Columns were washed with 10 mM Tris (pH 7.5), equilibrated with 100 mM glycine (pH 2.5), and washed again with 10 mM Tris (pH 8.8). Following equilibration was made with 100 mM triethylamine (pH 11.5) and washed with 10 mM Tris (pH 7.5) until pH 7.5 was reached. The antisera were diluted 10 times with 10 mM Tris (pH 7.5) and passed through the columns, which were then washed with 10 mM Tris (pH 7.5). The majority of bound antibodies was eluted with 100 mM glycine (pH 2.5), and after a wash with 10 mM Tris (pH 8.8), the remaining antibodies were released with 100 mM triethylamine (pH 11.5). The antibody-containing fractions were pooled and dialyzed against PBS with 0.02% sodium azide. Cell lysates were prepared from the *B. saltans*, *C. fasciculata*, *L. tarentolae*, and *T. brucei* cells at 1×10^7 cells/10 μ l as described elsewhere (22), analyzed on a 8% SDS-PAGE gel, and blotted. The *E. coli* extracts containing recombinant His-tagged protein (30 kDa) were analyzed on 12% SDS-PAGE gels. Blots were probed with polyclonal antisera raised against either the fusion protein (BstopoII-1; 1:2000) or the synthetic peptide (BstopoII-2; 1:1000). The secondary anti-rabbit IgG antibody (1:4000) coupled to alkaline phosphatase was visualized according to manufacturer's protocols using the ECL kit (Amersham Biosciences).

Immunocytochemistry—Cells were partially purified from the feeder bacteria by differential centrifugation and overnight sedimentation and resuspended in PBS at a concentration 1×10^7 cells/100 μ l. 20 μ l of the cell suspension was spotted onto poly-L-lysine-coated slides, and cells were allowed to adhere for 20 min in a humidity chamber. The slides were submerged into 4% paraformaldehyde in PBS for 3 min at room temperature, and fixation was stopped by washing the slides with 0.1 M glycine, pH 8.6, in PBS for 5 min followed by two washes in PBS for 5 min. Cells were permeabilized in 0.5% Tween 20 in PBS, and the blocking solution (10% BSA and 10% goat serum) in a humid chamber for 30 min at room temperature. After washing once in PBST (0.05% Tween 20 in PBS) for 5 min, slides were incubated with the affinity-purified primary antibody diluted in PBST (BstopoII-1; 1:100; BstopoII-2; 1:20), and incubation was performed under the same conditions for 2 h. Slides were washed four times in PBST 5 min each and then incubated with the Cy3-conjugated goat anti-rabbit IgG antibody (1:400; Amersham Biosciences) for 1 h at room temperature in a humidity chamber. The incubation was followed by three washes in PBST and one wash in PBS 5 min each at room temperature. Finally, the slides were incubated in PBS containing 0.1 μ g/ml DAPI for 3 min at room temperature, rinsed with distilled water, and mounted into the

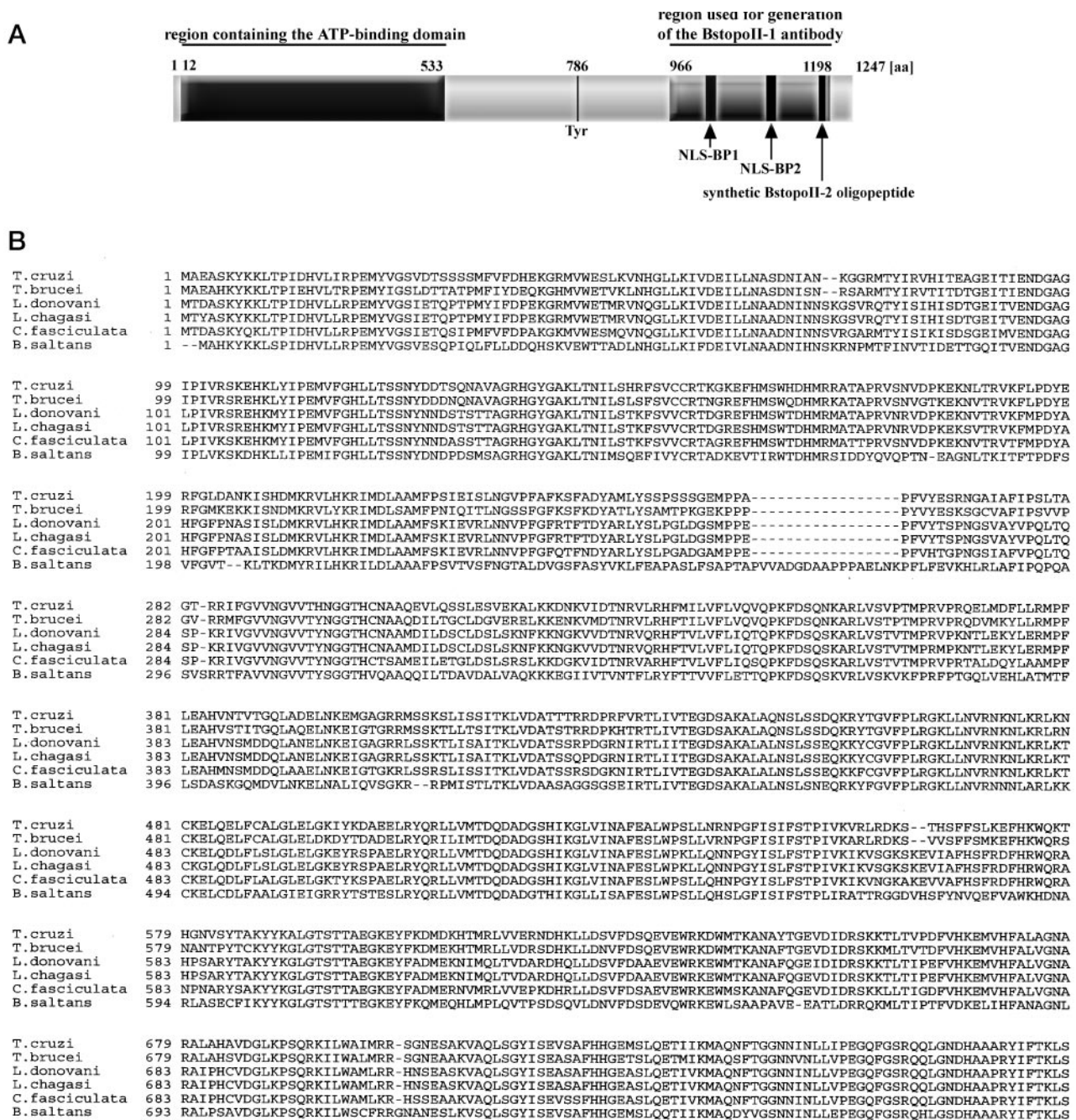


FIG. 1. A, schematic diagram of the *B. saltans* topo II protein. The PCR-amplified region used to screen the genomic library and the overexpressed region used to generate the BstopoII-1 polyclonal antibodies are shown with a bar. The conserved catalytic Tyr-786 is highlighted. The position and sequence of the synthetic oligopeptide against which the BstopoII-2 polyclonal antibodies were made is indicated. Also, positions of the predicted bipartite nuclear localization signals (NLS-BP1 and 2) are shown. B, multiple alignment of predicted protein sequences of topo II from *T. cruzi* (P30190), *T. brucei* (P12531), *C. fasciculata* (P27570), *L. chagasi* (O61078), *L. donovani* (AF150876), and *B. saltans* (AY083347). The amino acids are numbered to the left of the respective sequences. Conserved positions are in black, conservatively substituted in gray, and variable positions in white. Gaps were introduced to optimize the alignment.

antifade Dabco (Fluka). The slides were examined with a Zeiss Axioplan 100 microscope, and black and white images were recorded with a cooled charge-coupled device camera Mega F-View II (Soft Imaging Systems, Munster), pseudo-colored, and superimposed with the aid of an image-processing program Adobe Photoshop version 5.0.

In Situ Hybridization with the Minicircle Probe—The minicircle conserved region of *B. saltans* (BSM1) was amplified using the primers MB1 (GGGGTACCCAGCGTTTGGATGCTGTT) and MB2 (GGAAT-TCCCTCTTCCCTGGTACT) derived from the available minicircle sequences (14). Biotin labeling of BSM1 (1 unit of *Taq* polymerase, 5 μ M each primer, 0.1 mM dATP, dCTP, and dGTP, 0.065 mM dTTP, and 0.35 mM Biotin-N6-dTTP (Renaissance, PerkinElmer Life Sciences) was carried out under the following conditions: 94 $^{\circ}$ C for 1 min, 55 $^{\circ}$ C for 1.5 min, and 72 $^{\circ}$ C for 2 min (30 cycles) with a final extension of 72 $^{\circ}$ C for

10 min. The amplicon was gel-purified before use. The cells were processed and fixed as described for immunocytochemistry, but the fixation was stopped by washing the slides in the TN buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl). They were then placed in a humidity chamber for the following incubations: 30 min at 37 $^{\circ}$ C in 100 μ g/ml RNase A; 5 min at room temperature in 2 \times SSC; 10 min at room temperature in 4% formaldehyde; dehydrated and finally air-dried. After denaturation in the PCR buffer with Mg²⁺ for 3 min at 94 $^{\circ}$ C and immediate dehydration in icy methanol and air drying, the slides were incubated in the hybridization mix (50% formamide, 10% dextran sulfate, 12.5 μ g of salmon sperm DNA, 0.125% SDS, and 1 ng of probe per slide; the probe was denatured at 76 $^{\circ}$ C for 15 min, cooled on ice, and supplemented with 2 \times SSC before use) overnight at 37 $^{\circ}$ C in a humidity chamber. They were washed in 2 \times SSC for 5 min at 39 $^{\circ}$ C, 50% formamide in 2 \times

T. cruzi	778	SLARILFPSEDEPLLDYVTEEGQVPEPNHYVPIILPPLLCCNGSVGIGGFGFASNI PPFHPLDVSAAVSRMINGEAAKVVRRLVPAWVGQGEVRRGPEGE-
T. brucei	778	KVARLLFPSEDDPLLDYIIEEGQVPEPNHYVPIILPPLLCCNGSVGIGGFGFSSNI PPFHRLDVSAAVSRMINGSERAKSVRRLVPAWVGQGEVRRGPEGE-
L. donovani	782	RFARLLFPSEDDPLLDYMDDEEGTFFVEPHHYVPIILPMLCCNGAVGIGGFGFATTPSFHPLDVSAAVSRMINGSERAKQVVRNLVPAWVGQGTVRRGPNDE-
L. chagasi	782	RFGRLLFPSEDDPLLDYMDDEEGTFFVEPHHYVPIILPMLCCNGAVGIGGFGFATTPSFHPLDVSAAVSRMINGSERAKQVVRNLVPAWVGQGTVRRGPNDE-
C. fasciculata	782	RFARLLFPSEDDPLLDYIIEEGTFFVEPNHYVPIILPPLLCCNGAVGIGGFGFATTPSFHPLDVSAAVSRMINGSERAKQVVRNLVPAWVGQGTVRRGPEKE-
B. saltans	793	PLARLLFPKEDDELLQFVEDEGKYVEPRFYVPII PMLLVNGSVGIGGFGFACTVQMHHPDLVVTVRMRMKNTPQALEGLRPAWVGSGSVVQKTTVDDE-
T. cruzi	877	-----FIAAGSYQYVVDG--RVHVTEIPWTLSEIAPFRDHSVLASKDVQRIADYSGANHVDDIDLELTNGAMTTYAECESELSTQRIYINGTVF
T. brucei	877	-----FIAVGTYYCKGG--RVHVTEIPWTLSCVSEAFREHISYLAATKDIVNRDIADYSGANHVDDIDVEVAQAVNTYAECESELGLTQRIHNGTVF
L. donovani	881	-----FIAVGKYTAHPNG--RFHISEIPWMTSIEAPRLHISLASSADVVQRIADYSGANHIDIDLIVRDGSLTTWAECESTDALAQRIYINGTVF
L. chagasi	881	-----FIAVGKYTAHPNG--RFHISEIPWMTSIEAPRLHISLASSADVVQRIADYSGANHIDIDLIVRDGSLTTWAECESTDALAQRIYINGTVF
C. fasciculata	881	-----YIAVGKYTAHRNG--RLHVSELPMWTSIEAPRSHISLASSADVVQRIADYSGANHIDIDLIVRSGMSTTWAECESTDALASQRIYINGTVF
B. saltans	893	EGREVSTTHVAMGRPTTRKVKGFVVRVSELPWDRAIEQLRGRVAKLAEESKVLRIADYSGANHVSDVVLAADSFPASPREIEKDFLLTSNVANNAAVY
T. cruzi	965	SPTGVLTPLEGDLAPVLQWHYDRRLDLYKKRRQRLGLLEAELAREKSTLKFVTHFREGKIDIVNATDD-SLAKTCSKLGMRVDDSDYDVLRRKPIITFFY
T. brucei	965	SPNGTSLPESDLTPVLQWHYDRRLDLYKKRRQRLGLLEAELAREKSTLKFVTHFREGKIDIVNATDD-TLEKVCCKLGLVRRVDDSDYDYLRRKPIITFFY
L. donovani	969	SPTGTLSPIDSDLSVVLQWHYDRRLDLYKKRRTRKIGLMEMDLARLQSTRKFFVEHFRQGDIFLNAATDD-TLHKTCKVGLVRRVDDSDYDYLRRKPIITFFY
L. chagasi	969	SPTGTLSPIDSDLSVVLQWHYDRRLDLYKKRRTRKIGLMEMDLARLQSTRKFFVEHFRQGDIFLNAATDD-TLHKTCKVGLVRRVDDSDYDYLRRKPIITFFY
C. fasciculata	969	SPDGTSLPIDADLSVVLQWHYDRRLDLYKKRRTRQIGLLEMDLARLQSTRKFFVEHFRQGDIFLNAATDD-TLTKTCKVGLVRRVDDSDYDYLRRKPIITFFY
B. saltans	993	NPBMVLSPLGSESVESIVQWHYEQRLAMYTKRKRERQLLLNQLKLLKSTLTFETLRSGDLDFRKMADENALVKQCSKELKLRHRENSFDYLRTRRPVTFY
T. cruzi	1064	KTSLENLNRKISETEKRIEALKKTAPVQMWLDELDRDFRAFEHENTAVATILKERR-----VNPPTGD-VSRNLQQRLEEEVKVSSSGGKSV
T. brucei	1064	KTSFENLLKKAETERRIEALKKTTPVQLWLGELDQDFRFDQHEKMMVEAILKERR-----QRSPPSD-LLPGLQPRLEVEEAKG---GKKFE
L. donovani	1068	RTSTEKLQADIAKTAQIEELKRTTPVKMWLTDELDFKDTQYERVLINSIQKEQR-----SSITGGVELPALRQPLMLLEASAKDAT--AYRV
L. chagasi	1068	RTSTEKLQADIAKTAQIEELKRTTPVKMWLTDELDFKDTQYERVLINSIQKEQR-----SSITGGVELPALRQPLMLLEASAKDAT--AYRV
C. fasciculata	1068	KTSTEKLQADIAKTKQDSIAVLKQTPVKMWLTDELDFKDTQYERVLINSIQKEQR-----PASITGGVEPALRQPLMLLEAPAKGASSSYRV
B. saltans	1093	NNNVEKLKRDIEHCKSSI KSLESKSEVELWETDLAAFEKAPTAHEDALLRIEADRRPVFTAPEVDNLSQORS-----NEKWK
T. cruzi	1154	MRVVRKRYVPPPSKRPVHGQSVGGGGGGSSVSSAAAVVAHVAKKAARARSQMKMLLDVVARQVAVRVLPRLPWFLE---
T. brucei	1150	MRVQVRKRYVPPPTKR---GAGGRSDGDDGATAAGAAAAGVGRGKGGKPGRAGGVRRMVDLALAKRVTRLLPRLLE---
L. donovani	1157	HACQYKPPPSKRRPGESVGGARPSDSAARTVGRKLVGSRVFNKKNKPMRKNVSVLSTRVAQFAGAQLGRLLPHVLM---
L. chagasi	1157	HACQYKPPPSKRRPGESVGGARPSDSAARTVGRKLVGSRSEFNKKNKPMRKNVSVLSTRVAQFAGAQLGRLLPHVLM---
C. fasciculata	1159	HICRYBEPASKRRKPEDTYGGALSSGGSTRNVGKRLTGARG--AKKKVVRTRTRTKMSLGRTRVAEFAGAQLGRLLPQLPRLLE---
B. saltans	1171	FFRRKKGSSSSSSGGAGGGGAGEMVVRFPVPLPNQPPAAAAKAQAKAVAKFVGGVLMVATRLQVGVVGRLLH---

FIG. 1—continued

SSC for 10 min at 39 °C, 2× SSC for 5 min at room temperature (twice), and the TN buffer for 1 min at room temperature. Detection was by the Renaissance Tyramide Signal Amplification kit (PerkinElmer Life Sciences) as follows: incubation in the TNB buffer (TN buffer + blocking reagent) for 30 min at 37 °C; streptavidin-horseradish peroxidase conjugate (diluted 1:100 in the TNB buffer) for 30 min at room temperature; wash in the TNT buffer (TN buffer + 0.05% Tween 20) trice for 5 min at room temperature; incubation with biotinyl tyramide (diluted 1:50 in the amplification diluent) for 10 min at room temperature; wash in the TNT buffer trice for 5 min at room temperature; incubation with streptavidin-Texas Red (diluted 1:500 in the TNB buffer) for 30 min at 37 °C, and final wash in the TNT buffer trice for 5 min at room temperature. DNA was visualized with DAPI (0.1 μg/ml) or YOYO1 (1 nM) staining, and the slides were examined with a Zeiss AxioPlan 100 microscope or a Zeiss LSM410 confocal microscope, respectively.

RESULTS

Isolation of the Topo II Gene and Sequence Analysis—To isolate the protein-coding region of the kinetoplast-associated type II topoisomerase, we designed degenerate oligonucleotides based on the multiple alignment of available topo II sequences of the trypanosomatids *T. brucei*, *C. fasciculata*, and *T. cruzi*, and PCR-amplified a conserved 1.5-kb-long ATP-binding region located in the N-terminal part of the gene (Fig. 1A). The PCR product of expected size was cloned, sequenced, and found to have a high similarity to the topo II of trypanosomatids. Next, we prepared a genomic library from the total DNA of *B. saltans* and more than 50,000 plaque-forming units were screened with the amplicon as a probe. Recombinant phage DNA from two positive phages (6–1, and 7–2) was purified and shown by Southern hybridization to contain parts of the gene of interest. Fragments generated by *EcoRI* (3.3 kb long), *SalI* (1.2 kb long), and *SacI* (3.5 kb long) were subcloned into either pGEM7 or pGEM11 vectors and sequenced. The sequence analysis showed that the cloned fragments overlap and contain the entire Bstopo II gene.

The BstopoII protein-coding region is 1247 amino acids long with the predicted molecular mass being 139 kDa. Protein data base search with the predicted polypeptide using BLAST revealed homology with several topo II genes. On the amino acid level significant similarity with the homologs was observed throughout most of the open reading frame, while the C-terminal domain of BstopoII is unique. The most closely related sequences (42–49% identity) were the topo II genes of the

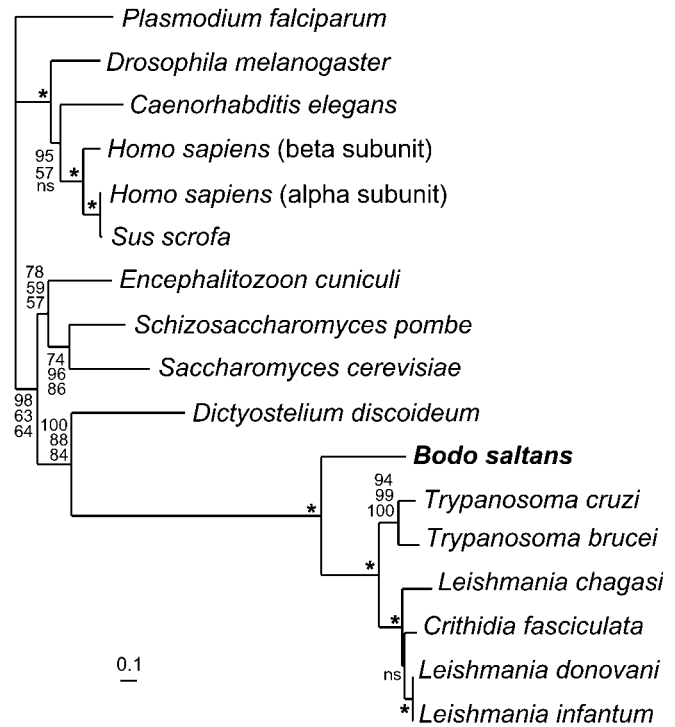


FIG. 2. Maximum likelihood tree inferred from the amino acid sequences of topo II of selected eukaryotes. From 1052 sites used for analysis, 154 sites were constant. Tree was rooted using *P. falciparum* as an outgroup. Quartet-puzzling support (1000 replicates) for maximum likelihood (upper value) and bootstrap support for maximum parsimony with gaps excluded (1052 characters; 810 parsimony informative; 4222 steps-long tree) (middle value) and included (1820 characters; 1160 parsimony informative; 6645 steps-long tree)(lower value) are shown. * indicates nodes with 100% support by all methods used. ns, no support.

trypanosomatids *T. brucei*, *T. cruzi*, *Leishmania* spp., and *C. fasciculata* (Fig. 1B). Mutual identity among the trypanosomatid topo II is much higher, ranging between 61 and 76%. On the amino acid level BstopoII has an identity of about 30% with other eukaryotic type II topoisomerases, represented by the *Saccharomyces cerevisiae*, human and *D. melanogaster* topo II

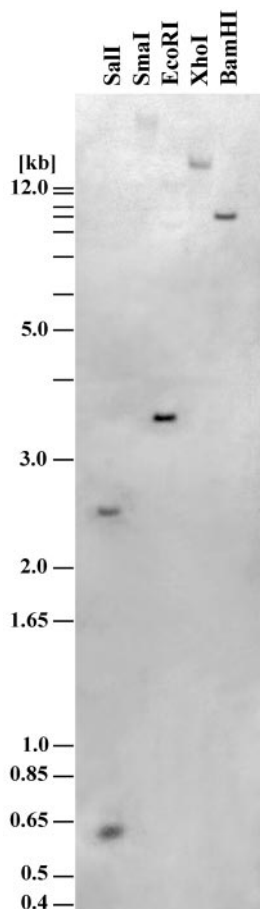


FIG. 3. Southern blot analysis of the *B. saltans* genomic DNA digested with restriction enzymes that cleave inside (*SalI*) and outside (*SmaI*, *EcoRI*, *XhoI*, and *BamHI*) the topo II gene. 10 μ g of digested DNA was fractionated on a 0.75% ethidium bromide-stained agarose gel, blotted, and hybridized as described under "Experimental Procedures." The position of molecular mass markers is indicated to the left.

genes. The identity with the subunit B of the prokaryotic DNA gyrase dropped to about 13% (data not shown).

In a phylogenetic analysis based on the amino acid sequences of selected eukaryotic topo II genes, including all homologs available from flagellates, *B. saltans* constituted the earliest lineage within the long branch leading to the kinetoplastids (Fig. 2). Maximum parsimony and likelihood analyses produced trees with generally the same topology and high support for the branching order. The analyses differed only in respect to the mutual relationship among *C. fasciculata* and *Leishmania* spp. that cannot be unambiguously resolved with the available data (Fig. 2). The topology of an unrooted tree analyzed with the above methods confirmed the position of *B. saltans* on the basis of the Kinetoplastida clade (data not shown).

The studied protein-coding region contains features characteristic for the eukaryotic topo II genes, like the ATP-binding domain, the conserved Tyr-786 in the catalytic site, and the typical motif TEGDSAKA. Topo II is relatively short in trypanosomatids, and the Bstopo II gene is no exception. We did not find a mitochondrial target signal, which is also absent from genes coding for mitochondrially located topo II of other flagellates. Interestingly, within regions of high conservation, the *B. saltans* sequence contains two unique insertions 17 and 10 amino acids long. Moreover, the Prosite data base search identified two putative bipartite nuclear localization signals that are composed of basic amino acid residues. The terminal 100 amino acids have no similar-

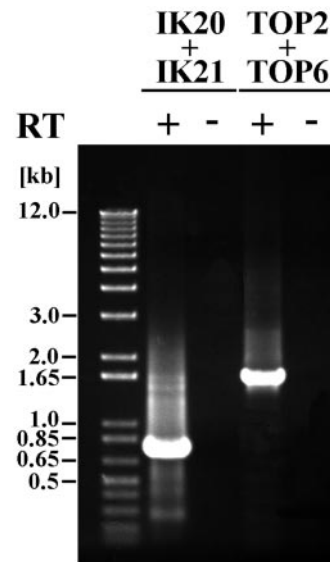


FIG. 4. Results of the RT-PCR analysis. 10 μ l of each PCR reaction was loaded on a 0.75% ethidium bromide-stained agarose gel. Above the slots, the primer pair used is shown (see "Experimental Procedures"). The (+) and (-) symbols indicate the addition and omission of reverse transcriptase. The position of molecular mass markers is indicated to the right.

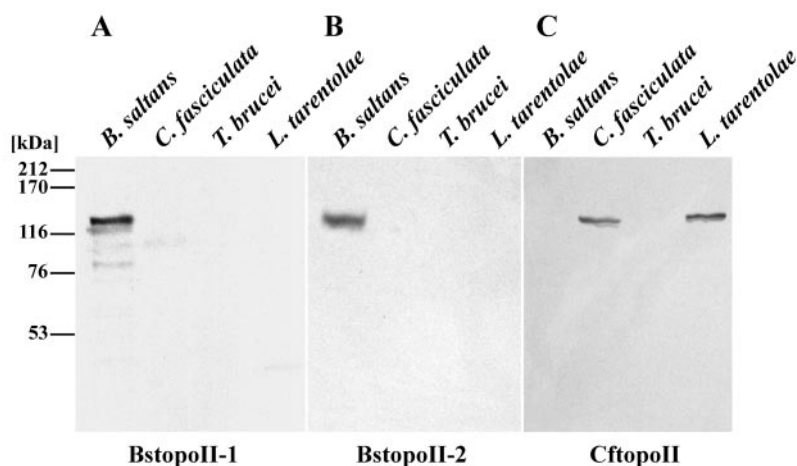
ity with other topo II genes (Fig. 1B). We have performed Southern hybridization to determine the copy number of the Bstopo II gene. The presence of a single hybridizing band in total DNA cut with the restriction enzymes *BamHI*, *EcoRI*, *SmaI*, and *XhoI* or two hybridizing bands after the *SalI* digestion (this enzyme cuts once in the region covered by the probe) probed with the unique C-terminal domain strongly suggests that it is a single-copy gene (Fig. 3). This is in accordance with other eukaryotes where topo II is also present in a single copy.

The cloned fragment contained also 1.9 kb of the region 5' to the start codon of the topo II protein-coding sequence and 150-bp long region behind the stop codon. In the adjacent regions no element involved in cycling of topo II, known from the related *C. fasciculata* (23), was identified. Several attempts to detect the topo II mRNA by Northern hybridization were not successful. Therefore, we have resorted to the RT-PCR approach to detect this apparently rare transcript. Two primer pairs were used that spanned most of the coding region. In both cases, a single amplicon of expected size was obtained, while the omission of reverse transcriptase rendered the reaction negative (Fig. 4). Thus, we have confirmed that the topo II gene is transcribed.

Expression of Topo II—We have first attempted to detect the topo II protein in *B. saltans* using the monoclonal antibodies against *C. fasciculata* topo II (anti-CftopoII) that was shown to be localized exclusively in the kinetoplast (8). While the antibody recognized a single specific protein of expected size (~130 kDa) in the lysate of *C. fasciculata* and *L. tarentolae*, under stringency conditions it failed to detect the protein in *B. saltans* and *T. brucei* (Fig. 5C).

Therefore we prepared specific polyclonal antibodies raised against the *B. saltans* topo II protein. To avoid possible cross-reactivity with the ATP-binding domain-containing proteins or with a different topo II, we decided to prepare antibodies against the C-terminal domain of BstopoII, which is a least conserved region. Affinity purified polyclonal antibodies raised against the His-tagged 30-kDa recombinant protein purified from the bacterial lysate showed a peptide of ~130 kDa (Fig. 5A), compatible with the expected size based on the deduced

FIG. 5. Western blot analysis of *B. saltans*, *T. brucei*, *L. tarentolae*, and *C. fasciculata*. Lysates of $\sim 10^7$ cells per lane were loaded on 8% SDS-PAGE gels, which were hybridized with the BstopoII-1 (A), the BstopoII-2 (B), and the CftopoII (C) antisera. The position of molecular mass markers is indicated to the left.



amino acid sequence. Moreover, several significantly weaker bands in the lower size range were also visible, while there was no cross-reactivity with cell lysates of the other kinetoplastid flagellates. We assume that the weakly cross-reacting proteins are derived from feeder bacteria rather than results of partial proteolysis since they were not recognized by the second antibody (see below).

To verify the specificity of the 130-kDa band, a second rabbit polyclonal antibody was generated against a synthetic 15-amino acid-long oligopeptide synthesized according to a region in the C terminus of BstopoII (Fig. 1A). In Western analysis of the *B. saltans* lysate, this affinity-purified antiserum reacted with a protein of the same size as the first one and no cross-hybridization with other proteins was observed (Fig. 5B).

Subcellular Localization of Topo II—From results obtained by Western analysis of the cell lysates we concluded that both antibodies are specific for the *B. saltans* topo II protein. The region against which these antibodies were prepared is unique for the target gene, and the homology search revealed no similarity with other genes available in GenBankTM. We have used the affinity-purified antisera to investigate the cellular localization of the protein. After about a 1-week-long cultivation, the flagellates were purified by several rounds of differential centrifugation and sedimentation to remove most of the feeder bacteria. The cells were smeared and counter-stained with DAPI to visualize both the kinetoplast and the nucleus (Fig. 6). This staining revealed a prominent kinetoplast that occurred as a single globular nucleoid located in the paraflagellar position, close to the kinetosomes of the two flagella. In most cells, the kinetoplast was distinguishable by its slightly oval or circular shape and evenly intense staining. The nucleus was usually irregular in shape, larger than the kinetoplast, while the intensity of DAPI staining was lower and uneven throughout the nucleus (Fig. 6).

In cells treated with the secondary antibody, the cyanin signal was localized throughout the kinetoplast and followed that of DAPI. The topo II protein seems therefore to be evenly spread throughout the kinetoplast (Fig. 6A). Unexpectedly, both antisera recognized the topo II protein also in the nucleus of *B. saltans*. At lower concentrations, rather diffuse staining of varying intensity was observed in the nuclear chromatin (Fig. 6A). However, when the blocking solution was supplemented with 10% BSA and higher concentration of the antisera (BstopoII-1, 1:100; BstopoII-2, 1:20) were used, a characteristic labeling pattern emerged. About a dozen distinct loci located on the periphery of the rounded nucleus became visible (Fig. 6B). The antibody generated against the overexpressed protein (BstopoII-1) recognized the nuclear topo II with higher intensity.

Localization of the kDNA Minicircles—We have PCR-amplified and biotin-labeled a 350-bp-long region that is highly conserved in the *B. saltans* minicircles (14) and used it as a probe for *in situ* hybridization. As shown in Fig. 7, the probe hybridized only with the kinetoplast bundle that is located in the anterior part of the mitochondrion between the flagellar kinetosomes and the nucleus. No signal was detected elsewhere in the mitochondrial lumen thus proving that all minicircles are confined to the kDNA globule. When the cells were counter-stained with DAPI or YOYO1, the kinetoplast appeared as a prominent oval to circular structure, staining more intensely than the nucleus (Fig. 7, A and B).

DISCUSSION

In most eukaryotes mitochondrial DNA occurs as a circular molecule(s) (24), replication and transcription of which will introduce topological stress that must be relieved by topoisomerases. While activities of topo II have been described in the eukaryotic mitochondrion, little is known about its biochemistry and localization within the organelle (25). Kinetoplastid flagellates harbor in their single mitochondrion the most complex organellar DNA known, composed of thousands of circular molecules (4, 5, 26). Due to this fact, their mitochondrial topo II is quite abundant and thus belongs to the most studied mitochondrial topoisomerases. Moreover, it seems that, at least in the mitochondrion of *C. fasciculata*, there are two topoisomerases II (7, 8) that may perform slightly different functions during replication of the kDNA network. In this study, we were interested in the localization of topo II in the mitochondrion of *B. saltans*, the primitive status of which among kinetoplastids (16–18) was confirmed by phylogenetic analysis of the topo II sequence. In all trypanosomatids studied thus far, the kinetoplast occurs as a disc-shaped structure with a high concentration of DNA that is located in the paraflagellar position (26, 27). The kDNA of *B. saltans* has a similar intracellular localization; however, it forms a single globular nucleoid with a bundle-like structure (18).

We have cloned and sequenced the entire topo II gene along with its 5'- and 3'-untranslated regions. The alignment with the *C. fasciculata* mitochondrial topo II (22) (genomic sequence is available only for one mitochondrial topo II), and other available trypanosomatid topo II genes revealed a level of identity similar to that observed in other bodonid *versus* trypanosomatid gene alignments (17, 28). We have identified neither a mitochondrial targeting signal nor a cycling element that confers cycling to topo II in *C. fasciculata* (23). To detect the topo II protein in *Bodo*, we have first tried polyclonal antibodies generated against the *C. fasciculata* topo II (8). Under high stringency conditions we were able to show that it recognized

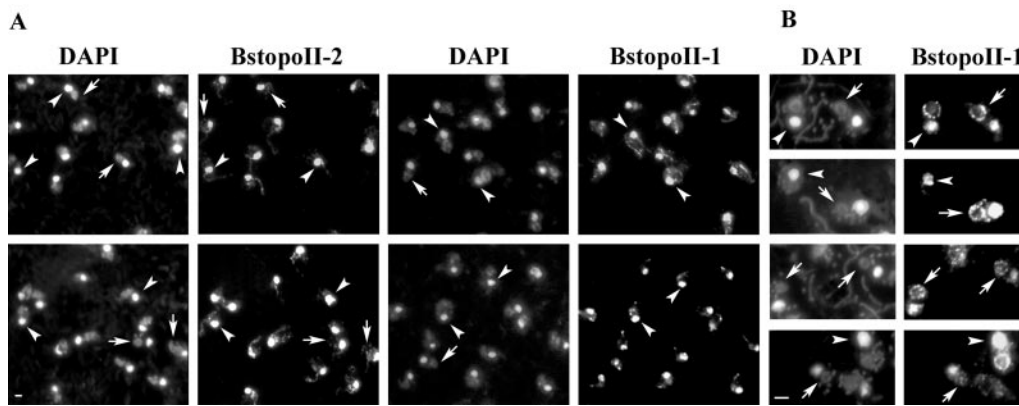


FIG. 6. Immunofluorescence analysis of the paraformaldehyde-fixed *B. saltans* cells. The left and right panels show the DAPI- and antibody-staining of the same cells, respectively. The arrowheads point to the kinetoplast, while the arrows indicate the nucleus. Panel A shows cells treated with either the BstopoII-1 or BstopoII-2 antisera under conditions when the nucleus is less intensely labeled (see “Experimental Procedures”). Panel B shows cells treated with a higher concentration of the BstopoII-2 antisera. Note distinct loci on the nuclear periphery indicated by arrows. Bar indicates 1 μm .

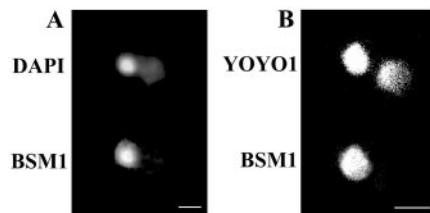


FIG. 7. Localization of the kDNA minicircles in *B. saltans* detected by *in situ* hybridization with the BSM1 probe. An example of the same cell stained with DAPI, labeled with the BSM1 probe, and examined in the Zeiss Axioplan 100 microscope is shown in panel A. The bar indicates 1 μm . A *B. saltans* cell stained with YOYO1, labeled with the BSM1 probe, and examined at a higher magnification (bar indicates 1 μm) in the confocal microscope Zeiss LSM410 is shown in panel B under conditions that the nucleus is not stained with BSM1.

the topo II protein only in *C. fasciculata* and closely related *L. tarentolae*. Two polyclonal antibodies directed against the unique C terminus of the *B. saltans* topo II did not react with total cell lysates of other flagellates and are therefore highly specific for the topo II protein of *Bodo*.

In the current kDNA replication model, topo II mediates decatenation of individual minicircles from the kDNA network. After their replication as free molecules, the progeny minicircles are reattached by another topo II action (6, 8, 29). The localization of topo II was studied in detail in the kinetoplast of *C. fasciculata* and *T. brucei*. Although both species are representatives of two different kDNA replication types (30, 31), their topo II is confined to two discrete antipodal sites that flank the kDNA network (8, 29, 32). The kDNA structure of *B. saltans* is significantly different from the kDNA network, since it is composed of relaxed non-catenated circles. It is well known that aggregated relaxed molecules are efficiently interlocked into catenanes by topo II (33, 34) and that by neutralizing the DNA phosphate charge, polycations such as spermidine and histones promote catenation (35). Several histone-like kinetoplast-associated proteins are apparently involved in packaging of the minicircles in the kDNA disk of *C. fasciculata* (36, 37). However, within the elongated mitochondrion of *B. saltans*, topo II is localized evenly throughout the globular kDNA bundle, which is in sharp contrast to the polar distribution of the enzyme in the trypanosomatid kDNA. The lack of catenation in the presence of topo II is a unique feature. We can only speculate whether the minicircular DNA is naked and therefore not located close enough for topo II to interlock the circles or that some yet unidentified protein(s) prevents the action of the enzyme.

The massive decatenation and reattachment of minicircles, performed by topo II in the trypanosomatids, is a function dispensable for the replication of *Bodo* kDNA. However, when the intact kDNA network of *C. fasciculata* was added to a partially purified lysate of *B. saltans* from which all nuclear and kDNA has been removed, a decatenating activity was detected, similar to that present in *C. fasciculata* (data not shown). Although in these preliminary experiments we did not prove that the decatenating activity pertained to the enzyme under study, it seems that in *Bodo* topo II may be responsible for the segregation of minicircle dimers and small catenanes formed by the replication of free minicircles. We suggest that the abundant presence of topo II in the non-catenated kDNA testifies for this function, which was also proposed for the *T. brucei* topo II (6). With the biotin-labeled minicircle probe we have shown *in situ* that all minicircles present in the *Bodo* kDNA as free molecules (14, 19) are indeed located within the single DNA globule. From the evolutionary perspective the appearance of massive catenation and network formation may have coincided with a possible gain of function in the more recently evolved trypanosomatid topo II.

Interestingly, the same topo II is also present in the nucleus of *B. saltans*. This conclusion is based on Southern hybridizations of the total DNA probed with the unique C-terminal domain that revealed a single band and by the immunolocalization experiments in which both antibodies reacted specifically with a nuclear protein. In this cellular compartment, however, the topo II protein occurs in multiple discrete loci, which seem to be associated with the nuclear membrane. The mitochondrial topo II protein was originally not found outside the organelle (8, 29), and only recent use of the anti-CftopoII and anti-TctopoII antibodies with *C. fasciculata* and *T. cruzi* cells lead Fragoso *et al.* (38) to suggest that the same enzyme may also be present in the nucleus. In both species the topo II signal co-localized with the propidium-stained nuclei, but no observation on subnuclear distribution was presented (38). Moreover, the polyclonal antiserum raised against the total overexpressed topo II of *L. donovani* reacted with the protein both inside the kinetoplast and the nucleus. The authors speculated that the same enzyme is responsible for activities in both cellular compartments, the division of which is synchronized in kinetoplastid flagellates (11).

The localization of topo II in the nucleus of *B. saltans* is reminiscent of distribution of its homolog in the nuclei of higher eukaryotes. In the nucleus of yeast, *Drosophila*, and mammalian cells the enzyme was co-localized with distinct replication centers (39–42). Our results suggest that in the nucleus of

B. saltans, an early branching eukaryote, such an intranuclear association, has already been established.

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Mitochondrial and Nuclear Localization of Topoisomerase II in the Flagellate *Bodo saltans* (Kinetoplastida), a Species with Non-catenated Kinetoplast DNA
Ivana Gaziová and Julius Lukes

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