Tryptosomatids are protozoan parasites that cause important diseases in humans and livestock. They have an unusual mitochondrial DNA, known as kinetoplast DNA (kDNA), which has a remarkable structure (see Refs. 1–3 for reviews). kDNA consists of several thousand circular DNA molecules, which are all catenated into a giant network. In the trypanosomatid *Crithidia fasciculata*, the subject of this study, electron microscopy reveals that an isolated network is an elliptically shaped monolayer of interlocked DNA circles, about 10 μm in size or smaller. With a single-stranded M13 DNA template and the four rNTPs as substrates, the enzyme makes heterogeneous oligonucleotides of which the vast majority are about 10 nucleotides in size or smaller. These localization studies have significant implications for our understanding of the mechanism of kDNA replication.

**A Mitochondrial DNA Primase from the Trypanosomatid *Crithidia fasciculata***

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We have purified to near homogeneity a DNA primase from a mitochondrial fraction of the trypanosomatid *Crithidia fasciculata*. The enzyme is a single polypeptide chain of 28 kDa. Using a poly(dT) template and ATP as a substrate, the enzyme makes oligonucleotides of which the vast majority are about 10 nucleotides in size or smaller. With a single-stranded M13 DNA template and the four rNTPs as substrates, the enzyme makes heterogeneous oligonucleotides in the same size range. These oligonucleotides efficiently prime the synthesis of DNA by the Klenow DNA polymerase. Immunolocalization with antibodies against the purified enzyme confirms that the primase is mitochondrial. Furthermore, the enzyme localizes to specific regions of the cell's single mitochondrion, above and below the condensed kinetoplast DNA. The primase does not co-localize with the mitochondrial topoisomerase II and DNA polymerase β, both of which are associated with two protein complexes positioned on opposite sides of the kinetoplast disc. These localization studies have significant implications for the mechanism of kinetoplast DNA replication.

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Trypanosomatids are protozoan parasites that cause important diseases in humans and livestock. They have an unusual mitochondrial DNA, known as kinetoplast DNA (kDNA), which has a remarkable structure (see Refs. 1–3 for reviews). kDNA consists of several thousand circular DNA molecules, which are all catenated into a giant network. In the trypanosomatid *Crithidia fasciculata*, the subject of this study, electron microscopy reveals that an isolated network is an elliptically shaped monolayer of interlocked DNA circles, about 10 μm in size. Each cell has one network, which is condensed into a disc-shaped structure within its single mitochondrion. The network contains two kinds of DNA circles known as minicircles and maxicircles. In *C. fasciculata*, the network contains about 5000 minicircles, each 2.5 kilobases, and 25 maxicircles, each 38 kilobases. Maxicircles have a genetic function resembling that of mitochondrial DNAs in other eukaryotes, encoding ribosomal RNAs and proteins required for mitochondrial energy transduction. Most maxicircle transcripts undergo editing, a process by which uridine residues are inserted or deleted from the sequence, thus forming an open reading frame. Minicircles encode small guide RNAs, which control the specificity of editing (see Refs. 4 and 5 for reviews on editing).

The network structure of kDNA must require unusual mechanisms for its replication. For example, minicircles do not replicate while topologically linked to the network, but instead they are released from the network by a topoisomerase. After release they replicate as free minicircles, using a mechanism, and the progeny minicircles are then reattached to the network periphery. When all minicircles have replicated, the network has doubled in size. Then it splits in two, and the two progeny networks segregate into daughter cells during cell division (see Refs. 3, 6, and 7 for reviews).

A current goal in our laboratory is to study proteins and enzymes that are involved in the maintenance and replication of kDNA. These proteins are interesting not only in their own right, but also because in at least some cases they are assembled into mitochondrial protein complexes, which can be easily visualized by immunofluorescence. For example, both a topoisomerase II (8) and a DNA polymerase β (9) co-localize within two discrete protein complexes (each roughly 0.4 μm in diameter), which are situated in antipodal positions at the edge of the kinetoplast disc (the disc is approximately 1 μm in diameter and 0.4 μm thick). Since minicircles thought to be replication intermediates (as visualized by fluorescence in situ hybridization) are also present in these two complexes (9), it is likely that these structures are involved in minicircle replication (see Ref. 3 for review).

In this paper we report the purification of a *C. fasciculata* mitochondrial DNA primase, the enzyme responsible for making short RNA molecules that initiate the synthesis of a DNA strand. We found that the primase is a single polypeptide of 28 kDa, and we describe some of its enzymatic properties. Using immunofluorescence, we found unexpectedly that the primase does not co-localize with the topoisomerase II and DNA polymerase β in the two protein complexes that flank the kinetoplast disc. Instead it localizes above and below the disc. This finding has significant implications for our understanding of the mechanism of kDNA replication.

**Experimental Procedures**

**Materials**—Ribo- and deoxynucleoside triphosphates were purchased from Boehringer Mannheim, and [α-32P]dATP (3000 Ci/mmol) and [α-32P]dGTP (3000 Ci/mmol) were from NEN Life Science Products. Poly(dT), from Pharmacia Biotech Inc., had an average length of 200–300 nucleotides.

**Primase Activity Assay**—A coupled assay was used to follow the enzyme during purification (10). In this assay, using ATP as a substrate, the primase synthesized non-radioactive oligo(dA) primers complementary to a poly(dT) template. The primers were then extended by Klenow DNA polymerase and [α-32P]dATP, and acid-insoluble radioactivity was measured. The 25-μl reaction mixture contained 50 mM Tris-HCl, pH 7.5, 50 mM NaOAc, 5 mM MgCl2, 0.1 mg/ml BSA (the 10 mg/ml stock solution had been heated at 60 °C for 15 min), 5% (v/v) glycerol, 1 mM DTT, 0.5 μg of poly(dT), 1 mM ATP, 50 μM [α-32P]dATP.
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(about 2400 cpm/pmol), 0.5–0.5 unit of primase, and 0.5 unit of Escherichia coli DNA polymerase I large fragment (New England Biolabs). After 30 min at 30 °C, products of the reaction were precipitated by sequential addition of 10 μl of BSA (10 mg/ml), 10 μl of 0.1 sodium pyrophosphate, 35 μl of H2O, and 20 μl of 10% (v/v) trichloroacetic acid. The precipitated product was collected onto GF/C filters (Whatman) under vacuum and washed, first with 0.1 M HCl and 0.1 M sodium pyrophosphate, and then with 95% (v/v) ethanol. Radioactivity on the filters was measured by liquid scintillation counting. One DNA primase unit is defined as the amount required for conversion of 1 pmol of [3H]dAMP/μl into an acid-insoluble form under assay conditions. The activity is linear with enzyme concentration in the range of 0.1–1 unit. The standard buffer conditions and temperature (30 °C) were used in all primase reactions described in this paper.

Large Scale Growth of C. fasciculata and Preparation of Mitochondrial Fraction—Parasites were grown in a 150-liter Fermentation fermenter (in the Department of Biochemistry, Johns Hopkins School of Hygiene and Public Health) at 28 °C in medium containing 1.8% Delton AE80M peptone, 0.45% yeast extract, 0.45% NaCl, 0.9% glucose, and 10 mg/g liter hemin (11). Alternatively, they were grown at room temperature in 6-liter flasks (each containing 4 liter of medium) with vigorous shaking (200 rpm). Cells at a density of about 4 × 10^7/ml were harvested by centrifugation either using a Sharples continuous flow centrifuge or a Sorvall GSA rotor (5000 rpm, 10 min, 4 °C). They were washed in STE buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% (v/v) glycerol, 25 mM Tris-HCl, pH 7.5, 2 μg/ml leupeptin). Insoluble material was removed by centrifugation (Sorvall GSA rotor, 12,500 rpm, 30 min, 4 °C).

Crude mitochondrial lysate (Fraction 1, 500 ml) was loaded onto a 50-ml Q-Sepharose (Pharmacia) column at a flow rate of 1 ml/min. DNA primase activity does not bind to this column; however, this step was essential for removal of nucleic acids in the lysate. The flow-through fraction (Fraction 2, 450 ml) was loaded onto a 50-ml S-Sepharose (Pharmacia) column at 2 ml/min, which was washed with 250 ml of buffer A, then with 250 ml of 0.2 M KCl in buffer A, and finally with 300 ml of 0.5 M KCl in buffer A. DNA primase activity was eluted at the 0.5 M KCl step (Fraction 3, 300 ml). Fraction 3 was then dialyzed and loaded onto a 30-ml double-stranded DNA-cellulose column at 0.5 ml/min. The column was washed with 200 ml of buffer A and eluted with a 240-ml gradient of 50 mM to 1 M KCl in buffer A at 0.5 ml/min. The DNA primase activity eluted between 0.25 and 0.4 M KCl. The active fractions were pooled (Fraction 4, 40 ml), dialyzed, and loaded onto a 1-ml Poros HS column (PerSeptive Biosystems) at 0.5 ml/min. After washing with 20 ml of buffer A, the column was eluted with a 25-ml gradient of 50 mM to 1 M KCl in buffer A at 0.5 ml/min. The activity eluted at about 0.3–0.4 M KCl. Pooled active fractions (Fraction 5, 4 ml) were diluted with 4 ml of 4X KCl and loaded onto a phenyl-Sepharose column (HR5/5, Pharmacia) at 0.5 ml/min. The column was eluted with a 35-ml reverse gradient, 2 ml to 0 M KCl in buffer A, at 0.5 ml/min. The DNA primase activity eluted at about 1 M KCl. The active fractions (Fraction 6, 1 ml) were dialyzed against buffer A containing 40% glycerol to reduce the volume to about 300 μl. The final chromatographic procedure was gel filtration on a 30-ml Superose 12 FPLC column (Pharmacia) using buffer A at a flow rate of 0.5 ml/min. The active fractions were pooled and dialyzed against buffer A containing 40% glycerol (v/v) and stored at −80 °C (Fraction 7, 0.8 ml).

In a second purification, we used a mitochondrial fraction isolated by Method B, and the purification steps were identical to those described in the previous paragraph with the following exceptions: 1) a 150-ml phosphocellulose column, eluted with a 1200-ml gradient from 0 to 1 M KCl in buffer A, substituted for the DNA cellulose column; 2) column sizes (except for the Poros HS column) and volumes of elution buffers were increased proportionally to the amount of protein; 3) a 10-ml phenyl-Sepharose column substituted for the phenyl-Sepharose, and this column was loaded after the Poros HS column, and 4) the final Superose 12 FPLC step was omitted because the protein was pure after Poros HS chromatography.

Antibodies—Rabbit antibody to mitochondrial DNA polymerase β was a gift from Dr. Al Torri (13). Antibody to the DNA primase was prepared by immunizing female BALB/c mice by intraperitoneal injections with purified DNA primase (Fraction 7, 2–5 μg/injection). The initial inoculations were in Freund’s complete adjuvant, and four subsequent boosts were at 3-week intervals with Freund’s incomplete adjuvant. The serum (at 1:1000 dilution) was screened by Western blot for specific recognition of the 28-kDa protein. The antibody recognizes the homogeneous primase on a Western blot (Fig. 1, lane 12; see lane 10 for Coomassie-stained gel) and also recognizes a polypeptide of the same size in a preparation of isolated mitochondria (Fig. 1, lane 11; see lane 9 for Coomassie-stained gel). Preincubation of 5 μl of primase antisemur with 5 μl of primase solution (30 min, 4 °C) resulted in 70% loss of primase activity in the standard assay. A control experiment with DNA polymerase β antisemur resulted in loss of only 15% of primase activity. In an immunodepletion assay, antisemur bound to protein A-Sepharose beads with less than 0.1% of the complete 74% of the primase activity. In a control experiment with DNA polymerase β antisemur, only 10% of the primase activity was depleted.

Immunolocalization of DNA Primase—Log phase C. fasciculata cells (2 × 10^7 cells/ml) were washed with 5 mM NaHPO₄, 5 mM NaH₂PO₄, 130 mM NaCl (PBS) (Sorvall GSA rotor, 10 min, 4000 rpm, 4 °C) and resuspended in PBS at the same cell concentration. Cell suspension (10 μl) was spotted onto 10-well slides pretreated with a 1/10 dilution of

2 J. Shlomai, personal communication.
The purified enzyme was stored at 80 °C in buffer containing 40% glycerol. It maintains over 50% of its activity after 6 months of storage.

As shown in Fig. 2, the DNA primase activity (panel A) co-eluted from the Superose 12 column with the 28-kDa protein as indicated by the intensity of the silver-stained band on SDS-PAGE (panel B). Primase activity and the 28-kDa protein both peak in fraction 31. The 28-kDa protein also co-eluted with primase activity on the phenyl-Superose and Poros HS columns, and the ratio of activity to silver staining of the band was comparable to that of Fraction 7. These data provide strong evidence that the 28-kDa protein is responsible for activity. To assess the size of the native enzyme, we also compared its behavior in gel filtration (Superox 12 FPLC) with that of two reference single-chain proteins, carbonic anhydrase (29 kDa) and BSA (67 kDa). The elution position of the DNA primase was the same as that of carbonic anhydrase (see legend of Fig. 2A), providing strong evidence that the primase is a 28-kDa monomer.

**Requirements for DNA Primase Activity**—Maximal primase activity requires ATP and a divalent cation (Mg2+). ATP and Mg2+ inhibit primase activity. We determined whether in our standard assay (using a poly(dT) template) the incorporation of [32P]dAMP into an acid-insoluble form depends on the activity of primase, we varied the ATP

**FIG. 1. SDS-PAGE analysis of DNA primase purification and Western blot to demonstrate specificity of the anti-primase antibody.** Aliquots of each fraction were electrophoresed on a 12% SDS-polyacrylamide gel, which was then stained with silver (lanes 1–8) or Coomassie Blue (lanes 9 and 10). Lanes 1–7, fractions from the purification summarized in Table I, using mitochondria isolated by Method A. Lane 1, mitochondrial lysate, 7.5 μg of protein; lane 2, Q-Sepharose flow-through, 5 μg of protein; lane 3, S-Sepharose, 3 μg of protein; lane 4, DNA-cellulose, 1.5 μg of protein; lane 5, Poros HS, 0.6 μg of protein; lane 6, phenyl-Superose, 0.2 μg of protein; lane 7, Superose 12 gel filtration, ~0.1 μg of protein. Lane 8 shows purified DNA primase (Poros HS fraction, ~0.15 μg of protein, 10% SDS-PAGE) from a different purification using mitochondria isolated by Method B. Lane 9, mitochondrial lysate, 5.0 μg of protein; lane 10, same as lane 8. Lanes 11 and 12 were identical to lanes 9 and 10 but DNA primase was detected on a Western blot using anti-DNA primase antibody. The mitochondrial preparation used in lanes 9 and 11 was prepared by Michele Klingbeil and Tina Saxowsky using a new unpublished method involving cell breakage by the Stanstead Cell Disruptor and Percoll purification of the mitochondria. For Western blotting, proteins were transferred to PVDF Western blotting membranes (Boehringer Mannheim). The membrane was first blocked with 20% horse serum in TBS buffer (25 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.5 mM EDTA) for 1 h and then incubated for 1 h at room temperature with anti-primase antibody diluted 1:1000 in blocking buffer. After washing three times for 10 min with TBS and 0.05% Tween 20, the membrane was incubated for 1 h at room temperature with 1:1000 diluted anti-mouse IgG monoclonal antibody conjugated with alkaline phosphatase (Boehringer Mannheim). After washing three times for 10 min with TBS containing 0.05% Tween 20, the blot was developed with bromochloroindolyl phosphate/nitro blue tetrazolium. The size markers on the left of the figure refer to lanes 1–7.

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**Table I. Purification of DNA primase**

| Fraction                     | Total protein | Total activity | Specific activity | Purification |
|------------------------------|---------------|----------------|-------------------|--------------|
| 1. Mitochondrial lysate       | 1750          | 543            | 0.31              | 1.0          |
| 2. Q-Sepharose flow-through   | 1440          | 552            | 0.38              | 1.3          |
| 3. S-Sepharose                | 420           | 332            | 0.79              | 2.5          |
| 4. DNA-cellulose              | 10            | 118            | 11.8              | 38           |
| 5. Poros HS                   | 0.64          | 90             | 141               | 455          |
| 6. Phenyl-Superose            | 0.02          | 66             | 3,300             | 10,600       |
| 7. Superose 12 FPLC          | 0.01          | 45             | 4,500             | 14,500       |

a. Protein was assayed with the Coomassie Protein Assay Reagent (Pierce) according to the manufacturer’s instructions.

b. These values may have significant error because of the low concentration of protein available.

**RESULTS**

**Purification of Primase**—Table I presents a summary of the purification. Starting from the mitochondrial lysate supernatant (prepared by Method A), the DNA primase was purified 14,500-fold and the yield of activity was about 8%. Fig. 1 shows an analysis of Fractions 1–7 by SDS-PAGE and silver staining. Fraction 7 appears to contain only a single homogeneous protein of 28 kDa.

The final yield of primase from mitochondria prepared by Method A was only about 10 μg of protein (Table I). We considered the possibility that this mitochondrial isolation procedure had a low yield, due at least in part to the extensive washing of the mitochondrial fraction in Method A. We therefore did a second purification using a mitochondrial fraction isolated by Method B. In the preparation shown in Table I, we started with only 1750 mg of mitochondrial protein in a mitochondrial lysate. Using Method B in a second purification, we started with 27,200 mg of mitochondrial protein from 450 g of cells. Using this procedure we obtained about 300 μg of purified DNA primase in about 10% yield. Its specific activity was comparable to that shown in Table I. Fig. 1 (lane 8) shows an SDS-PAGE analysis of the final product. This most pure fraction, or the Fraction 7 enzyme in Table I, was used in all experiments described in this paper. The purified enzyme was stored at −80 °C in buffer containing 25 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.5 mM EDTA, 1 mM DTT, 40% glycerol. It maintains over 50% of its activity after 6 months of storage.
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**TABLE II**

Characteristics of DNA primase

| DNA primase activity | %   |
|----------------------|-----|
| Complete systema     | 100 |
| −Mg2+                | 21  |
| −KCl                 | 49  |
| −ATP                 | 0   |
| +EDTA (5 mM)         | 0   |
| −Rifampicincb        | 0   |
| 100 µg/ml            | 59  |
| 50 µg/ml             | 80  |
| +N-Ethylmaleimideb   | 48  |
| 10 mM                | 95  |
| 5 mM                 |     |

a The standard assay conditions with a poly(dT) template, described under “Experimental Procedures.”
b Added at beginning of reaction with no preincubation.

concentration from 0 to 4 mM. As shown in Fig. 3A, there was no incorporation in the absence of ATP and incorporation was maximal at 1 mM (closed symbols). In a control experiment (open symbols), there was little effect of ATP on the incorporation of \( ^{32}P \)dAMP when primase was omitted and oligo(dA) was used as a primer. In a related experiment we used single-stranded M13 DNA as a template (Fig. 3B). Again there was dependence upon the presence of rNTPs (compare lower line to upper line), and the magnitude of \( ^{32}P \)dAMP incorporation depended upon the primase concentration.

**The Products of the DNA Primase Reaction**—To characterize the products, we conducted reactions (for times up to 45 min) containing poly(dT) template, \( [\alpha-\text{32P}]\text{ATP} \) as substrate, and DNA primase. Analysis of the reaction products on a 20% polyacrylamide gel revealed a ladder of fragments ranging up to about 10 nucleotides in size (Fig. 4A). However, with a long exposure of the autoradiogram, there were very small amounts of larger oligonucleotides, up to about 15 nucleotides in size. We also found in a similar experiment using M13 DNA as a template and the 4 rNTPs that the primase synthesized a very heterogeneous population of oligonucleotides in the same size range (data not shown).

We next used a chase to determine whether the oligonucleotides synthesized by primase can prime DNA synthesis (Fig. 4B). We first synthesized primers using \( [\alpha-\text{32P}]\text{ATP} \) as a substrate (lane 1), and the products are the characteristic ladder of fragments up to about 10 nucleotides in size. We then added Klenow DNA polymerase and increasing concentrations of dATP to the reaction. As shown in lanes 2–4, the primers were elongated and the products appeared in or near the slot. The products of DNA primase can also be elongated by the purified C. fasciculata mitochondrial DNA polymerase \( \beta \) (15). However, because of the low processivity of this enzyme, the primer extensions occurred with much lower efficiency (data not shown).

**Intracellular Localization of DNA Primase**—Four lines of evidence support the mitochondrial localization of this primase. First, in a small scale purification (from about 25 g of cells), we used Percoll gradient-purified mitochondria to purify the primase through the phenyl-Superose fraction. The primase obtained from the purified mitochondria had activity and chromatographic behavior similar to that obtained from the crude mitochondrial fraction. Second, the activity in the crude mitochondrial fraction was latent, requiring release from vesicles by treatment with a buffer containing 0.25% Nonidet P-40 and 0.5 mM KCl. In a control assay in which the detergent/KCl treatment was omitted, the amount of detectable primase activity was only about 10% of that obtained with detergent/KCl. Third, a Western blot of proteins from a mitochondrial fraction revealed a 28-kDa polypeptide that reacted with anti-primase antibody (Fig. 1, lanes 9 and 11). Finally, immunolocalization experiments proved conclusively that the primase is localized within the single mitochondrion of this parasite.

For immunolocalization we used a polyclonal antibody against the primase. The results indicate that the primase is indeed situated within the mitochondrion in discrete sites near the kDNA network. Fig. 5A shows two C. fasciculata cells visualized by Nomarski optics. Fig. 5B shows the same cells stained with DAPI, which brightly stains the kinetoplasts (the nuclei stain poorly under these conditions and are barely visible). The kinetoplast is a disc-shaped structure, oriented per-
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Fig. 4. The products of DNA primase. Panel A, to analyze the primase products, the reaction (25 μl) contained poly(dT) template, DNA primase (0.5 unit), and 50 μM [α-32P]ATP as substrate. After incubation for the designated time, the reaction was stopped by adding 2 μl of 0.5 M Na2-EDTA, 1 μl of glycogen (20 mg/ml, Boehringer Mannheim), 10 μl of 2 M NaOAc, and H2O to 100 μl. The reaction products were precipitated by adding 400 μl of isopropanol. After washing with 70% ethanol and drying in a Speed-Vac, the products were suspended in 5 μl of H2O and 5 μl of sequencing gel sample buffer and loaded onto a 20% polyacrylamide gel containing 8 M urea. After electrophoresis at 40 watts for about 3 h, the gel was autoradiographed. The size of the products (shown at the right of each panel) was not determined rigorously but was estimated from the number of bands in the ladder. Panel B, to determine whether the primase products can initiate DNA synthesis, primers were synthesized using [α-32P]ATP as substrate and poly(dT) as template (lane 1). After incubation at 30 °C for 20 min, Klenow polymerase (0.5 unit/25-μl assay) plus various concentrations (50 μM, 0.5 mM, and 1 mM) of dATP were added to the reaction (lanes 2–4, respectively). After an additional incubation of 20 min, the reaction products were precipitated and electrophoresed as described under panel A.

pendicular to the flagellum, and this image shows the edges of the discs. Fig. 5C shows the primase in the same cells, localized above and below the kinetoplast disc. Somewhat more fluorescence is observed on the side of the disc nearest the flagellum, a distribution observed in many cells. We were concerned that the images of primase and DAPI staining might have been misaligned and that both components of the primase fluorescence may have been on the same side of the kinetoplast disc. This possibility is highly unlikely, given that the two cells are oriented in opposite directions and that the distances between the DAPI stains and the primase stains in the two cells are identical. Therefore we conclude that the primase forms a sandwich-like structure around the kinetoplast disc. DNA primase is localized in the same positions in the majority of log phase cells (90% or more). In another experiment, Fig. 5 (panels D–G) shows images of a single cell, which is visualized by Nomarski optics (panel D), DAPI staining (panel E), immunolocalization of the primase (panel F), and immunolocalization of the mitochondrial DNA polymerase β (panel G). It is clear that the two enzymes have distinctly different locations. The primase is situated above and below the disc (panel F), whereas the DNA polymerase β is situated in two protein complexes (as described previously; Ref. 9) on opposite sides of the kinetoplast disc (panel G).

Discussion

Starting with a mitochondrial fraction from C. fasciculata, we have purified a DNA primase to near homogeneity. The purified enzyme has a molecular mass of 28 kDa, and its active form is a single polypeptide chain. Assuming that the recovery of mitochondria used for purification was 100%, we can make a rough estimate that there are about 11,000 molecules of primase within the parasite’s single mitochondrion. Using a poly(dT) template, the primase produces a ladder of homopolymeric products, with a maximum size of about 10 nucleotides. Using single-stranded M13 DNA as a template, the primase makes products in roughly the same size range (data not shown). However, due to sequence heterogeneity, the M13 products are not resolved into a uniform ladder by gel electrophoresis, a finding which implies that initiation on M13 occurs at multiple sites.

The primase products can be efficiently elongated by Klenow DNA polymerase in the presence of the appropriate dNTPs. However, when poly(dT) is used as a template, the only primers that are efficiently extended by Klenow polymerase are the 10-mers and, to a lesser extent, the 9-mers (see Fig. 4B, lanes 3 and 4). Shorter oligonucleotides are not elongated efficiently by the DNA polymerase. A similar effect has been observed with mammalian primases (16). A chase experiment, in which non-radioactive ATP was added after synthesis of primers with [α-32P]ATP, did not result in the shorter oligonucleotides being extended (data not shown). It is possible that many of the
shorter oligonucleotides had dissociated from the dT template and therefore could not serve as intermediates in primer synthesis. In another experiment, we found that the primers were poorly extended by the C. fasciculata mitochondrial DNA polymerase β, a result not surprising given that that enzyme may not be the major replicative enzyme in this organelle (see below).

Intracellular enzyme localization studies have been valuable in clarifying our understanding of kDNA replication. The C. fasciculata kinetoplast system is ideal for these studies because the non-replicating cell has only one kDNA network, which resides within its single mitochondrion. The network in vivo is condensed into a characteristic disc-shaped structure, about 1 μm in diameter and 0.4 μm thick, well within the resolution of fluorescence microscopy. Previous immunolocalization studies had demonstrated that a mitochondrial topoisomerase II (8) and a mitochondrial DNA polymerase β (9) co-localize within two protein complexes, which are situated in antipodal positions adjacent to the kinetoplast disc (see localization of DNA polymerase β in Fig. 5G). Based on the presence of two enzymes involved in replication, and other evidence (9), we had hypothesized that these two protein complexes are involved in minicircle replication. Another topoisomerase II (7), as well as some histone-like DNA binding proteins (17), localize within the kinetoplast disc. An hsp70 heat shock protein surrounds the disc or if it penetrates the upper and lower regions of the disc. We now report that the primase has a distinct localization, and it is known to have a preference for gap filling. The progeny minicircles could then be attached to the network periphery adjacent to these protein complexes, in another topoisomerase reaction (20). The localization of the primase above and below the disc raises the possibility that replication may not actually occur within the antipodal protein complexes. Instead, minicircle replication could occur in the region above or below the kinetoplast disc. A replicative DNA polymerase in this location (not yet discovered but possibly related to DNA polymerase γ) could have the major responsibility for DNA synthesis. The minicircle progeny could then migrate to one of the two protein complexes where many of the gaps in the discontinuously synthesized strand could be repaired immediately prior to network attachment (21). Another topoisomerase II (7) is ideally situated to carry out this reaction, and it is known to have a preference for gap filling. The progeny minicircles could then be attached to the network periphery by topoisomerase II. In an alternative scheme, the covalently closed minicircle, immediately after release from the network, could associate with primase and other proteins to form a replication initiation complex. It could then migrate to one of the two antipodal protein complexes to complete its replication. Further studies, including the localization of additional replication enzymes, will be needed to distinguish between these possibilities.

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What is the significance of the primase localization to the mechanism of minicircle replication? A current view of this mechanism is shown in Fig. 6. The diagram shows a section through the kinetoplast disc, with interlocked minicircles forming a monolayer (see Ref. 3 for further information about this arrangement and about the replication scheme). Covalently closed minicircles are released from the central region of the network by a topoisomerase II, which is situated within the disc. Ultimately these free minicircles migrate to one of the two protein complexes (containing topoisomerase II and DNA polymerase β) that flank the kinetoplast disc. The newly replicated progeny minicircles, containing gaps, are attached to the network periphery adjacent to these protein complexes, in another topoisomerase reaction (20). The localization of the primase above and below the disc raises the possibility that replication may not actually occur within the antipodal protein complexes. Instead, minicircle replication could occur in the region above or below the kinetoplast disc. A replicative DNA polymerase in this location (not yet discovered but possibly related to DNA polymerase γ) could have the major responsibility for DNA synthesis. The minicircle progeny could then migrate to one of the two protein complexes where many of the gaps in the discontinuously synthesized strand could be repaired immediately prior to network attachment (21). The DNA polymerase β is ideally situated to carry out this reaction, and it is known to have a preference for gap filling. The progeny minicircles could then be attached to the network periphery by topoisomerase II. In an alternative scheme, the covalently closed minicircle, immediately after release from the network, could associate with primase and other proteins to form a replication initiation complex. It could then migrate to one of the two antipodal protein complexes to complete its replication. Further studies, including the localization of additional replication enzymes, will be needed to distinguish between these possibilities.
