Distinct Genes Encode Type II Topoisomerases for the Nucleus and Mitochondrion in the Protozoan Parasite Trypanosoma brucei

Received for publication, June 1, 2005, and in revised form, November 9, 2005 Published, JBC Papers in Press, November 28, 2005, DOI 10.1074/jbc.M505977200

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Topoisomerases are essential for orderly nucleic acid metabolism and cell survival and are proven targets for clinically useful antimicrobial and anticancer drugs. Interest in the topologically intricate mitochondrial DNA (kinetoplast or kDNA) of Trypanosoma brucei brucei and related kinetoplastid protozoan parasites has led to many reports of type II topoisomerase that participate in kDNA metabolism (we term the T. brucei brucei gene TbTOP2mt). We have now identified and characterized two new genes for type II topoisomerases in T. brucei brucei, termed TbTOP2α and TbTOP2β. Phylogenetically, they share a common node with other nuclear topoisomerases, clearly distinct from a clade that includes the previously reported kinetoplastid genes, all of which are homologs of TbTOP2mt. Southern blot analysis reveals the new genes are single copy and positioned ∼1.7 kb apart. Cognate mRNAs are expressed in African trypanosomes, but only a single message is detected in Leishmania or Crithidia. TbTOP2α encodes an ATP-dependent topoisomerase that appears as a single ∼170-kDa band on immunoblots and localizes to the nucleus; RNA interference leads to pleomorphic nuclear (but not kDNA) abnormalities and early growth arrest. The role of TbTOP2β is unclear. Although transcribed in trypanosomes, TbTOP2β is not detected by β-specific antisense, and RNAi silencing results in no obvious phenotype. These studies indicate that African trypanosomes and related kinetoplastid human pathogens are unusual in having independent topoisomerase II genes to service their nuclear and mitochondrial genomes, and they highlight TbTOP2α as a promising target for the development of much-needed new therapies.

Trypanosoma brucei sp. are hemoflagellates from the order Kinetoplastida that cause African trypanosomiasis (also known as sleeping sickness) in humans and a related disease in cattle (1, 2). These diseases are fatal if untreated, and current therapies are antiquated, toxic, and limited by the emergence of drug-resistant parasites (3). New therapies are clearly needed. The parasite has two major life cycle forms as follows: insect forms with sexual and asexual stages, present in the tse-tse fly vector, and mammalian host-specific asexual bloodstream forms (4, 5). Kinetoplastids are phylogenetically ancient eukaryotes that have unusual DNA in both the nucleus and mitochondrion. The total nuclear DNA content of T. brucei brucei is ~2.6 × 10^7 bp per haploid genome. Chromosomes, which do not condense during mitosis, are divided into the following three classes: megabase chromosomes (11 pairs), intermediate chromosomes (1–5, ploidy uncertain), and minichromosomes (~100, unpaired) each carrying a single variable surface glycoprotein gene (6). Sequence and analysis of the megabase chromosomes of T. brucei brucei were reported very recently (7). The mitochondrial DNA, termed kinetoplast DNA (kDNA), is a topologically complex structure that consists of thousands of minicircles and tens of maxicircles interlocked in one massive disk-shaped network. Maxicircles contain genes for proteins that participate in oxidative phosphorylation. Minicircles encode guide RNAs, ~70-bp transcripts, which are required for the correct editing of maxicircle transcripts (kDNA structure and editing reviewed in Refs. 8–10).

The DNA topoisomerases alter the topological state of DNA without affecting its sequence and are proven targets for a variety of important antimicrobial and anticancer agents (topoisomerases reviewed in Refs. 11–13). Type II enzymes, which make double-stranded breaks in the DNA substrate via a phosphodiester linkage of active site tyrosines with the DNA backbone, can be further divided into type IIA and IIB subfamilies, which share common mechanistic features but vary in structure (13). Because there is no evidence to date for fully functional type IIB enzymes in either mammalian or kinetoplastid cells, we hereafter refer to the type IIA simply as type II. In mammalian cells, the type II enzymes are represented by two isoforms, topoisomerase Iα and -β, both of which localize to the nucleus and play a role in mitosis and/or meiosis. Topoisomerase II activity serving the mitochondrial genome of mammalian cells has been described only recently (14) and appears to be a truncated form of topoisomerase IIB.

Curiously, the situation is quite the opposite for trypanosomatids, in which a mitochondrial topoisomerase II is well described, but little if anything is known about a nuclear activity. To date, topoisomerase II activity has been purified from Trypanosoma cruzi and Trypanosoma equiperdum (15), Leishmania donovani (16, 17), and Crithidia fasciculata (18, 19), and several type II-encoding genes have been cloned (20–24), including a single gene on chromosome 9 of T. brucei brucei that we designate TbTOP2mt (25). The above genes share a high degree of identity (∼65%) with one another at the amino acid level, and when studied in their products immunolocalize predominantly, if not exclusively, to the mitochondrion (26–28). RNA interference (RNAi) of TbTOP2mt leads to progressive loss of kDNA networks (28) without a detectable effect on nuclear DNA, indicating that the nucleus is not a major site of action for

8 This work was supported by National Institutes of Health Grant AI28855. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) DQ309462 and DQ309463.

9 The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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2 The abbreviations used are: kDNA, kinetoplastid DNA; RNAi, RNA interference; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; DAPI, 4′,6-diamidino-2-phenylindole; dsRNA, double-stranded RNA; PBS, phosphate-buffered saline; BSA, bovine serum albumin.
the product of this gene. Our previous studies with topoisomerase inhibitors suggested that trypanosomes might have more than one type II enzyme (29); however, separate enzymatic activities were not detectable when cell extracts were fractionated, and the source for nuclear topoisomerase II activity has remained unknown.

In this study we describe two new topoisomerase II genes from *T. brucei brucei*, *TbTOP2a* and *TbTOP2b*. The product of *TbTOP2a* localizes to the nucleus, demonstrates ATP-dependent type II topoisomerase activity, and its silencing leads to growth arrest and severe defects in nuclear (but not mitochondrial) DNA. *TbTOP2b* expression is detected at the mRNA level in several species of African trypanosomes but presents no apparent phenotype when silenced. The role of *TbTOP2b*, if any, remains unclear. Our finding makes *T. brucei brucei* unusual in having at least two independent topoisomerase II genes serving the nucleus (*TbTOP2a*) and mitochondrion (*TbTOP2mt*).

**EXPERIMENTAL PROCEDURES**

**Cell Growth—** *T. brucei brucei* bloodstream forms (MiTat 1.2, strain 427 and TREU 927) and *T. brucei gambiense* (IL 1852) (30) were grown axenically (31) in phenol red-free HMI-9 medium (32) and procyclic (insect form) strain 427 at 28°C in SDM-79 (33) supplemented with 10% fetal bovine serum (Invitrogen). *L. donovani* promastigotes (MHOM/S.62/1S-CL2D) were cultured at 26°C in medium 199 (Sigma) supplemented with 10% fetal bovine serum. *C. fasciculata* were grown at 26°C in brain heart infusion medium (Difco) with 20 µg/ml hemin (Sigma) added. For RNAi, *T. brucei brucei* procyclic strain 29-13 (34) containing stably integrated constructs for T7 RNA polymerase and Tet repressor was maintained at 28°C in the following RNAi medium: SDM-79 supplemented with 10% Tet-approved fetal bovine serum (Clontech), 15 µg/ml G418, and 50 µg/ml hygromycin. All cell cultures, induced and uninduced, were maintained between 5 × 10^6 and 10^7 parasites/ml. Proliferation was monitored by hemocytometer counts of motile parasites. *T. equiperdum* (BoTat 24) and dyskinetoplastic *T. brucei brucei* (IsTat DK6-1) (35) were isolated from infected animals (36). All sequencing and RNAi experiments were done with procyclic strain 427.

Identification of Topoisomerase II Genes—*T. brucei brucei* mRNA purified with Oligotex® mRNA mini kit (Qiagen) was used in first strand cDNA synthesis with poly(dT) primer (ProSTARTM First Strand RT-PCR kit; Stratagene). For *TbTOP2a*, 5′-rapid amplification of cDNA ends (RACE) was performed using splice leader sequence (37) (GCG-TTCAG/HH11032) and gene-specific sequence GCGTTCCA-H11032 antisense primer. Resulting PCR products were sequenced and strands were sequenced.

The product of 3′-RACE with gene-specific sequence GCGTTCCA-H11032 translated region was determined from poly(dT) cDNA by sequencing nucleotide sequence from the EST as antisense primer (CATTAG-TTCAG/HH11032-G). Also included were putative topoisomerase II sequences from *T. brucei brucei* (AAP33503); *Leishmania chagasi* (Homo sapiens) and (β) (Q02880); *Leishmania major* (AAD34021); *Leishmania infantum* (AAFS6535); *Leishmania major* (ABC27310); *Mus musculus* (NP_033435); *Nicotiana tabacum* (AAP33503); *Plasmodium falciparum* (P41001); *Saccharomyces cerevisiae* (P06786); *Schizosaccharomyces pombe* (CAA20107); *TbTOP2a* (ABC17641); *TbTOP2b* (ABC17642); *TbTOP2mt* (P12531); and *T. cruzi* (P30190). Also included were putative topoisomerase II sequences from *GeneDB* (www.genedb.org), *Leishmania major* (LmjF15.1290) and *T. cruzi* (Tc011.077650230.70), and from Plasmodb (www.plasmodb.org). *P. falciparum* GyrB+A (PFLE1915w joined with PFLE1220). Default parameters (gap opening penalty, 10; gap extension penalty, 0.2; Gonnet series protein weight matrix) were used for multiple alignment with ClustalX (39). Gap-containing columns were removed manually. Phylogenetic analysis using parsimony (and other methods) (40), with default parameters, was used for tree reconstruction (maximum parsimony method) and bootstrap analysis (1000 replicates).

**RNAi Plasmid Constructs**—All constructs for RNAi were made in pLew 100 (34); RNAi sequences were identical to probe regions described above. To make constructs for Liniker and Beta targets, inserts were PCR-amplified from genomic DNA, using primers containing HindIII/Sphi (forward insert) or BamHI/MluI (reverse insert) sites at their 5′- or 3′-ends, respectively. Digested and gel-purified forward inserts were ligated into HindIII-Sphi-digested pET-29a plasmid

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AGTCAGAATCACCAG as sense primer and oligo(dT)35, as antisense primer. The intergenic region separating *TbTOP2a* and *TbTOP2b* was PCR-amplified from genomic DNA using primers specific to the coding regions of the α (CAGATGATTTGACCTTGACT, sense primer) and β gene (AGAAATATCCTGATGTTTGCGACGCC, anti-sense primer). The latter antisense primer was used to obtain the 5′-untranslated region for the β gene by 5′-RACE analysis with sense primers encompassing each putative splice site upstream from the first ATG codon. Finally, the entire cDNA of the *TbTOP2b* was amplified with specific primers, cloned into pBlueScript® II KS (+) plasmid (Stratagene), and sequenced.

**Southern and Northern Blotting—** Genomic DNA from procyclic strain 427 was purified, cut with restriction endonucleases, separated by electrophoresis, and transferred to Hybond N+ membranes (Amerham Biosciences). Total RNA was purified from cell lysates with RNeasy® mini kit (Qiagen), separated on 1% agarose-formaldehyde gels, and transferred to Hybond N+ membranes. 32P-Labeled probes were prepared by random priming (MegaPrime DNA labeling kit; Amerham Biosciences) of the following DNA templates: Alpha (nucleotides 3630–4299 of *TbTOP2a* coding sequence), Beta (nucleotides 3724–4373 of *TbTOP2b* coding sequence), Linker (nucleotides 1601–2150 of *TbTOP2a*, 90.6% identity with nucleotides 1724–2273 of *TbTOP2b*), *TbTOP2mt* (nucleotides 3001–3650 of *TbTOP2mt* coding sequence), α-tubulin (nucleotides 1–650 of *T. brucei brucei* α-tubulin gene, AL929605) (Fig. 1A), and LmT2A (nucleotides 781–1065 of L. major putative topoisomerase II, CAB73210). Membranes were hybridized (38), washed, and exposed to a PhosphorImager plate. The plate was scanned (Fujix BAS 1000 PhosphorImager) and visualized with Image Gauge version 3.45 (Fuji Photo Films Company). Contrast and brightness were adjusted with Adobe Photoshop.

**Phylogenetic Analysis**—Amino acid sequences of type II topoisomerases, obtained from GenBank™, included in the analysis were as follows: *Abigailopsis thaliana* (S53599); *Bodo saltans* (AL599217); *Caenorhabditis elegans* (NP_496536); *C. fasciculata* (P27570); *Dictyostelium discoideum* (P90520); *Drosophila melanogaster* (P15348); *Encephalitozoon caninum* (CAD25222); *Escherichia coli* GyrB+A (NP_312661 joined with NP_311141); *Giardia intestinalis* (AAP33503); *Homo sapiens* (P11388) and (β) (Q02880); *Leishmania chagasi* (O61078); *Leishmania donovani* (AAD34021); *Leishmania infantum* (AAFS6535); *Leishmania major* (ABC27310); *Mus musculus* (NP_033435); *Nicotiana tabacum* (AAN85208); *Plasmodium falciparum* (P41001); *Saccharomyces cerevisiae* (P06786); *Schizosaccharomyces pombe* (CAA20107); *TbTOP2a* (ABC17641); *TbTOP2b* (ABC17642); *TbTOP2mt* (P12531); and *T. cruzi* (P30190). Also included were putative topoisomerase II sequences from *GeneDB* (www.genedb.org), *Leishmania major* (LmjF15.1290) and *T. cruzi* (Tc011.077650230.70), and from Plasmodb (www.plasmodb.org). *P. falciparum* GyrB+A (PFLE1915w joined with PFLE1220). Default parameters (gap opening penalty, 10; gap extension penalty, 0.2; Gonnet series protein weight matrix) were used for multiple alignment with ClustalX (39). Gap-containing columns were removed manually. Phylogenetic analysis using parsimony (and other methods) (40), with default parameters, was used for tree reconstruction (maximum parsimony method) and bootstrap analysis (1000 replicates).

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(Novagen). Next, MluI-digested reverse inserts were ligated into MluI-HpaI-digested pET-29a containing the forward insert. Finally, the entire construct, including 525-bp fragment of pET-29a (flanked by Sphi and MluI sites) serving as a stuffer, was released and ligated into HindIII-BamHI-digested pLew 100. For the construct targeting Alpha, primers with HindIII/XbaI (forward insert) and BamHI/BstEII (reverse insert) linkers were used, with a 560-bp stuffer of luciferase gene present in original pLew 100 plasmid.

Transfection, Generation of Clonal Lines, and RNAi Induction—10 7 cells (strain 29-13) were washed in cytomix (120 mM KCl, 0.15 mM CaCl 2, 10 mM KH 2PO 4, 25 mM HEPES, pH 7.6, 2 mM EDTA, 5 mM MgCl 2), resuspended in 0.5 ml of cytomix containing 5 g of NotI-linearized pLew 100 construct, and transfected in 4-mm cuvettes using BTX ECM 830 square wave electroporator (1.7 kV, three pulses of 100 μs length and 200-ms interval). Cells were immediately transferred into 5 ml of RNAi medium; 24 h later 2.5 μg/ml phleomycin was added, and cells were grown for 3 weeks to obtain stable cultures. Clonal lines were generated by limiting dilution in conditioned medium (41). At ~2 weeks, detectable clones were transferred into nonconditioned medium, grown for several days to ensure stability, and dsRNA expression was induced by addition of 1 μg/ml tetracycline.

Preparation of Antigens and Antibodies—Fragments of TbTop2α (nucleotides 3940–4239) and TbTop2β (nucleotides 4090–4395) were PCR-amplified from genomic DNA with addition of NdeI and XhoI restriction sites to the 5′- and 3′-end, respectively. Amplified fragments were digested with NdeI and XhoI, gel-purified, and ligated into pET-29a (+) vector (Novagen) cut with the same enzymes. The resulting constructs, each with an additional LE(His), C-terminal tag, were transformed into E. coli strain RosettaTM (Novagen). Protein expression was induced at A 600 = 0.7 with 0.08 mM isopropyl-β-D-1-thiogalactopyranoside (Sigma) for 18 h at 16 °C. The cells were collected by centrifugation, lysed in CelllyticTM B-II (Sigma), and processed according to the manufacturer’s instructions. The His-tagged, soluble, and overexpressed proteins were purified on TALONTM Co2+ affinity resin in native conditions (BD Biosciences) and sent for commercial polyclonal antibody generation in rabbits (Covance Research Products, Inc.).

Western Blotting—Lysates from 10 9 cells were separated by SDS-PAGE on 7.5% polyacrylamide gel, transferred to Duralose-UV membrane (Stratagene), and blocked overnight at 4 °C with 3% bovine serum albumin (BSA fraction V; Sigma) in 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20, 0.001% Thimerosal (TTBS). The blot was incubated with rabbit anti-TbTop2α or anti-TbTop2β serum at 1:5000 dilution or rabbit polyclonal anti-BiP antibodies (1:10000; a generous gift from Dr. Jay Bangs). Primary antibodies were detected by peroxidase-conjugated goat anti-rabbit secondary antibodies (1:40,000; Sigma) and visualized by chemiluminescence (ECL Plus; Amersham Biosciences). Images were adjusted for contrast and brightness only in Adobe Photoshop. Western blots of yeast lysates (5 μg total protein/sample) were carried out as above.

Flow Cytometry—Cells (10 7) were harvested by centrifugation, washed once in 5% glycerol/PBS, fixed at 4 °C overnight in 75% methanol, 5% glycerol, washed once with 5% glycerol/PBS, and then incubated in 5% glycerol/PBS, 10 μg/ml RNase A for 30 min at 37 °C. After addition of propidium iodide (10 μg/ml final concentration), 10,000 cells were analyzed on FACSScan flow cytometer (BD Biosciences) using a 585 ± 21 nm bandpass filter. Forward and side scatter gates were set to exclude cell debris and clumps. Cells were also gated on height versus width of the red fluorescent signal with implementation of doublet discriminator module to exclude doublets. Data were analyzed with CELLQuest version 3.3 software (BD Biosciences). For kDNA analysis, live parasites were incubated for 20 min with 1 μg/ml dihydroethidium (Molecular Probes) in SDM-79 medium. Cells were washed once and resuspended in 5% glycerol/PBS.

Microscopy—Cells were fixed using a modified procedure of Field et al. (42). Briefly, 10 6 cells were washed once in 1 ml of PBS, 1 mg/ml glucose, resuspended in 0.1 ml of PBS, diluted with an equal volume of ice-cold 6% paraformaldehyde in PBS, and placed on ice for 15 min. Paraformaldehyde was inactivated by addition of 1 ml of 0.1 M glycine in PBS; cells were pelleted (5 min, 800 × g), washed with 1 ml of PBS, 0.1 M glycine, and resuspended in 0.2 ml of PBS. Cells were applied to slides (Electron Microscopy Sciences) coated with 0.01% poly-L-lysine (Sigma), allowed to adhere for 15 min, immersed in methanol (~20 °C, 30 min), rehydrated by three 5-min washes in TTBS, and treated with primary anti-TbTop2α antibody (1:2000 in 3% BSA, TTBS) for 1 h. Next, the samples were washed in TTBS (three times for 5 min), exposed to secondary Alexa Fluor® 594 goat anti-rabbit antibody (1:5000 in 3% BSA, TTBS; Molecular Probes) for 1 h, washed (twice for 5 min) in TTBS, stained with 2 μg/ml 4′-6-diamidino-2-phenylindole (DAPI), washed once in TTBS, mounted with Vectashield mounting medium (Vector Laboratories), and coverslips were sealed with clear nail polish. Fluorescence microscopy and digital image acquisition were carried out using an Axiostar microscope (Carl Zeiss, Inc.) equipped with a Retiga Exi Mono Cooled camera (QImaging) operated with IPLab software (Scanalytics, Inc.). All images were acquired using a ×100, 1.30 NA Plan-Neofluar oil immersion objective. Contrast and brightness were adjusted in Adobe Photoshop. For quantitative analysis, sets of micrographs of DAPI-stained cells were counted (>100 parasites/set) and scored in a blinded fashion by four independent observers. The nuclear morphology was defined as follows: absent (0N); abnormally small (<1N); normal (1–2N); and abnormally large (>2N). 0K, 1K, and 2K indicate number of kDNA networks per cell.

Yeast Plasmids and Strain—The multiple cloning site of pRS426(URA) (43) was replaced with a KpnI/SacI fragment of pRS316G(URA) (44) (both kindly provided by Kara Cerveny and Rob Jensen) containing the GAL1 promoter, multiple cloning site, and a portion of URA3 promoter with transcription stop site and polyadenylation signal, resulting in plasmid pRS426GU(URA). Entire open reading frames of TbTop2α and -β were PCR-amplified from cloned cDNAs with primers incorporating Sall and BamHI sites at the 5′ and 3′ termini, respectively. Coding sequence of the human TOP2α gene was amplified from plasmid pMJ1 (45) (generous gift from Neil Osheroff) with the addition of Sall and Xmal sites at the 5′- and 3′-end, respectively. PCR products were digested, ligated into pRS426GU(URA), and digested with corresponding restriction enzymes to produce pRS426GU-TbTop2α, pRS426GU-TbTop2β, and pRS426GU-HsTop2α constructs. S. cerevisiae strain JCW28 (MATa his3-D200 leu2-Δ1 trpl-Δ1 Δura3-Δ2 top2-4 Δtop1-Δe) (46) was transformed simultaneously with YEpTopA-PCD(PGP(1TRP)) (47), which contains E. coli topA gene linked to the yeast GPD promoter (strain and plasmid generously provided by Marc Gartenberg), and one of the topoisomerase II-containing pRS426GU(URA) plasmids. Transformants were selected on SD—ura, trp plates.

Decatenation Assay—Individual double-transformants were grown in liquid SD—ura, trp medium at 26 °C to an absorbance of 0.8 at 600 nm and then switched to SGal—ura, trp medium to induce protein expression. After 18 h cells were harvested, washed with cold water and then cold buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM 2-mercaptoethanol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture (100×, Sigma P8340)), and resuspended in 0.1 ml of buffer I. 50 μl of glass beads were added, and cells were lysed.
by vortexing (10 20-s bursts followed by 40 s on ice). Cell debris was removed by centrifugation. 100 ng of crude lysate was added directly to the decatenation assay buffer (25 mM Tris-HCl, pH 7.5, 80 mM KCl, 2 mM ATP, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 30 μg/ml BSA, 10% glycerol, 0.1 μg/ml C. fasciculata kDNA; final volume 20 μl) and incubated for 30 min at 37 °C. ATP was omitted from decatenation buffer where indicated. Reactions were separated in 0.8% agarose with 0.5 μg/ml ethidium bromide, and photographed under UV illumination.

**kDNA Replication Intermediates in TbTOP2α RNAi Cells**—Total DNA from 10⁶ parasites with or without induced dsRNA was isolated on days 1–6 post-induction. Half of each sample was digested with HindIII and, along with undigested DNA, separated in 1.5% agarose on days 1–6 post-induction. Half of each sample was digested with HindIII and, along with undigested DNA, separated in 1.5% agarose under UV illumination.

**RESULTS**

**Identification and Sequence Analysis of Two New Topoisomerase II Genes**—An expressed sequence tag (EST) screen of the *T. brucei brucei* genome revealed a 362-bp tag tentatively assigned as topoisomerase II (48). The sequence was identified on the basis of its significant similarity to *S. cerevisiae* topoisomerase II, rather than the previously reported *TbTOP2mt*. Although this EST predicted a new topoisomerase II gene, it actually led us to the discovery of two tandemly arranged new type II topoisomerases, the overall sequence of *TbTOP2α* can be divided into four distinct regions (50) as follows: N-terminal ATPase domain, linker, DNA cutting, and rejoining domain with active site localization signals starting at amino acids 1174, 1207, and 1403 (see supplemental Fig. 1), with no discernible mitochondrial targeting sequence. It contains regions highly homologous to topoisomerase II domains from several data bases (KOG0355, 0.0; smart00433, 3e⁻¹⁰⁸; COG0187, 9e⁻¹⁰⁸; smart00434, 3e⁻⁹⁸; and cd00187, 1e⁻⁹¹). Like other type II topoisomerases, the overall sequence of *TbTOP2α* can be divided into four distinct regions (50) as follows: N-terminal ATPase domain, linker, DNA cutting, and rejoining domain with active site tyrosine, and finally a C-terminal variable region (Table 1).

Indication of the second new topoisomerase II gene, termed *TbTOP2β*, came from Northern analysis when two distinct transcripts were detected with the Linker probe (Fig. 1A). Further studies identified the single-copy 4,398-bp open reading frame (Fig. 1B) and its ~6.6-kb transcript that is more abundant in bloodstream forms than in procyclic forms (Fig. 1C). The 167-kDa predicted protein has a pl of 6.2, a bipartite nuclear localization signal starting at amino acid 1300, a leucine zipper motif starting at 1182, and a toprim domain at 469–591. *TbTOP2β* has 74% identity with *TbTOP2α* (Table 1 and supplemental Fig. 1). The presence of these two distinct genes was further confirmed by PCR amplification and sequencing of the entire chromosomal locus, which revealed that *TbTOP2α* is positioned upstream and separated by an ~1.7-kb intergenic spacer from *TbTOP2β*. We highlight this point, since to date GeneDB contains only one putative gene on chromosome 11, termed Tb11.01.3390, which is a chimera of the α and β sequences.

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Type II Topoisomerases in T. brucei brucei

TABLE 1

<table>
<thead>
<tr>
<th>Domains*</th>
<th>Regions within coding sequence, amino acids</th>
<th>α-β identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase</td>
<td>1–489</td>
<td>89</td>
</tr>
<tr>
<td>Linker</td>
<td>490–645</td>
<td>91</td>
</tr>
<tr>
<td>Catalytic (active site tyrosine)</td>
<td>646–1164 (Tyr&lt;sup&gt;772&lt;/sup&gt;) 686–1205 (Tyr&lt;sup&gt;949&lt;/sup&gt;)</td>
<td>84</td>
</tr>
<tr>
<td>Variable C terminus</td>
<td>1165–1455 1206–1465</td>
<td>23</td>
</tr>
</tbody>
</table>

* Assignment based on pairwise alignments (ClustalX, default parameters) with sub-units of T4 bacteriophage topoisomerase II as follows: gp39 (NP_049621), gp52 (NP_049875), and gp60 (NP_049618) (50).

![FIGURE 2. Maximum parsimony tree based on amino acid sequences of selected type II topoisomerases.](image)

![FIGURE 3. Presence of TbTOP2α and TbTOP2β-related transcripts in various kinetoplastids.](image)

bloodstream form MiTat 1.2 (strain 427), the strain used in most of our studies; TREU 927, whose genome was sequenced (7); and IsTat DK6-1, a mutant with acriflavin-induced total loss of kDNA. Also included were bloodstream form T. brucei gambiense, which causes sleeping sickness in humans, and T. equiperdum, an equine parasite that is sexually transmitted and hence does not pass through an insect vector. Finally, L. donovani (a causative agent of visceral leishmaniasis in humans) and C. fasciculata (a parasite confined to insects) were also included in the analysis. Northern blot with the Linker probe, which recognizes both TbTOP2α and TbTOP2β mRNAs, indicated that two transcripts are present in all tested species of Trypanosoma (Fig. 3). Furthermore, across the organisms there is a striking similarity in the size of the two transcripts and in the abundance of the alpha message. Beta transcript levels are more variable. Because the Linker probe did not hybridize with L. donovani or C. fasciculata RNA, the blot was stripped and rehybridized with the LmT2A probe specific for a 5′ region of L. major—(above and Fig. 2), which detected a single transcript of ~5.8 kb in L. donovani and gave a very faint signal ~6 kb in C. fasciculata (Fig. 3).

RNA Interference of TbTOP2α and TbTOP2β—The RNAi approach was chosen to investigate the roles of the new topoisomerase II genes in T. brucei brucei. A region of the coding sequence common to both genes (Fig. 1A, Linker), as well as 3′ regions unique to the individual genes (Fig. 1A, Alpha and Beta) were cloned in opposing directions, separated by a stuffer, in the pLew 100 vector (34). This vector integrates in the rDNA intergenic region and allows for tetracycline-induced expression of stem/loop RNA under the control of a procyclin promoter.

Expression of Alpha-specific dsRNA led to growth arrest at day 3 post-induction, a significantly earlier onset than that seen upon silencing of TbTOP2mt (day 7 post-induction (28)). As reported previously for other essential trypanosomal genes (51), growth arrest persisted until day 9–10 post-induction, after which the culture was overtaken by cells not responding to RNAi. The growth arrest was associated with near-complete (~98%) depletion of the cognate mRNA without affecting transcript levels for α-tubulin (Fig. 4), TbTOP2β, or TbTOP2mt (not shown). An immunoblot of whole cell lysates taken during the course of

appear to be alleles of the same gene rather than α and β isoforms. Phylogenetic analysis of these and selected other topoisomerase II amino acid sequences places all previously reported topoisomerase II sequences from kinetoplastids, including TbTOP2mt, in a single clade (Fig. 2). TbTOP2α and TbTOP2β, along with the previously unreported type II topoisomerases we identified in the genome databases of L. major and T. cruzi, are grouped in a clade closely related to other eukaryotic, and predominantly nuclear, type II topoisomerases (Fig. 2). Noticeably, most of these nuclear genes radiate from a single central node, possibly implying a common ancestral origin and reinforcing their close phylogenetic relationship. In contrast, the genes previously identified in trypanosomatids, and now recognized to be mitochondrial, are clustered at a great distance from the central eukaryotic node and are positioned near the DNA gyrase branch, making them more closely related to the prokaryotic type II enzymes.

We tested for TbTOP2α and -β transcripts in several members of the Trypanosomatidae family. T. brucei brucei species were represented by...
induction demonstrated selective loss of the corresponding protein of ~170 kDa by day 2 (Fig. 5). Also, the nuclear signal detected in immunolocalization of TbTOP2α with α-specific antiserum in uninduced parasites was eliminated in cells in which dsRNA was expressed (Fig. 6A).

For TbTOP2β the results were quite different. Although RNAi silencing resulted in extensive degradation of cognate mRNA, there was no detectable effect on growth rate (Fig. 4). Polyclonal antibodies raised against a C-terminal fragment of TbTOP2β failed to react against trypanosomal lysate in Western blots and did not produce a signal in immunolocalization experiments, in either pre- or post-induction cells. Simultaneous knock-down of both TbTOP2α and β genes (mediated by Linker target sequence) led to reduction of both mRNAs and a growth arrest superimposable on that seen for TbTOP2α alone (Fig. 4).

**Cellular Morphology of TbTOP2α-deficient Parasites**—To investigate further the effects of TbTOP2α silencing, we monitored the morphology of induced and induced cells between days 2 and 5. Starting at day 3 post-induction, and progressing with time, we noted the presence of enlarged cells distinct from their uninduced counterparts (Fig. 6B), sometimes with multiple detached flagella (data not shown). Pronounced changes were also seen in nuclear size and shape. DAPI-stained parasites possessed apparently normal kDNA but showed pleomorphic nuclei that ranged from grossly enlarged and intensely stained, to fragmented and abnormally small, to apparently absent (Fig. 6A and B). Anucleate, kDNA-containing forms, termed “zooids,” have been observed in T. brucei cultures treated with rhizoxin or aphidicolin (52, 53). The RNAi silencing of cyclins (54) and cdc2-related kinases resulted in the appearance of zooids and of cells with enlarged nuclei (1–2N) (Fig. 6). In contrast, however, the same cells evidenced no changes starting at day 3 post-induction. A significant number of cells presented with abnormally increased (>2N) or decreased (<1N) DNA content (Fig. 7). Because kDNA accounts for only 4% of the total DNA in T. brucei (56), the irregularities in flow cytometric profiles can be attributed to the improper segregation and fragmentation of nuclear DNA. To demonstrate further that the silencing of TbTOP2α does not have an effect on replication of kDNA, we performed flow cytometry on live cells stained with dihydroethidium. This cell-permeant dye is oxidized to ethidium in the mitochondrion allowing for selective staining of mitochondrial but not nuclear DNA (57). There was no difference in flow-cytometric profiles between RNAi-induced and uninduced parasites (data not shown), affirming the nucleus as the primary site of action of TbTOP2α.

**Catalytic Activity**—A yeast expression system was chosen to determine whether the products of TbTOP2α and β are functional type II topoisomerases. The two trypanosomal genes and human topoisomerase IIα were each inserted into pRS426GU(URA) plasmid (which allows for galactose-inducible protein expression) and co-transformed into strain JCW28 (top2-4 Δtop1), along with YEtopA-PGPD(TRP) plasmid providing constitutive expression of E. coli topoisomerase I. Western blot of crude lysates of double transformants 18 h after induction revealed that cells carrying pRS426GU-TbTOP2α produced a protein of ~170 kDa, specifically recognized by TbTOP2α antiserum (Fig. 8A). The lysate from cells carrying pRS426GU-TbTOP2β showed a single

![FIGURE 4. RNAi of T. brucei brucei TbTOP2α and TbTOP2β. Clonal lines carrying stem/loop constructs targeting TbTOP2α (Alpha), TbTOP2β (Beta), or both (Linker) were grown in the absence (closed circles) or presence (open diamonds) of 1 μg/ml tetracycline (Tet). Cumulative cell density is the product of cell number and dilution factor. Insets are Northern blots of total RNA isolated from 10⁷ cells 2 days after treatment with or without tetracycline, probed with corresponding RNAi target sequence or with α-tubulin.](http://www.jbc.org/content/journal/jbc/281/6/3053.full.html)
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A product of ∼170 kDa, recognized exclusively by the anti-TbTOP2α serum (which had not detected protein on a Western blot of trypanosomal lysate). Cross-reacting bands were not present in TbTOP2α-containing yeast lysate probed with anti-TbTOP2β serum and vice versa. Additionally, neither antiserum detected protein on Western blots of lysates from cells carrying pRS426GU(URA).

A decatenation assay was employed to test whether the expressed proteins have catalytic activity. It relies on the ability of an enzyme to release nicked and covalently closed monomeric minicircles from kDNA networks. Decatenating activity was detected in lysates from yeast cells expressing TbTOP2α or HsTOP2α (Fig. 8B). (These cells were also able to grow at 35 °C, a nonpermissive temperature for the top2-4 mutation; data not shown). In both cases the activity was ATP-dependent, a hallmark of topoisomerase II as well as evidence against the possibility that decatenation was mediated by a type I topoisomerase acting on nicked minicircles. The observed difference in catalytic activity between TbTOP2α and HsTOP2α might reflect variations in the amount of expressed protein present in the lysate. As expected, topoisomerase II activity was absent in the pRS426GU(URA) transformant controls. Cells carrying pRS426GU-TbTOP2α provided no detectable type II activity, despite the fact that expression of the full-length protein was confirmed by Western blot (Fig. 8A).

DISCUSSION

We have identified, sequenced, and characterized two new genes for type II DNA topoisomerases in T. brucei brucei, termed TbTOP2α and -β. Phylogenetic analysis of these genes, their analogs from the genome data bases of T. cruzi, L. donovani, and previously reported topoisomer-
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Expression and catalytic activity. A, Western blot analysis of protein induction in yeast cells carrying pRS426GU-TbTOP2α (lane 1); pRS426GU-TbTOP2β (lane 2); or pRS426GU (lane 3) plasmids. Total cell lysates (5 μg per lane) were separated by SDS-PAGE, blotted, and probed with anti-TbTOP2α serum. The membrane was stripped and re-probed with anti-TbTOP2β serum. B, KDNA network decatenation by crude lysates (100 ng of protein per reaction) of yeast expressing recombinant topoisomerase II. Lane assignment is as follows: XhoI-linearized C. fasciculata kDNA (lane 1); substrate alone (lane 2); reactions containing lysate from pRS426GU (lanes 3 and 4); pRS426GU-TbTOP2α (lanes 5 and 6); pRS426GU-TbTOP2β (lanes 7 and 8) or pRS426GU-HsTOP2α (lanes 9 and 10) transformants. ATP was omitted where indicated. N, nicked; L, linear; C, covalently closed minicircle monomers.

FIGURE 8. Protein expression and catalytic activity. A, Western blot analysis of protein induction in yeast cells carrying pRS426GU-TbTOP2α (lane 1); pRS426GU-TbTOP2β (lane 2); or pRS426GU (lane 3) plasmids. Total cell lysates (5 μg per lane) were separated by SDS-PAGE, blotted, and probed with anti-TbTOP2α serum. The membrane was stripped and re-probed with anti-TbTOP2β serum. B, KDNA network decatenation by crude lysates (100 ng of protein per reaction) of yeast expressing recombinant topoisomerase II. Lane assignment is as follows: XhoI-linearized C. fasciculata kDNA (lane 1); substrate alone (lane 2); reactions containing lysate from pRS426GU (lanes 3 and 4); pRS426GU-TbTOP2α (lanes 5 and 6); pRS426GU-TbTOP2β (lanes 7 and 8) or pRS426GU-HsTOP2α (lanes 9 and 10) transformants. ATP was omitted where indicated. N, nicked; L, linear; C, covalently closed minicircle monomers.

ase II genes from kinetoplastids reveals two distinct clades (Fig. 2). One comprises the new genes, which contain nuclear localization signals and originate from the same node as nuclear enzymes from other eukaryotes. The second clade contains all the previously reported type II genes from kinetoplastids as follows: T. brucei brucei (25), T. cruzi (20), L. donovani (21), L. chagasi (24), L. infantum (23), and C. fasciculata (22), which share >65% identity with one another at the amino acid level and localize with genes from prokaryotes. The new topoisomerase genes and the phylogenetic analysis they make possible yield several important lines of information. First they provide the heretofore missing source for nuclear topoisomerase activity, which was implicated but not demonstrated by previous studies to be distinct from that of the mitochondrion (36). Northern analysis indicates that distinct genes for nuclear topoisomerase II activity can be expected for other kinetoplastids as well (Fig. 3). From a therapeutic perspective, TbTOP2α would appear to be more attractive than TbTOP2β as a target for drug development, based on the more rapid effect on cell proliferation after induction of cognate RNAi (3 versa 7 days). Second, although we cannot exclude the possibility of interchangeability between nuclear and mitochondrial activities, the aggregate results of our studies and the considerable preceding literature on TbTOP2mt and its orthologs are consistent with a single dominant enzyme in each compartment. The kinetoplastids are ancient and primitive eukaryotes whose mitochondrial DNA structure is unique and highly complex. It may be that the topological intricacy of kDNA network structure and replication requires a dedicated mitochondrial topoisomerase II activity, which bears phylogenetic similarity to prokaryote type II enzymes, and which was lost or replaced in other eukaryotes that have a much simpler mitochondrial genome structure. Third, clarification of the distinct TbTOP2α and -β sequences and their close proximity on chromosome 11 corrects the current version of the assembled T. brucei brucei genome sequence database which contains just one gene, a chimera of TbTOP2α and TbTOP2β.

Silencing the expression of TbTOP2α by RNAi leads to striking defects in cell and nuclear morphology (Fig. 6). Although insufficient topoisomerase II activity likely prevents the proper segregation of chromosomes, the emergence of cells that have no detectable nucleus, have only fragments of a nucleus, or have a grossly enlarged nucleus is compatible with the previously reported lack of checkpoints (54, 55) that would prevent cytokinesis in cells with incompletely segregated chromosomes. Chromosomes in kinetoplastids may not be as highly organized as those of other eukaryotes (e.g. they do not condense during mitosis); nevertheless, the irregular DAPI staining of the nuclear DNA seen in cells depleted of TbTOP2α suggests that the protein may play a structural, as well as a catalytic, role in the nucleus.

Why is there a TbTOP2β? One trivial explanation is that although TbTOP2α is expressed and functional by all criteria, TbTOP2β is simply a nonfunctional pseudogene that arose from a duplication event. Countering this, the Beta message appears in all five African trypanosomes that we examined and in all of these is of very similar size. Messenger RNAs in kinetoplastids are modified by trans-splicing at the 5′-end (37) as well as 3′-polyadenylation, so the presence and size similarity in these different organisms implies a functional significance. The phylogenetic conservation is particularly notable for T. equiperdum, which has diverged sufficiently from the T. brucei brucei species to have lost all but one of the ~250 minicircle sequences and to no longer be transmitted by an insect vector (8). Another possibility is that TbTOP2β is present at levels that are below the limit of detection by our antibodies and can be functionally substituted for by the α protein (but not vice versa). Alternatively, perhaps the β protein is only translated in cells undergoing meiosis or antigenic variation, which we did not study. It may be that TbTOP2β codes for an inactive protein. If so, conservation of the message is difficult to justify, and careful examination of the predicted amino acid sequence provides no obvious reason why TbTOP2β should be inactive. TbTOP2α and -β share residues necessary for the catalytic activity and differ significantly in few places, notably, the 41-amino acid N-terminal extension of TbTOP2β (which can be eliminated by assuming the subsequent methionine as a translation start site), and the variable C-terminal region. Whether either of these is
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responsible for the lack of catalytic activity of the TbTOP2β remains to be determined. For now the role of TbTOP2β is unknown.

These studies clarify our understanding of the type II DNA topoisomerases in trypanosomatids and related pathogenic protozoa and provide evidence for an important, and previously unrecognized, enzyme that functions in the nucleus. Given the great utility of topoisomerases as proven targets for drug therapy, nuclear topoisomerase II is an attractive candidate for much-needed new antitrypanosomal drug development.

Acknowledgments—We thank Rahul Bakshi, Jane Scocca, and Paul Englund for their comments on the manuscript; Suji Xie, Sean O’Hearn, and members of the Englund laboratory for their assistance; Jay Bangs for the anti-BP antibody; Lee Blosser for training and advice in flow cytometry; Marc Gartenberg and Neil Osheroff for yeast plasmids and strain; and Kara Cerveny and Rob Jensen for assistance with the yeast expression system.

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