Trypanosomatid parasites contain an unusual form of mitochondrial DNA (kinetoplast DNA [kDNA]) consisting of a catenated network of several thousand minicircles and a smaller number of maxicircles. Many of the proteins involved in the replication and division of kDNA are likely to have no counterparts in other organisms and would not be identified by similarity to known replication proteins in other organisms. A new kDNA replication protein conserved in kinetoplastids has been identified based on the presence of posttranscriptional regulatory sequences associated with S-phase gene expression and predicted mitochondrial targeting. The Leishmania major protein P105 (LmP105) and Trypanosoma brucei protein P93 (TbP93) localize to antipodal sites flanking the kDNA disk, where several other replication proteins and nascent minicircles have been localized. Like some of these kDNA replication proteins, the LmP105 protein is only present at the antipodal sites during S phase. RNA interference (RNAi) of TbP93 expression resulted in a cessation of cell growth and the loss of kDNA. Nicked/gapped forms of minicircles, the products of minicircle replication, were preferentially lost from the population of free minicircles during RNAi, suggesting involvement of TbP93 in minicircle replication. This approach should allow the identification of other novel proteins involved in the duplication of kDNA.

Trypanosomatid protozoa contain an unusual form of mitochondrial DNA consisting of thousands of circular DNA molecules catenated into a single enormous network termed “kinetoplast DNA” (kDNA) (24). These parasites are responsible for human diseases such as African sleeping sickness, visceral and dermal leishmaniasis, and Chagas disease. The trypanosomatid protozoa is among the earliest diverging eukaryotic organisms (40). Each parasite cell has only a single mitochondrion, which contains a single kDNA network that is condensed into a disk-shaped structure. The kDNA network is composed of two types of circular DNA: thousands of minicircles and 20 to 30 maxicircles. These circular DNAs are topologically interlocked to form a single DNA network. Each minicircle is interlocked with an average of three neighboring minicircles (8). The maxicircles usually range from 20 to 40 kb in size and encode a set of genes involved in the respiratory chain and mitochondrial rRNAs. Most maxicircle-encoded transcripts require specific addition and deletion of uridine residues by a process termed “RNA editing” to form translatable mRNAs (41). Minicircles from different species range from 0.9 to 10 kb in size and encode guide RNAs required for RNA editing of maxicircle transcripts (24).

kDNA replication involves the duplication of all minicircles and maxicircles in the network and division of the double-size network into two daughter networks. Replication of the kDNA takes place during a distinct period of the cell cycle close to the nuclear S phase (9, 34, 44), unlike in higher eukaryotes, where mitochondrial DNA replicates throughout the cell cycle (5, 14, 42). Minicircles are released from the network by a topoisomerase II prior to replication in the kinetoflagellar zone (KFZ), a specialized region of the mitochondrial matrix between the kDNA disk and the flagellar basal body (12). The free minicircles initiate replication as theta structures by a mechanism proposed to involve the universal minicircle sequence-binding protein (UMSBP), primase, and replicative polymerases (17, 20, 33). An additional protein, p38, has been shown recently to bind to the minicircle replication origin and appears to be essential for initiation of replication (23). The progeny free minicircles are proposed to migrate from the KFZ to the antipodal sites flanking the kDNA disk, where later stages of minicircle replication are catalyzed by proteins including DNA polymerase β, structure-specific endonuclease 1, DNA ligase kβ, and topoisomerase II (13, 18, 28, 38). The newly replicated free minicircles are partially repaired by filling the gaps between Okazaki fragments and sealing nicks before reattaching to the kDNA network at the antipodal sites. However, a single nick or gap remains in the newly synthesized strand of each daughter minicircle until all minicircles have been replicated (3, 4, 31, 32, 36). When all minicircles have been replicated, the remaining nicks/gaps in the minicircles are sealed and the disk is then segregated into two daughter networks by unknown mechanisms. Maxicircles also replicate by a theta mechanism but do not detach from the network during replication (7). However, much less is known about the maxicircle replication mechanism and the proteins involved.

More than 30 proteins have been found to be involved in kDNA replication or maintenance (19, 22). However, the complexity of kDNA replication and the frequency of occurrence of genes required for kDNA maintenance identified in a par-
tial screening of an RNA interference (RNAi) library suggest that at least 100 proteins are required to replicate and maintain kDNA (22). It is likely that many of these kinetoplastid proteins will be unrelated to known replication proteins in other eukaryotes. In earlier studies of kDNA replication genes in *Crithidia fasciculata*, we found that two or more copies of the consensus octamer (C/A)AUAGAA(G/A) are required in the 5′ and/or 3′ flanking regions of transcripts for S-phase expression of these genes (2, 6, 25, 35). Recently, Shlomai and coworkers (45) developed a computational tool to search the genome of *Leishmania major* and found that these posttranscriptional control elements identified S-phase-expressed genes in this kinetoplastid as well. We have screened this subset of *L. major* genes for predicted mitochondrial targeting as a means of identifying new kDNA replication proteins. We describe here a *Leishmania major* gene, LmA9, that encodes a protein that has a cell cycle-dependent localization to anti-podal sites flanking the kinetoplast disk and its*Trypanosoma brucei* ortholog, ThbP93, which we show to be an essential gene implicated in kinetoplast minicircle DNA replication.

**MATERIALS AND METHODS**

**Cloning of LmA9 and ThbP93 genes and plasmid constructions.** To generate LmA9 (LmJ9F 25,1360) fusion protein with a C-terminal influenza virus hemagglutinin (HA) tag in triplicate, the LmA9 coding region and 5′ flanking sequence was amplified by using *Leishmania major* genomic DNA as the template with the primers 5′-TAAAGCGTTCAAGGTGGACAGTGGAGGA-3′ and 5′-AAGGAATACGATCTACGCCTCCACCAG-3′. The PCR product was digested with HindIII and EcoRV and ligated into the corresponding sites of plasmid pMA1 (2). To generate a ThbP93 fusion protein with a C-terminal influenza virus HA tag in triplicate, the ThbP93 coding region was amplified by using *T. brucei* 29-13 genomic DNA as a template with the primers 5′-TCAAGGTCATATGCGGGCTACCCAC-3′ and 5′-AGTACGACAAATCAGAAGAACTTCTCGTG-3′. The PCR product was digested with HindIII and Nhel and ligated to the corresponding compatible sites of HindIII- and XbaI-digested expression vector pH54, a derivative of pLEW100 (43) that has the luciferase gene replaced by three copies of the HA tag. For ThbP93 (Th 927.3.1180) RNAI, a 1,000-bp fragment of ThbP93 coding region was amplified by using *T. brucei* 29-13 genomic DNA as the template with the primers 5′-GAAAAGCTTCTGCGGGCTTCTG-3′ and 5′-GGTTCTAAGAGCTAAGCGAC-3′. The PCR product was digested with HindIII/XbaI and incubated after the opposing T7 promoters in the inducible RNAi vector pJET7. A BLAST search of the *T. brucei* genome with this fragment showed no significant sequence identity elsewhere in the genome.

**Leishmania tarentolae and *Trypanosoma brucei* growth and transfection.** *L. tarentolae* was cultured at 28°C in brain heart infusion (BHI) medium (Becton, Dickinson and Co.) containing 10 μg/ml hemin (Sigma Co.,). Transfection was carried out as follows. Cells were washed twice in ice-cold BHI medium without hemin and resuspended at a density of 2.5 × 10^6 cells/ml. Ten micrograms of plasmid DNA was mixed with 0.4 μl of cells and electroporated by six pulses at 900 V with a 200-ms pulse length and 200-ms interval between pulses in 2-mm electroporation cuvettes in a BTX ECM830 square-wave electroporator. Cells were allowed to recover overnight in 10 ml of medium and then put under drug selection (2.5 μg/ml of phleomycin D1 [Invitrogen, Inc.]) the following day. Clonal cell lines were obtained by limited dilution in 50% conditioned medium and incubated at 28°C.

**Protein immunolocalization.** Immunofluorescent localization of HA-tagged fusion protein was performed as described previously (38). Briefly, cells at 4 × 10^7 per ml were harvested, resuspended in phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 1.5 mM Na_2HPO_4, 1.4 mM KH_2PO_4), spotted onto poly-L-lysine-coated slides, and allowed to adhere for 30 min in a humid chamber. The cells were then fixed in 4% paraformaldehyde in PBS for 10 min. Fixation was stopped by two 5-min washes in 0.1 M glycine in PBS, followed by incubation for 10 min in 0.025% Triton X-100 in PBS. The slides were kept in methanol at −20°C overnight, rehydrated by washing three times in PBS, and then blocked for 1 h at room temperature with 20% goat serum in PBS-0.05% Tween 20 (PBST). The slides were then incubated at room temperature overnight with a mixture of anti-HA monoclonal antibody HA.11 conjugated to Alexa Fluor 488 (Covance) at a 1:100 dilution, washed three times in PBST for 5 min each, and mounted in SlowFade Gold antifade reagent with DAPI (4′,6′-diamidino-2-phenylindole) (Invitrogen Co.). *T. brucei* cells undergoing RNAi were prepared in the same way, incubated with YL1/2 (Chemicon; rat monoclonal antibodies against *Sarcocystis muris* stained α-tubulin at a 1:400 dilution), washed three times in PBS, and stained with Alexa Fluor 568-conjugated secondary goat antibodies (Molecular Probes) to stain basal bodies. Cells were imaged using a Zeiss Axioskop II compound microscope with a 63× Placneufluor oil immersion objective lens. Images were captured using a Zeiss Axioscam digital camera and Zeiss Axiovision 3.0 software. Red, blue, and green images of the same field were captured independently and merged using Adobe Photoshop 7.0 on a MacIntosh computer running OSX v10.4.9. Contrast and brightness were adjusted in Adobe Photoshop for each image.

**RNA gel blotting and Northern blotting.** Cloned *T. brucei* cells stably transfected with NotI-cleaved ThbP93 RNAI vector were induced for RNAi by the addition of tetracycline (1 μg/ml). Aliquots of the culture were removed every 24 h and fixed in 4% paraformaldehyde in PBS for 10 min. Fixation was stopped by two 5-min washes in 0.1 M glycine in PBS, followed by incubation for 10 min in 0.025% Triton X-100 in PBS. The slides were kept in methanol at −20°C overnight, rehydrated by washing three times in PBST. The slides were mounted in SlowFade Gold antifade reagent (Invitrogen Co.) containing DAPI and analyzed by fluorescence microscopy for DNA phenotype. More than 150 cells were counted for each time point.

For Northern blots, cloned cells induced for RNAi by the addition of 1 μg/ml tetracycline for 40 h were collected and total RNA was extracted using an RNeasy mini kit (QIAGEN). RNA from 5 × 10^6 cells was loaded per lane, fractionated on a 1.2% agarose gel containing 7% formaldehyde, and analyzed by standard Northern blotting using a 32P-labeled probe made by random priming of the 1,000-bp fragment used for RNAi. A BLAST search of the genome did not detect additional sequences that might give rise to an off-target effect.

**Leishmania tarentolae synchronization and Northern blot expression.** *Leishmania tarentolae* cells were synchronized by hydroyurea treatment (34, 37). Cells were grown to late log phase (10^7 cells/ml) at 28°C in BHI medium supplemented with 10 μg hemin/ml and 100 μg streptomycin sulfate/ml prior to the addition of hydroyurea to 200 μg/ml for 6 h. Cultures expressing the epitope-tagged LmA9 protein also contained 40 μg hygromycin/ml. Growth was continued at 28°C in fresh medium lacking hydroyurea, and samples were removed every 30 min to determine the cell number and the number of dividing cells. Analysis of *L. tarentolae* P105 gene (Ltp105) expression during the cell cycle was performed by Northern blotting of RNA isolated from samples (5 × 10^6 cells) taken at 30-min intervals from a synchronous culture of *L. tarentolae*. RNA isolation and blotting were described as above. DNA probes were amplified from genomic DNA using the primers 5′-GGGCTAGACGTCGAGCGG-3′ and 5′-GGTTCTAAGAGCTAAGCGAC-3′. The probe was labeled with 32P by random priming for probing the Northern blot.

**Western blots.** *L. tarentolae* cells (2 × 10^7) stably expressing HA-tagged LmA9 fusion protein were harvested at 12,000 × g for 5 min. The cell pellets were washed three times with 1 ml PBS buffer, resuspended in 50 μl PBS, mixed with 50 μl 2× sample loading buffer (50 mM Tris-HCl, 4% sodium dodecyl sulfate [SDS], 16% glycerol, 5% 2-mercaptoethanol, 0.025% bromphenol blue, pH 6.8), and boiled for 5 min. Total lysates of 2 × 10^7 cells were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride transfer membrane (Perkin-Elmer) with transfer buffer (25 mM Tris, 20% methanol, 0.2 M glycine) at 100 V for 1 h. The membrane was incubated in Tris-buffered saline-Tween (2×BST) buffer (25 mM
Tris-HCl, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween 20, pH 8.0) for 5 min. The membrane was blocked with 5% (wt/vol) nonfat dry milk and 5% (vol/vol) goat serum in TBST for 1 h, and then incubated with anti-HA monoclonal antibody 12CA5 (Abcam, Inc.) in blocking buffer at a 1:1,000 dilution with gentle rocking for 1 h. After three washes in TBST for 5 min each, HA-tagged fusion protein bands were visualized using peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (Sigma) as secondary antibodies in blocking buffer at a 1:8,000 dilution and the SuperSignal West Pico chemiluminescent system (Pierce).

**RESULTS**

Identification of a putative kDNA replication/maintenance gene. A computational search of the *L. major* genome database identified a set of S-phase-expressed genes based on the presence of two or more sequences related to the octamer consensus (C/A)ATAGAA(A/G) in 5' and/or 3' flanking regions (45). We have searched this subset of genes using Mitoprot II software to identify genes that have a high probability of being mitochondrial. A gene, *LmP105* (LmjF25.1360), identified in this search has an open reading frame encoding a protein of 965 amino acids. *LmP105* has two putative posttranscriptional control elements, CATAGAA and CATAGAG, in the 5' flanking region within 400 nucleotides from the open reading frame. We have not yet mapped the *LmP105* splice acceptor site to determine whether these sequences lie within the 5' untranslated region (5' UTR); however, essential control sequences have been found 5' of the splice acceptor site in earlier studies in *C. fasciculata* (2). Mitoprot II predicts mitochondrial import of the protein with a probability of 0.98. The protein is highly conserved in kinetoplastids, as shown in Fig. 1, but has no significant similarity to genes of FIG. 1. Clustal alignment of predicted amino acid sequences of kinetoplastid genes related to *LmP105*. Identical amino acids are indicated by light shading. *Lm*, *Leishmania major*, AJ052544.1; *Tb*, *Trypanosoma brucei*, XP_843712; *Tc*, *Trypanosoma cruzi*, XP_814115.1.
investigate chronized by release from a hydroxyurea block in order to replication proteins, including topoisomerase II, polymerase H9252 ligase k Lm many cells in an exponential culture do not show localization of H9252 colocalizes with the polymerase C. fasciculata dependent localization like that of several (18). The results in Fig. 2D show that fasciculata C. antipodal sites of the kDNA disk, as previously reported for merase kDNA staining throughout the cell and never showed staining of the immunostaining of untransfected cells only shows background Although not all cells show localization of the LmP105 protein, LmP105 localization during the cell cycle (Fig. 3A). To determine the intracellular localization of LmP105, the coding sequence of LmP105, along with putative posttranscriptional control elements in the 5’ flanking sequence, was cloned and expressed with three copies of an HA epitope tag in Leishmania tarentolae (Fig. 2A). The tagged protein is predicted to have a molecular mass of 109 kDa but migrates on an SDS gel at a rate corresponding to a molecular mass of approximately 130 kDa, suggesting possible posttranslational modification of the protein. Figure 2B shows the immunolocalization of epitope-tagged LmP105 to antipodal sites flanking the kinetoplast disk. Although not all cells show localization of the LmP105 protein, immunostaining of untransfected cells only shows background staining throughout the cell and never showed staining of the kinetoplast (Fig. 2C). Coimmunolocalization with DNA polymerase β was also performed to further confirm the intracellular localization of LmP105. DNA polymerase β localized to antipodal sites of the kDNA disk, as previously reported for C. fasciculata (18). The results in Fig. 2D show that LmP105 colocalizes with the polymerase β at the antipodal sites. Since many cells in an exponential culture do not show localization of LmP105, we suspected that the protein may have a cell cycle-dependent localization like that of several C. fasciculata DNA replication proteins, including topoisomerase II, polymerase β, ligase kα, SSE1, and UMSBP (1, 13, 18, 39).

Cell cycle dependence of LmP105 kinetoplast localization. L. tarentolae cells expressing epitope-tagged LmP105 were synchronized by release from a hydroxyurea block in order to investigate LmP105 localization during the cell cycle (Fig. 3A). The lack of a complete doubling of the cell number by 5.5 h in the synchronized culture likely reflects the presence of cells initially that were unable to recover from the hydroxyurea treatment. It was noted earlier that hydroxyurea is selectively lethal to L. tarentolae cells in S phase (37), and these cells would contribute to the total cell count. Cells were scored for the presence of dividing cells containing two nuclei (doublets) as an indicator of cell cycle position. LmP105 was immunolocalized in cells harvested at 30-min intervals. Figure 3B shows an absence of kinetoplast localization in dividing cells with two nuclei. The lower panels of Fig. 3B show typical images of dividing and nondividing cells. Figure 3C shows the frequency of kinetoplast localization of LmP105 during the cell cycle. The frequency of kinetoplast localization of LmP105 was at a relative maximum from 0 to 1 h after release from the hydroxyurea block, a time corresponding to the initial S phase upon release from the block, and was at a minimum at 3 h, which corresponds approximately to G2/M. Cells undergoing division were never observed to show kinetoplast localization of LmP105.

S-phase expression of the LtP105 transcript in synchronized L. tarentolae. The possible S-phase expression of LtP105 was investigated by Northern blotting of RNA isolated from a synchronous culture at 30-min intervals after removal of the hydroxyurea block. As shown in the autoradiogram of the blotted RNA (Fig. 4A) and in the quantitation of the phosphorimage (Fig. 4B), the LtP105 levels cycle in the same manner as the cyclic localization of the LmP105 protein in L. tarentolae cells.

Kinetoplast localization of TbP93 and RNAi. TbP93, the ortholog of LmP105 in T. brucei, shares 46% identity with other organisms nor does it have any motifs that would give a clue as to its function.

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Kinetoplast localization of TbP93 and RNAi. TbP93, the ortholog of LmP105 in T. brucei, shares 46% identity with
LmP105 at the amino acid sequence level. To further characterize the role of TbP93/LmP105 in kinetoplast DNA replication/maintenance, the TbP93 coding sequence was cloned and inserted into a tetracycline-inducible expression vector with three copies of the influenza virus HA epitope tag and electroporated into T. brucei 29-13 cells. Immunolocalization of the epitope-tagged protein shows localization of the protein to the kinetoplast (Fig. 5).

To address the function of TbP93, the coding sequence was cloned into the tetracycline-inducible RNAi vector P2T7 and electroporated into T. brucei 29-13 cells. Figure 6 shows the growth curve and Northern blot analysis of a typical RNAi clone. Cells grew normally in the absence of tetracycline, whereas after 5 days of tetracycline treatment to induce RNAi, cell growth was arrested. A Northern blot of the TbP93 transcript shows a disappearance by 40 h after the addition of tetracycline.

Shrinkage and loss of kDNA by TbP93 RNAi. To observe both the nucleus and kDNA during RNAi induction, cells were removed from the culture every 48 h after tetracycline addition and stained with DAPI. RNAi of TbP93 resulted in a shrinkage and loss of kDNA. T. brucei cells had a normal-size kDNA prior to addition of tetracycline (Fig. 7A). After 8 days of RNAi induction, many cells showed a shrinkage or loss of kDNA.
kDNA. The typical cell phenotypes were categorized into three groups: the normal group, which included one nucleus and one normal-size kinetoplast; the small group, which included one nucleus and one small kinetoplast; and the “no” group, which included one nucleus and no kinetoplast. Figure 7B shows typical examples of these three types in cells stained with DAPI and with basal body antibodies. The percentage of each phenotype is shown in Fig. 7C. The percentage of cells with normal-size kDNA decreased during RNAi, and the percentage of cells with small or no kDNA increased during RNAi. The absence of kDNA was judged based on a lack of detectable DAPI fluorescence adjacent to the basal body. However, the possibility of a kDNA remnant too small to be detected cannot be excluded.

Effect of TbP93 RNAi on free minicircle replication intermediates. The shrinkage and loss of kDNA during RNAi suggest involvement of TbP93 in kDNA replication. Since minicircle replication occurs free of the kDNA network, we have therefore examined the population of free minicircles during RNAi by Southern blotting of total DNA using a minicircle probe. Prior to RNAi (day 0) there are similar levels of covalently closed (nonreplicating) and nicked/gapped free minicircles. The latter species, the products of replication, decreases during RNAi in parallel with the shrinkage and loss of kDNA (Fig. 8). The upper bands on the Southern blot are due to kDNA and to nonspecific hybridization to chromosomal DNA. The abundance of free covalently closed minicircles also declines during RNAi but less so than that of the nicked and gapped free minicircles. The preferential loss of nicked/gapped minicircles, the products of minicircle replication, during RNAi implies a role for TbP93 in minicircle replication.

DISCUSSION

We have shown in a series of earlier studies that transcripts of several genes involved in DNA replication in Crithidia fasciculata vary during the cell cycle, with maximal levels during S phase (2, 6, 16, 25–27, 29, 30, 34, 35). Furthermore, octamer consensus sequences (C/A)ATAGAA(A/G) present in flanking sequences of these genes were found to be essential for S-phase expression of these transcripts. A computational search of the Leishmania major genome database for similar posttranscriptional control sequences identified 132 genes, of which one-third encode known DNA metabolism genes and the remaining two-thirds are not annotated (45). A test of
cell cycle. The absence of HA-tagged LmP105 fluorescence in dividing cells is similar to that observed in C. fasciculata for polymerase β (18) and structure-specific endonuclease 1 (13). Since the absence of a fluorescence signal at the antipodal sites in dividing cells is a common feature of at least three proteins found there, there may be a common, but yet unknown, basis for the disappearance of the signal during cell division.

To address the function of this kinetoplast protein, we turned to the T. brucei ortholog TbP93 to take advantage of the use of RNAi to knock down expression of the gene. TbP93 was found to be essential for cell growth and for maintenance of the kDNA. During RNAi, the kDNA became progressively smaller and was lost in a large fraction of the cells. Further analysis of free minicircles showed a fivefold loss by day 9 of RNAi of the relative abundance of nicked/gapped minicircles, the products of minicircle replication. Taken together, these results suggest that LmP105/TbP93 has a direct role in kDNA replication. In the absence of a known motif in LmP105/TbP93, further characterization at a biochemical level will be required to address the function of these genes’ proteins.

If LmP105 and TbP93 are directly involved in minicircle replication, it is surprising that they localize to the antipodal sites of the kDNA disk rather than to the KFZ, where minicircle replication appears to take place. The antipodal sites have been identified in earlier studies as sites where at least partial repair of nicks/gaps in minicircles takes place after the nascent minicircles have been reattached to the kDNA network. Recent studies suggest that the antipodal sites are more complex than previously thought and may contain subdomains. For example, T. brucei ligase kβ and topoisomerase II do not precisely colocalize at the antipodal sites (11). p38, a recently described protein that can bind to the minicircle replication origin and which has been implicated in early minicircle replication, also localizes to the antipodal sites of the kDNA disk (23). The puzzling localization of LmP105 and TbP93 and that of p38 suggest that the role of the antipodal sites may be less restricted than previously thought.

Over 30 proteins implicated in kDNA replication or maintenance have been identified (22). The highly unusual structure of kDNA and its complex replication and maintenance mechanism may require a large number of replication proteins, many of which are possibly unique to kinetoplastids. An RNAi library-based screen has been reported to identify genes for kDNA replication and maintenance (22). It is estimated that over 100 genes may be involved in kDNA replication and maintenance. Here we demonstrate another approach based on a bioinformatics screen. By combining the search for posttranscriptional control elements (C/A)A TAGAA(A/G) for S-phase gene expression (45) and the prediction of mitochondrial targeting, a new gene involved in kDNA replication was identified. Our work shows this is an alternative means to identify kinetoplast replication genes that may not contain a known DNA replication-related motif.

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REFERENCES

1. Abu-Elnene, K. R. Robinson, M. E. Drew, P. T. Englund, and J. Shlomai. 2001. Intramitochondrial localization of universal minicircle sequence-binding protein, a trypanosomatid protein that binds kinetoplast minicircle replication origins. J. Cell Biol. 153:725–734.

2. Avliyakulov, N. K., J. C. Hines, and D. S. Ray. 2003. Sequence elements in both the intergenic space and the 3’ untranslated region of the Crithidia fasciculata maxicircle are required for cell cycle regulation of KF43 mRNA. Eukaryot. Cell 2:671–677.

3. Birkenmeyer, L., H. Sugisaki, and D. S. Ray. 1987. Structural characterization of site-specific discontinuities associated with replication origins of minicircle DNA from Crithidia fasciculata. J. Biol. Chem. 262:2384–2392.

4. Burka, L., J. C. Rauch, I. White, P. T. Englund, and N. R. Cozzarelli. 1999. The kinetoplast structure-specific endo/exo/endonuclease domain of bacterial DNA polymerase beta during replication. Proc. Natl. Acad. Sci. USA 96:8455–8460.

5. Cunningham, I. 1977. The topology of the kinetoplast DNA network. Cell 11:719–727.

6. Brown, L. M., and D. S. Ray. 1997. Cell cycle regulation of RPA1 transcript levels in the trypanosomatid Crithidia fasciculata. Nucleic Acids Res. 25:3291–3298.

7. Carpenter, L. R., and P. T. Englund. 1995. Kinetoplast maxicircle DNA replication in Crithidia fasciculata and Trypanosoma brucei. Mol. Cell. Biol. 15:5794–6003.

8. Guttes, E. W., P. C. Hanawalt, and S. Guttes. 1967. Mitochondrial DNA synthesis and the mitotic cycle in Phisium polycephalum. Biochim. Biophys. Acta 142:181–194.

9. Hill, K. L., N. R. Hutchings, D. G. Russell, and J. E. Donelson. 1999. A novel protein targeting domain directs proteins to the anterior cytosome face of the flagellar pocket in African trypanosomes. J. Cell Sci. 112:3091–3101.

10. Hines, J. C., and D. S. Ray. 1997. Periodic synthesis of kinetoplast DNA topoisomerase II during the cell cycle. Mol. Biochem. Parasitol. 88:249–252.

11. Johnson, C. E., and P. T. Englund. 1999. A refined localization of the mitochondrial DNA primase in Crithidia fasciculata. Mol. Biochem. Parasitol. 102:205–208.

12. Johnson, C. E., and P. T. Englund. 1998. Changes in organization of Crithidia fasciculata kinetoplast DNA replication proteins during the cell cycle. J. Cell Biol. 143:911–919.

13. Klingbeil, M. M., M. E. Drew, Y. Liu, J. C. Morris, S. A. Motyka, T. T. Saxowsky, Z. Wang, and P. T. Englund. 2001. Unlocking the secrets of trypanosome kinetoplast DNA network replication. Protist 152:255–262.

14. Klingbeil, M. M., S. A. Motyka, and P. T. Englund. 2002. Multiple mitochon-
drial DNA polymerases in Trypanosoma brucei. Mol. Cell 10:175–186.

15. LaCoutur, D. J., S. Bruse, K. L. Hill, and J. E. Donelson. 2000. Double-stranded RNA interference in Trypanosoma brucei using head-to-head pro-
moters. Mol. Biochem. Parasitol. 111:67–76.

16. Liu, B., Y. Liu, S. A. Motyka, E. E. Agbo, and P. T. Englund. 2005. Fellowship of the rings: the replication of kinetoplast DNA. Trends Parasitol. 21:363–369.

17. Liu, B., H. Molina, D. Kalume, A. Pandey, J. D. Griffith, and P. T. Englund. 2006. The role of p38 in replication of Trypanosoma brucei kinetoplast DNA. Mol. Biochem. Parasitol. 20:5382–5393.

18. Lukeš, J., D. L. Guibride, J. Totok, P. Ziková, R. Benne, and P. T. Englund. 2002. Kinetoplast DNA network: evolution of an improbable structure. Eukaryot. Cell 1:499–502.

19. Mahmood, R., J. C. Hines, and S. S. Ray. 1999. Identification of cis and trans elements involved in the cell cycle regulation of multiple genes in Crithidia fasciculata. Mol. Cell. Biol. 19:6174–6182.

20. Mahmood, R., B. Mittra, J. C. Hines, and D. S. Ray. 2001. Characterization of the Crithidia fasciculata mRNA cycling sequence binding proteins. Mol. Cell. Biol. 21:4453–4459.

21. Mahmood, R., and D. S. Ray. 1998. Nuclear extracts of Crithidia fasciculata contain factors that bind to the 5’ untranslated regions of TOP2 and RPA1 containing sequences required for their cell cycle regulation. J. Biol. Chem. 273:23729–23734.

22. Melendy, T., C. Sheline, and D. S. Ray. 1988. Localization of a type II DNA topoisomerase to two sites at the periphery of the kinetoplast DNA of Trypanosoma brucei. J. Biol. Chem. 263:1890–1893.

23. Mittra, B., C. Chen, P. T. Englund, and S. H. Biel. 2004. Presence of a poly(A) binding protein and two proteins with cell-cycle-dependent phosphorylation in Crithidia fasciculata mRNA cycling sequence binding protein II. Eukaryot. Cell 3:1185–1197.

24. Nuss, L. I., P. Kappler, K. Abe, and J. Shlomai. 2006. Binding of the universal minicircle sequence binding protein at the kinetoplast DNA replication origin. J. Biol. Chem. 281:1152–1167.

25. Passon, S. G., W. Brown, L. M. Brown, and D. S. Ray. 1994. Periodic expression of nuclear and mitochondrial DNA replication genes during the trypanosomatid cell cycle. J. Cell Sci. 107:3515–3520.

26. Pasion, S. G., J. C. Hines, X. Ou, R. Mahmood, and D. S. Ray. 1996. Sequences within the 5’ untranslated region regulate the levels of a kineto-
plast DNA topoisomerase mRNA during the cell cycle. Mol. Cell. Biol. 16:6724–6735.

27. Ryan, K. A., and P. T. Englund. 1989. Synthesis and processing of kinetoplast DNA minicircle mRNAs in Trypanosoma equiperdum. J. Biol. Chem. 264:5574–5579.

28. Nuss, L. I., P. Kappler, K. Abe, and J. Shlomai. 2006. Binding of the universal minicircle sequence binding protein at the kinetoplast DNA replication origin. J. Biol. Chem. 281:13746–13747.

29. Simpson, L., L. C. Bray, and M. B. Ichikow. 1971. The synthesis of mitochondrial DNA in Crithidia fasciculata. Proc. Natl. Acad. Sci. USA 101:4361–4365.

30. Sinha, K. M., J. C. Hines, N. Downey, and D. S. Ray. 2004. Mitochondrial DNA ligase in Crithidia fasciculata. Proc. Natl. Acad. Sci. USA 101:4361–4365.

31. Williams, D. H., and E. Moustacchi. 1971. The synthesis of mitochondrial DNA during the cell cycle in the yeast Saccharomyces cerevisiae. Biochim. Biophys. Res. Commun. 42:195–201.

32. Böttcher, E., S. Leal, C. Ochert, and G. A. Cross. 1999. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in Trypanosoma brucei. Mol. Biochem. Parasitol. 99:89–101.

33. Ihara, Y., and K. Galloway. 1990. Timing of nuclear and kinetoplast DNA replication and early morphological events in the cell cycle of Trypanosoma brucei. J. Cell Sci. 95:49–57.

34. Zeik, A., I. Onn, R. Bezalel, H. Margalit, and J. Shlomai. 2005. Assigning functions to genes: identification of 5-phase expressed genes in Leishmania major based on post-transcriptional control elements. Nucleic Acids Res. 33:4235–4242.