The Structure of Replicating Kinetoplast DNA Networks

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Abstract. Kinetoplast DNA (kDNA), the mitochondrial DNA of *Crithidia fasciculata* and related trypanosomatids, is a network containing ~5,000 covalently closed minicircles which are topologically interlocked. kDNA synthesis involves release of covalently closed minicircles from the network, and, after replication of the free minicircles, reattachment of the nicked or gapped progeny minicircles to the network periphery. We have investigated this process by electron microscopy of networks at different stages of replication. The distribution of nicked and closed minicircles is easily detectable either by autoradiography of networks radiolabeled at endogenous nicks by nick translation or by twisting the covalently closed minicircles with intercalating dye. The location of newly synthesized minicircles within the network is determined by autoradiography of networks labeled in vivo with a pulse of [^{3}H]thymidine. These studies have clarified structural changes in the network during replication, the timing of repair of nicked minicircles after replication, and the mechanism of division of the network.

KINETOPLAST DNA (kDNA)[1] is the mitochondrial DNA of trypanosomes and related parasitic protozoa. kDNA in the trypanosomatid *Crithidia fasciculata* consists of ~5,000 minicircles (each ~2.5 kb) and ~25 maxicircles (each ~37 kb). A unique feature of kDNA is that all of these circles are topologically interlocked into a massive network (for reviews see references 15, 16, and 20). As viewed by EM, the isolated *C. fasciculata* network is a two-dimensional elliptically shaped array of interlocked rings, ~10 by 15 μm in size. Within the mitochondrial matrix, the network is condensed into a disk ~1.0 μm in diameter and 0.3 μm thick (7). Each cell has one network within its single mitochondrion.

Kinetoplast maxicircles resemble other mitochondrial DNAs in that they encode rRNAs and a few mitochondrial proteins involved in energy transduction (16). However, maxicircles are unusual in that many of their transcripts are processed by RNA editing, involving addition or deletion of uridine residues at specific sites. Both maxicircles and minicircles encode guide RNAs which control the specificity of editing (for reviews of editing see references 1, 17, and 20).

Because of its network structure, kDNA replicates by an unusual mechanism (for reviews see references 14 and 15). Replication occurs during a discrete S phase of the cell cycle, at roughly the same time as that of nuclear DNA (2). Before replication, during the G1 phase, all 5,000 minicircles are covalently closed and the networks are known as Form I (4). When the S phase begins, minicircles are released from the network by a topoisomerase II (5). It is thought that the free minicircles migrate to either of two complexes of replication proteins which are situated on opposite sides of the kinetoplast disk (7, 10). There they replicate as Θ structures, and the progeny free minicircles, containing nicks or gaps, are reattached to the network periphery (4, 5, 18). To distribute the minicircles uniformly around the periphery, there appears to be relative movement of the kinetoplast and the two complexes of replication proteins; the kinetoplast probably rotates between the two fixed complexes (11). Finally, when all of the minicircles have replicated the network becomes double size, containing ~10,000 minicircles which are all nicked or gapped (4) (in the remainder of this paper, we shall refer to the interruptions in the strands of newly replicated minicircles as "nicks"). These networks are known as Form II. Formation of the progeny networks involves repair of all of the minicircle nicks and splitting of the double-size network in two.

We have now used EM to examine the structures of isolated *C. fasciculata* networks at various stages of replication. Some of these studies involve autoradiography of networks that were labeled either in vitro by nick translation (to mark the location of nicked circles) or in vivo with [^{3}H]thymidine (to indicate the sites of newly synthesized and attached minicircles). These studies have yielded new insight into the structure of partly replicated kDNA networks. They have also been informative about the final stages of replication when minicircle nicks are repaired and the double-size network divides in two.

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1. *Abbreviations used in this paper:* BHI, brain heat infusion; kDNA, kinetoplast DNA.

Materials and Methods

Cell Culture, Metabolic Labeling, and kDNA Isolation

C. fasciculata cells were grown in brain heart infusion (BHI) medium (37 g/liter) supplemented with hemin (20 μg/ml) at room temperature to mid log phase (2–3 × 10^7 cells/ml). We used two methods for kDNA network isolation. In one case, used especially for cells labeled in vivo with [^{3}H]thymidine (109–115 Ci/mmol, 1 mCi/ml) for the purpose of network autoradiography, the DNA was isolated by a small scale procedure described elsewhere (11). In other experiments, the DNA was isolated by a large scale technique (using 500-ml cultures). For the latter, in some experiments the DNA was radiolabeled by adding 5 mCi of [^{32}P]orthophosphate to the culture ~24 h before harvesting. The culture was then chilled in ice, centrifuged at 5,000 rpm for 10 min in a Sorvall GSA rotor at 4°C, and the cells were resuspended in 18 ml of 10 mM Tris-HCl, pH 8.0, 5 mM EDTA. The cells were then lysed by addition of 1 ml SDS (10%) and 0.1 ml proteinase K (20 mg/ml) followed by incubation at 37°C for 60 min. The lysate (18.8 ml) was then loaded on top of a 20-ml sucrose solution (20% in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA [TE]) and centrifuged at 19,000 rpm for 60 min at 5°C in a Beckman SW28 rotor (Beckman Instruments, Inc., Fullerton, CA). Most of the supernatant was discarded, except for 2 ml from the bottom of the tube which was used to redissolve the pellet containing kDNA. After adding 16.8 ml of TE, the DNA was further purified by a second centrifugation through 20% sucrose. The final pellet was redissolved in 2 ml of the 20% sucrose supernatant and dialyzed against TE overnight at 4°C. The kDNA was then precipitated by addition of 15 ml TE, 1.7 ml 3 M sodium acetate, and 10 ml isopropanol. The pellet was then washed with 70% ethanol, dried, and resuspended in 7 ml of TE. The yield of kDNA was ~52 μg, and when ^{32}P labeled, the specific radioactivity was ~5,000 Cerenkov cpm/μg. As judged by gel electrophoresis or EM analysis, this kDNA contained virtually no contaminating RNA or nuclear DNA.

The kDNA was then fractionated in a CsCl-propidium diiodide gradient to resolve networks from different stages of replication (4). kDNA (3 ml, containing 22 μg kDNA) was mixed with 0.1 ml of 1 M Tris-HCl, pH 8.0, 3.4 ml of water, 5.74 g of CsCl, and 0.7 ml of propidium diiodide (5 mg/ml). The refractive index was 1.3808. This solution was transferred to a 50 Ti rotor tube which was then filled with paraffin oil. It was centrifuged at 44,000 rpm for 25 h at 4°C in a 50 Ti Beckman rotor. The gradient was then collected from the bottom of the tube in 57 fractions and DNA concentrations were assayed either by scintillation counting (if radiolabeled) or by electrophoresis of 10 μl samples on a 0.9% agarose gel using TBE buffer containing 0.5 μg/ml propidium diiodide. In the latter case, the kDNA network concentration was estimated from the intensity of fluorescent material remaining in the slot. Recovery of DNA from the gradient was usually >95%. Four fractions were usually pooled (Form I networks, early replicating networks, late replicating networks, and Form II networks). The pools were then extracted with water saturated *n*-butanol to remove dye and dialyzed against TE overnight at 4°C.

EM and EM Autoradiography

The kDNA was prepared for EM by a microtechnique developed in our laboratory (12) which is a modification of the formamide method of Davis et al. (3). The EM autoradiography procedure has been previously described (11). Briefly, the grids were single-angle shadowed with Pt-Pd and then coated with carbon. A film of photographic emulsion was deposited on top of each grid, and the coated grids were then exposed at 4°C in the dark for up to 4 mo until development. The grids were then analyzed using a Zeiss 10 A/B high resolution electron microscope. Magnifications were estimated by photographing a diffraction grating replica (2,190 lines/mm).

Results

Autoradiographic Analysis of kDNA Networks at Different Stages of Replication

In this experiment we radiolabeled networks from different stages of replication in a nick translation reaction using DNA polymerase I and ^{3}H-labeled deoxyribonucleoside triphosphates. This treatment introduces ^{3}H-nucleotides at the sites of the endogenous nicks in minicircles. We then spread the networks on grids in the presence of ethidium bromide, coated them with photographic emulsion, and, after exposure and development, examined them by EM. The silver grains would indicate regions of the network which contained nicked minicircles (Fig. 1). Fig. 1 *A* shows a pre-replication Form I network. As shown by the absence of silver grains on this network, all of its minicircles must be covalently closed. Confirming this point, close inspection reveals that nearly all the minicircles were twisted by the intercalation of ethidium bromide; minicircles in the absence of this dye, even when covalently closed, appear relaxed (13). Fig. 1 *B* shows an early replicating network. Strikingly, there is a narrow and uniform ring of silver grains around the periphery of this network, marking the location of the nicked minicircles which had been attached to the network after replication. Minicircles in the central region of this network must be covalently closed. Fig. 1 *C* shows a network from a later stage of replication, with a much broader ring of silver grains and a smaller central zone. Fig. 1 *D* shows a larger network uniformly covered with silver grains. This is a double-size Form II network, from the final stage of replication, in which all the minicircles are nicked.

Further EM Analysis of Replicating Networks

Fig. 2 shows networks from different stages of replication, spread in the presence of ethidium bromide and without autoradiography. An enlargement is shown at the right of each panel. Fig. 2 *A* shows a pre-replication Form I network. Most of its minicircles, both on the periphery and in the interior, are twisted by the intercalating dye, and therefore must be covalently closed. One characteristic of Form I networks is a dense "line" around the periphery (marked by *large arrows* on the enlargement). This line appears to be a bundle of DNA fibers, and it may consist of minicircles, and possibly some maxicircles, stretched tightly around the periphery.

Fig. 2, *B* and *C* show partly replicated networks. When spread in the presence of ethidium bromide, the peripheral and central zones are easily distinguished. Especially in *B* the relaxed circles in the peripheral zone (examples marked by *open arrows*) are clearly differentiated from the twisted circles in the central region (examples marked by *closed arrows*). The network in *C*, having a smaller central zone, is at a more advanced stage of replication. A dense "line," like that around the periphery of Form I networks, frequently marks the boundary between the central zone and the peripheral zone. This line is marked by large arrows on the enlargements of *B* and *C*.

Fig. 2, *D*, *E*, and *F* show networks near the final stages of replication. These are Form II networks, in which all or virtually all of the minicircles are nicked. As exemplified by the network in *D*, about half of Form II networks have holes in the middle, presumably a consequence of the release of minicircles for replication. The hole in that network is rather large, but some in other networks appear smaller. The network in *D* has not quite finished replication, as a few covalently closed minicircles, twisted by ethidium bromide, are detectable in the central region (see *arrows* in enlargement). We assume that the holes in the network are gradually repaired by minicircle catenation. *E* shows a network in which the hole appears to be partly repaired, and that in *F* appears uniformly catenated with no sign of a hole. We have

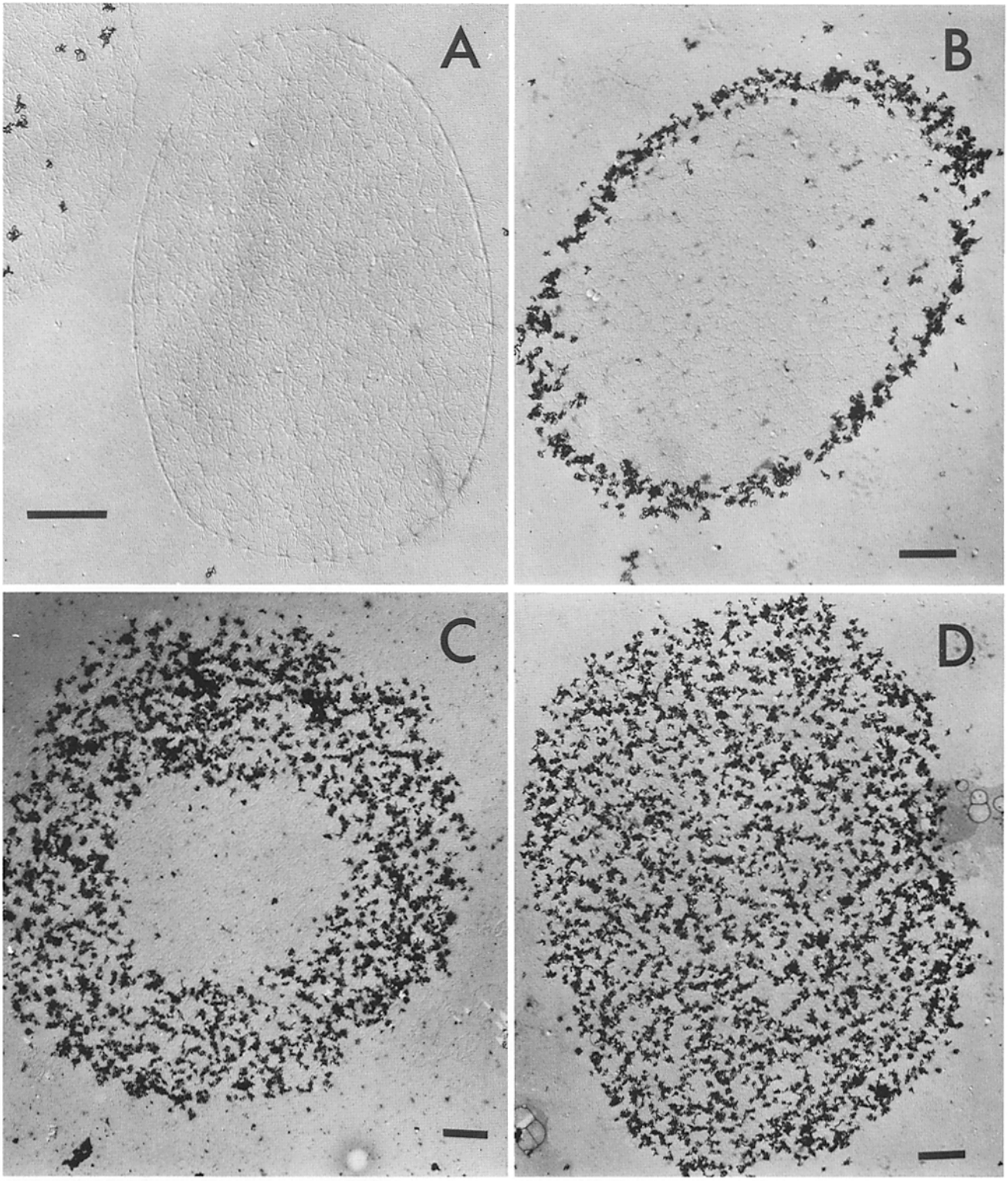


Figure 1. EM autoradiography of isolated kDNA networks radiolabeled by nick translation at endogenous minicircle nicks. The networks in *A*, *B*, and *D* had been fractionated according to stage of replication using a CsCl-propidium diiodide gradient, and that in *C* was from a solution of unfractionated networks. The nick translation reactions (50 μl) contained 100–200 ng of kDNA networks, 50 mM Tris-HCl, pH 7.5, 10 mM $MgSO_4$, 20 μM each of dCTP, dATP, and dGTP, 37.5 μCi of [^{3}H]dTTP (105 Ci/mmol; New England Nuclear), and 1 U of *E. coli* DNA polymerase I (GIBCO BRL, Gaithersburg, MD). The reactions were conducted at 16°C for 60 min and unincorporated nucleotides were removed by overnight dialysis against TE at 4°C. The radioactive kDNA was then spread on EM grids (in the presence of 100 μg/ml ethidium bromide) and autoradiographed. Exposure was 4 mo for *A*, 11 d for *B* and *D*, and 3 d for *C*. *A* shows a Form I network, *B* and *C* show replicating networks, and *D* shows a Form II network. Bars, 2 μm.

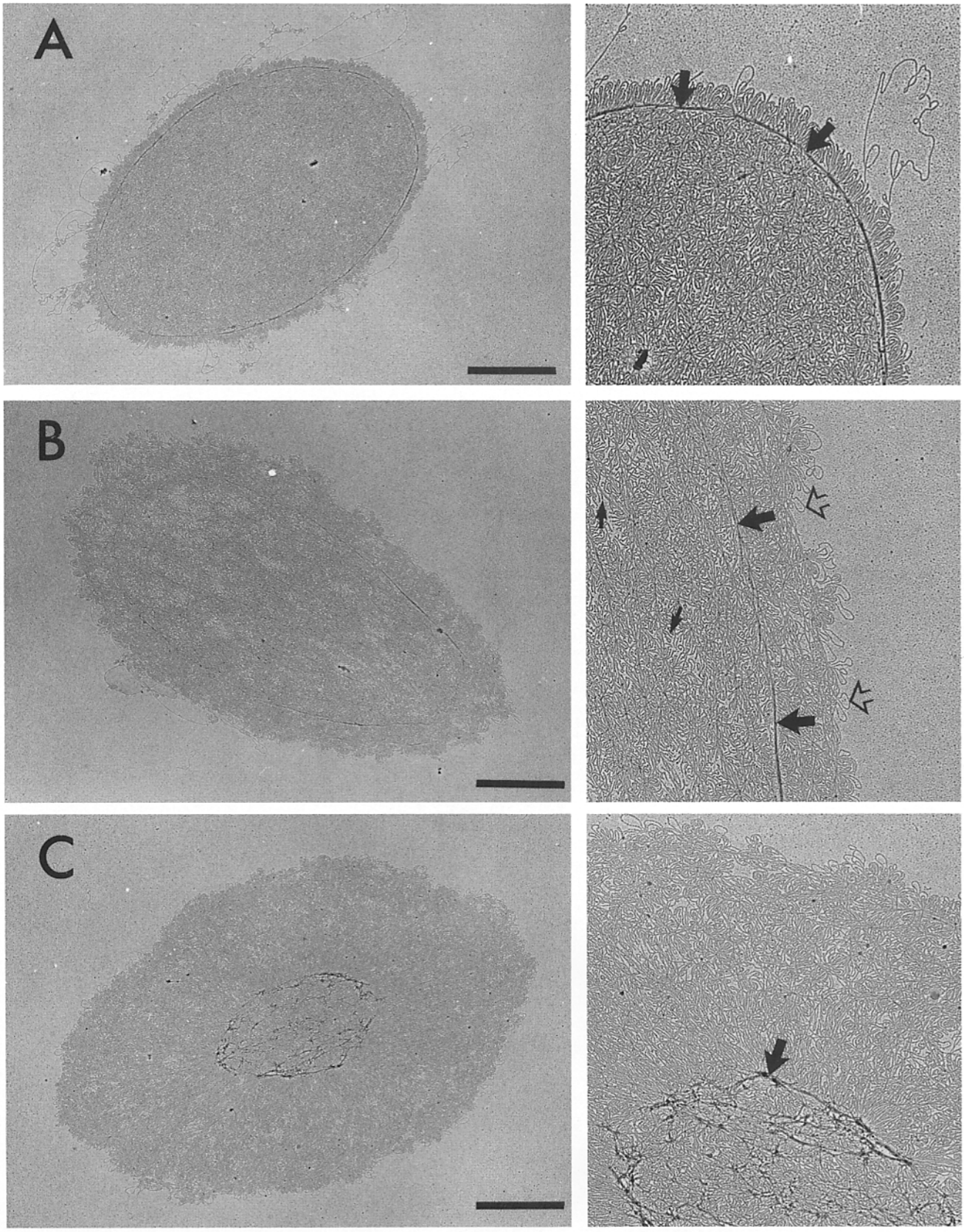
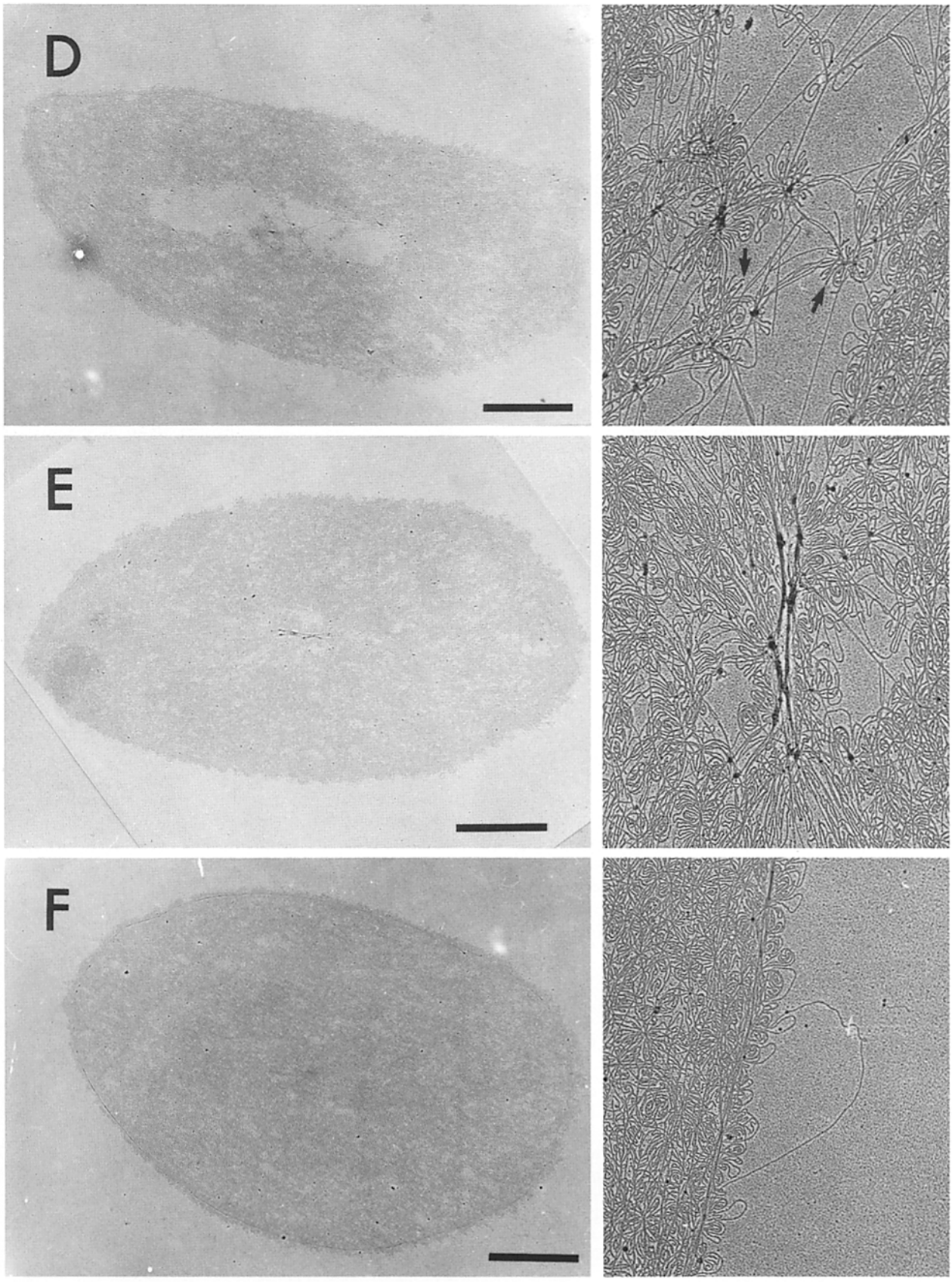


Figure 2. EM analysis of kDNA networks at different stages of replication. kDNA networks were metabolically labeled with [^{32}P]orthophosphate as described in Materials and Methods and, for reasons not related to this experiment, treated with 200 μg/ml bromodeoxyuridine for 30 min before harvesting; identical results were obtained if the cells were not treated with bromodeoxyuridine. The kDNA was isolated using the large-scale technique described in Materials and Methods with the following modification. After proteinase K treatment, the lysate was extracted with 20 ml phenol and then with 40 ml chloroform. The lysate was then treated with ribonuclease A (0.2 mg/ml) plus ribonuclease T1 (200 U/ml) for 30 min at 37°C. The lysate was then subjected to centrifugation on a sucrose column as described in Materials and Methods. The kDNA networks were fractionated according to stage of replication on a CsCl-propidium diiodide gradient.



The pooled fractions of Form I, replicating, and Form II networks were spread for EM in the presence of 100 μg/ml ethidium bromide. *A*, Form I network; *B*, early replicating network; *C*, later replicating network; *D*, *E*, and *F*, Form II networks. To the right of each panel is a section of the same network shown at higher magnification. Large arrows (*A*) point to the line around the periphery of a Form I network; a similar line, also marked by large arrows, separates the central and peripheral zones in replicating networks (*B* and *C*). Open arrows indicate relaxed minicircles in replicating network, and small closed arrows indicate twisted minicircles (*B*). Small closed arrows in *D* also indicate residual twisted minicircles. Bars, 2 μm.

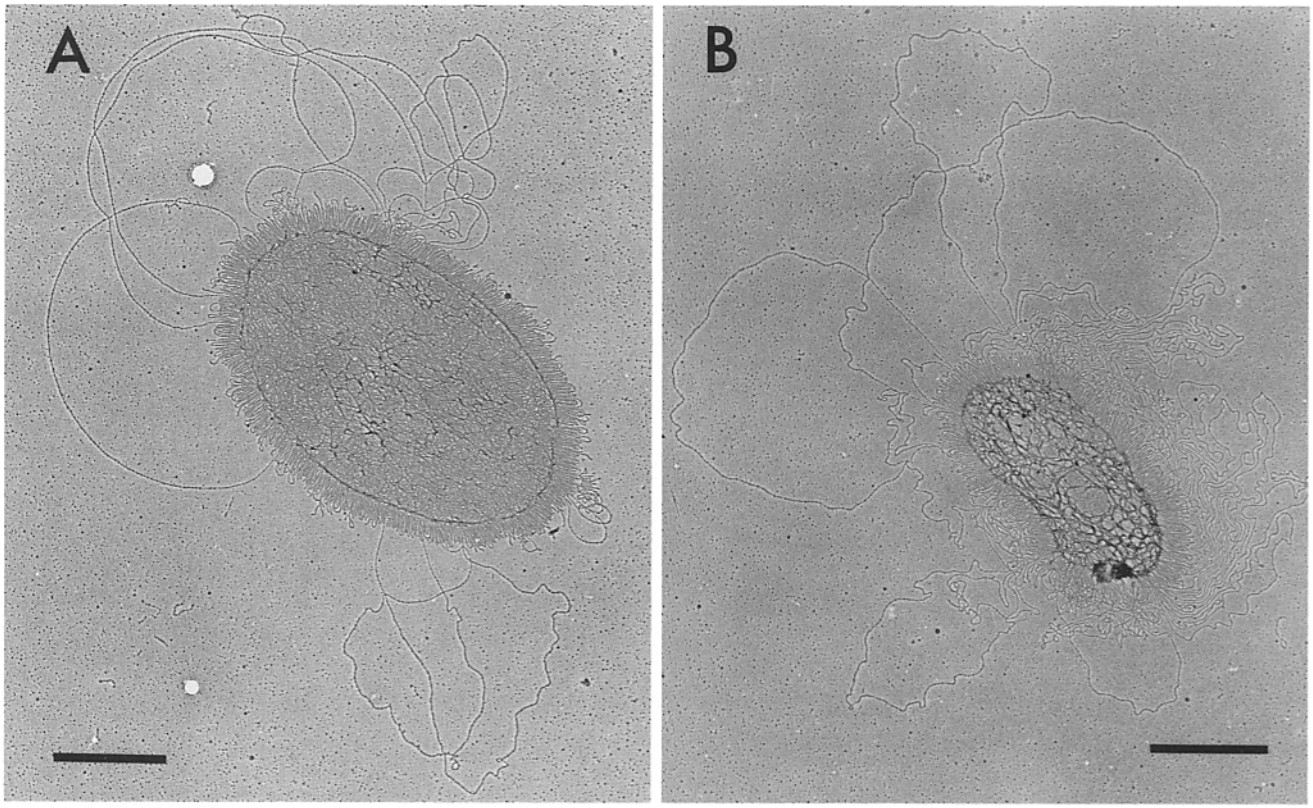


Figure 3. Extrusion of maxicircles from networks spread for EM in the presence of propidium diiodide. This dye, at 50 μg/ml, tightly condenses networks containing covalently closed circles (13). Networks were isolated from exponentially growing cultures by the small scale procedure (11). Bars, 2 μm.

not established precursor-product relationships for networks like those in Fig. 2, *D*, *E*, and *F*, but from their structures it is likely that the network in *D* is from an earlier stage and those in *E* and *F* are from later stages.

Comparison of the EMs in Fig. 2 shows that networks gradually increase in size during replication, due to the addition of newly synthesized minicircles. However, the actual size in these photographs is slightly misleading, due to the presence of intercalating dye during spreading for EM. The dye causes condensation of regions of networks which contain covalently closed circles (13).

In *C. fasciculata* there are ~25 maxicircles in a Form I network and about twice that number in a Form II (9). However, under the spreading conditions used in Fig. 2, we see relatively few maxicircles protruding from the network's edge. Examples of maxicircle loops are seen in the networks in Fig. 2, *A* and *F* (see enlargements). The other maxicircles must be threaded through the network interior. In Form II networks containing a hole in the center (e.g., Fig. 2 *D*), we frequently see long strands, presumably maxicircles, traversing the hole. If networks are spread for EM in the presence of 50 μg/ml propidium diiodide, regions containing covalently closed minicircles tightly condense (13). Under these conditions, many maxicircles are extruded from the network (Fig. 3).

The minicircles in the central region of replicating networks often seem more loosely packed than those in the peripheral region, presumably because minicircles have been released from the central region for replication. This loosening is most consistently seen in networks which have not been fractionated on CsCl-propidium diiodide gradients. Residual propidium from these gradients can result in compaction of the regions of networks which contain covalently closed minicircles (13), frequently obscuring any loosening. We therefore examined unfractionated networks (which had never been exposed to propidium diiodide) in the presence of 500 μg/ml ethidium bromide, as even this high concentration of ethidium does not compact the DNA so tightly. As shown in the replicating networks in Fig. 4, *A* and *B*, the twisted minicircles in the central region are loosely packed, resulting in the appearance of small holes in the network structure. The example in Fig. 4 *C* was never exposed to intercalating dye. Although we have no proof that it is a replicating network like those in Fig. 4, *A* and *B* (since we cannot tell whether minicircles in its central zone are nicked or covalently closed), its loose central region and its relatively large size (~16 by 22 μm) suggest that it is.

Repair of Minicircle Nicks and Gaps in Form II Networks

Previously we had observed double-size Form II networks with exclusively nicked minicircles and unit-size Form I networks containing only covalently closed minicircles (4). Therefore, we could not determine whether repair of minicircle nicks precedes network division, or vice versa. We have now observed double-size networks which are mosaics, containing both nicked and closed minicircles (Fig.

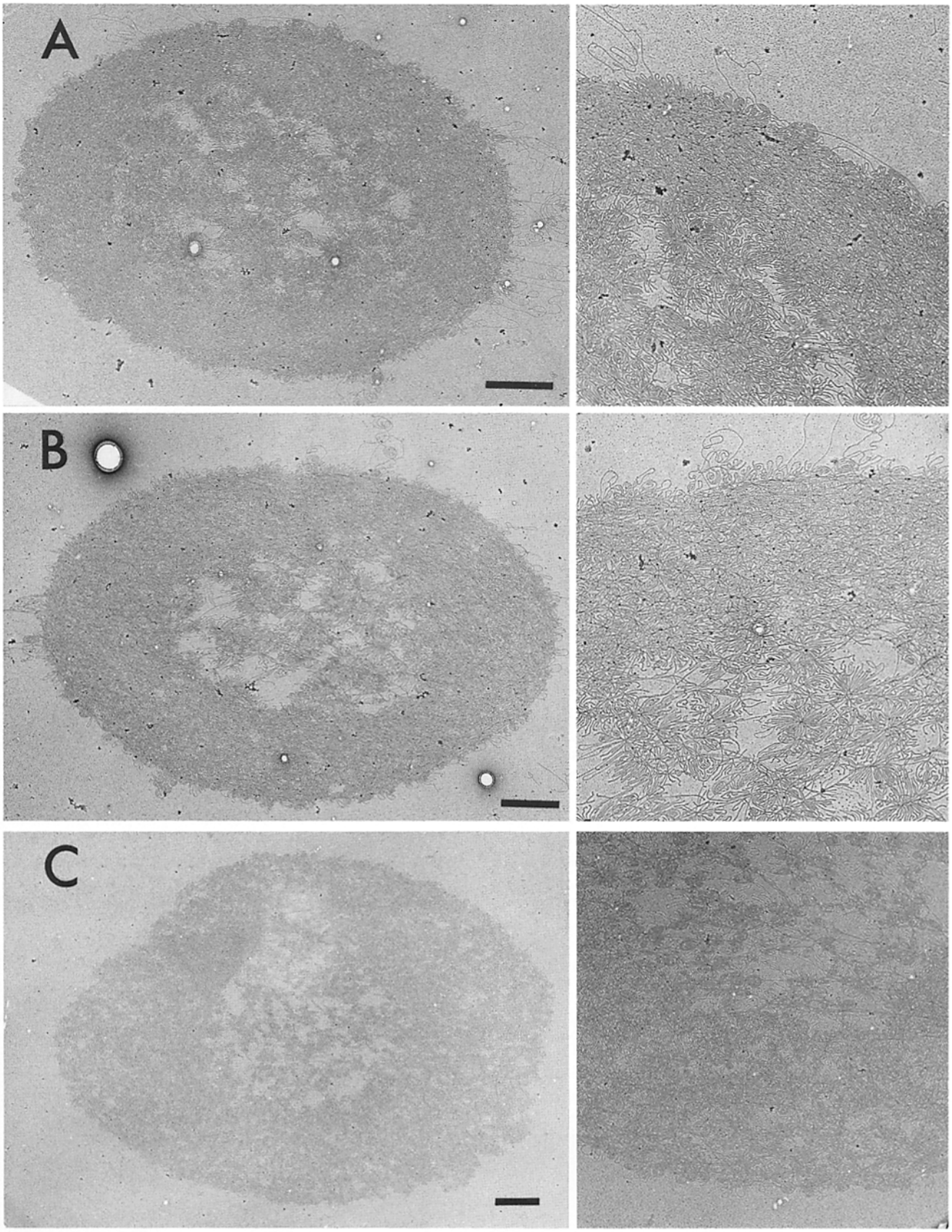


Figure 4. Structure of the central zone of replicating networks. kDNA was isolated from logarithmically growing cells by the small scale technique (11) and spread on the EM grid in the presence of 500 μg/ml ethidium bromide (*A* and *B*) or in the absence of dye (*C*). The network in *C* was treated with SstI, an enzyme that cleaves maxicircles but not minicircles, for reasons unrelated to this experiment. Bars, 2 μm.

5). These networks must be replication intermediates in which many minicircles have been covalently closed just prior to network splitting. Fig. 5 *A* shows a double-size network, labeled by nick translation and viewed by EM autoradiography after an exposure time of 4 mo, in which many of the minicircles are covalently closed (as shown in the enlargement, many minicircles are twisted by the ethidium bromide). The pattern of silver grains on this network demonstrate that the residual nicked minicircles are randomly distributed throughout this network. As a control, the neighboring network shown at the lower right in Fig. 5 *A* (and shown completely in Fig. 1 *A*) is a unit size Form I network which is completely devoid of silver grains. Another network from the same preparation, shown in Fig. 1 *D*, is a Form II network heavily covered with silver grains, even after an exposure time of only 11 d. Fig. 5 *B* shows another example of an autoradiograph of a mosaic network, and Fig. 5 *C* shows an EM of a network spread in the presence of ethidium bromide without autoradiography. In the latter double-size network, most of the minicircles have been repaired and are covalently closed. This network also appears to be pinched in the middle, possibly a step in its division into two Form I networks.

How Do Double-Size Networks Divide?

We addressed this issue by labeling asynchronous *C. fasciculata* cultures in vivo for 60 min with [^{3}H]thymidine. We then examined the isolated networks by EM autoradiography. Since newly synthesized minicircles are attached around the network periphery, we see many networks with a peripheral ring of silver grains (11). Fig. 6 *A* shows an example of a 60-min-labeled network which is approximately double-size and therefore in a late stage of replication. ~15% of the networks in this population also contained silver grains in a horseshoe pattern (examples are in Fig. 6, *B*, *C*, and *D*), and networks with a similar pattern of silver grains were observed earlier using light microscope autoradiography (18). These horseshoe networks are all about the size of Form I networks, and because virtually all of their minicircles were twisted in the presence of ethidium bromide, they must be covalently closed. It is likely that the horseshoes are networks which have just undergone division, arising from the split of a double-size network like that in Fig. 6 *A*. Horseshoe networks frequently had a flat edge in the region which is unlabeled, implying that scission of a double-size network is due to a straight cut down its center.

Discussion

These studies have clarified our understanding of the structure of kDNA networks at different stages of replication. As demonstrated by EM autoradiography of nick translated networks (Fig. 1) or EM in the presence of ethidium bromide (Fig. 2), the nicked minicircles form a uniform ring around the entire network periphery. As replication proceeds, the peripheral zone enlarges and the central zone contracts. Other autoradiographic studies, of networks labeled in vivo with [^{3}H]thymidine, show that the newly synthesized minicircles also have a similar peripheral location (11, 18, 19 and Fig. 6 *A*). In many networks, the zone of nicked minicircles is much larger than the zone of newly synthesized minicircles radiolabeled with a pulse of [^{3}H]thymidine in vivo (11). The reason is that the nicked minicircles include all of those replicated during the S phase whereas the newly synthesized minicircles include only those radiolabeled during the pulse.

Minicircle replication probably takes place in one of two protein complexes (containing topoisomerase II, DNA polymerase, and possibly other enzymes), situated on opposite sides of the kinetoplast disk in vivo (7, 10). The uniform distribution of newly synthesized nicked minicircles around the network periphery, as observed in Figs. 1 and 2, is thought to be due to relative movement of the kinetoplast and the two complexes; it is most likely that the kinetoplast rotates between two fixed complexes (11). Based on this model one would expect that networks at the very earliest stage of replication would contain nicked minicircles present only at two peripheral sites on opposite sides of the network. We detected few if any networks of this type, either by EM autoradiography after nick translation or by EM in the presence of intercalating dye, possibly because their steady-state concentration is very low.

kDNA replication involves release, by topoisomerase II, of covalently closed minicircles from the central zone of the network. Since this process must involve unlinking of the released minicircle from all of its neighbors, forming a hole, it is not surprising that the central region of replicating networks is frequently more loosely packed with minicircles than is the peripheral region (Fig. 4). Loosening is more or less uniform throughout the central region, implying that minicircles are released from random positions. We do not yet know if there is some repair of these holes during the S phase, also by topoisomerase action. Finally, when replication is nearly complete, there is frequently a large hole in the center of the double-size network (see example in Fig. 2 *D*). Replication of that particular network is not quite complete, as there are a handful of covalently closed minicircles, twisted by the intercalating dye, around the hole in the central region (marked by arrows in the enlargement of Fig. 2 *D*). After all of the covalently closed minicircles are released, the hole must be repaired. We speculate that the networks in Fig. 2, *E* (with smaller holes) and *F* (with no hole) are from stages later than that in Fig. 2 *D*.

When are the minicircle nicks repaired? In a previous investigation we detected double-size Form II networks containing only nicked minicircles and unit-size Form I networks containing only covalently closed minicircles (4). Now we have detected many examples of mosaic networks, which are double size and have a mixture of nicked and covalently closed minicircles (Fig. 5). We never observed a unit-size mosaic network, indicating that minicircle repair is always complete before network division. Based on EM autoradiography of networks labeled by nick translation (Fig. 5, *A* and *B*), the nicked and covalently closed minicircles appear to be randomly distributed, implying that gap repair and ligation occurs at random sites throughout the network. The mosaic network in Fig. 5 *C* appears to be pinching off in the middle, possibly a step in its division.

Many double-size networks isolated from *Trypanosoma brucei* resemble dumbbells, in which the nascent daughter networks are held together by only a few interlocked minicircles (6, 8). We never have observed dumbbell-shaped networks in *C. fasciculata*, although we do see double-size networks which may be just beginning to pinch off in the middle (4; and see the example in Fig. 5 *C*). However, using EM

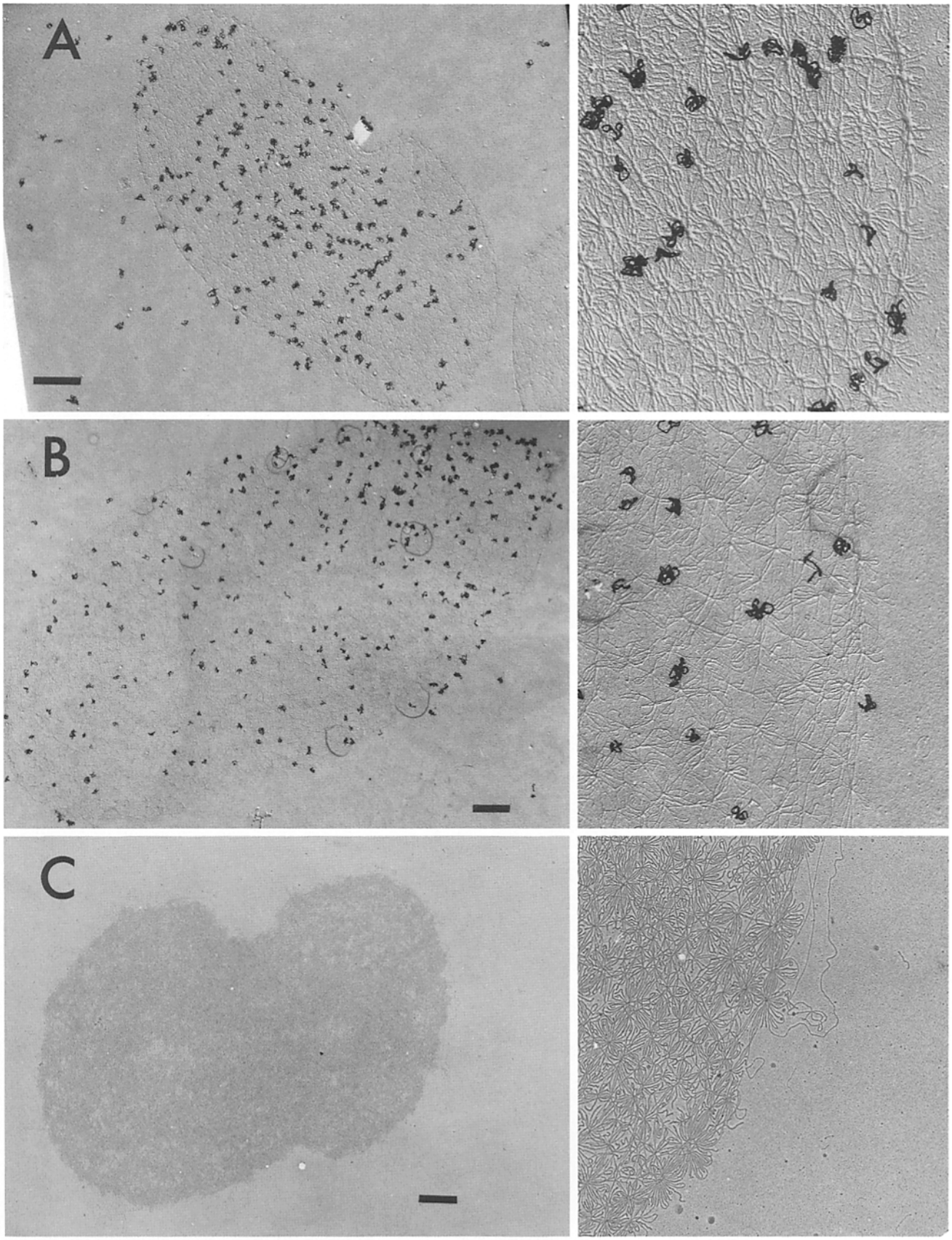


Figure 5. EM analysis of mosaic networks. The networks in *A* and *B* were labeled by nick translation; they are from the same preparation used for *A*, *B*, and *D* of Fig. 1. The network in *C* was neither nick translated nor autoradiographed. The network in *A* derived from the Form I peak of a CsCl-propidium diiodide gradient and that in *B* derived from the fraction of late replicating networks. All networks were spread in the presence of 100 μg/ml ethidium bromide, and at the right of each panel is a segment shown at higher magnification. The network shown in part at the lower right of *A* is the same network shown in Fig. 1 *A*. Exposure times were 4 mo. Bars, 2 μm.

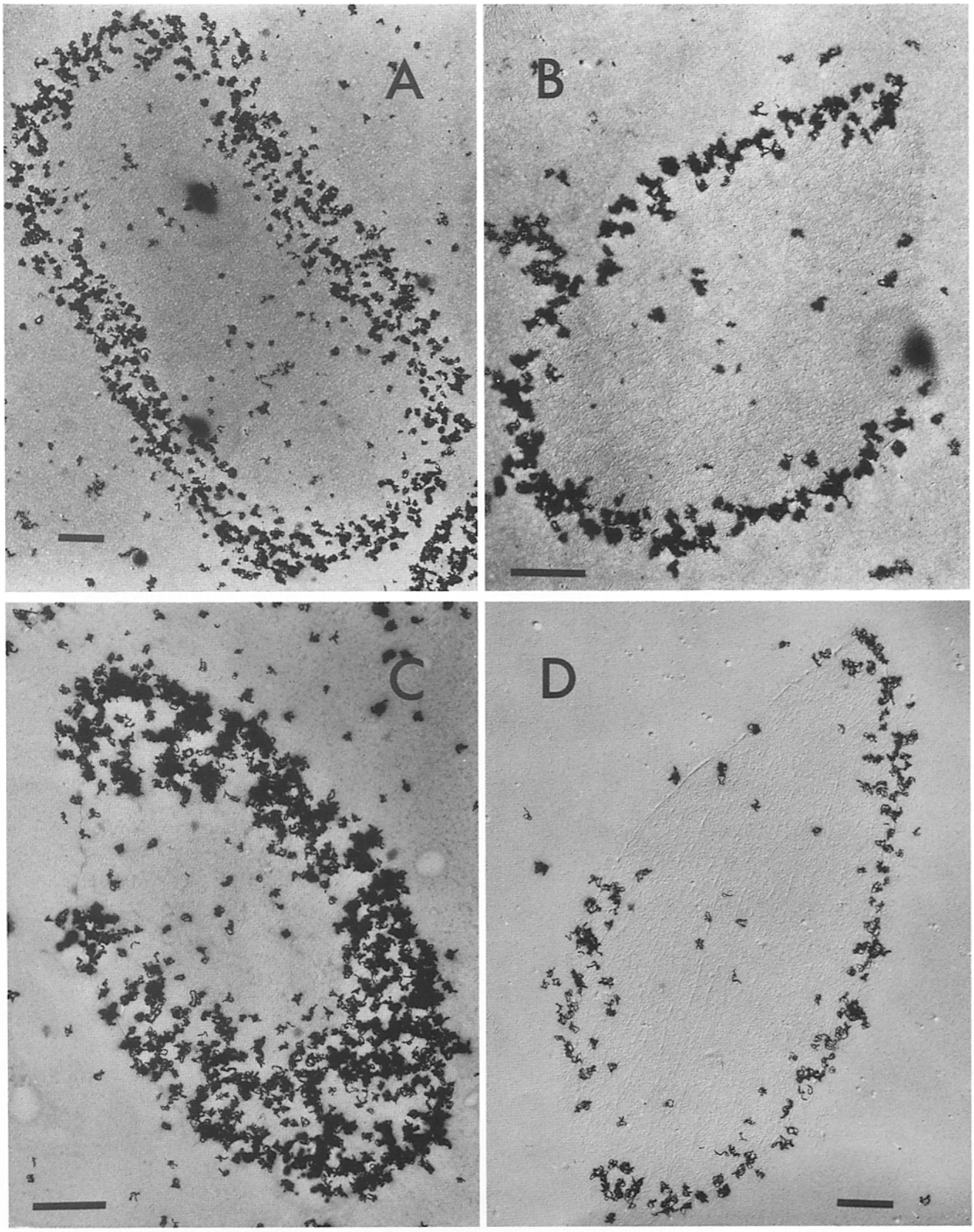


Figure 6. EM analysis of horseshoe networks. *C. fasciculata* cells were labeled in vivo with [^{3}H]thymidine for 60 min (*A* and *B*), 150 min (*C*), and 135 min (*D*). The kDNA was isolated and subjected to EM autoradiography. Intercalating dye was added during spreading for EM (10 μg/ml propidium diiodide for networks in *A–C* and 1 μg/ml ethidium bromide for that in *D*). *A* shows a nearly double-size network and *B–D* show unit size networks. Exposure times were 40 h (*A* and *B*), 1 mo (*C*), and 18 d (*D*). Bars, 2 μm.

autoradiography we could easily detect many networks which had just undergone division. When cells are labeled in vivo (e.g., for 30–150 min with [^{3}H]thymidine), many networks in the final stage of replication contain a ring of silver grains around the periphery (marking the sites of newly synthesized minicircles) (11; and see example in Fig. 6 *A*). Cutting a network of this type, along its minor axis, should give rise to a network with silver grains shaped like a horseshoe. Horseshoe networks are abundant in these preparations, accounting for ~5% of the total networks labeled for 30 min and 15% of those labeled for 60 min. They are always unit size, and based on EM in the presence of intercalating dye, all of their minicircles are covalently closed. They usually have a flat edge in the region of the periphery which is devoid of silver grains, implying that scission of a double-size network occurs by a straight cut along or at a small angle to the minor axis of the double-size network. Since mature Form I networks are elliptically shaped (see example in Fig. 2 *A*), there must be a remodeling process which converts the flat edge into a curved one.

The organization of maxicircles in *C. fasciculata* networks is still a mystery. There are ~25 maxicircles in a Form I network, and about twice that many in a Form II (9). Unless propidium diiodide is present during spreading (Fig. 3), maxicircles are only occasionally visible as elongated loops on the edge of the networks (examples are in Fig. 2, *A* and *F*), indicating that most are buried within the network structure. This situation contrasts with that in *T. brucei* networks, in which maxicircle loops are much more frequently visible. A striking feature of *T. brucei* networks is that large numbers of maxicircles are clustered in the center of the dumbbell-shaped dividing networks (6, 8). Although we have not observed this arrangement in *C. fasciculata* networks, we frequently detect long strands, presumably maxicircles, running through the hole in double-size networks (see example in Fig. 2 *D*). Therefore, *C. fasciculata* maxicircles are present and possibly could be concentrated in the center of networks that are preparing to divide. It is certain that new experimental approaches will be needed to evaluate the organization of maxicircles within kDNA networks and to detect changes which occur during their replication.

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