Gapped Minicircles
A NOVEL REPLICAION INTERMEDIATE OF KINETOPLAST DNA

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Kinoplast DNA, the mitochondrial DNA in trypanosomatids, is a network of thousands of interlocked circles. Most of these circles are minicircles and a few are maxicircles. Minicircles replicate, after decatenation from the network, by a Cairns-type mechanism. The minicircle progeny then reattach to the network (Englund, P. T. (1979) J. Biol. Chem. 254, 4895–4900). We have now discovered a novel intermediate in Crithidia fasciculata minicircle replication. It is a highly gapped 2.5-kilobase free minicircle with nascent fragments of only 20 to 110 nucleotides. These fragments are nonligatable, and some remain nonligatable even after gap filling with DNA polymerase. Solution hybridization studies show that the nascent fragments are predominantly, if not exclusively, heavy strand.

Kinetoplast DNA, the mitochondrial DNA in trypanosomes and related parasitic protozoa, is unlike any other DNA found in nature. It is a network of thousands of interlocked circles, and one network resides in the matrix of the cell’s single mitochondrion. The network contains two types of circles. There are about 5000 minicircles (2.5 kb) in the case of Crithidia fasciculata and a few dozen maxicircles (37 kb in C. fasciculata). For reviews on kinetoplast DNA, see Refs. 1–5.

The function of maxicircles is clear. They are the genetic equivalent of mitochondrial DNA in other eukaryotes. Their transcripts are rRNAs and mRNAs coding for proteins involved in electron transport and ATP synthesis (6–14). However, the function of minicircles is much more puzzling, as most evidence suggests that these molecules are not transcribed (7, 15, 16). Even within a network, minicircles are usually, but not always, heterogeneous in sequence (17–20), and there is little or no homology between minicircle sequences from different species (17, 21).

During the past several years we have been studying the replication of kinetoplast DNA. This complex process involves a doubling of the number of minicircles and maxicircles and then a distribution of the progeny circles, in the form of two networks, to the daughter cells. We have found that minicircles do not replicate while catenated to the network (22). Instead, they are released as covalently closed minicircles, presumably by a topoisomerase. A Type II topoisomerase, which could be involved in this process, has been purified from C. fasciculata extracts by Shlomai and Zadok (23). These free minicircles then replicate by a Cairns-type mechanism (24) to form two progeny circles which are nicked (22, 25, 26). These progeny circles are then recatenated to the network, presumably in another topoisomerase reaction. When all the minicircles have replicated and reattached, the network is double size, and all minicircles are nicked. At this time, the structure constricts in the middle and splits in two (27, 28). Sometime during this cleavage all the minicircles become covalently closed. Finally, during cell division one progeny network, which is presumably identical to its parent, segregates into each daughter cell.

In contrast to the minicircle replication mechanism, maxicircles replicate as rolling circles which remain interlocked in the network (29). Only after the entire genome has replicated is the branch of the rolling circle cast off to form a linearized free maxicircle. This molecule then recircularizes and reattaches to the network.

In this paper we describe a novel intermediate involved in minicircle replication. This intermediate is a highly gapped free minicircle in which the newly synthesized strand is composed of fragments only 20–110 nucleotides long. These fragments are separated by gaps. The intermediate represents only one of the two daughter minicircles because the nascent fragments of this gapped molecule are all H strand. The other daughter molecule, with a nascent L strand, has a different structure.

EXPERIMENTAL PROCEDURES

RESULTS

Gel Electrophoresis of Free Minicircles—“Free minicircle” replication intermediates can be isolated from C. fasciculata lysates by sedimentation through sucrose gradients (22). Previous electron microscopic studies had shown that this preparation consisted predominantly of covalently closed minicircles and nicked (or gapped) minicircles (22). It also contained a few single-stranded minicircles and Cairns-type replication

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2 P. A. Kitchin, V. A. Klein, and P. T. Englund, manuscript in preparation.

3 “Experimental Procedures” are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-1671, cite the authors, and include a check or money order for $1.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
judged from a photograph of the ethidium-stained gel (not shown). With \[^{\text{3}}\text{H}\text{thymidine (see "Experimental Procedures"). Samples (20 NN} \times \text{all these molecules are minicircle derived.}

Once; electrophoretic heterogeneity of the smear DNA, as well as its behavior during a pulse-chase experiment, strongly suggested that it was a major replication intermediate. One possibility, that the smear DNA consisted solely of Cairns-type replication intermediates, was unlikely because such molecules are very rare (<0.3% of total free minicircles) in electron micrographs of free minicircle preparations (22, 26), and the fluorescence of the ethidium-stained smear DNA indicated that a substantial amount of DNA was present (Fig. 1). Another possibility, that the smear DNA could be single-stranded fragments (of 2.5 kb or less) formed by branch migration of Cairns-type replication intermediates, was ruled out by comparison of the electrophoretic mobility of smear DNA with that of single-stranded markers (Fig. 1A, lanes 4 and 6). If the smear DNA consisted of single-stranded circular or linear DNA then it would be larger than 2.5 kb. However, this analysis did not eliminate the possibility that the smear DNA was composed of heterogeneous linear double-stranded DNA in the size range 2.0–2.5 kb.

We decided to investigate the molecular structure of the DNA comprising the smear. Our experiments, described in the following paragraphs, revealed an unexpected structure for these molecules. They are predominantly gapped minicircles with extremely short nascent H strands. Variability in gap size and number presumably accounts for the electrophoretic heterogeneity of these molecules.

**Electron Microscopy of Smear DNA**—We used agarose gel electrophoresis to purify the nicked, linearized, and smear components of free minicircles. Fig. 1C shows the characterization of the purified components, and this gel shows that the smear DNA appears to be virtually free of contaminating linearized (Band III) minicircles. These preparations were then examined by electron microscopy using the aqueous spreading technique (30). Micrographs of these components, spread with \(\phi X 174\) RF as an internal size marker, are shown in Fig. 2. This analysis revealed that the smear DNA contained about equal amounts of molecules which resembled individual minicircles, as well as linear molecules of approximately the same length (Panel B). It also contained some longer linear molecules, which may be contaminating nuclear DNA. There were no molecules which resembled the Cairns-type replication intermediates that had been observed previously in total free minicircles (22, 26). Unexpectedly, some of the minicircles in the smear DNA appeared to be smaller than those in the nicked minicircles (compare Fig. 2, A and B), and this was confirmed by contour length measurements (see histograms in Fig. 3). Panel A in Fig. 3 shows a histogram of contour measurements of total free minicircles which were isolated from a sucrose gradient and were not fractionated by gel electrophoresis. The larger of these molecules are about 2.5 kb, but there is a distinct shoulder formed by smaller molecules. Panel B shows purified nicked free minicircles. These molecules are close to 2.5 kb and are more homogeneous in size. Panel C shows purified smear DNA. Most of these molecules have an apparent size ranging from 1.2–2.1 kb, and they include only a few molecules of 2.5 kb. The average apparent size is 1.8 kb (31 units on the arbitrary scale on Fig. 3). These circles presumably account for the shoulder in the histogram of total free minicircles in Panel A. This apparent size reduction and heterogeneity could be an indication that the "smear" DNA circles contain multiple short gaps. Under aqueous spreading conditions, such small single-stranded regions would collapse into a more condensed form (30), thus causing the circles to appear smaller than their true size. Previous electron microscopy of total free minicircles using the formamide spreading technique (30) had not revealed the size heterogeneity shown in Fig. 3A (22). With formamide spreading the single strands in the gaps would not collapse, and all circles would appear to be full size.

**Size Measurements on the Nascent Fragments in the Smear DNA**—To measure the size of the nascent fragments in smear

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4. P. A. Kitchin, and P. T. Englund, unpublished observations.
DNA isolated from cells labeled with $[^3H]$thymidine for 20 min, we analyzed total free minicircles and the gel-purified components by alkaline agarose gel electrophoresis and fluorography (Fig. 4). In total free minicircles (lane 1) there are some 2.5-kb strands (equivalent to a full-size linearized minicircle strand, indicated by the arrow) and a heterogeneous population of strands of less than 0.6 kb. The 2.5-kb strand is derived principally from the nicked minicircles (lane 3). In contrast, the purified smear DNA is composed almost entirely of very small fragments (lane 5) which appear distinct from those associated with the linearized (Band III) minicircles (lane 4).

To measure more precisely the size of nascent fragments from the smear DNA and to eliminate the possibility that these small fragments had been produced by alkaline cleavage at ribonucleotides in larger fragments, we denatured total free minicircles with formamide and analyzed them on a sequencing gel. The results, shown in Fig. 5, reveal that the nascent fragments are about 20 to 110 nucleotides in length. Assuming that the gaps are small and that the average nascent fragment
length is 60 nucleotides, there may be as many as 40 breaks in the 2.5-kb nascent strand of smear DNA. The radioactivity that barely enters the gel (>0.4 kb) is probably due to the due to the 2.5-kb strands that were observed in Fig. 4, lane 1.

**S1 Nuclease Treatment of Smear DNA**—If smear DNA consists of gapped minicircles with single-stranded regions, then these molecules should be extremely sensitive to nuclease S1. We treated $^3$H-labeled free minicircles with varying levels of S1 nuclease, fractionated the products by agrose gel electrophoresis, and then fluorographed the gel (Fig. 6). We found that very-low levels of S1 (0.001 to 0.05 unit/0.8 μg of DNA) selectively eliminated the smear DNA but that even higher levels (up to 0.5 unit) had only a slight effect on the nicked (Band II) or linearized (Band III) minicircles. In another experiment (not shown), we found that when smear DNA was treated with low levels of nuclease S1 (0.05 unit/0.8 μg of DNA), it was degraded to DNA fragments in the size range of 0.3–2.5 kb, and <5% of the radioactivity was made acid soluble. Note that the fastest migrating components of the smear DNA are the most sensitive to S1. These especially sensitive molecules presumably have the most gaps or have gaps which are largest in size.

**Treatment of Smear DNA with DNA Polymerase and Ligated**—If the smear DNA is indeed composed of gapped minicircles, then it should be possible to fill in the gaps and convert the gapped minicircles to nicked minicircles. We treated purified smear DNA with T4 DNA polymerase and/ or T4 DNA ligase, separated the products by agarose gel electrophoresis, and then fluorographed the gel (Fig. 7). The addition of T4 DNA ligase alone had no apparent effect (lane...
analyzed upon a 0.8% agarose gel containing ethidium bromide. This gel was then fluorographed. I, covalently closed minicircles; II, nicked minicircles; III, linearized minicircles; S, smear DNA; nDNA, nuclear DNA.

To further investigate the effects of T4 DNA ligase, we treated total free minicircles with varying amounts of the enzyme and analyzed the products of the reaction on an alkaline agarose gel (Fig. 8). This experiment shows that T4 DNA ligase has only a small effect on the short nascent strands associated with the smear DNA; most are nonligasable. We also analyzed by alkaline agarose gel electrophoresis the nascent fragment length of smear DNA that had been treated with both T4 DNA polymerase and T4 DNA ligase (data not shown). In the presence of both enzymes, the nascent strands were elongated to lengths ranging from 500 nucleotides to full minicircle length (2.5 kb). This result suggests that even after gap filling some strands have termini which are not ligasable.

Hybridization of Pulse-labeled Smear DNA with Minicircle H and L Strands—To determine whether the nascent strand of the smear DNA was H strand or L strand, we performed solution hybridization with purified H or L strands prepared from network minicircles (see "Experimental Procedures" and Fig. 9 for strand separation methodology). The solution hybridization experiments involved incubating the denatured smear [3H]DNA under reannealing conditions and then mea-

3. However, the addition of T4 DNA polymerase alone converted the smear DNA to nicked and linearized minicircles (lane 2). The addition of ligase to the polymerase caused a slight increase in the production of nicked minicircles (lane 4). In none of the incubations were any covalently closed minicircles produced. All four dNTPs were incorporated in roughly equal amounts by T4 DNA polymerase into the smear DNA during this incubation (measured with [3H]dNTPs, data not shown). The experiment shown in Fig. 7 is consistent with the hypothesis that about half of the smear DNA is in the form of intact gapped minicircles. The other half is probably in the form of gapped linearized molecules which may be broken gapped circles.

Fig. 6. S1 nuclease analysis of total free minicircles. Free minicircles (~0.8 μg) were digested with nuclease S1 (Bethesda Research Laboratories) for 10 min at 22 °C (total volume, 20 μl; 30 mM Na acetate, pH 4.6, 50 mM NaCl, 1 mM ZnSO₄, 5% glycerol) and analyzed upon a 0.8% agarose gel containing ethidium bromide. This gel was then fluorographed. I, covalently closed minicircles; II, nicked minicircles; III, linearized minicircles; S, smear DNA; nDNA, nuclear DNA.

Fig. 7. Conversion of the smear DNA to linearized and nicked minicircles in vitro. Purified smear DNA (~50 ng) was incubated with T4 DNA polymerase (1.8 units, Worthington) and/or T4 DNA ligase (1 unit, Bethesda Research Laboratories) for 4 h at 22 °C in 66 mM Tris-HCl, (pH 7.1, 6.6 mM MgCl₂, 10 mM DTT, 400 μM ATP, 70 μM each of dATP, dCTP, dGTP, and dTTP. The products were analyzed on a 0.8% agarose gel containing ethidium bromide, and the gel was then fluorographed. Most of the mass of the smear DNA, as judged by ethidium fluorescence, was also converted to nicked and linearized minicircles (gel photograph not shown). Lane 1, smear DNA; lane 2, plus T4 DNA polymerase; lane 3, plus T4 DNA ligase; lane 4, plus both enzymes; lane 5, decatenated kDNA showing nicked and covalently closed minicircle markers; lane 6, linearized minicircle marker (SstII digest of kDNA networks). I, covalently closed minicircles; II, nicked minicircles; III, linearized minicircles; S, smear DNA.

Fig. 8. Alkaline agarose gel electrophoresis of total free minicircles after treatment with T4 DNA ligase. Total free minicircles (~1 μg) were incubated with T4 DNA ligase as described in the legend to Fig. 7. The products were analyzed on a 1% alkaline agarose gel which was then fluorographed. M, linearized minicircle marker. Arrow indicates position of a covalently closed minicircle. The position of marker DNA (eX174 RF digested with HaeIII) was measured from a photograph of the ethidium-stained gel (scale is in kb). Note that ligase treatment did produce a full-length linear nascent strand as well as cause the covalent closure of some (<1%) circles (arrow). This suggests that some of the components in total free minicircles have nascent strands that are fully ligasable, while others still possess a single nonligasable position. These strands are not derived from the smear DNA, and their significance will be discussed in another publication.
the "tracer." We first empirically found tracer concentrations completely, H strand.

which driver strand was complementary to the tracer. Where its SI sensitivity is virtually the same as that of causes no stimulation of reannealing. However, addition of L nascent strand in smear DNA is predominantly, if not com-

minicircles once). During a 3-h incubation, tracer alone rean-

neals only a few per cent. However, addition of either H strand or L strand driver causes extensive reannealing in the presence of excess amounts of unlabeled driver H or L minicircle strand DNA. The amount of single-stranded DNA remaining was measured with an S1 nuclease assay. The data is plotted as the mean value of duplicate determinations, which did not differ by more than 10%. The arrow indicates the total amount of $^3$H radioactivity present in the assay. A—A, tracer DNA alone; O—O, plus H minicircle strand driver DNA; □—□, plus L minicircle strand driver DNA. The recovery of radioactivity was 100% for the experiment in panel A and 95% for that in panel B.

Discussion

Previous studies from our laboratory have shown that kinetoplast minicircles do not replicate while attached to the network. Instead, they are released from the network, presumably by a Type II topoisomerase, and then they replicate as free minicircles. The minicircle progeny are then reattached to the network (22).

Little is known about the mechanism of free minicircle replication. We have observed some minicircle Cairns forms by electron microscopy and presumed that these are replication intermediates. Unfortunately, these molecules constitute less than 0.3% of the minicircle population, and, therefore, it has not been possible to obtain sufficient material for a thorough structural analysis. Based on staining of these molecules with Escherichia coli single-strand binding protein, we suggested that they have one single-strand branch. However, since this conclusion was based on the observation of a very small number of molecules, it must be considered tentative. Using the same staining procedure, we also observed a small number of single-strand minicircles in the population (26).

To learn more about the minicircle replication process, we decided to search for additional replication intermediates. We fractionated free minicircles (isolated by sucrose gradient centrifugation) by electrophoresis on an agarose gel containing ethidium bromide. We found bands that corresponded to covalently closed minicircles and nicked minicircles (Fig. 1), and their presence confirmed the previous conclusions from electron microscopy (22). We also detected linearized minicircles. However, we do not yet know if these molecules were broken during isolation or whether they were linear in vivo. The other major component detectable on these gels was a smear of ethidium-staining material which migrated between the nicked and covalently closed minicircles. Characterization of the smear DNA is the subject of this report.
There is strong evidence, based on a \([^{3}H]\)thymidine pulse-chase experiment, that the smear DNA is a replication intermediate (Fig. 1B). These molecules become the most highly labeled of all free minicircle components during a pulse. Subsequently, the radioactivity associated with the smear DNA disappears completely during a chase, presumably as these molecules are ultimately reattached to the network. Furthermore, they are a relatively abundant component of free minicircles as judged from the intensity of their ethidium staining on an agarose gel (Fig. 1, lane 2) and from the electron micrographs of total free minicircles (Fig. 3A).

Structural studies indicate that the smear DNA consists of minicircles in which the nascent H strand is heavily gapped. Variation in gap size and number probably accounts for their electrophoretic heterogeneity. Fragments of the nascent H strand have a size range of 20 to 110 nucleotides. We have not yet been able to measure directly the average gap size. However, the absence of numerous long stretches of clearly identifiable single-stranded regions in the electron micrographs of aqueous spread molecules (Fig. 2) or in molecules stained with single-stranded binding protein (26) suggests that most of the gaps could be relatively small. However, neither of these observations preclude the possibility that there are longer gaps present in smear DNA. Due to their gapped structure, these molecules are extremely sensitive to nuclease S1 (Fig. 6). The large number of gaps also presumably accounts for the apparent shrinking of these molecules when they are observed by electron microscopy (Figs. 2 and 3). Collapse of the single-stranded sequences in the gaps, under the conditions of aqueous spreading, presumably causes this effect.

Treatment of the smear DNA with T4 DNA polymerase in vitro causes an almost quantitative conversion of the radioactivity to approximately equal amounts of nicked and lineariized minicircles (Fig. 7). We do not know the significance of the linear molecules generated in this reaction. They probably arose from the gapped linear molecules already present in the smear DNA and thus may represent heavily gapped closed circles (Fig. 7). These experiments indicate that the minicircle progeny with nascent L strands contain only a single nick or small gap in their nascent strands. These 2.5-kb nascent L strands can be observed by alkaline agarose gel electrophoresis of total free minicircles (e.g. Fig. 4, lane 1). There are two reasons why the nascent H strand in free minicircles contains much more radioactivity than the nascent L strands. The L strands have a much lower thymine content than H strands (see Fig. 9), and the minicircles with a nascent L strand are reattached to the network much faster than those with nascent H strands.²

We do not yet know how the gaps were formed in the minicircles with a nascent H strand. It is possible that the gaps were introduced after the continuous replication of the H strand. However, this seems unlikely, and a more attractive possibility is that H strand replication is discontinuous. In this case, the gaps may have been created by removal of RNA primers. However, our earlier observations of Cairns-like structures with single-stranded branches (26) suggested that lagging strand initiation was delayed, as observed during the replication of other mitochondrial DNAs (36), until the lagging strand origin of replication was exposed. The presence of single-stranded free minicircles (26) further suggested that replication may be totally asymmetric and that lagging strand initiation did not occur until its template had been displaced as a single-stranded circle. The latter model would predict that the replication of both strands could be continuous. It is, therefore, surprising to find that the structure of the progeny minicircles with a nascent H strand is highly reminiscent of a discontinuous mechanism of replication. Further studies to resolve this paradox and to learn more about the molecular mechanism of minicircle replication are now in progress.

Acknowledgments—We thank Shirley Metzger for expert help in preparing this paper. We are grateful to Dr. Leroy Liu for Type II topoisomerase and to Simon Walker for expert help with the computer programs.

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**EXPERIMENTAL PROCEDURES**

Isolation of Free Minicircles

The DNA was grown to 4x10⁶ cells/ml in 400 ml H medium at 37°C (21). The cells were labeled with [3H]thymidine (100 000 cpm/ml) from 19:00 h to 21:00 h. The labeled cells were centrifuged at 10,000 rpm for 30 min and the supernatant was removed. The cell pellets were washed twice with 1 ml of fresh medium. The cells were then harvested by centrifugation, dispersed in 2 ml of 0.05 M EDTA (pH 7.5) solution containing 0.1 M NaOH, 100 µg/ml ethidium bromide and were subjected to lysis by sonication. The cell debris was removed by centrifugation and the supernatant was mixed with 1 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 0.025 M MgCl₂, 0.05 M EDTA and 0.05 M NaOH. The mixture was then divided into two equal parts and mixed with 5 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 0.025 M MgCl₂, 0.05 M EDTA and 0.05 M NaOH. The mixture was then divided into two equal parts and mixed with 5 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 0.025 M MgCl₂, 0.05 M EDTA and 0.05 M NaOH. 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