Kinetoplast DNA Replication: Mechanistic Differences between *Trypanosoma brucei* and *Crithidia fasciculata*

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Abstract. Kinetoplast DNA, the mitochondrial DNA of trypanosomatid parasites, is a network containing several thousand minicircles and a few dozen maxicircles. We compared kinetoplast DNA replication in *Trypanosoma brucei* and *Crithidia fasciculata* using fluorescence in situ hybridization and electron microscopy of isolated networks. One difference is in the location of maxicircles in situ. In *C. fasciculata*, maxicircles are concentrated in discrete foci embedded in the kinetoplast disk; during replication the foci increase in number but remain scattered throughout the disk. In contrast, *T. brucei* maxicircles generally fill the entire disk. Unlike those in *C. fasciculata*, *T. brucei* maxicircles become highly concentrated in the central region of the kinetoplast after replication; then during segregation they redistribute throughout the daughter kinetoplasts. *T. brucei* and *C. fasciculata* also differ in the pattern of attachment of newly synthesized minicircles to the network. In *C. fasciculata* it was known that minicircles are attached at two antipodal sites but subsequently are found uniformly distributed around the network periphery, possibly due to a relative movement of the kinetoplast disk and two protein complexes responsible for minicircle synthesis and attachment. In *T. brucei*, minicircles appear to be attached at two antipodal sites but then remain concentrated in these two regions. Therefore, the relative movement of the kinetoplast and the two protein complexes may not occur in *T. brucei*.

KINETOPLAST DNA (kDNA)[1] is the mitochondrial DNA in trypanosomes and related parasitic protozoa. kDNA has a highly unusual structure, consisting of a network of topologically interlocked circles. There is one network within each cell's single mitochondrion. Each network contains several thousand minicircles (2.5 kb in *Crithidia fasciculata* and 1.0 kb in *Trypanosoma brucei*) and a few dozen maxicircles (37 kb in *C. fasciculata* and 20 kb in *T. brucei*). Maxicircles encode rRNAs and mitochondrial proteins involved in energy transduction (such as subunits of cytochrome oxidase); their transcripts undergo extensive RNA editing. Minicircles encode small guide RNAs which control the specificity of editing. See references 2, 24, 27, 30, and 32 for reviews on kDNA.

Isolated networks, as viewed by EM, usually appear as elliptically shaped sheets of interlocked DNA rings. Nonreplicating networks in *C. fasciculata* are ~10 by 15 μm in size (22) while those in *T. brucei* are smaller (see below). Inside the cell the network is condensed into a disk-like structure, with the disk oriented perpendicular to the axis of the flagellum. In *C. fasciculata* the disk (when not replicating) is ~1 μm in diameter and 0.3 μm thick (11) and in *T. brucei* it is ~0.6 μm in diameter (see below) and 0.1 μm thick (measured from electron micrographs in reference 4).

kDNA replication in *C. fasciculata* occurs during a discrete S phase (5). During replication covalently closed minicircles are released from the central region of the network, by topoisomerase action, and they are thought to migrate to one of two complexes of replication proteins situated on opposite sides of the kinetoplast disk (11, 20). After replication of the free minicircles within a complex, their progeny, which contain nicks or small gaps (in this paper we shall refer to both types of interruptions as "nicks"), are attached to the network adjacent to each complex. Therefore, minicircle attachment occurs at two discrete sites on the network periphery (21, 29). However, EM analysis (22) and fluorescence in situ hybridization (11) indicate that newly replicated minicircles become uniformly distributed around the network periphery. To explain this paradox, we have provided evidence for a relative movement of the kinetoplast disk and

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[1] *Abbreviations used in this paper*: CCD, charged-coupled device; kDNA, kinetoplast DNA.

the two complexes of replication proteins; the disk may rotate between the two complexes (21). As a consequence of this movement, the partially replicated networks resemble donuts, with nicked minicircles on the periphery and covalently closed minicircles (forming the donut hole) in the center. As replication proceeds the central zone shrinks, the peripheral zone enlarges, and the network overall grows in size. When it finally reaches double size and consists exclusively of nicked minicircles, the nicks are then repaired and the network splits in two (22). At cell division, the two progeny networks partition into the two daughter cells. Less is known about *C. fasciculata* maxicircle replication. They replicate simultaneously with minicircles, and the partially replicated maxicircles resemble rolling circles which remain linked to the network. When replication of a maxicircle is complete, the branch of the rolling circle is cleaved off. After circularization, the free maxicircle reattaches to the network (13).

Replication of *T. brucei* kDNA has been studied much less extensively. As in *C. fasciculata*, kDNA replication occurs only during an S phase (36). Biochemical analysis of *T. equiperdum*, an African trypanosome closely related to *T. brucei*, has revealed that the mechanism of replication of free minicircles is similar to that in *C. fasciculata* (reviewed in reference 27). EM analyses of isolated *T. brucei* networks undergoing replication revealed a high frequency of dumbbell shaped double size networks (10, 15). In the dumbbell forms most of the maxicircles are clustered in the network center. Dumbbell-shaped dividing networks and centrally clustered maxicircles have never been detected in isolated networks from *C. fasciculata*.

We have now compared the mechanism of kDNA replication in *T. brucei* and *C. fasciculata* using fluorescence in situ hybridization (with minicircle and maxicircle probes) and EM of isolated networks. We found important differences in the organization of maxicircles within the network and in the location of newly synthesized minicircles in partially replicated networks.

Materials and Methods

Fluorescence In Situ Hybridization

Log phase *C. fasciculata* were obtained from brain heart infusion cultures at 27°C (8). Log phase procyclic *T. brucei rhodesiense* (YTaT 1.1, a gift of Dr. Elisabetta Ullu) were isolated from cultures at 27°C (35). All of the conditions for fixation, hybridization, and detection of the probe were described previously (11). Briefly, the cells were fixed in PBS containing 3.5% paraformaldehyde and 0.5% glutaraldehyde and then permeabilized with Triton X-100. After mounting the cells on slides, the kinetoplast disk was reoriented by proteinase K treatment in the presence of 0.5% SDS so that its flat surface was parallel to the slide (11). After denaturation of the probe and target DNA, hybridization, and washing, the probe was detected by avidin-FITC or anti-digoxigenin-rhodamine. The final wash solution contained DAPI.

Probes

The *C. fasciculata* minicircle probe was a gel-purified 2.5-kb *XhoI* fragment of isolated kDNA networks (*XhoI* cleaves nearly all *C. fasciculata* minicircles once). The *C. fasciculata* maxicircle probes, a gift of Dr. Hans van der Spek (University of Amsterdam, Amsterdam, The Netherlands), were three clones representing different regions of the maxicircle (delimited by *HindIII*$_3$-*EcoRI*$_3$, *EcoRI*$_3$-*EcoRI*$_4$, and *HindIII*$_5$-*HindIII*$_1$; see reference 14 for map). The latter clone, containing the maxicircle variable region, had undergone rearrangement and deletion of a ~5-kb segment; together these clones cover approximately half of the total maxicircle sequence. The *T. brucei* minicircle probe, a gift of Laura J. Rocco (Johns Hopkins Medical School, Baltimore, MD), was derived from a plasmid, pTE1012, which contains a complete *T. equiperdum* minicircle (Pasteur Institute strain; BoTat 24 [1]). The minicircle was cleaved at its single *BstBI* site and cloned in the plasmid YIp5. This minicircle has a "conserved region" of ~125 bp which is nearly identical to that found in all *T. brucei* minicircles. Maxicircle probes for *T. brucei*, a gift from Dr. Ken Stuart (Seattle Biomedical Research Institute, Seattle, WA), were generated from three plasmids, pTKH38, pTKH128, and pTKHR34, containing ~16.3 kb of sequence (33).

DNA probes were labeled by nick translation with biotin (Sigma Chem. Co., St. Louis, MO or BRL, Gaithersburg, MD) or digoxigenin (Boehringer Mannheim Corp., Indianapolis) modified dUTP using standard protocols (3). It was important to tailor reaction conditions to produce probes less than 500 nucleotides in length; longer probes resulted in nonspecific signals.

Image Acquisition and Processing

Fluorescent images were acquired with a Photometrics PM512 cooled charged-coupled device (CCD) camera attached to a Zeiss Axioskop. This camera has a 512 × 512 grid of pixels onto which all images were projected. Image magnification was adjusted to allow approximately one entire trypanosome cell to be imaged on that grid. The same magnification was used for all imaging. More specifically, each image resulted from an area of 10 × 10 μm being projected onto the 512 × 512 pixel grid of the CCD. This corresponds to a distance of 0.02 μm per pixel, which is well below the limits of optical resolution. All input images were stored as 8 bit gray scales. The lowest 10 intensity values were set to black to eliminate any background. A one to one correspondence of all source image pixels to final image pixels was maintained during image manipulation.

Electron Microscopy of Isolated Networks

Logarithmically growing *T. brucei* (ILTat 1.3), obtained as a buffy coat from whole blood of an infected rat (7), were provided by Jayne Raper. kDNA was isolated from ~10^8 trypanosomes using the small scale method which we had developed for *C. fasciculata* (21). kDNA was spread on a grid using a microtechnique (23) which is a modification of the formamide method (6). Ethidium bromide (500 μg/ml) was added to both the spreading solution and the hypophase. The grids were analyzed using a Zeiss 10 A/B high resolution electron microscope. Exact magnifications were determined using a diffraction grating replica (2190 lines/mm).

Results

Localization of C. fasciculata Maxicircles in Kinetoplasts In Situ

We had previously used fluorescence in situ hybridization with a minicircle probe to examine the changes in structure of *C. fasciculata* kDNA during replication in situ (11). This technique was especially informative because nicked (already replicated) minicircles hybridized efficiently, whereas covalently closed minicircles (not yet replicated) hybridized little or not at all. We had demonstrated that prereplication kinetoplasts contained exclusively covalently closed minicircles which do not hybridize. During replication, the kinetoplasts resembled donuts because all of the nicked (hybridizing) minicircles were localized around the disk's periphery. After replication, they contained exclusively nicked minicircles, and therefore the entire structure was fluorescent. All of these forms are shown, as controls, in Fig. 1; DAPI fluorescence is shown in the upper image and minicircle hybridization fluorescence is shown in the central image in each panel.

We now have used probes to localize maxicircles within the kinetoplast disk and to determine if there are changes in their organization during replication. Maxicircle hybridization differed strikingly from that of minicircles in that it was concentrated in discrete foci (Fig. 1, lower images). Prior to replication there are ~8–10 foci (Fig. 1 *A*), and during replication the number of foci seems to increase (*B* and *C*). We

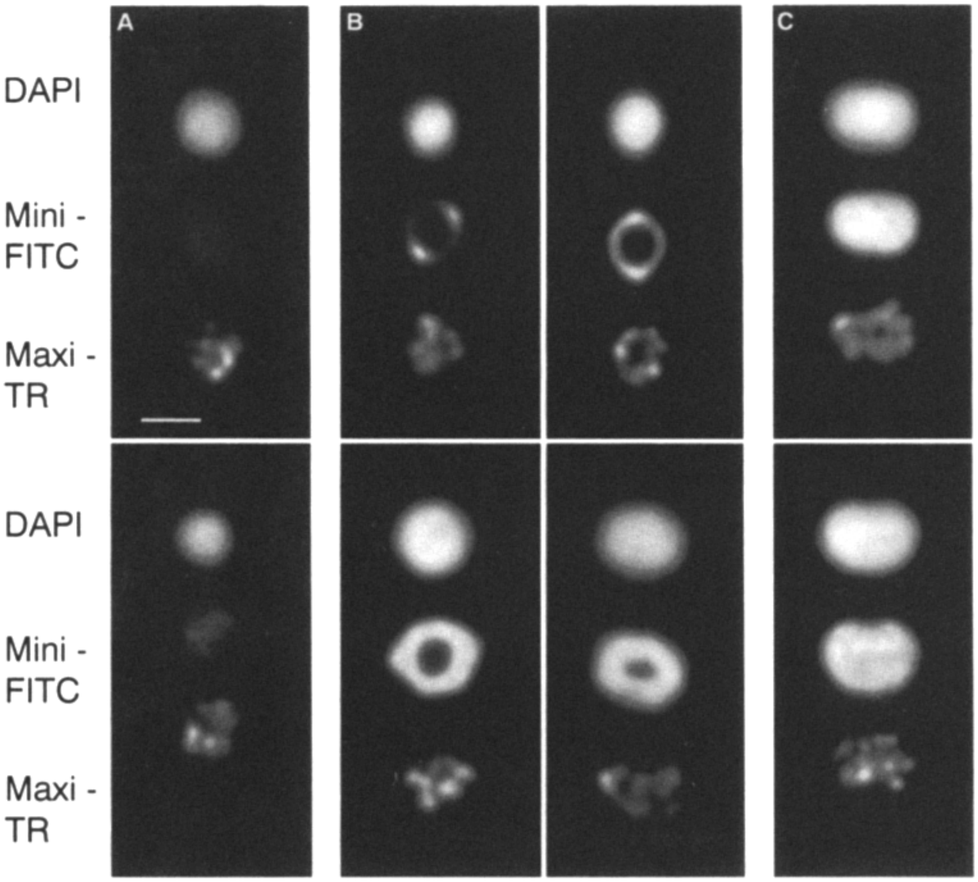


Figure 1. Visualization of kinetoplasts in fixed *C. fasciculata* cells. Kinetoplasts were stained with DAPI (upper image in each group), and also visualized by hybridization with a minicircle probe detected with fluorescein (FITC, middle image in each group) and a maxicircle probe detected with Texas red (*TR*, lower image on each group). (*A*) prereplication kinetoplasts. Because of the very low signal detected by minicircle hybridization, these images were obtained by allowing the CCD camera to accumulate photons 15 times longer than for those in *B* and *C*. (*B*) replicating kinetoplasts. (*C*) postreplication kinetoplasts. Bar, 1 μm.

could discern no consistent arrangement of the maxicircle fluorescence, except that all of the maxicircle probe seemed to hybridize to targets embedded within the kinetoplast disk. We used three different probes, representing different regions of the maxicircle, which together cover about half of the total sequence. The results from each probe were identical except that the signal intensity was proportional to the length of the probe. For the images depicted in Fig. 1, we pooled the three probes.

Efficient hybridization to minicircles requires nicking of the target DNA, and the same could be true for maxicircles. However, if we treated the fixed cells with DNase I (under conditions in which the minicircles in prereplication kinetoplasts develop maximum fluorescence [11]), there was no significant change in maxicircle fluorescence. Therefore, either the maxicircles are naturally nicked or are nicked during preparation of the cells for microscopy; alternatively, nicking may not be required to achieve maximum hybridization of these highly AT-rich 37-kb molecules.

Fluorescence In Situ Hybridization of T. brucei Kinetoplasts

We next analyzed kinetoplasts from an asynchronous culture of exponentially growing procyclic forms of *T. brucei* (Fig. 2). As with *C. fasciculata*, we hybridized the fixed cells with a rhodamine-labeled maxicircle probe (Fig. 2, lower images) and a fluorescein-labeled minicircle probe (Fig. 2, middle images). We also stained each kinetoplast with DAPI (Fig. 2, upper images). Each set of images represents a single kinetoplast, and they are arranged in a sequence to illustrate the changes in kinetoplast structure which occur during replication. We ordered the images both by the size of the DAPI images (showing the apparent division of the kinetoplast in the later stages) and by the pattern of minicircle fluorescence. In *A–G* there were increasing amounts of minicircle fluorescence (corresponding to increasing quantities of nicked minicircles generated during replication); in *H* and *I* there was much less minicircle fluorescence (due to repair of the nicked minicircles at the conclusion of replication).

A shows a *T. brucei* prereplication kinetoplast. The structure stains uniformly with DAPI, but there is only a small amount of minicircle hybridization because these circles are predominantly covalently closed. Maxicircle hybridization differs from that in *C. fasciculata*. Instead of being organized in multiple discrete foci, maxicircles are present in a mass which is distributed throughout the kinetoplast disk; in some images a slight heterogeneity in maxicircle fluorescence (*e.g.*, Fig. 2, *A*, *B*, and *C*) raises the possibility of foci which are too tightly packed to be optically resolved. Fig. 2, *B*, *C*, and *D* show kinetoplasts which appear to be from relatively early to mid S phase. Fluorescent (nicked) minicircles are concentrated in two enlarging antipodal zones, but the maxicircles are still distributed throughout most of the disk. Fig. 2 *E* shows a kinetoplast whose minicircles are nearly all replicated, as almost the entire structure hybridizes with the minicircle probe; in this kinetoplast the maxicircles are beginning to concentrate in the central region. Fig. 2 *F* shows a fully replicated kinetoplast in which all of the maxicircles have converged in the center between the two daughter

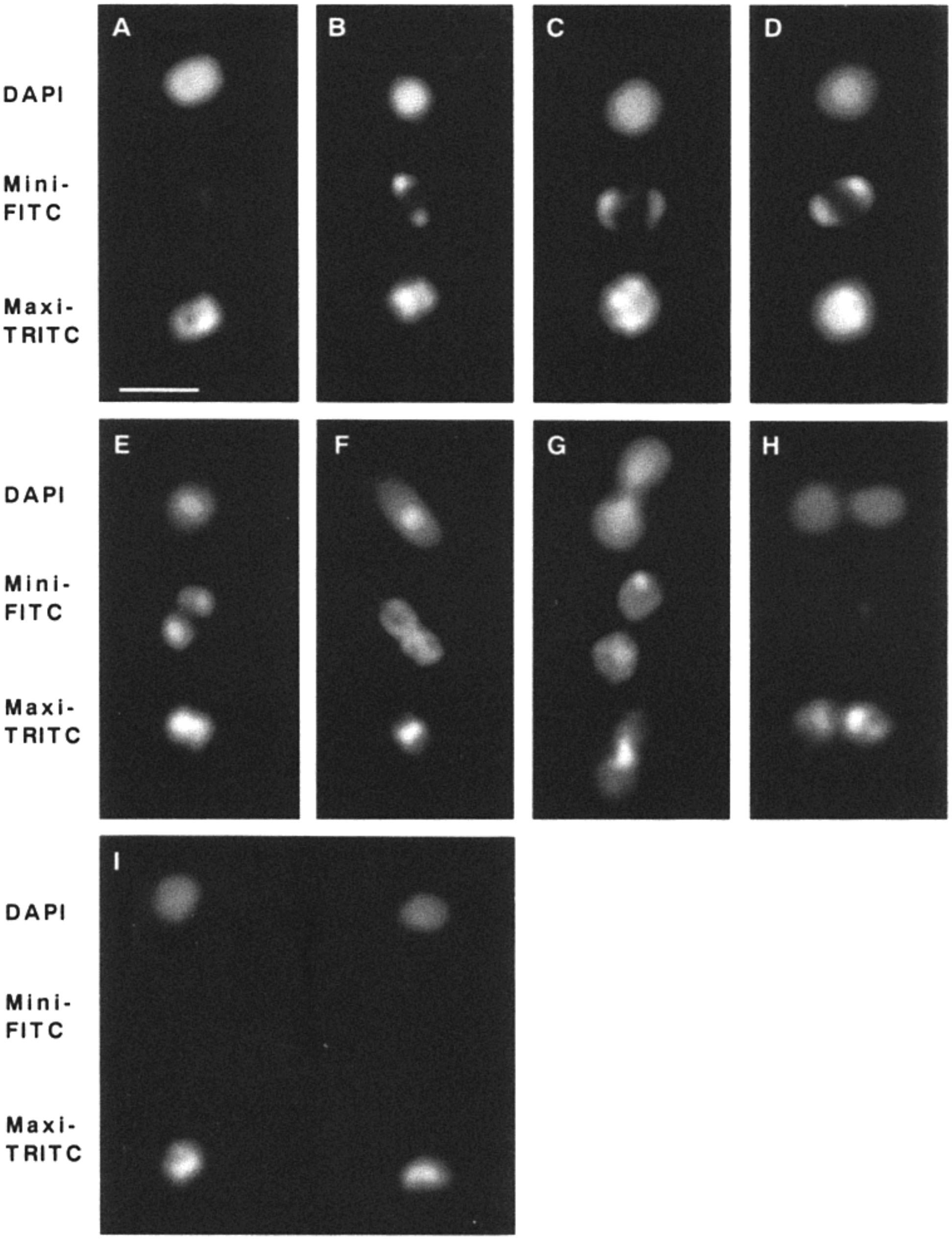


Figure 2. Visualization of kinetoplasts in fixed *T. brucei* cells. Kinetoplasts were stained with DAPI (upper image in each panel), and also visualized by hybridization with a minicircle probe detected with fluorescein (FITC, middle image in each panel) and a maxicircle probe detected with tetramethyl rhodamine (TRITC, lower image in each panel). *A* shows a kinetoplast prior to replication and subsequent panels show successive stages in the replication process. *I* shows two sister kinetoplasts soon after division; they are in a cell which still has a single nucleus. Bar, 1 μm.

kinetoplasts. Fig. 2 *G* shows a kinetoplast which is clearly beginning to divide, as shown by both the DAPI stain and the minicircle fluorescence; in this structure the maxicircles appear to be redistributing into the daughter kinetoplasts. Fig. 2 *H* shows another dividing kinetoplast in which the minicircle fluorescence has essentially disappeared, due to repair of nicks in the replicated molecules. Fig. 2 *I* shows sister kinetoplasts in a cell from a later stage in the cell cycle; virtually all of their minicircles are repaired and their maxicircles localize throughout the kinetoplasts.

In another *T. brucei* preparation which we assayed with a probe for minicircles but not for maxicircles, we measured the number of kinetoplasts in each stage of replication. About 64% were prereplication (resembling the kinetoplast in Fig. 2 *A*); ∼23% were in stages undergoing replication (corresponding to the kinetoplasts in *B–F*); ∼8% were dividing forms but with minicircles still nicked (resembling the kinetoplast in *G*); and ∼5% were at a later stage of division, in which minicircle nicks had been mostly repaired (resembling those in *H* and *I*).

There is a striking difference in the distribution of minicircle fluorescence in replicating kinetoplasts between *T. brucei* and *C. fasciculata.* In *C. fasciculata* 42% of kinetoplasts in a log phase population, as visualized by minicircle hybridization, resemble donuts ([11], see examples in Fig. 1 *B*). The donut-shaped structures are partly replicated kinetoplasts in which nonhybridizing covalently closed minicircles are concentrated in the center, forming the "donut hole." The same donut configuration was detected in EMs of isolated replicating networks (8, 22). Unexpectedly, we found few donut-shaped kinetoplasts in *T. brucei.* The few examples which we did observe (not shown) may resemble donuts because the two antipodal zones of hybridization are too close for optical resolution. In this regard, it is important that in a previous EM study of isolated *T. brucei* kDNA networks there were no donut-shaped structures reported (15), even under conditions in which we readily detected them in isolated *C. fasciculata* kDNA (22). Because the possible absence of donut-shaped kinetoplasts in *T. brucei* raises the possibility of profound differences in the mechanism of kDNA replication between the two parasites, we decided to study this point thoroughly. We examined isolated *T. brucei* networks by EM with the specific goal of searching for donut-shaped networks. These studies are described in the following paragraph.

EM Analysis of Isolated T. brucei Networks

We photographed 168 randomly chosen kDNA networks isolated from log phase *T. brucei*. To distinguish covalently closed from nicked minicircles, we spread the networks on the grid in the presence of 500 μg/ml ethidium bromide, a dye which twists covalently closed minicircles but not nicked minicircles. Fig. 3 *A*, shows a prereplication kinetoplast in which virtually all the minicircles are twisted, and therefore covalently closed; 58% of the networks were of this type. Fig. 3 *B* shows a network in an early stage of replication; covalently closed (twisted) minicircles are concentrated in the center and nicked (relaxed) minicircles are located in two noncontiguous peripheral zones, on opposite sides of the network. Fig. 3 *C* shows a later stage; again, the nicked minicircles are in two separate zones on opposite sides of the network and the covalently closed minicircles are concentrated in a smaller central zone. Fig. 3 *D* shows an even later stage, with two large lobes of nicked circles and a small central zone of closed circles. Partly replicated networks resembling those in Fig. 3 *B*, *C*, and *D* constituted ~17% of the total. Fig. 3 *E* shows a dumbbell-shaped network, with virtually all of its minicircles nicked and with the maxicircles concentrated in the central region. Networks of this type constituted ~21% of the total. Fig. 3 *F* shows a dumbbell form in which many of the minicircles are covalently closed, indicating that minicircle repair occurs before the final scission of the network; these types constituted ~4% of the total. In all of the partly replicated networks examined, we saw no examples of the donut shaped structures which are so common in replicating kDNA from *C. fasciculata* (22).

Discussion

We have provided for the first time information on the organization of maxicircles within the kinetoplast disk in situ. In *C. fasciculata* maxicircle hybridization occurs in discrete foci distributed more or less randomly throughout the kinetoplast disk (Fig. 1). There are no striking changes in maxicircle distribution in networks at different stages of replication; for example, the foci do not appear to concentrate in the central region of the kinetoplast in later stages of replication as they do in *T. brucei*. There are ~8–10 foci in prereplication kinetoplasts. Since a prereplication Form I network contains ~25 maxicircles (determined by EM measurements of the ratio of maxicircles to minicircles after decatenation of networks by topoisomerase II [18]), the in situ hybridization studies raise the possibility that each fluorescent focus contains several maxicircles. The apparent increase in the number of foci in replicating and after replication kinetoplasts is consistent with the fact that the maxicircle/minicircle ratio is relatively constant in prereplication (Form I), replicating, and after replication (Form II) networks (18) and that maxicircle replication occurs concurrently with that of minicircles (13). Although all the targets of maxicircle hybridization appear to be embedded within the kinetoplast disk, we cannot rule out the possibility that some maxicircle sequences are localized outside the disk; either they could have been lost during the fixation treatment or they could be from a portion of the maxicircle sequence not complementary to our probes. Further studies will be needed to clarify the significance of the maxicircle foci within the *C. fasciculata* kinetoplast.

In *T. brucei*, the location of maxicircles in situ appears different from that in *C. fasciculata*. In a prereplication kinetoplast (Fig. 2 *A*), fluorescence in situ hybridization reveals a maxicircle mass which fills the entire kinetoplast. There are no discrete maxicircle foci, and if they exist they must be packed too closely to be resolved by our microscopic technique. As replication in *T. brucei* proceeds, there are striking changes in maxicircle organization (Fig. 2). The maxicircles gradually concentrate in the center of the kinetoplast, until they are tightly packed in the central region between the two zones of nicked minicircles (Fig. 2 *F*). It is possible, as first suggested by Hoeijmakers and Weijers (15), that concentration of *T. brucei* maxicircles in the network center is due to their being left behind when minicircles are released from this region for the purpose of replication. In this regard, it is of interest that probably all of the maxicircles in a trypanosome network are actually interlocked with each other, forming a "network within a network" (28). As the double-size kinetoplast divides, the maxicircles gradually redistribute throughout the progeny kinetoplasts (Fig. 2, *G* and *H*). It is striking that these patterns of kinetoplast organization in situ closely resemble the EM photographs of isolated networks published previously (10, 15) and shown here in Fig. 3. In particular, EM has revealed dumbbell-shaped networks, with maxicircles clustered in the center, and also unit sized networks with maxicircle loops concentrated on one side. Hoeijmakers and Weijers suggested that the latter structures are newly segregated networks in which the maxicircles had not yet redistributed throughout the network (15). EM indicates that a typical isolated dumbbell-shaped network is ~13 μm by 4 μm in size (Fig. 3 *F*). The corresponding structure, viewed in situ by DAPI staining (Fig. 2 *G*), is ~1.5 μm by 0.6 μm in size. Therefore, even though the kDNA is markedly condensed in situ, it retains the overall structural organization of the isolated network. See references 11 and 17 for speculations on how a kDNA network condenses in vivo.

The second important difference between *C. fasciculata* and *T. brucei* kDNA replication concerns the location of newly replicated (nicked) minicircles in partly replicated networks. We found with *C. fasciculata*, using either EM of isolated networks (22) or fluorescence in situ hybridization (reference 11 and see Fig. 1 *B*), that the nicked minicircles are located in a uniform ring around the entire network periphery. This is not the case with *T. brucei*. By fluorescence in situ hybridization the nicked minicircles were almost always concentrated in two separate peripheral zones on opposite sides of the network (see Fig. 2, *B–E*). Although we did

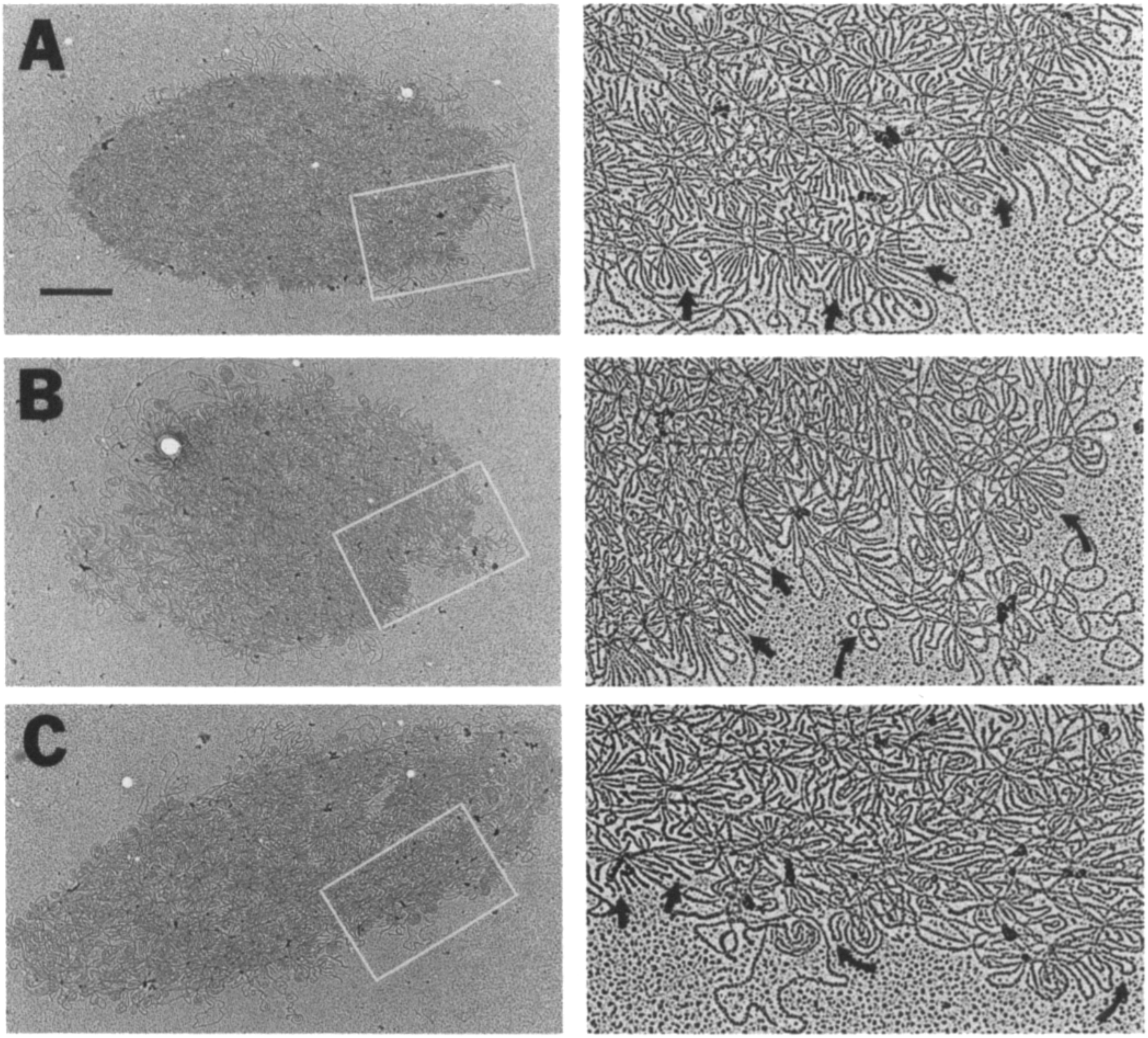
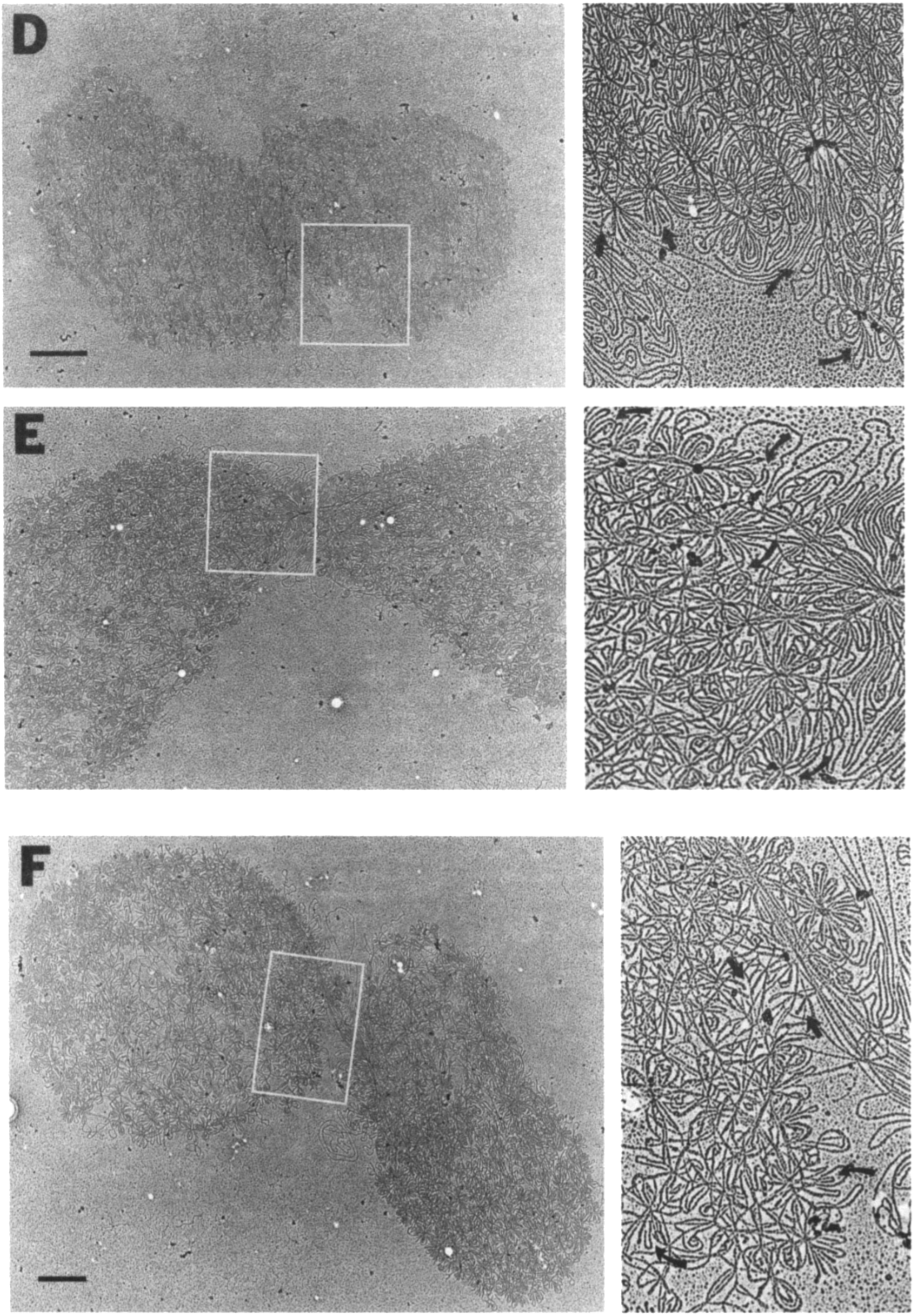


Figure 3. Electron micrographs of *T. brucei* networks isolated from log phase cells. The networks were spread in the presence of 500 μg/ml ethidium bromide to twist the minicircles which are covalently closed. To the right of each panel is an enlargement of the boxed area. (*A*) Prereplication Form I network. (*B–D*) Partially replicated networks containing both nicked and covalently closed minicircles. (*E*) Dumbbell-shaped network in which all minicircles appear to be nicked. (*F*) Double size network in which many minicircles have been repaired, resulting in covalent closure. In the enlargements, straight arrows indicate examples of twisted covalently closed minicircles and curved arrows indicate examples of relaxed nicked minicircles. The longer strands are segments of maxicircles. Magnifications were estimated by photographing a diffraction grating replica (2190 lines/mm). Bar, 1 μm.

observe occasional structures which could be interpreted as donut shaped (not shown), it is possible that in these structures we were unable to resolve the two independent zones of hybridization. We also observed no donut-shaped networks in a thorough examination of 168 isolated networks by EM (Fig. 3); in these studies we distinguished nicked from covalently closed minicircles by spreading the DNA in the presence of ethidium bromide. In all partly replicated networks, containing zones of both nicked and covalently closed minicircles, the nicked minicircles were concentrated in two separate regions on opposite sides of the network (Fig. 3, *B–E*). In a similar study, Hoeijmakers and Weijers had previously reported no donut-shaped networks (15).

Our EM photographs of isolated *T. brucei* networks (Fig. 3) agree closely with those published previously by Hoeijmakers and Weijers (15). The major difference is that they found all partially replicated networks (containing both nicked and covalently closed minicircles) to be about the same size as prereplication networks but to be apparently more densely packed. Even some networks in which all minicircles were nicked did not appear larger than prereplication networks. They concluded that the formation of the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dumbbell-shaped structures involved a rearrangement of the already replicated minicircles within the network. In our micrographs (Fig. 3), the networks clearly enlarge gradually during the course of replication. We cannot account for this slight difference from their results.

We can explain the difference in location of nicked minicircles in *C. fasciculata* and *T. brucei* networks in terms of a model based on our recent work on kDNA replication. *C. fasciculata* contains two complexes of replication proteins, situated on opposite sides of the kinetoplast disk. These complexes contain topoisomerase II (20), DNA polymerase (11), and possibly other enzymes involved in kDNA replication. These complexes also contain minicircles which have single stranded sequences, because they are detectable by in situ hybridization without prior denaturation. The minicircles in these complexes are probably free minicircle replication intermediates, as *C. fasciculata* minicircle θ-structures are known to have single-stranded regions (9). Therefore, the two protein complexes are the likely sites of minicircle replication (11). Newly synthesized minicircles are thought to be attached to the network rim adjacent to these complexes (21, 29).

Similar complexes of replication proteins have not yet been detected in *T. brucei*, mainly because there are no available antibodies against replication enzymes. In addition, fluorescence in situ hybridization, without denaturation of the target DNA (see reference 11 for conditions), did not lead to detection of these complexes (data not shown), possibly because *T. brucei* minicircle θ-structures may not have single-stranded sequences in the region complementary to our probe (26). Nevertheless, because partly replicated *T. brucei* networks have two separate antipodal zones of newly synthesized minicircles (Fig. 2, *B–E*, Fig. 3, *B–D*), we suspect that *T. brucei* also has two complexes of replication proteins.

We have previously provided evidence that *C. fasciculata* minicircles are uniformly distributed around the entire network because the kinetoplast may actually rotate between the two fixed complexes (21). If true, the localization of nicked *T. brucei* minicircles in separate antipodal zones would imply that its kinetoplast does not rotate. Its newly replicated minicircles apparently accumulate in the two peripheral zones adjacent to the putative replication complexes. Models comparing this feature of the replication of *C. fasciculata* and *T. brucei* kDNA networks are shown in Fig. 4.

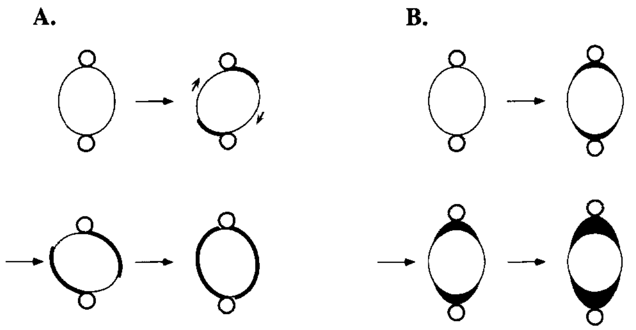

Figure 4. Comparison of models for kDNA replication in *C. fasciculata* and *T. brucei*. In both cases the kinetoplast disk is indicated by the elliptical structure and the two complexes of replication proteins, positioned on opposite sides of the disk, are indicated by the small circles. For *C. fasciculata* (*A*), newly synthesized minicircles are attached to the network adjacent to the replication complexes. They are distributed around the entire network periphery probably due to rotation of the kinetoplast disk (*small arrows*); the regions with newly synthesized minicircles are indicated by the bold line around the network periphery. Presumably after one row of minicircles is attached, the kinetoplast continues rotation in the same direction, resulting in minicircle attachment in a spiral pattern. This model is from reference 21. For *T. brucei* (*B*), the newly synthesized minicircles (shown in black) are also attached to the network adjacent to the replication complexes; however, apparently the kinetoplast disk does not rotate, causing the newly synthesized minicircles to accumulate in two antipodal zones. For both parasites, the central zone of the kinetoplast (with covalently closed minicircles) shrinks as replication proceeds.

We do not yet understand the significance of this striking difference in the pattern of minicircle attachment between *C. fasciculata* and *T. brucei*. A *C. fasciculata* kDNA network is much larger in size than that of *T. brucei* (although they may have comparable numbers of minicircles), raising the possibility that a smaller network can be replicated without kinetoplast rotation. Also, the fact that *T. brucei* (31) has a much more heterogeneous population of minicircles than *C. fasciculata* (34) could be related to this difference in replication mechanism. It is of interest that partially replicated kDNA networks from two other trypanosomatids resemble donuts; we found recently that replicating kDNA networks of *Leishmania donovani* and *T. cruzi*, as visualized by EM, have nicked minicircles distributed around the entire network periphery (T. Zimmer and P. T. Englund, unpublished observation). Since recent studies indicate that *T. brucei* preceded *C. fasciculata*, *T. cruzi*, and *L. donovani* in evolution (12, 16, 19), we speculate that a rotating kinetoplast developed in more recently evolved species.

Our current view of kDNA replication, expressed in the previous paragraphs, raises very interesting questions. If a minicircle replicates in one of the two protein complexes, its progeny minicircles would be attached to the replicating network at neighboring sites, especially in the case of *T. brucei*. Therefore the sister minicircles would likely segregate into the same daughter network at the time of cell division. This mechanism would raise serious problems for the inheritance of minicircles encoding essential guide RNAs, and parasite survival would apparently depend on multiple copies of crucial minicircles and redundant guide RNAs. Perhaps a rotating kinetoplast evolved for the purpose of facilitating segregation of sister minicircles. Furthermore, in the case of *T. brucei*, unless there was a mechanism for ensuring that equal numbers of minicircles were replicated in each of the two protein complexes, one might expect a gradual drift in the number of minicircles per network. Further investigation should resolve these issues.

In the accompanying paper, Robinson and Gull describe results comparable to those reported here, and they reach similar conclusions (25).

We thank Mike Delannoy for advice on the electron microscopy, Viiu Klein and Kristen Gaines for technical support, and Terry Shapiro and Laura Rocco for comments on the manuscript.

This research was supported by grants from National Institutes of Health (GM-40115 to D. C. Ward and GM-27608 to P. T. Englund) and the MacArthur Foundation (to P. T. Englund). AFT was supported by a National Institutes of Health postdoctoral fellowship (grant GM-13604) and D. Pérez-Morga by the Rockefeller Foundation (grant GAHS8937).

Received for publication 8 February 1994 and in revised form 26 April 1994.

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