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Synthesis and Processing of Kinetoplast DNA Minicircles in *Trypanosoma equiperdum*

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Kinetoplast DNA, the mitochondrial DNA in trypanosomes, is a giant network containing topologically interlocked minicircles. Replication occurs on free minicircles that have been detached from the network. In this paper, we report studies on the synthesis and processing of the minicircle L and H strands. Analysis of free minicircles from *Trypanosoma equiperdum* by two-dimensional agarose gel electrophoresis indicated that elongating L strands are present on theta structures. Hybridization studies indicated that L-strand elongation is continuous and unidirectional, starting near nucleotide 805 and proceeding around the entire minicircle. The theta structures segregate into monomeric progeny minicircles, and those with a newly synthesized L strand have a 8-nucleotide gap between nucleotides 805 and 814 (J. M. Ntambi, T. A. Shapiro, K. A. Ryan, and P. T. Englund, J. Biol. Chem. 261:11890–11895, 1986). These molecules are reattached to the network, where repair of the gap takes place. Of the molecules labeled during a 10-min pulse with [^{3}H]thymidine, gap filling occurred on half within about 15 min and on virtually all by 60 min; however, there was no detectable covalent closure of the newly synthesized L strand by 60 min.

Kinetoplast DNA (kDNA), the mitochondrial DNA of trypanosomes and related parasitic protozoa, is a network of thousands of topologically interlocked DNA circles. Most of these are minicircles of unknown function; a few dozen are maxicircles similar in structure and genetic function to mitochondrial DNA in other eucaryotic cells (see references 7, 12, 14, 18, and 19 for reviews on kDNA).

Before initiation of kDNA synthesis, minicircles are released from the network as individual covalently closed circles, presumably by a topoisomerase. These free minicircles then undergo replication, and their progeny are reattached to the network. Because two daughter molecules are reattached for every one removed, the network eventually doubles in size. The double-size network is then split into two progeny networks which segregate into daughter cells during cell division (see references 12 and 14 for reviews on kDNA replication).

There have been extensive studies on free minicircle replication intermediates in *Trypanosoma equiperdum* (10, 11, 13, 15) and *Crithidia fasciculata* (2, 3, 6, 8, 9, 17). Minicircles with a newly synthesized L strand differ strikingly from their sister molecules with a newly synthesized H strand. In *T. equiperdum*, minicircles with a newly synthesized L strand have a single L-strand gap between nucleotides 805 and 814; there are one or two ribonucleotides, presumably the remnant of a replication primer, linked to the L-strand 5′ end (10, 11). A small fraction of these molecules are topologically knotted (15). Minicircles with a newly synthesized H strand, which form a smear upon gel electrophoresis, contain multiple nicks or gaps in the newly synthesized strand (13). Some of the H-strand fragments map to specific locations on the minicircle sequence (13). On the basis of the structures of these two types of progeny minicircles, it has been assumed that the L strand is synthesized continuously and that the H strand is synthesized discontinuously.

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In this paper, we report new studies on the synthesis and processing of the L and H strands of *T. equiperdum* minicircles. We show that L-strand synthesis, which occurs on theta structures, is continuous and unidirectional around the entire minicircle. We have also detected the first two H-strand Okazaki fragments on theta structures. We report the kinetics of repair of the L-strand gap after the progeny minicircle is reattached to the network.

MATERIALS AND METHODS

Labeling and isolation of kDNA. *T. equiperdum* (Pasteur Institute strain BoTat 24) was grown in Wistar rats to parasitemias of approximately 10^8 cells per ml. Infected blood, obtained by cardiac puncture, was treated with heparin (final concentration, 10 U/ml). Blood from six rats, obtained over a 30-min period, was pooled at room temperature. The blood (60 ml, containing 6×10^9 parasites) was swirled gently at 37°C for 10 min before the addition of 6 ml of PSG (60 mM sodium phosphate [pH 7.4], 44 mM NaCl, 56 mM glucose) and [^{3}H]thymidine (final concentration, about 400 μCi/ml; 25 Ci/mmol) (11). After incubation at 37°C, cells in the whole blood were lysed by addition of an equal volume of 10 mM Tris hydrochloride (pH 8.0)–75 mM EDTA–1% sodium dodecyl sulfate–2 mg of proteinase K per ml. The lysate was incubated for 2 h at 55°C and then, after addition of RNase A (100 μg/ml and RNase T_1 (200 U/ml), for 30 min at 37°C. After phenol extraction, kDNA networks were isolated by differential centrifugation, followed by equilibrium centrifugation (11). Free minicircles were isolated from network-depleted DNA by zone sedimentation in a sucrose gradient (11). From cells labeled for 60 min with [^{3}H] thymidine, about 2,500 cpm/10^6 cells was recovered as networks and about 500 cpm/10^6 cells was recovered as free minicircles.

Gel electrophoresis. Agarose gels, denaturing polyacrylamide gels, two-dimensional agarose gels, Southern transfers, and electroelution from agarose gels were described previously (15). Densitometry was performed by using a Zeineh scanning densitometer (model SL-504-XL).

DNA probes and hybridization. Probes pJN6 (hybridizing

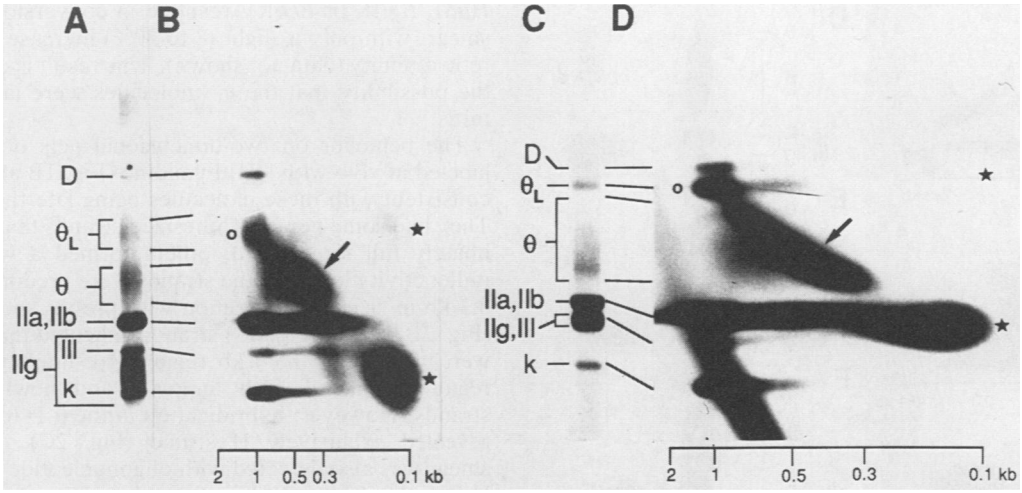

FIG. 1. Two-dimensional gel electrophoresis of free minicircles. Electrophoresis in the first dimension was in TBE (90 mM Tris, 92 mM boric acid, 2.5 mM EDTA [pH 8.3]) containing 0.5 μg of ethidium bromide per ml (A and B) or no ethidium bromide (C and D). Electrophoresis in the second dimension was in 30 mM NaOH–2 mM EDTA. Free minicircles (30,000 cpm), labeled in vivo with [^{3}H]thymidine for 10 min, were resolved in the first dimension (A and C) or in both dimensions (B and D). The fluorographs in panels A and C were exposed for 1/10 the time used for those in panels B and D. Scales indicate sizes of linear markers in the second dimension. Symbols: ←, diagonal of growing radioactive DNA on theta structures; *, small, newly synthesized strands on multiply gapped molecules (IIg) and late theta structures (θ_L); ○, 1-kb newly synthesized strand in the θ_L region of the gel. D, Structure probably containing two topologically interlocked minicircles; θ_L, late theta structures; θ, theta structures; IIa, nicked minicircles; IIb, uniquely gapped minicircles; III, linearized minicircles; IIg, multiply gapped minicircles; k, knotted minicircles (15).

to both minicircle strands), pKAR3-1 (hybridizing to the minicircle L strand), and pKAR5-1 (hybridizing to the minicircle H strand) were made as described previously (all contain a minicircle linearized at its *Bgl*II site) (15). Synthetic 20-base oligonucleotides hybridizing to the minicircle H or L strand were 5′ end labeled with T4 polynucleotide kinase (11). Each oligonucleotide probe is designated by the number of the 5′-terminal nucleotide (see reference 1 for nucleotide numbering system); the prefix H or L indicates the strand to which the probe hybridizes (e.g., probe H-720 hybridizes to the H strand). kDNA fragments on nylon filters were hybridized with ^{32}P-labeled oligonucleotide and polynucleotide probes as described previously (15). Filters were rehybridized after removal of old probe by submersion in boiling 1% sodium dodecyl sulfate for 10 min.

RESULTS

Synthesis of the minicircle L strand on theta structures. To investigate the mechanism of minicircle L-strand synthesis, we searched for *T. equiperdum* theta structures containing L strands undergoing elongation. We subjected purified free minicircles, labeled in vivo with [^{3}H]thymidine, to two-dimensional agarose gel electrophoresis. Figure 1A shows a fluorogram of the first dimension of a gel run in the presence of ethidium bromide; Fig. 1C shows a fluorogram of a similar gel run in the absence of ethidium bromide (ethidium causes significant differences in the resolution of the free minicircle components). These gels showed several forms of [^{3}H]thymidine-labeled free minicircles, including the previously characterized nicked (form IIa) (13, 15), uniquely gapped (form IIb) (11, 15), linearized (form III) (16), multiply gapped (form IIg) (13), and knotted (form k) (15) free minicircles. The covalently closed free minicircles are not labeled under these conditions. Two distinct smears, designated θ and θ_L, were candidates for theta-type replication intermediates (see Fig. 6 for scheme showing structures and interrelationships of these various replication intermediates).

The sizes of the newly synthesized strands in the molecules forming the θ and θ_L smears could be estimated from their mobilities in the second dimension (at alkaline pH). In alkali, the DNA denatures and the individual strands migrate according to size; the newly synthesized strands can then be detected by fluorography. The newly synthesized strands in the θ smear formed a diagonal characteristic of theta structures (Fig. 1B and D; Fig. 2A shows a shorter exposure of the fluorograph shown in Fig. 1D). Most of these strands were in the size range of about 0.3 to nearly 0.8 kilobases (kb), indicating that most of these theta structures were of intermediate maturity. The diagonal approached the second smear, θ_L, in which the newly synthesized strands were approximately 1 kb. The θ_L smear could have consisted of late theta structures (see below).

To determine whether the newly synthesized strands forming the diagonal were H or L strands, we hybridized a blot of a two-dimensional agarose gel (prepared from nonradioactive free minicircles) with radioactive probes specific for the L strand (Fig. 2B) or the H strand (Fig. 2C). The DNA forming the diagonal in the θ region hybridized exclusively with the probe specific for the L strand (Fig. 2B). There was only very faint hybridization to H-strand fragments, in the 0.1- to 0.3-kb size range, associated with molecules in the θ smear (Fig. 2C). To increase the sensitivity for detection of small H-strand fragments, we subjected a larger mass of theta structures, partially purified by gel electrophoresis and electroelution, to two-dimensional gel electrophoresis (Fig. 2D to F). Hybridization of the blot with oligonucleotide probes H-720 (Fig. 2D) and H-796 (not shown) revealed very small H strands (approximately 0.1 kb) associated with theta structures. Hybridization of the same blot with probes H-523 (Fig. 2E) and H-3 (Fig. 2F) reveals little if any hybridization. This lack of hybridization was not due to loss of DNA during removal of the various probes, since the H-796 hybridization was done last. In contrast to molecules in the θ region, fragments derived from θ_L (data

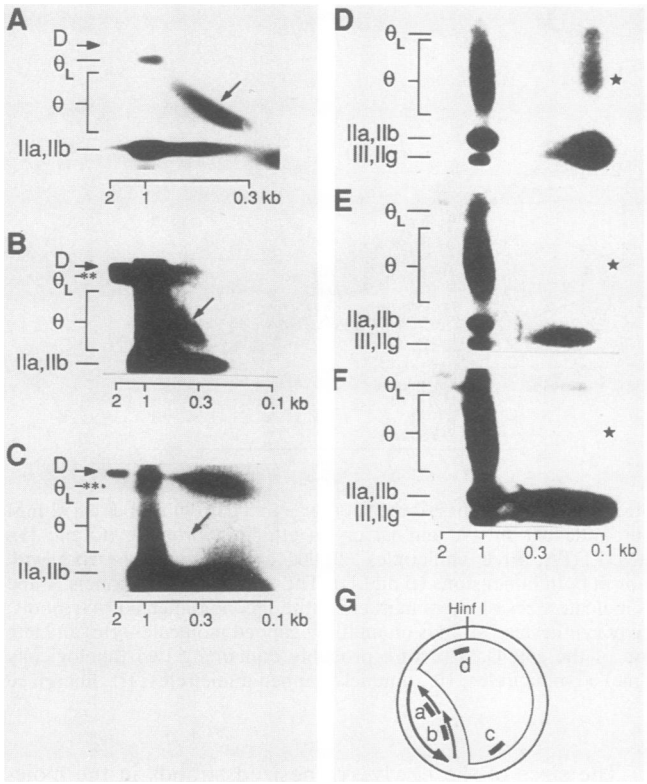

FIG. 2. Characterization of theta structures. (A) Shorter-exposure fluorograph of the two-dimensional gel shown in Fig. 1D. The free minicircles were labeled in vivo for 10 min with [^{3}H]thymidine. (B) Unlabeled free minicircles after electrophoresis as in Fig. 1D, transferred to a nylon filter and hybridized with pKAR3-1 (hybridizes with the L strand). (C) Same as panel B except hybridized with pKAR5-1 (hybridizes with the H strand). (D to F) Theta structures, purified by agarose gel electrophoresis and electroelution, that were resolved by two-dimensional agarose gel electrophoresis as in Fig. 1D and transferred to nylon filters (the preparation also contained some form IIa, IIb, III, and IIg molecules and a small amount of θ_L molecules.) and hybridized with oligonucleotides H-720 (D), H-523 (E), and H-3 (F). (G) Map showing the location of probes specific for the H strand (a, H-796; b, H-720; c, H-523; d, H-3). Scales indicate sizes of linear marker fragments in the second dimension. Note that the scales differ in different panels (e.g., A and B). The same blot was used for hybridizations shown in panels D to F. Symbols: ↙, diagonal of newly synthesized L strand on theta structures; *, small H-strand fragments; →, molecules that may be interlocked single-stranded circles; **, 2-kb molecules apparently comigrating with late theta structures (see text). Designation of molecules is the same as for Fig. 1.

not shown) and from the monomeric products of replication (form IIg [13] and those in the region of forms IIa and IIb [Fig. 2D to F]) hybridized extensively with all probes specific for the H strand.

θ_L smear and other oligomeric species. The θ_L molecules seen in Fig. 1 could have been late theta structures with parental strands still base paired, and the smearing could have been due to variable degrees of H- or L-strand synthesis. Alternatively, they could have two interlocked sister progeny minicircles, one of which is multiply gapped like those seen in smear IIg (Fig. 1A; 13), and the other could have a small gap like those in band IIb. Cleavage of the latter molecules with a single-hit reaction enzyme would result in conversion to 1-kb linearized minicircles and a smear. However, treatment of the electroeluted θ_L smear with either *Hin*fI, *Bgl*II, or *Eco*RV resulted in conversion to a broader smear, with only a slight (8 to 30%) increase in electrophoretic mobility (data not shown). This result is consistent with the possibility that the θ_L molecules were late theta structures.

The behavior on two-dimensional gels of θ_L molecules labeled in vivo with [^{3}H]thymidine (Fig. 1B and D) was also consistent with these molecules being late theta structures. They had some newly synthesized strands that were approximately full size (1 kb); others formed a weak smear of radioactivity in which the strands were predominantly 0.1 to 0.3 kb in size. Hybridization with probes specific for the L (Fig. 2B) and H (Fig. 2C) strands indicated that both strands were present in the 1-kb region, presumably because this region contained both template and newly synthesized strands. However, hybridization in the 0.1- to 0.3-kb region revealed exclusively H strand (Fig. 2C). This H-strand smear was also detected with oligonucleotide probes H-720, H-796, H-523, and H-3 (data not shown), indicating the presence of H-strand fragments from several regions of the minicircle. Therefore, by the θ_L stage of minicircle replication, synthesis of all regions of both the H and L strands may be nearly complete.

Another species, designated D in Fig. 1 and 2, probably consisted of two catenated minicircles that were products of theta replication. On two-dimensional gels (Fig. 1B and D), the newly synthesized [^{3}H]thymidine-labeled strands on these molecules were mostly 1 kb, although some smaller fragments (0.3 to 1 kb) were also present. Hybridization with strand-specific probes (Fig. 2B and C) indicated that the smaller fragments were H, whereas the larger could have been H or L. Two-dimensional gel electrophoresis of form D also produced a component in the 2-kb region (second dimension) that hybridized with both H- and L-strand-specific probes (Fig. 2B and C). These molecules were probably singly interlocked single-stranded parental H- and L-strand circles. The 2-kb component that comigrated with the θ_L structures and hybridized to the L-strand-specific probe (Fig. 2B) but not the H-strand-specific probe (Fig. 2C) was not related to the θ_L molecules. This component separated from θ_L molecules when the first-dimension gel buffer contained ethidium bromide (data not shown). The single-stranded parental circles that derived from the θ_L structures may not have separated from the 1-kb linear molecules because they were multiply interlocked or because one of the parental strands had a nick.

L-strand synthesis on theta structures is continuous and unidirectional. We previously showed that the 5′ end of the newly synthesized L strand is at a unique site near nucleotide 805 (11). To confirm that this location is the initiation site and to distinguish whether L-strand synthesis is discontinuous or continuous and unidirectional or bidirectional around the entire minicircle, we hybridized blots of two-dimensional gels with oligonucleotides specific for different regions of the L strand. Oligonucleotide L-777, which would hybridize near the 5′ end of a continuously synthesized L strand, recognized molecules in all regions of the diagonal in the θ region (Fig. 3A). Oligonucleotide L-381, which would hybridize near the middle of a continuously synthesized L strand, recognized molecules in the diagonal only if they were larger than about 0.5 kb (Fig. 3B). Oligonucleotides L-996 (Fig. 3C) and L-821 (not shown), which would hybridize near the 3′ end of a continuously synthesized L strand, recognized few if any of the newly synthesized L strands in the diagonal. These results indicated that molecules forming the diagonal in the θ region were a family of L strands of

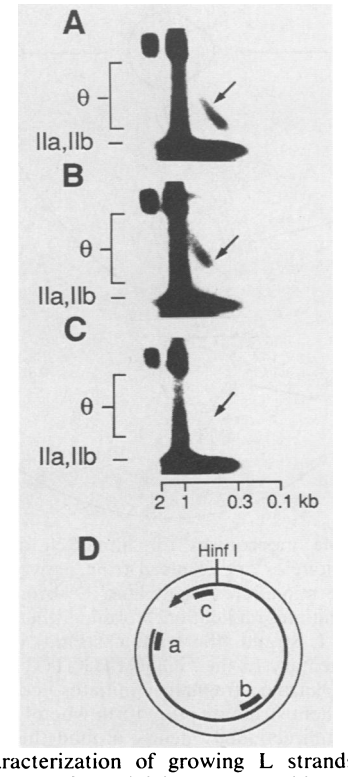

FIG. 3. Characterization of growing L strands on the theta structures. Unlabeled free minicircles were subjected to two-dimensional electrophoresis as in Fig. 1D. DNA was transferred to a nylon filter for hybridizations and autoradiography. (A) Hybridized with oligonucleotide L-777 probe; (B) same filter, hybridized with oligonucleotide L-381; (C) same filter, hybridized with oligonucleotide L-996; (D) map showing locations of the probes, all of which hybridized to the L strand (a, L-777; b, L-381; c, L-996). Designation of molecules is the same as for Fig. 1; scale represents sizes of linear markers in the second dimension.

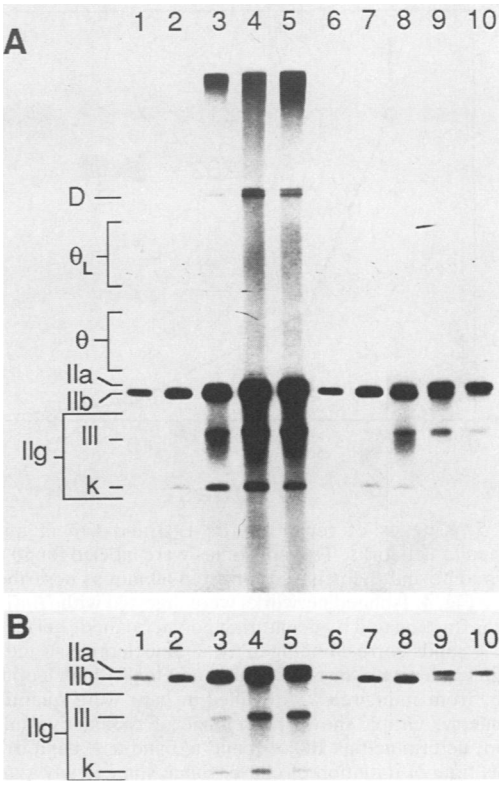

FIG. 4. Labeling of free minicircles in a pulse-chase experiment. Cells were labeled with [^{3}H]thymidine in blood (see Materials and Methods) for 5, 10, 20, 40, and 60 min (lanes 1 to 5) or for 5 and 10 min and then chased until 20, 40, and 60 min (lanes 6 to 10). Lanes 1 and 2 are the same as lanes 6 and 7. The chase was conducted by adding nonradioactive thymidine to a final concentration of 2.5 mM. Isolates free minicircles (5×10^7 cell equivalents per lane) were resolved on a 1.5% agarose gel as in Fig. 1A. (A) Fluorograph of the gel exposed for 10 days; (B) part of another fluorograph of the same gel exposed for 3 days. Designation of free minicircle species is the same as for Fig. 1.

increasing size with a common 5′ end in the region previously determined to be a site of initiation of L-strand synthesis. Therefore, L-strand synthesis initiates at a unique site and is continuous around the entire minicircle.

Behavior of theta structures in a pulse-chase experiment. To determine whether the molecules forming the θ and θ_L smears turn over rapidly, as would be expected of theta replication intermediates, we conducted a pulse-chase experiment (Fig. 4). Cells were labeled continuously with [^{3}H]thymidine for 60 min or subjected to a chase after 10 min of labeling. After one-dimensional gel electrophoresis, the free minicircles were detected by fluorography. During continuous labeling (Fig. 4, lanes 1 to 5), radioactivity in the θ and θ_L smears reached a steady-state level that constituted about 1% of the radioactivity in all of the free minicircles. As expected for an intermediate that rapidly turns over, radioactivity disappeared from these species during a chase (lanes 8 to 10). Presumably, the theta forms were converted ultimately to monomeric molecules.

This experiment also revealed the behavior of other free minicircle components during a pulse-chase. Monomeric minicircles with a newly synthesized L strand contain an 8-nucleotide gap between nucleotides 805 and 814 (10, 11). These minicircles include forms IIb and k, the latter of which is topologically knotted (11, 15). These forms were labeled efficiently early during continuous labeling (Fig. 4, lanes 1 and 2), and radioactivity from both disappeared during a chase (lanes 8 to 10). This loss in radioactivity could have resulted from reattachment of the minicircles to the network.

Monomeric molecules with a newly synthesized H strand are predominantly in the form of a smear of multiply gapped minicircles (form IIg; 13). These also disappeared rapidly during the chase (Fig. 4, lanes 8 to 10), possibly by being converted initially to nicked minicircles (form IIa). Radioactivity in the latter species increased (lane 9) and then declined (lane 10), presumably as these molecules were also reattached to the network.

Repair of the gap in the newly synthesized L strand. The free minicircles with a newly synthesized L strand, containing a gap between nucleotides 805 and 814, are eventually reattached to the network (10, 11). To evaluate the kinetics of gap repair after reattachment to the network, we conducted an in vivo pulse-chase experiment using [^{3}H]thymidine. After a 10-min labeling, the cells were chased for an additional 50 min. Isolated networks were then digested with *Hin*fI and subjected to denaturing polyacrylamide gel electrophoresis. The fragment between the *Hin*fI site and the unrepaired gap at the 3′ end of the newly synthesized strand is 202 nucleotides; filling in the gap (without sealing the nick) converts this fragment to 210 nucleotides. Complete repair of the gap (i.e., ligation of the final nick) would result in a 1-kb fragment after *Hin*fI digestion. In the control experi-

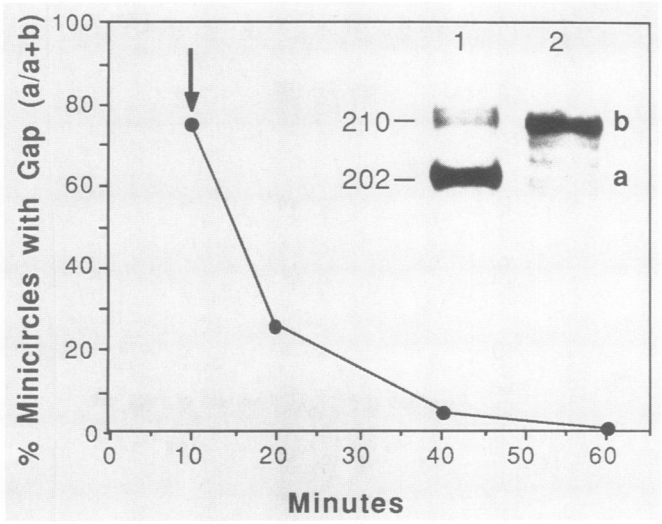

FIG. 5. Kinetics of repair of the L-strand gap in minicircles reattached to networks. Trypanosomes were labeled for 10 min with [^{3}H]thymidine and then chased for up to 60 min as described in the legend to Fig. 4. Isolated networks were digested with *Hin*fI, and the DNA was fractionated by denaturing polyacrylamide gel electrophoresis. The bands corresponding to the 202-nucleotide strand (band a, from minicircles with unrepaired gap) and the 210-nucleotide strand (band b, from minicircles with filled-in gap) were quantitated by densitometry. Graph shows percentage of radioactive minicircles with gap, determined as 100 × [band a/(band a + band b)]. Arrow indicates time of initiation of chase. Since some newly synthesized H strands contain a discontinuity at nucleotide 801 (13), some of band b could have derived from molecules with a newly synthesized H strand. There was no detectable radioactivity in a 1-kb *Hin*fI fragment of network DNA, indicating that no newly synthesized H or L strand was covalently closed during the 60-min experiment. Inset shows a control fluorograph in the region of a gel with the 202-nucleotide strand (band a) and the 210-nucleotide strand (band b); lane 1 shows strands from free minicircles labeled continuously for 40 min, and lane 2 shows strands from networks from the same cells.

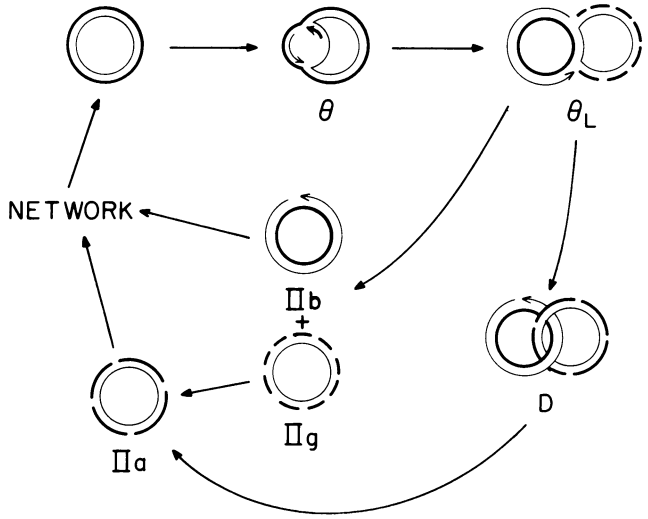

FIG. 6. Possible mechanism for minicircle replication in *T. equiperdum*. Minicircles are released from network to form covalently closed free minicircles (light line, L strand; heavy line, H strand). These initiate replication, forming theta structures (θ). Synthesis of the L strand (the leading strand) initiates by RNA priming complementary to the GGGGTTGGTGTA sequence. The first H-strand Okazaki fragment initiates complementary to ACGCCC. Late theta structures (θ_L) form when L-strand synthesis has proceeded unidirectionally nearly around the entire template; these probably contain a full complement of H-strand Okazaki fragments. Segregation then produces form IIb molecules (with a newly synthesized L strand and a gap opposite the GGGGTTGGT GTA sequence; 10, 11) and form IIg molecules (with the newly synthesized H strand in short fragments separated by small gaps; 13). Some of the form IIb molecules are knotted during or after segregation (15). The dimeric species, form D, may not be on the main segregation pathway, since its H-strand fragments are on average longer than those found in form IIg (unpublished experiments). Gap filling and partial ligation result in conversion of form IIg molecules to form IIa. The form IIa and IIb molecules are reattached to the network, where they eventually undergo covalent closure.

ment (Fig. 5, inset), free minicircles labeled continuously for 40 min had most of the radioactivity in the 202-nucleotide fragment and very little in the 210-nucleotide fragment. In contrast, network minicircles from the same cells had most of the radioactivity in the 210-nucleotide fragment (minor bands between 202 and 210 nucleotides probably derived from molecules in which the gap was partially repaired). The graph in Fig. 5 shows the kinetics of gap repair on minicircles reattached to the network either before or during the chase. As judged from the amount of radioactivity in the 202- and 210-nucleotide fragments, half of the molecules had their gaps repaired in about 15 min, and all were repaired at 60 min. However, there was no radioactivity detected in 1-kb minicircle *Hin*fI fragments (not shown); therefore, no radioactive network minicircles were covalently closed during the 60-min experiment.

DISCUSSION

These results, together with those of previous studies, clarify the mechanism of synthesis of the minicircle in *T. equiperdum*. Figure 6 shows a model of our current view of *T. equiperdum* minicircle replication. L-strand synthesis initiates by RNA priming complementary to the sequence GGGGTTGGTGTA (10, 11). This sequence is conserved in minicircles from all trypanosomatid species that have been examined, presumably because it is part of the replication origin. About two ribonucleotides are detectable on the L-strand 5′ end; since the 5′ ribonucleotide has a 5′ phosphate rather than a 5′ triphosphate, the primer is probably processed soon after synthesis (11). After initiation, L-strand synthesis proceeds continuously and unidirectionally around the entire minicircle (Fig. 3).

Elongation of the minicircle L strand in *T. equiperdum* occurs on theta structures. Two-dimensional agarose gel electrophoresis of these molecules (Fig. 1 and 2) indicates that the growing L strands are predominantly in the size range of 0.3 to nearly 1 kb. Smaller L strands could have been undetectable by the blotting procedures used, or they could have been lost by branch migration. Alternatively, if synthesis of the first 300 nucleotides of the L strand was rapid, the steady-state concentration of the earliest theta structures could have been too low to detect on the gel. The diagonal of newly synthesized L strands (Fig. 1B and D) asymptotically approaches the zone of nicked or relaxed minicircles rather than covalently closed minicircles. This distribution could imply that the theta structures are nicked or relaxed rather than supercoiled, as is found in many other replication systems.

Surprisingly, it was difficult to detect newly synthesized H strands on theta structures (Fig. 2). Recovery could have

been low because of the small size of these fragments. Nevertheless, use of oligonucleotide probes H-720 and H-796 clearly showed hybridization to theta-structure H-strand fragments in the 0.1-kb size range. In previous studies, H-796 was shown to hybridize to a 73-mer, a molecule with a 5′ end at nucleotide 727 (complementary to an ACGCCC sequence) and with a 3′ end at nucleotide 800 (within the GGGGTTGGTGTA sequence) (13). As we noted previously, the ACGCCC sequence is also conserved in minicircles in all species examined (14). On the basis of its position on the minicircle sequence, the 73-mer could be the first Okazaki fragment synthesized. Probe H-720 hybridizes to a 83-mer or a 138-mer, two overlapping fragments that map in a position expected for the second Okazaki fragment (13). Thus, at least the first two Okazaki fragments are synthesized on a theta structure. We do not know why we could not detect H-strand fragments from other regions of the minicircle on the theta structure; perhaps their synthesis is delayed.

We have not observed *T. equiperdum* theta structures by electron microscopy. However, these intermediates have been observed previously in both *C. fasciculata* (6) and *Trypanosoma cruzi* (4).

The θ smear on two-dimensional gels merges with a second smear, θ_L, which could contain late theta structures. In the θ_L molecules, a newly synthesized strand, presumably L, is approximately 1 kb, indicating that synthesis is virtually complete. Interestingly, the diagonal of newly synthesized L strands in the θ region does not fuse with the smear of 1-kb molecules in θ_L (shown most clearly in Fig. 2A); therefore, there may be a delay in the conversion of θ molecules to θ_L molecules. Newly synthesized H strands, ranging in size from 0.1 to 0.3 kb, are more easily detectable than on θ molecules; possibly their slightly larger size accounts for the higher recovery (Fig. 2C). Hybridization with oligonucleotide probes indicates that these H strands derive from several regions of the molecule. Pulse-labeled theta structures and late theta structures disappear during a chase, presumably because they segregate into monomeric products; topologically interlocked minicircle dimers (form D) could also be a segregation intermediate.

The progeny minicircles with a newly synthesized L strand have an 8-nucleotide gap, indicating that L-strand synthesis stops just before elongation is complete (10, 11). A small fraction of the progeny minicircles with a newly synthesized L strand are topologically knotted; the origin of the knots is unknown, but knotting could occur during segregation of late theta structures (15). The progeny minicircles with a newly synthesized H strand contain multiple H-strand fragments, ranging in size from 73 nucleotides to nearly 1 kb; the larger fragments may derive from ligation of smaller Okazaki fragments (13). Partial repair of molecules with a newly synthesized H strand may result in their conversion to nicked free minicircles (13).

Ultimately, all of the progeny free minicircles are reattached to the network. At least 75% of the minicircles with a newly synthesized L strand still contain the 8-nucleotide gap when reattached (Fig. 5), and some of these strands still have one or two ribonucleotides at the 5′ end (11). These gaps are filled in, half within about 15 min and virtually all by 60 min. However, there is no detectable ligation of the final L-strand nick within 60 min. If *T. equiperdum* minicircle replication resembles that in *C. fasciculata* (3, 5, 8), final closure may occur at the time of division of the double-size network into two progeny networks.

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