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Replication of DNA Minicircles in Kinetoplasts Isolated from *Crithidia fasciculata*: Structure of Nascent Minicircles

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We have previously described an isolated kinetoplast system from *Crithidia fasciculata* capable of ATP-dependent replication of kinetoplast DNA minicircles (L. Birkenmeyer and D. S. Ray, J. Biol. Chem. 261: 2362–2368, 1986). We present here the identification of two new minicircle species observed in short pulse-labeling experiments in this system. The earliest labeled minicircle species (component A) contains both nascent H and L strands and is heterogeneous in sedimentation and electrophoretic migration. Component A has characteristics consistent with a Cairns-type structure in which the L strand is the leading strand and the H strand is the lagging strand. The other new species (component B) has a nascent 2.5-kilobase linear L strand with a single discontinuity that mapped to either of two alternative origins located 180° apart on the minicircle map. Component B could be repaired to a covalently closed form by *Escherichia coli* polymerase I and T4 ligase but not by T4 polymerase and T4 ligase. Even though component B has a single gap in one strand, it had an electrophoretic mobility on an agarose gel (minus ethidium bromide) similar to that of a supercoiled circle with three supertwists. Treatment of component B with topoisomerase II converted it to a form that comigrated with a nicked open circular form (replicative form II). These results indicate that component B is a knotted topoisomer of a kinetoplast DNA minicircle with a single gap in the L strand.

Trypanosomes are flagellated protozoa with a single multilobed mitochondrion per cell. The mitochondrial DNA of these organisms consists of two types of circular molecules termed maxicircles and minicircles. The maxicircles, present at 20 to 40 copies per cell, are homogeneous within a single organism and contain mitochondrial structural genes analogous to those found in other organisms. Minicircles, present at 5,000 to 10,000 copies per cell, are essentially high-copy-number mitochondrial plasmids with no known function. The minicircles are nearly homogeneous in size in most species but may have extensive sequence heterogeneity. The entire complement of maxicircles and minicircles is usually contained in an extensively catenated network which is highly condensed in vivo. This condensed DNA network is localized near the base of the flagellum in a disk-shaped body termed the kinetoplast. The network DNA is referred to as kinetoplast DNA (kDNA). For recent reviews on kDNA, see references 7, 15, 16a, 17, and 18).

Unlike mitochondrial DNA of mammalian cells, kDNA appears to replicate coordinately with the nuclear S phase. Minicircles are released from the network before their replication as free molecules. In exponentially growing cells of the trypanosomatid *Crithidia fasciculata*, about 0.4% of the minicircles are free and the rest are network associated (6). Replication of the minicircles free of the network has permitted analyses of replication intermediates from in vivo sources and isolated kinetoplasts (1, 3, 6, 9, 10, 12–14).

Studies of the free replication intermediates have shown that the two daughter molecules that result from the replication of an individual minicircle have distinctly different structures (1, 3, 9, 10). The daughter molecule, with a newly synthesized L strand, has a single gap of 6 to 10 nucleotides in the nascent L strand. The sequence homogeneity of minicircles from *Trypanosoma equiperdum* and *C. fasciculata* Cf-C1 has permitted the mapping of a small gap at the origin of replication of one of the two strands of the minicircle (1, 3, 12). In both species, the gap overlaps a 12-nucleotide universal minicircle sequence (UMS) found in all minicircles sequenced to date. Evidence for the presence of a few ribonucleotides at the 5′ terminus within the gap suggests that this L strand is initiated by an RNA-priming mechanism and that the UMS may play a role in the initiation of one of the minicircle strands (3, 14). In *T. equiperdum* there is a single copy of the UMS, and the single gap therefore maps to a unique site (12). *C. fasciculata* contains two UMSs located within direct repeat sequences of about 170 base pairs on opposite sides of the minicircle (20). In minicircles replicated in isolated kinetoplasts from *C. fasciculata*, the newly synthesized L strand also contains only a single discontinuity which can be located at either UMS with about equal frequency (1, 3).

Minicircles containing nascent H strands are highly gapped and contain H-strand fragments about 40 to 90 nucleotides long (1, 10). The repair of the gaps in the H strand is initiated while the minicircles are free but it not completed until after reattachment to the network. In the course of maturation of the H strand in *C. fasciculata*, a half-length fragment has been observed (1). The termini of the two half-length H strands in such daughter molecules are located within the direct repeat sequence regions located 180° apart on the minicircle and about 100 base pairs away from the respective UMSs at the L-strand origins. The resistance to joining of these specific H-strand termini suggests that processing of these termini is required before their covalent closure.

Although a D-loop model of *Crithidia* minicircle replication similar to that proposed by Clayton (4) for dimeric mitochondrial DNA has been proposed (3), the early replication intermediates that give rise to the nicked and gapped daughter progeny have not previously been isolated and

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characterized. Such intermediates are present in cells in very small amounts and are therefore difficult to obtain in quantities sufficient to allow their detailed characterization. The ability to label minicircle DNA at much higher specific activity with labeled nucleotides in the isolated kinetoplast system has permitted us to overcome this limitation. We describe here the characterization of the minicircle species labeled in short pulse-labeling experiments and describe a novel replication product. This molecule appears to be a singly gapped knotted circle which can be unknotted by a type II topoisomerase.

MATERIALS AND METHODS

Materials. Brain heart infusion medium was purchased from Difco Laboratories. ATP, hemin, and streptomycin sulfate were from the Sigma Chemical Co. Unlabeled deoxynucleoside triphosphates were obtained from Pharmacia, Inc. Labeled [α-^{32}P]dTTP and [α-^{32}P]dATP were from New England Nuclear Corp. The enzymes used included T4 polymerase, T4 ligase, *E. coli* polymerase I, *Sac*II, and *Xho*I from Promega Biotec. *Mlu*I was obtained from Toyobo Co., Ltd. Topoisomerase II is a homogeneous kinetoplast type II topoisomerase from *C. fasciculata* (Melendy and Ray, manuscript in preparation). DNA molecular weight markers (1-kilobase [kb] ladder) were purchased from Bethesda Research Laboratories, Inc.

Cell growth. *C. fasciculata* Cf-C1 cells were cultured in Difco brain heart infusion medium supplemented with 10 μg of hemin per ml and 100 μg of streptomycin sulfate per ml at 28°C with shaking.

kDNA synthesis reaction. Kinetoplasts from *C. fasciculata* Cf-C1 cells were isolated and incubated in a DNA synthesis reaction for various periods of time at 30°C. Unless stated otherwise, all kinetoplast-labeling reactions and sucrose gradient sedimentation of reaction products were performed as described by Birkenmeyer and Ray (1). The following changes in the stated protocol were made. Pretreatment of the cells with 43.5 mM Tris and 43.5 mM EDTA was omitted, and 12.5 μM dTTP or dATP labeled with ^{32}P to 40,000 cpm/pmol was used in the synthesis reaction. The experiments shown in Fig. 1 and 3 to 7 were performed with minicircle DNA isolated in the absence of pronase digestion and ethanol precipitation. Instead, the DNA was isolated by digestion of the labeled kinetoplasts with 10 μg of RNase A per ml in 1% Sarkosyl N97 for 30 min at 37°C and concentrated after sucrose gradient sedimentation by using 5,000-molecular-weight cutoff dialysis tubing buried in solid polyethylene glycol 20,000 at 4°C.

Gradient purification of components B and B′. Pooled sucrose gradient fractions containing components B and B′ from 6 min of pulse-labeling with [α-^{32}P]dATP was sedimented through a second 5 to 20% linear sucrose gradient in 10 mM Tris (pH 7.6)–1 mM EDTA–1 M NaCl at 40,000 rpm in an SW41 rotor for 13 h at 4°C. Selected fractions were again pooled, concentrated, dialyzed versus 50 mM Tris (pH 7.5)–1 mM EDTA–50 mM NaCl–0.1% Brij 58, and sedimented through a third 5 to 20% linear sucrose gradient, this time in the presence of 100 μg of ethidium bromide (EtBr) per ml at 54,000 rpm in an SW60 rotor for 6 h at 4°C. Fractions from this gradient were pooled, extracted five times with water-saturated butanol, concentrated, and dialyzed as described above, and two pools were used as the purified components B (90% radiochemically pure) and B′ (15% radiochemically pure).

Neutral-alkaline two-dimensional electrophoresis. A DNA sample was electrophoresed in 0.089 M Tris–0.089 M boric acid–0.2 mM EDTA (TBE) through a neutral 0.8% agarose gel with or without 0.5 μg of EtBr per ml. The gel was then photographed, and marker lanes and first-dimension wells were removed. The gel was then equilibrated in alkaline running buffer (30 mM NaOH, 1 mM EDTA) for 1 h. Marker samples for the second dimension were ethanol precipitated and suspended in alkaline loading buffer (50 mM NaOH, 1 mM EDTA, 5% glycerol, 0.025% bromocresol green). The gel was rotated 90°, markers were loaded into preformed and aligned wells and electrophoresed at up to 5 V/cm with buffer recirculation. The gel was then soaked in 1 M Tris (pH 7.5)–0.15 M NaCl for 30 min, stained in 0.5 μg of EtBr per ml for 15 min, destained in distilled water for 45 min, and photographed. The gel was fixed in 7% trichloroacetic acid for 15 min, and blotted with Whatman 3MM paper backed with paper towels to reduce the gel thickness, and then vacuum dried before autoradiographic exposure on Kodak X-ray film at −70°C with a single Quanta III intensifying screen for 16 to 24 h.

Enzymatic reactions. Unless stated otherwise, all reactions were treated as follows. Each reaction contained 1,000 to 5,000 cpm (0.01 to 0.2 μg of DNA) in a reaction volume of 20 μl. The assay conditions for the enzymes used were those recommended by the manufacturer. The times and temperatures of the various reactions were as follows: *Mlu*I, *Sac*II, and *Xho*I, 37°C for 2 h; T4 polymerase, 37°C for 30 min; polymerase I, 16°C for 30 min; T4 ligase, 16°C for 2 h; DNase I, 37°C for 5 min; S1 nuclease, 37°C for 15 min; topoisomerase II, 30°C for 30 min. One nanogram of *Crithidia* topoisomerase II is sufficient to decatenate 50% of the minicircles from 0.2 μg of kDNA networks (Melendy and Ray, manuscript in preparation). After incubation, the reactions were quenched on ice and a 1/10 volume of 10× stop dye (100 mM Tris, 50% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol, 150 mM EDTA) was added. These samples were loaded directly onto 0.6% agarose slab gels with or without 0.5 μg of EtBr and electrophoresed for 12 h at 35 V. The gels were dried and autoradiographed as described above.

Solution hybridization. Gel-purified or gradient-purified ^{32}P-labeled intermediate components (5,000 to 20,000 cpm in 0.01 to 0.2 μg of DNA) were diluted to 1 ml with TE and boiled for 10 min along with a 100-fold excess of purified H- or L-strand DNA. Minicircle H and L strands were purified from M13 bacteriophage clones containing minicircle DNA as previously described (2). NaCl was added to 0.3 M, and reannealing was allowed to proceed for 30 min at 70°C, 30 min at 50°C, and 30 min at 30°C. The solution was made to be 1× S1 nuclease buffer (50 mM sodium acetate [pH 4.6], 4.5 mM $ZnSO_4$, 0.3 M NaCl, 10 μg of single-strand DNA per ml), and S1 nuclease was added to 35 U/ml. The DNA was digested for 15 min at 30°C and then quenched on ice after the addition of EDTA to 10 mM. Trichloroacetic acid was added to 10% along with 3 μg of carrier tRNA, and the DNA was precipitated for 1 h on ice before being filtered through GF/A filters and counted in a liquid scintillation counter.

RESULTS

Pulse-labeling of free minicircles. To identify potential early intermediates in minicircle replication, we analyzed free minicircles isolated by sucrose gradient sedimentation after pulse-labeling of isolated kinetoplasts with [α-^{32}P] dATP. Neutral gel electrophoresis of individual fractions of the free minicircles from 2.5 min of pulse-labeling (Fig. 1) revealed the presence of a highly labeled heterogeneous

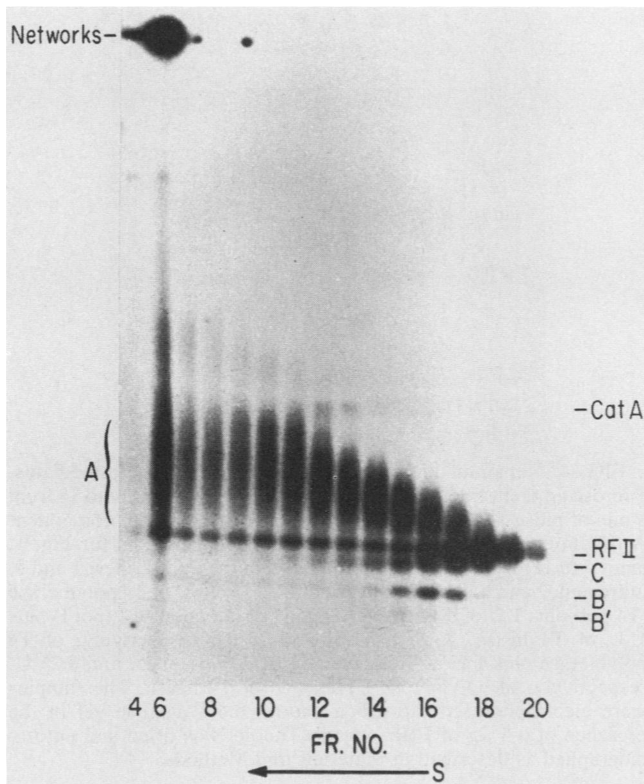

FIG. 1. Pulse-labeling of free minicircles in isolated kinetoplasts. Isolated kinetoplasts were labeled with [α-^{32}P]dATP for 2.5 min. Free minicircles were isolated and sedimented through sucrose gradients as described in Materials and Methods. Twenty-five microliters of each minicircle-containing fraction (1.1 ml each) was electrophoresed on a 0.6% neutral agarose gel. The gel was dried, and the pulse-labeled minicircles were detected by autoradiography. Sucrose gradient fraction numbers (FR. NO.) and the direction of sedimentation are shown beneath the autoradiogram.

species (component A) not seen in longer labelings. Component A sedimented throughout the gradient and migrated slower than nicked or gapped circles (replicative form II [RFII]). The faster-sedimenting molecules of component A migrated the slowest on the agarose gel, consistent with a monotonic increase in mass of the molecules in this population. The large amount of labeled material that remained in the well in fraction 6 was due to network DNA on the dense cesium chloride shelf. A species (component C) that migrated just ahead of RFII corresponds to the highly gapped molecules identified previously in longer-term labeling experiments both in isolated kinetoplasts (1) and in in vivo experiments in Englund's laboratory (9, 10). Two additional components (B and B') were apparent only in these shorter pulse-labeling experiments. Component B sedimented 1 to 2 fractions ahead of the peak of RFII, and B' sedimented about 2 to 3 fractions faster. These components also appeared to be homogeneous in their electrophoretic migration. It should be noted that covalently closed minicircles are normally relaxed and comigrate with RFII in the absence of EtBr. The species labeled catA has been identified as an interlocked dimer of nicked minicircles (data not shown).

Nascent-chain length determination. Two-dimensional agarose gel electrophoresis was used to determine the size(s) of the pulse-labeled nascent strand(s) of each of the minicircle species present in pooled gradient fractions. Pooled fractions

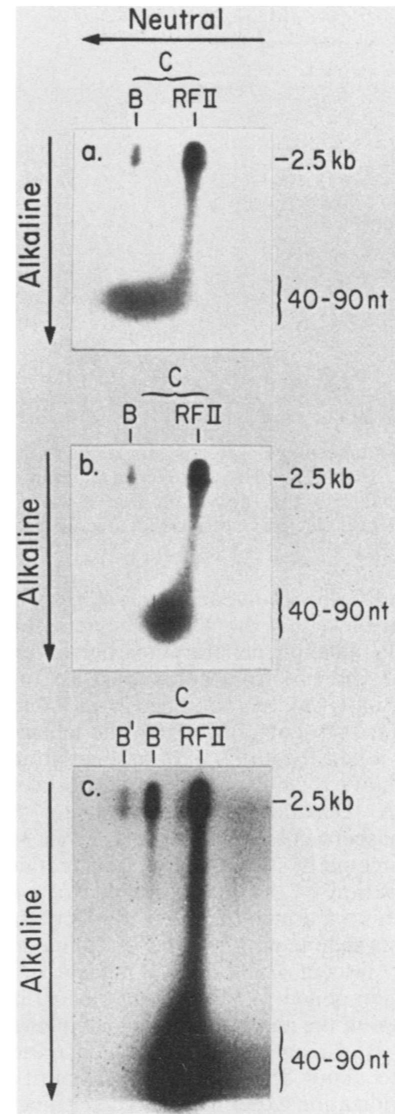

FIG. 2. Two-dimensional gel analysis of nascent chains from components B, B', C, and RFII. Pooled free minicircle intermediates (fractions 14 to 18 [Fig. 1]) from a 6-min pulse of [α-^{32}P]dTTP were electrophoresed on two-dimensional agarose gels. The gels were 0.8% agarose and were electrophoresed either with (a) or without (b and c) 0.5 μg of EtBr per ml in the first dimension. Panel c shows 6-min [α-^{32}P]dATP pulse-labeled intermediates in which component B' was also detected. The gels were processed and electrophoresed in the second dimension as described in Materials and Methods. nt, Nucleotides.

that contained RFII and components B and C were electrophoresed on neutral 0.8% agarose gels in the first dimension in the presence (Fig. 2a) or absence (Fig. 2b and c) of EtBr. The gels were then equilibrated in alkaline buffer, rotated 90°, and electrophoresed in a second dimension. The nascent strands released from component C were about 40 to 90 nucleotides long (Fig. 2a), whereas those released from component B were linear full-length nascent strands of 2.5 kb. The strands released from RFII were mostly unit length (2.5 kb), although the streak of labeled material between the 40- to 90-nucleotide fragments and the unit-length fragments reflects the presence of circular molecules in which the nascent strands were joined to various degrees. Since com-

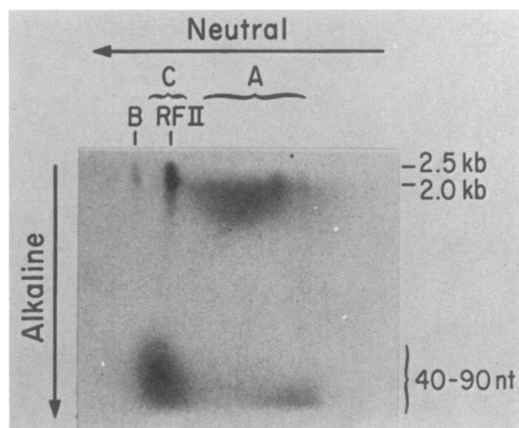

FIG. 3. Two-dimensional gel analysis of nascent chains from component A. Fractions 10 to 14 from a 2.5-min pulse-labeling experiment similar to that shown in Fig. 1 were analyzed by two-dimensional gel electrophoresis as for Fig. 2b. nt, Nucleotides.

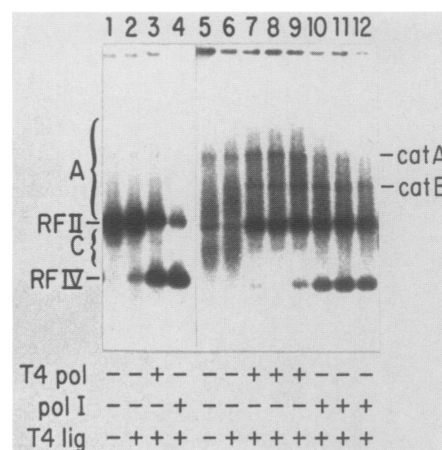

FIG. 4. Repair of nicks and gaps in replication intermediates. Sucrose gradient fractions enriched for RFII (fractions 14 to 18 from 6 min of pulse-labeling as for Fig. 2; lanes 1 to 4) or for component A (fractions 10 to 14 from 2.5 min of pulse-labeling as for Fig. 1; lanes 5 to 12) were repaired as described in the text. Lanes: 1 and 5, untreated; 2 and 6, 1 U of T4 ligase (T4 lig); 3, 15 U of T4 polymerase (T4 pol) plus 1 U of T4 ligase; 4, 0.25 U of polymerase I (pol I) plus 1 U of T4 ligase; 7 to 9, 1, 3, and 15 U, respectively, of T4 polymerase plus 1 U of T4 ligase; 10 to 12, 0.01, 0.05, and 0.25 U, respectively, of polymerase I plus 1 U of T4 ligase. The samples were electrophoresed through a neutral 0.6% agarose gel in the presence of 0.5 μg of EtBr per ml. The gel was dried and autoradiographed as described in Materials and Methods.

ponents B and C are resolved better in the absence of EtBr (Fig. 2b), it is clear that the 40- to 90-nucleotide fragments released upon alkaline denaturation derive entirely from component C and not from component B. In faster-sedimenting fractions, another homogeneous component (B′) migrated faster than component b in the neutral dimension and also had a unit length (2.5-kb) nascent strand (Fig. 2c).

Figure 3 shows a similar analysis of a pool enriched for component A. Denaturation of component A yielded a majority of nascent chains that were 1.5 to 2.3 kb long but also a small amount of strands 40 to 90 nucleotides long. The length distribution of the longer strands of component A observed in this experiment appears to reflect the pooling of only the faster-sedimenting species of component A, since even shorter nascent strands were present in the slower-sedimenting component A molecules detected in Fig. 2c.

Strandedness of the nascent strand of components B and C. Components B, C, and RFII were each enriched by gel purification to about 85% radiochemical purity for use in solution hybridization experiments to determine the identity of the nascent strand of each component. The radioactive labeling of both components B and C was asymmetric; 83% of the label in component B hybridized to H strands, and 17% hybridized to L strands. In contrast, 85% of the label in component C hybridized to L strands and 15% hybridized to H strands. The radioactivity in RFII hybridized about equally to each strand (55% hybridized to H strands and 45% hybridized to L strands). Thus, within the limits of the purity of each component, the 2.5-kb nascent strand of component B was identified as the L strand, while the nascent small fragments of component C were identified as exclusively H strands.

Repair of RFII and component A. Repair reactions with T4 ligase and either T4 polymerase or polymerase I were done on pools of intermediates enriched for either RFII or component A. More than 60% of RFII DNA could be covalently closed to yield relaxed covalently closed circles upon treatment with T4 ligase plus T4 polymerase or polymerase I (Fig. 4, lanes 1 to 4). (Relaxed covalently closed circles [RFIV] were the most rapidly migrating species in the presence of EtBr.) Lanes 5 to 12 show a titration of these two polymerases on component A with a fixed amount of T4 ligase. Component A was inefficiently repaired into catenated forms (catA and catB) by T4 polymerase and T4 ligase (lanes 7 to 9) and by polymerase I and T4 ligase (lanes 10 to 12). CatB is an interlocked dimer in which one minicircle is a covalently closed circle and the other is a nicked circle (data not shown). Enzymatic characterization of components B and C will be presented in a later figure.

Mapping the discontinuities of component B. Component B (90% radiochemically pure) was digested separately with three restriction enzymes (*Sst*II, *Mlu*I, and *Xho*I) that each cut *Crithidia* minicircles at only a single site and then electrophoresed on a 1% alkaline agarose gel. The autoradiograph of this gel is shown in Fig. 5. Before digestion, the label in the nascent L strand of component B was present in 2.5-kb linear strands, reflecting the presence of a single nick or gap in each molecule. Since four specific fragments were produced upon digestion with each restriction enzyme, there must be two specific sites at which a discontinuity can occur in component B. Further, each set of four fragments consisted of two pairs, each of which summed to 2.5 kb. Figure 6 shows a map of the H- and L-strand-specific discontinuities and their nucleotide locations. Also shown are the locations of the restriction sites for the enzymes used. These three sets of two pairs of fragments can result only from specific discontinuities that map to the previously described L-strand origins that overlap the UMS on opposite sides of the minicircle (3, 20). Although these results demonstrate that component B has a single nick or gap in the L strand at either of two alternative sites, these molecules migrate faster on an agarose gel than does a nicked or gapped RFII molecule. The anomalous migration of these singly nicked or gapped minicircles suggests an unusual structure for component B.

Enzymatic characterization of purified components B and B′. A pool enriched for components B and B′ was treated with T4 ligase alone or in combination with either T4 polymerase or polymerase I. The pool was also treated with DNase I, S1 nuclease, and topoisomerase II. In the absence of EtBr, component B approximately comigrated with a

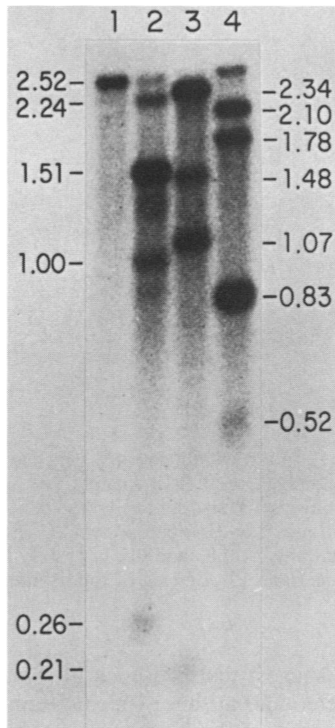

FIG. 5. Mapping of the discontinuities of component B. Component B was purified by resedimentation on sucrose gradients as described in Materials and Methods. Purified component B was digested with 8 U of *Sac*II (lane 2), *Mlu*I (lane 3), or *Xho*I (lane 4). Mock-treated, purified component B was electrophoresed in lane 1, and unlabeled marker DNAs were electrophoresed in adjacent lanes. After digestion, the samples were phenol-chloroform extracted, ethanol precipitated, and suspended in 20 μl of alkaline loading buffer. The samples were electrophoresed and autoradiographed as for Fig. 3 but only in the alkaline dimension. The sizes of the fragments are indicated on both sides of the gel.

covalently closed topoisomer of the 2.5-kb minicircle with three supertwists (Fig. 7a, lane 3). Under these conditions, RFII and RFIV comigrated while individual topoisomers of the covalently closed form migrated ahead of these species (lane 2). B′ migrated at a position of a minicircle with four to five supertwists, consistent with its being a knotted molecule with five nodes (11). RFIV and its covalently closed topoisomers all comigrated in the presence of EtBr (Fig. 7b) and were widely separated from RFII molecules (lane 5). Under these conditions, the migration rates of components C, B, and B′ were similar to those observed in the absence of EtBr (compare lanes 3 and 6), as expected for nicked or gapped circles. The inability of T4 ligase alone (lane 7) to close the single discontinuity in either B or B′ is consistent with the presence of a small gap rather than a nick in these molecules. Lanes 8 to 10 show that components B and B′ could be repaired to a covalently closed circle by polymerase I and T4 ligase but not by T4 polymerase and T4 ligase. Component C was efficiently repaired to RFII with T4 polymerase and T4 ligase, but only about 50% was converted to a covalently closed form. Components B, B′, and C were unaffected by DNase I nicking at a level sufficient to completely nick unlabeled RFIV molecules included in the sample (lane 11). Component A was also unaffected by DNase I nicking under these conditions (data not shown). Component C appears to have gaps between the small nascent fragments on the basis of the very high sensitivity of component C to S1 nuclease (lane 12). Under these conditions, about 50% of RFII and components B and B′ were converted to RFIII (lane 12). RFII and RFIII were not resolved under these gel conditions, which were optimized for separation of the intermediate species. However, the RFII and RFIII designations given were shown in all cases under different gel conditions. Finally, comparison of lanes 6 and 13 shows that B and B′ are knotted forms (topoisomers) of RFII on the basis of their conversion to RFII by topoisomerase II. Higher-order knots were also observed in very low abundance (data not shown).

DISCUSSION

The results presented here provide additional support for the minicircle replication model described previously (3) and identify two new minicircle species observed only in short pulse-labeling experiments. Component A is the most abundant species observed in short pulses with labeled dATP. The size distribution of single strands released from component A upon alkaline denaturation shows the presence of nascent chains of various lengths, ranging in size up to nearly unit length, and a small fraction of nascent chains of 40 to 90 nucleotides long. The observed labeling pattern and heterogeneity of component A suggest that these molecules represent partially replicated minicircles. In vitro repair of component A provides further support for its identification as a partially replicated minicircle. Repair of a small proportion of component A could be completed in vitro by T4 polymerase (or polymerase I) and T4 ligase to yield catenated minicircles. In the absence of a DNA topoisomerase, these catenated species were not resolved into individual daughter molecules.

The unusual structures of components B and B′ were unexpected. These species were both shown to be circular molecules with a single small gap in the L strand, and yet they migrated significantly faster than RFII on agarose gels. On agarose gels lacking EtBr, components B and B′ approximately comigrated with covalently closed minicircles with three and five supertwists, respectively, consistent with knotted molecules with three and five nodes, respectively (11). This property is similar to that seen in knotted trefoil DNA produced by topoisomerase II treatment of circular

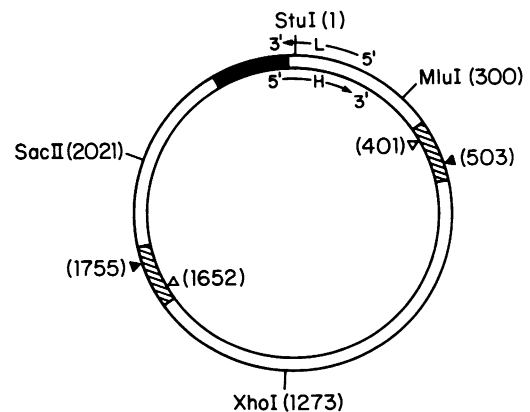

FIG. 6. Minicircle restriction map. Map showing the restriction enzymes used, with the nucleotide location given in parentheses. The *Stu*I restriction site is defined as position 1. The filled triangles represent the sites of the L-strand gaps, and the open triangles represent the sites of the H-strand nicks. The filled box designates the bent helix (16), and the cross-hatched boxes represent the conserved origin regions.

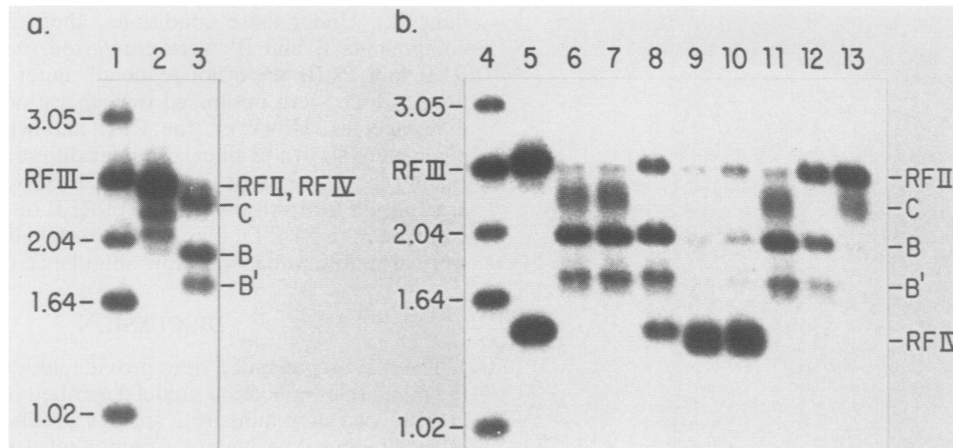

FIG. 7. Enzymatic characterization of purified components B and B′. Components B and B′ were partially purified as described in Materials and Methods. Samples were electrophoresed on neutral 0.6% agarose gels in the absence of EtBr (panel a) or in the presence of 0.5 μg of EtBr per ml (panel b). The lanes contained the labeled Bethesda Research Laboratories 1-kb ladder (only the 1.02-, 1.64-, 2.04-, and 3.05-kb fragments are shown) and labeled RFIII (lanes 1 and 4), labeled RFII and RFIV (lanes 2 and 5), components C, B, and B′ (lanes 3 and 6) treated with 1 U of T4 ligase (lane 7), 10 U of T4 polymerase and 1 U of T4 ligase (lane 8), 0.03 and 0.1 U of polymerase I and 1 U of T4 ligase (lanes 9 and 10, respectively), 5 ng of DNase I (lane 11), 0.02 U of S1 nuclease (lane 12), or 2 ng of topoisomerase II (lane 13).

DNA molecules (8). The conversion of components B and B′ to RFII upon treatment with a *C. fasciculata* type II topoisomerase indicates that these gapped molecules have a knotted structure. Knotted molecules have been seen previously as the products of topoisomerase I action on nicked circular DNA (5) or as the products of topoisomerase II action on covalently closed or nicked circular DNA under conditions of a high enzyme-to-DNA ratio (8). They have also been seen as a consequence of the action of phage lambda Int protein on supercoiled and nicked circular molecules (19). The knotted minicircles observed here may have resulted from the resolution of late Cairns-type structures with linking numbers of five or more. We also observed knotted minicircles in unreacted kinetoplasts (unpublished data), suggesting that these unusual structures also occur in vivo.

Component B contains discontinuities at specific sites identical to those observed in long-term labeling of RFII molecules with a nascent L strand, suggesting that component B is resolved into a singly gapped RFII molecule before reattachment to the network. Partial degradation of pulse-labeled networks with a restriction enzyme or by sonication yields labeled RFII and catenated species but no detectable B or B′ (unpublished data).

The conversion of components B and B′ to covalently closed molecules by DNA polymerase I and T4 ligase but not by T4 polymerase and T4 ligase or T4 ligase alone indicates that the gaps at the alternate L-strand origins are not terminated by either a 5′ phosphoryl ribo- or deoxyribonucleotide. Whatever structures exist at the 5′ termini of these gapped molecules, they can be removed by the potent 5′ exonuclease activity of polymerase I, leading to a structure that can then be covalently sealed by T4 ligase. Longer-term labeling experiments have yielded RFII molecules with ligatable nicks, consistent with the notion that limited gap filling and nick translation occur in isolated kinetoplasts (3).

The gaps in component C can be repaired to RFII by T4 polymerase, but efficient closure to RFIV requires the action of *E. coli* DNA polymerase I in addition to T4 ligase. This result suggests that some type of excision or unblocking is also involved in the conversion of component C to RFII. Earlier studies suggest that presence of a modified nucleotide (or ribonucleotide) at these specific termini (3).

A common feature of the 5′ termini of components B and C and RFII molecules is a requirement for excision of terminal nucleotides to ligate the termini efficiently. All minicircles are replicated and reattached to the network before repair of the specific discontinuities concomitant with the division of the kDNA network. The excision of these 5′ termini may represent a feature of the control mechanism that regulates network duplication.

A model of minicircle replication consistent with all of these data is presented in Fig. 8. This model is similar to the model proposed previously on the basis of the structures of the RFII replication products (3). Initiation begins at either of the alternate L-strand origins; only one of the possibilities is shown here. Elongation of the L strand continues, displacing the parental L strand. The H strand is initiated specifically within the now single-stranded origin region, and these specific initiations differ from those of the Okazaki-like fragments in their inability to be joined. At some point, when the nascent L strand nears full length and the nascent H strand is still highly gapped, component A is resolved into components B (or B′) and C. The gaps in component C are filled in, and the fragments are rapidly joined to give 1.25-kb nascent strands. Gap filling appears to be quite rapid on the basis of the very small amount of label present in fragments intermediate in size between 100 and 1,250 nucleotides.

Although the model shown here implies that component B is an essential intermediate in minicircle replication, this feature of the model remains to be tested. However, two lines of evidence suggest that the knotted circles are not simply in equilibrium with the pool of RFII molecules. (i) Component B was observed only in relatively large amounts in short pulses, and (ii) only molecules with a newly synthesized L strand were knotted. Alternatively, most of the daughter molecules with a newly synthesized L strand might be produced directly as unknotted RFII molecules, with only some of these replication products having a knotted structure. Structural studies of the late replication intermediates and the determination of the mechanism of segregation of the

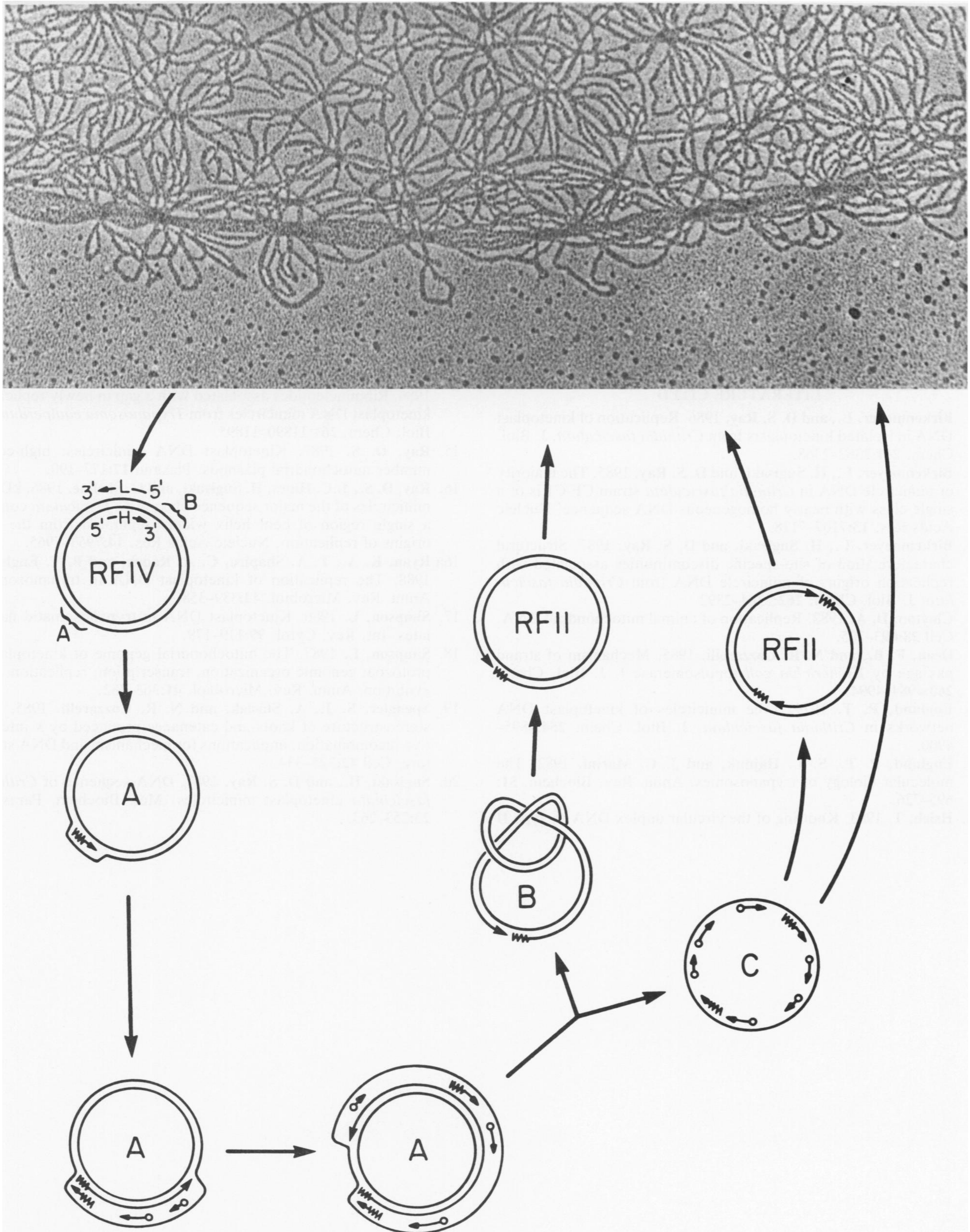

FIG. 8. A model for *C. fasciculata* minicircle DNA replication. A detailed description of the various steps in the model is provided in the text. Site-specific priming of nascent DNA strands is depicted as sawtooth lines. Random priming of Okazaki-like H-strand fragments is indicated by small open circles.

daughter minicircles will be essential for evaluating the possible role of knotted circles in minicircle replication.

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ADDENDUM

After the manuscript was submitted for review, a paper was published that reported similar results for component B and presented electron micrographs which confirm the knotted structure of this species (K. A. Ryan, T. A. Shapiro, C. A. Rauch, J. D. Griffith, and P. T. Englund, Proc. Natl. Acad. Sci. USA **85**:5844–5848, 1988).

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