Structural Characterization of Site-specific Discontinuities Associated with Replication Origins of Minicircle DNA from *Crithidia fasciculata*

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The kinetoplast DNA of trypanosomes is comprised of thousands of DNA minicircles and 20–50 maxicircles catenated into a single network. Replication intermediates of minicircle DNA from the trypanosomatid species *Crithidia fasciculata* contain site-specific discontinuities in both heavy (H) and light (L) strands. These discontinuities map to two small regions situated 180° apart on the minicircle; each region has two sites at which a discontinuity can occur, one on each strand. We have determined the position of these discontinuities on the minicircle DNA sequence and have characterized their structure. H-strand discontinuities occur within a 4–5-nucleotide sequence and consist of single nicks, only one of which appears to be a DNA-DNA junction. Characterization of the remaining H-strand nicks indicates a structure other than a typical DNA-DNA or DNA-RNA junction. Discontinuities on the L-strand can be either a nick or a short gap which overlaps a 12-nucleotide sequence universally conserved among minicircles from various trypanosomatid species. Up to 6 nucleotides are hydrolyzed from the 5' terminus facing the gap upon treatment with alkali, suggesting the presence of an RNA primer. Based on the structures of minicircle replication intermediates, we present a model for replication of minicircle DNA in which the site-specific discontinuities closely coincide with the origins of replication.

Trypanosomes contain a single large mitochondrion termed the kinetoplast. Within the kinetoplast are two distinct populations of double-stranded circular DNA, the minicircles for which there is no known function and the maxicircles which are the equivalent of conventional mitochondrial DNA. These circular molecules are highly catenated to form a large, disheveled network known as kinetoplast DNA (1–4). In the trypanosomatid *Crithidia fasciculata*, there are about 5000 minicircles (2.5 kb) per kinetoplast, making up 95% of the kinetoplast DNA network; the remaining 5% consists of about 25 maxicircles (37 kb) (2, 5, 6).

The DNA minicircles of most kinetoplastid species are heterogeneous in sequence. However, in the *C. fasciculata* strain Cf-C1 greater than 90% of the minicircles belong to a single class which is nearly homogeneous in DNA sequence (7). Therefore, it is possible to localize sequences of possible biological importance precisely and to determine the structure of minicircle replication intermediates at the nucleotide level.

The general mechanism for replication of kinetoplast DNA networks has been revealed through *in vivo* studies, primarily on *C. fasciculata*. Minicircles detach from the network, replicate as free molecules (8, 9), and then reattach to the network periphery (10, 11). The newly replicated network-associated minicircles contain both nicks and gaps. Thus, as replication progresses, a concentric wave of replication sweeps the kinetoplast DNA network, doubling its size, and transforming it from a covalently closed form to a nicked-relaxed form (5, 12).

*In vivo*, the free minicircle population contains a class of replication intermediates termed smear or gapped DNA; in these molecules the nascent H-strand has numerous nicks and short gaps (9, 13). However, no such intermediate has been observed for the L-strand which appears to be synthesized as a continuous DNA chain. Kinetoplasts isolated from *C. fasciculata* and incubated in an ATP-dependent DNA synthesis reaction give rise to minicircle DNA replication intermediates very similar to those described above. Furthermore, minicircle molecules containing nascent half-length (1.25 kb) H-strands are generated during the kinetoplast reaction (14, 15). Similar size nascent DNA chains of unknown strand specificity are associated with replicating kinetoplast DNA networks *in vivo* (13).

Minicircles replicated in the kinetoplast system contain discontinuities in both H and L nascent DNA strands. Each strand has two sites spaced 180° apart on the minicircle at which a discontinuity can occur. The sites of discontinuity on opposite strands do not overlap but are separated from one another by about 100 base pairs (14, 15). In *Trypanosoma equiperdum* minicircles, a gap of approximately 10 nucleotides is observed in one of the nascent DNA strands. This gap overlaps a 12-nucleotide sequence conserved between species and found in all minicircles sequenced to date (16).

Replication intermediates of animal cell mtDNA have been shown to contain site-specific discontinuities and to accumulate distinct size nascent strands (17, 18). Such structures are remarkably similar to those described above for minicircle

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1 The abbreviations used are: kb, kilobases; L-strand, light strand; H-strand, heavy strand.
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DNA replication intermediates and may reflect similarities between their modes of replication. Detailed characterization of the site-specific discontinuities found in C. fasciculata minicircles is presented here. These results show that the discontinuities coincide with sites of initiation of minicircle DNA replication, indicating that both chain initiation and termination occur at the replication origins.

EXPERIMENTAL PROCEDURES

Materials—Brain heart infusion medium was purchased from Difco and hemin from Sigma. The unlabeled deoxyribonucleotide triphosphates and ATP were obtained from Pharmacia and Sigma, respectively. Labeled [γ-32P]ATP was from ICN and [α-32P]dATP from New England Nuclear. Dideoxy nucleotide triphosphates were purchased from Takara Biomedicals. Diphosphorydine nucleotide was supplied by Bhring Diagnostics. Enzymes used included AluI, AuaII, HinfI and ATP were obtained from Pharmacia and Sigma, respectively. Labeled [γ-32P]ATP was from ICN and [α-32P]dATP from New England Nuclear. DNA ligase, T4 DNA ligase, and T4 polynucleotide kinase (Pharmacia).

Cell Growth—C. fasciculata Cf-C1 cells (7) were cultured in Difco brain heart infusion medium supplemented with 10 µg/ml of hemin at 37°C with 5% CO2.

Kinetoplast DNA Synthesis Reaction—Kinetoplasts from C. fasciculata Cf-C1 cells were isolated and incubated in a DNA synthesis assay for 30 min at 30°C in the presence of unlabeled deoxyribonucleotide triphosphates (14, 15). The reaction was quenched, and the free minicircle fraction was isolated on sucrose gradients as described (14). The free minicircle fraction was electrophoresed on an agarose gel, and the nick-ended circular form (RF IV) and the covalently closed relaxed circular form (RF IV) of free minicircles, the starting material for all subsequent experiments, were isolated separately by electrophoresis.

Electroelution of DNA—DNA samples were electrophoresed in neutral 0.8% agarose horizontal slab gels in Tris/borate/EDTA buffer at 37°C, and the acid neutralization step was omitted. The nascent DNA chains produced during the DNA synthesis reaction (14, 15). The nascent DNA chains produced during the DNA synthesis reaction were heated for 2 min at 70°C and quenched on ice before addition of the kinase enzyme. Recessed 3'-ends of double-stranded DNA were labeled and filled by incubation for 30 min at room temperature in 50 mM Tris, pH 7.2, 10 mM MgCl2, 0.1 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA, 1 µM [γ-32P]ATP (3000 Ci/mmol), and 5 units of T4 polynucleotide kinase (Pharmacia).

 Autoradiography—The polyacrylamide gels were dried prior to exposure on Kodak x-ray film at -70°C in the presence or absence of a single intensifying screen. Longer autoradiographic exposures were necessary to allow visualization of each step on the nucleotide ladder. Therefore, the exact size difference between fragments containing the same 5’- or 3’-end could be determined.

RESULTS

Site-specific Discontinuities Lie within Conserved Regions—Kinetoplasts isolated from the C. fasciculata strain Cf-C1 replicate minicircle DNA when incubated in a DNA synthesis reaction (14, 15). The nascent DNA chains produced during the reaction contain site-specific discontinuities which were localized on a restriction enzyme cleavage map (Fig. 1a) of the major minicircle class found in this strain (14). DNA sequence analysis has shown that the discontinuities are contained within imperfect direct repeats of approximately 170 base pairs. These direct repeats are present in all minicircles that have been sequenced from this strain.2 The regions containing the conserved direct repeats, designated A and B, are depicted in expanded scale in Fig. 1 b and c, respectively, along with a detailed restriction map for each region deduced from the DNA sequence. Most of the cleavage sites between and immediately adjacent to each pair of H- and L-strand discontinuity sites are seen to be conserved between regions A and B.

Determination of 3’-End Points of B Region Discontinuities—Nicked minicircle DNA (RF II), prepared from kinetoplasts reacted in the presence of unlabeled dNTPs, was cleaved with MluI or XboI and kinase-labeled at the resulting 5’-ends. A schematic of the B region discontinuities with the adjacent MluI and XboI sites cleaved and labeled on the H- and L-strands, respectively, is shown in Fig. 2a. In reality, an individual minicircle DNA molecule would not contain both discontinuities, since it would have only one nascent strand.

Utilizing the known sequence from the minicircle B region, oligonucleotide primers, a 21-mer and a 15-mer (see “Experimental Procedures”), were synthesized to be complementary

\(^2\) H. Sugisaki, and D. S. Ray, manuscript in preparation.
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![Diagram of minicircle map positions of site-specific discontinuities](image)

**Fig. 1.** Minicircle map positions of site-specific discontinuities within regions A and B. a, the sites of H (open triangles) or L (closed triangles) strand discontinuity are shown on a linear restriction enzyme cleavage map of the major minicircle species in the C. fasciculata strain CF-C1 (7). Regions A and B described in the text are designated by brackets and are shown in expanded scale in b and c, respectively. Cleavage sites used for end labeling are followed in parentheses by the end (5' or 3') at which they were labeled. In b and c, only restriction sites which were cleaved during the course of these experiments are shown. Relative size scales in base pairs (bp) are shown by the bars.

The H-strand-sequencing ladder, primed from the 21-mer, and/or XbaI and 5'-end labeled (*). Only the MluI-XbaI fragment containing the B region is shown. Breaks in the lines represent H- and L-strand discontinuities. b, single-stranded minicircle DNA clones M13 CFK120 (L-strand) and M13 CFK120C (H-strand) are shown hybridized with their complementary oligonucleotide primers (see "Experimental Procedures"). Arrowheads indicate continuation of the circular template DNAs, and dots represent the remainder of the primers.

**Fig. 2.** Structure of DNAs used to localize 3'-end points of B region discontinuities. a, minicircle RF II DNA was cleaved with MluI and/or XbaI and 5'-end labeled (*). Only the MluI-XbaI fragment containing the B region is shown. Breaks in the lines represent H- and L-strand discontinuities. b, single-stranded minicircle DNA clones M13 CFK120 (L-strand) and M13 CFK120C (H-strand) are shown hybridized with their complementary oligonucleotide primers (see "Experimental Procedures"). Arrowheads indicate continuation of the circular template DNAs, and dots represent the remainder of the primers.

The H-strand-sequencing ladder, primed from the 21-mer, was electrophoresed on a sequencing gel alongside the minicircle sample that had been end-labeled only at the MluI site (Fig. 3a). A series of labeled H-strand DNA fragments is observed spanning a 4-5-base sequence and differing in length by 1-nucleotide increments. The two largest fragments are more abundant than the shorter ones, forming a sharp cut-off to the upper size limit of the fragments.

In an analogous experiment, the L-strand-sequencing ladder was electrophoresed alongside the minicircle sample that had been end-labeled only at the XbaI site (Fig. 3b). Two distinct groups of L-strand fragments are seen. The groups are about 11 nucleotides apart and each group contains two prominent fragments separated by 1 nucleotide. A small degree of sequence heterogeneity exists within the major minicircle class (7). There is a large subpopulation of the major minicircle class containing a single base deletion with respect to the minicircle clone M13 CFK120C in the region between the XbaI site and the L-strand discontinuity (data not shown). This most likely results in the L-strand doublets observed in Fig. 3b, the upper fragment of each pair being derived from M13 CFK120C-like molecules and the lower fragment from the subpopulation containing the one base deletion. Labeling of the other 5'-ends produced upon cleavage with MluI or XbaI also occurs in these experiments, but the fragments labeled are so large that they do not interfere with the analysis.

**Structural Characterization of B Region Discontinuities—**

The actual DNA structure(s) at the sites of H- and L-strand discontinuity in the B region was probed by enzymatic means. Fig. 4 shows DNA samples which were treated with T4 DNA ligase, E. coli DNA ligase, or T4 DNA polymerase prior to cleavage and then 5'-end-labeled at both the MluI and XbaI sites. All of the H-strand fragments and the larger pair of L-strand fragments disappear upon treatment with T4 DNA ligase, indicating that the discontinuities at those locations are nicks and not gaps (Fig. 4a). The smaller set of L-strand fragments is unaffected by T4 DNA ligase but is elongated about 7 nucleotides by treatment with T4 DNA polymerase, generating a pair of fragments intermediate in size between the larger and smaller L-strand fragments. Thus, the smaller L-strand fragments seem to border a short gap and terminate with 3'-hydroxyl groups capable of priming T4 DNA polymerase.

Stepwise treatment of the DNA sample with T4 DNA ligase and T4 DNA polymerase, in either order, generates L-strand fragments of only the intermediate size (data not shown). Therefore, the intermediate size fragments represent the full extent of L-strand elongation by T4 DNA polymerase, and the junction so formed can not be sealed by T4 DNA ligase. The polymerase treatment also appears to cause a slight amount of strand displacement at the H-strand nicks, resulting in reduced levels of all but the largest fragment (Fig. 4a) and generating a junction which is resistant to sealing by T4 DNA ligase (data not shown).

In Fig. 4b the DNA sample was treated with E. coli DNA ligase prior to cleavage and end labeling. The absence of the largest H-strand fragment indicates that only the 3'-most H-strand nick is suitable for ligation by the E. coli enzyme, whereas the others remain open. L-strand nicks are closed by E. coli DNA ligase and, as expected, L-strand fragments bordering the gap are not affected. Thus, the L-strand nicks and the 3'-most H-strand nick appear to be DNA-DNA junctions with juxtaposed 3'-hydroxyl and 5'-phosphoryl end groups.

E. coli DNA ligase differs from the T4 enzyme in that it can not ligate a nick in which the 5'-terminus is a ribonucleotide and the 3' terminus is a deoxyribonucleotide (24). Incubation of the DNA sample under alkaline conditions, after treatment with T4 DNA ligase and end labeling, did not regenerate any of the H-strand fragment (data not shown). Therefore, these H-strand nicks which are closed by T4 DNA ligase but not by E. coli DNA ligase do not represent simple DNA-RNA junctions, a structure which should be hydrolyzed under these conditions.

Elongation of the lower L-strand fragments by T4 DNA polymerase stops short of the L-strand nick sites (Fig. 4a). To examine the structure of the 5'-terminus facing the L-strand gap, the DNA samples in Fig. 5 were treated as indicated in the figure legend and 3'-end-labeled on the L-strand at the B region AvaII site designated in Fig. 1c. Due to the absence of the analogous AvaII site within the A region and to the asymmetric labeling obtained at the cleaved ends of
terminating nucleotide is shown  

AuaII  

was electrophoresed  

dried and autoradiographed. The chain-  

sequences are presented at  

the  

Each sequencing lane and actual se-  

site  

B region discontinuities. 


eing gel ("Experimental Procedures")  

lanes on a denaturing polyacrylamide sequenc-  

continuity can be determined. The sample in the  

accurate size markers from which the exact 5'-end(s) of the  

DNA sample in Fig. 44. The 3' termini of the interme-  

ment 3 nucleotides longer than the nicked fragment in the untreated sample. These results are consistent with the 5'-end continuity within the B region (Fig. 1C). 

In the untreated sample (Fig. 7), two predominant L-strand fragments are observed (arrowheads). They are separated by 11–12 nucleotides and their 3'-ends lie at 12 nucleotides (smaller fragment) and 23–24 nucleotides (larger fragment) past the 86-nucleotide marker. Exact placement of the 3'-end of the larger fragment is not possible in this experiment due to the extended distance between it and the marker. The  

ends of the H-strand discontinuities in the A region. DNA samples in Fig. 6 were treated as indicated in the figure legend and 5'-end-labeled on the H-strand at the RsaI site designated in Fig. 1b. The 3'-end of the 138-nucleotide marker lies directly adjacent to the H-strand discontinuity and was generated by cleavage with AuaII. Two fragments flank the position of the 138-nucleotide marker in the experimental samples. They are also present in the control sample derived from RF IV minicircle DNA and so are not due to strand discontinuities. To eliminate a minicircle DNA fragment which overlaps the size range of fragments to be analyzed, the indicated samples were digested with TaqI. 

Two H-strand fragments, terminating 9 and 10 nucleotides past the 3'-end of the 138-nucleotide marker, are observed in the untreated sample (Fig. 6). This places the 3'-ends of these H-strand fragments at the same relative sequence position as was determined for the two largest B region H-strand fragments (Fig. 9a). Incubation of the DNA sample with T4 DNA ligase resulted in the loss of both H-strand fragments, demonstrating the presence of a nick at the 3' terminus of each fragment. E. coli DNA ligase only sealed the nick at the end of the larger fragment, leaving the smaller fragment unaffected. The results are the same as those obtained from the H-strand discontinuities in the B region. 

Analysis of the 3'-ends of the L-strand discontinuities in the A region is presented in Fig. 7. The DNA samples in Fig. 7 were treated as indicated in the figure legend and 5'-end-labeled on the L-strand after cleavage at the AluI site designated in Fig. 1b. The 3'-end of the 86-nucleotide marker fragment was produced by digestion at theMspI site located between the AluI site and the L-strand discontinuity. A pair of larger fragments present in the same lane are derived from minicircle sequence outside the A region. The remaining DNA samples were digested withHinII, thus eliminating any confusion of the A region L-strand fragments with similar size fragments extending between the AluI site and L-strand discontinuity within the B region (Fig. 1c). 

In the untreated sample (Fig. 7), two predominant L-strand fragments are observed (arrowheads). They are separated by 11–12 nucleotides and their 3'-ends lie at 12 nucleotides (smaller fragment) and 23–24 nucleotides (larger fragment) past the 86-nucleotide marker. Exact placement of the 3'-end of the larger fragment is not possible in this experiment due to the extended distance between it and the marker. The
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**Fig. 4. Characterization of 3'-ends of B region discontinuities.** Minicircle RF II DNA was treated as indicated above each lane and then cleaved at both MluI and XbaI sites and 5'-end-labeled. The samples were electrophoresed and autoradiographed as in Fig. 3. Fragments derived from heavy (H) or light (L) minicircle strands are indicated by the brackets. Panels a and b represent separate experiments.

A larger fragment is absent in DNA samples treated with T4 or E. coli DNA ligase, indicating that its 3'-end lies at a nick. The previously stated substrate specificities of these enzymes suggest that this nick represents a DNA-DNA junction. Within the resolution of this experiment, the L-strand nick in the A region occurs at the same nucleotide sequence as was observed for the B region (Fig. 9, b and c, respectively).

Ligase treatment has no effect on the smaller L-strand fragment. However, this fragment is extended 6-7 nucleotides by T4 DNA polymerase, generating a pair of intermediate size L-strand fragments (arrows) similar to those seen in the B region (Fig. 4a). Therefore the 3'-end of the smaller L-strand fragment in region A appears to border a short gap.

Fig. 8 shows the analysis of the 5'-end(s) of the L-strand discontinuity in region A. Minicircle RF II DNA was digested with both XhoI and SacII and the resulting 0.75-kb minicircle fragment containing the A region (Fig. 1a) was gel purified for the subsequent analysis. This was done to ensure that the L-strand fragments obtained would be derived from the A region and not the B region. The 0.75-kb fragment was aliquoted into several tubes and treated with T4 DNA ligase where indicated. Intermolecular joining between fragments during such ligations will not affect the results. All DNA samples were then cleaved and 3'-end-labeled on the L-strand of the AuaI site designated in Fig. 4b. The 5'-end of the 98-nucleotide marker was generated by cleavage at the MspI site directly adjacent to the L-strand discontinuity.

In the untreated control (Fig. 8), a prominent L-strand fragment is observed. Its 5'-end lies 3 nucleotides past that of the 98-nucleotide marker and at the position expected for the L-strand nick. This fragment is absent after reaction with T4 DNA ligase confirming that its 5'-end indeed lies at a nick. Therefore, the L-strand nick can now be precisely localized. Its position with respect to the A region sequence is the same as for the L-strand nick within the B region (Fig. 9, b and c).

In addition to the nick fragment, a series of faint L-strand fragments are detected with 5'-ends lying mostly 6-7 nucleotides past the nick (arrowheads). These fragments are not affected by ligase treatment. However, they are absent after incubation with alkali and a new fragment is observed, terminating a little more than 1 nucleotide 5' of the nick. Thus, the structures of the A and B region L-strand discontinuities appear to be identical.

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| Enzyme | No. | T4 Lig. | T4 Pol. | E. Coli Lig. |
|--------|-----|---------|---------|-------------|
| MspI   |     |         |         |             |
| AvaII  |     |         |         |             |

**Fig. 5. Characterization of the 5'-ends of B region L-strand discontinuities.** Minicircle RF II DNA was treated with ligase where indicated. DNA samples in the two outside lanes are derived from minicircle RF IV DNA. All samples were cleaved with AvaII and 3'-end-labeled. Cleavage with MspI or incubation with alkali was performed after labeling. The samples were electrophoresed and autoradiographed as in Fig. 3. Fragments indicated by the arrowheads, and the 123- and 155-nucleotide marker fragments are discussed in the text.

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**Fig. 4. Characterization of 3'-ends of B region discontinuities.** Minicircle RF II DNA was treated as indicated above each lane and then cleaved at both MluI and XbaI sites and 5'-end-labeled. The samples were electrophoresed and autoradiographed as in Fig. 3. Fragments derived from heavy (H) or light (L) minicircle strands are indicated by the brackets. Panels a and b represent separate experiments.

A larger fragment is absent in DNA samples treated with T4 or E. coli DNA ligase, indicating that its 3'-end lies at a nick. The previously stated substrate specificities of these enzymes suggest that this nick represents a DNA-DNA junction. Within the resolution of this experiment, the L-strand nick in the A region occurs at the same nucleotide sequence as was observed for the B region (Fig. 9, b and c, respectively).

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In the untreated control (Fig. 8), a prominent L-strand fragment is observed. Its 5'-end lies 3 nucleotides past that of the 98-nucleotide marker and at the position expected for the L-strand nick. This fragment is absent after reaction with T4 DNA ligase confirming that its 5'-end indeed lies at a nick. Therefore, the L-strand nick can now be precisely localized. Its position with respect to the A region sequence is the same as for the L-strand nick within the B region (Fig. 9, b and c).

In addition to the nick fragment, a series of faint L-strand fragments are detected with 5'-ends lying mostly 6-7 nucleotides past the nick (arrowheads). These fragments are not affected by ligase treatment. However, they are absent after incubation with alkali and a new fragment is observed, terminating a little more than 1 nucleotide 5' of the nick. Thus, the structures of the A and B region L-strand discontinuities appear to be identical.
that the series of H-strand nicks are the result of a sequential closure of the nick on the right by both DNA-DNA or DNA-RNA junctions. Although their structure remains unknown, modified nucleotides may be present at these junctions. T4 DNA ligase seals all the H-strand nicks while E. coli DNA ligase seals only the nick on the right (Figs. 4 and 6). The inability of E. coli DNA ligase to seal the remaining nicks, and the resistance to alkaline hydrolysis of bonds formed at these nicks by T4 DNA ligase (data not shown), indicate that they do not represent simple DNA-DNA or DNA-RNA junctions. Although their structure remains unknown, modified nucleotides may be present at these junctions. Closure of the nick on the right by both DNA ligases is consistent with it being a DNA-DNA junction. The fact that this nick lies the furthest 3′ in the sequence suggests that the series of H-strand nicks are the result of a sequential 5′ to 3′ excision of modified nucleotides presumably involved in priming of H-strand DNA synthesis.

The sequence location and structure of L-strand discontinuities within the A and B regions are summarized in Fig. 9, b and c, respectively. Numbering of the nucleotides above the sequences is solely for reference. In region A, the L-strand discontinuity can be either a nick or a short gap. A molecule containing both a nick and a gap in region A is shown in Fig. 9b, but in reality such discontinuities would exist at separate sequences. Unlike the H-strand, the L-strand nick occurs at a single site (arrow). This nick is sealed by either T4 or E. coli DNA ligase and so appears to represent a DNA-DNA junction with a 3′-hydroxyl and 5′-phosphoryl group.

Molecules containing an L-strand gap have a 3′ terminus at nucleotide 20 and predominant 5′ termini at nucleotides 13 and 14. Most gaps, therefore, span 5–6 nucleotides, but for simplicity only molecules containing a 5-nucleotide gap are shown. T4 DNA polymerase elongates the 3′ terminus of the gap by 6–7 nucleotides indicating that the first 1–2 nucleotides at the 5′ terminus of the gap are strand displaced.

Alkaline hydrolysis causes the release of nucleotides from the 5′ terminus of the gap, down to and including nucleotide 9, generating a unique 5′-end that maps between nucleotides 8 and 9. The slightly retarded mobility of this DNA fragment on a sequencing gel could be due to the loss of its 5′-phospho-
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Fig. 8. Characterization of 5′-ends of A region L-strand discontinuities. DNA samples in all lanes are derived from minicircle RP II DNA. Treatment of the DNA samples is indicated above the lanes and described in the text. Cleavage with MspI or incubation with alkali was performed after cleavage with AucI and 3′-end labeling. The samples were electrophoresed and autoradiographed as in Fig. 3. Fragments designated by the arrowheads and 98-nucleotide marker fragment are discussed in the text.

rny group upon alkaline hydrolysis or to the presence of an otherwise unusual nucleotide at the 5′-end. Whether all nucleotide linkages in the 5′-terminal region are alkali-sensitive, or just the linkage between nucleotides 8 and 9, and that the transition from DNA to RNA occurs at the junction of nucleotides 8 and 9 and that the primer excision mechanism results in the removal of the first deoxynucleotide encountered (i.e. nucleotide 8).

A 12-nucleotide universal minicircle sequence found in all minicircles sequenced to date (from T. brucei (25), T. equiperdum (26), Leishmania tarentolae (27), T. lewisi (28), and C. fasciculata (15)) is designated by the boxed regions in Fig. 9, b–d. The L-strand nick, the primer, and the 5′ terminus of the gap all fall within this sequence. This conserved sequence has been shown to overlap a 10-nucleotide gap in a nascent DNA strand of minicircles from T. equiperdum (16). The 5′ terminus of the gap in this species corresponds precisely with the nick site in C. fasciculata, but the presence of a primer-like structure was not reported. This may reflect more efficient primer excision due to the difference in species or to the fact that minicircle replication intermediates analyzed from T. equiperdum were generated in vivo. More recent results indicate the presence of one or two ribonucleotides at the 5′ terminus of the gap in T. equiperdum (29). Our results indicate that the universal 12-nucleotide minicircle sequence is involved in initiation and termination of L-strand DNA synthesis in C. fasciculata.

A model consistent with all observed replication intermediates of the major class of minicircles in C. fasciculata strain Cf-C1 is presented in Fig. 10. The principal assumption, that site-specific discontinuities in nascent DNA strands coincide with origins of minicircle DNA replication, is strongly supported by results reported here and by Ntambi and Englund (16). In most aspects the model is analogous to the D-loop replication model proposed for unicellular dimeric mouse mtDNA (17). Such dimeric mtDNA molecules contain four replication origins, two on each strand, with origins on the same strand located 180° apart on the circular genome. This is very reminiscent of the minicircle structure in C. fasciculata. Furthermore, except for the discontinuous synthesis of the second strand (i.e. H-strand), replication intermediates analogous to those observed in minicircles are also observed in
The dimeric form of mouse mtDNA. These include site-specific discontinuities and strand-specific nascent chains of several distinct sizes (17).

We propose that replication begins by initiation of L-strand synthesis within region A or B (Fig. 10a, a'). Molecules containing newly initiated L-strands within both regions are most likely very rare or nonexistent. Elongation of the nascent L-strand displaces the parental L-strand, exposing the adjacent origin of H-strand synthesis. This allows initiation of H-strand DNA synthesis which is then elongated about 100 nucleotides to the site of L-strand initiation where further synthesis may be blocked by the duplex nature of the molecule. The displaced parental L-strand also serves as template for multiple, apparently random, secondary initiations of H-strand synthesis (Fig. 10b, b'). Priming of these Okazaki-like fragments would differ from the site-specific priming of H-strand synthesis in that their primers are readily excised and the fragments joined. As L-strand synthesis proceeds around the minicircle, the second origin of H-strand synthesis is uncovered. A second site-specific initiation of H-strand DNA synthesis occurs at this point, and the chain is elongated until it encounters and is joined to the 5'-end of one of the Okazaki-like H-strand fragments (Fig. 10c, c'). When L-strand synthesis nears completion, the daughter molecules segregate. Thus, multiple discontinuities are present in the nascent H-strand of one daughter molecule (Fig. 10d, e') whereas the other contains a single discontinuity at the origin of L-strand synthesis in the A or B region (Fig. 10d, d'). Complete joining of the Okazaki-like H-strand fragments results in an intermediate containing two site-specific H-strand discontinuities, one at each origin of H-strand synthesis (Fig. 10f). Excision of the primers and sealing of the H-strand breaks is not simultaneous but stepwise, generating molecules with a single H-strand discontinuity at the origin of H-strand synthesis in the A or B region (Fig. 10g, g'). The final step is the excision of the single remaining H- or L-strand primer and repair of the discontinuity, producing a newly replicated covalently closed minicircle.

This model is consistent with all observed site-specific discontinuities, and the highly discontinuous nature of H-strand synthesis. It also provides an explanation for the existence of minicircle replication intermediates containing half-length nascent H-strands. Electronmicroscopic analysis of replicating minicircles reveals θ-type structures (8, 30, 31). In some of these structures, one arm of the replication bubble appears to be at least partially single-stranded (8, 31). This type of structure is predicted by the model during early stages of replication. A few examples of what appear to be single-stranded minicircles have been observed in electronmicrographs of the free minicircle fraction (31). Such a structure is not predicted by our model, but could arise from a replicating molecule in which H-strand synthesis has not been primed, thereby displacing a single-stranded parental L-strand circle. Further characterization of these rare circles is needed to determine whether they are, in fact, completely single-stranded or are just extensively gapped double-stranded molecules. It is possible that the spreading and staining procedures used (31) may not clearly distinguish between these two alternatives.

It is not now what prevents the discontinuities in newly replicated minicircles from being closed before all molecules have been replicated and reattached to the network. However, the presence of what appear to be modified nucleotides within the primers at these sites may be involved.

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