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Kinetoplast DNA Replication

Al F. Torri, Laura Rocco Carpenter, and Paul T. Englund

Department of Biological Chemistry
Johns Hopkins University School of Medicine
Baltimore, Maryland 21205

The order Kinetoplastida is composed of several families of flagellated protozoa which are among the most ancient of the eukaryotes. The most prominent members of this order are parasites that are responsible for major tropical diseases. These parasites include *Trypanosoma brucei*, the African trypanosome; *Trypanosoma cruzi*, the South American trypanosome; and several species of *Leishmania*. Other kinetoplastids include *Crithidia fasciculata*, an insect parasite, and species of *Phytomonas*, which are parasites of plants. All of the kinetoplastids possess a distinctive disk-shaped structure, known as the kinetoplast, within their single mitochondrion. The kinetoplast is composed of the cell's mitochondrial DNA, known as kinetoplast DNA (kDNA). kDNA has a structure unlike that of any other DNA in nature. It consists of two types of circular DNA molecules, maxicircles and minicircles, which are topologically interlocked into a single, massive network. Figure 1 shows an electron micrograph of part of a kDNA network from *C. fasciculata* (for reviews on kDNA, see Ray 1987; Simpson 1987; Stuart and Feagin 1992; Shapiro and Englund 1995).

There is substantial information on the genetic function of maxicircles and minicircles. Maxicircles resemble conventional mitochondrial DNA from mammals or yeast, in that they encode rRNAs and proteins involved in mitochondrial energy transduction (such as cytochrome oxidase subunits). A unique feature of maxicircles is that many of their transcripts undergo RNA editing, a process by which uridine residues are inserted or deleted at specific sites (for review, see Hajduk et al. 1993). In some cases, editing occurs on a massive scale, with half of the nucleotides in the coding region of the mRNA being uridines introduced by editing. Minicircles play a crucial role in RNA editing, encoding small guide RNAs that control editing specificity.

In this review, we discuss kDNA structure and its replication mechanism, focusing primarily on the organism *C. fasciculata*. Currently, there is no system to perform kDNA replication in vitro and only a few

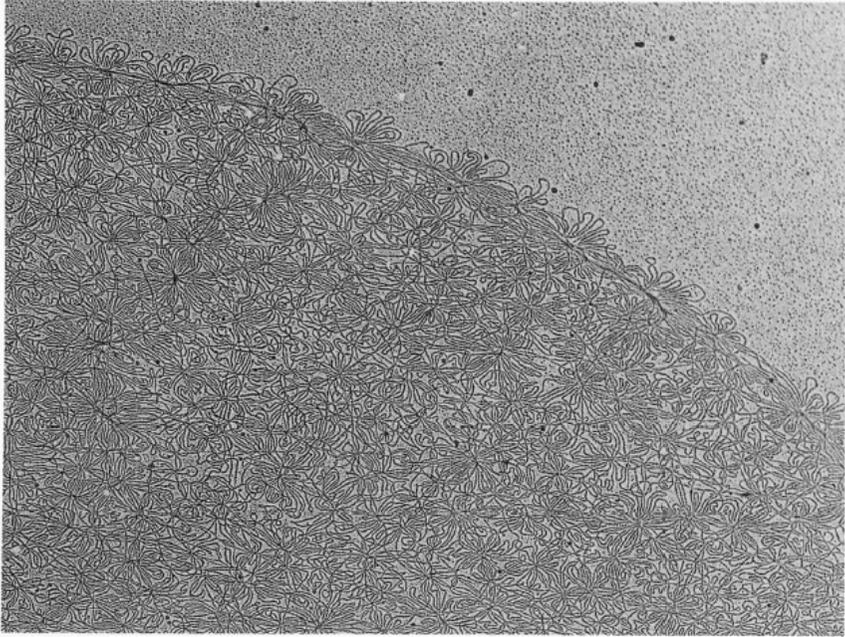


Figure 1 Part of a *C. fasciculata* kDNA network, shown by electron microscopy. Each small loop represents a 2.5-kb minicircle.

proteins involved in replication have been studied. Nevertheless, there is considerable information about the novel mechanism by which a DNA network undergoes replication, and this constitutes the major focus of this chapter (for reviews on kDNA replication, see Ray 1987; Shlomai 1994; Shapiro and Englund 1995).

STRUCTURE OF kDNA

A *C. fasciculata* network has about 25 maxicircles (38 kb) and 5000 minicircles (2.5 kb). Electron microscopy of isolated kDNA reveals that the network is a planar structure, about 10 μm by 15 μm in size, with an elliptical shape (Pérez-Morga and Englund 1993a). The minicircles are arranged in a monolayer, with a structure resembling that of chain mail (see diagram in Fig. 2A). The maxicircles are also catenated to the network, although it is not clear how they are organized. Treatment of the network with a topoisomerase II results in complete decatenation of the minicircles and maxicircles (Marini et al. 1980). In networks not undergoing replication, all of the minicircles are covalently closed (Englund 1978). However, unlike DNA circles in other cells, the mini-

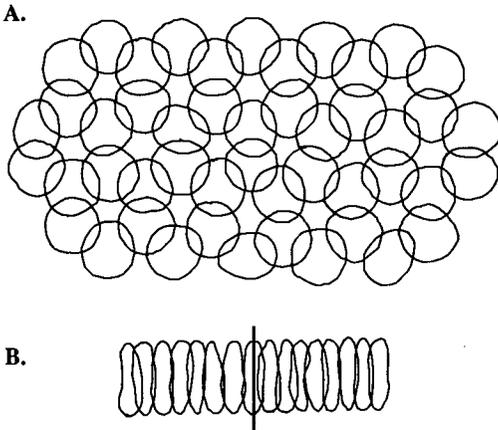


Figure 2 (A) Diagram of a segment of an isolated *C. fasciculata* kDNA network, in which each minicircle lies flat in a plane. (B) Diagram of a section through the kinetoplast disk in vivo (Delain and Riou 1969). Instead of lying in a plane as in A, each minicircle stands perpendicular to the plane. The vertical line shows the disk axis. (Reprinted, with permission, from Shapiro and Englund 1995.)

circles are relaxed rather than negatively supercoiled (Rauch et al. 1993). Minicircles in a *C. fasciculata* network not undergoing replication are each linked to an average of three neighbors (Chen et al. 1995). Unlike some other kinetoplastid parasites, which have a network with many different minicircle sequence classes, *C. fasciculata* networks have a single predominant minicircle class and only several minor classes (Sugisaki and Ray 1987).

Electron micrographs of thin sections and confocal fluorescence microscopy (using an acridine stain) indicate that in vivo the *C. fasciculata* network is condensed into a disk about 1 μm in diameter and 0.4 μm thick (Ferguson et al. 1992). Figure 2B shows a model for the organization of the network inside the mitochondrial matrix. The network in vivo is a monolayer, but in contrast to the isolated networks, in which minicircles lie in the plane of the network, the minicircles in vivo stand erect, perpendicular to the plane of the network. The minicircles stretch out and the strands run parallel to the disk's axis. The thickness of the kinetoplast disk is roughly half the circumference of an individual minicircle.

REPLICATION OF kDNA

During each cell cycle, every minicircle and maxicircle undergoes a single round of replication. In contrast to mitochondrial DNA in mam-

malian cells, which replicates throughout the entire cell cycle (Clayton 1982), kDNA replicates only during a discrete S phase, approximately coincident with the S phase of nuclear DNA (Cosgrove and Skeen 1970).

Network Replication

Problems in Replicating a DNA Network

One unique problem in replicating kDNA is topological. How do you replicate a minicircle that is interlocked to several neighbors? The kinetoplastid solves this problem by unlinking the minicircles from the network in a topoisomerase-mediated reaction (Englund 1979). The free minicircles can then undergo replication by a standard mechanism (discussed below), unhindered by the constraints of catenation. The network is never completely decatenated; instead there are several hundred free minicircles at any time during the S phase that are undergoing replication. After minicircle replication, the progeny are attached, in another topoisomerase reaction, to the network periphery. Another problem associated with kDNA replication is bookkeeping. How does the kinetoplastid keep track of which minicircle has replicated and which has not? The parasite solves this problem by maintaining nicks or gaps in minicircles after their replication (in this chapter we refer to these interruptions as "nicks") (Englund 1978). These distinguish minicircles that have replicated from those covalently closed molecules that have not. Covalent closure of the minicircles occurs late in the cell cycle, after all have undergone replication.

Complexes of Replication Proteins

Once minicircles are released from the network, presumably from random sites in the network's interior, they migrate to one of two protein complexes that are situated on opposite sides of the kinetoplast disk (see Fig. 3A). These complexes contain a topoisomerase II (Melendy et al. 1988), a DNA polymerase- β , and minicircles that are probably replication intermediates (Ferguson et al. 1992). They probably also contain the other enzymatic machinery needed for minicircle replication. Since the polymerase- β is likely involved in repair of gaps in newly replicated minicircles (see below), a replicative polymerase is probably also present in these structures. After replication, the nicked progeny minicircles are attached to the network adjacent to these complexes, as shown in Figure 3B.

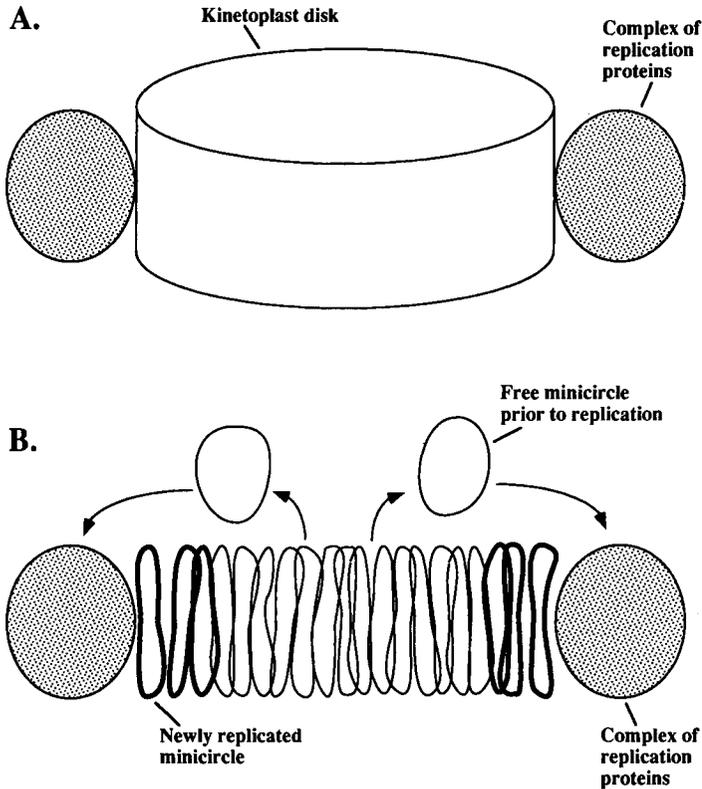


Figure 3 (A) Diagram of the kinetoplast disk, showing the two antipodal complexes of replication proteins. (B) Model for kDNA replication in vivo. The diagram shows a section through the kinetoplast disk. Minicircles in bold have undergone replication and are nicked. See text for details. (Reprinted, with permission, from Shapiro and Englund 1995.)

Structure of Networks Undergoing Replication

Visualization of partly replicated *C. fasciculata* networks either by electron microscopy after isolation (Pérez-Morga and Englund 1993a) or by fluorescence in situ hybridization (Ferguson et al. 1992) reveals that newly replicated minicircles, containing nicks, are uniformly distributed around the network periphery. Figure 4 shows networks at different stages of replication that had been labeled in vitro at their endogenous nicks with $[^3\text{H}]\text{dTTP}$ and DNA polymerase. The labeled DNA was spread on a grid, coated with photographic emulsion, and, after exposure and development, examined by electron microscope autoradiography (Pérez-Morga and Englund 1993a). The silver grains mark the location

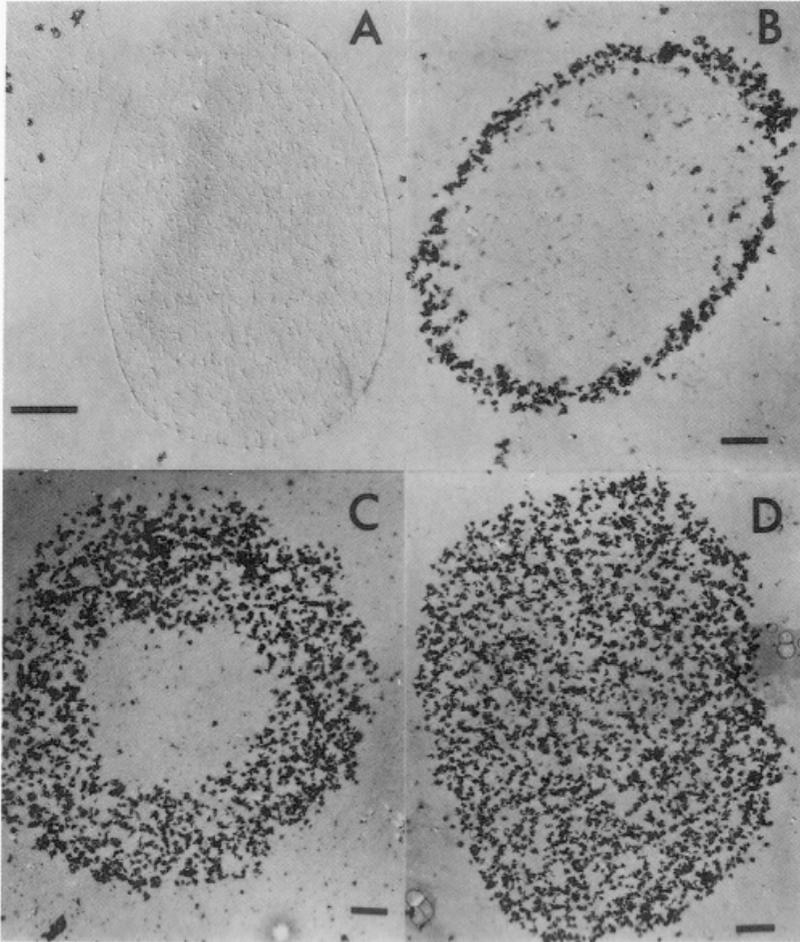


Figure 4 Electron micrographs of isolated kDNA networks at different stages of replication. The networks were radiolabeled with [^3H]dTTP at endogenous nicks using DNA polymerase and then subjected to autoradiography. The silver grains indicate the locations of nicked minicircles. (A) Prereplication network. (B) Network in an early stage of replication. (C) Network in a later stage of replication. (D) Fully replicated network with all minicircles nicked. Bars, 2 μm . (Reprinted, with permission, from Pérez-Morga and Englund 1993a [copyright Rockefeller University Press].)

of nicked minicircles. Panel A shows a prereplication network containing 5000 minicircles; there are no silver grains because the minicircles are all covalently closed. Panel B shows a network in an early stage of replication. The silver grains are distributed in a narrow and uniform ring

around the network's periphery. Panel C shows a later stage, resembling a donut, with a thicker ring and a smaller unlabeled region in the center (the donut hole). Finally, in Panel D, the network is uniformly labeled. At this stage the network contains 10,000 minicircles, double the number that were present in the prereplication network, and all are nicked. The further processing of this double-size network is discussed below.

The Spinning Kinetoplast Model

The uniform distribution of nicked minicircles around the network periphery presented a dilemma. If minicircles are attached to the network adjacent to the two protein complexes, how do they become distributed around the network periphery? This question has been addressed by autoradiography of *C. fasciculata* networks labeled *in vivo* with pulses of [³H]thymidine (Pérez-Morga and Englund 1993b). In cells labeled for 1–3 minutes, silver grains were concentrated in two peripheral positions, on opposite sides of the network (Simpson and Simpson 1976; Pérez-Morga and Englund 1993b), exactly as expected if minicircle attachment occurred adjacent to the two protein complexes (see example in Fig. 5A). In cells labeled for longer periods (20–60 min), the silver grains were distributed around the network periphery, forming donut-shaped structures (not shown). A clue to the mechanism by which minicircles are distributed around the network came from inspection of networks labeled for 6 minutes (Pérez-Morga and Englund 1993b). As shown in Figure 5B, the silver grains were concentrated in two zones, but together they nearly filled the entire periphery. Surprisingly, the silver grains in both zones form a density gradient. This gradient is due to the rising specific radioactivity of the dTTP during the 6-minute period of the labeling, as the exogenous [³H]thymidine mixes with the internal pool of DNA precursors. The presence of two labeling zones is consistent with the labeled minicircles being attached at two sites. The fact that the silver grains have approximately the same density on opposite sides of the network indicates that the two minicircle attachment sites are always positioned opposite each other. The gradient of silver grains indicates that minicircles are attached sequentially around the network periphery. This result led to the surprising conclusion that there is a relative movement of the kinetoplast disk and the two protein complexes. The kinetoplast disk could be fixed, and the complexes could move around its periphery. Alternatively, the complexes could be fixed, and the kinetoplast disk could rotate between them (see model in Fig. 6). Although much more information is needed before either model can be

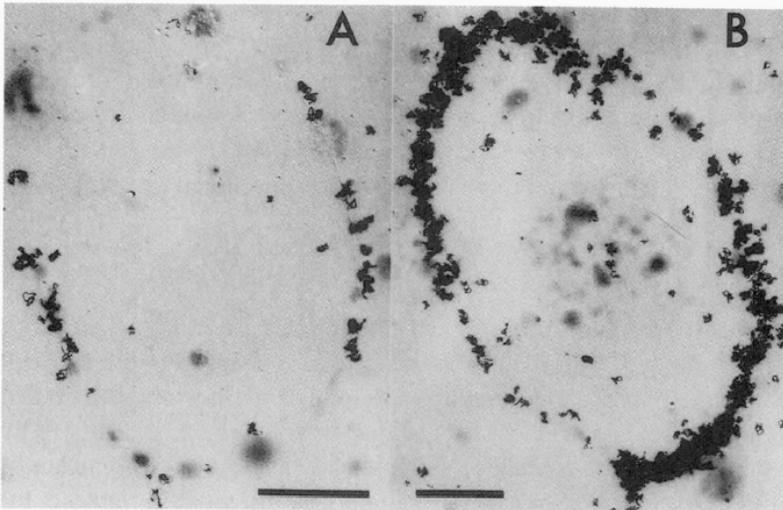


Figure 5 Electron micrographs of kDNA networks labeled *in vivo* with [^3H]thymidine. (A) 3-min label. (B) 6-min label. After isolation the networks were subjected to autoradiography. Bars, 3 μm . (Reprinted, with permission, from Pérez-Morga and Englund 1993b [copyright Cell Press].)

proven, some preliminary findings favor the latter possibility (Pérez-Morga and Englund 1993b). We assume that the spinning kinetoplast model is correct for the rest of this discussion. Since nearly the entire network periphery is labeled in 6 minutes, the kinetoplast must rotate approximately once every 12 minutes. Presumably, it rotates continuously in the same direction and at the same rate, allowing minicircle attachment in a spiral pattern. It probably takes about seven turns of the kinetoplast disk to replicate the entire network.

The Final Stages of Network Replication

At the end of S phase the network contains 10,000 minicircles, twice the number prior to replication, and all are nicked. At this time the nicks are repaired, and all the minicircles become covalently closed (Pérez-Morga and Englund 1993a). Then the structure splits in two. This scission is probably mediated by a topoisomerase that unlinks neighboring circles between the two nascent progeny networks, but how this reaction is controlled is completely unknown. The progeny networks, each identical to the parent, then segregate into the two daughter cells.

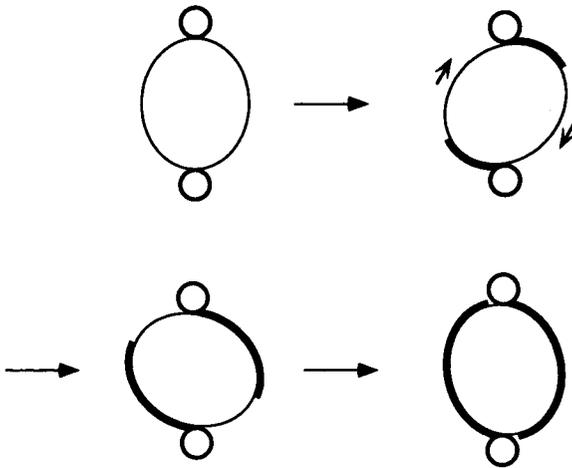


Figure 6 Model showing the rotation of the *C. fasciculata* kinetoplast *in vivo*. The kinetoplast is depicted as an ellipse and the two complexes of replication proteins as small circles. The bold lines represent a row of newly replicated minicircles. Small arrows indicate the direction of rotation of the kinetoplast. See text for discussion. (Reprinted, with permission, from Pérez-Morga and Englund 1993b [copyright Cell Press].)

Replication Mechanism for Minicircles and Maxicircles

Minicircles

Minicircle replication has been studied extensively in *C. fasciculata* and *Trypanosoma equiperdum*. Replication begins after the covalently closed minicircle is decatenated from the network, and it is thought to occur in one of the two protein complexes (discussed above). Minicircle replication occurs via θ intermediates and is unidirectional (Englund 1979; Ryan and Englund 1989b). Leading-strand synthesis initiates by RNA priming complementary to the sequence GGGGTTGGTGTA, a sequence found with only minor variation in a conserved region of minicircles from all kinetoplastids that have been examined (Ntambi et al. 1986; Birkenmeyer et al. 1987). *C. fasciculata* has two such sequences, positioned 180° apart, and either can serve as the site for the initiation of replication (Birkenmeyer et al. 1987). A second sequence, usually ACGCCC, is located 70–90 nucleotides downstream from the origin; the first Okazaki fragment initiates complementary to this 6-mer (Ryan and Englund 1989a). In both *C. fasciculata* and *T. equiperdum*, the leading strand is synthesized continuously around the molecule and the lagging-strand Okazaki fragments are roughly 100 nucleotides in size (Kitchin et

al. 1984; Birkenmeyer and Ray 1986; Birkenmeyer et al. 1987; Ryan and Englund 1989a). Nothing is known about the priming mechanism either for the leading strand or for the Okazaki fragments, and, in fact, RNA primers have not yet been detected on the Okazaki fragments. As mentioned above, covalent closure of the progeny minicircles occurs only after all minicircles have replicated. A unique gap opposite the GGGGTTGGTGTA sequence in the continuously synthesized strand and discontinuities flanking the first Okazaki fragment are the last interruptions to be repaired (Birkenmeyer et al. 1987; Ryan and Englund 1989a,b).

Maxicircles

Recent studies, involving electron microscopy of *C. fasciculata* maxicircles released from networks by restriction enzyme cleavage, revealed branched molecules derived from replication intermediates. Analysis of these molecules indicated that maxicircle replication initiates at a unique site and proceeds unidirectionally as a θ -structure (Rocco Carpenter and Englund 1995). The maxicircle replication origin is within the "variable region," a noncoding segment of the molecule that contains many repetitive sequences. Maxicircles and minicircles replicate simultaneously. However, in contrast to minicircles, the maxicircles replicate while still linked to the network (Hajduk et al. 1984).

Enzymes and Proteins Involved in kDNA Replication

Little is known about the enzymes and proteins involved in the replication of kDNA, and the lack of an *in vitro* replication system has complicated the search for these proteins. In this section we describe a few well-characterized proteins implicated in kDNA replication.

DNA Polymerase- β

An unusual DNA polymerase has been purified from a *C. fasciculata* mitochondrial fraction (Torri and Englund 1992) and shown by immunofluorescence to localize to the two protein complexes adjacent to the kinetoplast (Ferguson et al. 1992). On the basis of the recovery of activity during purification, this enzyme is present in roughly 60,000 copies per cell. This polymerase is small (42 kD), nonprocessive, deficient in proofreading exonuclease activity, and error-prone (Torri et al. 1994). It prefers a DNA substrate with small gaps. These properties differ from those expected for a conventional mitochondrial DNA polymerase- γ and

are instead typical of a DNA polymerase- β (pol- β). Pol- β has previously been found only in the nucleus of eukaryotic cells and is thought to be involved in DNA repair. Analysis of the *C. fasciculata* mitochondrial DNA polymerase sequence reveals similarities to human pol- β ; however, the mitochondrial enzyme possesses a cleaved amino-terminal pre-sequence that closely resembles mitochondrial import signals on other *C. fasciculata* proteins (Torri and Englund 1995). Therefore, this *C. fasciculata* enzyme is the first example of a mitochondrial pol- β . This enzyme may have been specially imported into the mitochondrion to assist in repairing the many small gaps between Okazaki fragments in newly replicated minicircles. The fact that this is a repair polymerase implies that the true replicative enzyme, which may resemble a polymerase- γ , remains to be discovered.

Topoisomerases

Two type II topoisomerases have been identified in the mitochondrion of *C. fasciculata*. One of these enzymes, containing four 60-kD subunits (Shlomai and Zadok 1983; Shlomai et al. 1984), appears to be localized within the kinetoplast disk (Shlomai 1994). From its intracellular location, this enzyme could be involved in releasing covalently closed minicircles from the network so that they can be replicated. The other topoisomerase, a dimer of 132-kD subunits (Melendy and Ray 1989), localizes to the two protein complexes (Melendy et al. 1988). This topoisomerase II most likely facilitates minicircle replication, and it could be responsible for attaching the newly replicated nicked minicircles onto the network periphery.

Origin-binding Protein

A *C. fasciculata* single-strand-specific DNA-binding protein interacts specifically with the GGGGTTGGTGTA sequence that is part of the minicircle origin of replication (see above) (Tzfati et al. 1992). It is a homodimeric protein, with 13.5-kD subunits, and its sequence predicts five CCHC zinc fingers (Abeliovich et al. 1993). This protein may be involved in replication initiation, binding to the origin after strand separation. In addition, it could function after the minicircle has completed replication, binding to and preserving the unique gap in the newly synthesized leading strand. In this way it would prevent covalent closure and ensure that the minicircle replicated only once per generation. Removal of this protein after all minicircles have replicated would then allow gap repair and covalent closure. The localization of this protein within the kinetoplast has not yet been reported.

Other kDNA-binding Proteins

Although they are probably not involved directly in kDNA replication, a family of highly basic proteins that could be involved in condensing and organizing kDNA in vivo has recently been reported. These proteins, 15 kD to 21 kD in size, were isolated by reversible cross-linking to *C. fasciculata* kDNA in vivo using formaldehyde (Xu and Ray 1993). One of these proteins, p16, has been shown by immunoelectron microscopy to localize within the kinetoplast disk (D. Ray, pers. comm.).

CONCLUSION

Although many features of the replication of the kDNA network are now understood, there are important issues that are still unexplored. One concerns the spinning kinetoplast. Does the kinetoplast really rotate, and if it does, what mechanisms are responsible? A second issue concerns the nature of the two complexes of replication proteins. Are all proteins required for minicircle replication present in these complexes, and how are they organized? Are the complexes permanent fixtures of the cell or do they assemble only during the S phase? Can we isolate them intact? These questions provide exciting opportunities for future investigation.

ACKNOWLEDGMENTS

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