A Second Mitochondrial DNA Primase Is Essential for Cell Growth and Kinetoplast Minicircle DNA Replication in *Trypanosoma brucei*†

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The mitochondrial DNA of trypanosomes contains two types of circular DNAs, minicircles and maxicircles. Both minicircles and maxicircles replicate from specific replication origins by unidirectional theta-type intermediates. Initiation of the minicircle leading strand and also that of at least the first Okazaki fragment involve RNA priming. The *Trypanosoma brucei* genome encodes two mitochondrial DNA primases, PRI1 and PRI2, related to the primases of eukaryotic nucleocytoplasmic large DNA viruses. These primases are members of the archaeanucleoprotein disc located close to the base of the flagellum, with the kDNA connected to the basal body through a transmembrane filament system termed the tripartite attachment complex (32). Each cell contains a single kDNA network that is free of the network (11). The newly replicated minicircles are then rejoined to the periphery of the network, and the network eventually doubles in size and is divided by unknown mechanisms to yield two daughter networks (22, 25).

Minicircles replicate by a unidirectional theta-type intermediate from an origin sequence that is specified by the universal minicircle sequence (UMS) (1, 2, 33, 34, 46). One strand (leading strand) is synthesized continuously, and the other strand (lagging strand) is synthesized discontinuously (3, 20, 21, 37, 41). The leading strand is initiated within the UMS by an RNA priming mechanism and is elongated past a nearby hexamer sequence where the first Okazaki fragment is initiated. A DNA-binding protein (UMSBP) binds both to the UMS and to the hexamer sequence in their single-stranded forms (33). More recently, a second kinetoplast replication protein, p38, has also been found to bind specifically to these two sequences (26). Origin recognition by these proteins is thought to represent the first step in initiating minicircle replication.

As mitochondrial replication proteins have become identified, it is apparent that kinetoplast replication proteins are novel in several aspects. The replication proteins are not contained in a single replication complex but have distinct localizations at several different sites within and around the kDNA. A type II DNA topoisomerase was localized initially to antipodal sites flanking the kDNA disc (31) and was shown to function in the attachment of replicated minicircles to the kDNA disc (47). Minicircle replication intermediates and other replication proteins were subsequently localized to the antipodal sites (12, 18). These include a β-type DNA polymerase (38), a structure-specific endonuclease (SSE1) (8, 10), a DNA ligase (ligase kβ) (42), RNase H (9), two DNA helicases (PIF1 and PIF5) (28, 29), a H1 histone-like protein (KAP4) (49), a minicircle origin binding protein (p38) (26), a type IA topoisomerase (39), and a DNA primase (PRI1) (16). At least...
partial repair of discontinuities in nascent minicircles is proposed to occur at these sites (15).

A region at the flagellar face of the kDNA disc termed the kinetoflagellar zone (KFZ) has now been identified as the place where minicircle replication appears to initiate (2, 7). Fluorescence in situ hybridization showed that minicircle replication intermediates are present in the KFZ as well as at the antipodal sites. Replication proteins localized to the KFZ include UMSBP (2), two DNA polymerases (POLIB and POLIC) (23) that are related to bacterial polymerase I, and DNA ligase k3 (43), which localizes to both faces of the kDNA disc but more so to the KFZ face. Proteins present throughout the kDNA disc include H1 histone-like proteins KAP1, -2, and -3 (49, 50). A mitochondrial RNA polymerase is essential for kDNA replication and may also be localized throughout the kDNA as, might be expected in view of its role in transcription (14). Two additional polymerase I-like mitochondrial DNA polymerases (POLIA and POLID) localize throughout the mitochondrial matrix (23).

The multiplicity of mitochondrial replication proteins in trypanosomes is strikingly different from that in most eukaryotes. Only a single mitochondrial DNA polymerase (polymerase γ) is required to replicate the mitochondrial DNA in other eukaryotes (19), while trypanosomes have six mitochondrial DNA polymerases (23). Similarly, trypanosomes contain six mitochondrial DNA helicases related to the yeast Saccharomyces cerevisiae PIFI (ScPIFI) helicase (27). Trypanosomes also have two mitochondrial DNA ligases (6, 42, 43) that are unrelated to the single mitochondrial DNA ligase in higher eukaryotes.

Maxicircle replication has been more difficult to analyze since replication occurs within the kDNA network while apparently still being interlocked with minicircles and other maxicircles (35, 40). Electron microscopic analysis of maxicircles released from networks by a DNA topoisomerase and digested with a restriction enzyme that makes only a single cut in the DNA showed that maxicircles also replicate by a unidirectional theta-type mechanism (4). Although the maxicircle replication origin has not been defined at the DNA sequence level, it appears not to contain the UMS, since the only UMS-related sequences are located far from the mapped origin region. RNA interference (RNAi) of a mitochondrial RNA polymerase results in selective loss of maxicircles, but its role is still unknown. Maxicircle replication is regulated by the T. brucei mitochondrial DNA primase PRI1 (16). RNAi of PRI1 functions to prime maxicircle replication and possibly that of minicircles, as well. We describe here a second essential mitochondrial DNA primase in T. brucei, which we term PRI2.

**MATERIALS AND METHODS**

*Trypanosome growth.* T. brucei procyclic cell line 29-13 expressing T7 RNA polymerase and the tetracycline repressor (48) was grown at 28°C in SM medium (5) containing 15% heat-inactivated fetal bovine serum (Invitrogen), 32 μg/ml G418, 50 μg/ml hygromycin, and 2.5 μg/ml phleomycin. YTAT 1.1 cells were grown in the same medium with 20 μg/ml G418.

**RNA isolation and Northern analysis.** A total of 5 μg of RNA isolated from cells using a PureLink RNA isolation kit (Invitrogen) was fractionated on a 1.2% agarose-0.22 M formaldehyde gel and transferred to a Hybond N membrane (Amersham). PRI2 mRNA was detected by probing membranes with a 32P random-primed 821-bp DNA fragment used as the RNAi target. PRI1 and tubulin mRNAs were detected as described previously (16).

**Endogenous epitope tagging of PRI2.** Primers Q30 and Q31 were used to amplify a portion of the PRI2 coding region from T. brucei 29-13 cell genomic DNA having PspOMI sites at both ends. This PCR product was digested and cloned into the EagI site of pHS756 (16) to create pHS78. Primers Q69 and Q70 were used to amplify a region of the PRI2 3′ untranslated region (UTR) from genomic DNA with SacI sites at both ends. This second PCR product was digested and cloned into the SacI site of pHS758 to create pHS583. This final plasmid was digested with NruI and AfeI and transfected into YTAT 1.1 cells. Recombinants were selected with 20 μg/ml G418, and clones were produced by limiting dilution.

**Microscopy.** Cells harvested during PRI2 RNAi, induced or uninduced, were resuspended in phosphate-buffered saline (PBS), spotted onto poly-l-lysine-coated slides, and allowed to adhere for 5 min in a humid chamber. The cells were fixed in 4% paraformaldehyde in PBS for 10 min, washed twice in 0.1 M glycine in PBS followed by 10 min in 0.025% Triton X-100 in PBS, and then stored in methanol at −20°C. Cells were rehydrated twice in PBS for 10 min and then mounted in Prolong Gold antifade reagent plus DAPI (4′,6-diamidino-2-phenylindole) for monitoring the loss of the kDNA. For protein immunolocalization, cells were adhered for 30 min to coverslips, treated for 2.5 min in 4% paraformaldehyde, permeabilized for 10 min in 0.025% Triton X-100, and stored at −20°C in methanol. Upon removal from −20°C methanol, cells were rehydrated by washing them twice in PBS and then blocked for 30 min at 37°C in 20% goat serum in PBS containing 0.05% Tween 20 (PBST). Next, the slides were incubated at 37°C for 2 h with the anti-HA (hemagglutinin) monoclonal antibody 12CA5 (Babco) at a 1:100 dilution and then washed four times in PBST. The slides were then incubated for 1 h at 37°C in goat anti-mouse antibody conjugated to Alexa Fluor 594 at a 1:150 dilution, washed 4 times in PBST, and mounted in Prolong Gold antifade reagent with DAPI. Cells were imaged using a Zeiss upright light microscope (Zeiss Axio Imager Z1) with a 100× oil immersion objective. All images were rendered using the Zeiss AxioVision software and a Zeiss digital charge-coupled camera (AxioCam MRM).

**RNA interference.** Primers Q9 and Q10 were used to amplify an 821-bp fragment of the PRI2 gene from T. brucei 29-13 cell genomic DNA. The PCR product was cloned into pCRII-TOPO (Invitrogen). The BamHI-to-HindIII and PRI2 fragment of the p29-13 cell genomic DNA were grown in the same medium with 20 μg/ml G418, and clones were produced by limiting dilution. Three clones were evaluated for RNAi loss and growth defect. One clone was selected for further analysis. Cell growth was monitored by limiting dilution. Three clones were evaluated for RNAi loss and growth defect. One clone was selected for further analysis.

**DNA isolation and Southern blot analysis.** Cloned T. brucei cells stably transfected with the RNAi vector were induced by the addition of 1 μg/ml tetracycline and harvested at various intervals. Total DNA and free minicircles were isolated as described previously (24). Analysis of free minicircle replication intermediates was performed by electrophoresis of DNA samples (from 5 × 106 cell equivalents or, in some experiments, 2 × 109 cell equivalents) on a 1.5% agarose or Southern blotting using a pool of five independently cloned T. brucei minicircles as the minicircle probe. For analysis of total minicircles and maxicircles, the DNAs (from 2 × 109 cell equivalents) were digested with HindIII and XbaI prior to electrophoresis and Southern blotting. A maxicircle probe was prepared by PCR amplification and cloning of a 2.6-kb fragment of the coding region of the maxicircle using primers MXF1 and MXFR1. This sequence corresponds to nucleotides 4088 to 6706 of the T. brucei maxicircle sequence (GenBank accession no. M94286). The trypanosome hexasome transporter (THT) probe (used as a
loading control for chromosomal DNA) was amplified from total DNA as described previously (24).

Fractionation of maxicircle DNAs. The relative amounts of nicked/gapped (N/G) and covalently closed (CC) forms of maxicircle DNA in the total DNA samples (2 \times 10^6 cell equivalents) were determined by decatenation of the DNA with human topoisomerase II (US Biochemicals), followed by electrophoresis on a 0.6% agarose gel and Southern blotting as described above. Maxicircle and chromosomal DNAs were detected as done for the other Southern blot analyses. Electrophoresis conditions were used as determined previously (16).

DNA primase assay. Coupled reactions (25 \mu l) contained 250 ng poly(dT), 15 mM Tris-HCl at pH 8.0, 10 mM MgCl2, 1 mM dithiothreitol (DTT), 16 mM ATP as indicated, and 1 \mu g of recombinant PRI2 lacking an additional long amino-terminal extension (PRI2-NT). After 40 min at 30°C, Escherichia coli Klenow DNA polymerase (1.5 unit) was added, and incubation was continued for an additional 40 min at 30°C. Where indicated, PRI2-NT was heat inactivated (20 min at 75°C), and then primers were reannealed (75°C to 25°C in 20 min) after the first incubation. Prior to the second incubation, RNaseH (2.5 units) was added, or not, for 30 min at 37°C. When RNaseH was included, additional heat inactivation and annealing (20 min at 65°C) was added prior to addition of Klenow DNA polymerase and [\alpha-32P]dATP (24,400 cpm/pmol). Reaction mixtures were terminated by being spotted onto Whatman DE81 filter discs, washed three times in 500 ml of ammonium formate at pH 7.8 and once in 500 ml of 95% ethanol, and air dried prior to scintillation counting.

Western blotting. Protein fractions were electrophoresed on SDS-polyacrylamide gels and transferred to membranes as described previously (24). Mouse anti-HA antibodies (12CA5; Babco) were used at a 1:1,000 dilution, with peroxidase-conjugated anti-mouse immunoglobulin G (Sigma) at a 1:8,000 dilution as a secondary antibody. Bands were visualized using the SuperSignal West Pico chemiluminescent system (Pierce).

Poly(rA) synthesis reactions. A 200-\mu l reaction mixture contained the same components and concentrations as those in the coupled reactions (minus dATP and Klenow DNA polymerase) and included [\alpha-32P]ATP (100 \mu M, 18,000 cpm/FIG. 1. Multiple sequence alignment. Predicted kinetoplastid PRI2 proteins from Trypanosoma brucei (Tb; 927.1.4010) region 751 to 1249, Trypanosoma cruzi (Tc; 00.104705306529.410) region 254 to 722, Trypanosoma vivax (Tv; 486_010173) region 574 to 1063, Leishmania braziliensis (Lbr; M12_V2.0640) region 19 to 580, Leishmania major (Lmj; F23.0680) region 1 to 543, and Leishmania infantum (LinJ; 12_V3.0590) region 1 to 545 were aligned using the ClustalW progressive multiple sequence alignment algorithm (45) in MacVector 7.2 (Accelrys). Only the region of each sequence containing the conserved RNA recognition motif (RRM) and PRICT-2 domains is shown. Amino-terminal sequences are shown in Fig. S1 in the supplemental material. The RRM is indicated by an open bar above the sequence, and the PriCT-2 domain is indicated by a filled bar above the sequence. Conserved amino acid residues are shown in shaded boxes. Aspartate and histidine residues conserved in all catalytically active members of the archaebukaryotic primase superfamily are indicated by asterisks underneath. GeneDB accession numbers for protein sequences are indicated in parentheses.
After incubation at 30°C for the indicated times, 50- or 75-μl aliquots were removed and extracted with phenol and then with chloroform, followed by phenol-chloroform extracted and precipitated. Binding reaction mixtures containing 3 nM Q82 (50,000 cpm), 15 mM Tris at pH 8.0, 10 mM MgCl2, 12% dATP and divided into either two or three equal portions. Klenow DNA polymerase (1.5 units) or RNaseH (1.5 units) was added as indicated with further incubation at 30°C for 40 min. Reactions were stopped by adding an equal volume of sequencing gel loading buffer. Samples were electrophoresed on a 14- by 16- by 0.5-cm 6% submerged horizontal polyacrylamide-7 M urea gel at 120 V and exposed to film at −80°C. Stained DNA was visualized using a UV transilluminator. Oligonucleotides used. The oligonucleotides used in this study are as follows: Q9, TCCAGGGCCCTTCATTTCGTTCTTCCAGGAC; Q10, ACAAACCCCGTACGAAGACCGCCACGCTACCTTCAAATATT; Q30, ACAACCCGCGGCGGCGGAACGCGCCGTCACCCACCCCCTCAA; Q31, CTGCAACCGCCGGCGCAGGCGCCAGTACCCACCCAGG; Q32, AACGGGAGTAATCCTCCTC; Q69, GATGCCGAGCTCAGCGG; Q70, ACGATGCGAGCTCAGCAGGTTT; Q82, TGTTAGCGGGCCCATTAAGGCAGCCAGCGTACCTTACCC; MXF1, CACAGCACCCGTTTCAGCACAG; and MXR1, TCTCCTCC AGTCTTGTCGCC; Q84, CAATTAATCCTGCGTATAAAT; Q91, GATGACGATCCGACGAGAGCTAG; Q92, GATGACGATCTCACCGAGCTAG; Q93, CGTCGAGAATGACGTTCTG; Q94, AGCGAGAGCTCAGCAGG; Q95, GAGATGGACGACGCTAG; Q96, GAGATGGACGACGCTAG; Q97, GAGATGGACGACGCTAG; and Q98, GAGATGGACGACGCTAG.

Electrophoretic mobility shift assays. The 30-nucleotide oligodeoxynucleotide Q82 was 32P end labeled with [γ-32P]ATP and T4 polynucleotide kinase and then phenol-chloroform extracted and precipitated. Binding reaction mixtures containing 3 nM Q82 (50,000 cpm), 15 mM Tris at pH 8.0, 10 mM MgCl2, 12% glycerol, and either no protein or 1.5, 3.75, 9.4, 23.4, 58.6, or 146.5 nM PRI2NT or PRI2NTCS were incubated for 15 min at 30°C. Samples were loaded onto a 12- by 16- by 0.5-cm 6% submerged horizontal polyacrylamide gel in 5 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer at pH 10.0, electrophoresed for 1.5 h at 75 V, dried, and exposed to X-ray film.

RESULTS

Identification of the PRI2 gene. The T. brucei PRI2 gene was identified initially through a search of the GeneDB database for Leishmania major and T. brucei genes containing both a predicted mitochondrial targeting sequence and sequence elements required for S-phase expression (16, 24). PRI1 contains a C-terminal PriCT-2 motif and is related to primases of eukaryotic nucleoplasmic large DNA viruses (17). A further search of GeneDB revealed a putative second gene containing the PriCT-2 motif, with weak sequence similarity to PRI1. Primases PRI1 and PRI2 each have an RNA recognition motif (RRM) and a C-terminal PriCT-2 motif (Fig. 1) characteristic of the iridovirus family. They also contain the conserved residues (Fig. 1, asterisks) essential for catalysis by members of the AEP superfamily. Although PRI1 and PRI2 are only distantly related to each other, they are each highly conserved in kinetoplastid parasites. In Leishmania species, the PRI2 genes encode proteins having 543 to 580 residues. The Trypanosoma sequences shown in Fig. 1 exhibit high sequence identity with the Leishmania sequences in the C-terminal region but also have long N-terminal extensions. The predicted T. brucei PRI2 protein carries an N-terminal extension of an additional 750 amino acids. Trypanosoma cruzi and Trypanosoma vivax PRI2 proteins carry extensions of 253 and 573 residues, respectively. The trypanosome N-terminal extensions have little sequence similarity with one another (see Fig. S1 in the supplemental material) and show no significant similarity to proteins in other species.
To examine the possibility that the apparent long amino-terminal sequence of the *T. brucei* predicted PRI2 protein might result from misidentification of the initiation codon, we examined the size of the PRI2 mRNA and protein. An RNA of approximately 7.0 kb detected on a Northern blot (see Fig. S2 in the supplemental material) is knocked down by PRI2 RNAi and is clearly large enough to encode the predicted full-length PRI2 protein. We directly examined the size of the PRI2 protein by *in vivo* epitope tagging of the protein in *T. brucei* YTat 1.1 cells with three copies of the hemagglutinin peptide. A Western blot (see Fig. S3 in the supplemental material) of cells expressing the endogenously epitope-tagged PRI2 protein showed a protein of approximately 150 kDa consistent with expression of the full-length protein. Examination of these cells by immunofluorescence (Fig. 2) showed an antipodal localization of PRI2 in approximately one-third of the cells in an exponential population, an observation similar to that of several other kinetoplast replication proteins, including SSE1 endonuclease (10), topoisomerase II, and polymerase β (18), which have cell cycle-dependent localization.

**RNAi of PRI2.** To address the function of PRI2, we cloned an 821-bp fragment of the PRI2 gene into the vector pJH533 that produces a stem-loop RNA upon induction with tetracycline (16). The cloned fragment corresponds to residues 976 to 1249 of the PRI2 coding sequence. The resulting construct was transfected into *T. brucei* 29-13 cells and cloned, generating the PRI2 RNAi strain. A cloned cell line was induced by the addition of tetracycline and monitored for cell growth and PRI2

**FIG. 4.** Loss of kDNA during PRI2 RNAi. Cells at 0 and 2 days of PRI2 RNAi were stained with DAPI and observed by fluorescence microscopy. N, nucleus; k, kinetoplast. Bar, 5 μm.

**FIG. 5.** Effect of PRI2 RNAi on minicircle and maxicircle DNAs. (A) Kinetics of loss of total minicircles and maxicircles during PRI2 RNAi. After digestion with HindIII and XbaI, total cellular DNA (2 × 10⁶ cell equivalents per lane) was fractionated on a 1.5% agarose gel containing 1 μg/ml ethidium bromide. Southern blots were probed for minicircle DNA, a maxicircle fragment (Maxi), and a hexose transporter fragment as a loading control (Load). A 1.0-kb minicircle fragment (Mini) represents the full-length minicircle. (B) Phosphorimager quantitation of the relative levels of maxicircle and minicircle DNAs shown in panel A. (C) Loss of N/C and CC maxicircles during RNAi. Southern blot of topoisomerase II-treated total DNA (2 × 10⁶ cell equivalents/lane) during PRI2 RNAi. The gel was run, Southern blotted, and probed with a maxicircle DNA probe (C) and then stripped and reprobed with the THT sequence as a chromosomal DNA loading control (D). (E) Phosphorimager quantitation of the levels of N/G (open circles) and CC (filled circles) maxicircles relative to the levels at day 0. Each lane was normalized relative to the loading control. Error bars show the standard errors of the means.
mRNA levels for 7 days. The PRI2 mRNA level was reduced by more than 95% after 48 h of RNAi (see Fig. S2 in the supplemental material), and cell growth was strongly inhibited by 4 days of RNAi (Fig. 3). Approximately two-thirds of the cells lacked kinetoplasts after 2 days of RNAi (Fig. 4), and remaining kinetoplasts were dramatically smaller.

Loss of kDNA during PRI2 RNAi. Southern blotting of restriction digests of total DNA was performed to quantify the loss of minicircle and maxicircle DNAs during PRI2 RNAi (Fig. 5A and B). Minicircles and maxicircles were lost at similar rates, with approximately 80% loss within 36 h. This rapid and equivalent loss of minicircles and maxicircles differs from that observed during PRI1 RNAi, in which total maxicircle DNA was lost at a higher rate than that of minicircle DNA.

Analysis of CC and N/G maxicircles during PRI2 RNAi requires the release of these species from their topological association with the kDNA network. To examine the fate of these species during RNAi, we decatenated the kDNA networks in total DNA isolated at different times during PRI2 RNAi and separated CC and N/G species by electrophoresis on a 0.6% agarose gel containing ethidium bromide (Fig. 5C). Just as for minicircles, the N/G molecules are replication products, and the CC molecules are precursors (15, 27). Both CC and N/G species were rapidly lost and each at the same rate during PRI2 RNAi (Fig. 5E). No preferential loss of N/G was observed, as has been observed in the case of the TbPif2 helicase, which is essential for maxicircle replication.

Free minicircles represent only a few percent of the minicircle content in the mitochondrion and are the result of decatenation from the kDNA network and replication that is free of the network. The free minicircles are detected by Southern blotting of undigested whole-cell DNA. Covalently closed (CC) minicircles represent the free unreplicated minicircles, and nicked/gapped (N/G) minicircles represent free minicircles that have undergone replication but have not been covalently closed. Free replication intermediates migrate in a smear ahead of the N/G species on an agarose gel (Fig. 6A).

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Purification and activity of PRI2ΔNT and PRI2ΔNTCS. After several unsuccessful attempts to express a recombinant form of full-length PRI2, we decided to express just a C-terminal portion of the protein containing the primase domain. A sequence corresponding to amino acid residues 732 to 1249 was cloned into plasmid pH604 to create an amino-terminal fusion to 10 histidine residues to allow purification by metal chelate chromatography. This construct deletes the N-terminal domain and is termed PRI2ΔNT. The His-tagged PRI2ΔNT construct was then mutagenized to introduce D870A and D872A mutations, residues predicted to be essential for catalysis in AEP family primases. The resulting construct is termed PRI2ΔNTCS. Both PRI2ΔNT and PRI2ΔNTCS were expressed in E. coli and purified by successive chromatography on metal chelate columns and then heparin Sepharose columns. In both cases, a major protein with an apparent molecular mass of approximately 60 kDa eluted from the heparin Sepharose columns (Fig. 7A and B). An assay of the column fractions showed that only the PRI2ΔNT protein had primase activity, indicating that the aspartate residues are required for catalysis (Fig. 7C).

To determine whether the loss of activity of PRI2ΔNTCS was due to impaired DNA binding of the enzyme, we utilized an electrophoretic mobility shift assay to compare the DNA-binding properties of PRI2ΔNT and PRI2ΔNTCS. Each enzyme was incubated, at increasing concentrations, with a 32P-labeled, 30-nucleotide, single-stranded oligodeoxynucleotide.
The reaction mixture was subsequently electrophoresed on a horizontal 6% polyacrylamide gel in order to separate primase-DNA complexes from free DNA. The autoradiograph of the gel (Fig. 7D) shows that both proteins bind DNA. Remarkably, the CS mutant protein is fully capable of binding DNA, and the binding is similar to, if not stronger than, that of PRI2/H9004 NT.

Therefore, as predicted for members of the AEP superfamily, the PRI2 aspartates D870 and D872 are essential for catalysis but not for DNA binding.

**Primase activity of the PRI2NT protein.** Peak fractions from the heparin column purification of PRI2NT were assayed for primase activity in a coupled assay with Klenow DNA polymerase on a poly(dT) template and various concentrations of ATP. Synthesis of poly(dA) required ATP and increased with the ATP concentration up to 0.8 mM (Fig. 8A). No poly(dA) synthesis was observed in the absence of ATP. Poly(dA) synthesis was also stimulated by first preincubating the template with PRI2NT and ATP for 40 min prior to adding Klenow polymerase and [α-32P]dATP (Fig. 8B). Synthesis of poly(dA) was inhibited when the preincubated template was treated with RNaseH prior to incubation with Klenow polymerase and [α-32P]dATP (Fig. 8C). These results are similar to those obtained previously with the PRI1 primase and indicate that the product of the PRI2NT reaction is a 3' hydroxyl terminated oligoriboadenylate in a hybrid structure with poly(dT).

The lengths of RNAs synthesized by PRI2NT show a striking difference from the lengths of those synthesized by PRI1. The products of the PRI2NT reaction were analyzed by polyacrylamide gel electrophoresis at 40, 80, and 120 min in reaction mixtures containing [α-32P]ATP and the poly(dT) template (Fig. 9, lanes 1 to 3). Whereas PRI1 primase synthesizes poly(A) oligoribonucleotides ranging in lengths of up to 70 to 80 residues, PRI2NT synthesized poly(A) oligoribonucleotides of up to 1,000 residues under the same conditions. Further incubation in the absence of PRI2NT and the presence of Klenow DNA polymerase and dATP resulted in elongation of the poly(A) with deoxynucleotides (Fig. 9, lanes 4 to 6). We note that the lengths of the poly(A) oligoribonucleotides synthesized by PRI2NT approach the length distribution of the poly(dT) template, suggesting that the primase may be processive and that the maximum length achieved is limited only by the template length. To further examine the poly(A) products of the primase reaction, we treated an equal aliquot of the sample shown in Fig. 9, lane 3, with RNaseH. The 32P-labeled poly(A) was degraded by RNaseH treatment (Fig. 9, lane 7), indicating that the poly(A) product was contained in a hybrid structure.

**DISCUSSION**

The overall basic mechanisms involved in the replication of kinetoplast DNA minicircles and maxicircles are understood in various detail, and many mitochondrial replication proteins have been identified and implicated in specific steps in replication. However, the detailed biochemical mechanisms of mi-
nicircle and maxicircle DNA strand initiation are still largely unknown. DNA strands are normally initiated in eukaryotic and prokaryotic cells by the synthesis of RNA primers by enzymes termed DNA primases. Initiation of DNA replication in mammalian mitochondria, however, utilizes the mitochondrial RNA polymerase for initiation (13). The

\[ T. brucei \]

mitochondrial RNA polymerase is required for kinetoplast replication but its precise role is still unknown and could be indirect (14). We recently described a mitochondrial DNA primase PRI1 that is highly conserved in kinetoplastid parasites and is essential both for kDNA replication and for cell growth. In the present work, we describe a second mitochondrial DNA primase PRI2 that is also highly conserved in kinetoplastids but is distinct from PRI1.

In Leishmania species, PRI2 predicted protein sequences encode proteins of approximately 61 to 66 kDa, containing the RRM and PriCT-2 domains characteristic of the AEP superfamily. PRI2 of Trypanosoma species has an additional extensive N-terminal sequence of unknown function. Bacterial expression of the C-terminal domain of \[ T. brucei \] PRI2 (PRI2\_H9004\_NT) corresponded to the full-length Leishmania PRI2 proteins, produced an active protein with properties similar to those of the \[ T. brucei \] PRI1 primase. Mutation of two aspartate residues in PRI2\_H9004\_NT, predicted to be essential for catalysis by members of the AEP superfamily, abolished the primase activity but left the protein still able to bind DNA. Like PRI1, the PRI2\_H9004\_NT enzyme synthesized poly(rA) in a hybrid structure on a poly(dT) template. Synthesis of poly(dA) depended on the presence of PRI2\_H9004\_NT and ATP. A striking difference was the length of poly(rA) synthesized by the \[ T. brucei \] PRI2\_H9004\_NT, consistent with at least some product molecules possibly extending to the end of the template strand. This feature of PRI2\_H9004\_NT may be a consequence of the absence of the long N-terminal extension or the absence of factors that limit primer extension in the mitochondrion. The lengths of products synthesized on this homopolymer template are unlikely to reflect primer lengths formed in vivo.

The possible roles of the N-terminal extensions of the
Trypanosoma PRI2 proteins are unclear. These sequences vary widely in length and show very little sequence similarity other than in predicted N-terminal mitochondrial targeting sequences. The *T. brucei* PRI2 N-terminal sequence is 750 residues in length, whereas those of *T. vivax* and *T. cruzi* are 573 and 253 residues in length, respectively (see Fig. S1 in the supplemental material). The lack of sequence conservation and wide variation in lengths suggest that these sequences are not likely to carry a common function. The *T. brucei* N-terminal extension is also unusually rich in basic residues, resulting in a predicted pI of 11.9 for the full-length protein. Western blots of the *T. brucei* PRI2 epitope tagged at the C terminus indicate that the full-length PRI2 protein is expressed (see Fig. S3) and migrates with an apparent molecular mass of approximately 150 kDa, whereas the molecular mass of the PRI2 protein predicted from the gene sequence is only 129 kDa. Anomalous migration of highly basic proteins on an SDS-polyacrylamide gel is well documented (49). It will be of interest to determine whether these long N-terminal sequences are essential for kinetoplast replication.

Unexpectedly, we found that PRI2 localizes at or near antipodal sites flanking the kDNA. This result is similar to that observed for several proteins thought to be involved in the repair of nicks and gaps in newly replicated molecules. We would have expected PRI2 to localize to the KFZ, where minicircle replication is thought to initiate. Minicircle replication intermediates localize there, as well as DNA polymerases PolIB and PolIC, the PIF2 DNA helicase, and the origin recognition protein UMSBP. Proteins localized at antipodal sites include enzymes involved in the following later stages of minicircle replication: primer removal, gap filling, and ligation. We note, however, that a second origin recognition protein, p38, thought to be involved in initiation of minicircle replication, also has an antipodal localization. In light of the small percentage of minicircles that are free of the network and available for replication, the amounts of these two proteins that might be needed for initiation on the free minicircles in the KFZ could possibly be below the limit of detection.

RNA interference of PRI2 resulted in the inhibition of cell growth, with shrinkage and loss of kinetoplast DNA. The initial accumulation of free CC minicircles during PRI2 RNAi, together with the rapid loss of replication intermediates and free N/G minicircles, is consistent with a requirement for the PRI2 primase in minicircle replication. Since the minicircle leading strand initiates by RNA priming and the first Okazaki fragment initiates nearby by RNA priming, inhibition of either priming event could block initiation of minicircle replication. Thus, PRI2 could potentially prime either one or both strands at the origin. In either case, preventing initiation in the origin region on CC minicircles released from the kDNA network would lead to the accumulation of free CC minicircles relative to that of the N/G-replicated minicircles. Although a role for the PRI2 primase in maxicircle replication is not excluded by these results, the primary role for PRI2 appears to be in initiating minicircle replication. It is of interest to note that the PRI1 primase and the DNA helicase PIF2 have been implicated in maxicircle replication, and the PRI2 primase and DNA helicases PIF1 and PIF5 have been implicated in minicircle replication. Further studies should address the possibility that minicircles and maxicircles utilize different sets of the mitochondrial replication proteins.

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