Kinetoplast DNA maxicircles: Networks within networks

(trypanosomes/topology)

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ABSTRACT  Kinetoplast DNA (kDNA), the mitochondrial DNA of trypanosomes, is an enormous network of interlocked minicircles and maxicircles. We selectively removed minicircles from Trypanosoma equiperdum kDNA networks by restriction enzyme cleavage. Maxicircles remained in aggregates that were resistant to protease or RNase and contained no residual minicircles, but were resolved into circular monomers by topoisomerase II. Maxicircles thus form independent catenanes within kDNA networks. Heterogeneity in the size, composition, and organization of maxicircle catenanes reflects changes that occur during kDNA replication. The rosette-like arrangement of maxicircle catenanes is distinctly different from that of minicircle catenanes. Trypanosome kDNA networks reveal unique topological complexity: they are composed of entirely dissimilar catenanes that are in turn extensively interlocked with one another.

Kinetoplastid protozoa are pathogenic to humans, livestock, and commercially important plants. A distinguishing morphologic feature of these organisms is their massive mitochondrial DNA (termed kinetoplast DNA, or kDNA), which repeatedly proved to be structurally and functionally fascinating. The single mitochondrion of each cell contains one kDNA network composed of thousands of interlocked minicircles and tens of maxicircles (reviewed in refs. 1–3). Depending on the species, minicircles are about 1 kb each and there may be hundreds of different sequence classes in a network; maxicircles are about 20 kb each and of identical sequence. Maxicircles are analogous to other mitochondrial DNAs in that they code for mitochondrial rRNA and some mitochondrial proteins. The unique genetic role of minicircles has only recently been discovered: they code for guide RNA molecules that direct the editing of maxicircle transcripts to generate functional open reading frames (reviewed in refs. 4–6).

Replication of kDNA entails the production of two daughter networks identical in size and composition to the parent (reviewed in refs. 7 and 8). Not surprisingly, this process is highly complex. Covalently closed minicircles are individually deconcatenated, and they replicate free of the network. Daughter minicircles are segregated from one another and attached to the periphery of the network. Much less is known about maxicircle replication, which involves a rolling-circle mechanism (9). In trypanosomes, the growing network evolves into a dumbbell shape and then divides (10). The numerous topological interconversions necessary to synthesize and remodel kDNA are catalyzed by topoisomerases. A mitochondrial type II DNA topoisomerase was demonstrated in Trypanosoma equiperdum by drug inhibition studies (11). Two distinct type II topoisomerases have been isolated from Crithidia fasciculata and immunolocalized to the kinetoplast (refs. 12 and 13; J. Shlomai, personal communication). In trypanosomes, the location of mitochondrial topoisomerase II with respect to kDNA is unknown.

Early EM studies showed that selective removal of maxicircles from kDNA networks by restriction enzyme digestion left residual catenanes of minicircles (14, 15). Treatment with topoisomerase II established unequivocally that each kDNA network contained several dozen unit-length circular maxicircles (16). However, the arrangement of maxicircles within the minicircle scaffold has remained speculative (10). In nonreplicating networks, maxicircle loops protrude from the margins of the matrix. In replicating networks from trypanosomes (but not from Crithidia), maxicircles are strikingly apparent as a cluster between the two lobes of the separating daughter networks.

T. equiperdum maxicircles are homologous with those of Trypanosoma brucei (17), but the minicircles are distinctive because they are all of one sequence (18). We identified an enzyme that would accomplish the statistically unlikely cleavage of minicircle, but not maxicircle, DNA. In this paper we describe the maxicircle product produced when Spe I selectively removes all minicircles from T. equiperdum kDNA networks. This product is a catenate of interlocked maxicircles. Maxicircles therefore form networks within kDNA networks.

MATERIALS AND METHODS

Trypanosomes and kDNA Isolation. T. equiperdum trypanosomes (Pasteur Institute strain BoTat 24; African trypanosomes closely related to T. brucei) were grown in female Wistar rats, isolated from blood by means of DEAE-cellulose, and lysed with SDS. The lysate was treated with RNase and proteinase K, phenol-extracted, and dialyzed (11). Dialysate was layered over sucrose cushions (20% in 10 mM NaCl/100 mM EDTA/10 mM Tris-HCl, pH 8.0) and centrifuged (19,000 rpm, 4°C, Beckman SW28 rotor, 1 hr). The pelleted networks were collected, dialyzed, and centrifuged (12,000 rpm, 4°C, Savant microcentrifuge, 1 hr).

Enzyme Digests. Unless indicated otherwise, digestion was for 3 hr at 37°C; buffers provided by the enzyme supplier were supplemented with 0.5 mM dithiothreitol. DNA was phenol-extracted and ethanol-precipitated before reactions. Networks (3 μg/ml) were decatenated with topoisomerase II from T4 phage [gift of Ken Kreuzer (19); 2.5 units/ml] for 1 hr at 37°C in 60 mM KCl/10 mM MgCl2/0.5 mM dithiothreitol/0.5 mM Na2EDTA, 0.5 mM ATP/40 mM Tris-HCl, pH 7.8, with bovine serum albumin (50 μg/ml). To analyze for residual minicircles, networks (3 μg/ml) were digested at 37°C for 3 hr with Spe I (50 units/ml) and for 1.5 hr after addition of more Spe I (25 units/ml). An aliquot was taken for gel analysis and the pH was adjusted to 9.5 with KOH prior to addition of A exonuclease (33 units/ml). The mixture was incubated at 37°C for 1 hr and the reaction was quenched with phenol.

Abbreviation: kDNA, kinetoplast DNA.
Gel Electrophoresis. Maxicircle DNA was fractionated in 0.6% agarose gels (18–21 hr; 3 V/cm; 24°C) in 90 mM Tris/22 mM boric acid/2.5 mM EDTA, pH 8.3, containing ethidium bromide (1 μg/ml). Size markers for maxicircles were HindIII or BglII digests of λ DNA. Nicked and covalently closed circular maxicircles were identified by repairing decatenated networks with ligase, DNA polymerase, and dNTPs; or by nicking decatenated networks with DNase I (data not shown). Markers for minicircles were Hae III or Hpa II digests of 6X174 replicative-form DNA. Electrophoresis of minicircle DNA and transfer to GeneScreen (NEN) have been described (11).

Probes and Hybridization. Plasmid pTKH128 (gift of Ken Stuart; ref. 20) contains a 7-kb HindIII T. brucei maxicircle fragment inserted into pBR322. The insert was excised with HindIII, purified by gel electrophoresis, and radiolabeled by the random primer method. Preparation of probes from full-length cloned T. equiperdum minicircle DNA was described previously (11). Membranes were prehybridized at 55°C for 30 min in 500 mM sodium phosphate, pH 7.2/10 mM EDTA/1% bovine serum albumin/7% SDS (50 μl/cm²) and then hybridized for 12–18 hr at 55°C in prehybridization solution (25 μl/cm²) containing 10 pmol of 32P-labeled probe (0.6–1.5 × 10⁶ cpm/pmol). Filters were washed four times (15 min each, 55°C) in 600 mM NaCl/60 mM sodium citrate, pH 7.0/0.5% SDS.

EM. DNA was prepared for microscopy by the formamide technique (21). Samples on parlodion-coated copper grids were stained with uranyl acetate and rotary shadowcast with platinum/palladium. Micrographs were taken on a Zeiss transmission model EM 10A electron microscope. DNA contour lengths were determined with a map measurer (model 11-11, Alvin Co., Windsor, CT).

RESULTS

Spe I Yields Linear Minicircles and Maxicircle Aggregates. To test whether Spe I selectively cleaves minicircles, purified kDNA networks were digested with enzyme and the sample was split to analyze minicircle and maxicircle products. The DNA was separated by gel electrophoresis, blotted, and probed with cloned 32P-labeled minicircle or maxicircle DNA (Fig. 1 A and B, respectively). As predicted from the sequence, the minicircle products of Spe I migrated as a single 1-kb band (Fig. 1A, lane 4) that represented full-length linearized molecules, clearly distinct from nicked and covalently closed circular monomers generated by topoisomerase II (Fig. 1A, lane 3). However, the maxicircle products of Spe I (Fig. 1B, lane 4) were not circular monomers such as those liberated by topoisomerase II (Fig. 1B, lane 3). Instead, maxicircle products remained largely at the origin, indicating that they were intermediate in size between intact networks that were too massive to enter the gel matrix (Fig. 1B, lane 1) and linearized maxicircles (Fig. 1B, lane 2). A faint smear extending from the slot to the level of nicked monomers (Fig. 1B, lane 4, and longer exposures) suggested that the Spe I products were heterogeneous. Full-length linearized maxicircles, produced by BamHI or Pst I digestion of intact networks, were not cleaved by Spe I, confirming that Spe I has no recognition sequence in T. equiperdum maxicircles (data not shown).

Visualization of Maxicircle Aggregates. To obtain further information about the maxicircle products, purified networks were treated with Spe I and prepared for EM. The micrographs reveal discrete clusters of maxicircles in a field of linearized minicircles (Fig. 2). Maxicircle structures occurred in a range of sizes, consistent with their behavior in agarose gels (Fig. 1B, lane 4). To determine the number of maxicircles in each structure, DNA contour lengths were measured. From two independent experiments (including different kDNA network preparations, Spe I digests, spreads, EM and film processing), 55 structures (29 plus 26) were evaluated. In each experiment, every maxicircle structure within a single grid space was photographed and analyzed. The mean contour length of a single maxicircle was determined from 12 circular monomers (8.0 ± 0.3 μm, a value not statistically different between experiments). The monomer content of each structure was calculated from the total contour length of the structure divided by the contour length of a monomer.

In addition to diversity in size, heterogeneity was evident in the composition of individual maxicircle structures, which contained both relaxed and supercoiled loops of DNA. In smaller aggregates (e.g., trimer, 18-mer), supercoiled loops were more common, whereas the largest structures (e.g., 35-mer, 45-mer) were composed entirely of relaxed DNA. The overall proportion of supercoiled DNA did not change when the DNA was spread for EM in the presence of ethidium bromide (100 μg/ml) (data not shown). This suggests that the relaxed loops seen in Fig. 2 are parts of circles that are not covalently closed and, further, that the covalently closed circles are virtually all supercoiled.

Another qualitative difference between small and large structures is in their general organization. The larger structures are striking for their highly organized rosette-like appearance, with loops emanating from dense central cores. In
FIG. 2. EM visualization of maxicircle aggregates. Purified *T. equiperdum* kDNA networks were incubated with Spe I and prepared for EM. Micrographs display linearized minicircles (open arrow) and maxicircle aggregates of various sizes. Monomer contents were determined from the total contour length of each structure, divided by the contour length of a monomer. Solid arrows, supercoiled maxicircles; curved arrow in 35-mer, maxicircle attached only to the periphery of the catenane; small arrow in 45-mer, loop of single-stranded DNA. (Bar = 1 μm.)

most cases, the loops seem to enter and exit at the same point. When several dozen micrographs are arranged by increasing size of maxicircle aggregate, the rosette-like character increases, and in the largest structures almost all DNA passes through a core. These cores are not apparent in the smaller aggregates (Fig. 2, compare 18-mer with 35-mer or 45-mer). In four large structures containing a total of 136 maxicircles, <2% of the DNA is in circles attached solely at the periphery of the catenane (curved arrow in 35-mer, Fig. 2). Occasional loops of DNA appear to be single-stranded (small arrow in 45-mer, Fig. 2). In the five structures suitable for analysis, the maxicircles appear to be joined by a single interlock.

Size Distribution of Maxicircle Aggregates. The 55 maxicircle structures range from monomers to 45-mers, but 90% of total maxicircle mass is in aggregates containing ≥11 monomers (Fig. 3A). Smaller structures may be generated by mechanical damage, or may reflect a true occurrence in vivo. Although it is impossible to distinguish between these possibilities, damage almost certainly contributes some of the small structures: in experiments with kDNA networks isolated several months before preparation for microscopy, or with Spe I digests that were vortexed, >20% of maxicircles were in aggregates of ≤9.

The kDNA networks in these experiments derive from unsynchronized trypanosomes. This is reflected in the population of intact networks (Fig. 3B), which contains structures ranging from single size (nonreplicating; 10–13 μm in circumference) to double size (late replicating; 27 μm in circumference). Maxicircle aggregates containing ≥16 monomers mimic this size distribution, suggesting that intact kDNA networks contain 16–45 maxicircles, in reasonable agreement with previous estimates (15, 16).

Aggregates Are Interlocked Maxicircles. The isolation procedure yields networks that appear pure on the basis of EM, UV absorption spectrum, and electrophoresis in agarose gels. However, to determine whether maxicircles are aggregated by residual protein or RNA, Spe I digests were further processed prior to electrophoresis. Extensive treatment with
proteinase K, followed by phenol extraction and ethanol precipitation, did not release monomers from maxicircle aggregates (Fig. 4, compare lanes 1 and 2). Digestion with RNases A and T1 also had no effect (lane 3). Similarly, these treatments did not alter the appearance of the aggregates on EM (data not shown). However, topoisomerase II released circular monomers from Spe I digests (lane 4), indicating that the aggregates were catenanes.

Another possible explanation for the maxicircle catenanes is that maxicircles were linked together by residual minicircles. This is highly unlikely, because (i) maxicircle catenanes were not affected by exhaustive digestion with Spe I and (ii) no interlocked minicircles were detected in electron micrographs of 43 maxicircle catenanes containing a total of 550 maxicircles. Nevertheless, to test more rigorously for residual catenated microparticles, intact networks were digested first with Spe I to produce maxicircle catenanes and linearized minicircles (Fig. 5, lanes 1). To reduce the strong signal from linearized minicircles, the Spe I digest was treated with λ exonuclease. The exonuclease had no effect on maxicircle catenanes (Fig. 5A, compare lanes 1 and 2) but converted linearized minicircles into shorter fragments and revealed a small population of nicked minicircle monomers (Fig. 5B, lane 2). These minicircles were resistant to Spe I, perhaps because the nick was positioned in or near the Spe I recognition sequence; they were also resistant to λ exonuclease, which does not act at a nick (22). Finally, after removal of the exonuclease, maxicircles were released from the catenanes by Pst I or topoisomerase II (Fig. 5A, lanes 3 and 4, respectively). These treatments, which dismantle maxicircle catenanes, caused no increase in nicked circular minicircles (Fig. 5B, compare lanes 3 and 4 with lane 2), the expected

Fig. 4. Maxicircles are not aggregated by protein or RNA. Spe I digests were treated as indicated to determine whether maxicircles were aggregated by residual protein or RNA. After treatment, the DNA was separated by agarose gel electrophoresis, blotted, and probed with 32P-labeled maxicircle DNA. Each lane contained 15 ng of kDNA. Lanes: 1, untreated; 2, proteinase K digestion and phenol extraction; 3, RNase A and T1 digestion; 4, incubation with topoisomerase II; 5, digestion with Pst I, which cleaves maxicircles once. N, C, L, and arrow as in Fig. 1.

Fig. 5. Maxicircle aggregates contain no detectable minicircle DNA. Isolated kDNA networks were sequentially digested with enzymes, as indicated. Samples were taken after each reaction, divided, and analyzed for maxicircle (A) and minicircle (B) DNA, as described for Fig. 1. (A) Purified kDNA networks were digested with Spe I (lane 1) and then λ exonuclease (lane 2). The DNA was phenol-extracted and ethanol-precipitated prior to incubation with Pst I, which cleaves maxicircles but not minicircles (lane 3), or with topoisomerase II (lane 4). Each lane contained 15 ng of kDNA. (B) Lanes 1–4, as in A; lanes 5–12, Spe I-digested kDNA (0.00005, 0.0001, 0.00025, 0.0005, 0.001, 0.01, 0.1, and 1.0 dilutions, respectively, of the amount of DNA in lane 1) (15 ng). N, L, C, and arrows as in Fig. 1.
product if maxicircle catenanes contained interlocked minicircles. (In a similar experiment, covalently closed minicircle monomers were not released when Spe I digests were treated directly with topoisomerase II.)

Based on serial dilutions of Spe I-digested networks (Fig. 5B, lanes 5–12), the nicked circles in lane 2 represent <0.05% of all minicircles, or two minicircles per kDNA network [assuming that each network contains 5000 minicircles (15)]. Therefore, the signal in lane 2 should have doubled in lane 3 or 4 if a maxicircle catenane each contained just two residual minicircles. No increase was detected. Lack of residual minicircles in maxicircle catenanes was confirmed by two further experiments. First, Spe I digests were treated with Bgl II, which cuts minicircles once and maxicircles at three sites. Maxicircles were entirely digested, but full-length minicircles were not evident. Second, Spe I digests were incorporated into agarose plugs and the free minicircles were selectively removed by electrophoresis. The maxicircle catenanes (which remained in the plugs) were then treated with Pst I or Bgl II prior to electrophoresis through an agarose gel. Maxicircle DNA was fully digested but newly released minicircle DNA was not detected.

**DISCUSSION**

These experiments provide definitive evidence that maxicircles are not simply threaded, in a random fashion, through a scaffold of minicircles. Instead, they are linked to one another within the catenane of minicircles. Vigorous efforts to remove protein or RNA did not dismantle maxicircle catenanes (Fig. 4), and multiple different experimental strategies, with sensitivity ranging down to 0.2 minicircle per maxicircle structure, failed to detect residual minicircles (Fig. 5). *T. equiperdum* kDNA networks are therefore composed of pure (minicircle or maxicircle) catenanes that are extensively interlocked with one another, clearly an intriguing topological arrangement. Whether this arrangement occurs in other kinetoplastids remains difficult to assess because *T. equiperdum* is unique in having minicircle DNA of one sequence class.

The component catenanes of kDNA networks obviously differ from one another in the size and sequence of their monomers; further, they differ in the number of monomers per catenane (thousands of mini-versus tens of maxicircles). We can now appreciate that these catenanes are dissimilar in their topological organization. Whereas minicircle catenanes (14, 15) resemble a chain-link fence or fishnet, large maxicircle catenanes are rosettes, with a dense central core and protruding loops (Fig. 2). In networks from *C. fasciculata*, the “unit structure” of minicircle catenanes is a circle linked to three other circles (23). Thousands of these unit structures linked together comprise the uniform grid of the minicircle catenane. However, this unit structure cannot explain the core and loop arrangement of large maxicircle catenanes. One possible model for maxicircles requires that each circle be linked, at least once, to virtually every other circle; less likely is a central circle, to which all other circles are linked.

Several characteristics suggest that the heterogeneity of maxicircle catenanes represents different stages of replication. (i) The size distribution of maxicircle catenanes parallels that of intact networks in various stages of replication. Maxicircle catenanes containing 16–20 monomers are most prevalent (Fig. 3A), analogous to the occurrence of single size networks (10–12 μm in circumference; Fig. 3B). In both populations the maximum size is about twice the most common size, consistent with the largest structures representing double-size forms in the final stage of replication. (ii) The topological arrangement of maxicircles in isolated catenanes mirrors their arrangement in intact networks. The rosette-like appearance of the largest catenanes bears striking similarity to the clustered maxicircles seen in the final stages of trypanosome network replication (10). Small maxicircle catenanes, like single-size networks, lack the dense core(s) of DNA unmistakably present in large structures. (iii) Smaller catenanes contain some covalently closed (nonreplicating) maxicircles, whereas the largest structures are composed entirely of noncovalently closed maxicircles.

During the remarkable process of kDNA replication, two entirely dissimilar catenanes are synthesized within each other, at the same time and in the same place, and perhaps by the same replication machinery. Assembly of these interlocked catenanes is a consequence of topoisomerase action, which must be highly coordinated and tightly regulated. One obvious control mechanism is suggested by the two topoisomerases found in *C. fasciculata* mitochondria. If trypanosome mitochondria also have two type II topoisomerases, perhaps one is devoted principally to maxicircle catenanes and the other to minicircle catenanes; or one may catenate, and the other decatenate, circular monomers. In trypanosomes, the maxicircle catenanes that coalesce late in replication may provide a mechanism, obviously distinct from that for minicircles, to ensure equal distribution of maxicircles into the daughter networks. Topoisomerase(s) may play a structural role in this process, possibly mediating the migration of maxicircles to the center of the dividing network.

The unique topology of kDNA provokes important questions on the advantages conferred by, the functional consequences of, and the mechanisms for transcribing and replicating these huge interlocked catenanes.

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