EFFECTS OF RNA INTERFERENCE OF TRYPANOSOMA BRUCEI STRUCTURE-SPECIFIC ENDONUCLEASE-I ON KINETOPLAST DNA REPLICATION

Yanan Liu, Shawn A. Motyka, and Paul T. Englund

From the Department of Biological Chemistry, Johns Hopkins Medical School, Baltimore, MD 21205

Running title: RNAi of Trypanosome Structure-Specific Endonuclease-I

Kinetoplast DNA, the mitochondrial DNA of trypanosomatid protozoa, is a network containing several thousand topologically interlocked DNA minicircles. Kinetoplast DNA synthesis involves release of minicircles from the network, replication of the free minicircles, and reattachment of the progeny back onto the network. One enzyme involved in this process is structure-specific endonuclease-I. This enzyme, originally purified from Crithidia fasciculata, has been proposed to remove minicircle replication primers (Engel, ML and Ray, DS, Nucleic Acids Res, 26, 4773-8, 1998). We have studied the structure-specific endonuclease-I homolog from Trypanosoma brucei, showing it to be localized in the antipodal sites flanking the kinetoplast DNA disk, as previously shown in C. fasciculata. RNA interference of structure-specific endonuclease-I caused persistence of a single ribonucleotide at the 5' end of both the leading strand and at least the first Okazaki fragment in network minicircles, demonstrating that this enzyme in fact functions in primer removal. Probably due to the persistence of primers, RNA interference also impeded the reattachment of newly replicated free minicircles to the network and caused a delay in kinetoplast DNA segregation. These effects ultimately led to shrinkage and loss of the kinetoplast DNA network and cessation of growth of the cell.

Trypanosoma brucei is a protozoan parasite that causes sleeping sickness in humans and similar diseases in livestock. Trypanosomes, being one of the earliest branching eukaryotes, have unusual biological properties. Their mitochondrial DNA, known as kinetoplast DNA (kDNA), is one of their most amazing features (see (1-3) for reviews on kDNA). kDNA is a giant network consisting of topologically interlocked DNA rings. Within the cell the network is condensed into a disk-shaped structure located within a distended portion of the mitochondrial matrix adjacent to the flagellar basal body. There is a transmembrane filament system linking the kDNA network to the basal body that facilitates segregation of daughter networks following replication (4,5).

The T. brucei kDNA network contains several thousand minicircles that are heterogeneous in sequence (each 1 kb) and a few dozen maxicircles (each 23 kb). Maxicircles, like mitochondrial DNAs of higher organisms, encode rRNAs and subunits of respiratory complexes. Minicircles encode small guide RNAs that control editing of maxicircle transcripts. Editing is a remarkable process involving addition or deletion of uridylicate residues at specific sites in the transcript to form functional mRNA (reviewed in (6,7)).

kDNA replication has been studied in T. brucei and also in the related parasite Crithidia fasciculata (reviewed in (1,3,8)). In both species kDNA synthesis occurs in approximate synchrony with nuclear DNA replication, but division of the kinetoplast occurs prior to that of the nucleus (9,10). Prior to kDNA replication, during the G1 phase of the cell cycle, all minicircles in the network are covalently-closed. When kDNA replication begins, minicircles are individually released from the network into the kinetoflagellar zone (KFZ) (11), a specialized region between the kDNA disk and the mitochondrial membrane adjacent to the flagellar basal body. Some replication proteins are localized in the KFZ, and these are...
responsible for initiation of replication of the free minicircles. These proteins include the minicircle origin binding protein (UMSBP) (12), DNA primase (13), and two DNA polymerases (14). The free minicircles replicate unidirectionally as theta-structures yielding daughter minicircles that probably segregate in the KFZ. The progeny minicircles, containing nicks or gaps, then migrate to two antipodal sites that flank the kDNA disk (11). Within these sites subsequent steps of replication occur, catalyzed by enzymes specifically localized there. These reactions are thought to include primer removal by a structure-specific endonuclease-I (SSE1) (15-17) and repair of some (but not all) of the minicircle gaps by a DNA polymerase β (18,19) and a DNA ligase (20,21). Finally, the progeny minicircles, still containing at least one nick or gap, are attached to the edge of the kDNA network by an antipodal topoisomerase II (22,23). As replication proceeds, there is enlargement of the network's peripheral zones (containing nicked or gapped minicircles, already replicated) and a shrinkage of the central zone (containing covalently-closed minicircles, not yet replicated) (24-26). When replication is complete, the minicircle copy number has doubled. Finally the double-size network splits in two, the remaining gaps are repaired (24-26), and the progeny networks segregate into the daughter cells during cell division.

_T. brucei_ and _C. fasciculata_ differ in the pattern of attachment of gapped minicircles to the network. In _T. brucei_, gapped minicircles are attached at two antipodal sites and remain concentrated in the two polar regions (24-27). In _C. fasciculata_, gapped minicircles are also attached at two antipodal sites but subsequently distribute uniformly around the network periphery, probably because of a relative movement of the network disk and the sites of minicircle attachment (28,29).

The subject of this paper is the structure-specific endonuclease-I (SSE1), a mitochondrial enzyme discovered in _C. fasciculata_ by Engel and Ray (15-17). As mentioned above, this enzyme localizes to the antipodal sites. Enzymatic studies on purified _C. fasciculata_ SSE1 protein showed that it has RNase H and DNA endonuclease activities (15) similar to those of the FEN family of enzymes (30,31). It is homologous to the 5' exonuclease domain of _E. coli_ DNA polymerase I (16). These findings suggested that SSE1 may function in removal of RNA replication primers and studies with model substrates provided further evidence for this role (17). In this paper we describe experiments using RNAi to demonstrate that SSE1 indeed functions in primer removal _in vivo_. Furthermore, we found that SSE1 RNAi affects segregation of the kDNA network after its replication.

**MATERIALS AND METHODS**

*Trypanosomes and RNAi--*Procyclic _T. brucei_ (strain 29-13, a gift from Dr. George Cross, Rockefeller University) were cultured at 27 °C in SDM-79 medium containing 10% fetal bovine serum (32). A stem-loop construct for RNAi experiments, prepared as described in (32), contained two opposing copies of a 744 bp sequence (starting at the codon for amino acid 105 and extending into the 3' UTR). The two copies of the SSE1 sequence were separated by a spacer sequence (32). Trypanosomes were transfected with the RNAi construct, selected by phleomycin (2.5 µg/ml), and then cloned by limiting dilution. Expression of dsRNA was induced by tetracycline (1 µg/ml) (32).

*RNA purification and Northern analysis--*Total RNA was isolated from mid-log phase cells (10 ml) using the Purescript RNA Isolation Kit (Gentra Systems) and then fractionated on a 1.5% agarose gel containing 7% formaldehyde. After transfer to a nylon membrane, the SSE1 mRNA was detected with a 32P-labeled probe made from the same fragment used in the stem-loop construct (32).

*DNA purification and analysis--*Total DNA was purified as described previously (23) as was kDNA (29). kDNA networks (isolated from 2.5 x 10^8 cells) were decatenated in a 20 µl reaction (60 min, 37 °C) containing 50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM MgCl₂, 500 µM DTT, 500 µM EDTA, 30 µg/ml BSA, 1.0 mM ATP, and 10 units human topoisomerase IIα (a gift of Dr. Leroy Liu, Robert Wood Johnson Medical School). Covalently-closed minicircles from decatenated networks were
isolated from an agarose-ethidium bromide gel like that used in Fig. 3.

All restriction enzymes and buffers were from New England Biolabs. For alkali treatment, isopropanol-precipitated DNA was dissolved in 0.3 M NaOH containing 50 mM EDTA, incubated overnight at 37 °C, neutralized with HCl, and isopropanol precipitated again. The kDNA, with or without alkali treatment, was redissolved in 10 µl of denaturing gel sample buffer (98% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol FF), heated for 6 min at 100 °C, chilled, and then loaded immediately on the gel. The kDNA, with or without alkali treatment, was redissolved in 10 µl of denaturing gel sample buffer (98% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol FF), heated for 6 min at 100 °C, chilled, and then loaded immediately on the gel. The 9% 40.5 cm polyacrylamide denaturing gel containing 8 M urea was cast and run in TBE buffer (1600 volts, ~6 hr).

The DNA was then electrotransferred to a nylon membrane (Perkin Elmer Life Sciences). The oligonucleotide probe for detection of the first Okazaki fragment is 5'-AAAATAGCACGGGATTTGTGTATG-3' and that for the 5' end of the leading strand is 5'-CATACACAAATCCGGTGCTATTTT-3'. Both sequences are within the minicircle conserved region (33) and therefore should recognize all of the minicircles. The probes were 5'-radiolabeled using [γ-32P]ATP (3000 Ci/mmole, NEN) and T4 polynucleotide kinase (New England Biolabs). Southern hybridization was carried out overnight at 55 °C in 5x SSPE (0.75 M NaCl, 50 mM sodium phosphate, pH 7.4, 5 mM Na2EDTA), 1% SDS, 1x Denhardt’s solution (34). Wash conditions were as described (34).

Localization of SSE1—For intracellular localization, a single genomic allele of SSE1 was GFP-tagged at its C-terminus using a PCR-based method (35). Briefly, a cassette encoding GFP and a drug resistance marker, blasticidin S deaminase, was amplified using the pXSGFPM3FUS vector (36) as template. Oligonucleotide primers contained 30 nucleotides homologous to the vector template plus 90 nucleotides homologous to the site of integration (upstream of the SSE1 stop codon for the forward primer and downstream of the stop codon for the reverse primer). The PCR product (4 µg) was electroporated into procyclic trypanosomes and transformants were selected with blasticidin S (10 µg/ml). Integration at a single SSE1 allele was confirmed by PCR. GFP-tagged SSE1 was detected using Alexa Fluor 488-conjugated rabbit anti-GFP antibody (Molecular Probes). Immunofluorescence was carried out as described (23).

In situ and in vitro labeling of kDNA networks with fluorescently-labeled dUTP—In situ fluorescent labeling of the nicks and gaps in network minicircles with terminal deoxynucleotidyl transferase (TdT) was performed as described (37). Briefly, cells were centrifuged, washed, and resuspended in PBS. Aliquots (5 x 10^5 cells in 25 µl PBS) were spotted on poly-lysine-coated slides and were allowed to adhere for 10 min. Then cells were fixed in 4% paraformaldehyde in PBS for 5 min and washed twice in PBS containing 0.1 M glycine for 5 min. After permeabilization in methanol overnight at –20 °C, the cells were rehydrated with three 5 min washes in PBS. The cells were then incubated for 20 min at room temperature in 25 µl TdT reaction buffer (Roche) containing 2.0 mM CoCl2. Slides were then incubated for 60 min at room temperature in a 20 µl reaction solution containing TdT reaction buffer (Roche), 2.0 mM CoCl2, 10 µM dATP, 2.5 µM Alexa Fluor 488-dUTP (Molecular Probes), and 10 units of TdT (Roche). The reaction was stopped with three 5 min washes in 2x SSC, 0.9x PBS, followed by two 5 min washes in PBS before proceeding with immunofluorescence.

In some experiments, following TdT labeling, the basal bodies in T. brucei were detected by immunofluorescence using a monoclonal antibody YL1/2 against yeast tyrosinated tubulin (Chemicon International, Inc.) (34,38). Antibody labeling was as described (34), except that the secondary antibody was Alexa Fluor 568-conjugated rabbit anti-rat IgG (Molecular Probes).

TdT labeling of gapped minicircles in isolated networks was carried out on the surface of a slide (27). Briefly, 1 µl of the isolated kDNA networks (~100 µg/ml, in sterile H2O) was mixed with 19 µl PBS and settled on a poly-lysine-coated slide for at least 30 min at room temperature. Then the slide was incubated with TdT and Alexa Fluor 488-dUTP (reaction conditions described above), followed by DAPI.
staining (2 µg/ml). In labeling isolated networks or permeabilized cells (see above) with TdT, the intensity of labeling was almost always either very strong or absent; only rarely did we observe weak labeling that could have derived from a network with nicks or gaps that were partly repaired. The latter were still defined as TdT positive.

Slides were examined on an Axioskop microscope (Carl Zeiss, Inc.). Images were captured with a CCD camera (Retiga™ Exi, QImaging Corp.) using IPLab software (Scanalytics, Inc.).

RESULTS

Intracellular localization of T. brucei SSE1 --We identified the T. brucei homolog of C. fasciculata SSE1 (16) by searching the T. brucei genome database (Tb10.100.0220 in www.genedb.org). The predicted T. brucei SSE1 protein (295 amino acids) is 63% identical to its C. fasciculata counterpart (297 amino acids), and there are no other genes closely related to SSE1 in the T. brucei genome. To localize T. brucei SSE1 intracellularly, we targeted an in-frame GFP sequence to the C-terminus of one of the SSE1 chromosomal alleles (35). We then detected the tagged protein using an anti-GFP antibody and fluorescence microscopy. The T. brucei SSE1 is concentrated in the antipodal sites flanking the kDNA disk (Fig. 1A), the same location previously reported for the C. fasciculata enzyme (16).

RNAi of SSE1--To verify the function of SSE1, we constructed a stem-loop vector for RNAi and transfected it into trypanosomes. After cloning, we induced synthesis of the stem-loop RNA by adding tetracycline to the cell culture. Cell growth was inhibited after 6 days of RNAi (Fig. 1B), indicating that SSE1 is essential for procyclic T. brucei. A northern blot indicated that RNAi caused a nearly complete loss of SSE1 mRNA after 1 day; the large smear of dsRNA is probably composed of fragments of the stem-loop RNA (Fig. 1C).

By DAPI staining and fluorescence microscopy, we evaluated the effect of RNAi on the size of the kDNA disk. We found that RNAi of SSE1 ultimately caused shrinking and loss of the kDNA with about 90% of the cells having no kDNA by day 10 (Fig. 1D). This loss indicates that SSE1 is essential for kDNA replication or maintenance. Surprisingly, we found during the course of RNAi that there was a transient accumulation of large kDNA, peaking at day 2. As discussed below, these networks are apparently double-size replication intermediates, and we previously observed accumulation of larger kDNA during a preliminary RNAi study of SSE1 using the pZJM vector (32). These large kDNAs were present in only ~20% of uninduced cells (day 0 in Fig. 1D), in agreement with previous results (26). However, they increased to 50% at day 2. Subsequent to day 2, kDNA networks became smaller and they eventually disappeared.

Effect of RNAi on replication primers--The enzymatic properties of purified SSE1 had implied a function in primer removal (15,17). To test this possibility in vivo, we compared the size of minicircle primers in uninduced cells with those in cells induced for RNAi for 2 or 4 days. Although this analysis was complicated by the extensive heterogeneity of T. brucei minicircles (33), we were able to map primers at the 5' ends of the leading strand and the first Okazaki fragment because both of these sequences reside within a conserved minicircle region containing the replication origin (reviewed in (3)).

The first Okazaki fragment in the closely-related parasite T. equiperdum is ~73 nucleotides (39), and the same is true in T. brucei (Y. Liu, unpublished experiment). Furthermore, the gaps flanking the T. equiperdum fragment are among the last to be repaired prior to covalent closure of network minicircles (39,40). To evaluate primers on the first T. brucei Okazaki fragment (see map in Fig. 2A), we isolated kDNA networks, digested them with Taq I, and fractionated the fragments on a denaturing 9% polyacrylamide gel. We then transferred the DNA to a nylon membrane for hybridization with a 32P-labeled probe complementary to the first Okazaki fragment (Fig. 2B). In cells not undergoing RNAi (day 0) or having undergone RNAi for 2 days, we detected a ~66 nucleotide fragment, and this major species persisted after alkali treatment. The treatment conditions (0.3 M NaOH, 50 mM EDTA, overnight at 37 °C) were considerably
more stringent than those used to cleave at ribonucleotides embedded in mammalian mitochondrial DNA (41). However, after 4 days of RNAi, there were 2 fragments, with the larger one (~67 nucleotides) being reduced in size by alkali treatment to that of the smaller one; therefore the ~67 nucleotide fragment probably contains a single 5' ribonucleotide. In another experiment alkali treatment suggested a single 5' ribonucleotide on this fragment at day 2.

We used a similar strategy to evaluate primers on the leading strand, cleaving the isolated networks with HpyCH4 V which cuts about ~69 nucleotides from its 5' end (see map in Fig. 2D). We hybridized the blot with a 32P-labeled probe complementary to the 5' terminal region of the leading strand. At day 0 we detected a fragment of ~69 nucleotides that was not alkali-sensitive, indicating that there are no ribonucleotides at the 5' end of the leading strand in cells not undergoing RNAi (Fig. 2E). After 2 or 4 days of RNAi, we detected fragments of ~69, ~70, and ~71 nucleotides, with only the latter fragment being sensitive to alkali. This result provided evidence that the leading strand on network minicircles in RNAi cells contains a single 5' ribonucleotide. The ~70 nucleotide fragment, which is insensitive to alkali, must contain a 5' terminal deoxyribonucleotide. Therefore, SSE1 in uninduced cells must be capable of removing not only a 5' ribonucleotide but also a penultimate deoxyribonucleotide. The latter cleavage is similar to that detected when a model substrate, containing 5' ribonucleotides mimicking a primer, was treated with recombinant SSE1 (17).

Following kDNA replication and during or soon after segregation of the daughter networks, all of the minicircle gaps are repaired ((24,26,27) and see below in Fig. 5A). Given the persistence of 5' ribonucleotides following RNAi, we tested whether ribonucleotide primer remnants could be ligated into covalently-closed minicircles. We isolated kDNA networks from cells undergoing RNAi for 0, 2, or 4 days, decatenated them by treatment with topoisomerase II, and isolated covalently-closed minicircles by gel electrophoresis. To determine in the RNAi cells whether 5' ribonucleotides on the leading strand or first Okazaki fragment (identified in Figs. 2B and 2E) had been ligated into covalently-closed circles, we digested them with Taq I or HpyCH4 V (see maps in Figs. 2A and 2D), and then treated them with alkali. We did not detect, in either case, the fragments that would have been predicted if ribonucleotides had been incorporated into covalently-closed circles (the position of the predicted fragments is marked by arrows in Figs. 2C and 2F). We did, however, detect a similarly-sized fragment when kDNA networks, with some minicircles still gapped, were digested with the restriction enzymes (lanes NW in Figs. 2C and 2F). Thus we concluded that the ribonucleotides detected at the 5' ends of the leading strand and the first Okazaki fragment could not be ligated into covalently-closed minicircles.

**Effect of RNAi on free minicircle replication intermediates**--Covalently-closed minicircles are released from the network for replication as theta-structures (42). The progeny free minicircles, containing nicks or gaps, then reattach to the network. In *T. brucei* the steady-state levels of covalently-closed and nicked/gapped free minicircles are roughly comparable (23). These two species, as well as networks (which remain in the slot), are easily separated by agarose gel electrophoresis with ethidium bromide in the gel and buffer (Fig. 3A). To analyze the effect of SSE1 silencing on free minicircle species, we induced RNAi and took daily samples for 9 days (the day 0 sample is uninduced) (Fig. 3A). We electrophoresed total DNA and detected kDNA species on a Southern blot with a probe specific for minicircles. As shown in Fig. 3A, we found in uninduced cells, as expected, comparable levels of covalently-closed and nicked/gapped free minicircles. The level of covalently-closed free minicircles remained relatively constant during 5 days of RNAi and then precipitously declined (see quantitative data in Fig. 3B). In contrast, the level of nicked/gapped free minicircles increased 2- to 3-fold during days 1 to 5 of RNAi. Then they declined in concentration, and all free minicircle species were undetectable on day 7. The accumulation of gapped free minicircles raises the possibility that SSE1 silencing delays attachment of newly replicated minicircles to the network.

**Effect of RNAi on network size**--Visualization of kDNA in DAPI-stained cells
(Fig. 1D) suggested that RNAi caused a transient increase in kinetoplast size, peaking at day 2. To address this issue quantitatively, we isolated networks from cells undergoing RNAi, stained them with DAPI, and used NIH Image software to measure the surface area of these planar structures on fluorescence micrographs. The results are presented in bar graphs in Fig. 4B, and representative images of DAPI-stained networks are shown in Fig. 4A. To identify which networks were undergoing replication we also fluorescently-labeled (with TdT and a fluorescent dNTP) the gapped minicircles that are present in replicating kDNA. We found in cells not undergoing RNAi that kDNA networks had a bimodal size distribution (Fig. 4B, upper graph). Those in the larger peak had an average surface area of about 15 µm², and most were TdT-negative (white section of each bar). Many of the larger-sized networks were TdT-positive (designated by the lower black section of each bar), indicating that they are undergoing replication. There was a different distribution after 2 days of RNAi (Fig. 4B, middle graph). The average size of the main peak was close to that of cells not undergoing RNAi (compare with upper graph), but there was a much higher percentage of bigger networks. Nearly all of the larger networks were TdT positive. After 4 days of RNAi (Fig. 4B, lower graph) the networks had clearly undergone shrinking, and most were TdT positive. Fig. 4B, lower graph, likely underestimates the shrinking because smaller networks may have been selectively lost during the sedimentation step of network purification.

The accumulation of larger (approximately double-size) TdT-positive networks after 2 days of RNAi raises the possibility that incomplete primer removal increases the efficiency of covalent closure of minicircles which in turn delays segregation of the double-size network. However, the subsequent appearance of smaller networks that are TdT-positive indicates that the double-size networks can eventually segregate. We address these issues in the following section.

RNAi causes a delay in gap-repair and network segregation--We fixed and permeabilized trypanosomes and then labeled the minicircle gaps in situ with TdT and a fluorescent dNTP. Next we stained the cells with DAPI and visualized them by fluorescence microscopy, focusing on cells in which the kDNA network had replicated and initiated segregation. Fig. 5A shows cells not undergoing RNAi. The left-hand images show a dividing kinetoplast, and the middle images show a kinetoplast at a slightly more advanced stage of division; in both cases the daughter networks are TdT positive. The right-hand images, however, show typical daughter networks that have segregated by about 3.5 µm; in these cases there is no TdT labeling, indicating that minicircle gaps are sealed soon after the daughter networks have segregated. Fig. 5B shows a different situation in cells that have undergone RNAi for 2 days. As shown in images in Fig. 5B, the kDNA remains TdT positive even after segregation by about 4.0 µm. We detected no examples, either with or without RNAi, in which one kinetoplast was TdT positive and the other TdT negative.

To quantitate the effect of RNAi on network segregation, we evaluated fluorescent images of 40 cells with two kinetoplasts. We randomly chose 20 cells that were TdT positive and 20 that were TdT negative. As a function of time of RNAi (day 0, day 2, and day 4), we measured the distance between the two kinetoplasts. The results are summarized in the scatter plot in Fig. 5E, with each circle representing a cell with one nucleus and two kinetoplasts, and each square representing a cell with two nuclei and two kinetoplasts. The filled symbols represent kinetoplasts that are TdT positive. These results clearly demonstrate that minicircle gap repair is delayed, but not blocked completely, in cells in which SSE1 had been silenced by RNAi.

We next provided evidence that this effect of RNAi is not due to an alteration in one aspect of the kDNA segregation machinery. Segregation of daughter kinetoplasts is mediated by a linkage between the kDNA network and the flagellar basal body (4,5), and therefore we labeled cells with DAPI, TdT, and an antibody that recognizes the basal body. We found, as expected in cells not undergoing RNAi (Fig. 5C), that the segregating kinetoplasts were each associated with a basal body. The same is true for cells subjected to RNAi for 2 days (Fig. 5D).
To obtain an overview of the changes in kDNA during 4 days of RNAi, we scored 1000 cells each day, evaluating whether they are TdT positive or negative. As shown in Fig. 6, RNAi causes a dramatic increase in the fraction of cells that are TdT positive. These findings confirm that RNAi silencing of SSE1 causes an increase in the number of cells with gapped minicircles (TdT positive), providing evidence that persistence of RNA primers prevents gap repair.

DISCUSSION

From its enzymatic properties and intracellular localization in the antipodal sites (see Introduction), it had been assumed that SSE1 functions in the late stages of minicircle replication to remove RNA primers. We have now used RNAi silencing of SSE1 to provide direct evidence that this enzyme functions at least in the final stages of primer removal in vivo. We also found that RNAi had other effects that are likely a consequence of the persistence of primers. As discussed below, these include an apparent delay in attachment of newly replicated minicircles to the network and alterations in the segregation of networks that have completed replication. The former effect could contribute to the gradual shrinking and loss of the kDNA network. Another factor contributing to kDNA loss could be the depletion of covalently-closed minicircles on networks; these molecules, when released from the network, are the substrates for replication. Since procyclic trypanosomes require respiratory proteins that are encoded by maxicircle transcripts whose editing is guided by minicircle transcripts, RNAi-mediated kDNA loss causes inhibition of growth of the parasite. 

RNAi of SSE1 causes persistence of minicircle replication primers--The primary effect of SSE1 RNAi is likely on primer removal (Fig. 2). We found that a restriction fragment derived from the 5' end of the leading strand of newly replicated network minicircles had no alkali-labile 5' ribonucleotides in cells not induced for RNAi (Fig. 2E, day 0). In contrast, after RNAi for 2 or 4 days, some of the leading strand fragments had a single 5' ribonucleotide. Comparison of the sizes of the fragments with and without RNAi revealed that SSE1 must remove not only a ribonucleotide but also a deoxyribonucleotide. We also detected no 5' ribonucleotides on the first Okazaki fragment in the absence of RNAi (Fig. 2B, day 0), whereas a single ribonucleotide was found on some fragments after RNAi.

The inability to detect alkali-sensitive primers on the first Okazaki fragment in networks from cells not undergoing RNAi agrees with previous studies on T. equiperdum (43) and C. fasciculata (44). However, our finding that network minicircles from uninduced cells have no leading strand 5' ribonucleotides differs from previous studies on C. fasciculata, which has up to six 5' ribonucleotides on the leading strand (44), and on T. equiperdum, which has one or two (43). Although T. brucei, the subject of this study, is related to T. equiperdum, there are many possible reasons that could explain the difference in persistence of leading strand primers. For example, it could be due to differences in life cycle stage or the cultivation of the parasites. Our T. brucei cells were procyclic forms cultured at 27 °C, whereas the T. equiperdum cells were bloodstream forms isolated from a rat.

SSE1 is unlikely to act alone in primer removal. Studies on nuclear replication in other eukaryotes suggest involvement of multiple proteins. In addition to FEN1 (which has activity similar to that of SSE1), these include RNaseH, dna2p, and replication protein A (30,31). C. fasciculata has a mitochondrial RNaseH (45), and a homolog of that enzyme is encoded in the T. brucei genome (46,47). Further studies will be needed to evaluate its role, and that of other proteins, in minicircle primer removal. Our data (Figs. 2B, 2E) suggest that a substantial fraction of the fragments derived both from the leading strand and the first Okazaki fragment contain no 5' ribonucleotides. It is possible that these ribonucleotides had been removed by RNaseH, by residual SSE1 in the RNAi cells, or by some other enzyme.

As mentioned above, we detected no 5' ribonucleotides in network minicircles in the absence of RNAi, indicating that in these cells primer removal occurs in free minicircles or immediately after attachment of the free minicircle to the network. With regard to the lagging strand in T. equiperdum (39) and in T.
*brucei* procyclic forms (B. Liu, and PTE, unpublished), most of the Okazaki fragments are joined prior to minicircle attachment to the network, and one step in joining must be primer removal. Removal of primers (or at least the final stages of primer removal) almost certainly occurs in the antipodal sites where SSE1 is localized (16). The persistence of 5' ribonucleotides in the leading strand of network minicircles of *C. fasciculata* or *T. equiperdum* implies either that a small fraction of the SSE1 is localized within the kDNA disk or that some other enzyme removes these primer remnants. RNase H1 is a candidate for the latter possibility as in *C. fasciculata* it is located within the kDNA disk (45).

RNAi of SSE1 impedes the attachment of newly replicated free minicircles to the network-Fractionation of free minicircle species by gel electrophoresis, as a function of time of RNAi, reveals a 2- to 3-fold increase in gapped free minicircles with little change in the level of covalently-closed free minicircles until after day 5 (Fig. 3). This result implies that detachment of covalently-closed minicircles from the network proceeds normally, but reattachment of gapped minicircles is slowed down. This delay likely contributes to the eventual shrinking and disappearance of the kDNA network. However, gapped minicircles eventually attach to the network because minicircle gaps are easily detectable by TdT labeling of isolated networks (Fig. 4). The free minicircle analysis also revealed an accumulation of oligomeric minicircles at days 4 and 5 (indicated by *). These could be fragments derived from shrinking networks or multimeric replication intermediates whose segregation was delayed.

**RNAi of SSE1 delays network segregation**--Although RNAi ultimately causes cells to lose kDNA, we found that DAPI staining revealed a transient accumulation of cells with larger-than-normal kinetoplasts (Fig. 1D, peaking at day 2). To prove that this enlargement is due to an increase in size of the kDNA network, we isolated networks from cells undergoing RNAi, stained them with DAPI, and visualized them by fluorescence microscopy. By measuring network surface areas (Fig. 4), we found in cells not induced for RNAi a distribution of unit-size, intermediate-size, and double-size networks similar to that reported earlier for wild type trypanosomes (26). As expected, most of the double- and intermediate-size networks, but only a small fraction of unit-size networks, were TdT-positive. In contrast, 2 days of RNAi results in a much larger fraction of networks that appear approximately double-size, most of which are TdT-positive. This finding suggests that the less efficient removal of 5' ribonucleotides, which in turn sustains minicircle gaps, causes a delay in segregation of double-size networks. The increased fraction of smaller networks after 4 days of RNAi, suggests that segregation can ultimately proceed after the delay.

We provided direct evidence that RNAi delays gap repair in networks undergoing segregation. Fluorescence microscopy indicated that in cells not undergoing RNAi the kinetoplasts converted from TdT-positive to TdT-negative soon after segregation (Figs. 5A and 5C). In contrast RNAi for 2 or 4 days slowed the conversion of TdT-positive kinetoplasts to TdT-negative. It is important to note that many RNAi cells eventually do become TdT-negative, implying that residual SSE1 or some other enzyme removes 5' ribonucleotides from network minicircles. We provided evidence that a mitochondrial DNA ligase is not able to covalently-close minicircles containing 5' ribonucleotides (Figs. 2C and 2F). Finally, as shown in Fig. 6, the overall trend during RNAi is to become TdT-positive with 76% of the cells attaining that status in 4 days.

RNAi silencing of SSE1 has one other remarkable effect on the structure of kDNA networks. When analyzed by TdT labeling with a fluorescent nucleotide, isolated networks from cells not undergoing RNAi have the well-described polar labeling expected from attachment of minicircles adjacent to the antipodal sites (24-27). We were therefore very surprised to find that most of the TdT-labeled networks from cells having undergone RNAi for 2 or 4 days had uniform labeling around the network periphery, resembling rings (Y. Liu, unpublished experiments). Ring intermediates are found in *C. fasciculata*, *T. cruzi*, *Phytomonas serpens*, and *Leishmania tarentolae* (27), and have been attributed to a relative movement between the kinetoplast disk and the
antipodal sites (29). The appearance of rings suggests that RNAi silencing of SSE1 causes a fundamental change in the kDNA replication mechanism. These findings, and their implications, will be published elsewhere.

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FOOTNOTE

1kDNA, kinetoplast DNA; SSE1, structure-specific endonuclease-I; KFZ, kinetoflagellar zone; UMSBP, universal minicircle sequence binding protein; TdT, terminal deoxynucleotidyl transferase; PBS, phosphate-buffered saline; GFP, green fluorescent protein; RNAi, RNA interference.

FIGURE LEGENDS

Fig. 1. Localization and RNAi of *T. brucei* SSE1. *A*, left panel shows immunofluorescence of GFP-tagged SSE1 using an anti-GFP antibody. Right panel shows DAPI staining of the kDNA disk (K) and the nucleus (N). Merging of these images confirms that the SSE1 is concentrated in the antipodal sites flanking the kDNA disk. Scale bar, 2 μm. *B*, growth curves of cloned cells transfected with the SSE1 stem-loop construct and either uninduced or induced for RNAi by addition of 1 μg/ml tetracycline. About 3 weeks after induction cell growth resumed, indicating that some cells had developed resistance to RNAi. Cells/ml on the Y-axis is the measured value times a dilution factor. *C*, northern analysis of total RNA extracted from cells either uninduced for RNAi or induced for 1 day. The significance of the two bands of SSE1 mRNA is not known. *D*, effect of RNAi on the apparent size of the kDNA disk. Cells were fixed and stained with DAPI. For each time point 1000 cells were scored by eye. Inset images are examples of two cells with large kDNA, normal kDNA, small kDNA, and no kDNA.

Fig. 2. Effect of SSE1 RNAi on replication primers. *A*, map of first Okazaki fragment (~73 nucleotides, showing Taq I site). * indicates putative 5' ribonucleotide(s). *B*, analysis of primers on first Okazaki fragment. *C*, analysis of incorporation of lagging strand 5' ribonucleotides into covalently-closed minicircles. Networks from uninduced (day 0) and RNAi-treated cells (days 2 and 4) were decatenated and covalently-closed minicircles were isolated. The covalently-closed minicircles were digested with Taq I and hydrolyzed with alkali as indicated. Other conditions were same as in Panel B. Arrow marks ~66 nucleotide fragment as in Panel B and * indicates other Taq I fragments derived from minicircle population that serve as loading control. NW, lane containing intact networks from uninduced cells digested with Taq I and not treated with alkali. *D*,
map of the 5' end of leading strand, showing location of the HpyCH4 V site. * indicates putative 5' ribonucleotide(s). 

E, analysis of primers on the leading strand. kDNA networks were digested with HpyCH4 V, and samples were hydrolyzed with alkali as indicated. Samples were electrophoresed, transferred, and hybridized with a 32P-labeled oligonucleotide probe complementary to the 5' end of the leading strand. 

F, analysis of incorporation of leading strand 5' ribonucleotides into covalently-closed minicircles. This experiment was similar to that in Panel C except DNA was digested with HpyCH4 V. The upper panel shows a segment from near the top of the gel that verifies that adequate kDNA was present in each lane. Arrow indicates ~69 nucleotide HpyCH4 V fragment. Size markers (M) for Panels B and C were 75 nucleotide sequences corresponding to the first Okazaki fragment (beginning with 5'-GGGCGTGCA) (33), and markers for Panels E and F were 69 nucleotide sequences corresponding to the 5' end of the leading strand (beginning with 5'-TACACCAAC) (33).

Fig. 3. Effect of SSE1 RNAi on free minicircle replication intermediates. A, total DNA (10^6 cell equivalents/lane) was fractionated on a 1.5% agarose gel with 1 µg/ml ethidium bromide both in the gel and in the gel buffer (23). After transfer to a membrane, the kDNA was detected by a 32P-labeled minicircle probe (23). The gel photograph was prepared on a Fuji Phosphorimager. M, markers of covalently-closed, gapped, and linearized free minicircles. The nuclear DNA band was due to non-specific hybridization. Lanes 0-9, DNA samples from the cells at indicated number of days of RNAi. * indicates oligomeric minicircles, either replication intermediates or network fragments. B, effect of RNAi on levels of gapped free minicircles and covalently-closed free minicircles, estimated by phosphorimaging of the gel shown in A.

Fig. 4. Network surface area as a function of time of RNAi. Isolated kDNA networks were labeled in vitro with TdT and Alexa-Fluor-488-dUTP to tag gapped minicircles that had undergone replication. Then they were stained with DAPI and visualized by fluorescence microscopy. A, examples of DAPI-stained networks at days 0, 2, and 4 of RNAi. B, network surface areas were measured by NIH Image Software (http://rsb.info.nih.gov/nih-image/Default.html) and TdT positive networks (lower black segment of bar) were scored. Networks were counted only if they appeared to be not folded or collapsed (i.e., if DAPI staining was not uniform). About 75% were counted in upper panel, 70% in middle panel, and 65% in lower panel. Bar, 10 µm.

Fig. 5. Delay in gap repair and segregation after RNAi. Cells without RNAi (A) or subjected to RNAi for 2 days (B) were fixed, permeabilized, and then labeled at minicircle gaps with Alexa-Fluor-488-dUTP and TdT. After staining with DAPI, the cells were visualized by fluorescence microscopy. In all examples shown the daughter kinetoplasts are beginning or undergoing segregation. Upper panel in each image is DAPI-stained (blue) and shows nucleus (N) and kinetoplast (K). Lower panels show TdT-labeled kinetoplasts (green). Panels C and D show similar experiments in which cells were also stained with an antibody recognizing basal bodies (red). Scale bar, 1 µm. Scatter plot in Panel E shows distance between sister kinetoplasts (measured center to center) in 40 cells with two kinetoplasts (20 were TdT positive and 20 were TdT negative) like those in Panels A and B. Filled circles and squares represent kinetoplasts that are TdT positive, open circles and squares represent kinetoplasts that are TdT negative. 1N2K, cell with one nucleus and two kinetoplasts; 2N2K, cell with two nuclei and two kinetoplasts.

Fig. 6. Effect of RNAi on kDNA gap repair. Trypanosomes, either uninduced (day 0) or induced for RNAi, were fixed and permeabilized, TdT-labeled with a fluorescent dNTP, and stained with DAPI. For each time point, 1000 cells were evaluated by fluorescence microscopy.
Fig. 1

A. Anti-GFP and DAPI images showing cell nuclei labeled with GFP and DAPI respectively. The images indicate cellular structures labeled "K" and "N".

B. Graph showing the log of cell counts over days for RNAi treatments labeled "-RNAi" and "+RNAi".

C. Gel electrophoresis showing bands at 2.4 kb and 1.4 kb labeled as SSE1 mRNA and dsRNA.

D. Graph showing the percentage of cells with different kDNA sizes (normal, small, large, no kDNA) over days.
Fig. 4

A.

B.

No RNAi

RNAi 2 days

RNAi 4 days

Network Number

Area (μm²)
Fig. 5

A. no RNAi

B. RNAi 2 days

C. no RNAi

D. RNAi 2 days

E. Distance between kDNAs (μm)

- 1N2K, TdT+
- 2N2K, TdT+
- 1N2K, TdT-
- 2N2K, TdT-
Fig. 6

Days after RNAi induction

Percent of cells

TdT+

TdT−
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Yanan Liu, Shawn A. Motyka and Paul T. Englund

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