Trypanosoma brucei Has Two Distinct Mitochondrial DNA Polymerase β Enzymes*

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In higher eukaryotes, DNA polymerase (pol) β resides in the nucleus and participates primarily in DNA repair. The DNA polymerase β from the trypanosomatid Crithidia fasciculata, however, was the first mitochondrial enzyme of this type described. Upon searching the nearly completed genome data base of the related parasite Trypanosoma brucei, we discovered genes for two pol β-like proteins. One is ~70% identical to the C. fasciculata pol β and is likely the homolog of this enzyme. The other, although ~30% identical within the polymerase region, has unusual structural features including a short C-terminal tail and a long N-terminal extension rich in prolines, alanines, and lysines. Both proteins, when expressed recombinantly, are active as DNA polymerases and deoxyribose phosphate lyases, but their polymerase activity optima differ with respect to pH and KCl and MgCl₂ concentrations. Remarkably, green fluorescent protein fusion proteins and immunofluorescence demonstrate that both are mitochondrial, but their locations with respect to the mitochondrial DNA (kinetoplast DNA network) in this organism are strikingly different.

Trypanosoma brucei belongs to a family of parasitic protozoa that are responsible for a growing human health problem. T. brucei is the causative agent of African sleeping sickness, a disease that threatens millions of people in sub-Saharan Africa, and related species are responsible for diseases such as Chagas disease and Leishmaniasis.

Trypanosomes are among the earliest diverging eukaryotes that contain a mitochondrion (1, 2), and perhaps because of this niche in evolution they exhibit some unique biological hallmarks. A prominent example is the fascinating structure and organization of their mitochondrial genome, known as kinetoplast DNA (kDNA). The kDNA contains several thousand DNA minicircles catenated together into a large network resembling medieval chain mail in its topology. In vivo this network is tightly condensed into a disk-shaped structure that associates with the basal body of the single flagellum of the parasite (see Ref. 3 for a review on kDNA).

With the topological constraints imposed by the unusual network organization of this mitochondrial DNA, it is difficult to imagine a priori how this genome might be replicated in an orderly fashion. Several proteins involved in this process have been identified, and they have distinct localizations with respect to the kDNA disk. This organization suggests the events of replication are spatially regulated, and knowledge of the functions and locations of these proteins has led to a fairly detailed model for kDNA replication (for recent reviews see Refs. 4 and 5). Briefly, minicircles are released from the network, presumably by a topoisomerase, prior to their replication. Release is vectorial into the kinetoflagellar zone, the space between the kDNA disk and the mitochondrial membrane nearest the flagellar basal body (6). In the kinetoflagellar zone, they encounter the origin binding protein UMSBP (7), a DNA primase (8), two Pol I-like DNA polymerases (Pol IB and Pol IC) (9), and likely other proteins that promote replication initiation. Replication via a theta structure intermediate is unidirectional and as such results in sister progeny containing either a single gap in the continuously synthesized strand or multiple gaps between Okazaki fragments on the discontinuously synthesized lagging strand.

It is not known whether minicircle replication is completed in the kinetoflagellar zone, but eventually newly replicated minicircles migrate to two discrete sites flanking the kDNA disk known as the antipodal sites. Here they encounter SSE1 (10), an enzyme with RNase H activity that can remove RNA primers (11), and DNA polymerase β (12), which presumably fills in most of the gaps between Okazaki fragments. Once this processing takes place, the newly replicated minicircles are reattached to the periphery of the kDNA network by a topoisomerase II (13) that also localizes to the antipodal sites (14). Interestingly, one or two gaps are retained on the newly replicated minicircles after they have been reattached to the network (15, 16). The residual gaps are thought to represent a counting mechanism that distinguishes molecules that have completed replication from those that have not yet begun the process (and are still covalently closed). Once the entire network has been replicated, the remaining gaps are filled, and the network is divided for segregation of the progeny into daughter cells.

Although much of what we know of kDNA replication has come from biochemical studies of purified proteins from the organism Crithidia fasciculata, the advent of the T. brucei genome project has facilitated a genomics approach to identify other replication proteins. In a search for the mitochondrial pol β homolog from T. brucei, we also anticipated finding a conventional nuclear enzyme as well. We report here the discovery of...
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Identification and Cloning of Full-length Genes—A TBLASTN search of the T. brucei genome data bases (www.tigr.org/dtd/mdb/tbdb and (www.sanger.ac.uk/Projects/T_brucie) using the C. fasciculata pol β protein sequence as the query pulled out two distinct sets of sequences. These sequences were used to design primers, and the full-length coding sequences for both genes were acquired from PCR amplification of genomic DNA from the T. brucei cell line 29-13. The primers used are indicated in subsequent sections. All of the PCR products used 30 cycles of 94°C melting (30 s), 55°C annealing (30 s), and 72°C extension (3 min). The accession numbers are AY354517 for pol β and AY354516 for pol β-Pak, the second pol β.

MATERIALS AND METHODS

Identification and Cloning of Recombinant Proteins—The coding sequence for T. brucei pol β lacking the putative mitochondrial localization signal (first 11 amino acids, based on homology to the C. fasciculata pol β) was amplified using the primers B-ND(F) (5'-CATATGCTAGACGACACCTTCTTC-3') and B-Bam(R) (5'-GGATCCATATGCTGGCCTTTGCTTC-3') and cloned into the pET14b vector. This purification yielded 0.8 mg of protein. The protein preparation analyzed by SDS-PAGE and stained with Coomassie Blue.

The entire coding sequence for T. brucei pol β-Pak was PCR-amplified using the primers PCX(F) (5'-CTCGAGATCTTGGTTCGCCCC-3') and P-Xho(R) (5'-CTCGAGATCTTTTGGATCTTCGTTT-3') and cloned into the XhoI site of the pET14b vector. This construct was electroporated into BL21(DE3), expression of the tagged protein in a 2-liter culture was induced for 3.5 h at 37°C with 1 mM isopropylthio-β-D-galactoside. The cells were harvested by centrifugation, resuspended in 30 ml of buffer A (50 mM Hepes, pH 7.6, 5 mM EDTA, 50 mM KCl, 1 mM PMSF) supplemented with 1 mg/ml lysozyme (Sigma), and incubated on ice for 30 min. The cells were lysed by sonication (six 10-s bursts at full power using a Branson sonicator). The lysates were centrifuged (30,000 × g, 15 min, 4°C), and the resulting supernatants were applied to a 5-ml DEAE-cellulose column that had been equilibrated in buffer A. The column was washed with 15 ml of buffer A containing 200 mM KCl. The 400 mM KCl fraction, which contained pol β-Pak, was applied to a 1-ml Ni²⁺-NTA column that had been equilibrated in buffer C (50 mM Hepes, pH 7.6, 100 mM KCl, 20 mM imidazole, and 1 mM PMSF). The column was washed with 10 ml of buffer C, and protein was eluted with 5 ml of buffer C supplemented with 250 mM imidazole. Through all of the chromatography steps, the pol β-Pak protein was identified by SDS-PAGE and Western blot using a rabbit antibody against the His tag (Santa Cruz Biotechnology; 1:1000 dilution). The purified protein was dialyzed against buffer C lacking imidazole and concentrated to ~200 μg in a Vivaspin 6 concentration device (Sartorius; 10,000 molecular weight cut off). Glycerol was added to 50%, and the resulting protein preparation was stored at −20°C. This purification yielded 0.8 mg of protein. The protein preparation analyzed by SDS-PAGE and Coomassie Blue staining is shown in Fig. 1A (lane 2).

Western Blot—Protein samples were boiled and were subjected to 8% polyacrylamide gel electrophoresis containing only the full-length protein. All of the immunizations were conducted following standard protocols at Calico Biologicals (Reamstown, PA). The sera were collected after four boosts.

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Polymerase Activity Assays—The Schiffer base trap and dRP lyase reactions were described previously (19). The substrate concentration was 2 μM, and the enzyme concentrations were 0.5 μM. For each 10-μl reaction, 1 μl was spotted on polyethyleneimine cellulose, and the remainder was resolved by SDS-PAGE.

Polymerase activity assays were performed as described previously (19), except that the primer-template was 40 μg of a poly(dA):oligo(dT) mixture (Amersham Biociences). In determining optimal buffer conditions, only one component was varied from the standard buffer conditions (50 mM Tris-HCl, pH 9.0, 5 mM MgCl₂, 0.1 mM bovine serum albumin).
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RESULTS

T. brucei Has Two Genes Encoding pol β-like Proteins—Our search of the T. brucei genome data base identified two genes, tandemly linked on chromosome V, that encode candidate pol β proteins. A schematic of the proteins encoded by the two pol β genes is compared with that of the C. fasciculata protein in Fig. 2A. The smaller of the two open reading frames encodes a 46-kDa protein that is ~70% identical to the C. fasciculata pol β, including a putative mitochondrial targeting signal at the N terminus. The two proteins are likely homologs, and thus we have designated the trypanosome protein as T. brucei pol β. The other gene encodes a much larger protein of 87 kDa. Within the polypeptide domain, this protein shares ~30% sequence identity with both the C. fasciculata and T. brucei pol β proteins. Interestingly, this protein has a 19-amino acid C-terminal tail as well as a ~300-amino acid N-terminal extension rich in prolines, alanines, and lysines (PAK domain), both of which are absent in the other proteins. Because of the latter domain, we have designated this protein as T. brucei pol β-PAK. Although we initially expected this second pol β to be nuclear, it also contains a putative mitochondrial targeting signal at its N terminus based on sequence characteristics outlined previously (22).

The sequence of the PAK domain (Fig. 2B) has unusual properties. It accounts for ~30 kDa of the total protein, and 65% of its sequence is composed of the amino acids proline, alanine, and lysine. At neutral pH it is predicted to carry a charge of +48. Within this domain are three near perfect 60-amino acid repeats (shown in bold type). A BLAST search using only this sequence pulls out no homology from any organism, but its amino acid content is reminiscent of two proteins cloned from C. fasciculata, kinetoplast-associated protein 1 (23) and DNA primase. Interestingly, both of these proteins are mitochondrial and associate with the kDNA.

Both Procyclic and Bloodstream Form Parasites Express pol β and pol β-PAK—To determine whether expression of either pol β or pol β-PAK was dependent on the life cycle stage of the parasite, we used SDS-PAGE to fractionate whole cell lysates from both procyclic and bloodstream form parasites. The proteins were transferred to a polyvinylidene difluoride membrane and probed using sera containing specific antibodies generated against the recombinant proteins (Fig. 1B). The rat anti-pol β antibodies recognized a specific band of ~46 kDa in both procyclic and bloodstream form lysates, in agreement with the predicted molecular mass of this enzyme (46.1 kDa). The rabbit anti-pol β-PAK antibodies recognized a band of ~105 kDa in lysates from both life cycle stages. This protein runs with significantly lower mobility than expected for its predicted molecular mass (87.2 kDa). Its migration is likely hindered by the large positive charge of the PAK domain, and the possibility of post-translational modification cannot be ruled out.

Recombinant pol β and pol β-PAK Are Active as DNA Polymerases—Inspection of the sequences of T. brucei pol β and pol β-PAK indicates that amino acid residues important for DNA polymerase activity are conserved in both enzymes (data not shown). To ascertain whether they are indeed active, we assayed recombinant proteins using buffer conditions previously published for the C. fasciculata mitochondrial pol β (24). An enzyme titration indicated that the T. brucei pol β and C. fasciculata pol β have similar levels of polymerase activity (Fig. 3A). In contrast, T. brucei pol β-PAK displays less than half as much activity under these conditions.

To determine whether the compromised polymerase activity of pol β-PAK was an intrinsic property of the protein or a function of the buffer conditions used, we varied the concentrations of KCl and MgCl₂ or the pH of the assay (Fig. 3B). Again, the T. brucei pol β and the C. fasciculata pol β behaved similarly. The activities of both were highest at low KCl and MgCl₂ concentrations, and their activity declined dramatically at pH levels lower than 9.0. For T. brucei pol β-PAK, however, the optimal buffer conditions differed greatly from the pol β proteins. The activity of pol β-PAK was stimulated by higher concentrations of KCl and MgCl₂. Also, it was much more tolerant of changes in pH over the small range examined.

The buffer component whose concentration influenced the polymerase activity most markedly was KCl (Fig. 3C). In assays where pol β-PAK showed the greatest specific activity (200

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a C. Li, J. C. Morris, and P. T. Englund, unpublished data.
mm KCl), it was able to incorporate 54 pmol of dTMP/pmol protein/min, surpassing the greatest specific activity shown by the C. fasciculata pol β (50 μM KCl) of 33 pmol dTMP/pmol protein/min incorporated. These data confirm that pol β-PAK is not intrinsically less active as a DNA polymerase than pol β.

Recombinant pol β and pol β-PAK Have dRP Lyase Activity—Because the catalytic amino acids required for dRP lyase activity are also conserved, we wanted to test whether both pol β and pol β-PAK are active in this capacity. We incubated the recombinant proteins with a radiolabeled dRP-containing substrate for 0 or 5 min and then reduced the intermediates with NaBH₄ (see Fig. 3 in Ref. 19 for the dRP lyase mechanism showing intermediates). Portions of the reaction mixtures were resolved by SDS-PAGE (8% gel; Fig. 4A), and the E-DNA and E-dRP intermediates were visualized by autoradiography. For both pol β and pol β-PAK, at time 0 when enzyme and NaBH₄ were added simultaneously, the E-DNA intermediate accumulated (lanes 3 and 5). However, if the enzyme was incubated with substrate for 5 min prior to reduction, much of the initial E-DNA intermediate had been converted to the E-dRP intermediate (lanes 4 and 6) or the final unsaturated dRP product (see next paragraph). This pattern was similar to that observed for the C. fasciculata pol β used as a control (lanes 1 and 2).

We also resolved the free unsaturated dRP derivative, the final product of the reaction, by polyethyleneimine cellulose thin layer chromatography. For all of the proteins, accumula-

![Image](https://i.imgur.com/59x750.png)

**Fig. 3. Analysis of polymerase activity.** A, enzyme titration of the C. fasciculata mitochondrial pol β, T. brucei pol β, and T. brucei pol β-PAK in a standard polymerase assay. B, effect of buffer composition on polymerase activity of pol β and pol β-PAK. The protein concentrations were 0.8 μM, and either KCl concentration (left panel), MgCl₂ concentration (center panel), or pH (right panel) was varied. We have defined 100% as the maximal activity observed over the range of buffer component examined. The underlined values on x axis represent standard assay conditions. C, maximum specific activity (pmol dTMP/pmol protein/min incorporated) observed for each protein in each set of varied buffer conditions. The values represent the 100% maximal activity point as defined in each panel of B. C.f., C. fasciculata; T.b., T. brucei.

Accumulation of unsaturated dRP did occur when the reactions were allowed to proceed for 5 min prior to reduction, however, and this accumulation was protein-dependent (lanes 2, 4, 6, and 8). Thus, both pol β and pol β-PAK can function as dRP lyases.

**pol β and pol β-PAK Are Both Mitochondrial—**Although the predicted protein sequences of both T. brucei pol β-like genes included putative mitochondrial localization signals (22), we wanted to verify the intracellular targeting of these proteins. We cloned the complete coding sequence of each gene into the procyclin promoter. We then identified the location of the fusion products by fluorescence microscopy of live cells.

Imaging of live T. brucei cells expressing the pol β-GFP fusion protein revealed 5–10% of cells in which the GFP fluorescence accumulated at two bright spots flanking the kDNA disk (Fig. 5A shows two examples). All cells displaying this pattern of GFP had one nucleus and one kDNA and were likely dividing kDNA, a weak GFP signal corresponding to the kDNA was observed; however no accumulation of pol β-GFP was seen in cells in which the nucleus had also undergone division (data not shown). Because its expression is driven by the strong procyclin promoter, the pol β-GFP fusion protein is probably overexpressed relative to the endogenous protein, and at longer exposures we detected some spillover of GFP fluorescence into
the entire tubular mitochondrial matrix (data not shown). In cells in which the two brightly staining antipodal sites were not detected, this background fluorescence was still apparent. The antipodal localization in only a portion of GFP-expressing cells indicates that, like its *C. fasciculata* homolog (18), accumulation in this location is cell cycle-dependent. Unfortunately, although we have specific antibodies to this protein (Fig. 1B), we were unable to obtain immunofluorescence data despite many attempts with a myriad of fixation, permeabilization, and detergent extraction conditions.

When we imaged live *T. brucei* cells expressing the pol β-PAK-GFP fusion protein, we were surprised to find that this protein localized not in the antipodal sites but exclusively to the kDNA disk (Fig. 5B). Immunofluorescence experiments using antibodies affinity-purified against full-length recombinant pol β-PAK confirmed this localization (Fig. 5C).
DISCUSSION

In higher eukaryotes it is firmly established that pol β is a nuclear enzyme involved in DNA repair. The pol β from the trypanosomatid parasite *C. fasciculata* was the first example of a mitochondrial enzyme of this type (12, 17, 25). We demonstrate here that the related parasite *T. brucei* has two genes encoding pol β-like proteins, and remarkably, both localize to the mitochondrion. Interestingly, data base searches have yet to reveal a third pol β gene that might represent the nuclear homolog in this organism. Perhaps, like the malaria parasite *Plasmodium falciparum* (26), *T. brucei* lacks a nuclear pol β and has evolved other mechanisms to repair its nuclear DNA. Surprisingly, the pol β homolog from *Leishmania infantum*, also a trypanosomatid parasite, has been reported to localize to the nucleus (27). This localization needs further study given that the *Leishmania* protein is 93% identical to the *C. fasciculata* mitochondrial pol β (using the Clustal alignment program) and includes an N-terminal mitochondrial localization signal.

Although pol β and pol β-PAK share ~30% sequence identity, including the active site residues essential for both the DNA polymerase and dRP lyase activities, there are striking differences between these two enzymes. One significant difference is the long N-terminal extension rich in the amino acids proline, alanine, and lysine for which pol β-PAK is named. This region is highly positively charged and contains three 60-amino acid repeats of unknown significance. The PAK domain could possibly assist in targeting this protein to the kDNA disk or anchoring it in this location. Alternatively, this domain could serve a regulatory role in modulating the enzymatic activities of this protein *in vivo* (see below). Because of the conservation of gene synteny between *T. brucei* and related organisms, we were able to identify pol β-PAK homologs upstream of the pol β genes in both the *Leishmania major* and *Trypanosoma cruzi* genomes. Interestingly, although the coding sequences of the second gene include N-terminal extensions in both species, neither the length nor the sequence of the PAK domain is well conserved. Additionally, the short C-terminal tail is also missing in these homologs. We failed to identify a third pol β that might represent the nuclear enzyme in genome searches of these organisms as well.

*T. brucei* pol β and pol β-PAK have different buffer preferences in *in vitro* polymerase assays. Although pol β prefers low concentrations of KCl and MgCl₂, and its activity declines rapidly at pH below 9.0, the polymerase activity of pol β-PAK is stimulated by higher KCl and MgCl₂ concentrations and is less sensitive to lower pH. The high salt content preferred by pol β-PAK *in vitro* could stabilize the enzyme by facilitating an important conformational change or affecting the aggregational state of the enzyme.

The size of pol β-PAK, as well as its propensity for salt activation, raises the possibility that this enzyme corresponds to a second *T. brucei* mitochondrial polymerase activity observed previously (28). Although there are some differences (for instance, in optimal MgCl₂ concentration), it should be noted that these previous analyses were done on crudely fractionated mitochondrial extracts. The second active fraction could have contained pol β-PAK, one or more of the pol I-like polymerases (9), or other proteins that might affect polymerase activity.

Finally, although both pol β and pol β-PAK reside in the mitochondrion, they have different sub mitochondrial localizations with respect to the kDNA disk. These data suggest that these two pol β proteins have distinct and nonredundant roles in kDNA replication or maintenance. We propose the following model to invoke the need for two pol βs (Fig. 6). Prior to replication, all of the minicircles in the network are covalently closed (*black circles*). Minicircles are released from the network and replicated, giving rise to one daughter molecule with a single gap (*blue circles*) and one with multiple gaps (*red circles*). Although the singly gapped molecules are capable of network reattachment with little or no alteration (29, 30), the multiply gapped circles undergo some processing in the antipodal sites prior to their reattachment to the kDNA network. In this processing step, pol β is thought to fill many of the gaps between Okazaki fragments, leaving only one or two on the molecules that are ultimately reattached (16). As replication proceeds, the central zone of covalently closed minicircles dwindles, and the peripheral zones of gapped molecules grow until finally the network consists entirely of gapped molecules. At this point, pol β-PAK is perfectly poised to fill these remaining gaps. Once the gaps are repaired, the network splits in two and the progeny networks are segregated to daughter cells. Because gap repair does not occur until minicircle replication is complete, there must be a regulatory mechanism to suppress pol β-PAK activity until the appropriate time. We are currently testing this model by determining the *in vivo* functions of these two enzymes using an RNA interference-based strategy.

In higher eukaryotes, only one DNA polymerase (pol γ) has been rigorously shown to be involved in mitochondrial DNA replication and maintenance (31). Thus, the initial discovery of a mitochondrial pol β in a trypanosomatid organism was certainly novel, and our report of a second mitochondrial pol β enzyme is even more so. Also, although the canonical pol γ is lacking in these cells, trypanosomes instead import a family of four pol I-like DNA polymerases into their mitochondrion (9). Currently, then, the tally of mitochondrial DNA polymerases in trypanosomes stands at six, an unprecedented multitude. The unusual and complicated structure of the kDNA, as well as the importance of its integrity to the parasite, seems to demand a more sophisticated system to ensure that this mitochondrial genome is properly maintained.

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