

Stem-Loop Silencing Reveals that a Third Mitochondrial DNA Polymerase, POLID, Is Required for Kinetoplast DNA Replication in Trypanosomes[∇]

Julian Chandler, Anthula V. Vadoros, Brian Mozeleski, and Michele M. Klingbeil*

Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01003

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Kinetoplast DNA (kDNA), the mitochondrial genome of trypanosomes, is a catenated network containing thousands of minicircles and tens of maxicircles. The topological complexity dictates some unusual features including a topoisomerase-mediated release-and-reattachment mechanism for minicircle replication and at least six mitochondrial DNA polymerases (Pols) for kDNA transactions. Previously, we identified four family A DNA Pols from *Trypanosoma brucei* with similarity to bacterial DNA Pol I and demonstrated that two (POLIB and POLIC) were essential for maintaining the kDNA network, while POLIA was not. Here, we used RNA interference to investigate the function of POLID in procyclic *T. brucei*. Stem-loop silencing of POLID resulted in growth arrest and the progressive loss of the kDNA network. Additional defects in kDNA replication included a rapid decline in minicircle and maxicircle abundance and a transient accumulation of minicircle replication intermediates before loss of the kDNA network. These results demonstrate that POLID is a third essential DNA Pol required for kDNA replication. While other eukaryotes utilize a single DNA Pol (Pol γ) for replication of mitochondrial DNA, *T. brucei* requires at least three to maintain the complex kDNA network.

Trypanosoma brucei and related trypanosomatid parasites (*T. cruzi* and *Leishmania* spp.) cause fatal and disfiguring diseases and, subsequently, significant medical and economic stress worldwide, with nearly 500 million people at risk for these vector-borne diseases (7). Current drug treatments are toxic, and no vaccines are available (45). Trypanosomatids are also divergent eukaryotes with a number of unusual biological properties, but one of their most interesting features is their mitochondrial DNA, known as kinetoplast DNA (kDNA). Unlike any DNA structure in nature, kDNA is a network containing thousands of catenated circular DNA molecules (minicircles and maxicircles). Several dozen maxicircles (23 kb) and ~5,000 minicircles (1 kb) are condensed into a disk-shaped structure in a specialized region of the cell's single mitochondrion, which is linked to the flagellar basal body through a tripartite attachment complex (16, 36, 39).

The kDNA network is essential for the survival of both procyclic and bloodstream forms of the parasite (42); therefore, understanding kDNA replication and repair processes is an important aspect of trypanosome biology. Network replication is complex, requiring coordinated duplication of each minicircle and maxicircle in near synchrony with nuclear DNA replication (during S phase) (50). Currently, trypanosomatids are the only known eukaryotes to contain at least six mitochondrial DNA polymerases (Pols), namely, two Pol β -type enzymes (typically a nuclear repair protein) and four family A Pols related to bacterial DNA Pol I (21, 40). This is in striking contrast to what is the case for other eukaryotes, which contain

just one mitochondrial DNA Pol, Pol γ , for replication and repair transactions.

To overcome the topological constraints within the catenated network, a key feature of the replication mechanism is the topoisomerase II-mediated release of individual covalently closed (CC) minicircles into a specialized region called the kinetoflagellar zone (KFZ) (10). Here, the free minicircles initiate unidirectional theta structure replication. Several proteins considered to be involved in this process are also found in the KFZ, including universal minicircle sequence binding protein (UMSBP), DNA primase, and two of the family A DNA Pols (1, 21, 23). Minicircle progeny are subsequently reattached at the network periphery (antipodal sites) still containing at least one gap. This results in a spatial separation of replication events: early initiation and replication occur in the KFZ, followed by Okazaki fragment processing and reattachment at the antipodal sites. These latter events are catalyzed by structure-specific endonuclease 1, Pol β , DNA ligase $\kappa\beta$, and topoisomerase II (9, 12, 14, 18, 31). Two recently described proteins, p38 and p93, also localize to the antipodal sites and have been shown to play roles in minicircle replication (24, 26). When all the minicircles have been replicated and reattached, the final gaps are filled, presumably by Pol β -PAK, and the network splits into two progeny networks. Although far less is known about maxicircle replication, it is clear that maxicircles do not decatenate from the network during theta structure replication (3). For kDNA structure and replication reviews, see references 20, 25, 28, and 44.

Why would trypanosomes require so many mitochondrial DNA Pols? They could have redundant functions, or, more likely, each could have a specific role in kDNA replication and repair. For example, in addition to the distinct localizations, the biochemical properties of Pol β and Pol β -PAK differ significantly and suggest nonredundant roles in the later stages

* Corresponding author. Mailing address: Department of Microbiology, University of Massachusetts, 639 North Pleasant Street, Amherst, MA 01003. Phone: (413) 577-3823. Fax: (413) 545-1578. E-mail: klingbeil@microbio.umass.edu.

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of minicircle replication (40). Similarly, our previous studies using RNA interference (RNAi) indicate that both POLIB and POLIC (which localize to the KFZ, where minicircle replication initiates) have essential roles in maintaining the kDNA network, and one cannot compensate for the loss of the other. A third Pol I-like protein, POLIA, is not essential under normal growth conditions and may be specialized for kDNA repair processes (21). Lastly, silencing with the pZJM vector was insufficient to knock down the function of POLID, leaving open the possibility that it too is required for kDNA replication.

Here we studied the function of POLID by use of a stem-loop double-stranded RNA (dsRNA) trigger for RNAi. In contrast to what was seen with attempts using the intermolecular trigger generated from the pZJM vector, RNAi of TbPOLID was successful only when using a stem-loop vector, suggesting that the intramolecular trigger may be a better tool to study the function of the mitochondrial Pols. Our results demonstrate that POLID plays a distinct and essential role in maintaining the kDNA network. Knockdown of this mitochondrial Pol causes the loss of the kDNA network, consistent with a role in kDNA replication. The five other mitochondrial Pols were not able to compensate for the loss of POLID. Currently, three mitochondrial DNA Pols are required to maintain the integrity of the complex kDNA network.

MATERIALS AND METHODS

Plasmid constructs. (i) RNAi vector pZJMID2. A TbPOLID coding sequence (nucleotides 694 to 1193) was PCR amplified from *T. brucei* 927 genomic DNA by use of forward (5' - GAG TCT AGA CGT GAT TGC TTA GTA AGT TGG - 3') and reverse (5' - GAG AAG CTT GTA CGA ATC AGT GCC CAA GTG G - 3') primers containing XhoI and HindIII sites (underlined). The 500-bp fragment was ligated into the pZJM vector (48) to generate a pZJMID2 construct. NotI-linearized pZJMID2 was transfected into *T. brucei* 29-13 cells as described below.

(ii) RNAi vector pSLID. Briefly, the same region of the POLID coding sequence was used for the construction of the stem-loop vector pSLID, as previously described by Wang et al. (48). The resulting stem-loop plasmid contains two copies of the POLID fragment in opposite orientations separated by the stuffer fragment. EcoRV-linearized pSLID was transfected into *T. brucei* 29-13 cells as described below.

Trypanosome growth and transfection. *T. brucei* procyclic strain 29-13 (49) was grown in SDM-79 (2) medium with 15% heat-inactivated serum (Invitrogen), 15 μ g/ml G418 (Fisher), and 50 μ g/ml hygromycin (Invitrogen) at 28°C. Linearized RNAi constructs (15 μ g) were transfected in 4-mm cuvettes by use of a BTX 630 electroporator at a peak discharge of 1.6 kV with a resistance of 25 Ω . After recovery for 24 h in 10 ml of medium, cells were placed under drug selection at 2.5 μ g/ml phleomycin (Invivogen). Clonal cell lines were obtained by limiting dilution in conditioned medium supplemented with an additional 5% heat-inactivated serum. Induction of the dsRNA was initiated with 1 μ g/ml tetracycline. Cell densities were determined using a Coulter Counter (model Z2; Beckman Coulter), and cultures were diluted 1:10 when the density reached 4×10^6 to 9×10^6 cells/ml.

RNA purification and Northern analysis. Total RNA was isolated from 5×10^7 (mid-log-phase) cells by use of the Purescript RNA isolation kit (Gentra System) and fractionated on a 1.5% agarose/7% formaldehyde gel. RNA was transferred to a GeneScreen Plus membrane (NEN). The following oligonucleotides were used to generate PCR products (from *T. brucei* 927 genomic DNA) for use in randomly primed labeling reactions: for POLID, the same primers used to generate pSLID; for POLIC, forward IC (5'-TCT AGA CGT GAT TGC TTA GTA AGT TGG - 3') and reverse IC (5'-ACG CGT GTA CGA ATC AGT GCC CAA GTG G - 3'); and for POLIB, forward IB (5'-TCT AGA AAG ATG AGC GTG TCA ACG AGG - 3') and reverse IB (5'-ACG CGT GGT AAA CCG TGG CGC GAC GAG G - 3'). A 650-bp XhoI/HindIII fragment was liberated from the pZJM α vector and used as the template for the tubulin probe. Probes were generated using 50 ng of gel-purified products with the Random Primers DNA labeling system (Invitrogen) according to the manufacturer's in-

structions. Hybridization and washing conditions were as described previously (47). Quantitation was performed using a Molecular Dynamics PhosphorImager (Typhoon 9210; GE Healthcare) and normalized against the tubulin signal by use of ImageQuant 5.2 software.

Microscopy. Uninduced and RNAi-induced cells were harvested at the indicated time points, resuspended in phosphate-buffered saline, and adhered to poly-L-lysine-coated slides for 10 min. To avoid variations in fixation or staining, cells were harvested on the same day from staggered inductions. Briefly, cells were fixed with 4% paraformaldehyde, stained with 2 μ g/ml 4'-6'-diamidino-2-phenylindole (DAPI), and mounted in Vectashield (Vector Laboratories). For each time point, more than 300 DAPI-stained cells were scored by eye according to the size of the kDNA network and other changes in kDNA morphology based on fluorescence intensity. For cells classified with small kDNA, where the kDNA network was at least 50% smaller than those found in uninduced cells, and for those classified with no kDNA, there was no detectable extranuclear DAPI-stained spot even when focusing through several planes of vision. However, a remnant kDNA network too small to be detected cannot be ruled out. Only intact cells by differential interference contrast (DIC) were included in the analysis. Slides were viewed with a Nikon Eclipse E600 microscope. DIC and DAPI fluorescence images were captured using a Spot digital camera (Diagnostic Instruments).

DNA purification and analysis. Uninduced and induced cells were harvested at the indicated time points from staggered inductions. Total DNA was isolated from 1×10^8 cells by use of the Puregene DNA isolation kit (Gentra System). For analysis of network kDNA, total DNA was digested with HindIII and XbaI, fractionated on a 1% agarose gel, processed, and transferred to a nylon membrane. For analysis of free minicircles, total DNA was fractionated on a 1.5% agarose gel (20 by 25 by 0.5 cm, 60 V, 18 h) including ethidium bromide (1 μ g/ml) in the gel and running buffer, processed, and transferred to a nylon membrane (47). Minicircles were detected with a randomly primed ³²P-labeled probe made from a cloned full-length *T. equiperdum* minicircle sequence from the pJN6 vector (35). Maxicircles were detected with a randomly primed ³²P-labeled PCR product by use of the following oligonucleotides: forward (5'-CTA ACA TAC CCA CAT AAG ACA G - 3') and reverse (5'-ACA CGA CTC AAT CAA AGC C - 3'). Hybridization and washing conditions using maxicircle, minicircle, and tubulin probes were as previously described (47).

RESULTS

RNAi silencing of the TbPOLID gene. The aim of this study was to assess whether mitochondrial POLID is an essential protein for kDNA maintenance. To study the cellular function of POLID, two different regions of the coding sequence were cloned into tetracycline-inducible RNAi vectors and electroporated into *T. brucei* 29-13 cells. Previous experiments were unsuccessful despite three other related Pols being efficiently silenced using pZJM (21). A second 500-bp fragment in the 5' region of POLID (Fig. 1A) corresponding to amino acids 232 to 398 also resulted in the selection of ZJMID2 phleomycin-resistant cells that escaped mRNA degradation by the intermolecular dsRNA-specific trigger (data not shown). In most cases, RNAi inductions with pZJM result in significant decreases of the mRNA of interest. However, there are other examples where the pZJM vector is ineffective (11, 15, 48).

As an alternative, we used another tetracycline-inducible vector that produces an intramolecular stem-loop dsRNA based on an inverted repeat of the gene of interest separated by an unrelated stuffer fragment (SL vector) (47). The same 500-bp fragment in the 5' region of POLID was used (Fig. 1A), and the final construct (pSLID) was transfected into 29-13 cells. RNAi of TbPOLID caused growth inhibition starting 4 days after tetracycline induction of the stem-loop dsRNA, indicating that POLID is an essential mitochondrial DNA Pol (Fig. 1A). Three clonal cell lines were analyzed and all produced similar patterns of growth inhibition, with average doubling times of 13 h for the uninduced cells. The POLID RNAi

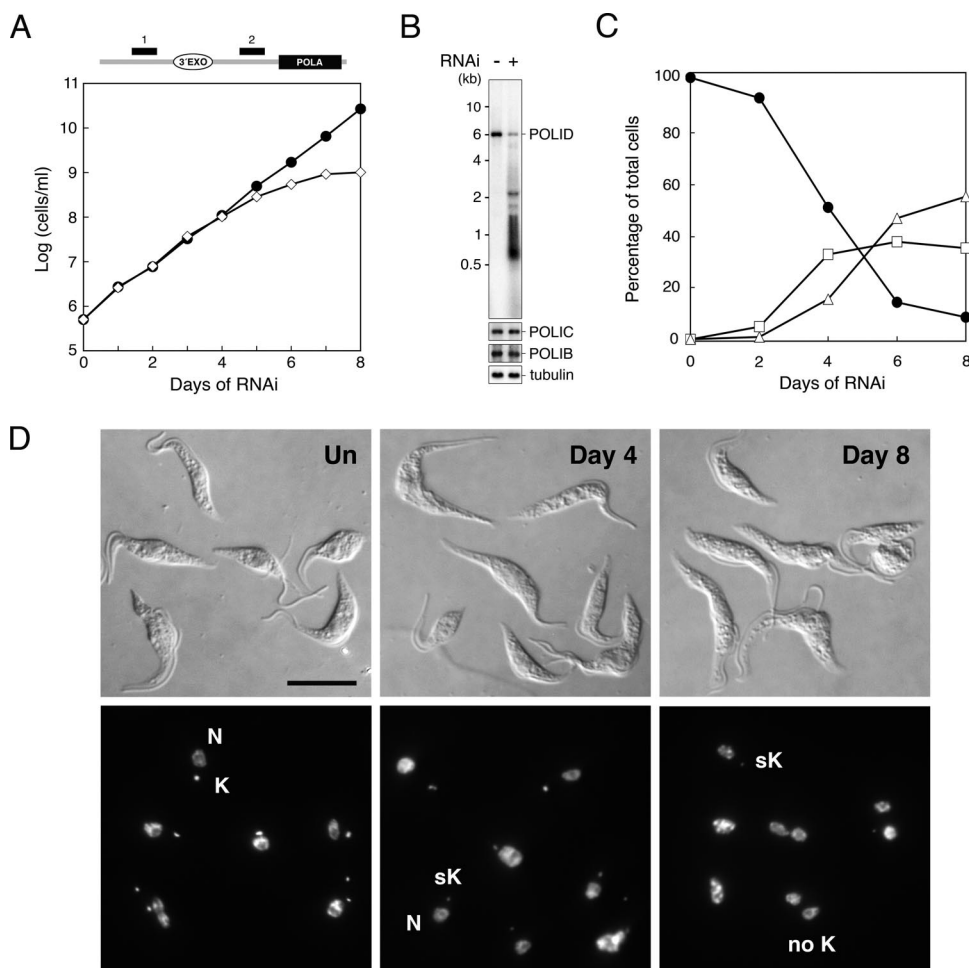


FIG. 1. Effects of *POLID* RNAi. (A) Diagram: protein domain structure of *POLID* with the region used to generate the pSLID and pZJMID2 vectors (1) and the region used to generate pZJMID1 (2) (21). Graph: clonal cell line C8P1 was grown in the absence (closed circles) or presence (open diamonds) of tetracycline (1 μg/ml) to express the stem-loop dsRNA. (B) Northern blot of total RNA from uninduced (–) and RNAi-induced (+) cultures hybridized for 48 h with radiolabeled probes specific for *POLID*, *POLIC*, and *POLIB*. Hybridization with α-tubulin probe was the loading control. RNA marker sizes are indicated on the left in kilobases. (C) Kinetics of kDNA loss as determined by visual analysis. Symbols: filled circles, normally sized kDNA; open squares, small kDNA; open triangles, no kDNA. More than 300 randomly selected cells were scored for each time point. (D) Effect of *POLID* RNAi on kinetoplast size. (Top) DIC images; (bottom) DAPI-stained fluorescent images. Abbreviations: N, nucleus; K, normally sized kDNA; sK, small kDNA; no K, no kDNA; Un, uninduced. Bar, 10 μm.

growth arrest is similar to that seen for RNAi of other replication proteins that result in kDNA loss (9, 21, 24, 26, 27, 41, 47).

Northern blot analyses revealed an 80% reduction of *TbPOLID* mRNA 2 days after the addition of tetracycline (Fig. 1B) that persisted through 8 days of RNAi, and the level continued to decrease through the 8 days of RNAi induction (data not shown). Additionally, mRNA levels for the two other essential mitochondrial Pols (*POLIB* and *POLIC*) did not change significantly following 2 days of RNAi induction (Fig. 1B), indicating that the loss of *TbPOLID* transcript does not lead to compensatory changes in the transcription of *TbPOLIB* and *TbPOLIC*. These findings are consistent with previous reports on the lack of transcriptional regulation of trypanosomatid genes (6, 37).

POLID RNAi leads to a progressive loss of kDNA. The condensed kDNA network is easily visualized in the cell by DAPI staining. To assess the effects of *POLID* depletion on

kDNA networks, DAPI-stained cells were examined by fluorescence microscopy (Fig. 1D). As expected uninduced cells contained a nucleus and a normally sized kDNA with the various stages of the cell cycle evident (one nucleus and one kDNA [1N1K], 1N2K, and 2N2K). Following 4 days of RNAi induction, cells began to display abnormally small kDNA networks, and after 8 days of RNAi a majority of the cells lacked a kDNA network. Those cells lacking kDNA still appeared to be proceeding through the cell cycle, as evidenced by cells containing two nuclei. Additionally, basal body staining with the YL1/2 antibody (which detects tyrosinylated tubulin) indicated that *POLID* RNAi does not inhibit the duplication or segregation of the basal bodies (data not shown). Figure 1C is a representative experiment showing the kinetics of kDNA network shrinking and loss, and this same pattern of kDNA network shrinking and loss was reproducible in two additional *POLID* RNAi inductions. The percentage of cells with normally sized kDNA declined during the course of the induction,

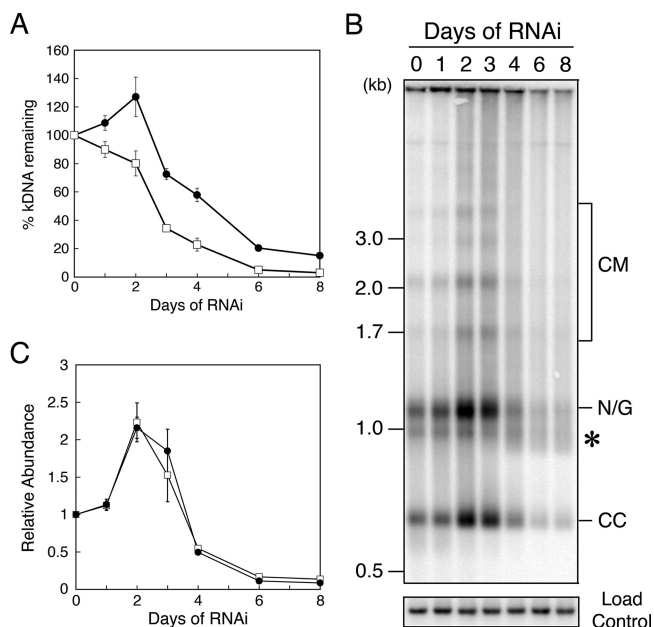


FIG. 2. POLID RNAi effects on kDNA abundance. C8P1 clonal cells were grown in the presence or absence of tetracycline and harvested at the indicated time points. (A) Total DNA (10^6 cell equivalents/sample) was digested, fractionated on a 1% agarose gel, and transferred to a nylon membrane. Southern blot analysis of changes in minicircle (closed circles) and maxicircle (open squares) content was determined with minicircle, maxicircle, and tubulin probes. Quantitation of kDNA species was performed using a PhosphorImager, and values are reported as percentages of those detected for uninduced cells. Values are the mean (\pm standard deviation) of three separate RNAi inductions. (B) Southern analysis of free minicircles. Total DNA (2×10^6 cells/lane) was fractionated on a 1.5% agarose gel and transferred to a nylon membrane. After a probe for minicircle DNA, the blot was stripped and probed for α -tubulin as the loading control. *, multiply gapped minicircle progeny; CM, catenated minicircles. (C) Quantitation of free minicircles by phosphorimaging. Values were corrected for the loading control. Symbols: closed circles, CC free minicircles; open squares, N/G free minicircles. Values are the mean (\pm standard deviation) of two separate RNAi inductions, one of which is shown in panel B.

while the percentage of cells containing small and no kDNA increased to 36% and 52%, respectively, by day 8. The shrinking and loss of the kDNA network parallels the growth inhibition that began at day 4 of POLID silencing.

Loss of kDNA species following POLID RNAi. The loss of the kDNA network during POLID RNAi suggests a role in kDNA replication. To assess the effects of POLID RNAi on the minicircle and maxicircle copy number, we used Southern blot analysis of total DNA digested with HindIII and XbaI probed with minicircle and maxicircle probes. Quantitation of these data revealed that maxicircle loss was evident within the first 2 days of POLID silencing, whereas minicircle abundance increased during the same time period (Fig. 2A). Maxicircle abundance declined to about 34% by day 3 and reached 5% by day 6. Minicircle abundance eventually declined to 70% by day 3 and subsequently to 20% by day 6 of the induction.

Effect of POLID RNAi on free minicircle replication intermediates. Minicircles are released from the kDNA network during replication; therefore, we next examined the effects of POLID silencing on the pool of free minicircle replication

intermediates (Fig. 2B). Free minicircles normally constitute a small fraction of the total kDNA (0.4% in an unsynchronized population) (13) but can be separated easily on an agarose gel. Southern blot analysis of undigested total DNA with a minicircle probe allows identification of the free minicircle species. In control cells, the major types of replication intermediates are CC monomers (Fig. 2B, day 0) that have not yet replicated and nicked/gapped (N/G) daughter progeny (Fig. 2B, day 0) that have segregated from one another but are not yet reattached to the kDNA network. The concentration of free-minicircle replication intermediates increased during the initial 2 days of POLID silencing. During the course of the induction, there were progressive increases in both CC and N/G species (2.3-fold increase, day 2) that closely paralleled one another (Fig. 2C). During the later days of the induction, the abundance of both replication intermediate species quickly declined, in agreement with the loss of minicircle content and possibly due to degradative processes. Additionally, during the later days of POLID RNAi, a heterogeneous broad band accumulated that resembled lagging-strand progeny containing multiple gaps (Fig. 2B) (21, 41). The increase in minicircles that enter the pool of free replication intermediates on day 2 could explain the apparent increase in minicircle mass.

DISCUSSION

We have shown previously that four Pol I-like proteins are mitochondrial and that two of these (POLIB and POLIC, which both localize to the KFZ) are essential for replicating the kDNA network (21). The other two Pols, POLIA and POLID, localize throughout the mitochondrion. All previously studied kDNA replication proteins localize to specific regions surrounding the kDNA disk, suggesting that the replication machinery is precisely organized around this structure. POLIA was not essential under normal growth conditions and is likely a kDNA repair protein. We initially hypothesized that POLID might also be a repair protein based on its localization, which is similar to that of POLIA. In this study, we used stem-loop RNAi to partially characterize POLID of *T. brucei* and to establish that POLID is also essential for replicating the kDNA network. The effect of the depletion of POLID cannot be overcome by the five other mitochondrial DNA Pols. Therefore, replication of the kDNA network requires at least three mitochondrial Pols, wherein each plays a specific role in network replication.

Two attempts to silence POLID using the pZJM vector failed even when different regions of the gene were used. The inducible pZJM vector uses opposing dual T7 promoters to synthesize an intermolecular dsRNA trigger (48). Typically, RNAi using pZJM causes effective knockdown for about 80% of the genes tested (33). This vector is more sensitive to leakage (synthesis of the dsRNA in the absence of tetracycline) and can lead to revertant cell lines (5). Possible mechanisms for this phenomenon include loss of the dsRNA cassette by recombination of the inverted repeats that flank the cloned fragment of the gene of interest. Alternatively, selection for RNAi-negative cells that no longer respond to the dsRNA challenge could explain such a result (46). In fact, Shi et al. have generated RNAi-deficient trypanosomes simply by using repeated cycles of electroporation with α -tubulin dsRNA, which re-

quired no prior mutagenesis steps (43). Currently, we do not know why pZJM has failed for the study of POLID function.

Our results indicate that the stem-loop vector is a valuable tool to silence POLID with an effective reduction in target mRNA (~80%) that is comparable to what has been seen in other *T. brucei* RNAi experiments. The stem-loop vector is slightly more efficient at knockdown of target mRNA levels and is less sensitive to the leakage often seen with pZJM (11). Additionally, Chanez et al. showed that silencing *T. brucei* dynamin-like protein with a stem-loop vector was more efficient (maximal depletion within 1 or 2 days of induction) than silencing with pZJM (required 3 or 4 days for depletion) (4, 32). Taken together, these findings suggest that the intramolecular dsRNA trigger is more efficient for studying the other mitochondrial Pols.

Consistent with a role in replication, the silencing of POLID resulted in growth inhibition and the subsequent loss of the kDNA network within 4 days of dsRNA induction (Fig. 1). During RNAi, the size of the kDNA network progressively decreased and was absent from a majority of the cells by day 8 of RNAi. The loss of the network was due to a decrease in both minicircle and maxicircle abundance. However, early during the POLID RNAi induction, the maxicircle copy number declines, with a consistent increase in minicircle abundance (day 2) that is also reflected as an increase in free minicircle replication intermediates. The basis for this trend is unclear, but it could represent a compensatory response to maintain total network DNA content. A similar trend was noted when silencing mitochondrial topoisomerase IA; then, maxicircle copy number increased while minicircle abundance declined (41). The transient accumulation of both CC minicircles and the N/G progeny is followed by a rapid decline that persists for the remainder of POLID silencing. Additionally, the appearance of multiply gapped progeny (Fig. 2B) indicates that minicircle replication is severely impaired.

If POLID was the sole Pol working at the minicircle replication fork, then silencing of this protein should produce a blockage in replication that results in the accumulation of CC minicircles only. However, POLID RNAi resulted in the accumulation of both unreplicated and replicated intermediates. Additionally, even though the knockdown of *POLID* mRNA was greater than 80%, a diminished level of protein may be sufficient to allow replication for an intermittent period until a minimal threshold is reached and kDNA network replication is then severely impaired. The protein stability of POLID is yet to be determined. It is also possible that functional uncoupling of two Pols at the replication fork occurs when POLID is silenced and the partner to POLID may be enzymatically optimized to perform either continuous or discontinuous replication. A precise role of POLID has not been determined at this time, leaving open the possibility that maxicircle replication may be a primary role for POLID and that the effect on minicircles may be secondary.

Previously, POLID localized throughout the mitochondrial matrix and colocalized with the matrix protein lipoamide dehydrogenase in an unsynchronized *T. brucei* cell population (21). Here we show that POLID plays an essential role in kDNA replication and represents an example of a protein involved in kDNA replication that does not exclusively localize to the specific regions surrounding the kDNA disk. This sug-

gests that POLID would need to redistribute closer to the kDNA to perform its essential role in replication. In other model systems, several replication proteins undergo dramatic relocalization by interactions with PCNA during S phase to perform their essential functions (22). Additionally, p38, a protein that binds to minicircle origins, would need to relocalize from the antipodal sites to the KFZ to perform its essential role in minicircle replication (26). Alternatively, POLID may be an abundant protein present at the disk and throughout the mitochondrial matrix.

Since the function of multiple DNA Pols in mitochondrial replication has never been documented for other eukaryotes, this distinction raises the question of how *T. brucei* utilizes three DNA Pols to replicate the kDNA network. The essential functions of the three DNA Pols suggest nonredundant roles and raise the possibility of different asymmetric kDNA replication fork complexes for the minicircle and maxicircle templates. In *Escherichia coli*, the chromosomal replicative complex is composed of two DNA Pol III enzymes as a dimeric replicase (29). In low-GC-content gram-positive bacteria such as *Bacillus subtilis*, the core of the replisome is composed of two different Pols: Pol III and PolC, with individual roles in leading- and lagging-strand synthesis (8, 19). Additionally, the eukaryotic nuclear replisome is also an asymmetric dimeric replicase composed of Pol delta and Pol epsilon, with distinct leading- and lagging-strand roles, respectively (34, 38).

The replication-priming mechanisms appear to be different for the minicircle and maxicircle templates. UMSBP binds to the minicircle origin of replication and likely recruits a specific protein complex containing primase and Pol I-like proteins for replication. No origin binding protein has been identified yet for maxicircles. However, RNAi of mitochondrial RNA Pol resulted in a selective loss of maxicircles over minicircles, demonstrating a preferred role in maxicircle replication (17). Mitochondrial RNA Pol along with another subset of Pol I-like proteins could function in maxicircle replication. Further studies using chimeric gene silencing could clarify any cooperative roles for the trypanosome mitochondrial Pols at a replication fork. For example, if POLID and POLIB are part of the same asymmetric replicase, then dual silencing of these two Pols could produce a rapid loss of replication intermediates of the preferred kDNA template. A triple replisome, as recently described by McInerney et al., also remains an open possibility (30).

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