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The replication mechanism of kinetoplast DNA networks in several trypanosomatid species

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SUMMARY

Kinetoplast DNA, a giant network of interlocked DNA circles, replicates by an unusual mechanism. Minicircles are released individually from the network by a topoisomerase II, and then, after replication, their progeny are reattached at antipodal positions on the network periphery. Studies to date have revealed two distinct variations on this model. In *Crithidia fasciculata* the newly replicated minicircles quickly become uniformly distributed around the network periphery, whereas in *Trypanosoma brucei* the minicircles accumulate near their two points of attachment. The kinetoplast DNA replication mechanism used by other related trypanosomatid species was until now unknown. Here we used a novel method, involving fluorescence microscopy of isolated networks, to investigate kinetoplast DNA replication in *Leishmania tarentolae, Leishmania donovani, Trypanosoma cruzi* and *Phytomonas serpens.* We found that all of these species have a replication mechanism resembling that of *C. fasciculata* and that the polar replication mechanism observed in *T. brucei* is so far unique to this species.

Key words: Kinetoplast DNA, Trypanosomatid, Replication

INTRODUCTION

Trypanosomatids are an ancient family of parasitic protozoans, many species of which cause disease in diverse organisms ranging from humans to plants. In this paper we focus on the complex structural organization and mode of replication peculiar to these parasites' remarkable mitochondrial DNA, termed kinetoplast DNA (kDNA).

kDNA is composed of several thousand circular, covalently-closed, non-supercoiled DNA molecules catenated to form a huge planar network which resembles chain mail. Each kDNA network contains two types of circular DNA molecules. Minicircles are present in several thousand copies, ranging from 0.5 to several kilobases (kb) in different species. They are usually heterogeneous in sequence but identical in size within a network. Their only known genetic function is to encode small guide RNAs which control the specificity of editing of maxicircle transcripts. Maxicircles, present in several dozen apparently identical copies, range between 20 kb and 38 kb in different species and are threaded through the catenated monolayer of minicircles. Maxicircles encode rRNAs and mitochondrial proteins involved in functions such as electron transport and ATP synthesis. In vivo the kDNA network is highly condensed into an organized disc-shaped structure. For reviews on kDNA, see Simpson (1987), Stuart and Feagin (1992), Shapiro and Englund (1995).

The network structure of kDNA dictates an unusual replication mechanism. Minicircles do not replicate while linked to the network, but instead are released by a topoisomerase II to replicate as free minicircles (Englund, 1979). Covalently-closed free minicircles undergo replication as θ-structures, and their progeny, which contain gaps, are then reattached to the network periphery (Pérez-Morga and Englund, 1993a). As network replication proceeds, the central region of unreplicated covalently-closed minicircles diminishes in size and the peripheral region, containing newly-replicated, gapped minicircles, enlarges. Once all the minicircles have replicated, all contain gaps and the minicircle copy number has doubled. The gaps are then repaired and the network splits in two, the latter process presumably mediated by a topoisomerase II which unlinks neighboring minicircles along the network cleavage line (Pérez-Morga and Englund, 1993b). See Ray (1987), Shlomai (1994) and Shapiro and Englund (1995), for reviews on kDNA replication.

Comparative studies on kDNA replication in *Crithidia fasciculata* and in the African trypanosomes *Trypanosoma brucei* or *T. equiperdum* have revealed two variations of this replication model. In *C. fasciculata* newly replicated minicircles are reattached to the network at two opposite sites on the network periphery, thought to be adjacent to two protein complexes known to contain topoisomerase II and a DNA polymerase β (Simpson and Simpson, 1976; Melendy et al., 1988; Ferguson et al., 1992; Pérez-Morga and Englund, 1993a). Despite the antipodal sites of minicircle attachment, the newly replicated gapped minicircles soon become uniformly distributed around the network periphery in a sequential manner. This uniform distribution is thought to be due to a relative movement of the kinetoplast disk and the two protein complexes. Although not proven, it has been suggested that the kinetoplast disk rotates between the two complexes.

Due to the distribution of the newly replicated minicircles in the network we will refer to this mechanism as the 'annular' replication mechanism. There is a different situation with the kDNA of the African trypanosome *T. brucei*. Newly replicated, gapped minicircles are again attached at two opposite sites on the network periphery. In this case, however, the progeny minicircles accumulate about these two sites and do not distribute around the network periphery (Hoeijmakers and Weijers, 1980; Ferguson et al., 1994; Robinson and Gull, 1994). This polar pattern gives rise to dumbbell-shaped networks, a distribution that could be explained if the kinetoplast disk did not spin, but remained stationary. We refer to this mode of replication as the 'polar' kinetoplast replication model.

In this paper our goal was to determine whether kDNA in several different trypanosomatid species replicated by the annular or polar kinetoplast replication model, or whether novel mechanisms exist. We addressed this issue by developing a new and simple experimental method which allows vivid, rapid and accurate visualization of the distribution of newly replicated minicircles in a partly replicated isolated network. The technique involves selective incorporation of fluorescein-labeled nucleotides into the endogenous gaps specific to newly replicated minicircles. Fluorescence microscopy then reveals the pattern and distribution of fluorescein localization, which indicates the mode and stage of replication of each member of large populations of networks. We found that the kDNAs of *Leishmania tarentolae, Leishmania donovani, Trypanosoma cruzi,* and *Phytomonas serpens* all replicate in a manner similar to that of *C. fasciculata*, implying a wide distribution of the annular model. *T. brucei* was the only example in which kDNA replicated by the polar model. In addition, the method we have described should prove useful, not only for diagnosing the mechanism of kDNA replication, but also for assessing the kDNA replication status in parasites in which the cell cycle has been interrupted by drugs or mutations.

MATERIALS AND METHODS

Parasite culture and kDNA isolation

C. fasciculata and *L. tarentolae,* originally gifts of Larry Simpson (UCLA), have been cultured in our laboratory for many years. *P. serpens* G-1 was from Dimitri Maslov (UC, Riverside). These cells were cultured in brain heart infusion (BHI) medium (37 g/l) supplemented with hemin (20 μg/ml) at room temperature to mid log phase. *T. cruzi* epimastigotes (Silvio X-10 clone) and *L. donovani* promastigotes (MHOM/SD/62/1S-CL2D) were from Terry Shapiro (Johns Hopkins School of Medicine) and were also grown to exponential phase as described (Bodley and Shapiro, 1995). *T. brucei* 427-60 procyclic forms were from Mary G. S. Lee (New York University) and were grown to exponential phase in SDM-79 medium containing 10% fetal calf serum, at 27°C. All kDNA was isolated as described (Pérez-Morga and Englund, 1993a).

Incorporation of fluorescein-labeled nucleotides

The fluorescent nucleotide, fluorescein-5(6)-carboxamidocaproyl-[5-(3-aminoallyl)-2′-deoxy-uridine-5′-triphosphate] (dUTP-F, Promega or Boehringer-Mannheim), was incorporated into the endogenously gapped minicircles in isolated kDNA networks using 3′ end labeling by *Escherichia coli* DNA polymerase I or terminal deoxynucleotidyl transferase (TdT). All labeling reactions were carried out in foil-wrapped tubes to avoid photo-bleaching. For DNA polymerase I, the reaction (50-100 μl) contained 50 mM Tris-HCl, pH 7.6, 10 mM

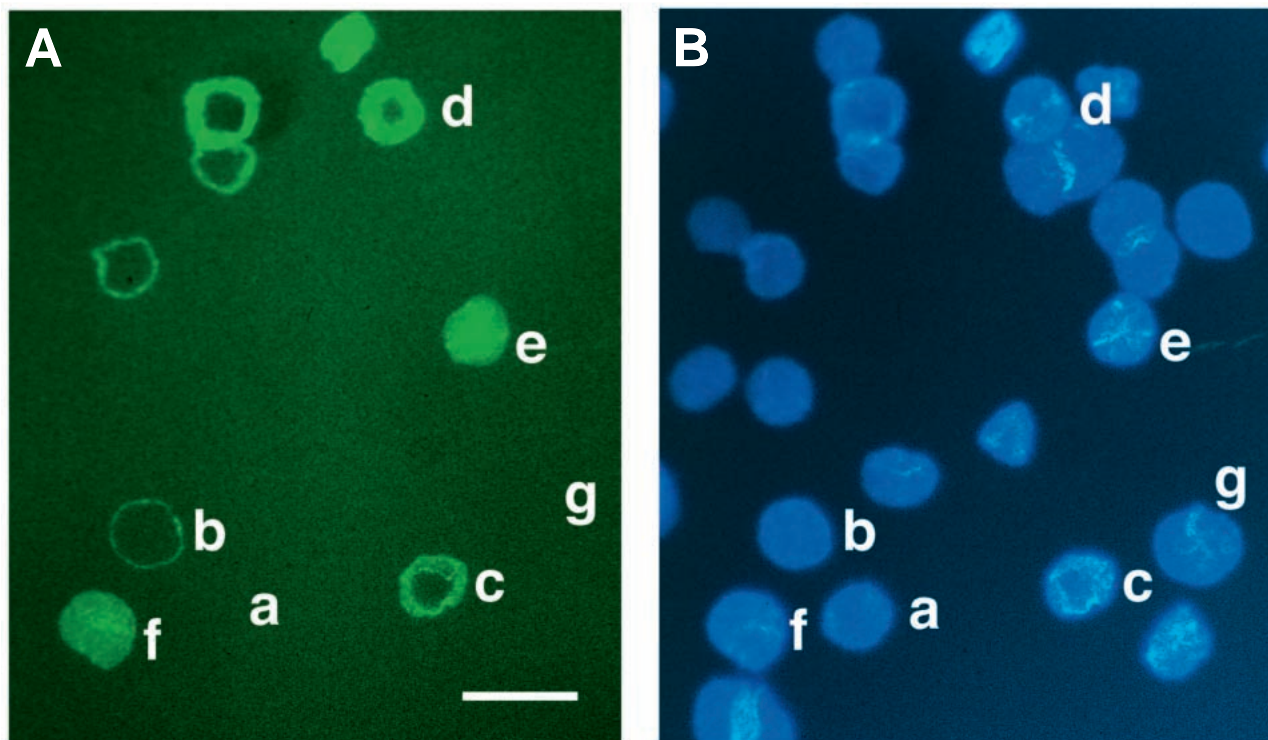

Fig. 1. Networks from asynchronous exponentially growing *T. cruzi.* Networks were labeled with dUTP-F using DNA polymerase I (A) and stained with DAPI (B). Lettered networks are discussed in the text. Bar, 16 μm. Magnification was determined by measuring sizes of a given image on film and on the printed figure, then multiplying this ratio by the microscope magnification factor of 250.

$MgCl_2$, 5 μM dUTP-F, 10 μM dATP (no additional deoxynucleotides were added), 0.2 unit/μl *E. coli* DNA polymerase I (New England Biolabs or Gibco-BRL), and 0.1-1.5 μg kDNA. The reaction was incubated for 2 hours at 16°C. The TdT reaction (50 μl) contained 180 mM potassium cacodylate, pH 6.6, 24 mM Tris-HCl, pH 6.6, 0.18 mM DTT, 0.1 mM EDTA, 0.225 mg/ml bovine serum albumin, 2.25 mM $CoCl_2$, 5 μM dUTP-F, 10 μM dATP, 10 units of TdT enzyme (Promega), and 0.1-1.5 μg kDNA, and was incubated for 30 minutes at 37°C. The TdT reaction has been widely used in situ to detect fragmentation of DNA in apoptotic cells. Both reactions were stopped

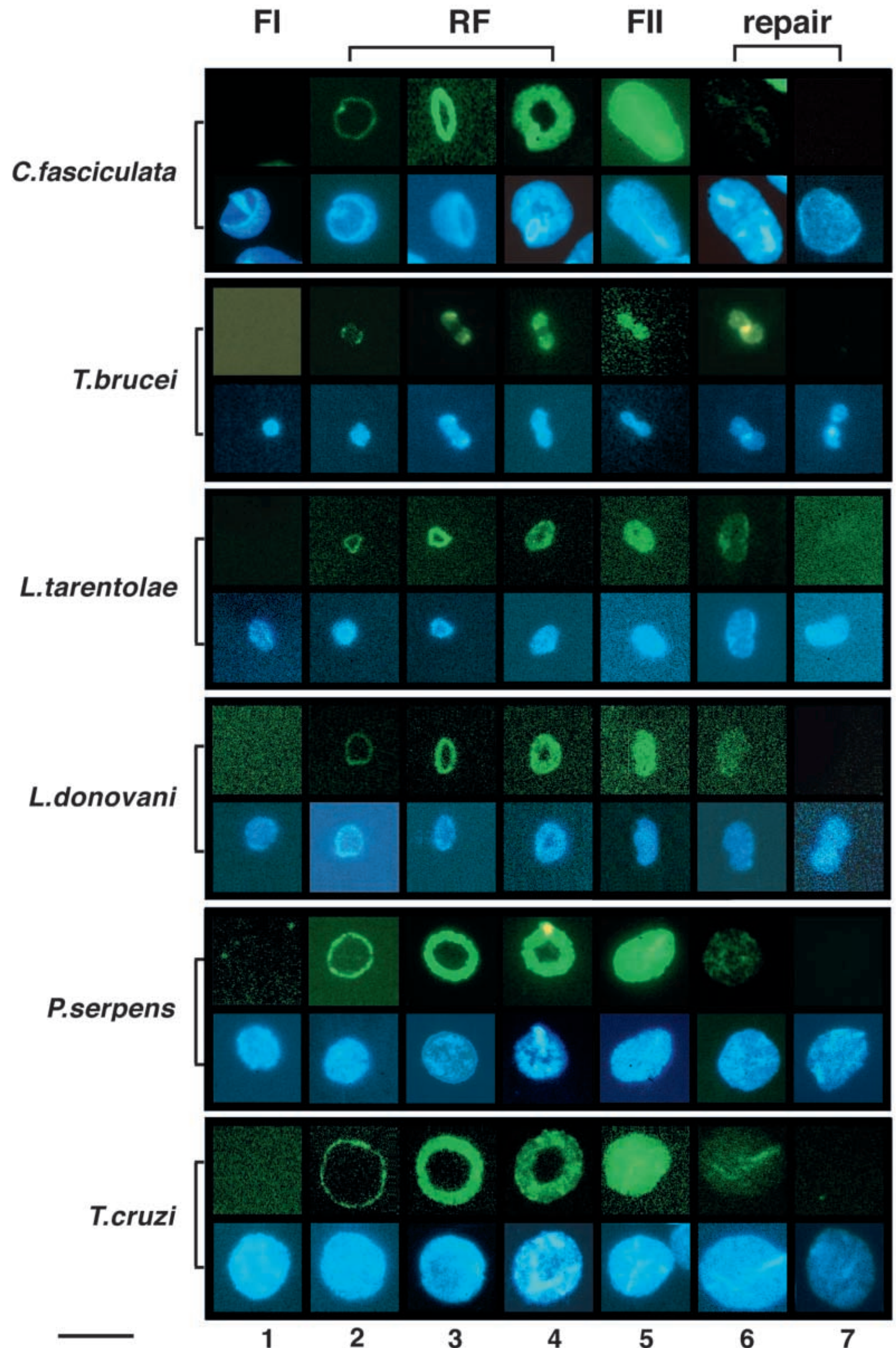

Fig. 2. Networks from different trypanosomatid species at progressive stages of replication. Networks were labeled with dUTP-F using either DNA polymerase I (*C. fasciculata, L. tarentolae, L. donovani, P. serpens, T. cruzi*) or TdT (*T. brucei*), and then stained with DAPI. They were assigned to a stage of replication, either replicative form (RF) or Form II (FII) by the criterion of increasing levels of fluorescein fluorescence; Form I (FI) or repaired networks, which expressed diminished or no fluorescein fluorescence, were distinguished on the basis of size which is larger for repaired networks. In the different species, 23%-73% of all networks were Form I and 14%-50% were replicating networks. No dumbbell shaped networks were observed in any species except *T. brucei*. Bar at lower left, 18 μm. Size measurements of networks from fluorescence images can be up to twofold smaller than those derived from EM images (data not shown); we attribute this to differences in settling and adhesion during spreading on the glass slide.

by chilling and the addition of 0.5 M Na_2EDTA to a final concentration of 25-50 mM. The samples were stored at 4°C. Fluorescence microscopy showed that the TdT reaction labeled kDNA more brightly than did the polymerase I reaction, but the TdT-labeled networks sometimes appeared to have slightly rough edges when compared to non-treated or DNA polymerase I-labeled kDNA.

Slide preparation and fluorescence microscopy

All steps were carried out in the dark or under low level illumination at room temperature. Glass slides were washed in 90% ethanol, air-dried, wiped with a tissue dipped in 0.01% poly-L-lysine (Sigma), and then air-dried for 30 minutes. Fluorescein-labeled kDNA (15-30 μl), mixed with 150 μl of sterile water and 10 μl of 0.2% Tween-20, was placed on the slide and then spread over the surface with the side of a plastic (yellow) pipette tip. The slide was set in a humid, dark box for 45 minutes at room temperature, allowing the kDNA networks to adhere to the slide surface. The slide was then washed twice, for 7 minutes each time, in phosphate buffered saline (PBS, 138 mM NaCl, 3 mM KCl, 2 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.2) for 15 minutes. In some experiments, formaldehyde (200 μl of 2.7%) was added for 30 seconds and then removed by draining; however, this treatment is not necessary. The slide was washed for 5 minutes in PBS containing 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) and then for 2 minutes in PBS. The slide was then treated with 20 μl of 1 mg/ml *p*-phenylenediamine in 50% glycerol, 500 mM NaCl, 50 mM Tris-HCl, pH 9.0, 4 mM NaOH. A cover slip was added and edges were sealed with clear nail polish. The slide was stored at 4°C in the dark. Analysis was within 24 hours on a Zeiss MC80 Axioskop fluorescence microscope, using DAPI and fluorescein filters and a 100× Phase 3 oil lens. Photographs were taken at standard exposure setting using Kodak Elite II ASA 400 slide color film. Slides were scanned on a Nikon LS1000 Slide Scanner with Nikon Scan 5.2 software. Images were imported into Adobe Photoshop 3.0 for processing. The images used to create Fig. 2 were treated identically at all stages to preserve relative magnification.

RESULTS AND DISCUSSION

A method for analysis of replication of isolated kDNA networks

Our initial goal was to develop a rapid and accurate method for analyzing the spatial distribution of covalently-closed and gapped minicircles in large numbers of isolated kDNA networks. Previous methods, using electron microscopy (EM) in the presence of intercalating dye (Englund, 1978; Pérez-Morga and Englund, 1993b) or fluorescence in situ hybridization in intact cells with minicircle probes (Ferguson et al., 1992), are laborious and time-consuming. We found that we could efficiently label isolated networks at the endogenous gaps in newly replicated minicircles by introducing a fluorescein-labeled nucleotide (dUTP-F) using either DNA polymerase I or terminal deoxynucleotidyl transferase. We could then examine large populations of networks by fluorescence microscopy and compare the distribution of fluorescein fluorescence with that of DAPI fluorescence, DAPI being a dye which uniformly stains all of the networks. Fig. 1 shows a field of networks isolated from an asynchronous, exponentially growing culture of *T. cruzi* which have been fluorescein-labeled (A) and DAPI-stained (B). All networks stain with DAPI but some, in the pre-replication stage, do not label with fluorescein because all the minicircles are covalently closed (an example is network a). Networks which are undergoing replication show fluorescein fluorescence only around the periphery. In these, a narrow peripheral fluorescent ring is seen in networks at an early stage of replication (e.g. network b). The ring becomes progressively thicker, and the non-fluorescent central region becomes smaller as networks advance through replication (e.g. networks c and d). After replication is complete, the Form II networks are uniformly labeled with fluorescein because all the minicircles (twice the original copy number) are gapped (e.g. network e). Subsequently these gaps are repaired so that networks gradually lose their fluorescence, and the network becomes larger in surface area. A possible example of a partly repaired network, with slightly reduced fluorescence intensity, is network f; another more definitive example is shown in Fig. 2, column 6. Some of the partially repaired networks (e.g. network f) appear larger than Form II networks (e.g. network e), probably because of topological changes similar to those occurring in *C. fasciculata* networks during replication (Chen et al., 1995). Finally, when all of the minicircle gaps are repaired the network, still large in size, completely loses its fluorescein fluorescence (e.g. network g). This species will undergo scission to form two unit size Form I networks. All of these replication intermediates are identical to those seen previously by EM in *C. fasciculata* (Englund, 1978; Pérez-Morga and Englund, 1993b; Chen et al., 1995). In these experiments we assumed that maxicircles make negligible contribution to the network fluorescence as they represent only a few per cent of the total DNA mass and in most species are thought to be dispersed throughout the kDNA.

We found that this technique provided good spreading of networks on the slide, making the different stages of replication readily distinguishable. For example, most of the networks in Fig. 1A, appear well spread and easily interpretable. In B, only a very few appear condensed or distorted. Only rarely did we find generally poor network spreading and we ignored those slides.

Analysis of the mode of kDNA replication of several members of the trypanosomatid family

We used this method to analyze the structure of kDNA networks isolated from asynchronous, exponentially growing cultures of several trypanosomatid species. Fig. 2 shows representative examples of networks at progressive stages of replication from *C. fasciculata, T. brucei, L. tarentolae, L. donovani, P. serpens,* and *T. cruzi.* For each parasite, the upper row of images represents fluorescein-labeling and the lower row DAPI-staining of the same network. The networks are arranged from left to right according to stage of replication, as determined by the distribution and amount of fluorescein labeling and by the size of the network. All images are at the same magnification and therefore reflect the relative sizes of the networks. *T. cruzi* kDNAs are the largest, with Form I networks about four times the diameter of those from *T. brucei.*

In the case of *C. fasciculata*, the observed forms agree with those detected previously by EM (Englund, 1978; Pérez-Morga and Englund, 1993b). Form I networks show the marked cup-shape characteristic of *C. fasciculata.* The network in column 2 shows a very fine rim of fluorescein fluorescence and represents a very early stage of network replication. Subsequent replicative stages have the characteristic replicating, Form II, and repaired structures discussed previously for Fig. 1.

T. brucei networks labeled with fluorescein show the same structures observed previously by EM (Hoeijmakers and Weijers, 1980; Ferguson et al., 1994; Robinson and Gull, 1994). In replicative forms the gapped minicircles are located at antipodal positions and there were no networks observed with a ring of fluorescein fluorescence around the periphery. Form II networks resemble dumbbells, uniformly labeled over the entire network. The intense DAPI fluorescence in the central region of repaired networks (e.g. the network in column 6) is likely due to the high concentration of maxicircles known by EM or fluorescence in situ hybridization to be localized in this position in this species (Hoeijmakers and Weijers, 1980; Ferguson et al., 1994).

In the other parasites studied, *L. tarentolae, L. donovani, P. serpens,* and *T. cruzi,* network replication intermediates resemble those of *C. fasciculata*. In all cases replicating networks have a peripheral ring of fluorescein fluorescence which enlarges at later stages of replication. We therefore conclude that all of the parasites in this study except for *T. brucei* utilize the annular mode of kDNA replication.

T. brucei has been considered the most ancient of the parasites studied in this report (Fernandes et al., 1993; Landweber and Gilbert, 1994; Maslov et al., 1996). By this criterion it would appear that kDNA replication by a polar model is the more primitive mechanism, and that the annular mode of replication arose later in evolution. Furthermore, in this scenario the annular mechanism very likely arose before the evolutionary separation of the species which we studied. However, recent work (Lukes et al., 1997) suggests that the Salivarian trypanosomes, which include *T. brucei*, are evolutionarily derived from the Stercorarian group, to which *T. cruzi* belongs. This suggests instead that *T. cruzi* is the more ancient species, and raises the possiblity that the polar mechanism of kDNA replication is a derivative of the annular mechanism.

It is interesting that there is no obvious correlation between the replicative mechanism and any other aspect of the network's structure, such as minicircle size or sequence heterogeneity, or network size. A crucial question which therefore remains to be solved concerns the biological role of the annular kinetoplast replication mechanism, which may involve rotation of the kinetoplast.

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