

Asymmetrical division of the kinetoplast DNA network of the trypanosome

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Trypanosome mitochondrial DNA is a network containing thousands of interlocked minicircles. Silencing of a mitochondrial topoisomerase II by RNA interference (RNAi) causes progressive network shrinking, allowing assessment of the minimal network size compatible with viability. We cloned surviving cells after short-term RNAi and found, as expected, that the number of surviving clones decreased with the duration of RNAi. Unexpectedly, a clonal cell line contained heterogeneously sized networks, some being very small. Several experiments showed that cells survived network shrinkage by asymmetrical division of replicated networks, sacrificing daughters with the small progeny network. Therefore, the average network size gradually increased. During the network shrinkage and early stages of recovery, there were changes in the minicircle repertoire.

Keywords: kinetoplast division/minicircle/RNAi/topoisomerase II

Introduction

Trypanosomes have attracted attention from the research community not only because of their pathogenicity, but also because of their unusual biology. As one of the earliest branching eukaryotes with mitochondria, these parasites have an amazing mitochondrial DNA termed kinetoplast DNA (kDNA). kDNA is a network consisting of thousands of DNA circles topologically interlocked in a planar array (for a review, see Shapiro and Englund, 1995; Klingbeil *et al.*, 2001). *In vivo*, the network is condensed into a disk-shaped structure within the matrix of the cell's single mitochondrion and is positioned near the flagellar basal body. There are two types of DNA circle catenated in a network: a few dozen maxicircles (each 23 kb, all identical in sequence) and several thousand minicircles (each 1 kb, heterogeneous in sequence). The maxicircles are homologs of mitochondrial DNAs in other eukaryotes, encoding rRNAs and mitochondrial proteins that function mainly in energy transduction (for a review, see Simpson, 1987). Maxicircle transcripts are edited by addition or deletion of uridine residues at specific sites, and minicircles encode guide RNAs (gRNA) that control editing specificity (for a review, see Estevez and Simpson, 1999; Madison-Antenucci *et al.*, 2002).

Our current knowledge of the mechanism of kDNA replication has come mainly from studies of *Crithidia fasciculata*, an insect parasite related to the trypanosomes (for a review, see Klingbeil *et al.*, 2001; Morris *et al.*, 2001). According to the current replication model, covalently closed minicircles are released vectorially from the kDNA disk (towards the flagellar basal body) by a topoisomerase II (topo II) (Englund, 1979; Drew and Englund, 2001). The free minicircles then initiate replication, ultimately forming singly and multiply gapped progeny (Kitchin *et al.*, 1984, 1985; Birkenmeyer and Ray, 1986; Birkenmeyer *et al.*, 1987). The partially or fully replicated minicircles migrate to two antipodal sites that flank the kDNA disk. In these sites, they are thought to undergo primer removal (Engel and Ray, 1999) and repair of some, but not all, of the gaps in the multiply gapped species (Shapiro and Englund, 1995). Then the gapped progeny minicircles are attached to the periphery of the network by a topo II (Melendy *et al.*, 1988; Wang and Englund, 2001). At this stage, the partially replicated network contains zones of unreplicated covalently closed minicircles and replicated gapped minicircles (Ferguson *et al.*, 1992; Pérez-Morga and Englund, 1993; Guilbride and Englund, 1998). As replication proceeds, the number of closed minicircles decreases and that of gapped minicircles increases until, at the end of replication, they have doubled in copy number and all contain gaps. The minicircle gaps are then repaired, and the network splits in two by a mechanism that is completely unknown. Although this model is based mostly on studies of *C. fasciculata*, a parasite that has been ideal for biochemical experiments, the availability of a nearly complete genome project and powerful genetic techniques provide compelling reasons to investigate kDNA replication in the African trypanosome *Trypanosoma brucei*.

Little is known about the factors that control the size of the kDNA network. To encode all the gRNAs necessary for its extensive RNA editing, *T. brucei* contains several hundred distinct minicircle species (Steinert and Van Assel, 1980; Stuart and Gelvin, 1980). The copy numbers of different minicircles vary and for some minicircles are thought to be low. Since minicircle segregation following replication may not be perfectly precise (Simpson *et al.*, 2000), a large network size may be necessary to minimize loss of essential minicircles during the network division (Savill and Higgs, 1999). However, it is not known whether the parasite can survive with a smaller network.

In a previous study, we used RNA interference (RNAi) to silence a *T. brucei* mitochondrial topo II (Wang *et al.*, 2000; Wang and Englund, 2001). In the absence of topo II, the free minicircle replication progeny cannot attach to the kDNA network, thus leading to the gradual shrinkage and loss of the network and eventual cell death (Wang and Englund, 2001). This finding provided a simple method to

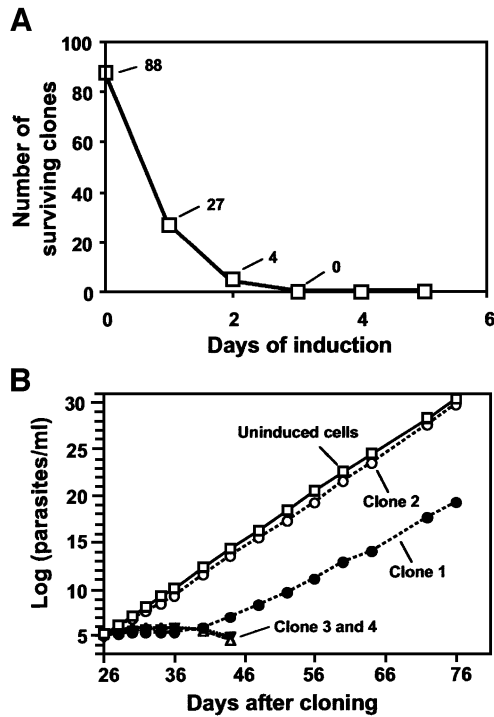


Fig. 1. Cloning of cells that survived short-term RNAi silencing of topo II. (A) Number of surviving clones obtained after tet removal. About 200 cells were diluted into two 96-well plates, and the numbers of surviving clones are plotted as a function of days after RNAi induction. (B) Growth curve of four day 2 clones after transferring to a culture flask (26 days after original cloning). Cells were diluted when the density reached 10^7 cells/ml, and values plotted on the graph are the product of the cell density and the dilution factor.

shrink the size of the network in a relatively controlled manner. We have now cloned cells after short-term RNAi for the purpose of determining the smallest size of a network compatible with viability. Not only did we answer this question, but we also made novel findings that have provided insight into the mechanism of network division and of the segregation of progeny minicircles.

Results

Cloning of trypanosomes after short-term RNAi of topo II

For the purpose of shrinking the kDNA network, we used RNAi to knock down expression of the mitochondrial topo II. We used an integrated stem-loop construct to express topo II-specific double-stranded (ds) RNA, which was induced by adding tetracycline (tet) to the culture (Wang and Englund, 2001). On consecutive days after induction, we washed cells with tet-free medium and cloned them by limiting dilution. After plating ~200 cells in two 96-well plates (at ~10 cells/ml, 0.1 ml/well), we obtained 88 clones from the uninduced control, giving a plating efficiency of 46% (consistent with our usual efficiency for wild-type cells of 30–50%) (Figure 1A). After 1 day of RNAi, we obtained only 27 clones in a similar plating. We found only four clones survived after RNAi for 2 days, and none after 3 or more days (Figure 1A). Since the kDNA network started to shrink as early as day 1 after RNAi (Wang and Englund, 2001),

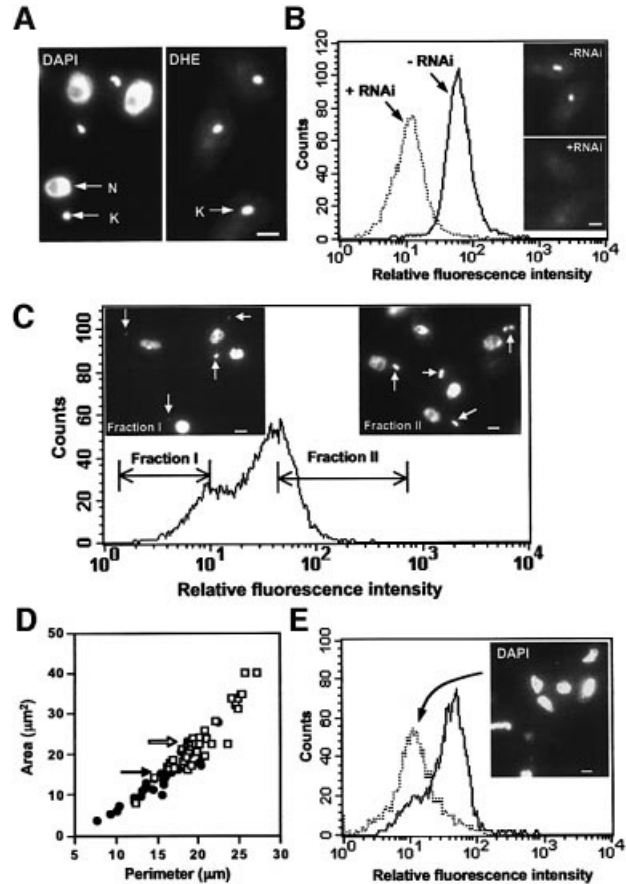


Fig. 2. Sizes of the kDNA networks evaluated by DHE staining and EM. (A) *Trypanosoma brucei* cells stained with DAPI (fixed cells) and DHE (live cells). Both the kDNA (K) and the nucleus (N) are indicated. (B) Flow cytometry profiles of DHE-stained cells either uninduced or induced for 9 days of RNAi silencing of topo II. After induction for 9 days, most cells are dyskinetoplastic (Wang and Englund, 2001). The inset shows images of uninduced cells (upper panel) and cells induced with tet to silence topo II for 9 days (lower panel), all stained with DHE. Phase microscopy showed that two cells are present in the lower panel. (C) Flow cytometry profile of clone 1 cells stained with DHE. We also sorted two populations of cells by FACS (fractions I and II) and then stained them with DAPI (inset). kDNA networks are indicated with arrows. (D) Size distribution of kDNA networks. The network area and perimeter from uninduced cells (squares) and clone 1 cells at day 56 (circles) were measured from EM images. Average areas are indicated by an open arrow (uninduced cells) and a solid arrow (clone 1 cells). (E) Flow cytometry profile of clone 1 cells before (solid line) or 10 days after (dashed line) tet induction. The inset shows an image of DAPI-stained clone 1 cells 10 days after tet induction. Bar = 2 μm in all images.

we presumed that progressive shrinking of the network accounted for the loss of cell viability.

When we transferred the cloned cells to culture flasks, we found that all the day 1 clones grew at the same rate as the uninduced cells. However, the day 2 clones grew significantly slower than uninduced cells even during the cloning process. Both uninduced cells and the day 1 clones, like wild-type cells, took 14 days to grow to turbid density in the plates, whereas all day 2 clones took 26 days to grow to a comparable density. After transferring the day 2 cells to culture flasks, we followed their growth pattern. Only one of the day 2 clones (clone 2) grew at the rate of uninduced cells, and two clones (clones 3 and 4) died ~15 days after transfer (Figure 1B). The most

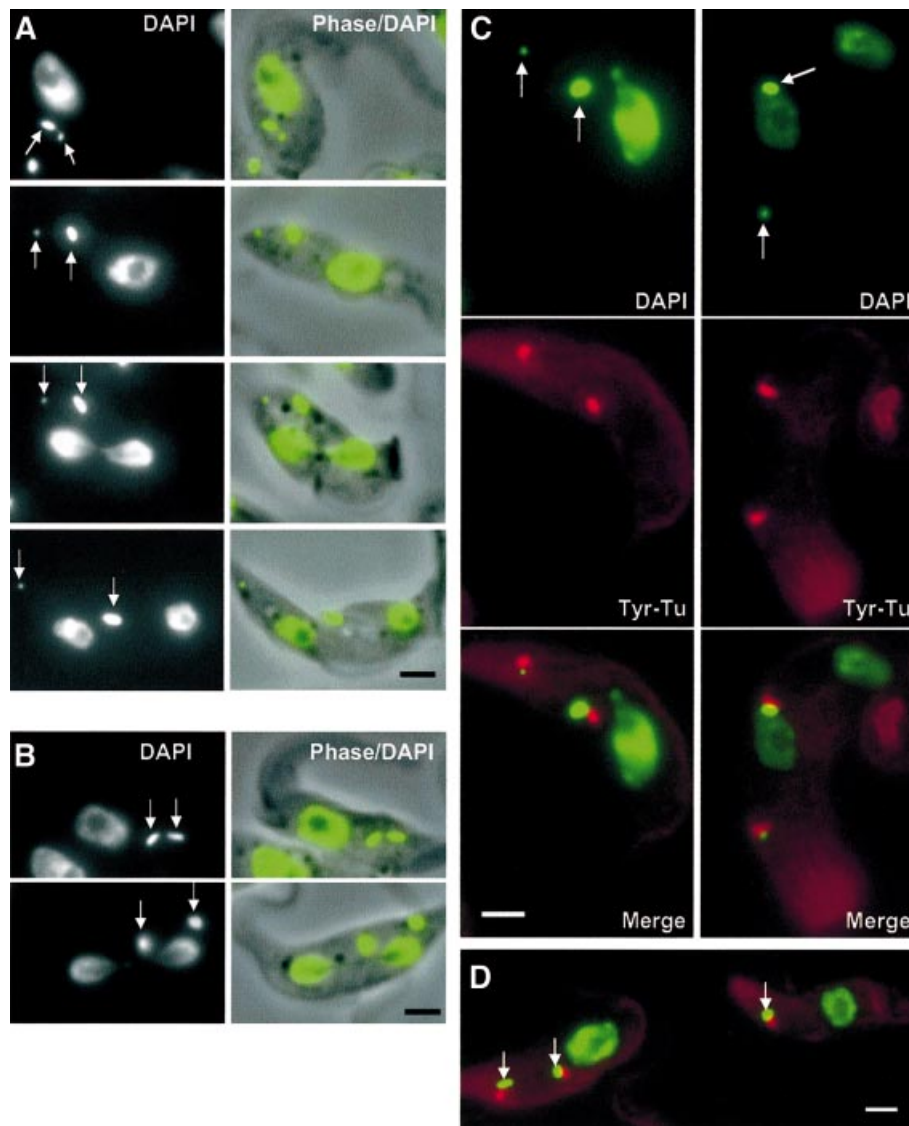


Fig. 3. Asymmetrical division of the kDNA network. **(A)** Images of clone 1 cells during kDNA network division (49 or 56 days after cloning). Left panels, DAPI staining. Right panels, overlay of DAPI staining (green) and phase images of the same cell. The two sister networks are indicated with arrows. From top to bottom are cells at progressive stages of network division. **(B)** Images of uninduced cells during network division. **(C)** Basal body images of cells undergoing asymmetrical kDNA division (54 days after cloning). Top panels, DAPI staining. Middle panels, basal bodies of the same cell shown by immunofluorescence using antibody for tyrosinated tubulin (Tyr-Tu). Bottom panels, merge of the above images. **(D)** The localization of the basal body stained with YL1/2 antibody (red) in uninduced cells as a control. The kDNA (indicated by arrows) and nucleus are stained with DAPI (green). Bar = 2 μm in all images.

interesting clone was clone 1. These cells grew very slowly, if at all, during the first 10 days in culture, and then resumed growth at a rate lower than that of uninduced cells (Figure 1B).

The kDNA network size in surviving clones

We made a preliminary assessment of kDNA size by fluorescence microscopy of 4',6-diamidino-2-phenylindole (DAPI)-stained cells. All the day 1 surviving clones, as well as clone 2 from day 2, appeared to have normal kDNA network size, as judged by DAPI staining (not shown). Surprisingly, clone 1 cells had heterogeneously sized kDNA, with some having a very small network (not shown). We therefore focused our attention on clone 1 and used these cells for most of the remaining experiments in this paper.

To assess more precisely the kDNA network size in a large number of cells, we developed a new method using dihydroethidium (DHE) to stain selectively the kDNA network. DHE is taken up by the mitochondrion, where it is oxidized to ethidium, which then stains the kDNA network. The staining is specific, with very little staining of the nucleus (Figure 2A, compare DAPI staining to DHE staining). As a control experiment, we stained uninduced and tet-induced cells that had lost kDNA by RNAi of topo II (for 9 days) (Figure 2B). The uninduced cells had clear kDNA staining (Figure 2B, upper panel in inset), whereas the induced cells did not stain (Figure 2B, lower panel in inset). We then examined the DHE-stained cells by flow cytometry and found that the induced cells, with little or no kDNA, shifted dramatically towards lower fluorescence intensity (Figure 2B).

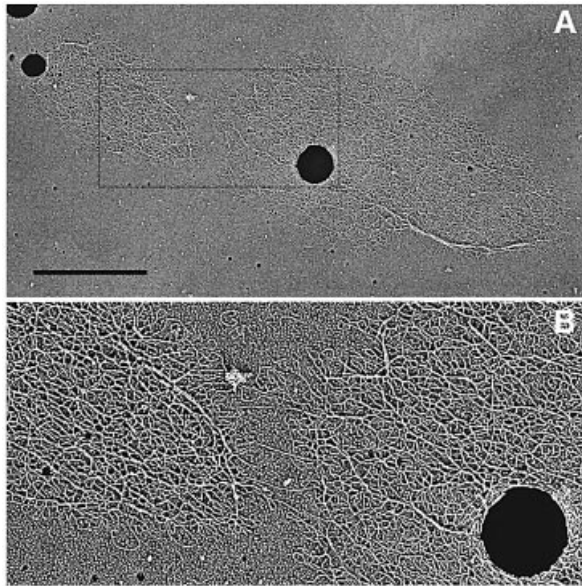


Fig. 4. kDNA network of clone 1 observed by EM. (A) EM image of a network undergoing division. The rectangle indicates an area that is shown at higher magnification in (B). Since the density of networks on the grid is very low, it is highly unlikely that this EM shows two individual networks that happen to be overlapping. Bar = 2 μ m.

We next analyzed clone 1 cells by DHE staining and flow cytometry (Figure 2C). Clone 1 cells (47 days after cloning) included two populations that differ in DHE staining intensity. In a separate experiment, we used fluorescence-activated cell sorting (FACS) to sort two fractions of cells that differed in DHE fluorescence intensity (see Figure 2C). We then stained the cells with DAPI for fluorescence microscopy (Figure 2C, inset). Cells with low DHE fluorescence intensity (Fraction I) indeed had very small kDNA networks. Cells with high DHE staining intensity (Fraction II) had larger sized networks (many of them are undergoing replication).

We also used electron microscopy (EM) to measure the size of kDNA networks. We measured the surface areas and perimeters of networks from clone 1 cells (56 days after cloning, indicated by circles) and found that they were significantly smaller than those of uninduced control cells (indicated by squares) (Figure 2D). Assuming that all the networks have an identical density of minicircles, there would be a corresponding reduction in minicircle copy number. The average network area (indicated by horizontal arrows) was reduced by 33%. This difference is statistically significant with $P < 0.001$ (determined by Student's t -test). These measurements may actually underestimate the difference between the two samples because some of the very small networks from the clone 1 cells could have been selectively lost during the centrifugation procedure used for network isolation (Pérez-Morga and Englund, 1993).

The very small sized kDNA networks in clone 1 cells were not due to further shrinking by a residual RNAi effect. One reason is that there was no dsRNA expression detectable by northern hybridization either before tet addition or after tet removal (Wang and Englund, 2001). Furthermore, topo II protein was present in clone 1 cells that had recovered from RNAi and was localized at its

normal position in the antipodal sites, as judged by immunofluorescence (not shown). More importantly, when we induced the clone 1 cells with tet (63 days after cloning), these cells still lost their kDNA and died at a rate similar to that of their parent RNAi cell lines. Flow cytometry of these cells, stained with DHE, showed the dyskinetoplasmic phenotype 10 days after induction (Figure 2E, dashed line). The inset image, of DAPI-stained clone 1 cells 10 days after induction, confirmed that most have little or no kDNA.

The asymmetrical division of the kDNA network

An intriguing question is how clone 1 cells, which derived from a single cell, develop heterogeneity in the size of their kDNA networks. One simple explanation could be that the cells somehow survived the stress of a shrinking network by unequal division of their replicated network. The daughter cell having the larger network would survive, and that with the smaller network or its progeny would be sacrificed. To test this possibility, we examined DAPI-stained clone 1 cells (49 or 56 days after cloning) by fluorescence microscopy. We found that networks in $\sim 5\%$ of the cells undergoing kDNA division indeed divided asymmetrically. This percentage is probably an underestimate because the asymmetrical division was recognized only when the two daughter networks are significantly different in size. Figure 3A shows examples of clone 1 cells at different stages of the cell cycle when the replicated kDNA network is dividing into two daughter networks. The top panel shows the kDNA dividing into two unequally sized daughters, which then move apart (second panel). After nuclear division (third panel), the cell begins to undergo cytokinesis (fourth panel). In a control experiment, using uninduced cells, the two daughter networks are always comparable in size (see examples in Figure 3B). The asymmetrical division of the network occurred more frequently early after cloning. We found unequal division in $\sim 5\%$ of dividing cells at day 49 or day 56, whereas it was hard to detect ($<1\%$) 11 weeks after culturing (data not shown).

The kDNA network is located close to the flagellar basal body and these two structures are physically linked. Furthermore, the division of the kDNA network is thought to be mediated by the segregation of basal bodies (Robinson and Gull, 1991). Thus, we decided to examine the basal bodies in asymmetrically dividing clone 1 cells by immunofluorescence using the monoclonal antibody YL1/2. This antibody binds to tyrosinated tubulin (Tyr-Tu) and strongly stains the basal body of *T. brucei* (Sherwin *et al.*, 1987). We found that the basal bodies of clone 1 cells had the same morphology and position as those in control cells within the resolution limits of light microscopy (Figure 3D). Even in cells undergoing asymmetrical division, the basal bodies separated normally (Figure 3C), suggesting that at least part of the machinery for network division was still intact. We also found that in most cases of asymmetrical division (26 of 27 random examples photographed), the small daughter network was associated with the new basal body, which is localized at the posterior end of the cell (Robinson *et al.*, 1995).

The asymmetrical division of the network was also confirmed by EM. Among ~ 60 isolated clone 1 kDNA networks examined by EM (56 days after cloning), we

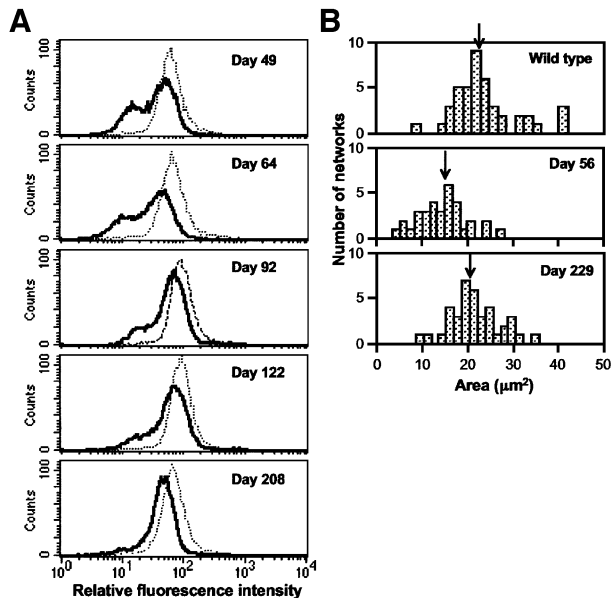


Fig. 5. Changes in clone 1 kDNA network size during long-term culturing. (A) The DHE staining profiles were followed by flow cytometry over nearly 7 months. Day 0 is the day when we initiated cloning. Uninduced cells (dashed lines) were examined at the same time as a control. (B) Distribution of kDNA network areas at different days after cloning. The network areas were measured from EM images, using those of uninduced cells as a control. The data for uninduced and day 56 are the same as those shown in Figure 2D. Average areas are indicated by arrows.

found one example of a network in the late stage of division in which the two developing progeny networks differed in size by a factor of about three (Figure 4A). An enlargement of the junction region between these two progeny networks confirmed that they were still linked (Figure 4B).

Changes in clone 1 kDNA networks during long-term culturing

If the kDNA networks in clone 1 cells divide asymmetrically, and if the cells with the smaller daughter network die, then cells with small networks should gradually disappear over time. Using DHE staining and flow cytometry to follow the kDNA of clone 1 cells for ~7 months, we found that the population of very small networks gradually declined (Figure 5A). By the end of 208 days, the cells with low DHE staining intensity had nearly disappeared and the major peak had a fluorescence intensity close to that of uninduced control cells.

We further measured the network size of long-term cultured clone 1 cells by EM (Figure 5B). Compared with uninduced cells (average area of $23 \mu\text{m}^2$), the clone 1 networks are significantly smaller at 56 days after cloning (average area of $15.5 \mu\text{m}^2$, 33% smaller than those from uninduced cells). By day 229, the networks had grown to 90% of normal size (average size of $20.4 \mu\text{m}^2$). Because the difference in network size between day 229 and uninduced cells was small, we evaluated its statistical significance with the Student's *t*-test ($P = 0.07$). We think this small difference is likely to be real because clone 1 cells at day 229 had lower DHE staining intensity than uninduced control in the flow cytometry experiments

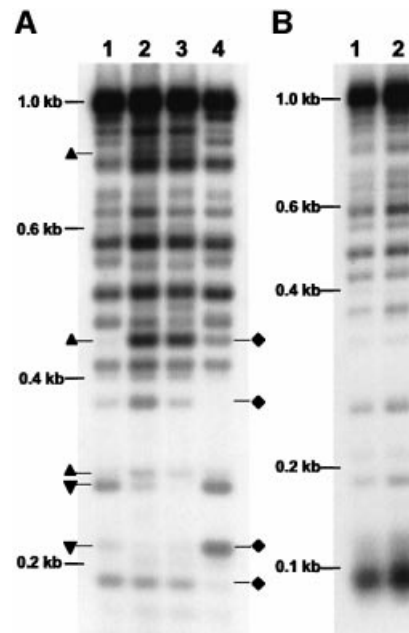


Fig. 6. Changes in minicircle composition. Total DNA was digested with *TaqI-SpeI*, fractionated on a 2% agarose gel (5×10^6 cell equivalents/lane) and detected on a Southern blot with an oligonucleotide probe for minicircles. (A) Lane 1, DNA from uninduced topo II RNAi cells. Lane 2, DNA from clone 1 cells at day 49 after cloning. Lane 3, DNA from clone 1 cells at day 149 after cloning. Lane 4, DNA from clone 2 cells at day 49 after cloning. The upright triangles indicate the minicircle classes that increased in clone 1, and the inverted triangles indicate those that decreased. The diamonds indicate the classes that changed in clone 2 (comparing lanes 1 and 4). (B) Lane 1, DNA from uninduced topo II RNAi cells. Lane 2, DNA from same cells continuously cultured for 236 days.

where 10 000 cells were counted (similar to day 208 in Figure 5A).

Change in minicircle repertoire during propagation of clone 1 cells

There are gradual changes in minicircle composition during long-term culturing of *Leishmania tarentolae* (a parasite related to the trypanosomes), probably due to imprecise segregation of minicircle progeny after replication (Simpson *et al.*, 2000). We speculated that an even more dramatic change might occur during the shrinking and subsequent asymmetrical division of the network. To test this possibility, we used a fingerprinting method in which we digested kDNA samples with *TaqI*, an enzyme that cleaves nearly all of the minicircles at least once (Borst *et al.*, 1980). We also used *SpeI* to increase the extent of digestion. Given the extensive minicircle heterogeneity, these enzymes produce a distinctive pattern of minicircle fragments when fractionated on a 2% agarose gel. We detected the fragments by Southern hybridization using an oligonucleotide probe (19mer) specific to the minicircle conserved region.

The results are shown in Figure 6A. Comparison of uninduced cells (lane 1) with clone 1 at day 49 (lane 2) showed that some minicircle fragments increased in abundance (upright triangles), whereas others decreased (inverted triangles). In contrast, there was much less change in clone 1 minicircles between day 49 (lane 2) and day 149 (lane 3). We detected a comparable drift in

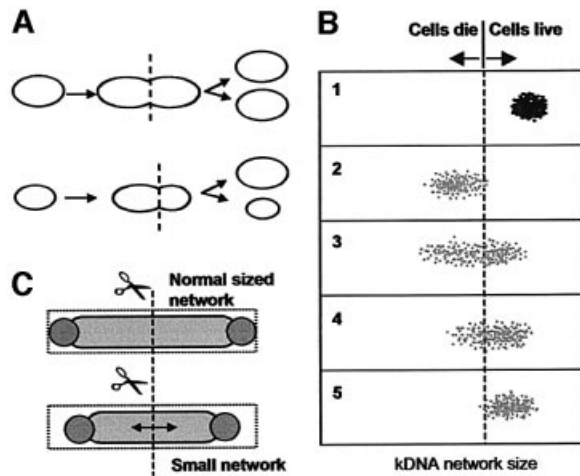


Fig. 7. Models for asymmetrical cleavage of kDNA network. (A) Diagram showing replication and symmetrical division of a normal kDNA network (upper panel) and asymmetrical division of a small network (lower panel). (B) Diagram to explain the change in kDNA network size. Each network is presented as a dot in all panels, and the vertical dashed line shows the threshold of network size for viability. Panel 1, the kDNA networks in wild-type cells. Panel 2, networks shrunk by 2 days of RNAi. Clone 1 might have a network just above the size threshold. Panel 3, networks produced by asymmetrical network division in clone 1. Panels 4 and 5, clone 1 cells after continuous culturing. The cell population with a small network is diminishing due to selection. See text for more details. (C) A hypothetical model to explain why shrunken networks can undergo asymmetrical division. Light gray structure, condensed kDNA network. Dark gray circles, antipodal sites where newly replicated minicircles are attached to the network. Dotted block, fixed space in replication machinery. Vertical dashed line, site where network scission takes place. The double-headed arrow indicates that the small network can move in fixed space.

minicircle composition in an independent clone (clone 2, 49 days after cloning, lane 4), in which different minicircle fragments were changed in concentration (indicated by diamonds) relative to uninduced cells (compare lane 4 with lane 1). In a control experiment, we found little or no change in uninduced cells cultured continuously for 239 days (Figure 6B).

Discussion

We reported previously that RNAi silencing of a *T. brucei* mitochondrial topo II causes a gradual shrinkage and ultimate loss of the kDNA network. Both minicircles and maxicircles are lost at about the same rate (Wang and Englund, 2001). We have now used this RNAi procedure as a tool to shrink the network in a relatively controlled manner for the purpose of determining the smallest network size compatible with cell viability [note that kDNA is essential for procyclic trypanosomes because it encodes proteins required for cellular respiration (Simpson, 1987)]. Because different cells in the population may lose kDNA at slightly different rates, we cloned cells from a mixture undergoing RNAi. We found that the number of viable clones decreased significantly in proportion to the duration of RNAi (Figure 1A), indicating that cells progressively passed the point beyond which they cannot survive a further loss of kDNA. After 2 days of RNAi, only four clones initially grew up (out of ~200 cells plated), and two of these died soon after, presumably

because their kDNA networks were too small for survival. We chose to study one of the surviving clones, clone 1, which grew more slowly than uninduced cells after a 2 week lag period (Figure 1B). We assumed that the kDNA network of this cell had shrunk to the point where it could barely survive.

We indeed found by EM and flow cytometry (Figure 2) that the clone 1 networks were smaller than those of uninduced cells, with an average size 33% less than that of wild type (at day 56 after cloning). However, we were surprised that the networks in clone 1 cells were heterogeneous in size even though the cell population from which they were derived was clonal (Figure 2). Further studies, based on DAPI staining of cells (Figure 3) and EM of isolated networks (Figure 4), revealed that the reason for this heterogeneity was that the smallest sized networks in the population derived from asymmetrical division of double-sized networks. In wild-type trypanosome populations, the double-size network divides symmetrically (or very nearly symmetrically), therefore the sizes of the progeny unit-size networks are close to that of the parent network (Figure 3B; Hoeijmakers and Weijers, 1980; Ferguson *et al.*, 1994; Robinson and Gull, 1994). In the RNAi-treated clone 1 cells, which initially had a network much smaller than that of wild type, asymmetrical division of the double-size network produces one daughter network that is larger than its parent, and thus this cell will have increased its chances of survival. The other daughter network is smaller than its parent and this cell or its progeny will die. Figure 7A illustrates symmetrical and asymmetrical network division. Consistent with this hypothesis, we found using flow cytometry (Figure 5A) that the fraction of smaller sized kDNA networks gradually decreases as clone 1 cells are propagated in culture.

As shown in Figure 7B, there must be a network size threshold for viability (vertical dashed line). When the network size is smaller than this threshold, viability cannot be maintained indefinitely as some essential minicircle classes would be lost during network division (or possibly the maxicircle copy number becomes too low). The networks of wild-type cells (panel 1) are larger than the threshold and are probably tightly distributed around the average size. RNAi of topo II makes networks shift toward a smaller size. After 2 days of RNAi, the networks for most cells are smaller than the threshold (panel 2). Clone 1 is probably at the edge of survival, but clone 1 progeny can recover by undergoing asymmetrical network division (panel 3). The progeny cells with small networks will eventually die, and cells with larger networks can propagate more efficiently. This selection process reduces the population of cells with small networks and shifts the average network toward a larger size (panels 4 and 5). At day 229 after cloning, the average size of clone 1 networks is ~90% of those of wild type, as judged by EM (Figure 5B) and confirmed by flow cytometry (see Figure 5A for day 208).

What is the mechanism of division of the replicated kDNA network and how is network size normally controlled? kDNA replication proteins are organized in discrete loci surrounding the kDNA disk (reviewed in Klingbeil *et al.*, 2001). We speculate that the wild-type network has evolved together with its replication machinery so that the kDNA fits precisely within the

available space within the machinery. When the network has grown to double size after replication, it is therefore positioned so that the scission apparatus (probably involving a topo II) will cleave the network almost precisely in the middle, forming two daughter networks with a size nearly identical to that of the parent network (Figure 7C, upper panel). However, after shrinking by RNAi, the network does not fit tightly inside the replication apparatus and its freedom of movement allows its scission to occur asymmetrically, giving two progeny of different size (Figure 7C, lower panel). Furthermore, the segregation of progeny kDNA networks is thought to be driven by an association of the network with the flagellar basal bodies (Robinson and Gull, 1991). We visualized this kDNA–basal body association by immunofluorescence even in the case of networks dividing asymmetrically (Figure 3C), suggesting that this abnormal network division is not due to faulty connections to the basal body. We also found that in almost all cells undergoing asymmetrical network division, the small progeny networks are associated with new basal bodies. A possible explanation for this correlation could be that the links between the network and the new basal body are weaker or fewer than those to the old basal body.

Trypanosomes with very small kDNA networks apparently die because they lose minicircles encoding guide RNAs essential for RNA editing. In fact, the kDNA is thought to be organized as a network because this novel structure prevents loss of minicircles by ensuring their proper segregation following replication (Borst, 1991; Lukes *et al.*, 2002). Despite the network structure, segregation is not perfectly precise, allowing minicircle composition of the network to drift during long periods of culturing (Hoeijmakers and Borst, 1982; Simpson *et al.*, 2000). However, there are opportunities for the minicircle repertoire to change dramatically during RNAi silencing of topo II, when minicircles are lost from the network, and during the subsequent recovery, when the networks are undergoing asymmetrical division. We found that this is indeed the case when we examined the minicircle repertoire by agarose gel electrophoresis of minicircle restriction fragments. Compared with uninduced cells, there were changes in minicircle composition between induction of RNAi and day 49 after cloning (Figure 6A), but, as expected, there was little further change in the same cells between days 49 and 149 (Figure 6A). A different cloned line, clone 2, had different changes (Figure 6A), indicating that minicircle loss is random. These changes must depend on RNAi and asymmetrical division because uninduced cells do not significantly change their minicircle composition during 8 months in culture (Figure 6B).

The original objective of this study was to determine the smallest network size compatible with cell viability. It now seems that the founding cell for clone 1 had an exceedingly small network, but that by chance it had a complete minicircle repertoire. Despite its small network, this cell had enough minicircle-encoded gRNA to conduct all of its RNA editing reactions. By asymmetric division and selection, the clone 1 networks grew in average size, and by the time we had enough cells to measure network size (56 days after cloning), they were on average 33% smaller than those of wild type. By day 229, they were only 10% smaller than wild type.

An interesting question is whether the kDNA networks in clone 1 will ever shift back to wild-type size. It is usually taken for granted that nature selected for the optimal network size during evolution. If the network was too small, there would be inadequate minicircle redundancy and critical minicircle classes would be subject to loss. If the network was too large, there would be too much DNA to package and replicate. We found that by day 229 (when the networks were 90% of wild type), there was no detectable difference between the growth rate of clone 1 and that of wild type (not shown), so there may be little or no selective advantage for wild-type size networks. Therefore, we cannot predict whether the networks will eventually grow back to wild-type size or, under laboratory culture conditions, they will stabilize at a size slightly smaller than wild type. We cannot rule out the possibility that RNAi induces some irreversible change in the network that prevents it from regaining its normal size.

Materials and methods

Trypanosome growth and generation of the topo II RNAi cell line

Procyclic *T.brucei* were grown at 27°C in SDM-79 medium supplemented with 15% fetal bovine serum (Wang *et al.*, 2000). Cell densities were determined by hemocytometer counting and the cultures were diluted 1:10 when the cell density reached 10^7 cells/ml. The RNAi cell line was generated by transfecting strain 29-13 [a gift from Elizabeth Wirtz and George Cross, Rockefeller University (Wirtz *et al.*, 1999)] with the topo II stem–loop construct as described previously (Wang *et al.*, 2000). dsRNA expression was induced by adding tet (1 µg/ml) to the culture.

Cloning of cells surviving short-term RNAi

The topo II RNAi cells were induced with tet and, on each subsequent day, 5 ml of mid-log phase culture ($\sim 5 \times 10^6$ cells/ml) were centrifuged and washed once with tet-free medium. The cells were counted and diluted with tet-free medium to a density of $\sim 10^6$ cells/ml, and 0.1 ml aliquots were added to each well of two 96-well plates. The plates were then incubated in a 27°C incubator with 5% CO₂ until the surviving clones grew to turbid density. These clones were then transferred to tet-free medium in culture flasks.

kDNA staining and flow cytometry

DAPI staining was performed as described previously (Wang and Englund, 2001). DHE (Molecular Probes) was dissolved in dimethylformamide at 10 mg/ml and used to stain live cells (kDNA in fixed cells does not stain). To stain cells, 1 µl of this solution was added to 1 ml of culture. After 10 min of incubation at room temperature, the cells were centrifuged, washed once with cytomix (van den Hoff *et al.*, 1992) and finally resuspended in 1 ml of cytomix. Flow cytometry was performed in the core facility at Johns Hopkins Medical School using a FACScan flow cytometer (Becton Dickinson Biosciences) equipped with an air-cooled argon laser. FACS was performed at the same facility on a FACStar Plus (Becton Dickinson Biosciences).

Immunofluorescence

We used a monoclonal antibody against yeast Tyr-Tu (MAB1864; Chemicon Inc.) to detect the basal bodies of *T.brucei* (Sherwin *et al.*, 1987). Cells were harvested by centrifugation, washed once in phosphate-buffered saline (PBS) and resuspended at 10^8 cells/ml in PBS prior to spotting on polylysine-coated slides (Guilbride and Englund, 1998). After adhering to the slide for 10 min, the cells were fixed in 4% paraformaldehyde in PBS for 5 min, washed twice in PBS containing 0.1 M glycine for 5 min each, washed once in PBS for 5 min, and then permeabilized by methanol for 1 h at –20°C. The slides were rehydrated with two 10 min washes in PBS, and then incubated for 60 min with antibody against Tyr-Tu [1:400 diluted in PBS with 1% bovine serum albumin (BSA)]. The slides were washed three times (10 min each) in PBS with 0.1% Tween-20. Fluorescein isothiocyanate-conjugated anti-rat IgG antibody (Sigma Co. catalog no. F1763; 1:400 diluted in PBS with 1% BSA) was then applied for 30 min and washed three times in PBS with

0.1% Tween-20 (10 min each). The slides were stained with DAPI (0.2 µg/ml for 1 min), washed twice with PBS (5 min each) and mounted with VECTASHIELD (Vector Lab. Inc.). All operations were performed at room temperature unless noted otherwise. Fluorescence microscopy and digital image acquisition were carried out as described previously (Drew and Englund, 2001) with a Zeiss Axioskop microscope (Carl Zeiss, Inc.).

Electron microscopy

The kDNA networks were purified and spread on EM grids as described previously (Pérez-Morga and Englund, 1993), and then photographed on a Philips CM120 electron microscope. EM images were then scanned and the perimeters and surface areas of the networks measured using IPLab software.

DNA purification and Southern analysis

Trypanosomal total DNA was purified as described previously (Wang and Englund, 2001). For restriction enzyme digestion, DNA was first digested with *SpeI* in NEBuffer 2 (supplemented with 0.1 mg/ml BSA) at 37°C overnight. *TaqI* was then added and the incubation continued at 65°C for 8 h. Enzymes and buffers were from New England BioLabs. The *SpeI*-*TaqI*-digested DNA was fractionated on a 2% agarose gel (20 × 25 × 0.5 cm, 65 V, ~18 h) with 1 µg/ml ethidium bromide in both the gel and the TBE running buffer. The oligonucleotide probe was made by 5'-end labeling of a 19mer (5'-TGCACGCCATAAGATTTT), a sequence within the minicircle conserved region. Hybridization was carried out overnight at 50°C in 5× SSPE, 1% SDS, 1× Denhardt's solution. Blots were washed twice for 1 min in 2× SSC, 0.1% SDS at room temperature, then washed once for 2 min in 2× SSC, 0.1% SDS at 50°C.

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