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A knotted free minicircle in kinetoplast DNA

(trypanosome/mitochondrial DNA/topoisomerase II)

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ABSTRACT **Kinetoplast DNA, the mitochondrial DNA of trypanosomes, is a network containing thousands of minicircles that are topologically interlocked. The minicircle replication intermediates are free molecules that have been released from the network. We report here that one form of free minicircles is a trefoil knot. Identification of this knotted structure is based on its electrophoretic and sedimentation properties, its response to treatments with restriction enzymes or topoisomerase II, and its appearance by electron microscopy. Except for its topology, the knotted minicircle closely resembles a previously described replication intermediate with a unique gap in the newly synthesized L strand.**

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 circles are minicircles, which have an unknown function; a few dozen are maxicircles, which have a structure and genetic function similar to those of mitochondrial DNA in other eukaryotic cells (see refs. 1–5 for reviews on kDNA).

During replication of kDNA, 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-sized network then splits into two progeny networks, which segregate into daughter cells during cell division (see refs. 4 and 5 for reviews on kDNA replication).

In this paper, we describe a knotted molecule found in the free minicircle population of *Trypanosoma equiperdum*. Except for its knotting, this molecule closely resembles a previously described minicircle with a unique gap in its newly synthesized L strand (6, 7). There have been several reports on the structures of other types of free minicircles (6, 8–13).

MATERIALS AND METHODS

Labeling and Isolation of kDNA. *T. equiperdum* (Pasteur Institute strain, BoTat 24) were grown in female Wistar rats. Free minicircles were pulse-labeled with [^{3}H]thymidine and were isolated by sedimentation in a sucrose gradient (6). The 1-kilobase (kb) minicircle nucleotide sequence is found in ref. 14. The strand containing the universally conserved minicircle sequence GGGGTTGGTGTA is designated the H strand and is synthesized discontinuously. The complementary strand, which is synthesized continuously, is designated L.

Gel Electrophoresis. Agarose gels (1.5%, 20 × 25 × 0.5 cm) were cast and run (13–16 hr, 3 V/cm) in TBE buffer (90 mM Tris/92 mM boric acid/2.5 mM EDTA, pH 8.3), which contained ethidium bromide (1 μg/ml) when indicated.

For two-dimensional electrophoresis, agarose gels (1.5%, 20 × 25 × 0.5 cm) were first run (12 hr, 1.9 V/cm) in TBE buffer. The gel was then soaked for 60 min in 1 liter of 50 mM NaOH/1 mM EDTA and subsequently soaked for 60 min in 1 liter of alkaline buffer (30 mM NaOH/2 mM EDTA). After electrophoresis in alkaline buffer (8 hr, 1.1 V/cm), gels were either equilibrated for 30 min in 90 mM NaCl/9 mM sodium citrate/30 mM NaOH prior to Southern transfer (15) of the DNA to nylon filters (GeneScreen, DuPont) or neutralized with 0.1 M Tris·HCl (pH 8) prior to fluorography (7).

Electroelution of DNA from agarose gel slices, unless otherwise indicated, was in TBE buffer in dialysis bags (3 hr, 120 V). After centrifugation of the eluate (10,000 rpm, 15 min, Sorvall HB-4 rotor, 4°C) and ethanol precipitation, the DNA was dissolved in 10 mM Tris·HCl, pH 8/0.5 mM EDTA.

DNA Clones and Probes. pJN6 contains a full-length minicircle, cut at its *Bgl* II site, inserted into the *Bam*HI site of pSP65. pJN6 probes were produced by the random-primer method (16). pKAR3-1 and pKAR5-1 have the minicircle insert from pJN6 in M13mp18 and M13mp19, respectively. A probe produced from pKAR3-1 hybridizes to the minicircle L strand and that from pKAR5-1 hybridizes to the H strand (17). Synthetic 20-base oligonucleotides that hybridize to the minicircle H strand were 5′ end-labeled with T4 polynucleotide kinase (6).

Hybridization. kDNA fragments on nylon filters (360 cm^2) were hybridized with ^{32}P-labeled oligonucleotides (10 pmol, 0.6–6 × 10^6 cpm/pmol) in 10 ml of 0.5 M sodium phosphate, pH 7.2/10 mM EDTA/1% bovine serum albumin/7% $NaDodSO_4$ at 45°C for 13 hr (18). Filters were washed four times (0.3 M NaCl/30 mM sodium citrate, pH 7/0.5% $NaDodSO_4$, 15 min each, 45°C). After hybridization with the pKAR3-1, pKAR5-1, or pJN6 probe, filters were washed at a final stringency of 15 mM NaCl/1.5 mM sodium citrate, pH 7/0.5% $NaDodSO_4$ at 65°C. Autoradiography was performed at −70°C with XAR-5 film (Kodak).

Electron Microscopy. DNA samples (2-μl drops, in 0.15 M NaCl/2 mM spermidine) were placed onto grids with freshly glow-charged carbon films. After washing in distilled water and then in 20, 50, 75, and 100% ethanol, the grids were air dried. They were rotary shadowed with tungsten in a fully cryopumped evaporator at 10^{-7} torr (1 torr = 133 Pa), and the DNA was visualized in the electron microscope at 20 kV (19).

RESULTS

A Knotted Free Minicircle Species. Free minicircles from *T. equiperdum* contain several components that can be separated by agarose gel electrophoresis (Fig. 1 *A* and *B*). These species can be detected either by hybridization with a minicircle probe (Fig. 1*A*) or by [^{3}H]thymidine label incorporated *in vivo* during a 10-min pulse (Fig. 1*B*). The major

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Abbreviation: kDNA, kinetoplast DNA.

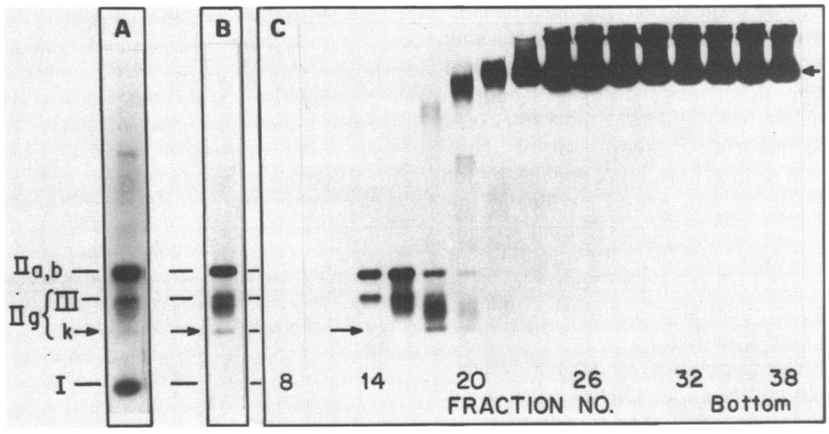

FIG. 1. Electrophoretic and sedimentation properties of *T. equiperdum* free minicircles. (*A*) Free minicircles (150 ng) were resolved on a 1.5% agarose gel in the presence of ethidium bromide (1 μg/ml). DNA was then partially depurinated with HCl, treated with NaOH (15), transferred to a nylon filter, and probed with radiolabeled pJN6. k, Knotted minicircle species; IIa, nicked minicircles; IIb, uniquely gapped minicircles; III, linearized minicircles; IIg, heavily gapped minicircles; I, covalently closed minicircles. (*B*) Free minicircles (5000 cpm, pulse labeled *in vivo* with [^{3}H]thymidine for 10 min) were resolved as in *A* and were detected by fluorography. (*C*) Centrifugation of network-depleted trypanosome DNA (from 2.7 × 10^7 cells, labeled 40 min with [^{3}H]thymidine) in a 5–20% sucrose gradient (25,000 rpm, 22 hr, SW28 rotor, 4°C; ref. 6). Even-numbered fractions were assayed by electrophoresis and by fluorography as in *B*. Free minicircles are in fractions 12–20; as visualized by ethidium bromide staining, the nonradioactive covalently closed free minicircles are in fractions 15–17. Arrow at lower left, minicircle species k; arrow at upper right, nuclear DNA.

species are 1-kb minicircles that are covalently closed (I), nicked (IIa), uniquely gapped (IIb), linearized (III), or multiply gapped (IIg) (K.A.R., unpublished results). Another species, designated k, does not comigrate with any available minicircle standard, and its electrophoretic properties do not suggest any obvious structure. The purpose of this paper is to demonstrate that species k is a knotted minicircle.

As judged by its weak hybridization signal, species k is a minor component of the free minicircle population (Fig. 1*A*). However, it is labeled *in vivo* during a 10-min pulse with [^{3}H]thymidine (Fig. 1*B*). In a shorter pulse (5 min; data not shown), ≈10% of the total radioactivity in minicircles was present in this species, and most of the remainder was in form IIb molecules (the uniquely gapped minicircle with a newly synthesized L strand; refs. 6 and 7). When a 10-min *in vivo* pulse labeling was followed by a chase with nonradioactive thymidine (2.5 mM), radioactivity in both form IIb and species k disappeared with a half-time of ≈20 min (data not shown).

The electrophoretic mobility of species k, either in the presence or absence of ethidium bromide, was that expected for a linear molecule of about 830 base pairs (bp) under the electrophoretic conditions used (Fig. 1 *A* and *B* and data not shown). However, its sedimentation in a sucrose gradient was not compatible with that of a 830-bp linear molecule (Fig. 1*C*); rather, species k sedimented faster than a 1-kb linearized or nicked minicircle (Fig. 1*C*).

Based on these electrophoretic and sedimentation properties, it is possible that species k is a minicircle that has a small size and/or a compact conformation. From its relatively high specific radioactivity after a short labeling *in vivo* with [^{3}H]thymidine, it must contain newly synthesized DNA.

Characterization of the Two Strands of Species k. To study the two strands of species k, [^{3}H]thymidine-labeled free minicircles were subjected to two-dimensional electrophoresis (Fig. 2). In the first dimension, at neutral pH, species k was separated from other free minicircle components. In the second dimension, in alkali, its denatured strands ran independently. The strand containing newly synthesized DNA was identified by its [^{3}H]thymidine label, and both strands were distinguished by hybridization with strand-specific probes. As shown in the fluorograph in Fig. 2*A*, the *in vivo*-labeled strand migrated as a full-size linear strand (about 1 kb) in the second dimension (boxed area in *A*). Probing of a similar gel with strand-specific probes showed that only the L strand migrated as a 1-kb linear strand, indicating that the radioactive strand is L (Fig. 2*B*). In contrast, the H strand migrated slightly faster, suggesting that it is either smaller than 1 kb (due to missing sequences) or that it has a more compact conformation (Fig. 2*C*). Control experiments did not reveal detectable mobility differences in the second dimension between the two complementary linear strands (H and L) or between a single-strand circle and a single-strand linear molecule.

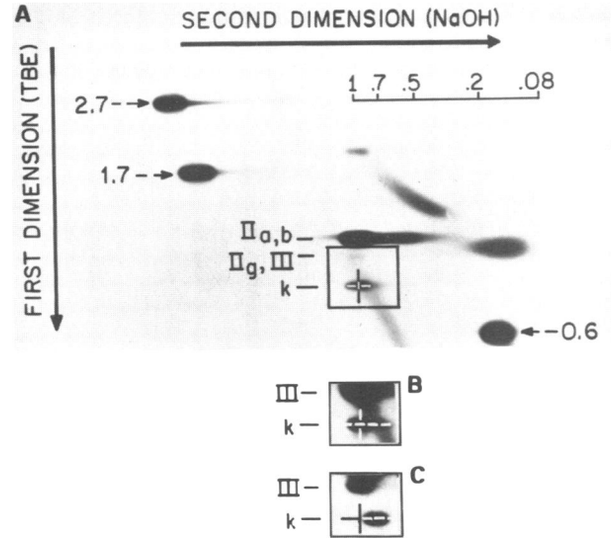

FIG. 2. Two-dimensional gel electrophoresis of free minicircles. Electrophoresis was in TBE (first dimension) and in 30 mM NaOH/2 mM EDTA (second dimension). (*A*) Fluorograph of gel of free minicircles (30,000 cpm, labeled *in vivo* with [^{3}H]thymidine for 10 min). Designation of molecules is the same as in Fig. 1. Small arrows indicate linear size markers loaded in a slot just to the left of the sample lane (2.7 kb and 1.7 kb) or to the right of the sample lane (0.6 kb). Scale represents sizes of linear strands in second dimension. Box containing species k indicates area of gel analyzed with strand-specific probes in *B* and *C*. (*B*) Free minicircles (600 ng, unlabeled) were resolved as in *A*, except DNA was transferred to a nylon filter and probed for the L strand with pKAR3-1. (*C*) The same filter as described in *B* was probed for the H strand with pKAR5-1. Crossed lines mark the position of 1 kb in the second dimension.

To explore the possibility that the accelerated electrophoretic mobility of the H strand at alkaline pH is due to missing sequences, blots of two-dimensional gels were hybridized with synthetic 20-base oligonucleotides encompassing nucleotides 777–796, 701–720, 504–523, 328–347, and 996–3 (see ref. 14 for nucleotide numbering system). All of these oligonucleotides hybridized to the H strand of species k (not shown). In addition, restriction enzymes that cleave the minicircles once at positions 1, 345, 557, and 851 cut species k (not shown). These experiments minimize the possibility of large deletions in the H strand of species k. Therefore, it is likely that the accelerated mobility of the H strand on alkaline gels is due to a compact conformation.

Treatment of Species k with Topoisomerase II or a Restriction Enzyme. To test the hypothesis that species k has some unusual topology that makes it compact, total free minicircles were treated with HeLa topoisomerase II. Species k was no longer visible in the gel after this treatment (Fig. 3*A*, lane 2), but the presence of the other free minicircle molecules made it impossible to identify the product of the topoisomerase reaction. Therefore, we isolated species k by gel electrophoresis and electroelution (the preparation also contained some covalently closed form I minicircles). When the electroeluted molecules (Fig. 3*A*, lane 3) were treated with topoisomerase II, they were converted into a species that comigrated with form II minicircles (Fig. 3*A*, lane 4). When the same electroeluted preparation of species k was digested with *Bgl* II, which cleaves the minicircle sequence only once, the sole product comigrated with the 1-kb linearized minicircle (Fig. 3*A*, lane 5). These results provide strong support for the hypothesis that species k molecules contain the entire minicircle sequence and have a compact topology.

Electron Microscopy of Species k. Electron microscopy of gel-purified species k revealed that many of the circles were knotted in the form of trefoils (Fig. 4). Of 50 circular molecules, 54% appeared knotted, 14% had questionable topology, and 32% appeared unknotted. Southern blotting indicated that the gel-purified preparation used for electron microscopy contained approximately 60% species k molecules and 40% covalently closed minicircles. In a control experiment, none of 78 gel-purified nicked and uniquely

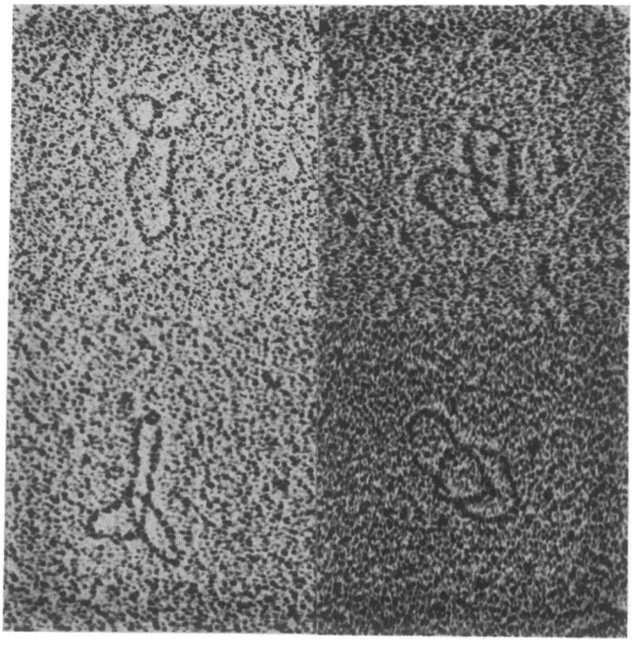

FIG. 4. Electron microscopy of gel-purified species k molecules. Free minicircle DNA from 2×10^{10} cells was centrifuged to equilibrium in a CsCl/ethidium bromide gradient. A broad peak containing the nicked minicircles and species k was collected and fractionated on a 1.5% agarose gel. Species k minicircles were electroeluted in an Elutrap cell (Schleicher & Schuell) with TBE buffer and concentrated by Centricon (Amicon) filtration. The samples were prepared for electron microscopy as described in *Material and Methods*. As shown by probing blotted gels, 60% of the DNA was species k and about 40% was covalently closed mincircles. Four different knotted molecules are shown. Contour lengths of knotted molecules were within 5% of those of gel-purified uniquely gapped and nicked minicircles.

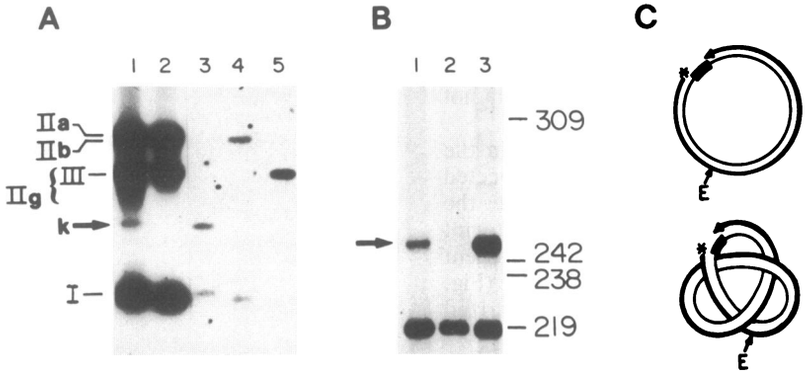

FIG. 3. Characterization of gel-purified species k. (*A*) Effect of topoisomerase II or *Bgl* II on species k. DNA was electrophoresed as described in Fig. 1, transferred to a nylon filter, and hybridized with radiolabeled pJN6 probe. Lane 1, untreated free minicircles (50 ng). Lane 2, same as lane 1, but treated with HeLa topoisomerase II (a gift from Liu Yang) in 50 mM Tris·HCl, pH 7.5/100 mM KCl/10 mM $MgCl_2$/0.5 mM dithiothreitol/0.5 mM EDTA/30 μg of bovine serum albumin per ml/1 mM ATP for 45 min at 30°C. Lane 3, gel-purified species k (electroeluted as described in *Materials and Methods* and still containing some covalently closed minicircles). Lane 4, same as lane 3, but treated with HeLa topoisomerase II as in lane 2. Lane 5, same as lane 3, but treated with *Bgl* II. The slight increase in mobility of covalently closed circles (compare lanes 1 and 2 and also lanes 3 and 4) could be due to reduced ethidium bromide binding after relaxation by topoisomerase II. (*B*) Mapping the 5′ end of the L strand of knotted minicircles. Knotted or form II DNA was dephosphorylated with bacterial alkaline phosphatase (Bethesda Research Laboratories) and rephosphorylated with polynucleotide kinase (Bethesda Research Laboratories) and [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq; ref. 6). DNA was resolved on a 6% polyacrylamide/7 M urea sequencing gel (6), and the gel was autoradiographed. Lane 1, knotted minicircles digested with *Eco*RV. Lane 2, same as lane 1, but DNA was subsequently treated with NaOH (0.3M, 8 hr, 37°C). Lane 3, form II DNA treated with *Eco*RV. Knotted and form II DNA had been electroeluted from an agarose gel as described in Fig. 1. The knotted minicircle DNA preparation resembled that in lane 3 of *A*; form II DNA contained forms IIa (nicked circles) and IIb (uniquely gapped circles). The 219-nucleotide control fragment, present in all lanes, was added in equal quantity to all samples to assess recoveries during alkali treatment and ethanol precipitations. Scale indicates sizes (in nucleotides) of marker fragments. (*C*) Diagram of uniquely gapped and knotted minicircle structures. The thick line represents the L strand; the thin line represents the H strand. *, Alkali-sensitive group at the 5′ end of the L strand at nucleotide 804; E, *Eco*RV site at nucleotide 557; ■, conserved sequence GGGGTTGGTGTA.

gapped molecules (forms IIa and IIb) appeared knotted (data not shown).

Mapping the 5′ End of the L Strand of the Knotted Minicircle. To further characterize the structure of the knotted minicircle, the 5′ ends present on a gel-purified preparation were labeled with ^{32}P by using polynucleotide kinase after bacterial alkaline phosphatase treatment. Form II minicircles (a mixture of IIa and IIb) were similarly labeled as a control. Digestion of each DNA with *Eco*RV produced a ^{32}P-labeled fragment of ≈247 nucleotides (Fig. 3*B*, lanes 1 and 3). Similarly, digestion of each of the end-labeled DNAs with *Mbo* II produced two fragments of about 148 and 147 nucleotides (data not shown). Hybrid selection showed that the labeled strand of the *Eco*RV fragment from knotted minicircles was the L strand (data not shown). These results indicate that both knotted and form II minicircles have a 5′ end of an L strand at or near position 804 (Fig. 3*C*), a site previously mapped as the 5′ end of the nascent L strand in uniquely gapped circles (6, 7). Treatment of 5′ ^{32}P-labeled knotted molecules with alkali results in loss of label from the 247-nucleotide *Eco*RV fragment (Fig. 3*B*, lane 2). Thus, the L strand in knotted molecules may have one or more ribonucleotides at its 5′ end, as does the uniquely gapped minicircle (6).

DISCUSSION

kDNA minicircles are replicated as free molecules after release from the network (8). Free minicircles are easily isolated, and several different types of these molecules have previously been characterized (6, 8–13). In this paper we describe a form of free minicircles that is knotted. Although the knotted minicircles constitute only a small fraction of the total free minicircle population (Fig. 1*A*), they are efficiently labeled *in vivo* during a pulse with [^3H]thymidine (Fig. 1*B*; unpublished data).

Evidence that species k is a knotted molecule is derived from the following observations. The knotted molecules migrate as a discrete band on agarose gels in the position expected for a linear molecule of 830 bp (Fig. 1 *A* and *B*), whereas they sediment faster than linearized or relaxed minicircles (Fig. 1*C*). When the two strands of the knotted minicircle are separated by alkaline gel electrophoresis (Fig. 2), the L strand migrates as expected for a full-length linear molecule, while the H strand, a knotted single-strand circle, migrates slightly faster. The knotted molecules are converted by *Bgl* II, an enzyme that cleaves minicircles once, to molecules that comigrate with linearized minicircles. They are converted by topoisomerase II to molecules that comigrate with uniquely gapped minicircles (Fig. 3*A*, form IIb). By electron microscopy, they appear to be trefoils (Fig. 4).

Except for their topology, the knotted molecules resemble the previously described uniquely gapped minicircles (form IIb; refs. 6 and 7). (*i*) Both molecules label to high specific radioactivity *in vivo* during a short pulse with [^3H]thymidine (Fig. 1*B*). (*ii*) Both molecules have a newly synthesized L strand, which is approximately full length (Fig. 2 *A* and *B*; refs. 6 and 7). (*iii*) Both molecules have an L strand whose 5′ end is at exactly the same site, a site that is complementary to the H strand sequence GGGGTTGGTGTA (Fig. 3*B*). This sequence, which is found in minicircles of all trypanosomatids, is believed to be the site where L-strand synthesis initiates (6). (*iv*) Both have an alkali-sensitive group at the 5′ end of the L strand, which presumably derives from an RNA replication primer (Fig. 3*B*; ref. 6). We have not shown that the knotted minicircles have the 10-nucleotide gap that is present between the 3′ and 5′ ends of the L strand of uniquely gapped molecules (6, 7). However, cleavage of knotted minicircles by S1 nuclease, at a rate indistinguishable from that of the uniquely gapped molecules, is consistent with the possibility that they are gapped (K.A.R., unpublished observations). Diagrammatic representations of the uniquely gapped minicircle and the knotted minicircle are shown in Fig. 3*C*.

Based on its electrophoretic properties and its appearance by electron microscopy, it is likely that species k is a trefoil knot. However, there is another minor species that may be knotted to a greater complexity (data not shown). Longer exposures of the fluorograph in Fig. 2 (3 times as long as for the figure shown) reveal a minor species that migrates slightly faster in the first dimension than species k, at the expected rate for a linear molecule of 790 bp. Its L strand, also labeled *in vivo* with [^3H]thymidine, comigrates in the second dimension with the L strand of species k. Its H strand, in contrast, migrates approximately 5% faster than the H strand of species k. Therefore, like species k, the behavior of this second species is consistent with a structure having a nick or gap in its L strand and a more complex knotting of its H strand. We have not detected any knotted species with a newly synthesized H strand. However, since some of the unknotted versions of these molecules are heterogeneous in electrophoretic mobility (form IIg in Fig. 1), any knotted species with a newly synthesized H strand might be difficult to detect.

In the kinetoplast system, there appears to be some specificity in the knotting of free minicircles, since the knotted molecules are predominantly trefoils and are derived from a particular subset of the free minicircle population (the uniquely gapped minicircles). In addition, the knotted molecules account for a relatively large percentage (10%) of the radioactivity found in free minicircles after pulse-labeling cells for 5 min. We have not shown that knotting is obligatory in minicircle replication; it is possible that only a fraction of minicircles with a nascent L strand are knotted during this process.

Mechanistically, knotting is likely to be a result of topoisomerase activity during replication of minicircles. The segregation of daughter molecules is an example of a specific event in replication, shown to be mediated by topoisomerase II activity (20, 21), which could be significant in the formation of knotted minicircles. In fact, in studies utilizing VP16-213 (etoposide), a selective inhibitor of type II topoisomerases in higher eukaryotes (22) and in *T. equiperdum* (T.A.S., unpublished results), our preliminary evidence suggests a role for topoisomerase II in both segregation and knot formation. When intact trypanosomes are treated for 30 min with VP16-213 and then lysed with $NaDodSO_4$, ≈15% of the total minicircle population is obtained as linearized molecules, with protein bound at both 5′ ends. Furthermore, production of monomeric free minicircles (including the knotted form) is inhibited, whereas dimeric forms accumulate, indicating that there is inhibition of both segregation and knot formation. Topoisomerase I has also been shown to be capable of catalyzing knot formation with nicked or gapped substrates (23). However, *in vivo* treatment of *T. equiperdum* with camptothecin, a topoisomerase I inhibitor (24), generates protein-bound linearized minicircles but does not change the amount of knotted molecule present in the free minicircle population (T.A.S., unpublished observations).

Although less interesting from the point of view of replication, it is possible that the knotted minicircles are simply in equilibrium with the uniquely gapped minicircles. Structural features that could favor knot formation include the 10-base gap between the 3′ and 5′ ends of the L strand (which would introduce flexibility into the molecule), the bent helix (about 90 bp downstream from the gap; ref. 25), and bound protein. Indeed, knotted DNA probably exists in trace quantities in other cell types. For example, it was reported recently that 1% of pBR322 plasmids in *Escherichia coli* are knotted forms (26).

In conclusion, the fact that kinetoplast minicircles can be knotted or topologically interlocked into giant networks suggests that the factors that regulate DNA topology in trypanosome mitochondria are different from those in other cells. Further studies will be needed to determine the origin and the biological role of knotted minicircles.

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