# The Configuration of DNA Replication Sites within the *Trypanosoma brucei* Kinetoplast

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*Abstract.* The kinetoplast is a concatenated network of circular DNA molecules found in the mitochondrion of many trypanosomes. This mass of DNA is replicated in a discrete "S" phase in the cell cycle. We have tracked the incorporation of the thymidine analogue 5-bromodeoxyuridine into newly replicated DNA by immunofluorescence and novel immunogold labeling procedures. This has allowed the detection of particular sites of replicated DNA in the replicating and segregating kinetoplast. These studies provide a new method for observing kinetoplast DNA (kDNA) replication patterns at high resolution. The techniques reveal that initially the pattern of replicated DNA is antipodal and can be detected both on isolated complexes and in replicating kDNA in vivo. In *Trypanosoma brucei* the opposing edges of replicating kDNA never extend around the complete circumference of the network, as seen in other kinetoplastids. Furthermore, crescent-shaped labeling patterns are formed which give way to labeling of most of the replicating kDNA except the characteristic midzone. The configuration of these sites of replicated DNA molecules is different to previous studies on organisms such as *Crithidia fasciculata*, suggesting differences in the timing of replication of mini and maxicircles and/or organization of the replicative apparatus in the kinetoplast of the African trypanosome.

THE mitochondrion of trypanosomes such as *Trypanosoma brucei* is unusual among the eukaryotes in that it exists in the form of a single elongated tubular structure that extends along the length of the cell body. The mitochondrial genome is contained within a discrete structure, the kinetoplast, which is always located in a distended portion of the mitochondrion near to the base of the flagellum (Robinson and Gull, 1991). The kinetoplast DNA (kDNA)[1] is usually represented as two types of circular DNA molecules, maxicircles and minicircles. In the African trypanosome *T. brucei* this involves a heterogeneous minicircle population of up to 10,000 minicircles, each ~1 kb in size, plus a homogeneous maxicircle population of 25–50 molecules each of ~20 kb in size. These circular DNA molecules which form the mitochondrial genome of *T. brucei* are concatenated into the massive, complex structure of the kinetoplast (Steinert et al., 1976; Fairlamb et al., 1978; Ryan et al., 1988). The kDNA is unusual in that maxicircle transcripts are edited through a system of addition or removal of uridine residues; a process involving guide RNA molecules (Blum et al., 1990; Stuart and Feagin, 1992). The trypanosome mitochondrial genome is also unusual in that replica-

1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; kDNA, kinetoplast DNA.

tion of this massive complex takes place only once during the cell cycle and is coordinated with nuclear "S" phase (Steinert and Van Assel, 1967; Cosgrove and Skeen, 1970; Simpson and Braly, 1970; Woodward and Gull, 1990). The segregation of replicated kDNA is mediated by the existence of a physical connection between the kinetoplast and flagellar basal body (Robinson and Gull, 1991).

The replication process within the kinetoplast differs between maxicircle and minicircle DNA molecules. Maxicircles are thought to replicate by a network-bound rolling circle method with the release of free, linearized, maxicircle progeny which subsequently reattach to the network (Hajduk et al., 1984; Ryan et al., 1988). Minicircles detach from the kDNA networks by the action of topoisomerase II and replicate as theta structures. The circular progeny are then reattached, initially to two opposing edges of the network, (Englund, 1978, 1979; Ryan et al., 1988). A number of workers have investigated the sites of DNA replication patterns within the kDNA networks (Simpson et al., 1974; Simpson and Simpson 1976; Englund 1978; Hoeijmakers and Weijers, 1980). Primarily, these studies have been based on autoradiographic detection of [³H]thymidine incorporation into kinetoplasts, or on electron microscope analysis of isolated, spread replicating kDNA networks. The results of the studies by Simpson and Simpson (1976) indicated that initially newly synthesized kDNA is localized in two foci 180° apart in isolated kDNA networks. Interestingly, Melendy et al. (1988) have demonstrated that a kinetoplast-specific

topoisomerase II enzyme is located on two opposing sites on the periphery of the replicating *Crithidia* kDNA in vivo. Moreover, Ferguson et al., (1992) have used a monoclonal antibody against a kDNA polymerase and biotin-labeled DNA probes on kDNA in vivo to reinforce the view that DNA replication within the network is associated with two peripheral regions coincident with the location of the enzyme machinery required for the process.

P'erez-Morga and Englund (1993*a,b*) have used [³H]thymidine in conjunction with electron microscopy of isolated and spread kDNA networks to allow visualization of the sites of attachment of newly synthesized minicircles in *C. fasciculata*. These high resolution studies again confirm the view that new synthesized molecules are concentrated in two peripheral zones on opposite edges of the network. This supports the view that initially the minicircle attachment sites and the sites of the replicative enzyme machinery are almost coincident. Interpretation of the resulting labeling patterns leads these workers to suggest that as mitochondrial "S" phase proceeds the kinetoplast body may rotate between the two fixed replicative enzyme complexes. Much of this current view of kDNA replication has come from studies on the insect trypanosome *C. fasciculata*. In this present study we have addressed the question of the configuration of the DNA replication sites within the kDNA of the African trypanosome, *Trypanosoma brucei brucei*. We have studied this by using immunological detection of newly synthesized, 5-bromodeoxyuridine (BrdU)-substituted DNA at the light microscope level. We have extended the resolution of this approach by developing a novel procedure that allows detection of such DNA at the electron microscope level. Our results obtained using isolated complexes and kDNA networks in vivo show that initially, as in *Crithidia*, there are two antipodal sites of newly synthesized DNA that are located on the edge of the kDNA complex. However, our results indicate that, in comparison with *Crithidia*, the pattern of DNA replication then develops in a rather different manner throughout kDNA "S" phase. Later phases show very different labeling patterns of replicated DNA suggestive of differences in either the timing of replication of minicircles and maxicircles and/or organization of the replicative apparatus in the kinetoplast of the African trypanosome.

## Materials and Methods

### Organisms

Growth of the 427 strain of procyclic *Trypanosoma brucei brucei* cells, labeling with BrdU and the preparation of cells for immunofluorescence detection of the BrdU-substituted DNA was performed essentially as per Woodward and Gull, 1990.

### Lowicryl Embedding and Immunolabeling

Cells were harvested by centrifugation, washed in PBS, and then the pellets fixed in 3.8% paraformaldehyde, 0.6% glutaraldehyde, in PBS (pH 7.2) for 3 h at room temp. The fixed cells were washed in PBS, dehydrated, and embedded in Lowicryl resin. The embedded cells were transferred to gelatin beam capsules suspended on copper wire loops and then resin polymerized by 360 nm ultraviolet light at −35°C overnight. The blocks were then left to harden at room temperature for 24 h before ultrathin sections were cut. The sections were cut using glass knives, placed onto acetone washed G200 nickel TEM grids, and transferred to 40-μl drops of 1.5 M HCl for 20 min. Sections were washed twice by floating onto 250-μl drops of double distilled water for 10 min, blocked in 1% BSA in PBS for 5 min, then transferred to first antibody diluted 1:10 in 0.5% BSA in PBS plus 0.05% Tween 20 for 60 min. Sections were then washed for 2 min, six times, in 1% BSA and transferred to gold-conjugated second antibody diluted 1:15 (10 nm goat anti-mouse, Sigma Chem. Co. Ltd., Poole, Dorset, U.K.). The sections were then washed four times, two min each, on 500-μl drops of 1% BSA in PBS. After a further six washes on 500-μl drops of 0.1% BSA in PBS the sections are washed twice in PBS, and fixed for 30 s in 2.5% glutaraldehyde in PBS. When dry the sections were stained by floating on a solution of 5% uranyl acetate in sterile double distilled water for 45–60 min. After staining the sections were washed and examined by electron microscopy.

### Incorporation of BrdU and Isolation of kDNA Networks

kDNA networks were isolated by a modification of the technique used by Fairlamb et al. (1978). 300 ml of an exponentially growing culture was labeled with 50 μM of BrdU and 50 μM deoxycytidine at 28°C for 60-, 90-, 180-, and 240-min periods. Cells were harvested by centrifugation at 1,000 g for 10 min, and resuspended in 5 ml of TNE buffer (0.1 M Tris, 0.1 M NaCl, 0.05 M EDTA [pH 7.2]). SDS and proteinase K were added to a final concentration of 1% and 0.5 mg/ml, respectively, and tubes were left overnight at 42°C. Solid CsCl was added to a final concentration of 1 g/ml and ethidium bromide was added, to give a final concentration of 1 mg/ml. The tubes were centrifuged at 1,000 g for 20 min at room temperature, the supernatant gently decanted and made up to a density of 1.5 g/ml with CsCl and then centrifuged at 100,000 g for 24 h at 20°C. The kDNA band was removed using a 5-ml sterile syringe and a 19-gauge sterile needle. The ethidium bromide was extracted with a 1:1 ratio with CsCl saturated isoamyl alcohol. After extraction the samples were dialyzed against double distilled sterile water overnight at 4°C.

### Immunofluorescence Labeling of Isolated kDNA Networks

50 μl of isolated kDNA (6 μg/ml) was added to 10 μl of 1% BSA and spread onto alcohol washed slides. The slides were left at room temperature in a moist chamber until almost dry (~35–40 min). The slides were then transferred to 75% ethanol, 4°C for 60 min and were then washed twice (5 min each wash) in filtered PBS. After washing the kDNA was denatured in 1.5 M HCl for 20 min at room temperature. 50 μl of first antibody, (anti-BrdU; Partec or Biocell Labs. Cardiff, UK) diluted 1:75 in PBS was then applied to the slides for 45–90 min at 25°C, in a moist atmosphere. After two 5-min washes in PBS the kDNA was incubated with 50 μl of second antibody, (rabbit anti-mouse; Dakopaks, Denmark) diluted 1:30 in PBS containing 0.5% BSA for 60 min at room temperature. The slides were then washed again in PBS and then processed for immunofluorescence as previously described. The polyclonal gold-conjugated second antibody used in immunogold-labeling experiments was preincubated with kDNA complexes. This was found to be essential in order to remove nonspecific binding to DNA or chromatin due to autoimmune type anti-DNA antibodies which are sometimes found in polyclonal immune-sera.

### Immunogold Labeling of Isolated kDNA Networks

Immediately after picking up the kDNA (spread by the spontaneous absorption method of Lang and Mitani, 1970), the grids were washed by transferring them to 500-μl droplets of sterile double distilled water 2 × 5 min. After washing, the grids were transferred to 250-μl drops of 20 mM glycine for 5 min then washed once more. The kDNA was denatured by floating grids on 0.07% NaOH 10 s followed by two 5-min washes in water. The grids are then blocked for 5 min in sterile, first antibody buffer, 20 mM tris, 20 mM NaCl, and 0.2% glycerol containing 10 μg/ml sterile BSA. After blocking the grids were transferred to 50-μl droplets of first antibody for 30 min (anti-BrdU; Biocell Labs.) diluted 1:150 in the above buffer, washed 4 × 5 min in first antibody buffer, and then transferred to 50-μl droplets of second antibody diluted 1:150 for 45 min. The grids were then washed 2 × 2 min in first antibody buffer, 2 × 2 min in 0.1% BSA first antibody buffer, 2 × 2 min in 20 mM Tris, 20 mM NaCl, 0.2% glycerol, 1 × 5 min in water. After washing, the antibodies were fixed to the kDNA with 1% glutaraldehyde for 10 s. After fixation the kDNA was dehydrated in isopentane and metal shadowed.

## Results

### Visualization of kDNA Replication In Vivo

The production of monoclonal antibodies to the thymidine analogue 5-bromodeoxyuridine (Gratzner, 1982) has made it possible to visualize DNA synthesis within cells using standard immunofluorescence techniques. We have shown previously that this technique is an efficient way of labeling DNA synthesis in both the nucleus and kinetoplast of the African trypanosome, *T. brucei* (Woodward and Gull, 1990). To examine the pattern of DNA replication within the kinetoplast in vivo we allowed a culture of procyclic form trypanosomes to incorporate BrdU for a short period and then processed the cells for immunofluorescence microscopy. Examination of the kinetoplast body of cells in "S" phase revealed that the newly synthesized DNA was located to two antipodal sites on the kDNA network (Fig. 1). We have also been able to develop this labeling technique to the electron microscope level of resolution allowing detection of areas of newly synthesized DNA at higher resolution. We have developed techniques that allow detection using both thin sectioning approaches and examination of spread kDNA. Examination of whole kinetoplasts in vivo using immunogold, thin section techniques after incorporation of BrdU revealed that the labeling of newly synthesized DNA was essentially located to the electron dense kinetoplast body and was not present in the immediately surrounding mitochondrial matrix. Moreover, the labeling could often be detected on the poles of the kinetoplast body indicating the presence of discrete, antipodal sites of initial kDNA synthesis in vivo (Fig. 1 *d*).

### Visualization of Replicated DNA in Isolated kDNA Networks Using Immunofluorescence Microscopy

More detailed structural information on the configuration of the sites of kDNA synthesis can be obtained when kDNA networks are isolated and spread in an extended, two-dimensional form. To identify specific regions of DNA replication within the isolated kDNA networks, procyclic cultures were incubated with BrdU and then complete kDNA networks were isolated and allowed to spread onto glass slides. These were then processed for immunofluorescence and viewed in the fluorescence microscope. Since the cell doubling time of *T. brucei* is 8.5 h and kinetoplasts replicate once every cell cycle (Woodward and Gull, 1990), we used incorporation times between 30 and 240 min in order to provide information on labeling patterns in a single cell cycle. When evenly spread on glass slides the kDNA networks appeared as circular-, elliptical-, and biolobed-shaped structures as seen in Fig. 2. Micrographs of these kDNA networks were then sorted by using the shape of the DAPI image and the increasing extent of immunofluorescence labeling as being representative of the stage of kDNA replication during kDNA "S" phase (Fig. 2). Initial DNA synthesis patterns reflected those seen in vivo in that the kDNA networks showed antipodal incorporation of the BrdU label (Fig. 2). A comparison of the DAPI images of the kinetoplasts and the corresponding BrdU images suggests that this general antipodal pattern of sites of newly synthesized DNA is maintained for some time. Later, it appears that arcs of labeled DNA extend from these antipodal sites along the edges of the
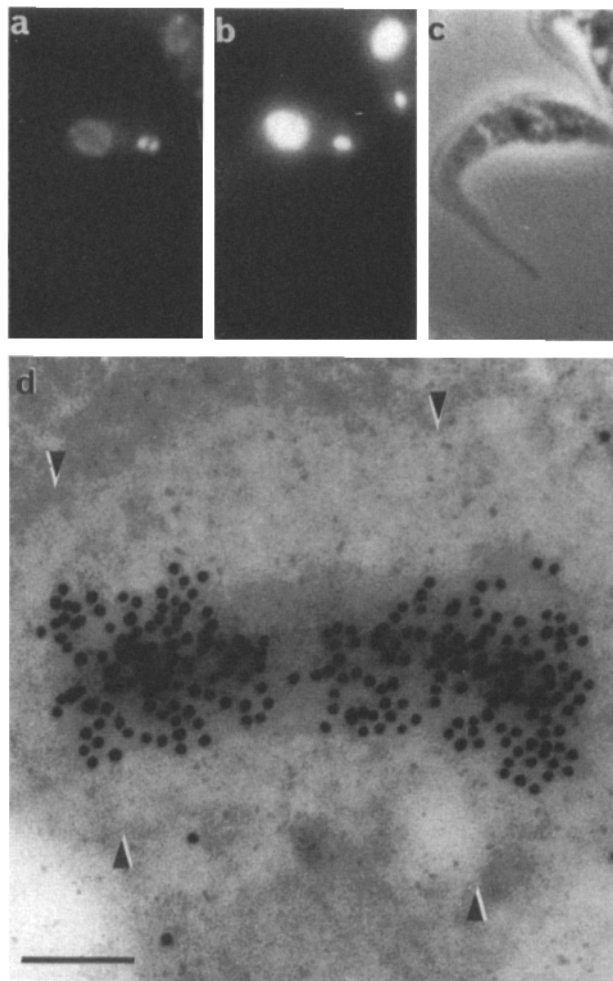


*Figure 1.* Immunofluorescence (*a*) and immunogold electron microscopical (*d*) detection of an antipodal incorporation of BrdU into replicating kinetoplast of *T. brucei* in vivo. Figs. 1, *a*, *b*, and *c* are immunofluorescence DAPI and phase images of the same cell. Fig. 1 *d* is a thin section electron micrograph of a mitochondrion (outlined by *arrowheads*) in a *T. brucei* cell. In both cases label is located at two poles of the kinetoplast. Bar, 0.1 μm.

kDNA networks (Fig. 2). It is apparently only late in the process of kDNA "S" phase that the full body of the network contains newly replicated DNA. However, even at this time when the network shows a clear bilobed form (Fig. 2) we find that the central portion shows no immunofluorescence detection of newly synthesized DNA while the DAPI image clearly indicates that this region does contain DNA. Later in the final stages of synthesis, very clearly bilobed segregating kDNA networks show a uniform distribution of both DAPI and immunofluorescence staining. This coincidence of labeling patterns indicates the presence of newly replicated DNA throughout the network at this late stage.

### Visualization of Replicated DNA in Isolated kDNA Networks Using Electron Microscopy

Isolated kDNA networks are easily visualized at the electron microscope level when carefully spread and metal shadowed and this approach has often been used to study minicircle and
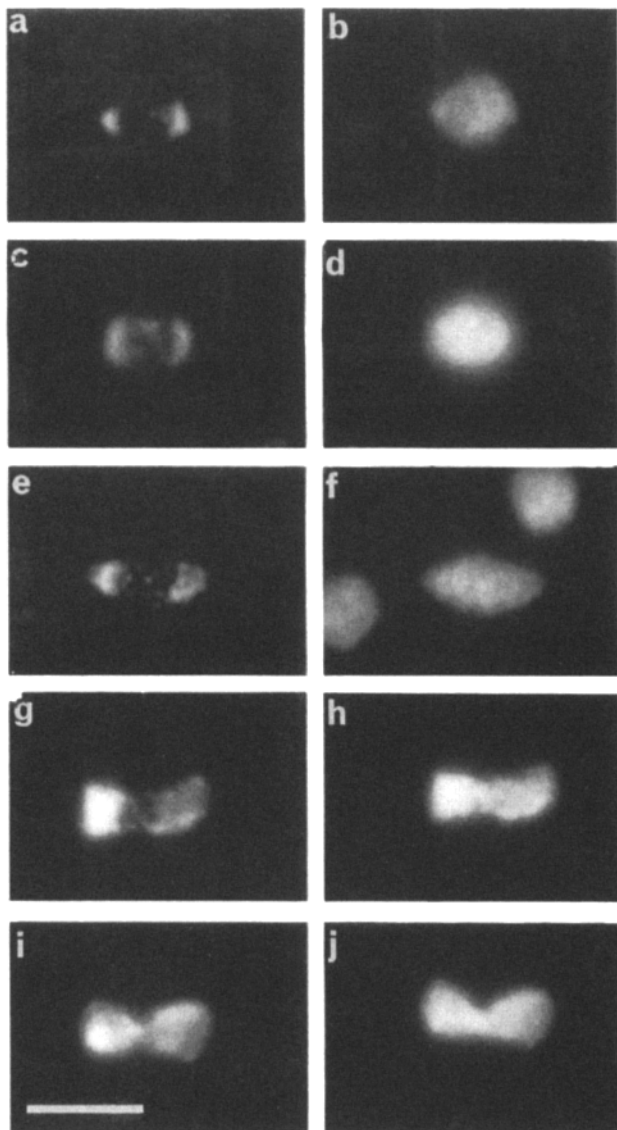
*Figure 2.* Immunofluorescence (*a, c, e, g,* and *i*) and DAPI images (*b, d, f, h,* and *j*) of isolated and spread kinetoplasts after incubation of *T. brucei* cells with BrdU. The kinetoplasts have been ordered in this figure in a manner representative of the progression through "S" phase and segregation. Bar, 5 μm.

maxicircle organization (Lang and Mitani, 1970). We reasoned that we might be able to extend the immunological detection of the BrdU substituted DNA to much higher levels of resolution if we could perform the immunogold labeling on such networks and then visualize them by metal shadowing/electron microscopy. Spread kDNA networks isolated from cells incubated with BrdU were prepared for transmission electron microscopy using the spontaneous absorption micromethod involving spreading the networks at the surface

of drops of a cytochrome C solution in a formaldehyde saturated environment. These methods gave excellent visualization of the networks with essentially the same structure as presented by other previous studies. The development of this method to include the immunolabeling step proved to be rather difficult since detection of the BrdU substituted DNA by the monoclonal antibody requires a denaturation step involving incubation of the spread network on a solution of alkali, which has a deleterious effect on the quality of visualization of the DNA network. However, we were able to achieve conditions whereby we could obtain immunogold detection of the newly synthesized DNA and still retain reasonable visualization of the ultrastructure of the kDNA network.

Figs. 3, 4, 5, and 6 show representative images of whole, spread kDNA networks metal shadowed after immunogold detection of the newly synthesized DNA. The areas of BrdU incorporation are seen as accumulations of 10-nm gold particles and these micrographs have been printed at a size that allows recognition of the gold particles, yet reveals entire networks. They have been ordered in their apparent "S" phase position by comparison of the overall network profile and ultrastructure by reference to previously published work (Hoeijmakers and Weijers, 1980).

Fig. 3 displays a spread kDNA network in which the region of gold labeling is observed at two opposing regions of the network, again confirming the antipodal pattern seen at the light microscope level and in vivo. The region of labeling does extend somewhat around the edges of the network from these polar regions. The central region between these two opposing sites remains unlabeled with only a few gold particles present. The label does not encroach completely around the whole of the periphery of the network. As the replication process continues the kDNA network grows and the lobes increase in size. The labeling pattern of the two lobes continues to maintain the antipodal, crescent shape (Fig. 4). In well labeled, elongated kDNA networks the immunogold technique allows the detection of newly replicated DNA molecules over most of the lobes of the network (Fig. 5). This type of labeling pattern appears akin to the conformation seen in Fig. 2 where the main body of both lobes of the replicating network are labeled and yet newly synthesized DNA appears to be absent from the central, mid-zone portion of the network. This pattern of labeling of kDNA isolated in the very late stages of segregation is emphasized in Fig. 6. Here, although both main lobes are clearly labeled with gold particles there is very little labeling of the very central mid-zone DNA. It is a characteristic of *T. brucei* kDNA that these very late segregating networks have maxicircle regions (termed clews) located at this mid-zone of the replicating lobes (Hoeijmakers and Weijers, 1980). Surprisingly, it is these maxicircle clews of late stage replicating kDNA networks that appear unlabeled while the rest of the network is heavily labeled (Fig. 6).

*Figures 3–6.* Immunogold detection of sites of BrdU substituted within isolated spread and metal shadowed kinetoplast complexes. These micrographs show representative images illustrating the initial antipodal sites of newly replicated DNA (Fig. 3) and the progressive labeling of the polar lobes (Figs. 4 and 5). Fig. 6 shows a kinetoplast complex in a very late stage of replication/segregation and shows both lobes well labeled but a central midzone (containing the maxicircles) remains essentially still unlabeled. Bars: (Fig. 3) 1 μm; (Fig. 4) 1 μm; (Fig. 5) 1 μm; (Fig. 6) 1 μm.

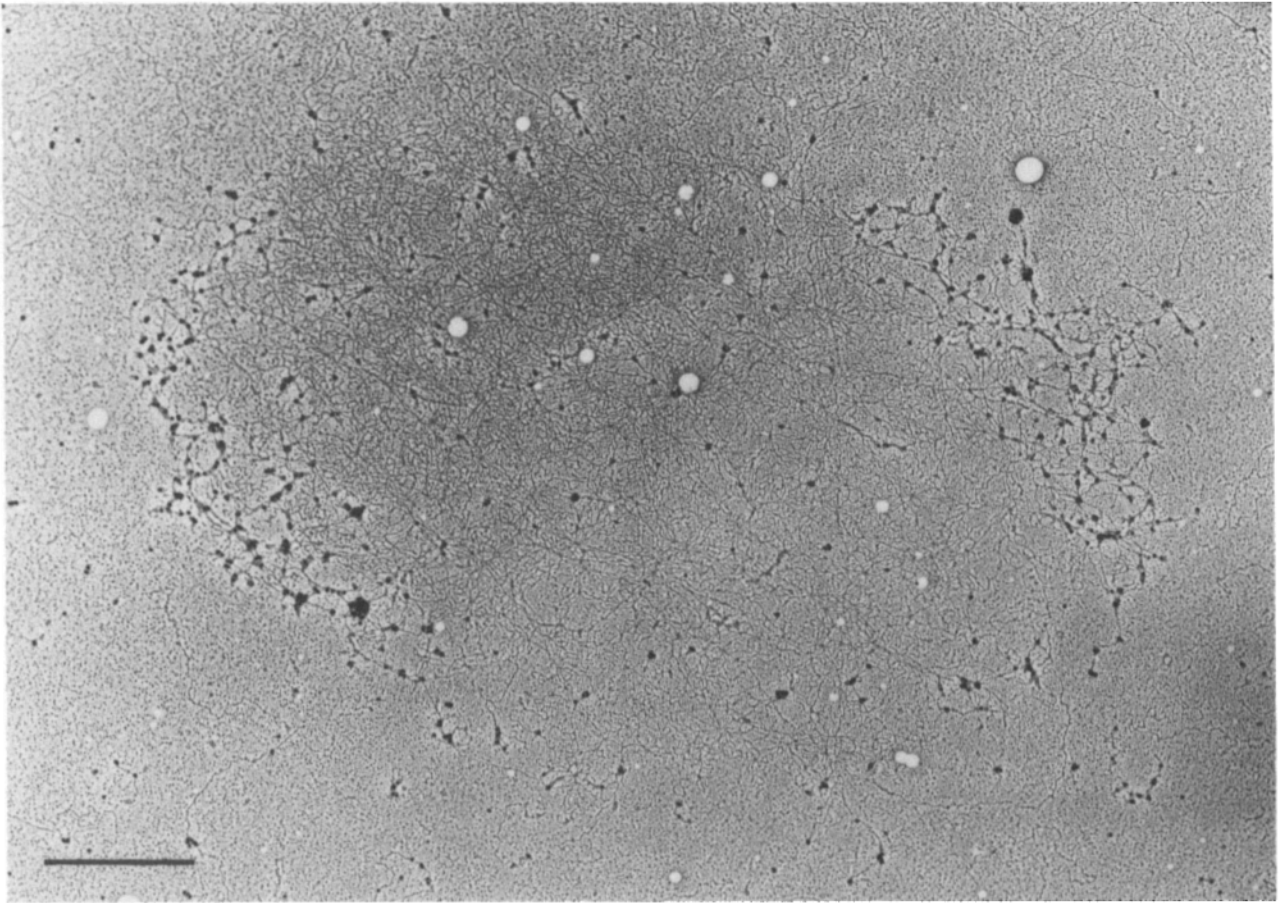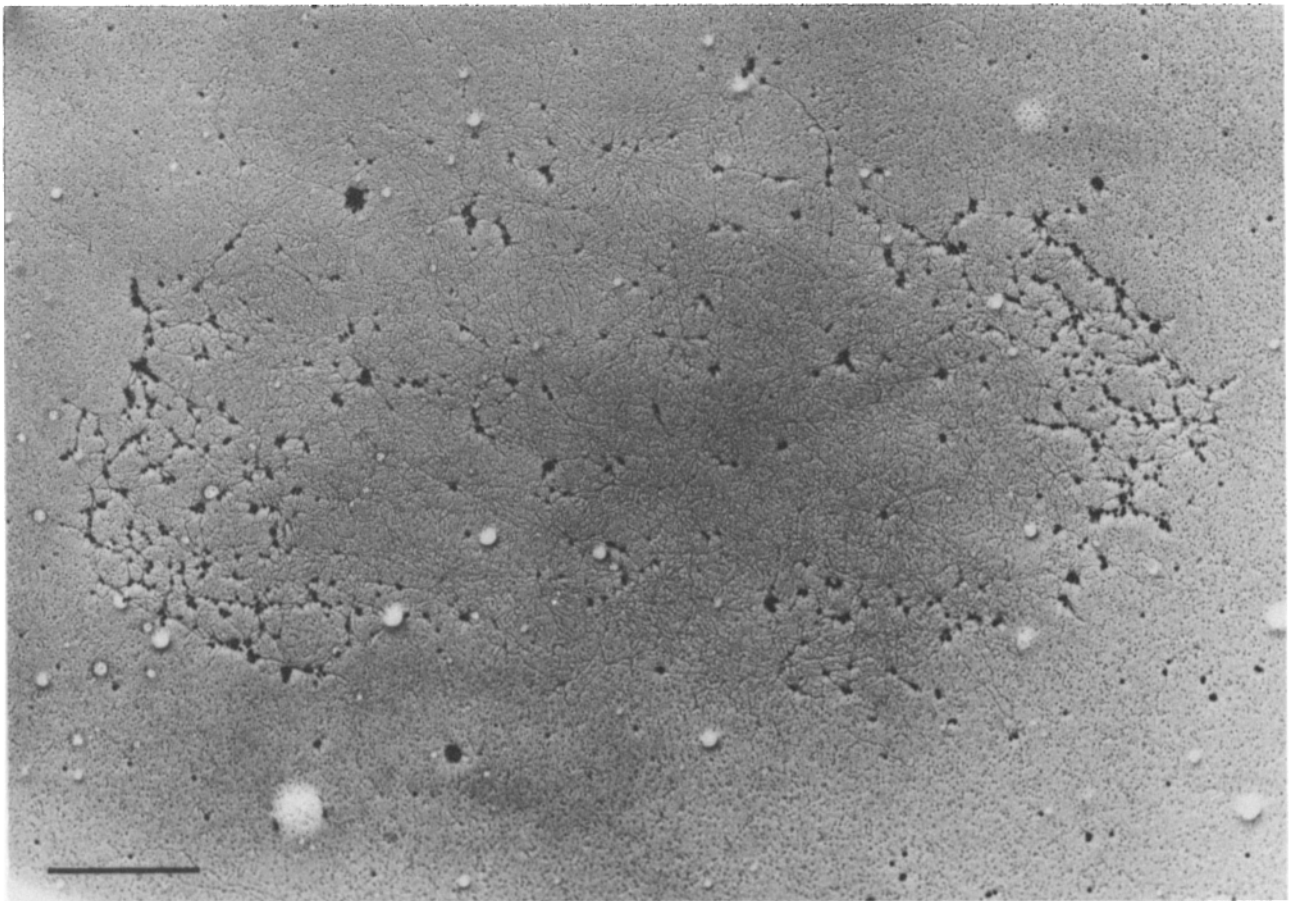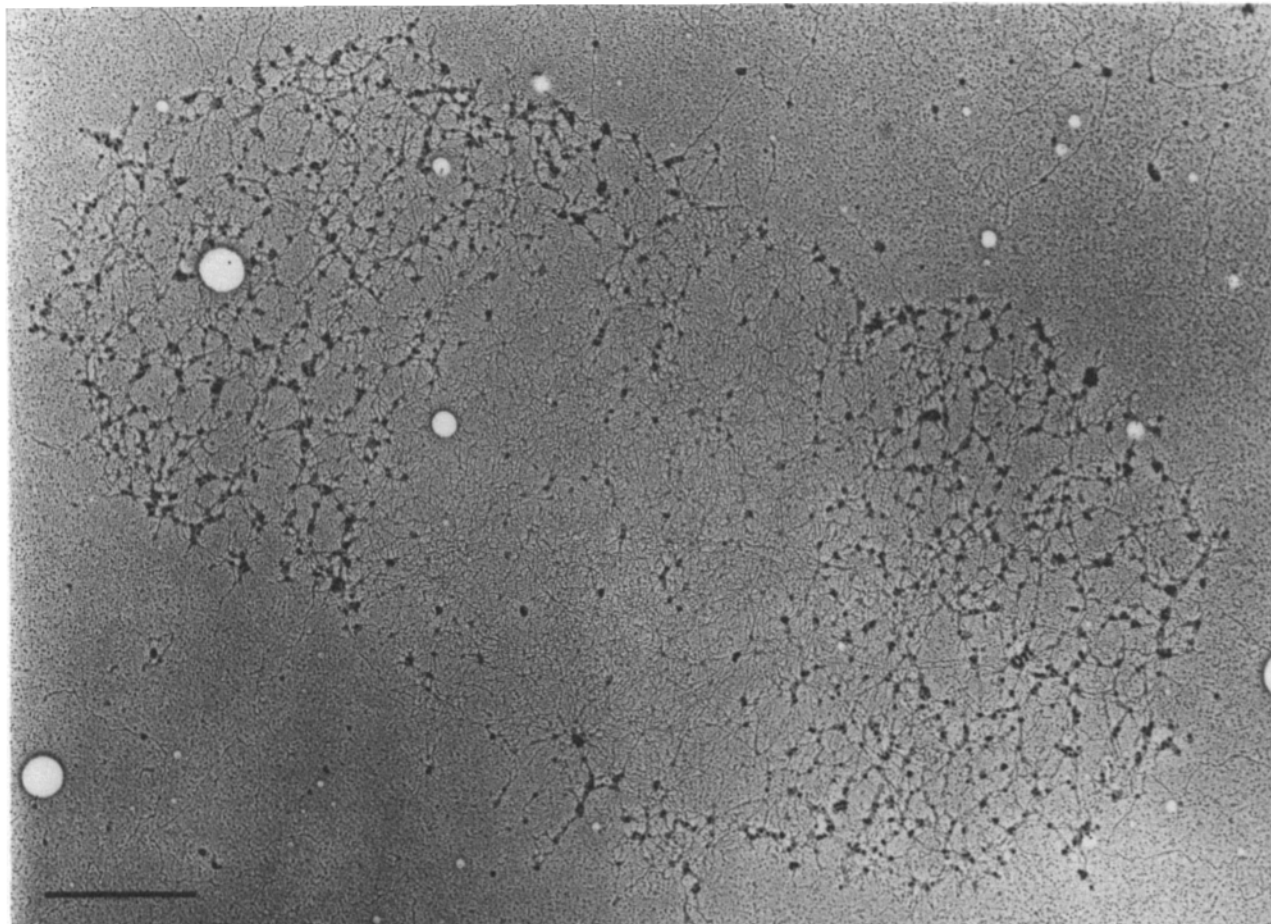*Figure 3.*



*Figure 4.*

*Figure 5.*

The various labeling patterns of newly replicated DNA within the *T. brucei* spread kDNA networks are outlined in diagrammatic form in Fig. 7.

## Discussion

We have been able to achieve detection of replicated DNA within the *T. brucei* replicating and segregating kDNA by application of the BrdU/immunolabeling approach. This has a number of advantages over previously used methods in that it has allowed greater resolution of the configuration of the position of the replicated DNA, both in vivo and after isolation of the kDNA networks. Moreover, we have been able to take this approach to the electron microscope level to facilitate the definition of the positions of replicated DNA molecules within this complex at high resolution. The initial pattern of antipodal sites of newly replicated DNA molecules (Fig. 7, stage 1) is very similar to that described previously for other kinetoplastids. The kDNA replication process in *C. fasciculata* and *Leishmania tarentolae* is initiated by replicated molecules relocating to the edges of the network at sites directly opposing each other (Simpson et al., 1974; Simpson and Simpson, 1976; P'erez-Morga and Englund, 1993*a,b*). Our labeling of *T. brucei* kDNA indicates that this pattern seen on isolated networks is a true reflection of the geometry of the process in vivo. After this initial stage of the replication process our labeling studies suggest that the

newly replicated DNA is then detected as two crescent arms extending from the initial antipodal sites (Fig. 7, stage 2). A pattern of replication emerges in *T. brucei* which do not completely encroach around the network. The latter stages of kDNA replication patterns observed in *T. brucei* do not appear to have been detailed for any other kinetoplastid. We have detected no real evidence of the "doughnut" type of labeling patterns that are so clearly present in the intermediate stages of replicating kDNA of *C. fasciculata* (Simpson et al., 1974; Simpson and Simpson, 1976; Englund, 1978; Ferguson et al., 1992). The interesting model of rotating kDNA networks and/or replicating machinery of *Crithidia* (P'erez-Morga and Englund, 1993*a,b*) clearly does not completely fit the replicating patterns that we have observed in *T. brucei*. The replication process appears then to proceed in *T. brucei* with the newly replicated DNA molecules essentially filling the main body of the elongating network. However in both the light microscope studies and at the electron microscope level it appears that these late networks (Fig. 7, stages 3 and 4) do not exhibit the presence of newly synthesized molecules at their midzones. Only in the final stages of kDNA replication in *T. brucei* does the central region become labeled, suggesting that the midzone replicates just prior to network segregation. These constricted networks appear to be much more of a prominent feature of *T. brucei* kDNA replication than other members of the kinetoplastids so far studied. We can detect very late networks (Fig. 7, stage
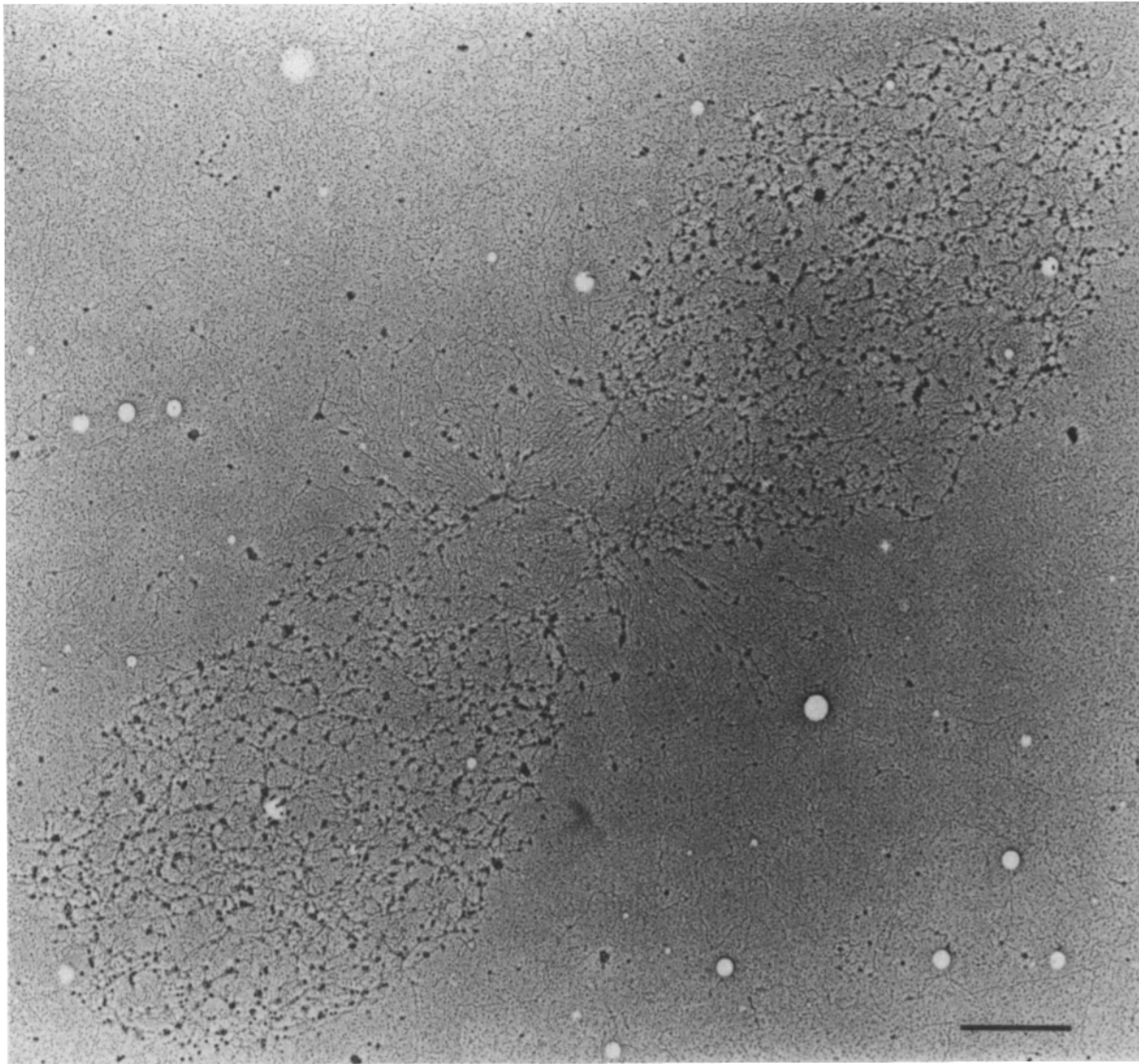
*Figure 6.*

5) which show an essentially uniform pattern of detection of newly replicated molecules over the entire kDNA network. Therefore, it appears that ultimately the entire complex contains replicated molecules. The nature of the lack of labeling at the midzones of the networks late in the replication process is intriguing. We have applied a novel technique to the process of detecting the newly replicated DNA. It may be that the immunological detection of the BrdU substituted DNA has a threshold level of detection that does not allow visualization of replicated molecules at this site. However, in the light microscope images it is clear there is a reasonable amount of DNA at these sites, judging from the DAPI image. The replicating *T. brucei* kDNA network has been studied previously by electron microscopy and it is clear that the central, midzone areas of these late replication complexes are the main sites of location of the maxicircle population (Hoeijmakers and Weijers, 1980). Indeed in our studies on the immunological detection of BrdU-labeled DNA at the electron microscope level we have been able to reveal the presence of the maxicircles at these sites. Again, these maxi-

circles are unlabeled in comparison to the rest of the network. Given the caveat of the sensitivity of the detection procedure, this raises the possibility that there is a slight difference in the timing of replication of the molecules of the complex. The absence of labeling at the constricted midzones may therefore be a reflection of the fact that maxicircle DNA replicates in "S" phase at a slightly later stage than minicircle DNA. Recently, we have been able to show that there is a physical connection between the kinetoplast and the basal body of the flagellum in *T. brucei* and that the segregation of the kinetoplasts is intimately associated with segregation of the basal bodies in the trypanosome cell cycle. Both the kinetoplast and basal body are single copy organelles in the trypanosome cell and it appears possible that the reason for the antipodal sites of location of newly synthesized DNA (and the replicative enzyme factories?) may be a reflection of a high order association with this segregational machinery.

In summary, it appears that the configuration of the sites of newly synthesized DNA molecules within the replicating
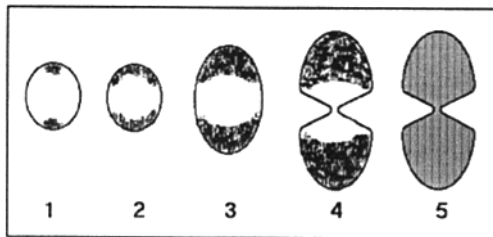
*Figure 7.* Diagram representing the replication pattern of DNA of *T. brucei*. The dark zones indicate the site of newly replicated DNA which are initially antipodal (stage 1), they become more extensive (stages 2 and 3) yet do not encircle the replicating kinetoplast. This pattern is maintained towards the bilobed stage 4 complex until finally reaching the stage 5 network. This latter stage network appears to have a relatively uniform distribution of newly replicated DNA.

kDNA network of the African trypanosome share some of the characteristics that are well established for the initial phases of replication in *Crithidia*. However, our application of an immunological procedure for detection of the sites of newly replicated DNA suggests that there may be important differences in the models derived from studies of other kinetoplastids.

The accompanying paper of Ferguson et al. (1994) reports results which are similar and complementary to those we have described in our studies.

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