5-13-2011

Mitochondrial DNA Polymerase IB: Functional Characterization of a Putative Drug Target for African Sleeping Sickness

David F. Bruhn
University of Massachusetts Amherst, dbruhn@mcb.umass.edu

MITOCHONDRIAL DNA POLYMERASE IB: FUNCTIONAL CHARACTERIZATION OF A PUTATIVE DRUG TARGET FOR AFRICAN SLEEPING SICKNESS

A Dissertation Presented

by

DAVID F. BRUHN

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2011

Molecular and Cellular Biology

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Approved as to style and content by:

_____
Michele M. Klingbeil, Chair

_____
John M. Lopes, Member

_____
Craig T. Martin, Member

_____
Steven J. Sandler, Member

_____
Barbara A. Osborne, Director
Molecular and Cellular Biology

DEDICATION

The following dissertation is dedicated to my wonderful family, especially my father and mother (Steven and Debbie Bruhn), my brother (Ryan Bruhn), and my sister (Nichole Phillipo). Your unwavering love and support have been with me throughout the years of my education. Mom and Dad, you are truly the two persons in this world I admire the most. Your example of hard work, respect, dedication, and giving 110% to every endeavor have been formative in my education as an individual and scientist.

ACKNOWLEDGMENTS

Having recently realized I am now in “twenty-first grade” I have many persons to acknowledge as contributing to the events and accomplishments leading up to this doctoral thesis. I am grateful to Thomas Nerbonne, choral director at Wachusett Regional High School, for teaching me the lifelong lesson of always reading several frames ahead and to Dean Peter Eden (Endicott University) my professor and undergraduate research mentor at Marywood University, for igniting my interest in scientific research. I am thankful to my current research mentor, Dr. Michele Klingbeil, for her support throughout my doctoral studies and for refining my ability to think as an objective, independent scientist. I am additionally grateful to my committee members, Drs. John Lopes, Craig Martin, and Steven Sandler for their guidance and advice.

Throughout my doctoral studies I have been tremendously fortunate to conduct research that truly fascinates me and to have the pleasure of working with individuals whom are outstanding as both scientists and friends. I’d like to acknowledge Anthula Vandoros for training me when I arrived to the Klingbeil lab and for numerous insightful conversations. Life in lab would not have been the same without Jeniffer Concepción, whose ability to add humor and positive energy to very focused research always helped me to “just keep swimming”. In addition to my labmates, the advice of senior MCB graduate students Ankit Vahia, Russ Neuner, and Emine Suphon Bakkal has been critical to my success at the University of Massachusetts. I am truly thankful to you and all the members of the MCB Community. I am additionally grateful to Adaris Rodríguez-Cortés for her love and support. Gracias por todo mi amor. Te amo muchisimo!

The best advice I have ever received “it doesn’t matter what you do, do it to the best of your ability” came from my parents, to whom I cannot describe my admiration and gratitude in words. The hard work and sacrifice you made to put Nikki, Ryan, and I first will never be forgotten. You have truly enabled me to set and achieve many goals in life, including the completion of my doctoral studies. Thank you!

ABSTRACT

MITOCHONDRIAL DNA POLYMERASE IB: FUNCTIONAL CHARACTERIZATION OF A PUTATIVE DRUG TARGET FOR AFRICAN SLEEPING SICKNESS

MAY 2011

DAVID F. BRUHN, B.S., MARYWOOD UNIVERSITY

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Michele M. Klingbeil

Trypanosoma brucei and related parasites are causative agents of severe diseases that affect global health and economy. *T. brucei* is responsible for sleeping sickness in humans (African trypanosomiasis) and a wasting disease in livestock. More than 100 years after *T. brucei* was identified as the etiological agent for sleeping sickness, available treatments remain inadequate, complicated by toxicity, lengthy and expensive administration regiments, and drug-resistance. There is clear need for the development of a new antitrypanosomal drugs. Due to the unique evolutionary position of these early diverging eukaryotes, trypanosomes posses a number of biological properties unparalleled in other organisms, including humans, which could prove valuable for new drug targets. One of the most distinctive properties of trypanosomes is their mitochondrial DNA, called kinetoplast DNA (kDNA). kDNA is composed of over five thousand circular DNA molecules (minicircles and maxicircles) catenated into a topologically complex network. Replication of kDNA requires an elaborate topoisomerase-mediated release and reattachment mechanism for minicircle theta structure replication and at least five DNA polymerases. Three of these (POLIB, POLIC,

and POLID) are related to bacterial DNA polymerase I and are required for kDNA maintenance and growth. Each polymerase appears to make a specialized contribution to kDNA replication.

The research described in this dissertation is a significant contribution to the field of kDNA replication and the advancement of kDNA replication proteins as putative drug targets for sleeping sickness. Functional characterization of POLIB indicated that it participates in minicircle replication but is likely not the only polymerase contributing to this process. Gene silencing of POLIB partially blocked minicircle replication and led to the production of a previously unidentified free minicircle species, fraction U. Characterization of fraction U confirmed its identity as a population of dimeric minicircles with non-uniform linking numbers. Fraction U was not produced in response to silencing numerous other previously studied kDNA replication proteins but, as we demonstrated here, is also produced in response to POLID silencing. This common phenotype led us to hypothesize that POLIB and POLID both participate in minicircle replication. Simultaneously silencing both polymerases completely blocked minicircle replication, supporting a model of minicircle replication requiring both POLIB and POLID. Finally, we demonstrate that disease-causing trypanosomes require kDNA and the kDNA replication proteins POLIB, POLIC, and POLID. These data provide novel insights into the fascinating mechanism of kDNA replication and support the pursuit of kDNA replication proteins as novel drug targets for combating African trypanosomiasis.

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CHAPTER 1

UNIQUE BIOLOGICAL PROPERTIES PROVIDE POTENTIAL NEW DRUG TARGETS FOR THE TREATMENT OF AFRICAN SLEEPING SICKNESS

1.1 Kinetoplastid Parasites and Neglected Tropical Diseases

Neglected Tropical Diseases (NTDs) are a group of chronic infectious diseases affecting some of the world's poorest populations (Payne & Fitchett, 2010). An estimated 2.7 billion persons (approximately 14-28% of the global population) are affected by NTDs (Hotez *et al.*, 2009). Of these at risk individuals, more than 1 billion are already infected with at least one NTD (Payne & Fitchett, 2010). More so than direct medical expenses, the greatest cost of NTDs is their economic burden (Boelaert *et al.*, 2010). Time lost from work during infection and the inability to return to work following infection (due to blindness and disfigurement) compound desperate economic conditions in already impoverished communities (Zhang *et al.*, 2010). The dire economic situation of peoples affected by NTDs has led to very little financial incentive for pharmaceutical investment in novel therapeutics. As a result, treatments for these truly socio-economical diseases remain hugely inadequate.

NTDs are caused by a variety of organisms, ranging from viruses to eukaryotic parasites. Parasites belonging to the order Kinetoplastida are responsible for 3 of 17 NTDs recognized by the World Health Organization. This group of single-celled eukaryotes are responsible for numerous diseases, including leishmaniasis (*Leishmania ssp.*), Chagas Disease (*Trypanosoma cruzi*), and Human African trypanosomiasis (HAT) (*Trypanosoma brucei*). In addition to devastating human diseases, kinetoplastid parasites also cause economically important diseases in livestock. *T. brucei*, for example, causes

sleeping sickness (trypanosomiasis) in humans and nagana in domestic animals. Given the lack of adequate treatment for HAT, it may be surprising that the cause of the disease and identification of its insect vector have been known for over a century (Cox, 2004, Bruce, 1895).

1.2 Human African Trypanosomiasis

Human sleeping sicknesses are caused by two subspecies of *T. brucei* that vary in their geographic distribution and clinical manifestation. *T. brucei gambiense* is found in Central and Western Africa, where it causes chronic sleeping sickness and extended mortality. Symptoms of chronic sleeping sickness can last for years. In Southern and East Africa, *T. brucei rhodesiense* is responsible for high mortality. This subspecies causes a rapidly progressing form of the HAT that leads to death within weeks or months of infection. Left untreated, infection with either *T.b. gambiense* and *T.b. rhodesiense* is absolutely fatal (Baral, 2010). Despite differences in epidemiological properties, *T.b. gambiense* and *T.b. rhodesiense* are morphologically indistinguishable and share a common lifecycle that is absolutely dependent upon development in its insect vector, the tsetse fly (Fig. 1.1) (Wang, 1995). Tsetse flies are large, bloodsucking insects found in 37 countries in the African continent (Van den Bossche *et al.*, 2010, Ilemobade, 2009). When an infected fly takes a blood meal from a mammalian host, metacyclic stage parasites are injected into subcutaneous tissues including blood and lymph systems. Here, the parasites begin to divide and differentiate into a long, slender form know as trypomastigotes, or bloodstream form (BF). In this early stage of the disease, known as the haemolymphatic phase, BF parasites are restricted to the blood and lymph system where they cause fever, headache, pruritus (itching), and joint pain (Steverding, 2008). In

untreated patients, proliferating BF parasites cross the blood-brain barrier and enter the cerebrospinal fluid. After passing the blood-brain barrier, the parasites disrupt normal neurological and endocrine function, resulting in coma and, eventually, death (Wang, 1995, Steverding, 2008, Baral, 2010). Some of the BF parasites proliferating in the blood differentiate into a non-proliferative, stumpy form. When a tsetse fly draws a bloodmeal from an infected mammal and ingests stumpy form parasites, the parasites differentiate into the epimastigote form in the fly midgut. The parasite's complex lifecyle is completed when epimastigotes migrate into the fly's salivary gland, where they differentiate into the mammalian-infectious metacyclic stage (Baral, 2010).

1.3 A Limited Arsenal of Anti-Trypanosomal Drugs

Like many NTDs, treatment options for HAT are antiquated and ineffective. The choice of treatment depends upon disease progression. Early, haemolymphatic stage, infections are treated using the drugs suramine and pentamidine. Suramine was introduced by Bayer in 1916 and is still used today (Steverding, 2008). Given the long history of suramin use, it is not surprising that suramin-resistant parasites have been reported both in the clinic and in a laboratory setting. Suramin treatments are extensive and last up to 30 days, complicating patient compliance (Brun *et al.*, 2010). Sleeping sickness patients in central and western Africa often suffer from co-infection with roundworm species (*Onchocerca ssp.*). The high activity of suramin against *Onchocerca ssp.* is known to lead to dangerous allergic reactions, precluding the use of the drug in areas affected by *T.b. gambiense* (Brun *et al.*, 2010). Early stage cases of *T.b. gambiense*, which represent 90% of total cases, are treated using pentamidine (Aksoy, 2011). Pentamidine is the treatment of choice for early stage *T.b. gambiense* infections, but the

course of administration is cumbersome and requires 7 consecutive days of intramuscular injections. Long-term culture of BF parasites in the presence of subcurative levels of pentamidine successfully selects for pentamidine-resistant parasites, raising concerns that drug resistant parasites could also be selected during patient treatment (Bernhard *et al.*, 2007). The same study showed that resistance to melarsoprol, introduced in 1949 to treat late-stage infections, could also be acquired *in vitro* (Bernhard *et al.*, 2007). Recent efforts to replace melarsoprol as the first-line drug to late-stage infections have been driven by concerns of reported clinical drug resistance and the highly toxic nature of this organoarsenic compound (Brun *et al.*, 2010, Delespaux & de Koning, 2007). Following administration of melarsoprol 5-10% of patient are afflicted with severe encephalopathy, 50% of which die from the treatment (Barrett *et al.*, 2007). A safer alternative to melarsoprol became available in 1990 with the introduction of eflornithine. Originally marketed as a topical cream used to remove undesired facial hair (Vaniqa®), eflornithine was found effective treatment for trypanosomiasis in the late 1980s (Pepin *et al.*, 1987). Largely the result of concerted WHO efforts, in the form of training programs and coordination of donations, the percentage of late-stage *T.b. gambiense* patients treated with eflornithine increased from 17% in 2003 to 62% in 2009 with a corresponding decline in the use of melarsoprol (Simarro PP, 2011). Although a safer alternative to melarsoprol, eflornithine treatment is cumbersome and requires intravenous administration every 6 hours for 14 days (Priotto *et al.*, 2009, Kennedy, 2008). Despite severely limited treatment options the number of cases of sleeping sickness continues to decline from the more recent epidemic, which peaked in the late 1990s with an estimated 300,000-500,000 annual infections (WHO, 1998, Kennedy, 2008). The recent decline in

the number of cases of sleeping sickness reflects the successful implementation of disease education, surveillance, combination therapies, control efforts, and drug administration programs (Aksoy, 2011, Simarro PP, 2011). These advances, however, by no means indicate a diminished need for the development of novel anti-trypanosomal drugs (Aksoy, 2011). Current treatments for both early and late-stage sleeping sickness are time and resource intensive, dependent upon patient compliance and continued drug donations from pharmaceutical manufacturers (such as Sanofi-Aventis and Bayer) (Aksoy, 2011). Given the severely limited number of treatments available for treating sleeping sickness and the constant threat of acquired drug-resistance, there is a clear and critical need for the development of the next generation of drugs for combating this medically and economically devastating disease.

1.4 Kinetoplast DNA: Structure and Function

Kinetoplastid parasites possess a number of unusual biological features without counterpart in nature that could be exploited for the development of novel drugs. One of the most notable features, after which the group is named, is its mitochondrial DNA network. Known as kinetoplast DNA (kDNA), this network is composed of thousands of circular DNA molecules catenated into a structure with topology likened to that of medieval chain mail (Morris *et al.*, 2001). Although isolated kDNA networks are of similar size to the cell from which they were extracted, *in vivo* the network is compacted into a disk-shaped structure close to the flagellar basal body. kDNA is found in *T. brucei*, *T. cruzi*, and *Leishmania ssp,* as well as non-infectious kinetoplastids. The relatively

recent availability of RNA interference (RNAi) and ease of cell culture has made *T. brucei* the model organism of choice for many kinetoplastid research groups.

The two DNA components of kDNA networks are known as maxicircles and minicircles. In each *T. brucei* network there are approximately 40-50 maxicircles, each ~23 kb in length (Morris *et al.*, 2001). Maxicircles are identical in sequence and are similar to other eukaryotic mitochondrial DNA in that they encode numerous proteins involved in respiration. This includes seven subunits of Complex I (NADH:Ubiquinone oxireductase 1,3-5, 7-9), one subunit of Complex III (apocytochrome b), three subunits of Complex IV (cytochrome oxidase I-III), and as well as a subunit of Complex V (Subunit A6). Maxicircles also encode proteins involved in mitochondrial translation, including ribosomal protein RPS12 and ribosomal RNAs 9S and 12S. In addition to these more typical mitochondrial genes, maxicircles encode five open reading frames of unknown function. Three of these are known as MURFs (maxicircle unidentified reading frames; MURF1, MURF2, MURF5) while the other two are GC-rich sequences called CR3 and CR4. Experiments elucidating the functions of these reading frames will increase comprehension of maxicircles products and, so, mitochondrial function in trypanosomes.

Predicting the function of maxicircle-encoded proteins is made both complicated and intriguing by the propensity of maxicircle transcripts to require post-transcription editing. This process, known as RNA editing, involves the insertion and deletion of uridines to create a final open reading frame that can be translated at the ribosome. RNA editing is restricted to maxicircle transcripts. Not all nascent transcripts are edited equally. Only portions of apocytochrome b and cytochrome oxidase subunit II are edited, whereas the entire length of other transcripts is edited (Fisk *et al.*, 2008). The later

scenario is called pan-editing and has long been hypothesized a means for parasites to increase mitochondrial protein diversity. Indeed a recent study revealed that alternative editing of cytochrome c oxidase III (COXIII) produces a protein (alternatively edited protein 1, or AEP1) required for segregation of replicated kDNA networks (Ochsenreiter *et al.*, 2008).

The insertion and deletion of uridines during RNA editing is by no means a random process. RNA editing is, rather, a highly coordinated process directed by trans-acting RNAs (guide RNAs) encoded by the minicircle component of the kDNA network. *T. brucei* kDNA networks include about 5000 minicircles, each ~1kb in length. Given that there are more than 250 sequence classes of minicircles in *T. brucei* and that each minicircle encodes for 3 guide RNA molecules, it seems the field has only scratched the surface in regards to the potentially vast pool of proteins produced by alternative editing of maxicircle transcripts. Nonetheless, the clear dependency of nascent maxicircle transcripts upon minicircle-encoded guide RNAs indicates that both species are required for proper mitochondrial physiology and, hence, parasite viability.

1.5 Replication of Kinetoplast DNA Networks

In addition to its unique topology, kDNA is distinct from other mitochondrial genomes in that it is only replicated once per cell cycle, in close coordination with nuclear S-phase. *In vivo*, kDNA networks are compacted into a disk-shaped structure by histone-like proteins and each of the network's 5000 minicircles is catenated to three other minicircleds. To overcome the challenges presented by complex topology and dense packaging of kDNA networks, trypanosomes use a network-free mechanism of DNA replication in which minicircles are releases, replicated, and then re-attached to the

network. kDNA replication initiates just prior to nuclear S-phase and begins with the vectoral release of minicircles from the network by a yet unidentified topoisomerase. Minicircles are released in a specialized region of the cell know as the kinetoflagellar zone (KFZ), which is located between the kDNA disk and the flagellar basal body. During release, minicircles encounter Universal Minicircle Sequence Binding Protein (UMSBP), a replication initiation protein that binds to a region conserved in all minicircles, and an RNA primase (PRI2), which synthesizes RNA primers. Primed minicircles are replicated unidirectionally as theta structures in the KFZ, where minicircle replication intermediates have been detected and two DNA polymerase (POLIB and POLIC) have been localized. Replication of each minicircle produces two daughter minicircles. Daughter minicircles migrate to antipodal sites, two protein-rich sites located 180 degrees apart on opposite sides of the kDNA disk. Here, Structure Specific Endonuclease I (SSEI) removes RNA primers and Okazaki fragments are processed. During Okazaki fragment processing, DNA Polymerase β and DNA ligase kβ fill all but a single gap in the backbone of progeny minicircles, which are then re-attached to the network periphery. The single gap remaining on replicated minicircles is proposed to serve as a book keeping mechanism (preventing re-replication) and is filled by DNA polymerase β-PAK and DNA ligase kα only once all network minicircles been replicated. Double-sized kDNA networks are physically attached to the cell's two flagellar basal bodies via a transmembrane filament system known as the tripartite attachment complex (TAC). As the cell proceeds through cytokinesis, movement of flagellar basal bodies separates the double-sized kDNA network into individual networks and pulls these into the two daughter cells. Maxicircle replication is far less understood, but it is known that

maxicircles replicate attached to the kDNA network and that an RNA primase (PRI1) participates in the initiation of maxicircle replication.

1.6 Polymerase I – Like Enzymes

Early biochemical and genetics attempts to identify a polymerase γ-like protein from trypanosomes were unsuccessful, despite the participation of polymerase γ as the sole enzyme replicative enzyme in mitochondrial DNA replication in other known eukaryotes (Klingbeil *et al.*, 2002). Surprisingly, classical (activity-based) purification from *T. brucei* mitochondrial extracts revealed two peaks corresponding to Pol β activity as well as additional, distinct peaks of polymerase activity in other fractions (Fuenmayor *et al.*, 1998). While the two classically purified Pol β enzymes (Pol β and Pol β-PAK) were proposed to contribute to Okazaki fragment processing, insight into the identities of the additional mitochondrial DNA polymerases came later, from analysis of the partially sequenced *T. brucei* genome. Sequence analysis confirmed the absence of a discernable polymerase γ homologue, and verified the presence of two distinct Pol β enzymes. Unexpectedly, however, this analysis revealed the presence of a four additional DNA polymerases predicted to localize to the parasite's single mitochondrion. Reflecting their 25-35% sequence identity to bacterial polymerase I, these DNA polymerases were named POLIA, POLIB, POLIC, and POLID and confirmed, by fluorescence microscopy, to localize to the mitochondrion (Klingbeil *et al.*, 2002). POLIA and POLID were reported to localize throughout the mitochondrial matrix while POLIB and POLIC were found to localize proximal to the KFZ, where minicircles are replicated. Sequence analysis revealed that all four Pol I-like proteins contained amino acids critical for polymerase activity, including two aspartic acid residues required for coordination of Mg^{2+},

stabilizing transition states during nucleotide incorporation (Klingbeil *et al.*, 2002). It was therefore possible that one, or all, of these Pol-I like polymerase participates in kDNA replication.

RNAi was used to individually silence each Pol I-like protein in order to dissect out their unique (and potentially overlapping) functions. Initial studies (conducted in procyclic parasites) indicated that POLIB, POLIC, and POLID are required for *T. brucei* cell growth. Silencing each essential polymerase resulted in a progressive loss of kDNA networks, indicating that each makes a non-redundant contribution to kDNA maintenance (Klingbeil *et al.*, 2002). Detailed analyses of the function of POLIC and POLID revealed a perturbation of the abundance of free minicircle species during RNAi, but did not indicate that minicircle replication is blocked in the absence of either enzyme. (Chandler *et al.*, 2008, Klingbeil *et al.*, 2002). These studies suggested that an additional Pol was functioning at the minicircle replication fork. POLIB, localizes to KFZ and possesses sequence motifs and residues predicted critical for both DNA Polymerase activity and proofreading exonuclease (Klingbeil *et al.*, 2002). It is, therefore, plausible that POLIB performs a critical role at the core of minicircle replication.

1.7 Purpose of Study

The following dissertation research was conducted with the goal of deepening the understanding of the molecular events underlying replication of kDNA, the most complex mitochondrial genome known. The proteins and molecular mechanism involved in kDNA replication are unparalleled in higher eukaryotes (including humans), suggesting that kDNA replication enzymes could be valuable targets for the development of novel anti-

parasitic drugs. Given this intended application and long-standing concerns that kDNA could be dispensable in the medically relevant lifecycle stage of *T. brucei*, a second major aim of this research was to determine if disruption of kDNA replication is lethal to the form of the parasite that proliferates in the blood of an infected host and causes disease.

1.8 Research Questions

The following dissertation research addresses three major questions:

1. What contributions does Mitochondrial DNA Polymerase IB make to minicircle replication?
2. Do two or more mitochondrial DNA polymerases participate in minicircle replication?
3. Are Mitochondrial DNA Polymerases IB, IC, and ID required for survival of bloodstream form Trypanosoma brucei?

1.9 Significance of Findings

In the absence of product markets attractive to the pharmaceutical industry, the pursuit of badly needed drugs to combat Neglected Tropical Diseases has largely fallen to the academic research community. Ninety-percent of candidate drugs that enter clinical trials fail and this failure is frequently attributed to a lack of understanding the compound's biological target and mode of action. Rational, target-based approaches to drug discovery, therefore, offer a means to improve the odds of a candidate drug successfully gaining approval for clinical use. To this end, the research presented in following dissertation is directed at expanding comprehension of the molecular

mechanism of kDNA replication. The current study is a significant addition to the scientific community in that it both deepens knowledge of the molecular biology of kDNA replication and encompasses *in vitro* validation of kDNA replication proteins as drug targets in disease-causing trypanosomes.

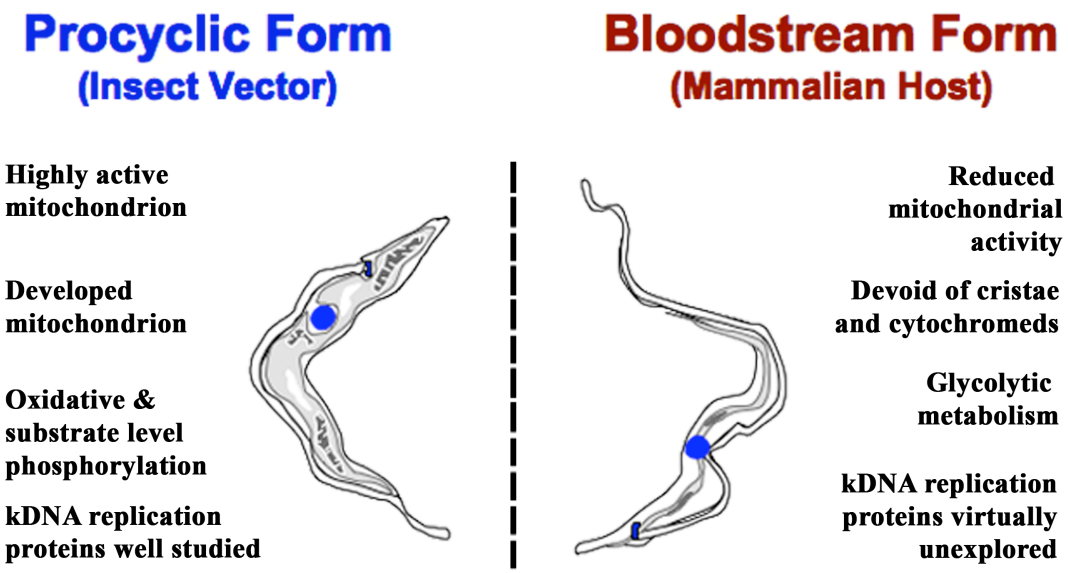

Figure 1.1 Representation of *Trypanosoma brucei* metabolism and morphology in two distinct life cycle stages
Diagram illustrating morphology of Procyclic and Bloodstream form *T. brucei*. Stage specific differences are described and referenced in the Introduction.

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CHAPTER 2

MITOCHONDRIAL DNA POLYMERASE POLIB IS ESSENTIAL FOR MINICIRCLE REPLICATION IN AFRICAN TRYPANOSOMES

2.1 Abstract

The unique mitochondrial DNA of trypanosomes is a catenated network of minicircles and maxicircles called kinetoplast DNA (kDNA). The network is essential for survival, and requires an elaborate topoisomerase-mediated release and reattachment mechanism for minicircle theta structure replication. At least seven DNA polymerase (pols) are involved in kDNA transactions, including three essential proteins related to bacterial DNA pol I (POLIB, POLIC and POLID). How *Trypanosoma brucei* utilizes multiple DNA pols to complete the topologically complex task of kDNA replication is unknown. To fill this gap in knowledge we investigated the cellular role of POLIB using RNA interference (RNAi). POLIB silencing resulted in growth inhibition and progressive loss of kDNA networks. Additionally, unreplicated covalently closed precursors become the most abundant minicircle replication intermediate as minicircle copy number declines. Leading and lagging strand minicircle progeny similarly declined during POLIB silencing, indicating POLIB had no apparent strand preference. Interestingly, POLIB RNAi led to the accumulation of a novel population of free minicircles that is composed mainly of covalently closed minicircle dimers. Based on these data, we propose that POLIB performs an essential role at the core of the minicircle replication machinery.

2.2 Introduction

The African trypanosome *Trypanosoma brucei* is the protist parasite responsible for the neglected tropical disease known as sleeping sickness and a related disease in livestock called nagana. With no vaccine for this fatal disease, current drug treatments that are toxic, and emerging drug resistant parasites, there is a concerted effort to identify and validate new molecular targets for development of the next generation of therapeutic interventions. *T. brucei* and closely related trypanosomatid parasites (*T. cruzi* and *Leishmania* ssp.) are interesting experimental organisms because of their impact on global human health and as early diverging eukaryotes. Their long and independent evolutionary history has resulted in a number of unusual biological properties that can be exploited as drug targets. One property without counterpart in nature is the kinetoplast DNA (kDNA) network that resides in the cell's single mitochondrion.

The structure and replication of kDNA have been reviewed extensively (Shlomai, 2004, Liu *et al.*, 2005). The trypanosomatid kDNA is a planar structure composed of relaxed circular DNA molecules (5000 minicircles and 25 maxicircles) that are topologically interlocked into a single network, and is considered the most complex among mitochondrial genomes. Several histone-like proteins help condense the network into a disk-shaped structure within a specialized region of the mitochondrial matrix, which is linked to the flagellar basal body through the transmembrane tripartite attachment complex (TAC) (Xu *et al.*, 1996, Lukes *et al.*, 2001, Ogbadoyi *et al.*, 2003). Maxicircles (23 kb) resemble other mitochondrial DNAs in that they encode rRNA and a few subunits of respiratory complexes (Lukes *et al.*, 2005). However, most maxicircle transcripts require editing, a process that involves minicircle encoded gRNAs that direct

the proper insertion or deletion of uridine residues to create open reading frames (Stuart *et al.*, 2005). Parasite survival requires the kDNA network (Sela *et al.*, 2008), therefore understanding the replication and repair mechanisms of this unique DNA structure is an important aspect of trypanosome biology.

For this single celled eukaryote, network replication requires the coordinated replication of each maxicircle and minicircle in close synchrony with nuclear S phase, and segregation of progeny networks into daughter cells via the basal body associated TAC (Woodward & Gull, 1990, Robinson & Gull, 1991). The topological complexity of replicating a catenated DNA network dictates some unusual features such as the elaborate topoisomerase II-mediated release-and-reattachment mechanism for minicircle replication, and at least seven mitochondrial DNA polymerases (pols) for kDNA transactions (Klingbeil *et al.*, 2002, Saxowsky *et al.*, 2003, Rajao *et al.*, 2009). In brief, individual covalently closed minicircles are released from the network into a region between the kDNA disk and the mitochondrial membrane near the flagellar basal body called the kinetoflagellar zone (KFZ) (Drew & Englund, 2001). The free minicircles initiate unidirectional theta replication at the conserved universal minicircle sequence (UMS) through interactions with several proteins that localize to the KFZ including UMS binding protein, DNA primase, and two family A DNA pols, POLIB and POLIC (Abu-Elneel *et al.*, 2001, Li & Englund, 1997, Klingbeil *et al.*, 2002). Replication of circular DNA molecules imposes additional topological complexities including supercoiling and formation of pre-catenanes that must be resolved for proper replisome progression and successful segregation of progeny molecules. The recent report on the essential role of mitochondrial topoisomerase IA for minicircle theta structure resolution highlights the

roles of kDNA modifying enzymes in network replication (Scocca & Shapiro, 2008). Okazaki fragment processing of progeny minicircles is mediated by structure specific endonuclease 1, pol β, and DNA ligase kβ at two antipodal sites flanking the kDNA disk (Hines *et al.*, 2001, Engel & Ray, 1999, Ferguson *et al.*, 1992, Downey *et al.*, 2005). Minicircles that still contain at least one gap are subsequently reattached to the network periphery by topoisomerase II localized at the antipodal sites (Melendy *et al.*, 1988). When all minicircles have replicated and reattached, the discontinuities are presumably closed by pol β-PAK and DNA ligase kα prior to segregation of progeny networks (Saxowsky *et al.*, 2003, Sinha *et al.*, 2006).

Although recent proteomic and bioinformatics approaches have led to the identification of several new minicircle replication proteins such as p38 and p93 (Li & Englund, 1997, Liu *et al.*, 2006, Li *et al.*, 2007), characterization of the processive minicircle replisome has remained elusive. Our lab studies four family A DNA pols that are related to bacterial pol I and localize to the mitochondrion. Three (POLIB, POLIC, and POLID) are essential for parasite growth, suggesting each performs a specialized function (Chandler *et al.*, 2008, Klingbeil *et al.*, 2002). The fourth, POLIA, is non-essential under normal growth conditions and may contribute to kDNA repair. The use of multiple DNA pols in mitochondrial DNA replication has never been documented in other eukaryotes; instead a single DNA polymerase, pol gamma, is used for replication and repair. Previous studies using RNA interference (RNAi) indicated that POLIC and POLID are required for kDNA maintenance, but suggested that an additional pol was the primary minicircle replicase. The third pol I-like protein, POLIB, localizes to the KFZ where free minicircle replication intermediates are detected and contains amino acid

residues critical for proofreading exonuclease and DNA polymerase activities suggesting it is an ideal candidate for the minicircle replicative DNA polymerase.

In the present study we report a detailed analysis of the *in vivo* role of POLIB and its contributions to the complex process of minicircle replication using a RNAi-based approach. POLIB silencing resulted in loss of the kDNA network and greatly disrupted the pattern of minicircle replication intermediates. Depletion of POLIB also led to the accumulation of both unreplicated minicircle monomers and fraction U, a new free minicircle species. Despite the presence of six other mitochondrial DNA pols, none were able to effectively compensate for the loss of POLIB. These data suggest that POLIB serves a critical role at the core of the minicircle replication machinery.

2.3 Material and Methods

2.3.1 Trypanosome growth

T. brucei procyclic cell line 29-13 expressing T7 RNA polymerase and tetracycline repressor was maintained at 27°C in SDM-79 medium (Wirtz *et al.*, 1999, Brun & Schonenberger, 1979) supplemented with heat-inactivated fetal bovine serum (15%), G418 (15 μg/mL), and hygromycin (50 μg/mL).

2.3.2 RNA interference

The pSLIB vector produces an intramolecular stemloop double-stranded RNA to target the mitochondrial DNA polymerase POLIB (Tb11.02.2300) and was constructed as previously described (Wang *et al.*, 2000). Briefly, 500 bp of *TbPOLIB* coding sequence (nucleotides 275-774) was PCR amplified from *T. brucei* 927 genomic DNA using primers with appropriate restriction enzyme linkers (forward: 5'-

AAGATGAGCGTGTCAACGAGG-3' and reverse: 5'-GGTAAACCGTGGCGCGACGGAGG-3') to generate the two fragments for subsequent cloning steps. This was the same region used for the pZJMIB vector previously reported (Klingbeil *et al.*, 2002). The resulting construct, pSLIB, contains two copies of the *TbPOLIB* fragment as inverted repeats separated by an unrelated stuffer fragment. EcoRV-linearized pSLIB (12 μg) was transfected into *T. brucei* 29-13 cells by electroporation, and the generation of stable cell lines by selection (2.5 μg/ml phleomycin) and limiting dilution were described in detail as previously reported (Chandler *et al.*, 2008). RNAi of *TbPOLIB* in clonal cell lines was induced by adding tetracycline (1.0 μg/ml). Cell growth was monitored using a Z2 model Coulter Counter (Beckman Coulter), and cultures were maintained between 2.0×10^5 - 1.0×10^7 cells/ml. To avoid variation in sample preparations, staggered RNAi inductions were performed to process all time points on the same day.

2.3.3 RNA isolation and Northern Analysis

Total RNA was isolated from 5×10^7 cells (mid-log phase) using the Purescript RNA isolation kit (Gentra Systems), fractionated on a 1.5% agarose/ 7% formaldehyde gel, and transfered to GeneScreen Plus membrane (NEN). Specific mRNAs were detected with ^{32}P-random primed labeled probes, and signals quantified as previously described (Chandler *et al.*, 2008).

2.3.4 Microscopy Analysis and Quantitation

Uninduced and POLIB RNAi induced cells were processed from staggered inductions. Conditions for fixation, permeabilization and staining cells as well as the microscope equipment and camera software were as previously described (Chandler *et al.*, 2008). For quantitative analysis of kDNA network morphology, more than 300 cells per timepoint were scored by eye for size of the kDNA and other changes to kDNA morphology based on 4'-6'-diamidino-2-phenylindole (DAPI) fluorescence. For cells classified with small kDNA (sK), the surface area of the network was at least 50% less than that found in uninduced cells. For those classified with no kDNA (nK), there was no detectable extranuclear DAPI staining even when focusing up and down through several focal planes.

2.3.5 DNA Isolation and Southern Blot Analysis

Total DNA was isolated from 1 x 10^8 cells using Purescript Genomic DNA isolation kit (Gentra Systems). For Southern blot analysis of minicircle and maxicircle content, total DNA was digested with HindIII and XbaI for 24 hr, fractionated on a 1% agarose gel containing 1.0 μg/mL ethidium bromide (EtBr). For analysis of free minicircle replication intermediates, total DNA was fractionated on a 1.5 % agarose gel for 16 hours at 2.4 V/cm including 1.0 μg/mL EtBr in the gel and Tris-Borate-EDTA (TBE) running buffer (buffer was recirculated). Fractionated DNA was processed using standard depurination, denaturation, and neutralization treatments, transferred to GeneScreen Plus membrane using capillary transfer and then UV cross-linked. Membranes were probed with maxicircle, minicircle, and α-tubulin specific random primed radiolabeled probes as previously described (Chandler *et al.*, 2008). Quantitation

was performed using a Typhoon 9210 Molecular dynamics Phosphoimager (GE Healthcare) with background intensity subtracted, and normalized against the tubulin signal using ImageQuant 5.2 software.

2.3.6 Neutral/Alkaline Two-Dimensional Analysis

Total genomic DNA isolated from cells after 0, 4, and 6 days of POLIB RNAi was fractionated using two-dimensional electrophoresis as previously described (Liu *et al.*, 2006, Ryan & Englund, 1989). Briefly, total DNA (3×10^7 cells) was fractionated in the first dimension as described above. After 18 hr (2.5 V/cm), the gel was washed 2 times in 50 mM NaOH, 1 mM EDTA, and then equilibrated with 3 washes in running buffer, (30 mM NaOH, 2 mM EDTA). DNA was then separated for 20 hr (0.8 V/cm) in recirculating running buffer, processed, transferred and cross-linked to GeneScreen Plus membrane. Hybridization with T4 polynucleotide kinase 5'-end labeled oligonucleotide probes 5'-AAAATAGCACGGGATTTGTGTATGGTGAAATCTGCACGCCC-3' or 5'-GGGCGTGCAGATTTCACCATACACAAATCCCGTGCTATT TT-3' detected heavy (lagging) strand and light (leading) strand minicircle intermediates, respectively.

2.3.7 Free minicircle isolation

Cells (8×10^9) were pelleted and washed in NET-100 (100 mM NaCl, 100 mM EDTA, 10 mM Tris-HCl, pH 8.0). Cells were lysed for 1 hour at 56°C in NET-100 containing 0.5% SDS and 200 μg/mL proteinase K and then treated with RNase A (500 μg/mL, 30 min at 37˚C). The lysates (3.5 ml/gradient) were loaded to the top of 34 mL linear 5%-20% sucrose gradients (TE, 1M NaCl) and centrifuged for 20 hours (4ºC at

25,000 x g) using a Beckman SW28 rotor. Fractions (1 mL) were collected from the top of gradient. To detect free minicircle species, 10 μl of each fraction was analyzed on a 1.5% agarose gel followed by Southern blotting using a minicircle probe. Fractions containing non-monomeric minicircle species (19-25) were pooled, concentrated using phenol:chloroform extraction (pH 8.0), ethanol precipitated and resuspended in TE. This concentrated fraction U was then treated with proteinase K (500 μg/mL) and SDS (0.5%) for 2 hours at 56°C. Proteinase K was brought to 1 mg/mL and the reaction incubated overnight at 56°C. Deproteinated fraction U was then treated with RNase A (500 μg/mL, 15 min at 37°C) followed by phenol:chloroform extraction, ethanol precipitation and hydration in 50 μl TE.

2.3.8 Minicircle Treatments

Minicircles (1.6x 10^7 cell equivalent) were treated with 5 units of Topoisomerase I (NEB, 30 minutes at 37ºC), 10 units of topoisomerase II human alpha (USB, 15 minutes at 30ºC), or 5 units of T7 endonuclease (NEB, 37ºC) according to manufacturers instructions. All reactions were stopped by the addition of EDTA to 0.9 μM and incubation with proteinase K (Invitrogen, 0.5 mg/mL) at 56ºC for 20 minutes.

2.3.9 **Allelic Tagging of POLIC**

The C-terminal 2226 bp of *TbPOLIC* coding sequence was PCR amplified from *T. brucei* 927 genomic DNA using primers with appropriate restriction enzyme linkers underlined (forward: 5'-GGG CCC GTT CGC TCT ACG CAG GAT ATC AGC-3' and reverse: 5'-CGG CCG CCT GGA CAA CTC CCC TAG TGA TG-3'). The resulting fragment was restriction enzyme digested with ApaI and EagI and ligated into the pC-

PTP-NEO, which had been restriction digested using ApaI and NotI. The neomycin resistance cassette was liberated from pPOLIC-PTP-NEO by digestion with NdeI and SpeI and replaced with the puromycin cassette, liberated from pN-PURO-PTP using these same restriction enzymes. The resulting construct, pPOLIC-PTP-PURO was linearized with AflII and transfected in the clonal cell line SLIB2C7. Stable transfectants were selected using 1 μg/mL puromycin.

2.3.10 **Microscopy Analysis and Quantitation**

Uninduced and induced cells for the indicated days of POLIB RNAi were harvested by centrifugation, washed in PBS, spotted onto poly-L-lysine coated slides, and fixed using 4% paraformaldehyde. After overnight permeabilization in methanol, cells were rehydrated with PBS. Staining of basal bodies was carried out using rat monoclonal YL1/2 primary antibody (1:400, 60 min) and secondary polyclonal goat anti-rat Alexa 488 secondary antibody (1:250, 60 min). YL1/2 recognizes the tyrosinated tubulin. Staining of POLIC-PTP was performed using rabbit monoclonal Anti-Protein A (1:5000, 90 min) primary antibody and goat anti-rabbit Alexa 594 secondary antibody (1:250, 90 min). Fixed cells were stained with 3 μg/mL DAPI and mounted in Vectashield. POLIC-HA localization was also examined and was comparable to POLIC-PTP localization (data not shown). Experiments presented are all based on POLIC-PTP. The pC-PTP-NEO and pN-PURO-PTP vectors were generously provided by Arthur Günzl (University of Connecticut Health Center).

2.3.11 Metabolic Labeling and Epitope Detection

SLIB2C7 cells in mid-log phase were cultured for 3 hours in presence of 50 μM Bromodeoxyuridine (BrdU) and 50 μM deoxycytidine under otherwise normal growth conditions. Cells were fixed and permeabilized as described above, then treated with 1.5M HCl (30 min) to expose the BrdU epitope. Slides were incubated with anti-BrdU AlexaFluor 594 conjugate (Molecular Probes, 1:50), washed in 1X PBS with 0.1M glycine, and then incubated with AlexaFluor 594 goat anti-mouse IgG (Invitrogen, 1:50).

2.3.12 Western Blot Analysis

Parasites were pelleted, washed in cytomix containing Protease Inhibitor Cocktail Set II (CalBioChem), and lysed by heating in sodium dodecyl sulfate (SDS) loading dye. Samples were separate using SDS-PAGE and transferred to PVDF membrane. Chemiluminescence detection of POLIC-PTP was performed using Peroxidase-Anti-Peroxidase soluble complex (PAP) reagent (Sigma) at a dilution of 1:2000. Detected membrane was incubated for 15 minutes at 37°C in 100 mM Glycine (pH 2.5) to remove PAP. Membrane was then incubated with primary antibody MCP72 (1:10000) followed by secondary chicken anti-rabbit IgG-HRP (Santa Cruz Biotechnology, 1:10000) to detect Hsp70, a loading control. Rabbit polyclonal antibody MCP72 was generously provided by Paul Englund (Johns Hopkins School of Medicine).

2.3.13 Live Cell Imaging with MitoTracker

Parasites were incubated in media containing 200 nM MitoTracker Red CM-H_2XRos (Molecular Probes) for 30 minutes and 50 μg/mL DAPI for 20 minutes covered

from light. Following incubation parasites were washed and then resuspended in cytomix, spotted on microscope slides, and immediately viewed.

2.4 Results

2.4.1 Stem-loop silencing of *TbPOLIB*

Previous *TbPOLIB* silencing experiments using the pZJM vector, that produces an intermolecular dsRNA trigger, led to a reduced growth rate in procyclic cells and a small increase in the percentage of cells with abnormal sized kDNA networks. While depletion of POLIB indicated an essential role for growth and suggested a role in kDNA maintenance, the resulting phenotype could not be fully characterized due to poor penetrance (Klingbeil *et al.*, 2002). Therefore, the aim of this study was to more fully assess the cellular function of TbPOLIB, particularly its role in minicircle replication.

A 500 bp fragment corresponding to 275-774 bp of the *TbPOLIB* coding sequence used to generate the previous pZJM construct was also used to generate the POLIB stem-loop construct (Fig. 2.1A). The final construct (pSLIB) was transfected into 29-13 cells. Ten clonal SLIB cell lines were analyzed and all produced a similar pattern of growth inhibition with average doubling times of 13 hr for the uninduced cells (data not shown). Detailed phenotypic analyses using clonal line SLIB2C7 are presented. Tetracycline induction of the intramolecular stem-loop dsRNA resulted in growth inhibition starting 4 to 5 days post-induction. This growth inhibition lasted for at least 10 days, while the growth rate of the uninduced population remained constant (Fig. 2.1B). The growth arrest for induced SLIB cells lagged behind the depletion of the target mRNA and was similar

to RNAi phenotypes of other replication proteins that result in kDNA loss (Downey *et al.*, 2005, Li *et al.*, 2007, Liu *et al.*, 2006, Scocca & Shapiro, 2008, Wang & Englund, 2001). Northern blot analyses revealed a 90% reduction of *TbPOLIB* mRNA 48 hr after tetracycline induction of dsRNA synthesis with no significant changes in the mRNA levels for the two other essential mitochondrial pols (Tb*POLIC* and Tb*POLID*) (Fig. 2.1B, inset). Further indication that the observed RNAi phenotype is specifically due to downregulation of *TbPOLIB*, was obtained by chromosomally tagging *TbPOLIC* in the SLIB2C7 clonal cell line (Fig. 2.2). Kinetics of growth inhibition of this tagged cell line was comparable with parental SLIB2C7 (Fig. 2.2A). Silencing of POLIB for up to 4 days resulted in no significant effect on abundance of POLIC-PTP as determined by Western Blot (Fig. 2.2B). Additionally, quantitation of more than 300 cells per timepoint revealed no change in POLIC-PTP distribution during POLIB RNAi (Fig. 2.2, C-H). These data are consistent with the previously determined essential role in growth, and demonstrate that SLIB2C7 is an ideal cell line for a detailed study of the *in vivo* role of POLIB.

2.4.2 Progressive loss of kDNA networks during *TbPOLIB* RNAi

The previous *POLIB* pZJM RNAi data resulted in a subtle shrinking kDNA defect and suggested a likely role in kDNA maintenance (Klingbeil *et al.*, 2002). To further assess the effects on kDNA networks, uninduced and induced SLIB RNAi cells were DAPI-stained and examined by fluorescence microscopy to manually score the size of the kDNA network (>300 cells per data point). Uninduced cells exhibited normal duplication and segregation of the nuclear (N) and kDNA (K) genomes with each cell cycle karyotype easily observed [1N1K, 1N1K*(replicating kDNA), 1N*2K (replicating nucleus) and 2N2K] (Fig. 2.3A, Day 0). In contrast, POLIB depleted cells exhibited

progressive shrinking and loss of the kDNA network (Fig. 2.3 A,B). Additionally, figure 3B shows the kinetics of kDNA loss with Day 6 post-induction displaying the greatest transition. At this timepoint, only 30% of the cell population still contained normal-sized kDNA, while the remaining population contained small kDNA (mean 34.7%) or no kDNA (mean 33.5%). While there was variation in the percentage of cells with small kDNA and no kDNA at Day 6 in the two separate RNAi inductions, the rapid decline of cells with normal-sized kDNA was consistent. Cells with no detectable kDNA continued to accumulate and constituted 54% of the population following 10 days of *POLIB* RNAi. Although multiple focal planes were analyzed for residual kDNA, the possibility of a network too small to be detected cannot be excluded. Those cells with abnormal sized kDNA still appeared to initiate the cell cycle properly. Detection with YL1/2 antibody, which is specific for tyronsinated α-tubulin, shows uninterrupted basal body duplication and segregation following staining (Fig. 2.4A). Cells with abnormally small kDNA also contained nuclei that still incorporated the thymidine analog, BrdU, while there was no detectable BrdU observed in the residual kDNA networks (Fig. 2.4B). Therefore, loss of kDNA is likely the primary defect following POLIB silencing, and not a secondary effect due to cell cycle disruption.

2.4.3 *TbPOLIB* RNAi affects minicircle replication

The kDNA network is a unique mitochondrial genome requiring duplication of two different DNA templates, minicircles and maxicircles. Silencing of some kDNA replicative proteins such as TopoII, POLIC, or p38 resulted in parallel loss of the two templates, while RNAi of mitochondrial RNA polymerase or the PIF2 helicase showed selective loss of maxicircles suggesting critical roles in maxicircle replication (Wang &

Englund, 2001, Klingbeil *et al.*, 2002, Liu *et al.*, 2006, Grams *et al.*, 2002, Liu *et al.*, 2009). Therefore, we investigated whether POLIB might display a preference for minicircle or maxicircle templates. Loss of kDNA mass was further evaluated by Southern blotting of restriction digests of total DNA isolated from a staggered induction (Fig. 2.5A). Minicircle and maxicircle loss was evident as early as Day 2 with a 25% decline in abundance for both molecules (Fig. 2.5B). Subsequent days of silencing revealed continued reduction in minicircle and maxicircle abundance. Following 8 days of POLIB silencing, maxicircle abundance declined 70% while minicircle abundance declined 85%. To further evaluate the role of POLIB in kDNA replication, we next focused our analysis to ask how POLIB contributes to minicircle replication.

A key feature of kDNA network replication is the release of covalently closed (CC) minicircles, unidirectional theta replication as free molecules, and then reattachment of minicircle progeny to the kDNA network. If POLIB were a replicative enzyme for minicircles, RNAi should alter the population of free minicircles so that CC precursors become the most abundant species. Although the replication intermediates are a small fraction of the total kDNA in an unsynchronized population (Englund, 1979), free minicircles are resolved in a specific and predictable pattern on a single-dimension (1D) agarose gel containing ethidium bromide (Kitchin *et al.*, 1985, Ryan & Englund, 1989). Following separation of total DNA, free minicircles were detected on a Southern blot using a minicircle probe that contains the UMS, a sequence found in all minicircles. In control cells, newly replicated nicked/gapped (N/G) progeny and replication precursors, CC monomers, are approximately equimolar (Fig. 2.5C, Day 0). The concentration of free minicircles changed minimally during the first 3 days of RNAi. At Day 4 there was

a modest decline in N/G molecules, and then in parallel with shrinking and loss of the kDNA network, free minicircle abundance rapidly declined. During the later time points, newly replicated progeny were barely detectable with a striking increase in the CC:N/G ratio (mean 4.5) after RNAi for 8 days (Fig. 2.5C, D). The fold increase in unreplicated CC molecules may be even greater since background hybridization resulting from general degradation of the kDNA network (smear running from the top of the gel to N/G species beginning at Day 4) overlapped with the N/G signal. The apparent decline in CC molecules seen in D6 is due to underloading (see tubulin loading control). Notably, a heterogeneous smear migrating between N/G and CC molecules accumulated following 4 days of POLIB RNAi (Fig. 2.5C, U) with a similar migration pattern to previously described partially replicated multiply-gapped (MG) free minicircle intermediates (Ryan & Englund, 1989). These data are consistent with a defect in minicircle replication.

2.4.4 POLIB participates in leading and lagging strand minicircle synthesis

Heterodimeric replicases with individual DNA pols exhibiting leading or lagging strand preferences are well documented from bacteria to mammals (Dervyn *et al.*, 2001, Inoue *et al.*, 2001, Nick McElhinny *et al.*, 2008, Pursell *et al.*, 2007). To investigate whether POLIB exhibits strand preference during minicircle replication and to further characterize the free minicircle population, we utilized neutral/alkaline two-dimensional agarose electrophoresis of free minicircle replication intermediates. Free minicircle molecules were separated first on an agarose gel containing ethidium bromide. Strands were denatured with 30mM NaOH and separated in the second dimension and were then detected separately with 5' end-labeled synthetic oligonucleotides specific for the

continuously synthesized leading strand (L) and the discontinuously synthesized lagging strand (H) (Liu *et al.*, 2006). Consistent with previous findings, the L strand probe recognized all free minicircle species in control cells including multiply gapped minicircles migrating below N/G species (Fig. 2.6A, MG). Additionally, the more slowly migrating theta structure intermediates (Fig. 2.6B, T) were detected by adjusting contrast on phosphoimager scans from the uninduced population. After probing for H strand (lagging strand), the levels of CC, N/G and covalently closed dimer (ccD) minicircles were comparable to L strand probing.

After 4 days of POLIB RNAi, there was a modest decline in N/G molecules (consistent with the 1D analysis) with a slight increase in CC:N/G ratio that was comparable in both L and H strand probings (Fig. 2.6C). Notably, there were no detectable theta structures by day 4, and the abundance of MG molecules greatly diminished (Fig. 2.6B, L strand D4, Fig. 2.6D). The pattern of free minicircle molecules was greatly altered by day 6 of silencing. Reduction in both N/G and CC minicircle abundance was comparable for L and H strand probings, and this reduction coincided with loss of the kDNA network. Quantitation of the free minicircle populations indicated an increase in the ratio of CC to N/G, consistent with the 1D analysis (Fig. 2.6C, Fig. 2.5D). The concurrent accumulation of unreplicated CC minicircles and decline in newly replicated progeny is consistent with a block in the synthetic phase of minicircle replication during POLIB silencing. The pattern and abundance of both L and H stand minicircle replication intermediates were equally effected by silencing, suggesting POLIB contributes to both leading and lagging strand replication (Fig. 2.6A, C).

2.4.5 Fraction U, a novel free minicircle species that accumulates during *POLIB* silencing

Unexpectedly, the two-dimensional (2D) analysis revealed that the heterogeneous minicircle population detected in 1D analyses (Fig. 2.5C, U) migrated with mobility distinct from the previously characterized MG and fraction S (Liu *et al.*, 2006, Ryan & Englund, 1989). Our partial characterization of this novel fraction isolated from POLIB-depleted cells suggests similar properties to a population of free minicircles composed of multiply interlocked covalently closed dimers recently reported by Liu and colleagues. This population, which they named fraction U, was produced in response to silencing either of two other kDNA replication proteins, TbPIF1 and TopoII (Liu *et al.*, 2010). Therefore, we have provisionally adopted the same name, fraction U, for the heterogeneous minicircle species that accumulates during POLIB RNAi. Fraction U migrates as a continuous arc originating from ccD, and is detected as early as Day 4 of POLIB silencing (Fig. 2.6A, B). Importantly, fraction U accumulates simultaneously with the decline in replicating molecules and contains nearly equimolar L and H strands (Fig. 2.6A, C-D).

To understand the properties of this new minicircle species, we used sucrose gradient sedimentation for a large-scale purification of free minicircles and analyzed each fraction on a neutral 1D gel by Southern blotting with a minicircle probe. The monomeric species CC, N/G, L and MG, sediment near the top of the sucrose gradient for both the uninduced and Day 6 POLIB RNAi samples. In contrast, fraction U sediments farther into the sucrose gradient than the monomeric molecules (Fig. 2.7A, D6). Sedimentation rate depends on both mass and molecular conformation. The faster sedimentation of fraction U is likely due to increased molecular mass, but may also

reflect a difference in molecular conformation. The day 6 sample contained more linearized minicircle than in uninduced cells reflecting that the residual kDNA from POLIB RNAi cells may be more fragile than the intact network in an uninduced population. Fraction U properties were further analyzed by comparing migration patterns in the absence and presence of ethidium bromide. Ethidium bromide introduces supertwists into otherwise relaxed minicircles. The unreplicated CC minicircles are highly supertwisted and migrate more rapidly than nicked and gapped progeny, facilitating the resolution of these two species. In the absence of ethidium bromide fraction U migrated more slowly than CC and N/G molecules, further suggesting that fraction U consists of minicircles more massive than these monomeric minicircles, possibly dimers (Fig. 2.7A, bottom panel). The majority of fraction U ran faster than N/G molecules in the presence of ethidium bromide, but significantly slower than CC molecules suggesting that fraction U molecules are covalently closed and capable of being supertwisted, but more massive than CC monomers (Fig. 2.7A, top panel).

To further evaluate fraction U, we pooled and concentrated fractions 19-25, and deproteinized the sample using proteinase K digestion and phenol/chloroform extraction. Fraction U was then treated with several DNA modifying enzymes and the products were separated on a 1D gel containing ethidium bromide (Fig. 2.7B). A short treatment with human Topo II converted the fraction U smear to mainly CC monomers strongly suggesting that a majority of fraction U is composed of covalently closed molecules. Topo II treatment also resulted in a population migrating similar to N/G monomers, which were neither detectable in untreated fraction U or in the original sucrose gradient fractions. Additionally, fraction U was resistant to treatment with Topo I from two

different sources. This suggested that the heterogeneous fraction U molecules were not significantly supercoiled, or that increasing levels of catenation in dimers could have obscured topoisomerase I mediated relaxation of supercoiling. Taken together these data indicate that fraction U is composed mainly of covalently closed dimers that accumulate when the synthetic phase of minicircle replication is impaired during POLIB silencing.

2.5 Discussion

Mitochondrial DNA replication and repair in other eukaryotes requires just a single DNA polymerase, pol gamma (Kaguni, 2004, Graziewicz *et al.*, 2006). In contrast, trypanosomes contain seven mitochondrial DNA polymerases from three different DNA polymerase families; four Family A proteins (Klingbeil *et al.*, 2002), two Family X proteins (Saxowsky *et al.*, 2003), and one from Family Y (Rajao *et al.*, 2009). While POLIB, POLIC and POLID are essential for growth with non-redundant roles in kDNA maintenance, it is unclear exactly how trypanosomes utilize the three pol I-like proteins for kDNA network replication. One hypothesis is that each pol may perform a specialized role; for example preference for the minicircle and maxicircle templates, or a strand specific role in synthesis (leading vs. lagging). Using stemloop RNAi, we report here a primary role for the essential POLIB as a replicative minicircle DNA polymerase. Importantly, we also report the accumulation of a novel, heterogenous free minicircle species when POLIB is depleted.

Consistent with an essential role in kDNA replication, POLIB RNAi resulted in growth inhibition and a corresponding loss of the kDNA network. The first indication that POLIB is required for minicircle replication comes from the loss of minicircles during silencing experiments. The subsequent decline in maxicircles could result from

kDNA network fragmentation or nucleotlytic breakdown. Our data clearly highlight a role for POLIB in minicircle replication, but do not rule out a possible contribution to kDNA repair or maxicircle replication. When other proteins involved in minicircle replication are silenced, maxicircle depletion also accompanies the minicircle loss defect (Klingbeil *et al.*, 2002, Liu *et al.*, 2006, Scocca & Shapiro, 2008, Wang & Englund, 2001). The observed delay in growth inhibition following 4 to 5 days of RNAi likely represents the time required for the numerous minicircle copies that encode essential gRNAs for maxicircle editing to drop below a critical threshold. Indeed cells depleted of POLIB for more than 4 days exhibited decreased fluorescence of MitoTracker Red CM-H_2XRos (Fig. 2.8). Decrease in MitoTracker fluorescence appeared to correlate with cells containing small or no kDNA.

Further evidence supporting a role in minicircle replication was obtained from analyzing free minicircle replication intermediates via 1D and 2D agarose gel electrophoresis. After 4 days of POLIB RNAi, the decrease in theta structure intermediates, decrease in multiply gapped molecules, and an initial accumulation of unreplicated covalently closed molecules were all indicative of a defect in minicircle replication. The pattern for replication intermediates was not altered greatly until day 4 of silencing, even though the minicircles abundance had decreased nearly 60%. This indicated that CC unreplicated precursors were still released from the kDNA network and initiated minicircle synthesis resulting in an increase in free minicircles without subsequent reattachment to the network. It is possible that during the earlier days of RNAi, one or more of the six other mitochondrial DNA pols were compensating for loss of POLIB, but could not effectively keep pace with cell cycle progression.

To address the possibility that POLIB is a strand-specific enzyme at the core of a heterodimeric replicase, we detected leading and lagging strand minicircle progeny using neutral/alkaline electrophoresis. Interestingly, probing for L or H strand progeny was nearly identical strongly suggesting that POLIB does not appear to have strand preference. An alternative explanation is that minicircle replication proceeds by a strand-coupled mechanism similar to what has been proposed for mammalian mtDNA replication (Holt, 2009), and could require another of the essential pol I-like pols. In this case, depletion of POLIB could then result in disruption of the replisome uncoupling the leading and lagging strand pols and any of their associated components. The possibility of a minicircle strand-coupled mechanism has never been investigated and warrants further investigation.

Previous TbPOLIB silencing experiments using the pZJM vector, that produces an intermolecular dsRNA trigger, led to a reduced growth rate in procyclic cells and a small increase in the percentage of cells with abnormal sized kDNA networks. While depletion of POLIB indicated an essential role for growth and suggested a role in kDNA maintenance, the resulting phenotype could not be fully characterized (Klingbeil *et al.*, 2002). Recently, we demonstrated that a stem-loop dsRNA trigger was the better tool to study the function of the related mitochondrial Pol I-like protein, TbPOLID (Chandler *et al.*, 2008). Therefore we chose to fully characterize TbPOLIB using the pSL vector. Similar to TbPOLID, stem loop silencing of TbPOLIB produced a more robust phenotype than reported using pZJM. Although the same region of POLIB was used for two separate constructs (pZJMIB and pSLIB) and produced at least a 90% knockdown, only the intramolecular stem-loop trigger achieved notable growth inhibition and a

marked kDNA phenotype. At this time we cannot definitively state why silencing the two Pol I-like proteins is more effective with a stem-loop dsRNA. Silencing with pZJM or other constructs that contain opposing T7 promoters is generally effective in *T. brucei.* However, cell lines generated using the pZJM constructs are more susceptible to leaky transcription problems (synthesis of dsRNA trigger in the absence of tetracycline induction) since the promoter of pSL is regulated by two tetracycline promoters whereas pZJM is regulated by only a single operator (Wang *et al.* 2000). Leaky transcription can lead to development of RNAi insensitive cell lines, termed revertants (Chen *et al.*, 2003). Similarly, trace amounts of tetracycline may be present in media (from fetal bovine serum) and could lead to the development of a sub-population of revertants. Chen *et al.* studied the molecular basis for generation of RNAi resistance and demonstrated that deletion of the target-specific portion of the integrated pZJM-derived construct occurred in revertant parasites. Alternative mechanisms for development of resistance likely also exist. The presence of RNAi insensitive cells may have contributed to the weaker response in pZJMIB studies and could be assessed using dilution cloning. Others have also reported that stem-loop dsRNAs are more efficient at knockdown of target mRNAs, and are less sensitive to leaky transcription (Durand-Dubief *et al.*, 2003, Chanez *et al.*, 2006). However, which proteins are silenced best with a stem-loop dsRNA will likely need to be determined empirically. Certainly, culturing parasites in tetracycline-free media and avoiding long term cultivation may help to avoid development of revertants and apparent incomplete penetrance of RNAi.

An unexpected finding from POLIB RNAi was the accumulation of a new free minicircle species, provisionally called fraction U. Although initially presumed to be

multiply-gapped minicircles, this free minicircle population migrated distinctly from any other species previously reported and continued to accumulate until it represented a majority of the free minicircle population. Most free minicircle species are replication intermediates that still contain discontinuities in the newly replicated strands with the exclusion of the non-replicated precursors, covalently closed monomers. Englund and co-workers recently described fraction U as a population of ccDs with multiple interlocks resulting from silencing either TbPIF1 or TopoII (Liu *et al.*, 2010). However, our enzymatic treatments with topoisomerase I and II indicate that fraction U produced following POLIB silencing may be a more heterogenous population of molecules composed mainly of ccDs and possibly pre-catenane dimers, even though electrophoretic analyses of the fraction U populations were quite similar. It is interesting to note that the formation and accumulation of fraction U minicircles has not been reported for any other kDNA replication proteins besides TopoII, TbPIF1, and POLIB.

Multicatenane (multiply interlocked) dimers are produced in theta structure replication of circular DNA (Sundin & Varshavsky, 1981, Sakakibara *et al.*, 1976). While a kDNA replication mechanism involving a multicatenane pathway was suggested in *T. brucei* (Scocca & Shapiro, 2008) the presence of multiply interlocked dimeric minicircle intermediates have not been demonstrated in wild type trypanosomes. Fraction U was occasionally detected in very low abundance in the uninduced population (data not shown), suggesting that this species may occur transiently during normal cell cycle progression. Naturally occurring multicatenane replication intermediates are often resolved quickly and, thus, challenging to detect in an unperturbed cellular population (Martinez-Robles *et al.*, 2009, Leonard *et al.*, 1982). While this observation is intriguing,

we have not yet succeeded in isolating sufficient amounts of these molecules from uninduced cells to indisputably confirm that these molecules are indeed fraction U.

Fraction U may accumulate in POLIB depleted cells as a result of minicircle replication fork stalling and restart with an alternative DNA polymerase. During normal replication, the replisome can encounter DNA lesions or structures that stall or collapse the progressing replication fork. Bacteria and eukaryotes utilize several well-characterized mechanisms for restarting stalled forks (Heller & Marians, 2006). However, there is no available data on the protein machinery or mechanism of mitochondrial DNA replication restart in any organism. In this case, loss of POLIB from the replisome following silencing would likely result in collapse of the replication fork, and disassembly of other essential proteins from the processive minicircle replisome. These proteins may include polymerase loading and processivity factors, a helicase to unwind the parental DNA duplex, and a topoisomerase to decatenate replication intermediates. The stalled minicircle replication fork may elicit a kDNA repair response similar to bacterial SOS and result in the recruitment of proteins including alternative DNA pols. One of the six other trypanosome mitochondrial DNA pols may load at the minicircle replication fork, successfully complete synthesis and preserve interactions with proteins required for final gap closure and ligation yet fail to efficiently recruit those enzymes required for resolution of pre-catenanenes. Replication of kDNA would still need to keep pace with nuclear replication every cell cycle, and we demonstrated that nuclei are capable of incorporating BrdU through day 5 of POLIB RNAi. However, these alternative DNA pols likely have differing affinities and lower processivity than POLIB, hence slowing down the entire minicircle replication process. Decreased

processivity would result in incomplete replication of all minicircles within the given time frame, fewer minicircles progeny reattaching to the kDNA network, and subsequent decline in minicircle copy number as seen starting as early as day 2 of POLIB silencing. Evidence for such slowing of a replication fork was recently demonstrated with translesion synthesis (TLS) proteins DNA pol II and DNA pol IV, which occupy the replication fork during DNA damage and proceed at a slower pace with lower fidelity (Indiani *et al.*, 2009). Additionally, two other eukaryotic DNA pols with low fidelity are implicated in TLS, pol nu (Takata *et al*., 2006, Arana *et al*., 2007) and pol theta (Arana *et al*., 2008). Interestingly, both these TLS pols and the essential pol I-like proteins of *T. brucei* are family A DNA polymerases.

Currently, the exact mechanism of fraction U formation is difficult to determine. However, it is attractive to hypothesize that disruption of putative minicircle replisome components could give rise to fraction U. Indeed, silencing the mitochondrial helicase, TbPIF1 and Topoisomerase II produces a population of free minicircles with similarity to our partially characterized fraction U resulting from POLIB silencing. Therefore, we are now poised to ask questions about other proteins that may be interacting with POLIB at the minicircle replication fork. Interestingly, the bacterial replisome is the target for small molecule inhibitors that disrupt the complex but do not necessarily inactivate the DNA polymerase (Georgescu *et al.*, 2008). Discovering inhibitors of the essential replicative minicircle DNA polymerase, POLIB, or any associated factors would offer new options for treatment of kinetoplastid diseases.

2.6 Acknowledgements

This paper is dedicated to the memory of Dr. David V. Pollack, a colleague and friend. We thank George Cross (Rockefeller University) for *T. brucei* 29-13 cell and the pLew100 vector, and Paul Englund (Johns Hopkins School of Medicine) for the pZJM, pJM326 and pJN6 vectors. Additionally, we are grateful to Drs. Paul Englund and Beiyu Liu for sharing results prior to publication, and insightful discussions. We thank Anthula Vandoros for excellent technical assistance, and members of the Klingbeil Lab for many thoughtful discussions, and appreciate comments on the manuscript by Drs. James Morris and Nicholas Downey. This research was supported by NIH grant AI066279 to MMK.

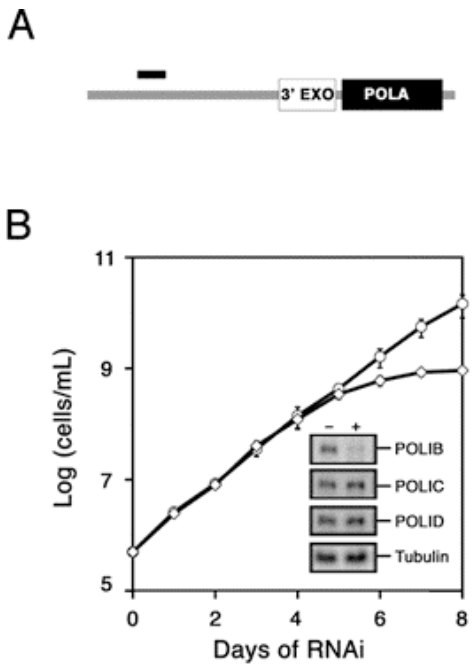

Figure 2.1: Effects of POLIB RNAi on trypanosome growth
(A) Diagram: Protein domain structure of TbPOLIB was predicted using the conserved domain database (version 2.16). The black bar indicates the 500 bp region of *TbPOLIB* used to generate the pSLIB vector. (B) Clonal cell line SLIB 2C7 was grown in the absence (open circles) or presence (open diamonds) of tetracycline (1 μg/ml) to express the stem-loop dsRNA. Cell density was plotted as the product of cell number and total dilution. Values are the mean (± standard deviation) of three separate RNAi inductions. Inset: Northern blot of total RNA from uninduced (-) and RNAi induced (+) 2C7 cells for 48 hr. Following probing for *POLIB* (4.2kb), the same blot was stripped and reprobed in succession with *POLIC* (6.1 kb), *POLID* (5.3 kb), and α-tubulin as the loading control.

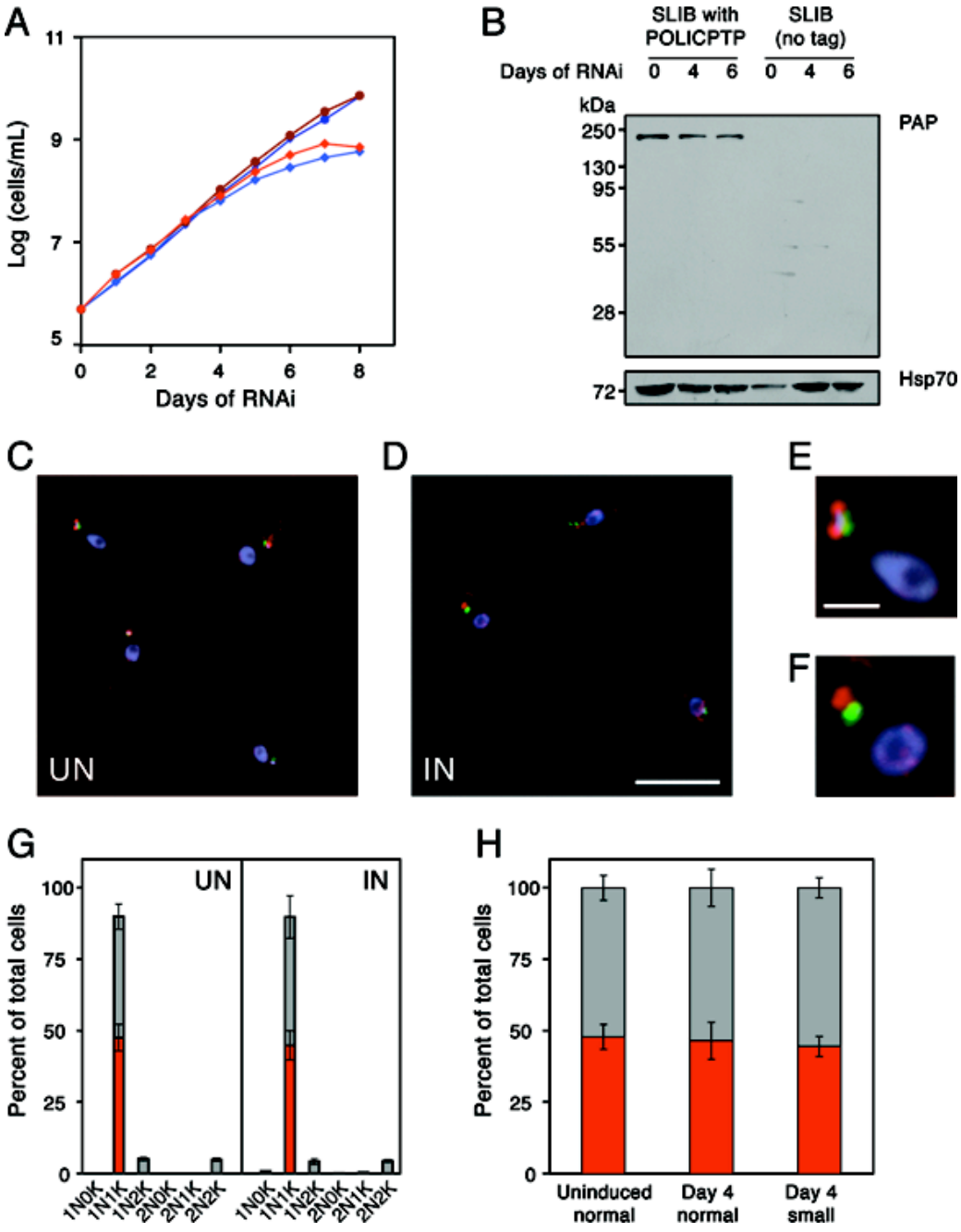

Figure 2.2: POLIC localization is not disrupted during POLIB silencing

(A) SLIB2C7 (red) or SLIB2C7 expressing POLIC-PTP (blue) were grown in the presence (diamonds) or absence (circles) of tetracycline. (B) Western blot of lysates from induced cells for indicated number of days of POLIB RNAi. POLIC-PTP was detected with Peroxidase-Anti-Peroxidase Soluble Complex (PAP) reagent. Membrane was stripped and the loading control, Hsp70, was detected. (C-D) Representative images of POLIC-PTP localization in uninduced (C) and Day 4 induced (D) POLIB cells. Basal bodies (green, YL1/2), POLIC-PTP (red, anti-protein A), and DNA (blue, DAPI). Bar, 10 μm. Enlarged images of uninduced (E) and induced (F) cells presented in C. Bar, 2 μm. (G, H) Quantitation of POLIC-PTP localization during POLIB RNAi. More than 300 randomly selected cells were scored for each time point. Cells were classified as positive (red bars) or negative (grey bars) for POLIC-PTP foci, karyotype, and size of kDNA. Values are the mean (± standard error) of two separate RNAi inductions. (G) Prevalence of POLIC-PTP foci scored by size of cell karyotype. (H) Prevalence of POLIC-PTP foci in uninduced and induced cells.

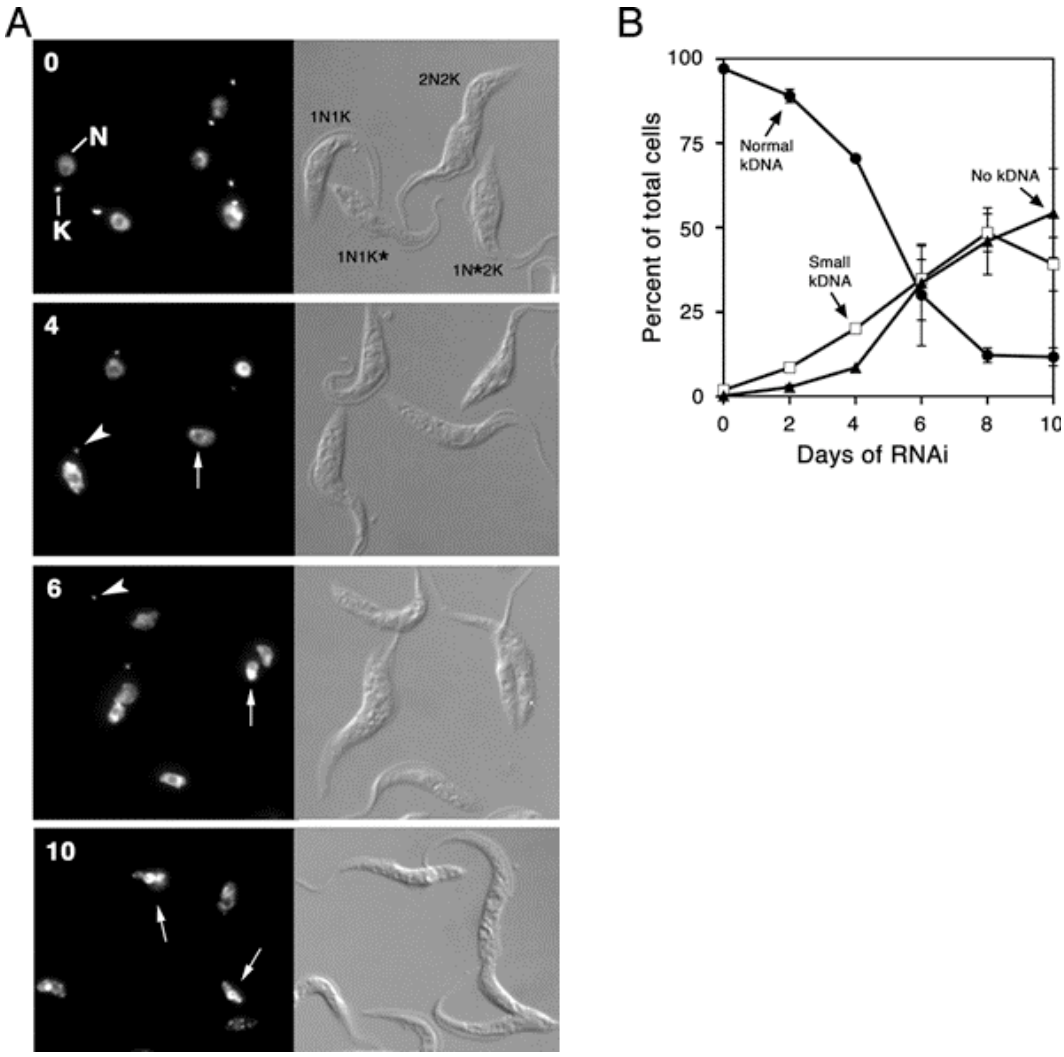

Figure 2.3: Effect of POLIB RNAi on kDNA morphology
(A) Effect of TbPOLIB RNAi on kinetoplast size. Left panel, DAPI-stained fluorescent images; right panel, DIC images. Representative images for the indicated timepoints showing progressive loss of kDNA are presented. N, nucleus; K, normal-sized kDNA; * replicating genome; arrowhead, small kDNA; arrow, no kDNA. Bar, 10 μm. (B) Kinetics of kDNA loss determined by visual analysis of DAPI-stained cells. Normal-size kDNA (filled circles); Small kDNA (open squares); No kDNA (filled triangles). More than 300 randomly selected cells were scored for each time point. Values are the mean (± standard deviation) of two separate RNAi inductions.

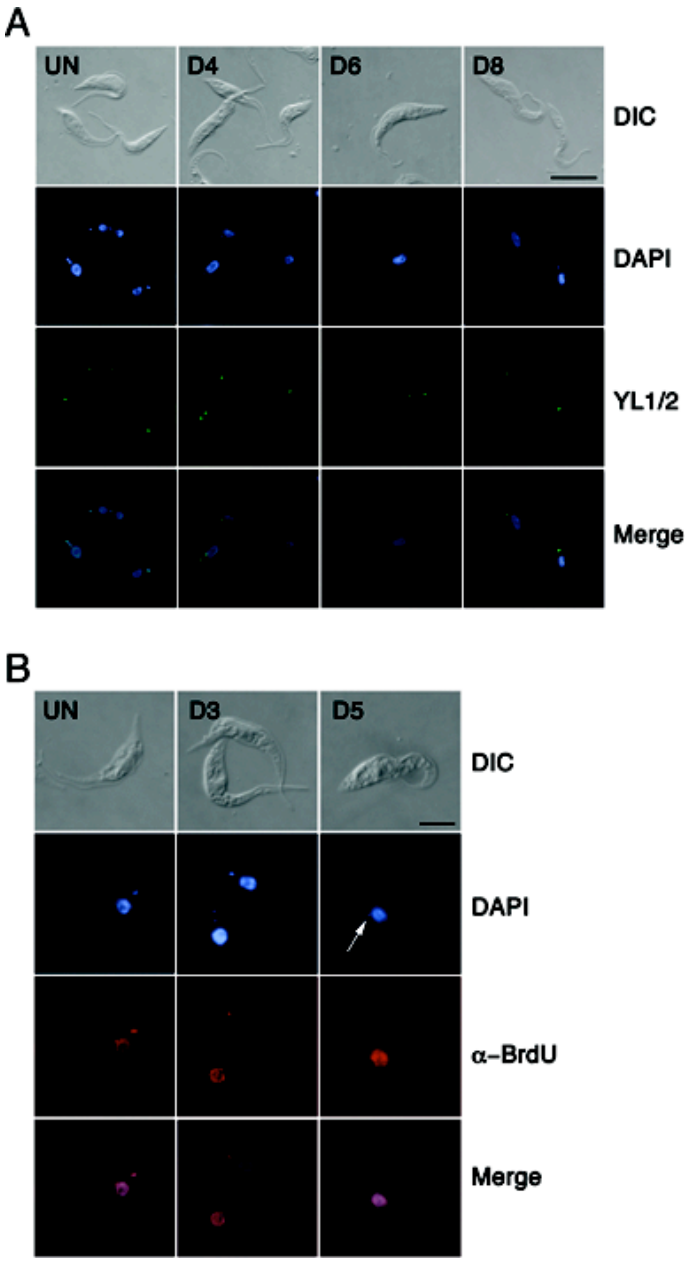

Figure 2.4: POLIB silencing specifically inhibits kDNA replication
(A) Representative images for the indicated timepoints showing normal basal body duplication and segregation during POLIB RNAi-mediated kDNA network loss. Bar, 10 μm. (B) Representative images of uninduced and RNAi induced cells cultured in the presence of bromodeoxyuridine and deoxycytidine. BrdU epitope was exposed by treating fixed cells with HCl prior to detection with anti-BrdU and DAPI staining. Arrow indicates a small kDNA. Bar, 5 μm.

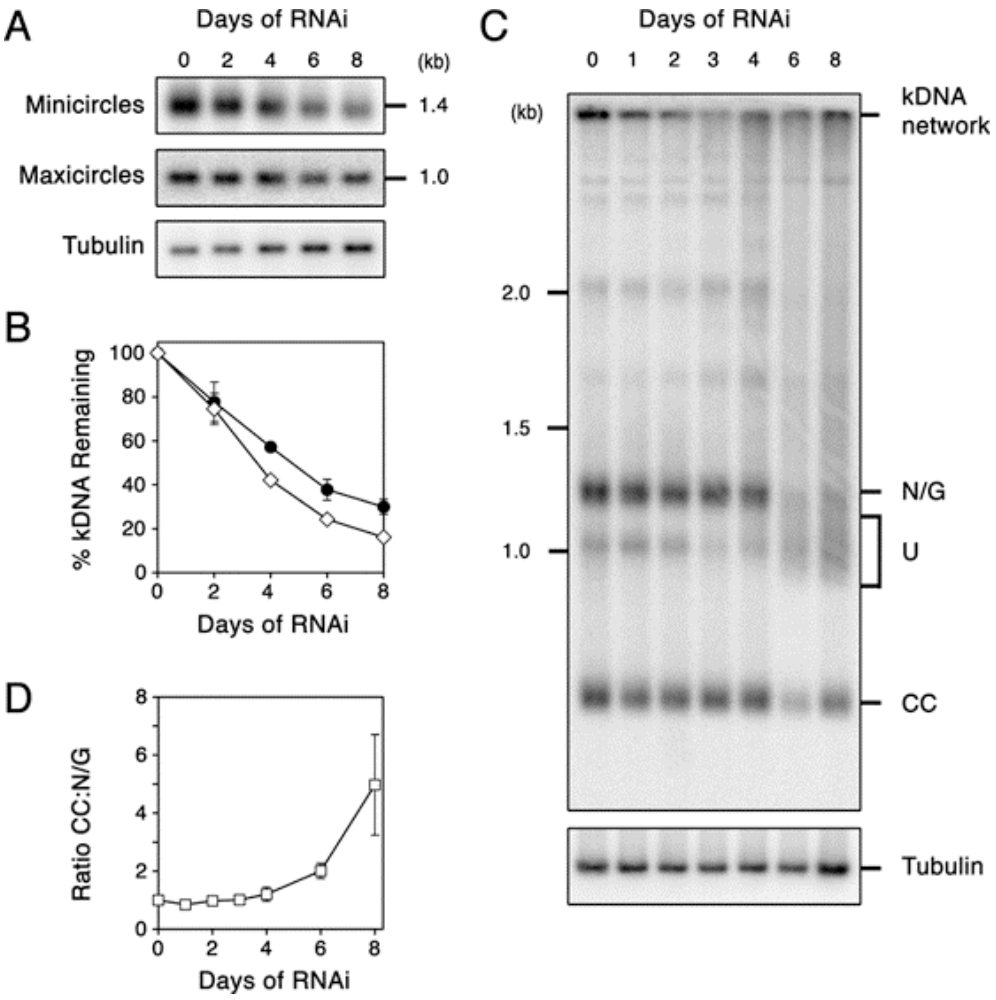

Figure 2.5: POLIB is required for kDNA replication

SLIB2C7 clonal cells uninduced or induced for RNAi were harvested at the indicated time points from a staggered induction. (A) Representative images of kDNA loss determined by Southern blot analysis. Total DNA (10^6 cell equivalents/sample) was digested with HindiIII and XbaI, fractionated on a 1% agarose gel and transferred to nylon membrane. Minicircle and maxicircle abundance was determined with minicircle and maxicircle specific probes. (B) Phosphoimaging quantitation total minicircle and maxicircle abundance. Values were normalized against the α-tubulin loading control. Minicircles (open diamonds); Maxicircles (closed circles). (C) Southern blot analysis of free minicircles. Total DNA (2 x 10^6 cells/lane) was fractionated on a 1.5% agarose gel in the presence of ethidium bromide (1 μg/ml), and transferred to nylon membrane. After probing for minicircle DNA, the blot was stripped and probed for α-tubulin as the loading control. CC, covalently closed; N/G, nicked/gapped; U, uncharacterized, heterogenous minicircle population. (D) Phosphorimager quantitation of free minicircles. The relative abundance of free minicircle molecules was estimated by plotting the ratio of signals from CC and N/G minicircles detected from southern blots similar to the one in Fig. 2.5C. Values presented in B and D are the mean (± standard deviation) of two separate RNAi experiments.

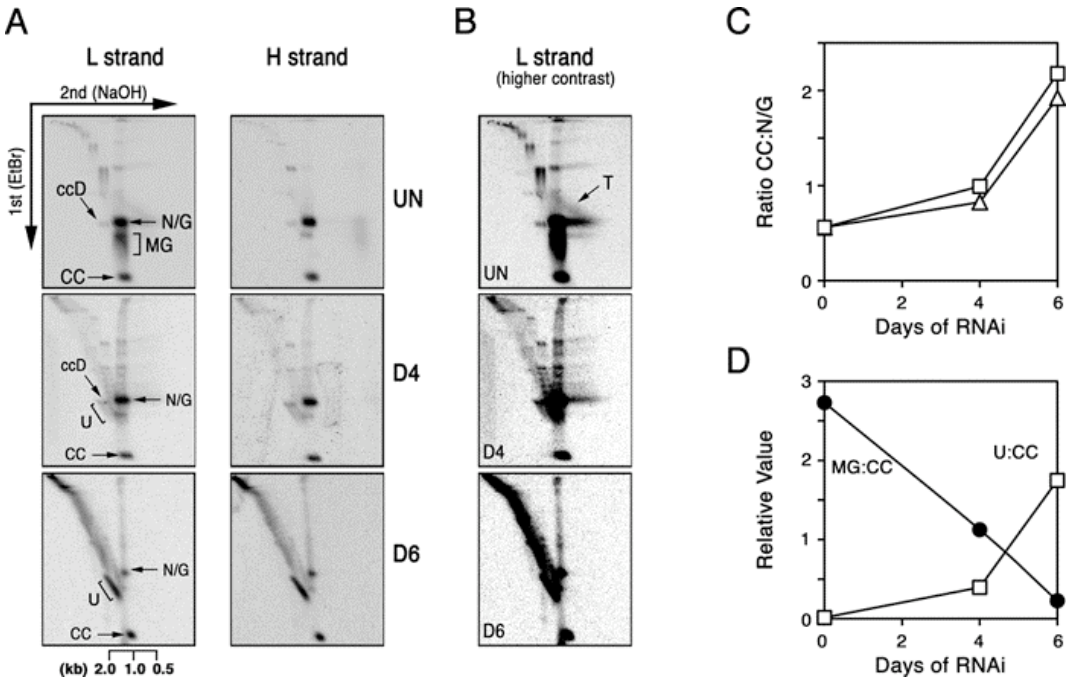

Figure 2.6: Silencing of POLIB inhibits minicircle replication
SLIB2C7 clonal cells induced or uninduced for RNAi were harvested at the indicated time points from a staggered induction. (A) Neutral/alkaline two-dimensional gel electrophoresis of free minicircles. Total DNA (3 x 10^7 cell equivalents) from uninduced and cells induced for the indicated number of days of RNAi were separated first in the presence of ethidium bromide and then under denaturing conditions. After transfer to nylon membrane, radiolabeled H and L strand oligomers were used for detection. Individual blots were probed first with L strand oligoprobe, stripped, and reprobed with the H strand oligoprobe. CC, covalently closed; ccD, covalently closed dimer; MG, multiply gapped; N/G, nicked/gapped; U, uncharacterized, heterogenous minicircle population. (B) Higher contrast images. Contrast was adjusted equally in images of membranes probed for L strand in (A) to visualize abundance of theta structures. T, theta structures. (C) Quantitation of changes in CC and N/G free minicircles. Phosphoimager quantitation was used to plot the relative abundance of unreplicated CC and newly-replicated N/G intermediates determined by scanning blots in Fig. 2.6A. L strand detection (open squares) and H strand detection (open triangles). (D) Quantitation of fraction U accumulation using leading strand probe. Phosphoimager quantitation of blots in Fig. 2.6A were used to plot the relative abundance of unreplicated CC, MG intermediates, and fraction U. Ratio of MG:CC (filled circles) and U:CC (open boxes).

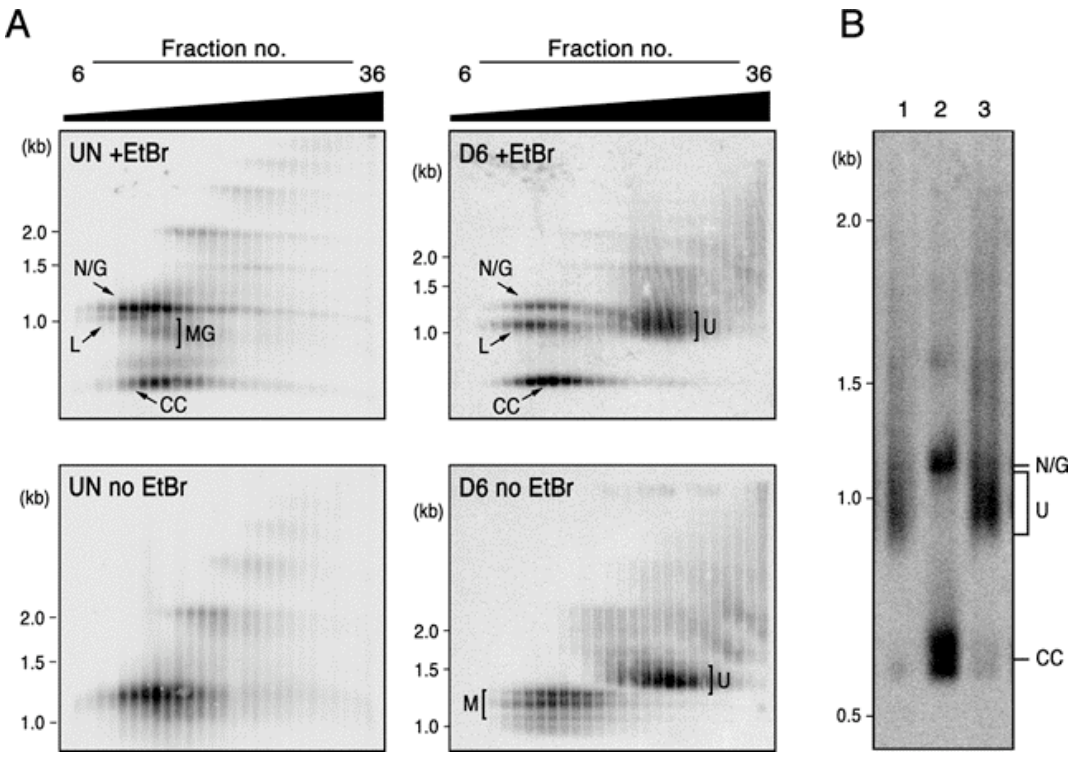

Figure 2.7: Fraction U is a heterogeneous, topologically complex minicircle population

(A) Sucrose gradient sedimentation of free minicircles. Total DNA from cell lysates of uninduced and induced for 6 days of *TbPOLIB* RNAi were sedimented through 5-20% sucrose gradients. Fractions 6-30, 32, 34, 36 (10 μl each) were separated on a 1.5% agarose gel in the presence or absence of ethidium bromide. DNA was transferred to nylon membrane and detected with a minicircle probe. CC, covalently closed; L, linearized; M, monomers; MG, multiply gapped; N/G, nicked/gapped; and U, fraction U. (B) Enzymatic treatments of concentrated fraction U. Fraction U (1.6 x 10^7 cell equivalents) was treated as indicated for each lane and separated on a 1.5% agarose gel containing ethidium bromide before transfer to nylon membrane and detection with a minicircle probe. CC and N/G minicircles purified from uninduced cells (not shown) were used as mobility markers. Lane 1, untreated; Lane 2, Topoisomerase II (10U, 30 min); Lane 3, Topoisomerase I (5U, 15 min).

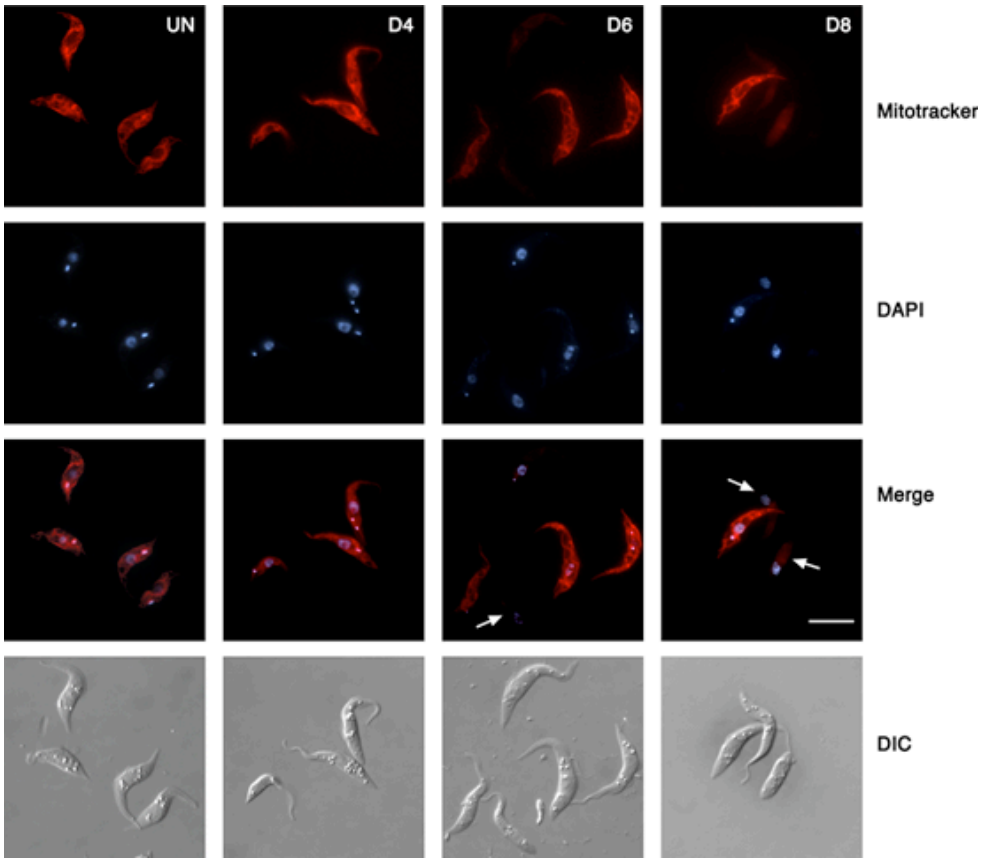

Figure 2.8: POLIB silencing results in decreased mitochondrial membrane potential
Live SLIB2C7 cells induced for indicated number of days of RNAi were incubated with MitoTracker Red CM-H_2XRos, which accumulates and fluoresces in cells dependant upon mitochondrial membrane potential, and DAPI. White arrows indicate cells with decreased accumulation of MitoTracker. Bar, 10 μm.

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CHAPTER 3

TWO MITOCHONDRIAL DNA POLYMERASES, POLIB AND POLID, PARTICIPATE IN MINICIRCLE REPLICATION IN *TRYPANOSOMA BRUCEI*

3.1 Abstract

Kinetoplast DNA (kDNA), the mitochondrial DNA of kinetoplastid parasites, is a catenated network of minicircles and maxicircles essential for parasite survival. kDNA replication is unparalleled in nature, involving multiple mitochondrial DNA polymerases and an elaborate mechanism of release, replication, and re-attachment of minicircles. Three family A DNA polymerases (POLIB, POLIC, and POLID) are essential for parasite growth and perform non-redundant roles in kDNA maintenance. Individual silencing of POLIB or POLID perturbs but does not block minicircle replication. A covalently closed species of minicircle dimers (Fraction U) was previously found to accumulate during POLIB RNAi and, as we show here, during POLID RNAi. Fraction U production is a unique phenotype restricted to RNAi of a subset of four kDNA replication proteins, thus suggesting that each enzyme makes a specialized contribution to a common pathway of minicircle replication. Here, we examine this possibility by simultaneously silencing POLID and POLIB. Silencing both polymerases together accelerated growth inhibition and loss of kDNA witnessed during individual polymerase silencing experiments. Additionally, dual gene silencing resulted in a near-complete inhibition of minicircle replication, which was never achieved with single-gene silencing. Our data are supportive of a model of minicircle replication requiring the specialized functions of POLIB and POLID.

3.2 Introduction

Trypanosoma brucei and related trypanosomatid protozoan parasites are agents of severe and wide-spread diseases affecting some of the world's poorest populations. *T. brucei* causes Human Africa Trypanosomiasis (HAT) in humans and nagana in cattle. Despite the tremendous economical and medical burden *T. brucei* exerts globally, there is no vaccine to prevent infections and available drugs are inadequate (Luscher *et al.*, 2007). Trypanosomatid parasites posses a number of biological properties without counterpart in their mammalian hosts that could provide new drug targets. One of the most intriguing and unique properties of *T. brucei* is its mitochondrial genome, termed kinetoplast DNA (kDNA). This giant network resides in the cell's single mitochondrion where is it compacted into a disk- shaped structure positioned near the flagellar basal body. The network consists of two types of circular DNA molecules, minicircles and maxicircles, which are catenated into a network that has been likened to medieval chain mail.

Maxicircles (23kb each, 25 per network) encode rRNAs and mRNAs for subunits of respiratory complexes (Lukes *et al.*, 2005). Maxicircle transcripts often require post-transcriptional editing, including the insertion and deletion of uridines to create a functional open reading frame. This essential process, RNA editing, is directed by templates (guide RNAs) encoded by minicircles (Stuart *et al.*, 2005). Therefore, expression of mitochondrial proteins is a combined effort between maxicircles and minicircles, making both necessary for parasite survival.

The catenated topology of kDNA presents a unique challenge for DNA replication. To overcome this topological challenge, trypanosomes employ a network-free minicircle replication mechanism. Minicircles are released from the network into the

kinetoflagellar zone (KFZ), a specialized region between kDNA disk and flagellar basal body. Here unreplicated, covalently closed (CC) minicircles encounter several replication proteins, including universal minicircle sequence binding protein (UMSBP), RNA primase, and at least two DNA polymerases (POLIB and POLIC) (Abu-Elneel *et al.*, 2001, Klingbeil *et al.*, 2002, Li & Englund, 1997, Liu *et al.*, 2006). Following theta structure replication, nascent minicircles possessing multiple gaps (MG) and nicks (N/G) are attached to the network periphery by a topoisomerase II (TopoIImt). Here, RNA primers are removed by structure specific endonuclease 1 (SSE1) and all but a single gap in the backbone is filled by DNA polymerase β and ligase Kβ (Engel & Ray, 1999, Hines *et al.*, 2001, Downey *et al.*, 2005). This gap is proposed to serve as a bookkeeping mechanism and is filled by DNA polymerase β–PAK and ligase kα only after all network minicircles have been replicated. The double-sized network is split into two daughter networks, which are attached to flagellar basal bodies through a filamentous structure known as the tripartite attachment complex (TAC) (Melendy *et al.*, 1988, Saxowsky *et al.*, 2003). As basal bodies are pulled apart during late cytokinesis, daughter networks are subsequently segregated into the two new cells.

The utilization of multiple DNA polymerases (pols) for mitochondrial DNA replication in trypanosomes is unprecedented in other higher eukaryotes, which reply upon a single mitochondrial DNA polymerase, Pol γ (Copeland & Longley, 2003). At least six mitochondrial DNA polymerases are required for kDNA maintenance, including three family A polymerases (POLIB, POLIC and POLID), two family X polymerases (pol β and pol β-PAK) and a family Y polymerase (pol κ). RNAi silencing of POLIB, POLIC, or POLID results in kDNA loss and subsequent lethality, indicating that each

performs a specialized function in kDNA replication. Individual silencing of each of these three polymerases altered minicircle replication intermediates but did not completely block minicircle replication, suggesting that two or more polymerases may work together as a minicircle replicase. Previously, we found that silencing of POLIB or POLID resulted in the production of a heterogeneous minicircle species with electrophoretic mobility similar to that of MG (leading strand) replication intermediates (Chandler *et al.*, 2008, Bruhn *et al.*, 2010). We recently demonstrated that, in the case of POLIB silencing, this heterogeneous population is a distinct species of multiply interlocked minicircle dimers known as fraction U (Bruhn *et al.*, 2010). Production of fraction U has only been reported upon depletion of two other kDNA replication proteins (a mitochondrial topoisomerase II and helicase), suggesting that this subset of kDNA replication proteins contribute to a common pathway in minicircle replication.

In the current study we discern that the heterogeneous minicircle species produced during POLID silencing is also fraction U. This shared phenotype suggests that POLIB and POLID both participate in minicircle replication. To test this hypothesis, we simultaneously silenced POLIB and POLID. Combinatorial gene silencing of both POLIB and POLID resulted in accelerated growth inhibition and loss of kDNA networks, as compared to silencing of either polymerase individually. A complete block in minicircle replication was achieved by silencing both proteins simultaneously. These data indicate that mitochondrial DNA polymerases IB and ID perform specialized functions in a common pathway of minicircle replication.

3.3 Material and Methods

3.3.1 **Plasmid Construction**

Nucleotides 694 – 1193 of the TbPOLID coding sequence (Gene ID Tb11.02.0770) were PCR amplified with forward (5'- GAG TCT AGA CGT GAT TGC TTA GTA AGT TGG -3') and reverse (5'- TAT GAG CCA TGG GTA CGA ATC AGT GCC CAA GTG G) primers containing XbaI and NcoI linker (underlined). The resulting product was purified, restriction enzyme digested with XbaI and NcoI, and ligated into vector pL4440 to generate pL4440-ID. Nucleotides 275 - 774 of the TbPOLIB coding sequence (Gene ID Tb11.02.2300) were PCR amplified by forward (5'- TAT GAG CCA TGG AAG ATG AGC GTG TCA ACG AGG -3') and reverse (5'- TAT GAG AAG CTT GGT AAA CCG TGG CGC GAC GAG G -3') primers containing NcoI and HindIII linkers (underlined). This fragment was digested and ligated into pL4440-ID to create pL4440-DB. The 1020 bp region containing both TbPOLID and TbPOLIB fragments, DB, was PCR amplified from pL4440-DB using forward (5'-GAG TCT AGA CGT GAT TGC TTA GTA AGT TGG-3') and reverse (5'- TAT GAG ACG CGT GGT AAA CCG TGG CGC GAC GAG G-3') primers containing XbaI and MluI linkers (underlined). Resulting fragment DB was restriction with XbaI and MluI and then ligated into pLEW100 to generate pLEW100-DB. Separately, fragment DB was digested with HindIII and NheI and ligated into pJM326 to create pJM-DB. To create the final stemloop vector, pLew100-DB was digested with XbaI and HindIII, and the vector backbone (6316bp) was gel extracted. pJM326-DB was digested with XbaI and HindIII, the insert (DB+stuffer ~1500bp) was gel extracted. The final pSLDB was acquired by ligation of vector backbone and insert.

3.3.2 Cell Culture and Transfection

T. brucei brucei procyclic strain 29-13 was grown in SDM-79 medium containing 15% heat-inactivated fetal bovine serum (Invitrogen), 15 μg/ml G418 (Fisher) and 50 μg/ml hygromycin (Invitrogen) at 28 °C. Parasites were cultured at densities between 5x105 and 1x107 parasites per milliliter. For transfections, 10 μg of linearized pSLDB was introduced by electroporation. Stable transfectants were subsequently selected with 2.5 μg/ml phleomycin (Invitrogen) and clonal cell lines obtained by limiting dilution described previously (Chandler *et al.*, 2008). To induce for RNA interference (RNAi), growth medium was supplemented with tetracycline (1 μg/mL). Single-gene silencing of POLID was performed using clonal cell line C8P1, the cell line used previously for functional analysis of this protein (Chandler et. al. 2008).

3.3.3 Microscopy Analysis

Parasites were harvested by centrifugation (5 minutes at 1000 rcf) and washed and resuspended in phosphate-buffered saline before settling by gravity to poly-L-lysine-coated slides (10 min. at room temperature). Adhered parasites were fixed with 4% paraformaldehyde, which was then neutralized by washing in phosphate-buffered saline containing 0.1M glycine. Fixed parasites were stained with 2 μg/ml 4'-6'-diamidino-2-phenylindole (DAPI) prior to mounting in Vectashield (Vector Laboratories). Slides were viewed with a Nikon Eclipse E600 microscope. Quantitation of kDNA was performed as described previously. For each timepoint, more than 300 cells were classified as possessing normal kDNA (the size in uninduced populations), small kDNA (networks

clearly < 50% the size of normal kDNA), or no kDNA. The later category was reserved for parasites that contained no distinguishable staining in the kinetoplast region in multiple focal planes. Images were captured using a Spot digital camera (Diagnostic Instruments).

3.3.4 DNA Isolation and Southern Blotting

Total DNA was isolated from 1×108 parasites using the Puregene Core Kit A (Qiagen). Southern Blotting was performed to assess kDNA loss during RNAi. Briefly, total DNA from 1×107 cells was digested with HindIII/XbaI, fractionated on a 1% agarose gel, and transferred to Genescreen plus membrane. Based molecular weight markers and known migration patterns of bands detected with minicircle, maxicircle, and tubulin fragments, the resulting membrane was cut into three sections to separated these bands. Individual strips were then detected with minicircle, maxicircle and α-tubulin-specific radiolabeled probes as described previously (Chandler *et al.* 2008).

3.3.5 Two-Dimensional Electrophoresis

Two-dimensional analyses of network free minicircles were performed as described previously (Bruhn *et al.* 2010). Total DNA from 3×107 cells was fractionated at 60V on a 1.5% agarose gel in (20 × 25 × 0.5 cm) in Tris-borate-EDTA (TBE) containing 10 μg/ml ethidium bromide. After 18 hours, the gel was washed with 500mL of 50mM NaOH/1mM EDTA (three washes, 20 minutes each) and equilibrated in 500mL of 30mM NaOH/2mM EDTA (two washes, 30 minutes each). The gel was rotated 90 degrees clockwise, relative to its original orientation, and the denatured DNA was

electrophoresed in 30mM NaOH/2mM EDTA for 20 hours at 25V. Subsequently, the gel was depurinated, denatured, and neutralized and the DNA transferred to membrane. DNA was detected with 32P end-labeled oligos that detect heavy strand (5'- GGG CGT GCA GAT TTC ACC ATA CAC AAA TCC CGT GCT ATT TT-3') or light strand (5'- AAA ATA GCA CGG GAT TTG TGT ATG GTG AAA TCT GCA CGC CC -3') minicircle replication intermediates. Images were acquired using a Molecular Dynamics PhosphorImager (Typhoon 9210; GE Healthcare).

3.4 Results

3.4.1 Fraction U accumulates during POLID RNAi

To determine if the heterogeneous minicircle population produced during POLID silencing was MG replication intermediates or fraction U, we examined changes in minicircle replication intermediates during POLID RNAi. Minicircle replication is a network-free process, making replication intermediates resolvable by two-dimensional electrophoresis. The continuous DNA backbone of unreplicated covalently closed (CC) minicircles makes these intermediates more susceptible to ethidium bromide induced supertwisting than gapped (N/G) nascent minicircle replication products. In the presence of ethidium bromide, CC minicircle migrate more rapidly into an agarose gel than N/G. Total DNA extracted from parasites induced for 0 or 6 days of POLID RNAi was fractionated first in the presence of ethidium bromide and then in a second dimension, under alkaline conditions. Resolved minicircles were then transferred to membrane and detected with oligo probes specific for leading and lagging strand minicircle replication

intermediates. As previously demonstrated (Bruhn *et al.*, 2010, Liu *et al.*, 2006), unreplicated (CC) and newly replicated (N/G) free minicircles were present in approximately equal amounts in uninduced populations with no detectable fraction U. In parasites induced for POLID RNAi, CC became slightly more abundant than N/G minicircles and fraction U represented a large portion of the pool of network-free minicircles (Fig. 3.1). Importantly, fraction U was not produced in response to silencing POLIC (data not shown) or other previously studied kDNA replication proteins. Production of fraction U during RNAi of this subset of essential kDNA replication proteins suggests that, although neither POLIB or POLID can effectively compensate for the loss of the other, these two polymerases perform specialized, non-redundant functions in a common pathway of minicircle replication.

3.4.2 Dual gene silencing of POLIB and POLID

To examine the possibility that POLIB and POLID both participate in minicircle replication, we generated the dual gene silencing vector pSLDB to simultaneously knockdown transcripts of both polymerases. Clonal cell lines derived from stable transfectants showed similar growth inhibition, knockdown efficiency, and doubling times. Clonal cell 1A10 was selected for further phenotypic analysis. Northern blot analysis of mRNA from uninduced and induced cells revealed over 80% reduction of both target mRNAs (TbPOLIB and TbPOLID) within 48 hours of RNAi induction (personal communication, J. Luo). Importantly, mRNA levels of the third essential mitochondrial DNA polymerase TbPOLIC remained unchanged during the induction. We further confirmed polymerase silencing by examining proteins levels of affinity-tagged POLID and POLIB during RNAi. Western blot analysis revealed that the abundance of

both proteins rapidly declined following RNAi induction, reduced by more than 95% after 2 days of silencing (personal communication, J. Luo). The powerful knockdown achieved with clonal cell line 1A10 suggests that this cell line is suitable our analyses. Inhibition of parasite growth was consistently observed 3 to 4 days following induction of RNAi (personal communication, J. Luo). The onset of growth inhibition by silencing POLIB and POLID together is earlier than when either protein is silenced alone. 1A10 divides more slowly than the clones used in single gene RNAi experiments, indicating that parasites induced for dual gene silencing complete fewer rounds of cell division, and presumably DNA replication, prior to growth inhibition than parasites induced for single-gene silencing (Fig. 3.2).

3.4.3 POLIB/POLID RNAi accelerates minicircle loss

Parasites depleted of individual kDNA replication proteins (including POLIB and POLID) exhibit network shrinkage and loss that can be visualized using DAPI staining. Since minicircles constitute more than 90% of the kDNA mass, we anticipated that microscopy analysis would reveal a more rapid loss of kDNA during dual gene silencing than when either polymerase was silenced individually. To examine this possibility, the sizes of kDNA networks were observed in cells induced for increasing durations of POLIB/POLID RNAi. Both nuclear and kinetoplast genomes are clearly visible in uninduced DAPI-stained parasite populations. Following moderate kDNA loss during the first few days of dual gene silencing, a dramatic loss of kDNA was seen beginning 3 days post induction for RNAi (Fig. 3.3A). This increase in the rate of network loss correlated with the onset of growth inhibition, which also occurs after 3 days of RNAi. To quantify kinetics of kDNA loss, DAPI-stained parasites were examined and scored as possessing

normal, small, or no kDNA by criteria defined in Materials and Methods. To eliminate possibility for bias, the identities of samples were withheld from the microscopist during the analysis. Two independent experiments confirmed that kDNA loss during POLIB/POLID RNAi was more rapid and complete than that elicited by silencing either protein individually (Fig. 3.3C). After just 4 days of POLIB/POLID silencing, the percentage of the population possessing normal-sized networks fell to less that 10%. This is markedly less than the normal-sized population seen after 3 days of silencing POLIB or POLID (50 and 70%, respectively) (Bruhn *et al.*, 2010, Chandler *et al.*, 2008). In single-gene silencing experiments, the normal-sized population did not fall below 10% until more than 6 days of RNAi. Consistent with these data, parasites completely lacking kDNA appeared earlier during dual gene silencing. For example, after 8 days of POLIB/POLID RNAi more than 70% of the population lacked detectable kDNA (Fig. 3.3C). This is a significantly greater portion of the population than that produced during the same duration of silencing either POLIB (~40%) or POLID (~50%) (Bruhn *et al.*, 2010, Chandler *et al.*, 2008). These data indicate that kDNA networks are lost more rapidly when POLIB and POLID are simultaneously silenced than when either gene is silenced individually.

To confirm these observations, we performed Southern blot analysis and probed with maxicircle and minicircle-specific radioprobes to monitor changes in their abundance during the timecourse of RNAi. We were initially surprised that plotting minicircle/maxicircle abundance as a function of days of RNAi did not reveal accelerated loss of these kDNA components, as compared to single gene silencing (personal communication, J. Luo). Considering that our single-gene silencing clones grow more

rapidly than our dual gene silencing clone, we found that plotting kDNA loss as a function of cumulative doublings (rather than days of RNAi) confirmed that silencing a second polymerase accelerated minicircle loss (Fig. 3.4A). For example, 7 cell doublings were required for minicircle abundance to fall below 20% of uninduced population during POLIB/POLID RNAi whereas more than 10 cell doublings were required to achieve this level of minicircle loss when either gene was silencing individually. Interestingly, POLIB/POLID RNAi did not increase the rate of maxicircle loss produced during POLID RNAi (Fig. 3.4B). This observation suggests POLID may be the primary polymerase in maxicircle replication and will be explored further elsewhere. Therefore, both our microscopy and Southern blot analyses indicate that simultaneous depletion of POLIB and POLID accelerates kinetics of minicircle loss.

3.4.4 Dual silencing of POLIB and POLID blocks minicircle replication

Individual silencing of POLIB or POLID perturbed the pool of minicircle replication intermediates, yet neither was sufficient to completely block production of nascent, N/G progeny minicircles; in each case the production of N/G minicircles declined but was not abolished. To determine if dual gene silencing was sufficient to produce such a block, we began by performing single dimension replication intermediate assays. The abundance of CC and N/G (which are present in approximately equimolar amounts in uninduced populations) was consistent until day 3 of the induction, when both species began to decline and fraction U began to accumulate (personal communication, J. Luo). Notably the production of fraction U seen during dual gene silencing occurs earlier than in either single gene silencing background, where fraction U only becomes evident 4-6 days post induction.

Single-dimensional analyses of minicircle replication intermediates are a powerful tool for comparing changes in the relative abundance of minicircle species during a timecourse experiment, but are limited in that they cannot resolve N/G minicircles from singly-interlocked covalently closed dimers (ccD). Due to this technical limitation an increase in fraction U (which emerges from unreplicated ccD) could be mistaken for the persistence of N/G minicircles in single-dimension analyses. To determine if a block in the production of N/G occurred during dual gene silencing but was masked by fraction U, we used two-dimensional analysis to more finely resolve these individual species. Indeed, two-dimensional analysis confirmed that fraction U production obscured a tremendous decline in the production of N/G minicircles during POLIB/POLID RNAi (Fig. 3.5A). Whereas CC and N/G are present in about equimolar amounts in uninduced cells, CC were about 6 times more abundant than N/G in parasites depleted of POLIB and POLID for six days (Fig. 3.5B). Occasionally during our analysis, N/G minicircles were completely undetectable in cells induced for POLIB/POLID RNAi (data not shown). The block in the production of N/G during POLIB/POLID RNAi strongly supports a model in which POLIB and POLID both participate in minicircle replication.

3.5 Discussion

The accommodation of multiple DNA polymerases at a replicative fork is well documented in the context of nuclear DNA replication of both prokaryotes and eukaryotes. Pol III holoenzyme in *E. coli*, the nuclear replisome, contains two copies of pol III core which are individually responsible for leading and lagging strand synthesis

(Johnson & O'Donnell, 2005). Several eukaryotes, such as *Saccharomyces cerevisiae,* coordinate three essential family B DNA polymerases, pol α, pol ε and pol δ, at the replicative core. Pol α synthesizes RNA primers and short DNA segments then pol ε and pol δ extend leading strand and lagging strands, respectively (Pursell *et al.*, 2007). Mitochondrial DNA replication in most known eukaryotes, however, relies upon a single mitochondrial DNA polymerase, pol γ (Copeland & Longley, 2003). To date, no mitochondrial replisome has been purified, yet *T. brucei* requires at least three mitochondrial DNA polymerases. Although the polymerase domains of POLIB, POLIC, and POLID are more than 90% identical, each protein appears to make a specialized contribution to kDNA replication. An increased comprehension of the characteristics that distinguish the unique functions of these three closely-related polymerases affords a rare opportunity to dissect mitochondrial replication fork dynamics.

While two-dimensional analysis of minicircle replication intermediates is a powerful tool that affords fine resolution and detection of minicircle replication intermediates, this methodology precludes comparative analysis of changes in the abundance of replication intermediates over the timecourse of an RNAi induction. Single-dimensional analysis (electrophoresis in the presence of ethidium bromide) remains the most appropriate method for detecting changes in the relative abundances of minicircle replication intermediates. In the study presented we found that, despite microscopy and Southern blot analyses indicating a decline in total minicircle content, the pattern of minicircle replication intermediates was not visibly different from our uninduced control during the first three days of RNAi. Lack of change in levels of free minicircle species during early RNAi is neither paradoxical nor inconsistent with the moderate kDNA loss

observed during this time. Rather, the presence of consistent levels of these network-free species maintained as total network minicircle mass declined, suggests that a larger percentage of residual minicircles were network-free.

Although POLIB/POLID silencing resulted in a block in minicircle replication, our single-dimension analysis of minicircle replication intermediates did not reveal an accumulation of unreplicated CC minicircles. There are two clear reasons why this may be so. First, CC minicircles may not be the only pre-replication product indicating a block in minicircle replication initiation. Metabolic labeling experiments were unsuccessful at labeling fraction U, suggesting that it may not be the end-product of active DNA replication (Liu *et al.*, 2010). Thus, fraction U could be a pre-replication molecule produced by aberrant topoisomerase activity as was recently described for a different free minicircle species, Fraction E. In this case, the accumulation of Fraction U and the persistence of CC minicircles during RNAi would indeed be indicative of an accumulation of unreplicated minicircles. More definitive studies regarding the production of fraction U are necessary to fully assess this possibility. A second explanation why an accumulation of CC minicircles was never witnessed during the timecourse of POLIB/POLID RNAi is that the progressive loss of networks masks an increase in the percentage of total minicircles existing separate from the network, including unreplicated CC minicircles. For example, parasites induced for 3 days of RNAi have lost ~50% of total minicircles but one-dimensional analyses show no changes in the abundance of CC minicircles, indicating that percentage of total minicircles existing apart from the network as CC has doubled, suggestive of a block in minicircle replication.

The most definitive proof that POLIB and POLID both participate in minicircle replication comes from our two-dimensional analyses. Multiply-gapped minicircles and Okazaki fragments are markers for leading and lagging strand minicircle replication, respectively, and are detectable in high-contrast images of minicircle from uninduced populations (Bruhn *et al.*, 2010). These markers for active minicircle replication either absent or below the limit of detection in two-dimensional analyses of parasites induced for POLIB/POLID RNAi, indicating disruption of minicircle replication. Additionally, production of nascent (N/G) minicircles is almost completely blocked in response to simultaneous silencing of POLIB and POLID, a phenotype never achieved when silencing either polymerase individually. The production of N/G molecules was almost beyond the limit of detection in two-dimensional analysis of replication intermediates within 6 days of dual gene silencing. Residual production of N/G minicircles could be the result of incomplete gene silencing. Efforts to assess minicircle replication intermediates beyond this timepoint of RNAi were unsuccessful due to the extent of kDNA loss and unavailable minicircle mass to analyze. Occasionally during our analysis, N/G were completely absent in RNAi induced populations, indicative of a complete block in minicircle replication.

Production of fraction U during RNAi of POLIB and POLID (but not POLIC) suggested that both proteins participate in minicircle replication, thus, posing the question of strand-specific functions. For example, POLIB (which possesses an exonuclease domain similar to the leading strand ε subunit) could participate primarily in light strand synthesis. Our analyses of replication intermediates during POLIB and POLID RNAi did not reveal obvious strand-preferences (Fig. 3.1) (Bruhn *et al.*, 2010). Nonetheless, these

data provide significant insight into minicircle replication fork dynamics. Silencing POLIB and POLID simultaneously produces a more severe phenotype than silencing either protein individually, suggesting that the two do not participate in a strand-coupled replication mechanism whereby inhibition of synthesis in one direction disrupts synthesis of the opposite strand (as seen with the T4 replisome) (Nelson & Benkovic, 2010). A more likely scenario is that under conditions of dysfunction (including RNAi), one polymerase substitutes for the other, but with limited efficiency; the result is a partially functioning, less processive replisome. Propensity for polymerase switching under conditions of dysfunction has been documented in other eukaryotes, where pol δ is capable of substituting for a polymerase-deficient pol ε (Garg & Burgers, 2005). Indeed, immunodepletion of either leading (pol ε) of lagging strand (pol δ) polymerase impairs but does not completely inhibit DNA synthesis in xenopus egg extracts, indicating partial yet inadequate polymerase substitution occurs (Fukui *et al.*, 2004). Similarly, *in vivo* depletion of either POLIB or POLID only partially inhibits minicircle replication. The complete inhibition of minicircle replication witnessed when both polymerases are depleted is consistent with a model of asymmetric minicircle replication where POLIB and POLID inefficiently replace one another under conditions of individual polymerase dysfunction.

In addition to its fascinating molecular biology, kDNA is a remarkable structure in that it is a validated target for antitrypanosomal drugs (pentamidine for humans and ethidium bromide in livestock). Indeed, we recently demonstrated that ablation of kDNA replication proteins is lethal to disease-causing *T. brucei* (Chapter 4). Studies that unravel molecular mechanisms of kDNA replication, such as that presented here, enable target-

specific screens for novel antitrypanosomals with improved fewer side effects than those currently available. Efforts to identify protein-protein interactions of POLIB and POLID are ongoing (and will be presented elsewhere) and will likely expand our pool of novel drug targets without human homologues and deepen our understanding of the replication of the most complex genome in nature.

3.6 Acknowledgements

We are grateful to Klingbeil Lab members for invigorating discussions as well as Dr. Aishwarya Swaminathan for critical analysis of this manuscript. This research was supported by NIH grant AI066279 to MMK.

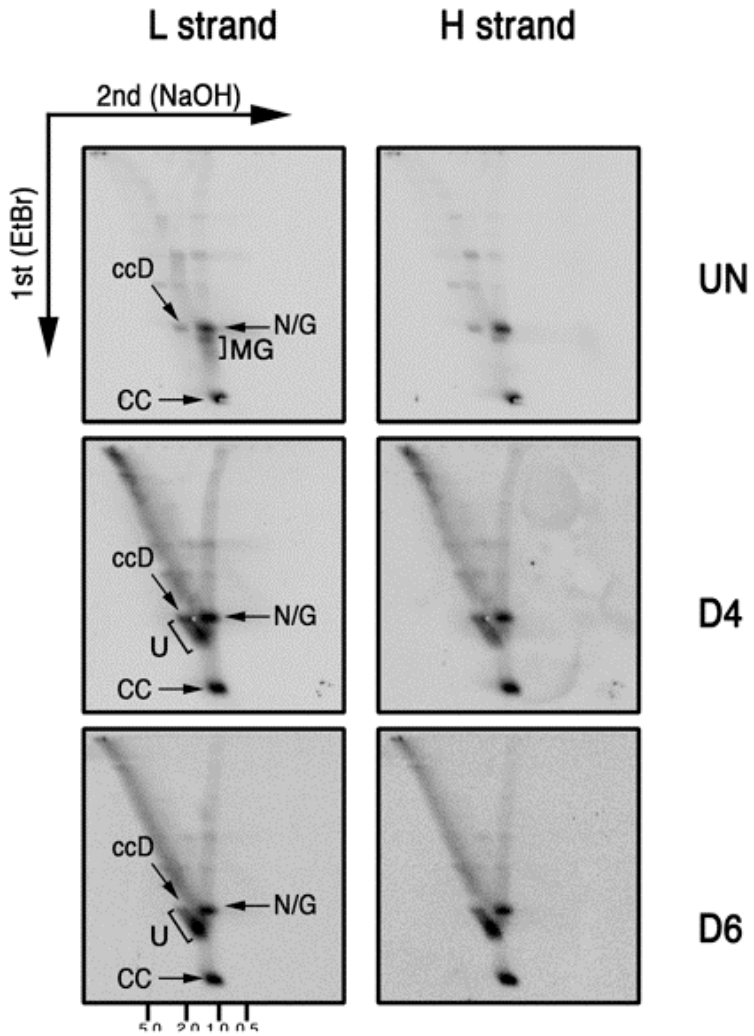

Figure 3.1: Fraction U is produced during POLID RNAi
Two-dimensional gel electrophoresis of free minicircles from SLID RNAi. Total DNA extracted from parasites induced for 0 or 6 days of POLID RNAi was harvested and fractionated in the presence of ethidium bromide and then under alkaline conditions. DNA was transferred to membranes and free minicircle detected using radio-labeled oligos specific for leading (L, light) and lagging (H, heavy) strand replication progeny. Abbreviations: ccD, covalently closed dimer; N/G, nicked/ gapped minicircles; MG, multiply gapped minicircles; CC, covalently closed minicircles; U, fraction U.

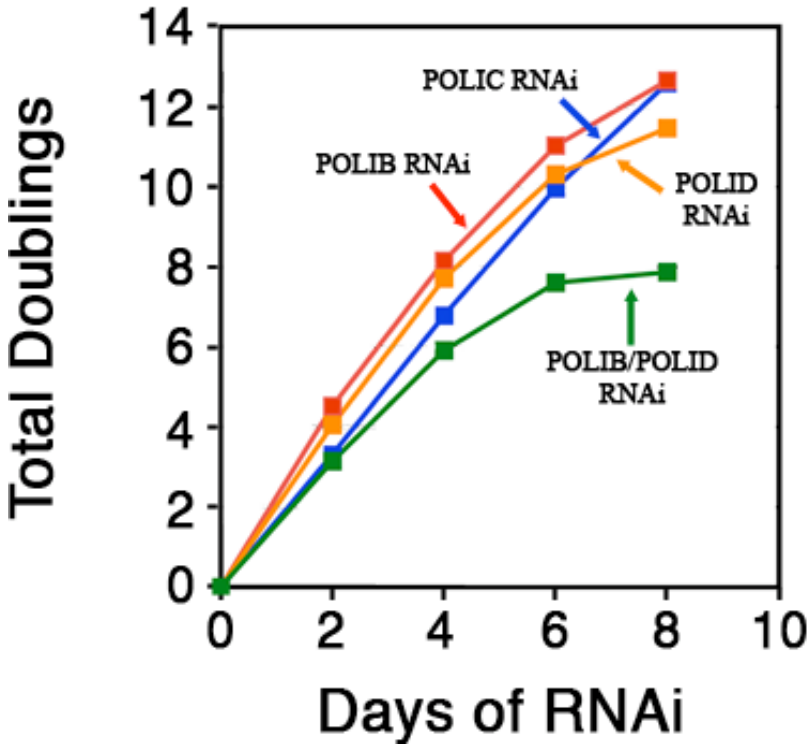

Figure 3.2: POLIB/POLID RNAi accelerates growth inhibition
Growth of parasites induced for RNAi of indicated polymerase(s) was plotted as a function of cumulative cell divisions following induction for RNAi. Data for POLIB, POLIC, and POLID were adapted from original publications (Klingbeil *et al.*, 2002, Bruhn *et al.*, 2010, Chandler *et al.*, 2008).

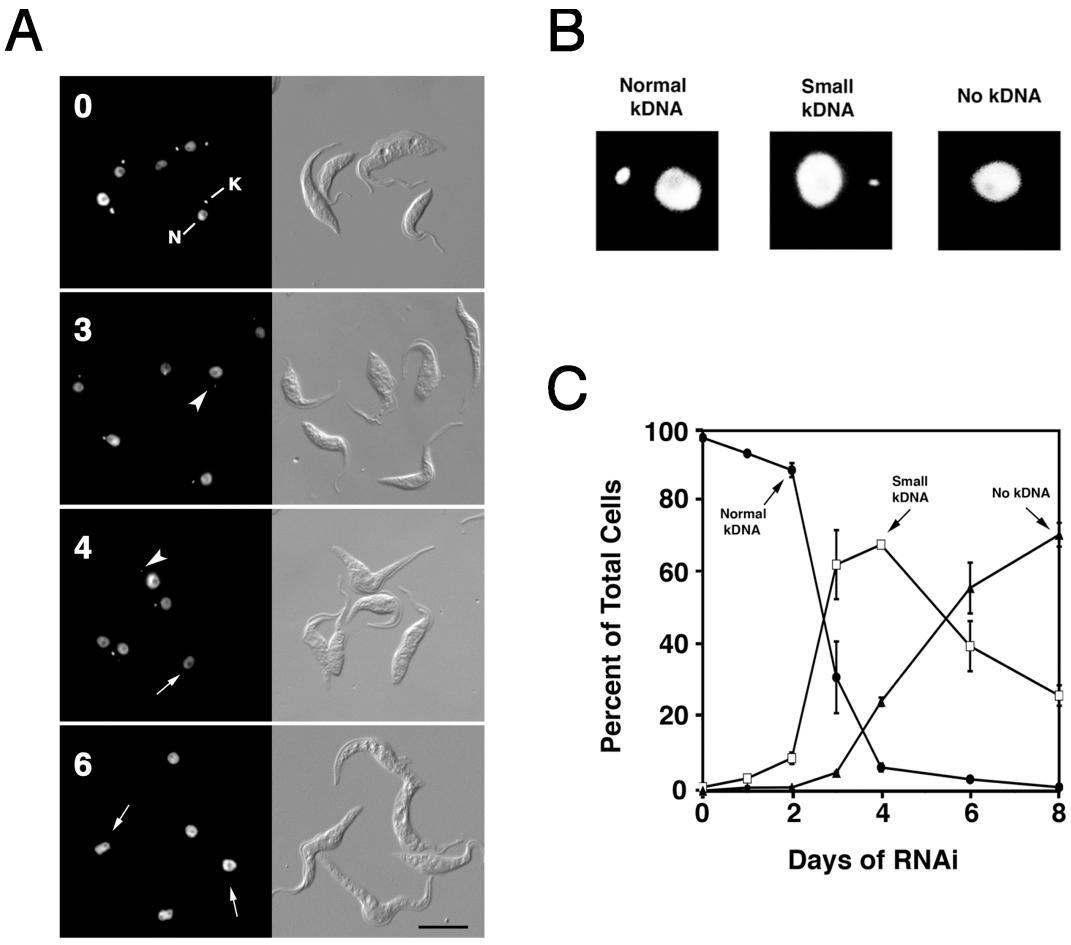

Figure 3.3: Dual gene silencing of POLIB and POLID causes accelerated loss of kDNA networks

(A) Representative DAPI (left) and DIC (right) images of parasites induced for indicated days of POLIB/POLID RNAi. Abbreviations and Symbols: N, nucleus; K, normally sized kDNA; arrowhead, small kDNA; arrow, no kDNA. Scale bar is 10 μm. (B) DAPI images showing examples of the three categories used to score parasites in panel C. (C) Microscopy quantitation of kinetics of kDNA loss. More than 300 cells per timepoint were scored as possessing normal sized kDNA (filled circles), small kDNA (open squares) or no kDNA (filled triangles). Graph displaying mean (+/- standard error) from two independent experiments.

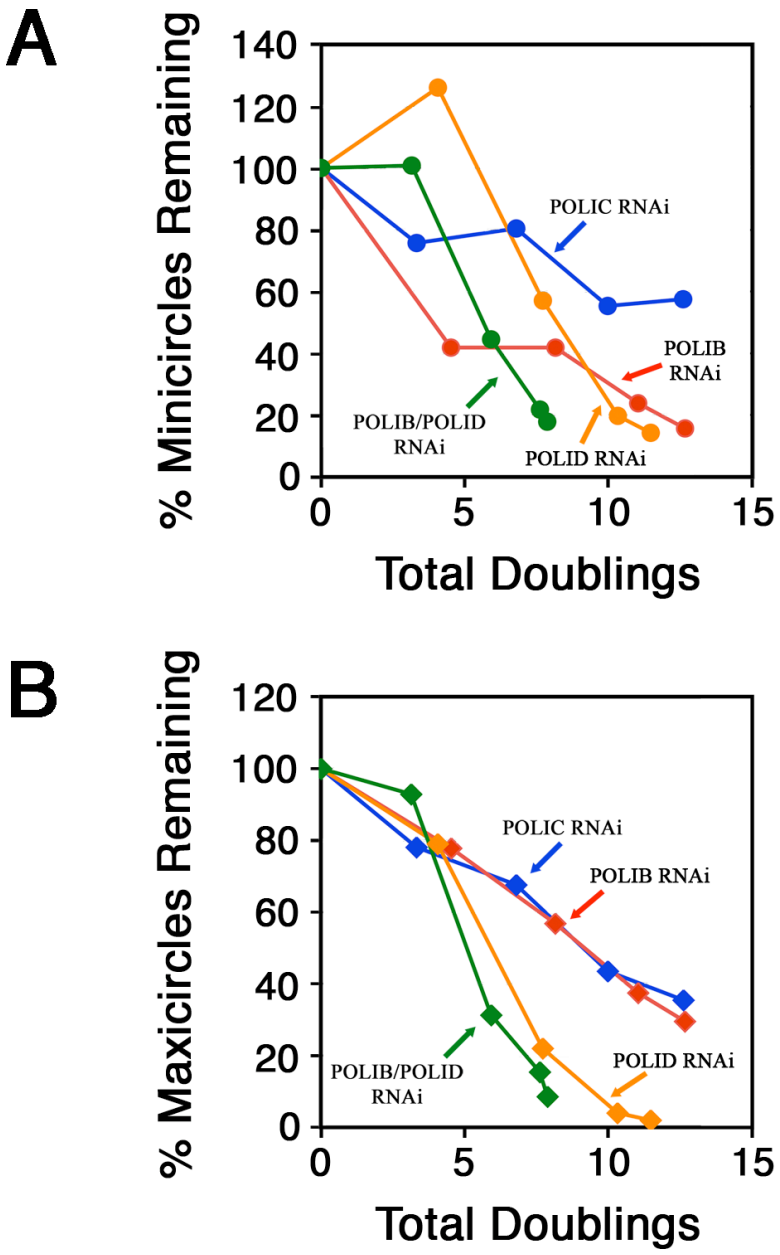

Figure 3.4: Loss of minicircles is accelerated during POLIB/POLID RNAi
Abundance of total (A) minicircle (B) maxicircle mass were determined from Southern Blot analyses and plotted as a function of cumulative cell divisions following induction for RNAi. POLIB/POLID data was adapted from Figure 3. Data for POLIB, POLIC, and POLID were adapted from original publications (Klingbeil *et al.*, 2002, Bruhn *et al.*, 2010, Chandler *et al.*, 2008).

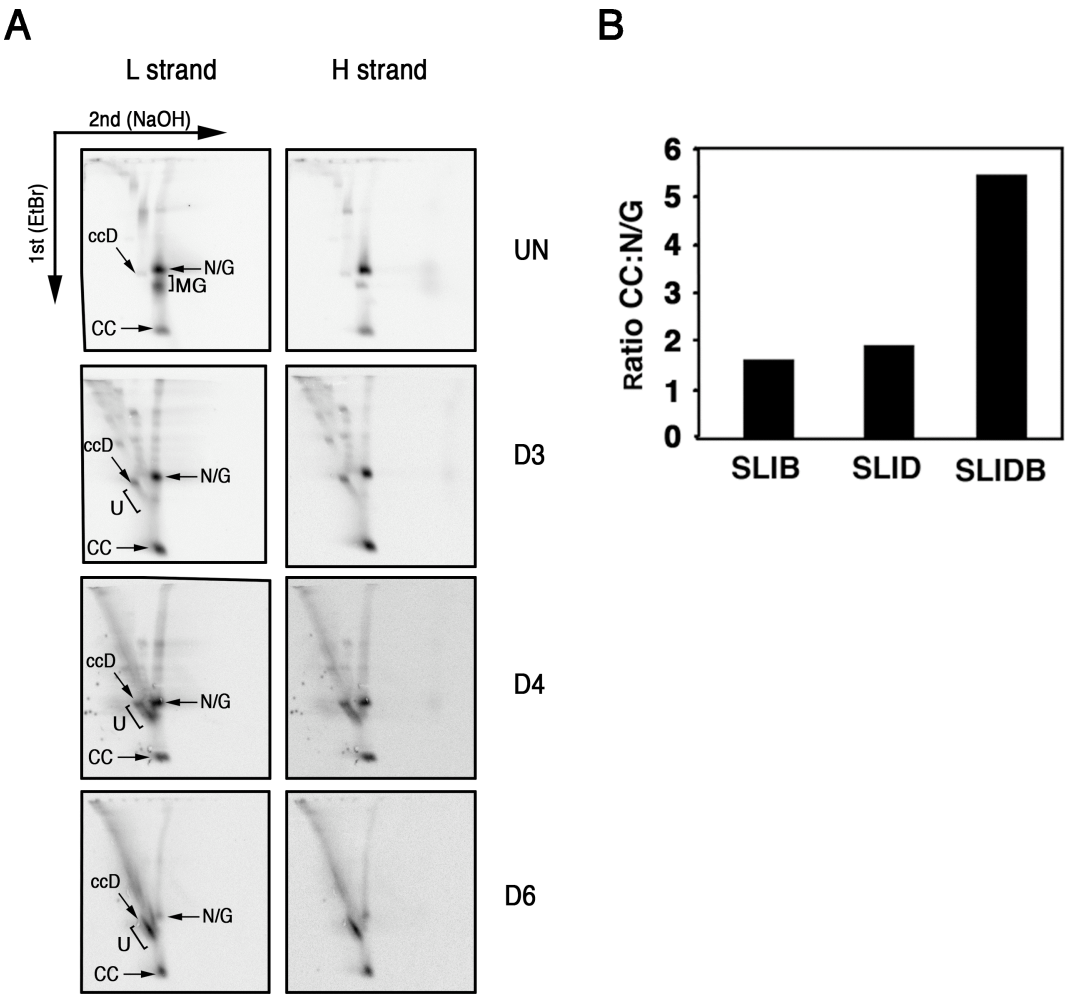

Figure 3.5: Dual gene silencing of POLIB and POLID blocks minicircle replication
Total DNA was isolated from parasites uninduced or induced for the indicated time of POLIB/POLID RNAi. (A) Two-dimensional analysis of free minicircles from parasites induced for indicated days of POLIB/POLID RNAi. Analyses were performed described for Figure 1. Abbreviations: ccD, covalently closed dimer; N/G, nicked/ Gapped minicircles; M.G., multiply gapped minicircles; CC, covalently closed minicircles; U, fraction U. (B) Quantitation of the relative abundance of CC and N/G minicircles during stemloop silencing of POLIB, POLID, or POLIB and POLID together. The relative abundance of minicircle species was estimated by plotting the ratio of signals from CC and N/G minicircles after 6 days of silencing POLIB (Bruhn *et al.*, 2010), POLID (SLID, Figure 1), or POLIB and POLID (Figure 3, panel A).

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CHAPTER 4

THREE MITOCHONDRIAL DNA POLYMERASES ARE ESSENTIAL FOR KINETOPLAST DNA REPLICATION AND SURVIVAL OF BLOODSTREAM FORM *TRYPANOSOMA BRUCEI*

4.1 Abstract

Trypanosoma brucei, the causative agent of Human African Trypanosomiasis, has a complex life cycle that includes multiple life cycle stages and metabolic changes as the parasite switches between insect vector and mammalian host. The parasite's single mitochondrion contains a unique catenated mitochondrial DNA network called kinetoplast DNA (kDNA) that is composed of minicircles and maxicircles. Long-standing uncertainty about the requirement of kDNA in bloodstream form (BF) *T. brucei* has recently eroded with reports of post-transcriptional editing and subsequent translation of kDNA-encoded transcripts as essential processes for BF parasites. These studies suggest that kDNA and its faithful replication are indispensable for this life cycle stage. Here we demonstrate that three kDNA replication proteins (mitochondrial DNA polymerases IB, IC, and ID) are required for BF parasite viability. RNAi silencing of each polymerase was lethal, resulting in kDNA loss, persistence of pre-replication DNA monomers, and collapse of the mitochondrial membrane potential. These data demonstrate that kDNA replication is indeed crucial for BF *T. brucei*. The contributions of mitochondrial DNA polymerases IB, IC, and ID to BF parasite viability suggest that these and other kDNA replication proteins warrant further investigation as a new class of targets for the development of anti-trypanosomatid drugs.

4.2 Introduction

Trypanosoma brucei is the protist parasite responsible for the fatal human disease Human African Trypanosomiasis (HAT) and a related disease in livestock called nagana. The few current pharmacological options to treat HAT are hampered by high toxicity and the emergence of drug resistant parasites (Baral, 2010). Therefore, there is an urgent need for the development of new anti-trypanosomal drugs. Trypanosomes possess a number of biological features without counterpart in humans that may provide sources of new targets for drug discovery efforts. One of the parasite's most remarkable properties is the unusual mitochondrial DNA network of trypanosomatids called kinetoplast DNA (kDNA). This DNA network is housed within the parasite's single mitochondrion and contains topologically interlocked circular DNA molecules called minicircles and maxicircles (Shlomai, 2004). Maxicircles are functionally similar to other eukaryotic mitochondrial DNA in that they encode proteins involved in respiratory complexes (Feagin, 2000). Nascent maxicircle transcripts require insertion and deletion of uridines in order to create a functional open reading frame (Hajduk & Ochsenreiter, 2010). This post-transcriptional process, known as RNA editing, is dependent upon minicircle-encoded guide RNAs (Hajduk & Ochsenreiter, 2010, Stuart *et al.*, 2005). Therefore, both minicircles and maxicircles are essential for mitochondrial physiology.

The topological complexity of the catenated kDNA network dictates a unique mode of replication in which minicircles are released from the network, replicated as theta structures, and reattached to the network periphery where Okazaki fragment processing occurs (Shlomai, 2004). A plethora of proteins involved in kDNA replication

have been studied in *T. brucei*, including five DNA polymerases (Bruhn *et al.*, 2010, Klingbeil *et al.*, 2002, Saxowsky *et al.*, 2003, Chandler *et al.*, 2008), six helicases (Liu *et al.*, 2009a, Liu *et al.*, 2009b, Liu *et al.*, 2010, Scocca & Shapiro, 2008), two DNA ligases (Downey *et al.*, 2005), two primases (Hines & Ray, 2010, Hines & Ray, 2011), a topoisomerase IA (Scocca & Shapiro, 2008), and a topoisomerase II (Wang & Englund, 2001). These studies provide compelling molecular evidence for essential function in the distinct steps of kDNA replication in procyclic form (PF) parasites, a life cycle stage found in its insect vector. However, analysis of kDNA replication protein functions in bloodstream form (BF) parasites, the life cycle stage found in the mammalian host and the target for disease intervention (Schnaufer *et al.*, 2005, Hannaert *et al.*, 2003), is an understudied area of trypanosome biology.

A striking feature of *T. brucei* is its ability to adapt to diverse environments encountered throughout the stages of its life cycle. Developmental regulation of mitochondrial activity appears to play a central role in these adaptations (Hannaert et al., 2003; Milman et al. 2007). PF parasites posses a highly active, branched mitochondrion and generate ATP through oxidative phosphorylation and mitochondrial substrate level phosphorylation (Tielens & van Hellemond, 2009). Conversely, BF parasites have a much-reduced mitochondrion, lack cytochromes and depend exclusively upon glycolysis for ATP production. A strictly glycolytic metabolism creates a seeming independence of BF parasites from maxicircle-encoded products and contributed to the assumption that kDNA is dispensable in the BF stage, thus diminishing the value of kDNA replication proteins as a source of new drug targets. This notion has been challenged by multiple lines of evidence, beginning with the demonstration that RNA editing is active and

essential in BF parasites and that maxicircle-encoded subunit A6 of ATP synthase complex (complex V) is required for generation of the mitochondrial membrane potential ($\Delta\Psi m$) (Schnaufer *et al.*, 2001, Fisk *et al.*, 2008, Schnaufer *et al.*, 2005). More recently, mitochondrial translation was found to be essential for BF *T. brucei* (Cristodero *et al.*, 2010). Further, inhibition of minicircle replication initiation appears to contribute to the trypanosome death elicited by treatment of infected animals with ethidium bromide (Roy Chowdhury *et al.*, 2010). These findings suggest that kDNA is by no means dispensable in this medically relevant life cycle stage.

Only a single kDNA replication protein, topoisomerase II (TbTopoII¬mt), has been examined in BF *T. brucei* thus far. RNA interference (RNAi) resulted in modest loss of kDNA networks (20-30%) accompanied by slowed parasite growth but not cell death (Timms *et al.*, 2002, Worthen *et al.*, 2010). The kDNA loss phenotype produced in BF parasites was significantly reduced compared to that produced in PF, where TbTopoII¬mt RNAi resulted in loss of kDNA in ~80% of the population (Wang & Englund, 2001). Silencing efficiency was not reported in these BF studies. Thus, it remains unclear if the slow growth phenotype reflected a diminished requirement for this kDNA replication protein in BF parasites or an inefficient knockdown that makes data interpretation difficult. Nonetheless, these data could indicate that TbTopoII¬mt is crucial for BF survival and strongly suggest that kDNA replication proteins are indeed required for viability of BF parasites.

We directly examined this hypothesis by individually silencing the family A mitochondrial DNA polymerases POLIB, POLIC, and POLID in BF parasites. Our previous studies of these polymerases indicated that all three are required for cell growth

and revealed non-redundant roles in PF kDNA replication but did not encompass studies in BF parasites (Bruhn *et al.*, 2010, Chandler *et al.*, 2008, Klingbeil *et al.*, 2002). Here we report that depletion of these proteins was lethal to BF parasites and resulted in loss of kDNA networks. Network loss appeared to result from inhibition of minicircle replication and was accompanied by depolarization of mitochondrial membrane potential and subsequent parasite death. These findings provide the first direct evidence that BF parasites require kDNA replication for viability. Therefore, kDNA replication proteins warrant further investigation as biological targets for the development of new anti-trypanosomal drugs.

4.3 Material and Methods

4.3.1 Trypanosome growth

Bloodstream form *T. brucei* single marker (SM), a derivative of Lister 427 engineered to express T7 RNA polymerase and tetracycline repressor, were maintained at 37°C with 5% CO¬2 in HMI-9 medium as previously described (Wirtz *et al.*, 1999). Cell densities were determined using a Neubauer hemocytometer, and cultures were maintained between 5 x 10^5 and 1 x 10^6 parasites/mL unless otherwise indicated. To avoid generation of revertants, clonal cells were maintained in culture for no longer than 21 days.

4.3.2 RNA interference

Vectors for RNAi were constructed as described previously (Shi *et al.*, 2000, Brandenburg *et al.*, 2007, Wang *et al.*, 2000) substituting pT7-stl, a derivative of pLew100 for initial cloning steps. Coding sequences corresponding to 500 bp fragments of POLIB (Tb11.02.2300), POLIC (Tb927.7.3990), and POLID (Tb11.02.0770) were PCR amplified from Lister 427 genomic DNA using gene-specific primers with appropriate linkers. The coding sequences and primers for POLIB and POLID were identical to those previously used for RNAi in PF with no reports of off-target effects (Bruhn *et al.*, 2010, Chandler *et al.*, 2008). Forward (CGAGAGACAACCGAATCATCC) and reverse (TGCATAGCACCTCACGC) primers were used to amplify the fragment for POLIC. Following linearization with EcoRV, the stemloop plasmids were transfected into SM parasites, using the Amaxa Nucleofector System as previously described (Burkard *et al.*, 2007) and stable clonal transfectants were selected using phleomycin (2.5 μg/mL) with limiting dilution. Clonal cell lines for silencing of POLIB, POLIC, and POLID were termed SMIB, SMIC, and SMID, respectively. RNAi was induced by the addition of 1.0 μg/mL tetracycline in growth medium. Staggered RNAi inductions were performed to minimize variation in sample preparation.

4.3.3 RNA isolation and Northern Analysis

Total RNA was extracted from 5 x 10^7 cells using the Purescript RNA isolation kit (Gentra Systems) and fractionated on a 1.5% agarose/ 7% formaldehyde gel. RNA was transferred to GeneScreen Plus membrane (NEN). Transcripts were detected with

^{32}P-random primed labeled gene-specfic probes as previously described (Bruhn *et al.*, 2010).

4.3.4 Clonogenic Assays

Parasites that were uninduced or induced for 10 days of RNAi were subjected to limiting dilution cloning in HMI-9 supplemented with appropriate antibiotics but lacking tetracycline using 96 well plates at 1 parasite/ml. Individual wells were examined five days later for the presence of motile parasites and plating efficiencies determined. Proliferating parasites were diluted in HMI-9 medium and maintained as described above.

4.3.5 Microscopy and Fluorescence Analyses

Parasites were pelleted at 800 x g, washed in room temperature trypanosome dilution buffer (TDB; 5 mM KCl, 80 mM NaCl, 1 mM MgSO4, 20 mM Na2HPO4, 2 mM NaH2PO4, 20 mM glucose, pH 7.7) and resuspended in TDB at a concentration of 2 x 10^7 parasites/mL. Parasites were allowed to settle by gravity onto poly-L-lysine coated microscopy slides, and fixed for 5 minutes in 1% formaldehyde dissolved in TDB. Following overnight permeabilization in ice-cold methanol, parasites were rehydrated with 3 washes in phosphate buffered saline (PBS, pH 7.4), stained with 6.7 µg/mL 4'-6'-diamidino-2-phenylindole (DAPI), and mounted in Vectashield (Vector Laboratories). Slides were viewed using a Nikon Eclipse E600 microscope and images acquired using a Spot digital camera from Diagnostic Instruments. Quantitation of kDNA network morphology was performed as previously described (Bruhn *et al.*, 2010, Chandler *et al.*,

2008). To eliminate any potential for bias, the identities of samples were withheld from the individual performing quantitation.

4.3.6 Neutral/Alkaline Two-Dimensional Analysis

Two-dimensional fractionation of total DNA was performed as previously described (Bruhn *et al.*, 2010, Liu *et al.*, 2006). Briefly, total DNA was separated in the first dimension for 18 hr in the presence of 1.0 μg/mL ethidium bromide and then equilibrated and electrophoresed in the second dimension for 20 hr in the presence of 50 mM NaOH. Following standard depurination, denaturation, and neutralization treatments, DNA was transferred to GeneScreen Plus membrane. Leading and lagging strand minicircle replication intermediates were detected using strand-specific T4 polynucleotide kinase 5'-end labeled oligonucleotide probes.

4.3.7 Analysis of Mitochondrial Membrane Potential

Detection of mitochondrial membrane potential was performed essentially as described previously (Brown *et al.*, 2006). Uninduced and induced parasites were sedimented, resuspended in HMI-9 at 2.5 x 10^{-6} cells/mL, and incubated for 30 minutes at 37°C with 5% CO2 in HMI-9 containing MitoTracker Red CM-H2XRos (Invitrogen) provided at 1μM for microscopy analyses or 2.5 μM for flow cytometry analyses. Cells were then washed 3 times in PBS and fixed for microscopy as described above or resuspended in 1mL of PBS for flow cytometry. Control cells were pre-treated for 60 minutes with 5 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) or the FCCP carrier (100% ethanol), washed and then resuspended in PBS. Changes in mitochondrial fluorescence intensity were analyzed using a Becton Dickinson LSR II

flow cytometer. FlowJo software (version 7.6.1) was used to analyze and graph experimental results.

4.4 Results

4.4.1 POLIB, POLIC, and POLID are required for viability of BF *T. brucei*

To determine if POLIB, POLIC, or POLID are required for *T. brucei* BF growth, inducible stemloop RNAi constructs for each of the polymerases were stably integrated and selected for in SM parasites, which express T7 RNA polymerase and tetracycline repressor protein. The individual clonal cell lines, referred to as SMIB, SMIC, and SMID, grew with average doubling times of approximately 8.5 hours which was slightly slower than the parental line (~8.2 hours per doubling). Induction for silencing of POLIB, POLIC, and POLID all resulted in slowed growth after 4 days of RNAi with subsequent parasite cell death (Fig. 4.1A-C). Northern blot analysis of parasites induced for 48 hours of RNAi revealed knockdown efficiencies ranging from 90 – 95% for each target transcript (Fig. 4.2). Notably, parasites non-responsive to RNAi, commonly referred to as "revertants," did not emerge after 10 days of the SMIB, SMIC, or SMID RNAi induction, as previously reported for silencing other essential proteins in BF parasites (Urbaniak, 2009, Chen *et al.*, 2003).

The low cell culture density required to cultivate BF parasites makes it challenging to observe cells that have potentially recovered or following prolonged periods of time in culture. Therefore we performed clonogenic assays by limiting dilution to further assess the contributions of these kDNA replication proteins to BF parasite

viability. Parasites cultures, that were uninduced or induced for 10 days of RNAi, were diluted to a single parasite per mL and plated in 96-well plates. Five days later, wells were examined for the presence of parasites. Parasites induced for RNAi prior to plating exhibited dramatically reduced plating efficiencies as compared to the uninduced controls, which were 80-90% viable. Parasites induced for silencing of POLIB, POLIC, and POLID clonally proliferated with efficiencies of 2.6%, 2.6%, and 0% respectively (Fig. 4.1D). These experiments demonstrate, for the first time, a lethal phenotype upon silencing of kDNA replication proteins in BF *T. brucei*.

4.4.2 POLIB, POLIC, and POLID perform essential kDNA maintenance roles in BF parasites

Functional studies have implicated numerous essential proteins for kDNA replication in PF parasites, yet only a single kDNA replication protein has been examined in disease-causing BF parasites. Silencing of this topoisomerase II (TbTopoII¬mt) in BF parasites resulted in a modest loss of kDNA compared to the extent of network loss observed when silencing this gene in PF (Timms *et al.*, 2002, Worthen *et al.*, 2010). Therefore, we sought to assess the role of three mitochondrial DNA polymerases in BF kDNA maintenance. Parasites were stained with DAPI, which intercalates into both nuclear and mitochondrial DNA, and viewed using fluorescence microscopy. While normal sized kDNA networks were clearly distinguishable in uninduced cultures, parasites induced for 4 days of polymerase silencing exhibited obvious network shrinkage and loss (Fig. 4.3A,C, E). To quantify these striking observations, parasites induced for silencing of POLIB, POLIC, or POLID were observed and scored according to network size. More than 300 parasites per timepoint were classified as possessing

normal-size networks, small kDNA (networks unambiguously less than one-half the size of normal seen in uninduced cells), or no kDNA if no extranuclear DAPI-staining was observed despite viewing multiple focal planes. Loss of kDNA resulted when each polymerase was silenced (Fig. 4.3B, D, F). For example, after 4 days of POLIB silencing the percentage of parasites possessing normal sized kDNA fell to less than 3% while parasites with no kDNA represented more than 85% of the cells at this timepoint. Kinetics of kDNA loss during silencing of POLIC and POLID were also rapid, with the majority of parasites completely lacking kDNA after 4 days of silencing. Interestingly, the kinetics of network loss seen during POLID silencing were almost indistinguishable from those produced during POLIB silencing, with less than 3% of the cells viewed possessing intact networks following 4 days of RNAi.

4.4.3 Dyskinetoplastid BF parasites produced during RNAi are not viable

Previously, viable *T. brucei* that lacked portions of kDNA (dyskinetoplastids) were reported following extended treatment with the highly mutagenic DNA-binding compounds acriflavin and ethidium bromide (Schnaufer *et al.*, 2002, Stuart, 1971). A small percentage of parasites surviving ten days of polymerase RNAi were viable in the clonogenic assays (Fig. 4.1D). To address the possibilities that the viable cells following silencing had become non-responsive to RNAi or were in fact dyskinetoplastid, parasites recovered from clonogenic assays were expanded and further analyzed. Recovered parasites from POLIB RNAi induced and uninduced control cultures were stained with DAPI. All recovered cells examined possessed kDNA networks, as evident from DAPI staining (Fig. 4.4A,C), and remained sensitive to RNAi induction with similar growth inhibition patterns as the parental cells (Fig. 4.4B, D). Similar results were seen for cells

recovered following POLIC RNAi (Fig. 4.5). These data suggest incomplete knockdown, rather than the development of insensitivity to RNAi or survival of parasites in the absence of kDNA.

4.4.4 Disruption of network-free minicircle replication precedes parasite death

During kDNA replication, minicircles are released as covalently closed (CC) monomers and replicated as theta structures to produce nicked and gapped (N/G) nascent minicircles (Drew & Englund, 2001). Discontinuities in the DNA backbones of nascent minicircles decreases susceptibility to ethidium bromide induced supertwisting; thus newly replicated N/G species exhibit decreased electrophoretic mobility compared to unreplicated CC minicircles. The pattern of minicircle replication intermediates is well established in PF, with CC and N/G present in approximately equimolar amounts (Bruhn *et al.*, 2010, Liu *et al.*, 2006, Scocca & Shapiro, 2008). To ensure that the network-free mode of minicircle replication utilized by PF parasites is conserved in BF parasites, we performed two-dimensional analysis of free minicircles from single-marker cells, the parental line of our RNAi clones. Hybridization with radiolabeled oligos that detect leading and lagging strand replication intermediates revealed that the pattern of free minicircles in BF parasites was virtually indistinguishable from that of PF (Fig. 4.6A). Higher contrast images of detected membranes revealed theta structures as well as leading (MG) and lagging strand (Okazaki Fragments) specific intermediates (Fig. 4.6B).

To provide further evidence that parasite cell death during RNAi resulted from inhibition of kDNA replication, we used two-dimensional electrophoresis to assess disruption of minicircle replication. Previously, silencing of POLIB in PF parasites resulted in the persistence of unreplicated CC minicircles accompanied by the

accumulation of a multicatenane dimeric minicircle species known as fraction U (Bruhn *et al.*, 2010). Analysis of minicircle replication intermediates produced during BF POLIB silencing revealed an increase in the abundance of unreplicated minicircles relative to newly replicated progeny beginning 4 days post induction for silencing (Fig. 4.6C). As in PF, the persistence of unreplicated minicircles was accompanied by the accumulation of fraction U. These data indicate that POLIB performs a conserved role in minicircle replication in both PF and BF parasites and that disruption of kDNA replication leads to parasite cell death.

4.4.5 Disruption of mitochondrial membrane potential accompanies loss of kDNA

Viability of BF trypanosomes requires an intact ΔΨm (Brown *et al.*, 2006). The ATP-synthase complex is responsible for ΔΨm generation in BF trypanosomes and Schnaufer and colleagues demonstrated ΔΨm depolarization and lethality upon RNAi silencing of the α subunit (Schnaufer *et al.*, 2005). Loss of kDNA networks during RNAi of mitochondrial DNA polymerases resulted in depletion of maxicircles (Bruhn *et al.*, 2010, Chandler *et al.*, 2008). We anticipated the same loss of maxicircles in BF parasites (including the maxicircle-encoded subunit 6 of the ATP-synthase complex) would subsequently lead to the collapse of ΔΨm and cell death. To determine if a collapse in ΔΨm could be contributing to lethality in parasites depleted of POLIB RNAi, we used the fluorescent dye MitoTracker Red CM-H2XRos. This cell permeable dye is provided in a reduced form that fluoresces when oxidized within a polarized mitochondrion. Fluorescence microscopy analysis of uninduced parasites revealed staining of the tubular mitochondrion, indicating an intact ΔΨm (Fig. 4.7A). Parasites induced for RNAi exhibited depolarization of ΔΨm, with MitoTracker fluorescence signal dramatically

declining within 4 days of POLIB depletion (Fig. 4.7A). To provide a more quantitative analysis of ΔΨm collapse, flow cytometry analysis of MitoTracker stained cells were performed. Fluorescence intensity of control cells pre-treated with the protonophore FCCP to uncouple ΔΨm was significantly decreased compared to cells that were uninduced or pre-treated with ethanol or DMSO, the solvents used for FCCP and Mitotracker, respectively. Fluorescence intensity of POLIB-depleted cells decreased over the time course of RNAi. Mean fluorescence intensity (MFI) of parasites induced for more than 3 days was similar to that of FCCP-treated negative control parasites. Together, our findings demonstrate that loss of kDNA during DNA polymerase silencing results in depolarization of ΔΨm, which contributes to cell death.

4.5 Discussion

Individual silencing experiments for three mitochondrial DNA polymerases, POLIB, POLIC, and POLID have previously revealed essential kDNA replication roles in the PF insect stage of the parasite. This stage relies on maxicircle-encoded proteins for its oxidative phosphorylation metabolism. Alternatively, the metabolism of the disease-causing BF stage of the parasite is exclusively glycolytic (Durieux *et al.*, 1991, Tielens & van Hellemond, 2009). Therefore, the loss of kDNA would be lethal to BF parasites only if the kDNA-encoded proteins function in cellular processes besides oxidative phosphorylation. Recent studies indicate that RNA editing proteins, A6 subunit of ATP synthase, and mitochondrial translation are essential in BF trypanosomes (Cristodero *et al.*, 2010, Schnaufer *et al.*, 2005). However, silencing of TbTOPOIImt, the enzyme involved in reattaching newly synthesized minicircles to the network, in BF parasites

resulted in only mild growth and kDNA loss defects. The goal of this study was to determine whether kDNA replication proteins were essential for BF viability. Here we report rapid loss of kDNA networks upon silencing of POLIB, POLIC, and POLID, and for each polymerase, loss of kDNA was followed by cell death. This marks the first time that ablation of kDNA replication proteins results in lethality of BF *T. brucei*.

Knockdown of each polymerase gene resulted in cell death 5-6 days post RNAi induction (Fig. 4.1). This likely indicates that loss of proteins encoded by kDNA, rather than depletion of enzymes required for its replication, is the primary cause of cell death. Proper mitochondrial function is required for numerous processes critical to cell physiology, including energy metabolism, calcium homeostasis and signaling, and generation of membrane potential (Brown *et al.*, 2006). This creates an attractive paradigm in which inhibition of a single kDNA replication enzyme could lead to the disruption of multiple cellular pathways, effectively creating a multi-potent effect from inhibiting a single target. Maintenance of the mitochondrial membrane potential is clearly amongst these pathways, as we demonstrate here (Fig. 4.7). Yet unknown functions for kDNA-encoded proteins likely exist and may prove essential in BF parasites. Alternative editing of maxicircle transcripts is hypothesized to increase mitochondrial protein diversity (Ochsenreiter *et al.*, 2008b). Indeed AEP1, a product of alternative editing of cytochrome oxidase III, was identified as a kDNA maintenance factor in BF *T. brucei* (Ochsenreiter *et al.*, 2008). Additionally, maxicircle coding sequence also contains three "maxicircle unidentified reading frames" (MURFS) and a series of GC-rich regions predicted to encode a series of highly hydrophobic proteins of unknown function (Schnaufer *et al.*, 2002). A more complete understanding of these

components and the repertoire of proteins produced by alternative editing of maxicircle transcripts may reveal additional indispensable functions of kDNA encoded components for BF parasites.

Our current functional analyses of the kDNA replication proteins POLIB, POLIC, and POLID indicate that the essential roles of these proteins in kDNA replication appear consistent in both life cycle stages examined. Silencing each of the polymerase resulted in loss of kDNA networks and was accompanied by changes in the repertoire of free minicircle species. For example, when silencing POLIB, unreplicated CC monomers persisted and fraction U accumulated with the BF results indistinguishable for those obtained when silencing POLIB in PF parasites (Fig. 4.6). Additionally the kinetics of kDNA loss for POLIB and POLID were nearly identical, again similar to the results obtained from the PF silencing experiments (Fig. 4.3). Interestingly, when comparing the rate of kDNA loss however, the BF parasites appear to lose their kDNA with faster kinetics. While it takes nearly 20 doublings for cells to lose their kDNA in PF POLIB silencing (52% no kDNA, 40% small kDNA), it took only 12 generations for BF parasites to lose their kDNA (90% no kDNA). Currently we do not understand why kDNA loss occurs more rapidly in BF parasites but life stage-specific cell cycle checkpoints have been identified and may contribute to these differences (Hammarton, 2007).

This study is the first in-depth analysis of kDNA replication protein function in BF parasites. Previous functional studies of kDNA replication proteins have been performed in PF, including those from our laboratory, yet the relevance of these analyses to drug development mandates essential function in disease-causing BF parasites. Focus

on the PF stage is largely the result of highly efficient stable transfection methods for this form of the parasite. While standard transfection methodologies yield efficiencies of 10^{-3} – 10^{-6} in PF *T. brucei*, the technique is remarkably less successful in BF parasites (10^{-7} – 10^{-8}) (Li & Gottesdiener, 1996, McCulloch *et al.*, 2004, Asbroek *et al.*, 1990, Carruthers *et al.*, 1993). However, the recent application of nucleofection to BF parasites increased stable transformation efficiency nearly 1000 fold, providing greater opportunity to examine kDNA replication protein function in this disease-causing life cycle stage (Burkard *et al.*, 2007). When silencing the three DNA polymerases, we found that parasites that survived RNAi (proliferated in clonogenic assays) still contained intact kDNA networks and remained responsive to induction for RNAi, as evident from growth kinetics and loss of kDNA during RNAi (Fig. 4.4, Fig. 4.5). Sustained sensitivity to induction for RNAi is noteworthy here, since RNAi resistant "revertant" parasites have been widely reported in both PF and BF *T. brucei* (Krazy & Michels, 2006, Chen *et al.*, 2003, Urbaniak, 2009, Milman *et al.*, 2007, Galland *et al.*, 2007, Moyersoen *et al.*, 2003). The reasons why revertants were not produced when silencing POLIB, POLIC, or POLID is beyond the scope of our analyses. Sustained RNAi sensitivity, however, was critical in determining the essential contribution each of these mitochondrial DNA polymerases makes to the viability of BF *T. brucei*.

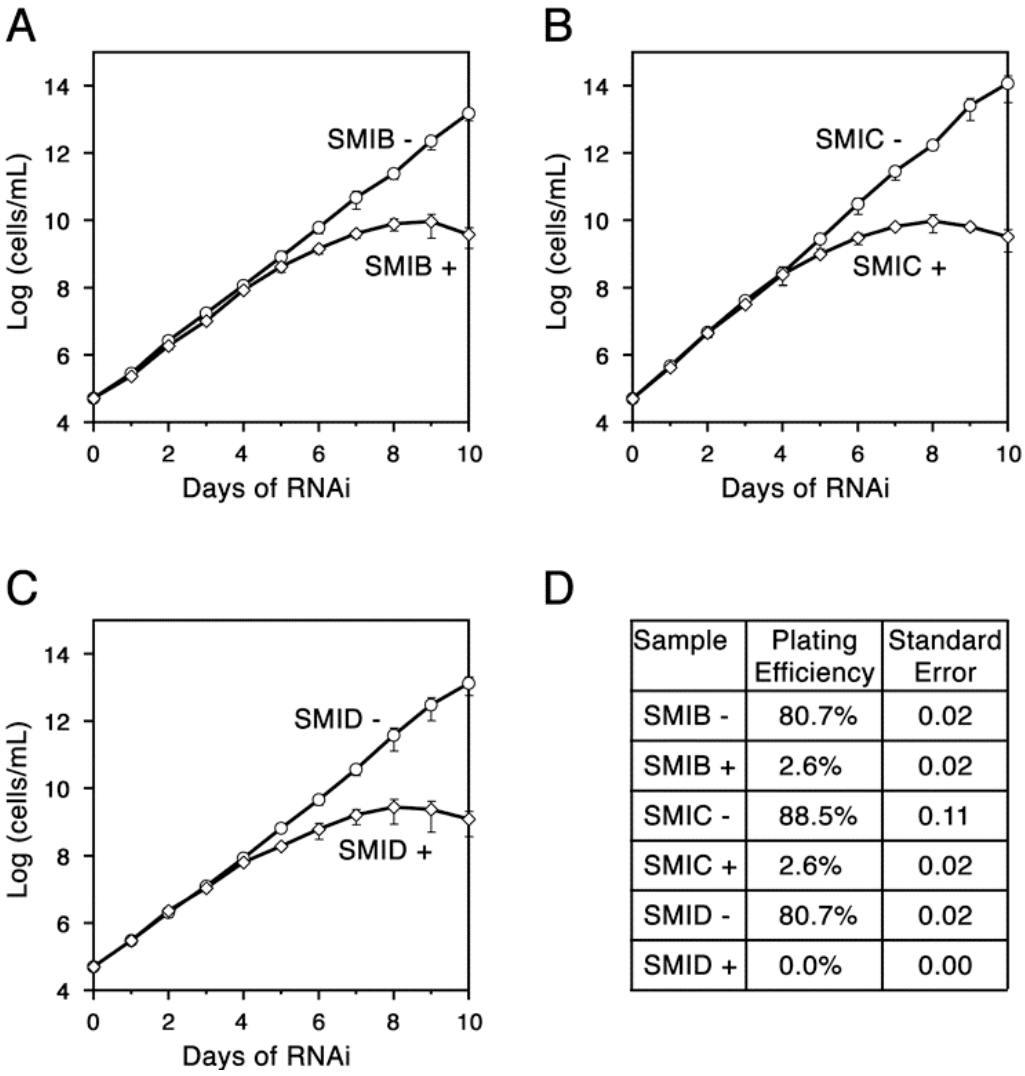
The demonstration that BF *T. brucei* cannot survive without kDNA is fundamental in evaluating kDNA replication proteins as drug targets. Yet, trypanosomes lacking functional portions of their kDNA genome exist in nature (*T. evansi* and *T. equiperdum*) and have been generated through prolonged culture in the presence of mutagenic conditions (Schnaufer *et al.*, 2002, Stuart, 1971). Although permissive in those

BF parasites, partial loss of kDNA locks the parasite into a monomorphic life cycle that is unable to survive in the tsetse fly vector and, therefore, spread from an infected host (Schnaufer, 2010, Lun *et al.*, 2010). Naturally dyskinetoplastid *T. equiperdum* and *T. evansi*, as well as an acriflavine-induced dyskinetoplastid strain of *T. brucei* were recently found to possess mutations in the nuclear encoded γ subunit of ATP synthase complex. These mutations are proposed to compensate for the loss of maxicircle-encoded subunit A6 of this complex (Lun *et al.*, 2010, Schnaufer, 2010, Schnaufer *et al.*, 2005). The compound ethidium bromide inhibits kDNA replication in BF parasites yet, viable dyskinetoplastid *T. brucei* have not emerged despite decades of the compound's use in treating infected animals suggesting that compensatory nuclear mutations occur at low frequency (Roy Chowdhury *et al.*, 2010). Additionally, RNAi of other proteins required for kDNA function (particularly RNA editing) fails to produce viable dyskinetoplastid parasites. Chemical inhibitors of kDNA replication proteins would likely inactivate target proteins even more rapidly than RNAi, thus decreasing the window of time for selection of these low frequency mutations.

Our current study adds to a rapidly growing body of literature indicating that kDNA is required for BF mitochondrial physiology and, thus, viability. Indeed two available treatments for sleeping sickness, pentamadine (in humans) and ethidium bromide (in livestock) appear to target kDNA (Shapiro & Englund, 1990, Roy Chowdhury *et al.*, 2010). The historical success of drugs targeting kDNA and our finding that mitochondrial DNA polymerases IB, IC, and ID are essential in BF parasites indicate that targeting kDNA replication proteins remains a promising approach for the discovery of new anti-trypanosomal drugs.

4.6 Acknowledgements

We thank Dr. Bibo Li (Cleveland State University) for performing transfections and providing cells lines used in this study and Christina Arieta (University of Massachusetts, Amherst) for assistance with flow cytometry. We are additionally grateful to Dr. Arthur Günzl (University of Connecticut Health Center) for his gift of the pT7-stl RNAi vector and the laboratory of Dr. Nagendra Yadava (Pioneer Valley Life Sciences Institute, Springfield, MA) for providing the FCCP membrane potential uncoupler. We appreciate useful comments and discussion on this manuscript from Drs. James Morris, Arthur Günzl, and Derrick Robinson. This research was supported by NIH Grant AI066279 to M.M.K.



Sample	Plating Efficiency	Standard Error
SMIB -	80.7%	0.02
SMIB +	2.6%	0.02
SMIC -	88.5%	0.11
SMIC +	2.6%	0.02
SMID -	80.7%	0.02
SMID +	0.0%	0.00

Figure 4.1: Effect of DNA polymerase RNAi on bloodstream form cell viability
(A-C) Clonal cell lines were grown in the absence (open circles) or presence (open diamonds) of tetracycline (1 μg/mL) to induce for RNAi. Cell density was plotted as the product of cell number and total dilution. Mean and standard error of three separate RNAi inductions are presented for clonal cell lines (A) SMIB A24, (B) SMIC A15, and (C) SMID A13. (D, Table) Results of clonogenic assays performed with parasites that were uninduced (-) or induced (+) for 10 days of RNAi prior to plating. Viability (plating efficiency) and standard error from two separate inductions are presented for each clonal cell line.

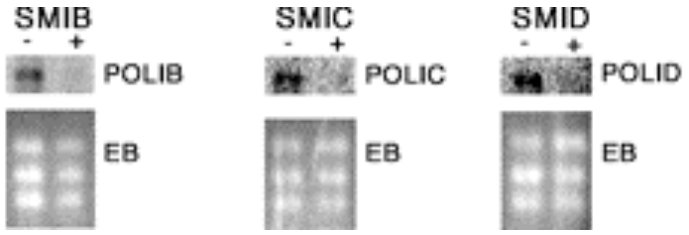


Figure 4.2: Knockdown of DNA polymerase mRNA in bloodstream form using stemloop RNAi

Northern Blot of RNA isolated from 5 x 10^7 parasites induced for 0 or 48 hours of RNAi of the gene indicated. Membranes were hybridized with 32-P labeled probes as described previously (Bruhn *et al.*, 2010).

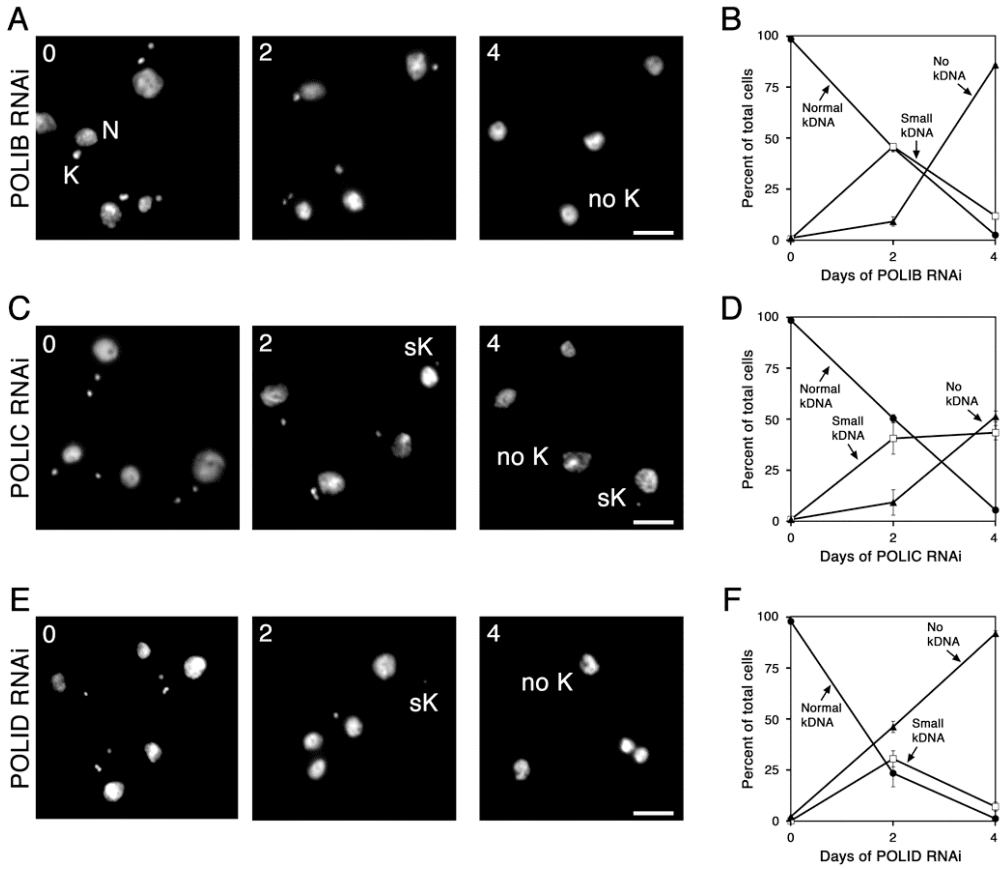


Figure 4.3: Kinetics of kDNA loss during bloodstream form DNA polymerase silencing

(A,C,E) Representative images of parasites induced for indicated number of RNAi for (A) POLIB, (C) POLIC, or (E) POLID. (B,D, F) Kinetics of kDNA loss were determined by classifying cells as possessing normal sized kDNA (closed circles), small kDNA (open squares), or no kDNA (closed triangles). The mean and standard error from two inductions are presented for parasites depleted of (B) POLIB, (D) POLIC, and (F) POLID. Abbreviations: N, nucleus; K, normal sized kDNA; sK, small kDNA; no K, no kDNA. Scale bar, 5 μM.

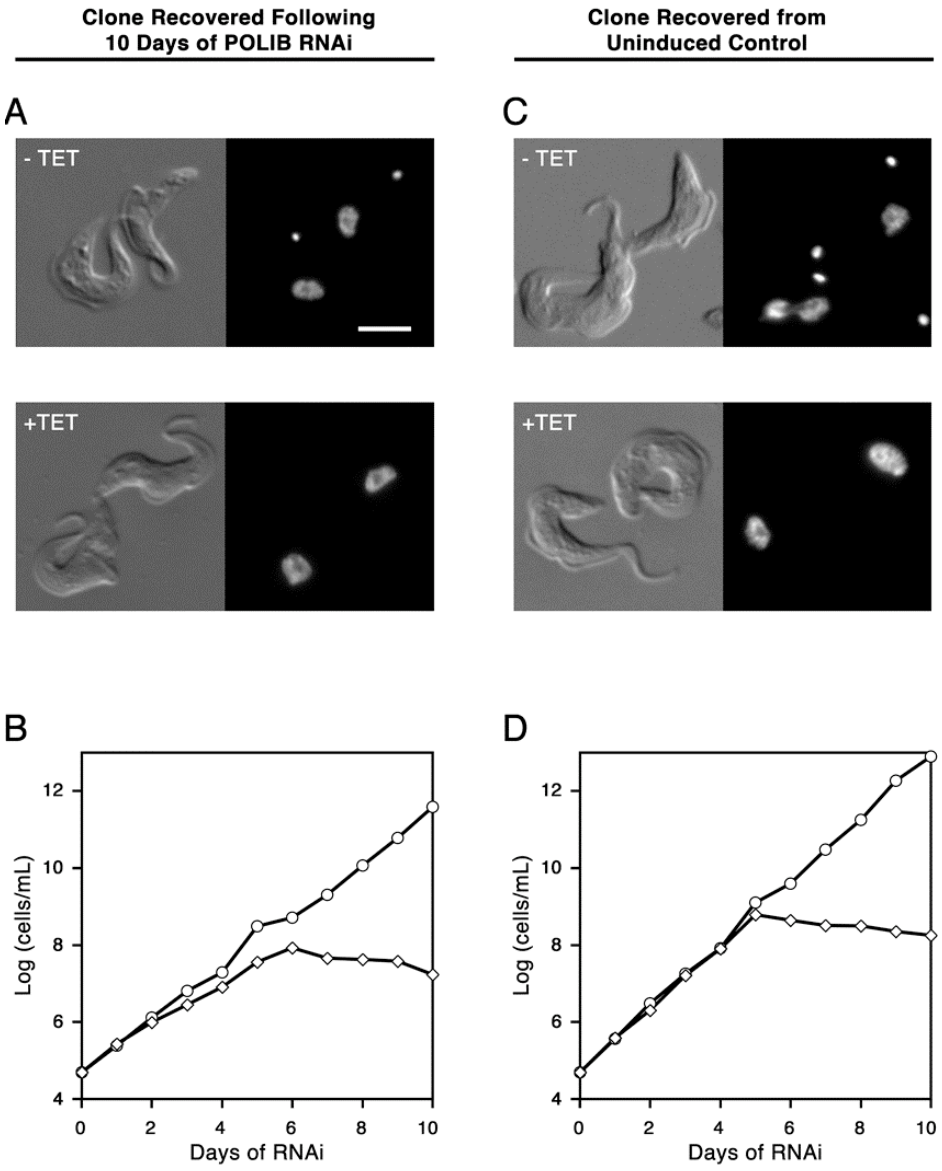


Figure 4.4: Analysis of parasites recovered from POLIB clonogenic assays
(A-B) Parasites viable in clonogenic assays of cultures induced for POLIB RNAi were recovered and examined for presence of kDNA and sensitivity to RNAi. (A) DIC and fluorescence microscopy images of DAPI stained parasites that were grown in the presence or absence of tetracycline. (B) Growth curve of parasites recovered from clonogenic assays of POLIB RNAi induced cultures. (C-D) Parasites viable in clonogenic assays of uninduced control cultures were recovered and assessed for RNAi sensitivity as in A-B. Scale bar, 5 μM.

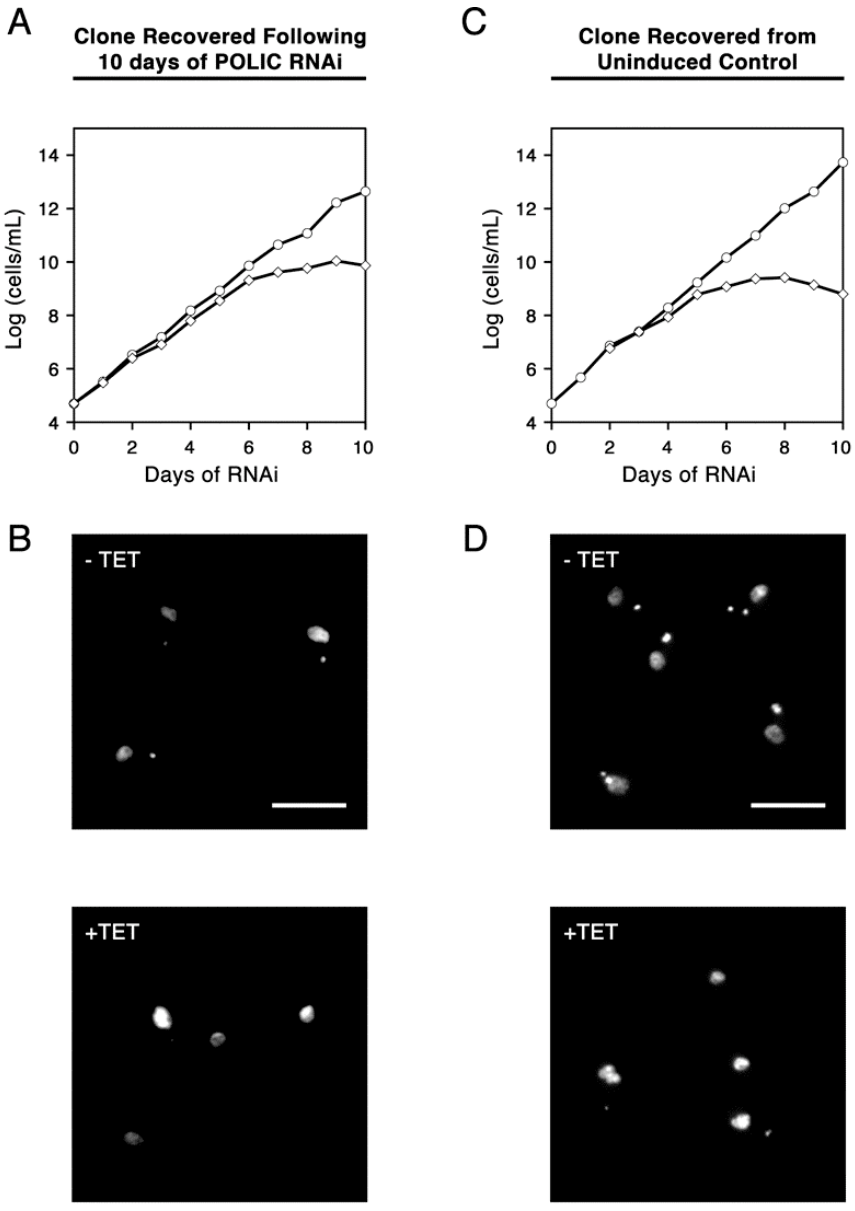


Figure 4.5: Analysis of parasites recovered from POLIC clonogenic assays
(A-B) Parasites viable in clonogenic assays following POLIC RNAi were recovered and examined RNAi sensitivity and presence of kDNA. (A) Growth curve of parasites recovered from clonogenic assays (B) Images of DAPI-stained parasites that were grown in absence (-) or presence (+) of tetracycline. (C-D) Parasites recovered from clonogenic assays of uninduced cultures were assessed for RNAi sensitivity as in A-B.

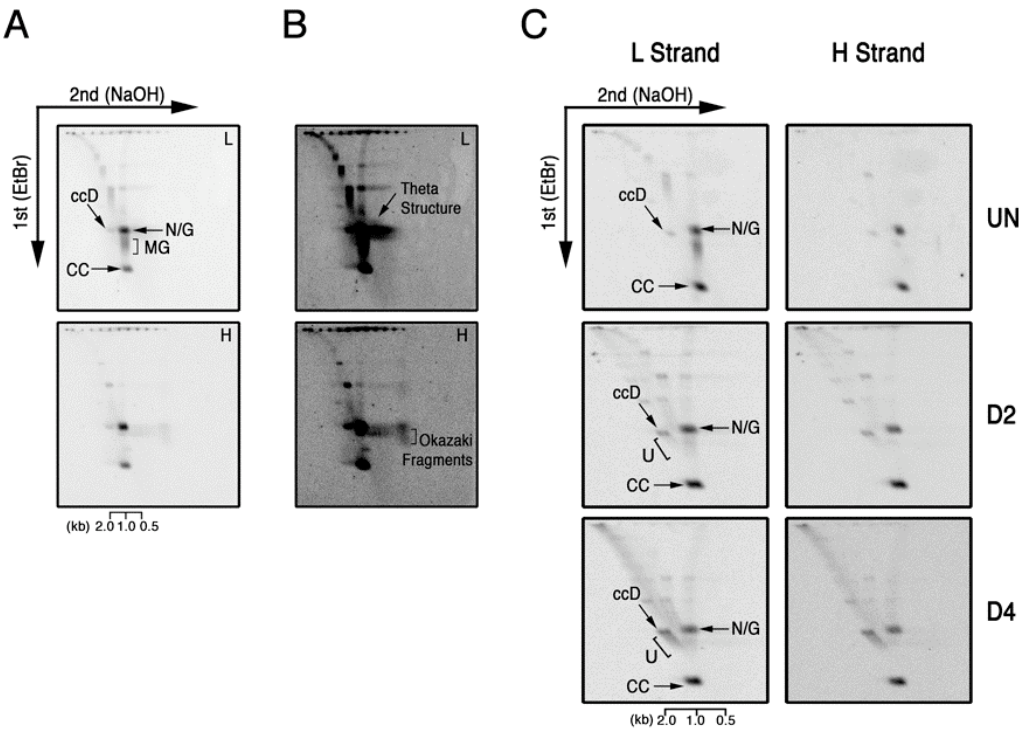


Figure 4.6: Analysis of minicircle replication intermediates in parental and POLIB-depleted parasites

Neutral/alkaline two-dimensional gel electrophoresis of free minicircles. (A) Total DNA from parental parasites was separated in the presence of ethidium bromide and then under denaturing conditions (NaOH) prior to transfer to membrane. Minicircle replication intermediates were detected with oligomers that specifically hybridize to leading (L) or lagging (H) strand intermediates. (B) Higher contrast images of membranes presented in panel A. Contrast was adjusted equally in images of membranes to visualize abundance of theta structures and Okazaki fragments (C) Two-dimensional analysis of parasites induced for indicated number of days of POLIB RNAi. Abbreviations: CC, covalently closed; ccD, covalently closed dimer; MG, multiply gapped; N/G, nicked/gapped; U, Fraction U.

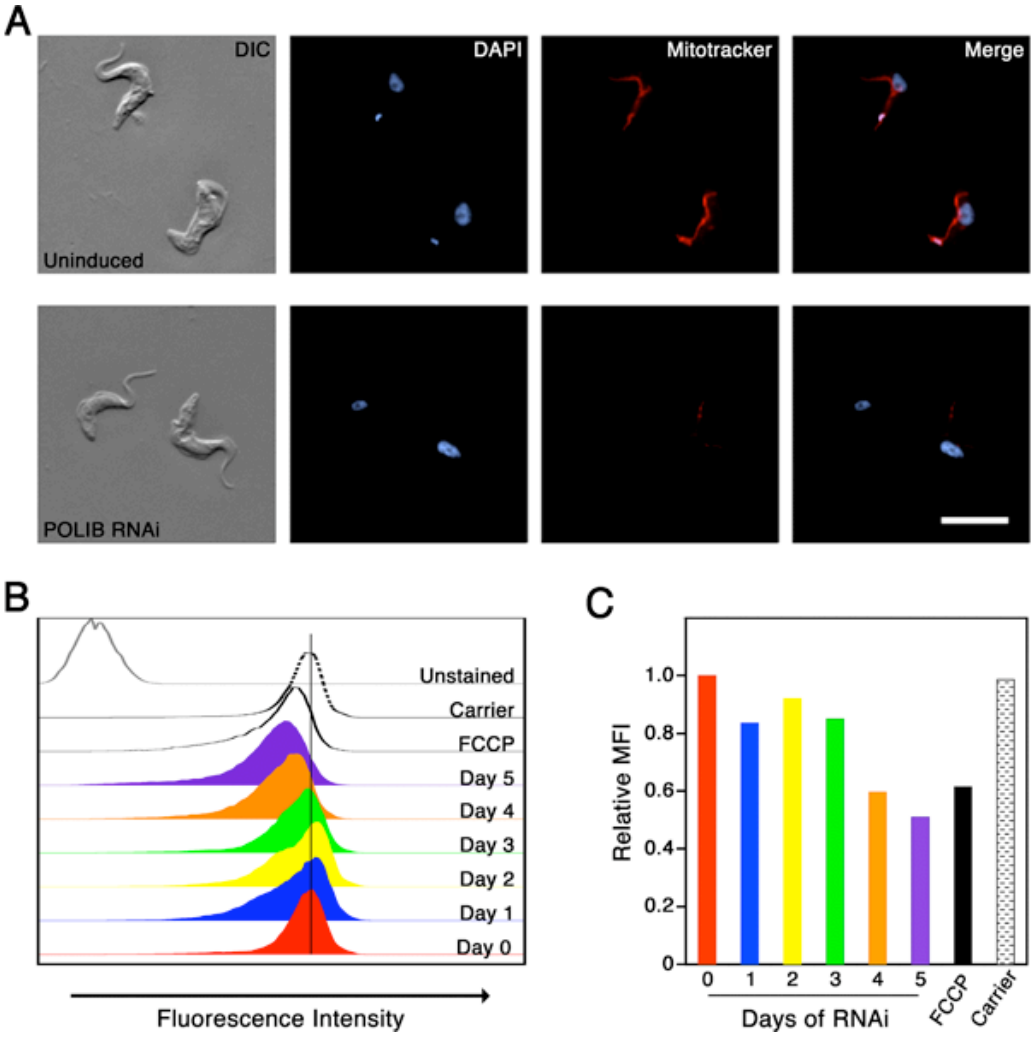


Figure 4.7: Disruption of mitochondrial membrane potential during DNA polymerase silencing

(A) Representative images of MitoTracker stained parasites that were either uninduced or induced for 4 days of POLIB RNAi. (B-C) Flow cytometry analysis of MitoTracker stained parasites. Unstained control; parasites treated with DMSO (used as solvent for MitoTracker solutions), FCCP; protonophore used as negative control, Carrier; parasites treated with ethanol (used as a carrier for FCCP). (B) Histogram showing fluorescence intensity of indicated samples. (C) Relative mean fluorescence intensity (MFI) of parasites presented in panel B. Unstained background was subtracted from raw MFI values prior to graphing adjusted MFI relative to that of uninduced cells.

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APPENDIX

A GROUNDWORK FOR *IN VIVO* FUNCTIONAL ANALYSES

5.1 Abstract

The mitochondrial genome of Kinetoplastid protists is known as Kinetoplast DNA, or kDNA. kDNA is a massive network composed of thousands of catenated minicircles and dozens of maxicircles. Each minicircle is attached to three others within the network, which is compacted into a disk-like structure close to the flagellar basal body. Replication of kDNA is a highly complex process that requires at least three mitochondrial DNA polymerases (Pols) related to bacterial polymerase I. Silencing of each polymerase (POLIB, POLIC, and POLID) is lethal and results in loss of kDNA networks. Thus it appears that each polymerase makes a specific contribution to kDNA replication. POLIB, POLIC, and POLID each possess conserved motifs of family A DNA polymerases and are predicted to adopt the canonical "right hand" polymerase structure. *In vivo* dissection of protein activities will reveal the unique contributions each makes to kDNA replication and, hence, cell viability. Several important tools and strategies for *in vivo* functional analyses have been established and are described here.

5.2 Introduction

Mitochondrial DNA replication in trypanosomes (kinetoplast DNA, kDNA) is a remarkably complex process, reflecting the highly unusual topology of kDNA - a network of over 5000 catenated DNA circles (Morris *et al.*, 2001). Minicircles, which are

1kb each and account for ~90% of kDNA mass, are released from the network, replicated as theta structures, and then reattached to the network. The other network component, maxicircles, are ~23 kb each and are replicated while still attached to the network. At least 5 DNA polymerases contribute to kDNA replication. Two Family B DNA polymerases (Pol β and Pol β PAK) participate in Okazaki fragment processing and three Family A DNA polymerases similar to prokaryotic DNA Pol I (POLIB, POLIC, and POLID) appear necessary for synthesis (Bruhn *et al.*, 2010, Chandler *et al.*, 2008, Klingbeil *et al.*, 2002, Saxowsky *et al.*, 2003). POLIB, POLIC, and POLID are essential for parasite survival. Silencing these Pol I-like polymerases results in loss of kDNA networks, suggesting each makes a unique contribution to kDNA replication.

Family A DNA polymerases are found in a wide range of organisms yet share a common "cupped right hand" overall structure composed of three subdomains (Lange *et al.*, 2011). The palm subdomain includes residues critical for catalysis and, along with the thumb subdomain, interacts with the primed DNA template (Marini *et al.*, 2003). The fingers subdomain switches between an "open" and "closed" state to form a binding pocket with appropriate geometry for substrate recognition and incorporation of the proper nucleotide (Loh & Loeb, 2005). The structure of family A DNA polymerases can be further described by a series of motifs and highly conserved amino acid residues critical for accurate and processive DNA synthesis (Fig. 5.1, Fig. 5.2A). Motif 1 is located at the tip of the thumb and contributes to processivity through its contacts with the phosphate backbone of the DNA template and primer. Contacts with the template phosphate backbone are also made by motif 2 (Loh & Loeb, 2005). Motif 2 consists of two beta strands that make additional contacts with the minor groove (Marini *et al.*,

2003). Motif 3, also known as motif A, is highly conserved in all DNA and RNA polymerases. Residues in this portion of the palm subdomain make contacts with the phosphate backbone and RNA primer bases (Kiefer *et al.*, 1998). Motif A contains 1 of 2 aspartic acid residues strictly required for polymerase activity, serving to coordinate divalent cations necessary for catalysis (Loh & Loeb). Motif 4 (motif B) contributes to enzyme substrate recognition (recognition of incoming dNTPs) and, hence, fidelity. The second critical aspartic acid residue is found in motif 5 (motif C), two antiparallel β strands located in the palm subdomain (Marini *et al.*, 2003). An additional motif located in the palm, Motif 6, is an alpha helix that interacts with the minor groove of the DNA template and appears to contribute to processivity and fidelity (Loh & Loeb, 2005). Variations in these conserved motifs can significantly alter enzyme properties that may lead to specialized cellular functions (Loh & Loeb, 2005, Marini *et al.*, 2003). Therefore the recognition of both novel and conserved residues in motifs of trypanosome mitochondrial DNA polymerases could be used to form testable hypotheses and provide valuable insight into individual polymerase characteristics and cellular functions.

Studies of Family A DNA polymerase structure-function relationships studies are of tremendous importance for interests ranging from development of polymerases with traits desired for laboratory applications (such as increased fidelity and velocity) to correlation of structural changes in mutated polymerases with development of cancer (Longley *et al.*, 2005, Lange *et al.*, 2011). Knowledge of family A motifs has many applications to the study of kDNA polymerases. The most immediate application is the *in vivo* dissection of the polymerase contributions to kDNA replication, namely determining which domains/activities of each polymerase are required for cell viability. Mutations

introduced to alter polymerase properties (such as fidelity) have been successfully used to dissect the leading and lagging strand-specific contributions of eukaryotic polymerases (ε and δ) and could be applied to dissecting the *in vivo* functions of *T. brucei*'s multiple mitochondrial polymerases (Burgers, 2009, Pursell *et al.*, 2007).

Analysis of primary sequences for POLIB, POLIC, and POLID confirmed 5 of 6 known family A DNA polymerase motifs are present. Aspartic acid residues critical for polymerase activity are conserved in all three proteins and homology modeling suggests that these residues are positioned within the active site of each polymerase's conserved right hand structure. Much progress has been made in generating an appropriate strategy and materials to examine specificity of RNAi and finely resolve the functions of mitochondrial DNA polymerases *in vivo*. This progress and materials are described here.

5.3 Material and Methods

5.3.1 Homology Modeling

Residues corresponding to the C-terminal 1200 residues of POLIB (Tb11.02.2300), POLIC (Tb927.7.3990), and POLID (Tb11.02.0770) were used as the query for the Protein Homology/analogY Recognition Engine (Phyre). Phyre is an ensemble homology modeling server that, based on the query sequence, identifies homologues with solved protein structures, uses multiple threading programs to generate a three dimensional model of the protein of interest. Importantly, Phyre does not model

regions of low homology. Provided atomic coordinates were imaged using PyMOL software. Relevant polymerase motifs in the resulting models for POLIB, POLIC, and POLID were colorized for future analysis and comparison with crystal structures deposited in the Protein Data Bank (PDB).

5.3.2 Trypanosome Growth

Procyclic *T. brucei* (29-13 line) were cultured as described previously, at 27°C in SDM-79 medium containing 15% heat-inactivated fetal bovine serum in the presence of G418 (15 μg/mL), and hygromycin (50 μg/mL) (Bruhn *et al*. 2010).

5.3.4 Inducible Overexpression

The overexpression plasmid pLew79MHTAP was used to create pLew79IBMHTAP, suitable for tetracycline inducible overexpression of POLIB with a C-terminal Myc-6His-Tandem Affinity Purification tag (Jensen et al., 2007). The full-length coding sequence for POLIB (including its predicted mitochondrial targeting sequence) was PCR amplified using Phusion Polymerase (NEB) and the gene specific primers 5'-CTCGAGATGCGGCTAAATAGCTGCTGG-3' and 5'-CTCGAGCACCGTAATTTCACTGTCAG-3' with appropriate XhoI restriction enzyme linkers (underlined). The resulting product and the empty vector were digested with XhoI. Digested backbone and insert were gel extracted without exposure to UV and ligated. The resulting plasmid, pLew79IBMHTAP was transformed into *E. coli*. Positive transformants (with POLIB in the desired orientation) were identified using restriction enzyme analysis. The positive plasmid chosen was sequenced and found to possess no mutations, which could be introduced during PCR. pLew79IBMHTAP was linearized

with NotI and transfected into 29-13 parasites. Phleomycin used to select for positive transfectants. The resulting parasite population was cultured in the presence of G418 (15 μg/mL), and hygromycin (50 μg/mL) and phleomycin (2.5 μg/mL). Tetracycline was added to culture media to induce for expression of POLIBMHTAP.

5.3.5 RNA Interference

A ~500 bp region of the POLIC 3' untranslated region (utr) was PCR amplified using forward and reverse primers 5'-AAGCTTGCGGAGGTGAGGAGTAGCGTCG-3' and 5'-TCTAGAGTGTAGTAATCAGGGCGACG-3' which amplified the target sequence and HindIII and XbaI linkers, respectively. For insertion into the plasmid pJM326, the resulting PCR product was digested with XbaI and HindIII and ligated into pJM326 that had been digested with NheI and HindIII. Ligation of cohesive compatible ends of XbaI and NheI resulted in destruction of these sites and the production of pJM326-ICutr. The same ~500bp region of the 3' UTR was PCR amplified as above, except the linker used for the forward primer was MluI. The resulting product digested with MluI and XbaI and ligated into the corresponding sites on pLew100, resulting in pLew100-ICutr. pJM326-ICutr was digested with XbaI and HindiIII and the liberated band corresponding to the ~500 bp region plus a ~500 bp stuffer was gel extracted and ligated into the XbaI/HindIII sites of pLew100-ICutr to generate the plasmid "stem loop –IC UTR" (pStl-ICutr). pStl-ICutr was linearized with EcoRV and transfected into 29-13 cells. Positive transfectants were selected using phleomycin and cultured in SDM79 media containing G418 (15 μg/mL), and hygromycin (50 μg/mL) and phleomycin (2.5 μg/mL).

5.3.6 Constitutive Expression of POLIC

Plasmid pC-POLICPTPpuro, previously generated by replacing the phleomycin resistance cassette of pC-POLICPTP with a puromycin resistance cassette was transfected into 29-13 cells or 29-13 cells containing pStlICutr (Bruhn *et al.*, 2010).

5.4 Results and Future Applications

5.4.1 Homology modeling reveals a conserved "right hand" structure for POLIB, POLIC, and POLID

Family A DNA polymerases share a series of structural elements that contribute to specialized polymerase characteristics, such as fidelity, processivity, substrate recognition/nucleotide selectivity. The motifs can be identified using bioinformatics analysis, including multiple sequence alignments with well-studied family A DNA polymerase. POLIB, POLIC, and POLID each possess five of the six family A motifs, including residues predicted critical for catalysis (Fig. 5.1A). Proper positioning of each motif within the right hand polymerase structure is crucial for their contributions to catalysis and protein-specific characteristics (Fig. 5.2A). Homology modeling was used to predict if the motifs conserved in POLIB, POLIC, and POLID are spatially oriented to make contacts with template and products. Threading using the Phyre Server predicted that each polymerase adopts the characteristic three-dimensional right hand structure (Fig. 5.2, B-D). Conserved motifs were predicted to occupy appropriate positions of polymerase subdomains (fingers, palm, thumb) with critical aspartic acids of motifs 3 and 5 located within the predicted catalytic site. Therefore, each of the examined *T. brucei*

mitochondrial DNA polymerase appears suited to contribute polymerase activity to kDNA replication and repair processes.

5.4.2 Overexpression of POLIB is not lethal to the parasites and may be used for dominant negative analyses.

Efforts to study the enzymatic properties of POLIB *in vitro* have been hampered by consistent production of POLIB as insoluble and truncated protein when using heterologous expression (*E. coli* and insect cells). Numerous attempts to favor soluble protein production of POLIB - including titration of IPTG for induction of expression, expression at decreasing temperatures, ethanol/cold shocks to upregulate bacterial chaperone expression, and even outsourcing production to a private company – failed to yield soluble POLIB. Given that these difficulties might arise from improper post-translational modification in a heterologous expression system, we chose to overexpress affinity-tagged POLIB in procyclic form *T. brucei* using the vector pLew79IBMHTAP. Overexpression of POLIBMHTAP was not cytotoxic, as parasites induced for more than 10 days of expression exhibited growth kinetics indistinguishable from uninduced controls (Fig. 5.3). Importantly, immunofluorescence microscopy (kindly performed by Jeniffer Concepción) indicated that POLIBMHTAP is successfully targeted to the mitochondrion. These pilot overexpression studies suggest that POLIBMHTAP is suitable for several desired applications including (1) immunoprecipitation to assay for enzymatic activity, (2) tandem affinity purification to identify interacting proteins (3) overexpression of mutant versions of POLIB to dissect enzymatic and essential activities.

5.4.3 Polymerase silencing by targeting untranslated regions

Previous constructs for RNAi of POLIB, POLIC, and POLID were designed to specifically target a 500bp C-terminal region. Although these target regions chosen lack homology to other sequences within the parasite's genome, the influence of off-target effects cannot be ruled out and, therefore, must be addressed. One effective strategy for examining potential off-target effects is to target endogenous transcripts for degradation by producing UTR-specific dsRNA while simultaneously driving transcription of the target gene with a heterologous UTR that does not share identity with the endogenous (target) UTR. An inducible RNAi vector suitable for this approach, pStl-utrIC was generated and transfected into 29-13 parasites, a procyclic cell line engineered to expression T7 RNA polymerase and tetracycline repressor protein. Integration of chromosomal tagging vectors replaces the endogenous gene's UTR with sequence lacking sufficient identity to the dsRNAi trigger. Thus, transcription from the tagged allele produces mRNA refractory to RNAi. If the phenotype produced during RNAi is the result of specific silencing (rather than off target effects), silencing the endogenous transcript in the presence of RNAi refractory transcript is anticipated to rescue the phenotype, with no distinguishable differences between uninduced and induced parasites.

5.4.4 Constitutive expression of POLIC (and mutants) refractory to RNAi

Silencing endogenous POLIC transcripts in the presence of an RNAi refractory POLIC transcript can also be used to dissect enzyme function *in vivo*. The family A polymerase domain of POLIC is the only predicted domain in the protein yet accounts for less than 20% percent of the total coding sequence. It is, therefore, possible that the

essential activity POLIC makes for kDNA replication (and/or cell viability) is not limited to nucleotide incorporation. Identifying the essential contribution(s) that POLIC will deepen understanding of kDNA replication processes while enabling the design of screens for antitrypanosomal compounds inhibiting the enzyme's essential function. An approach similar to that described above for examining specificity of RNAi can be taken to introduce an RNAi refractory allele of POLIC with ablated polymerase activity. Aspartic acid residues critical for polymerase activity have been identified and mutagenic PCR primers designed to mutate these residues to alanines (Fig. 5.4). A clonal population for UTR-targeted RNAi (pStl-ICutr) is actively being selected for at the time of this dissertation and will be transfected with either POLICPTP or POLICPTP(D1380A/D1592A). Induction for RNAi in resulting cell line (29-13/pStl-ICutr/POLICPTP(D1380A/D1592A) will be lethal is the polymerase activity of POLIC is amongst its essential function(s). Adequate constructs were designed and generated to examine specificity of POLIB and POLID RNAi and are available for future analyses.

5.5 Additional Discussion

An attractive application of family A DNA polymerase structure-function relationships is applying this knowledge to the design of target driven, cell-based high-throughput drug screens. Identification of pathogen-specific drug targets without counterpart humans decreases the chance for identification of compounds with off-target effects. Nonetheless, inhibitor screens performed on heterologously expressed proteins in a non-native environment are not strictly representative of the cellular conditions under

which the compound must succeed in clinical trials and following approval. The in-depth knowledge of the molecular biology and cellular functions of replicative polymerases (such as that acquired in this thesis work and related studies), however, can be applied to design cell-based inhibitors screens for a specific target polymerase. For example, mutations known to increase efficiency of fluorescent nucleotides could be introduced into a target kDNA replication polymerase and hit compounds identified by a readout of decreased fluorescence (Anderson *et al.*, 2005, Giller *et al.*, 2003, Ghadessy *et al.*, 2004). Several molecular tools for resolving the specialized activities of POLIB, POLIC, and POLID have been generated and hold future potential for answering a long list of intriguing questions about kDNA replication in *T. brucei*.

Trypanosomes are amongst the earliest diverging eukaryotes to posses a mitochondrion. Perhaps reflective of its unique evolutionary position, this group of eukaryotes utilizes DNA polymerases with apparently bacterial origin. Further, our studies are supportive of a minicircle replication mechanism in which prokaryotic-like DNA polymerases contribute to asymmetric, non-strand coupled synthesis bearing similarities to eukaryotic nuclear replication. While our findings do not rule the possibility that POLIC contributes to minicircle replication, they suggest that POLIB and POLID both participate at the minicircle replication fork. Biochemical experiments (including co-immunoprecipitation of POLIB with POLID and purification of replication complexes using tandem affinity purification) are ongoing in the Klingbeil laboratory. These future experiments will expand knowledge of minicircle replication dynamics gained through this dissertation research and deepen biological understanding of kDNA replication proteins, a new class of drug targets for combating African trypanosomiasis.

A

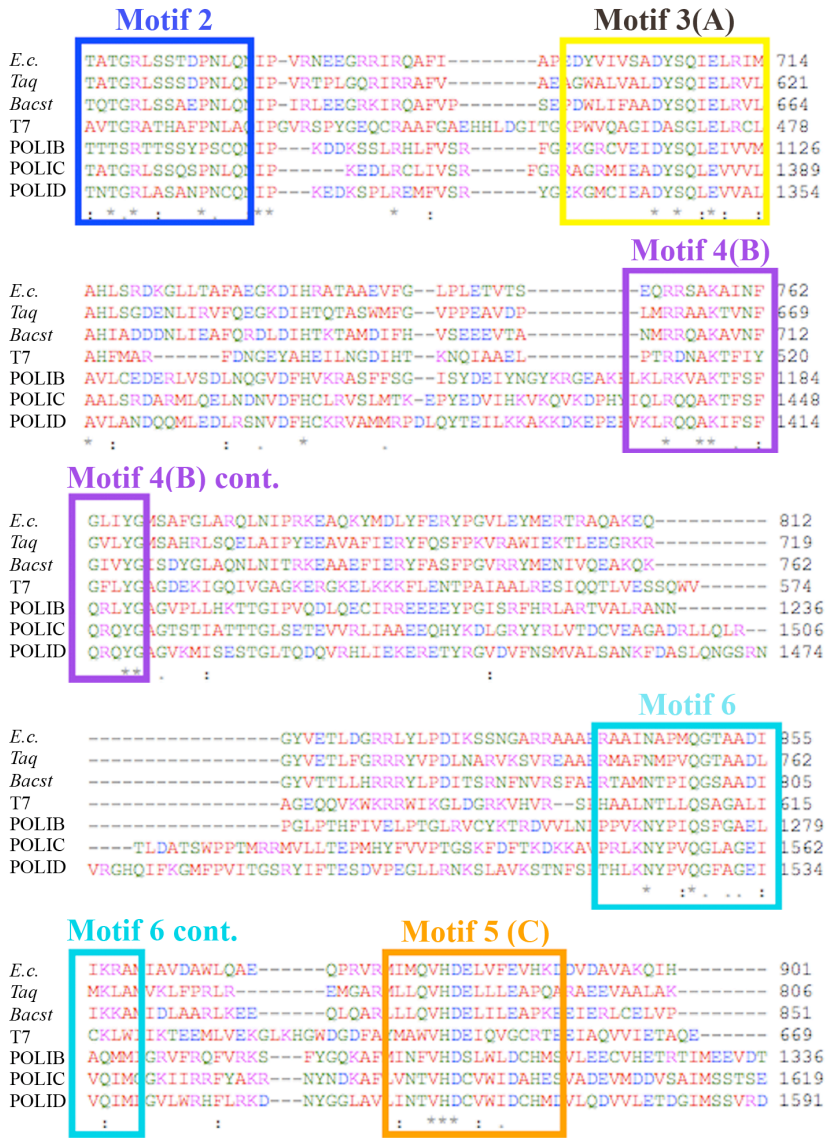


Figure 5.1: Conserved Family A DNA polymerase motifs in POLIB, POLIC and POLID

Multiple sequence alignment of Family A DNA polymerases performed with ClustalW. Residues corresponding to motifs 2-6 are indicated by colorized boxes. Abbreviations: E.c, *E. coli* Polymerase I; *Taq*, *Thermus aquaticus*; *Bacst, Bacillus Stearothermophilus*; T7, Bacteriophage T7; POLIB, *T. brucei* mitochondrial DNA polymerase IB; POLIC, *T. brucei* mitochondrial DNA polymerase IC; POLID, *T. brucei* mitochondrial DNA polymerase ID.

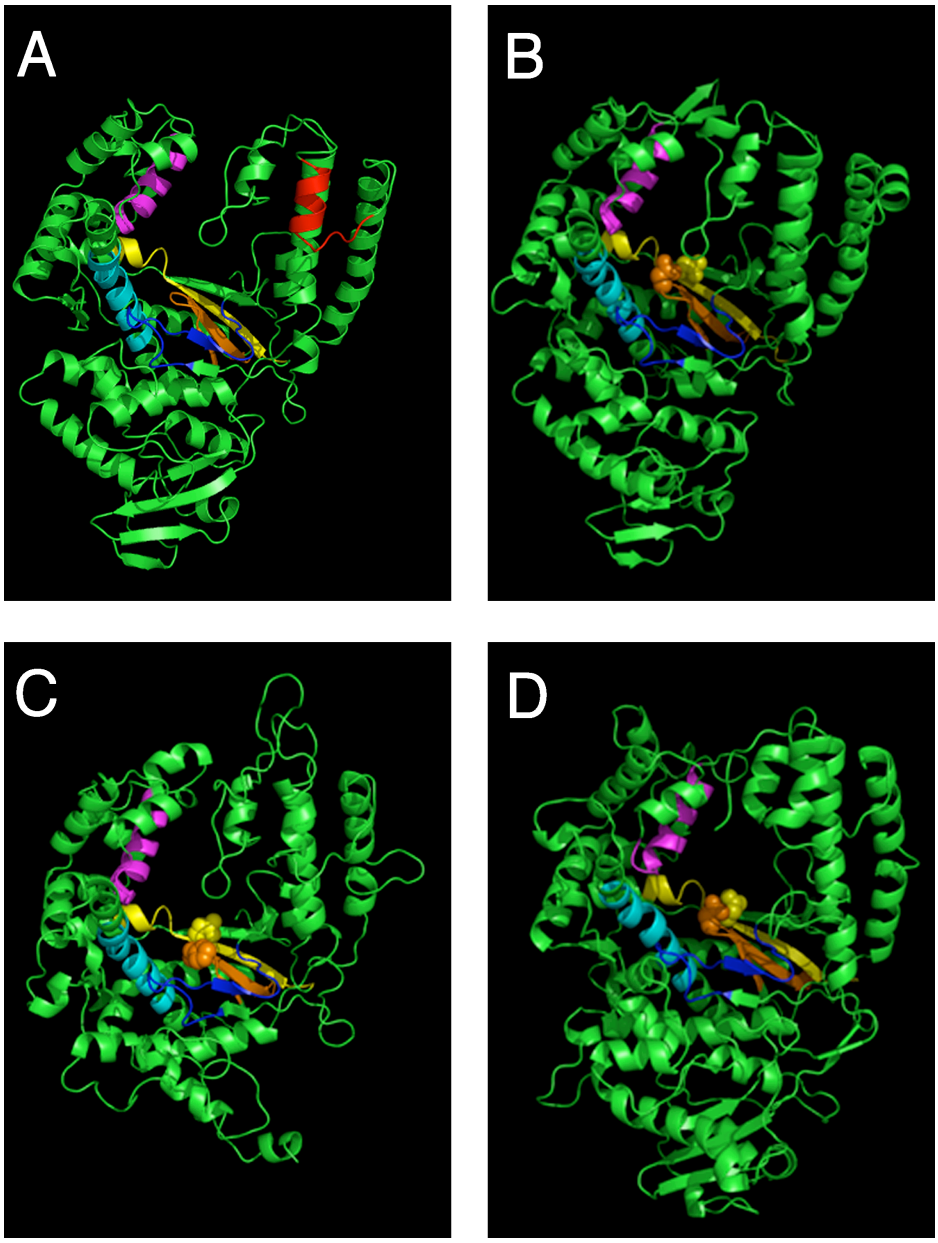


Figure 5.2: Predicted structures of mitochondrial DNA polymerases IB, IC, and ID
(A) Cartoon representation of the three dimensional structure of *Thermus aquaticus* DNA Polymerase I (PDB 4KTQ) produced using PyMOL. (C-D) Homology models were produced using the ensemble homology modeling server Phyre and shown as cartoon, produced in PyMOL. and critical aspartic acid residues shown as spheres. (B) Model of POLIB based on *Thermus aquaticus* DNA Polymerase I (PDB ID: 4KTQ). (C) Model of POLIB based on *Thermus aquaticus* DNA Polymerase I (PDB ID: 4KTQ). (D) Model of POLID based on *Geobacillus stearothermophilus* DNA POLYMERASE I/DNA COMPLEX (PDB ID: 3BDP). Motifs are colorized as in Figure 5.1.

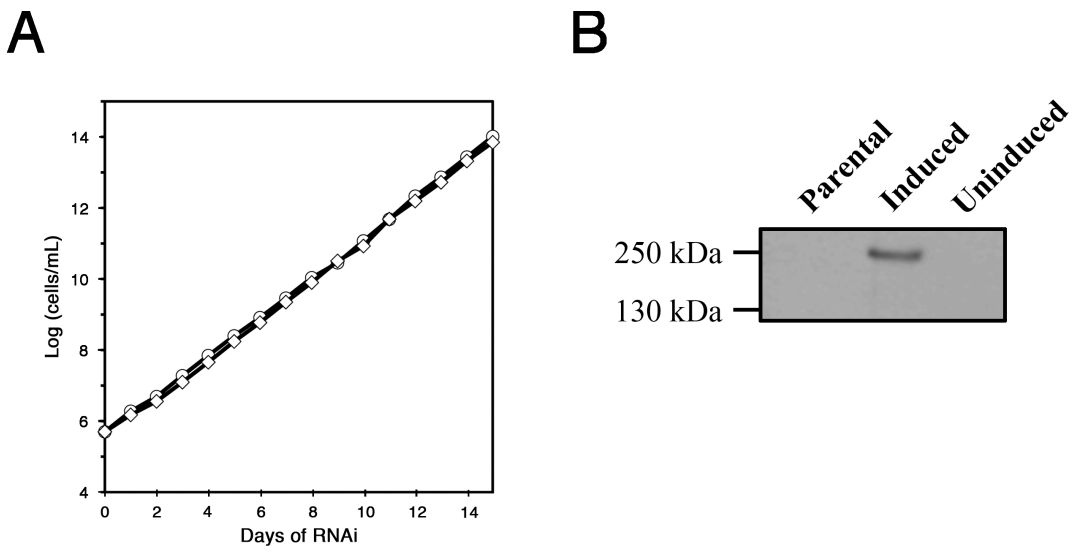


Figure 5.3: Overexpression of POLIBMHTAP
(A) The POLIBMHTAP population was grown in the absence (open circles) or presence (open diamonds) of tetracycline (1 μg/ml) to induce for overexpression of IBMHTAP. Cell density was plotted as the product of cell number and total dilution. (B) Western blot of lysates from parental (29-13) cells, cells transfected but not induced for overexpression, or cells induced for overexpression. POLIC-PTP (predicted M.W., 188 kDa) was detected with Peroxidase-Anti-Peroxidase Soluble Complex (PAP) reagent.

A

Location of **Asp** in Motif A:

```
POLIB     1110 KGRCVEIDYSQLEIVVM   1126
POLIC     1373 AGRMIEADYSQLEVVVL   1389
POLID     1338 KGMCIEADYSQLEVVAL   1354

POLIB AAGGGCCGTTGCGTGGAAATTGACTACTCGCAGCTGGAGATTGTTGTTATG
POLIC GCTGGTCGTATGATTGAGGCGGACTATAGTCAGTTGGAAGTTGTGGTGCTC
POLID AAGGGAATGTGTATTGAGGCAGATTATTCACAGCTTGAAGTCGTCGCATTG
```

B

Location of **Asp** in Motif C:

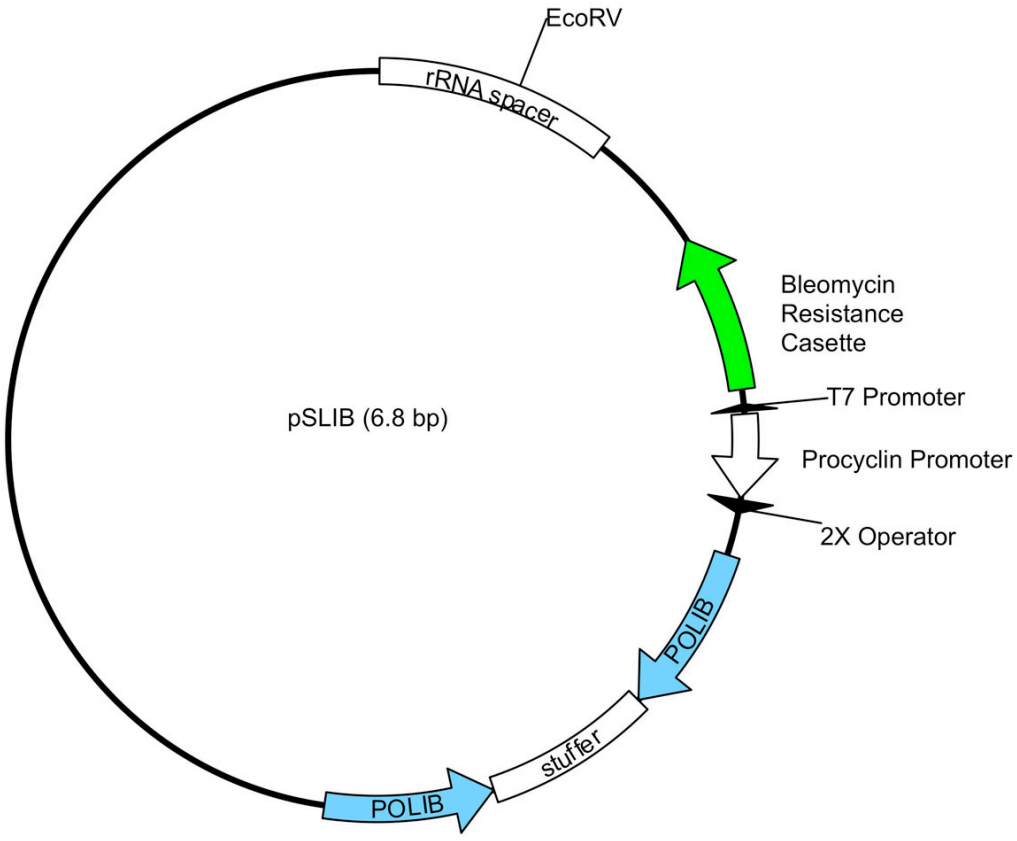
```
POLIB     1302 FMINFVHDSLWLDCHMS   1318
POLIC     1585 FLVNTVHDCVWIDAHES   1601
POLID     1557 VLINTVHDCVWIDCHMD   1561


POLIB TTTATGATAAACTTCGTACACGATTCGCTTTGGCTCGACTGCCACATGAGT
POLIC TTTCTTGTTAATACCGTTCACGATTGCGTTTGGATTGATGCCCACGAATCC
POLID GTACTAATCAACACCGTGCACGACTGCGTATGGATTGACTGCCACATGGAT
```

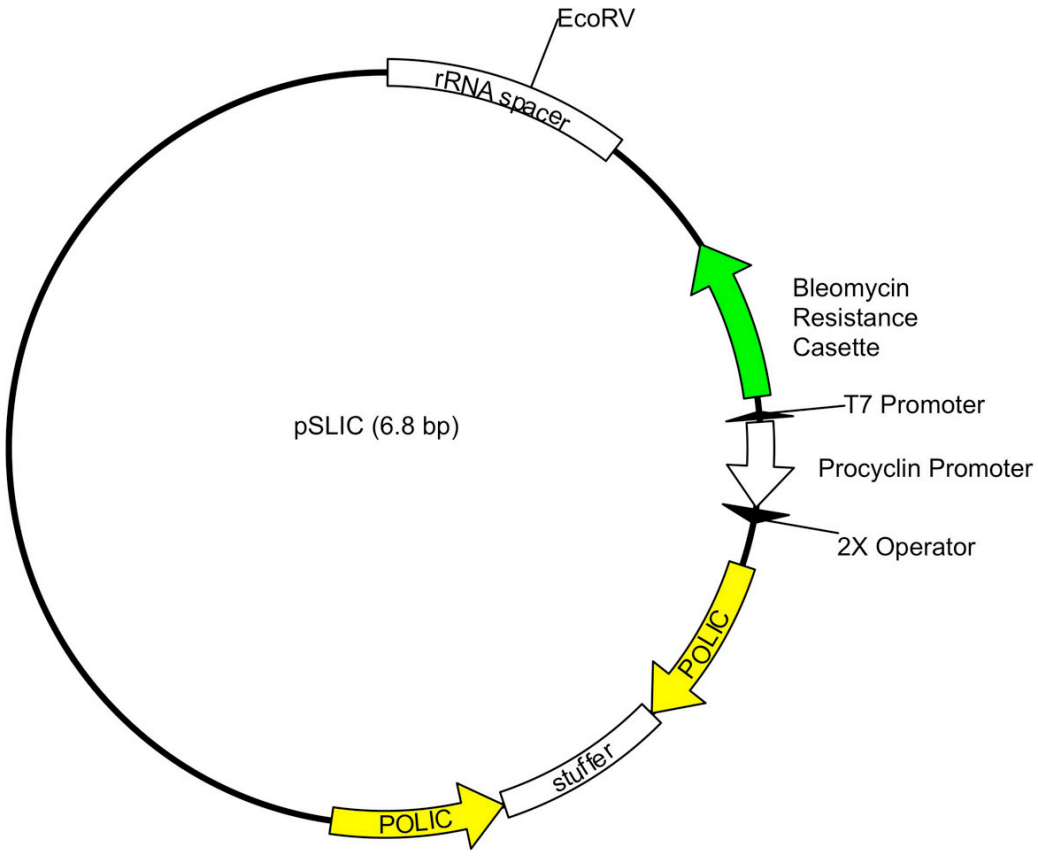
Figure 5.4: Location of critical aspartic acid residues in motifs A and C
(A) Residues flanking the critical aspartic acid residue in Motif A are shown, with the corresponding coding sequence displayed below. (B) Residues flanking the critical aspartic acid residue in Motif C are shown, with the corresponding coding sequence displayed below.

5.6 Plasmids Generated

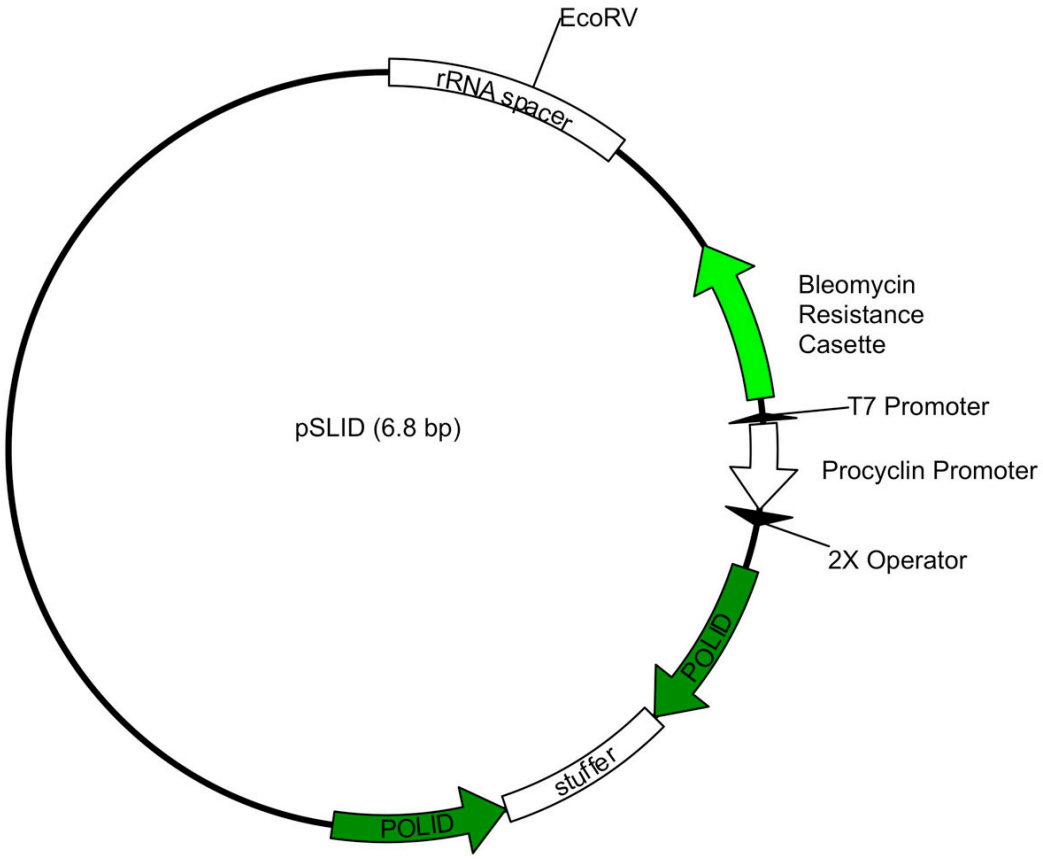
5.6.1 pSLIB



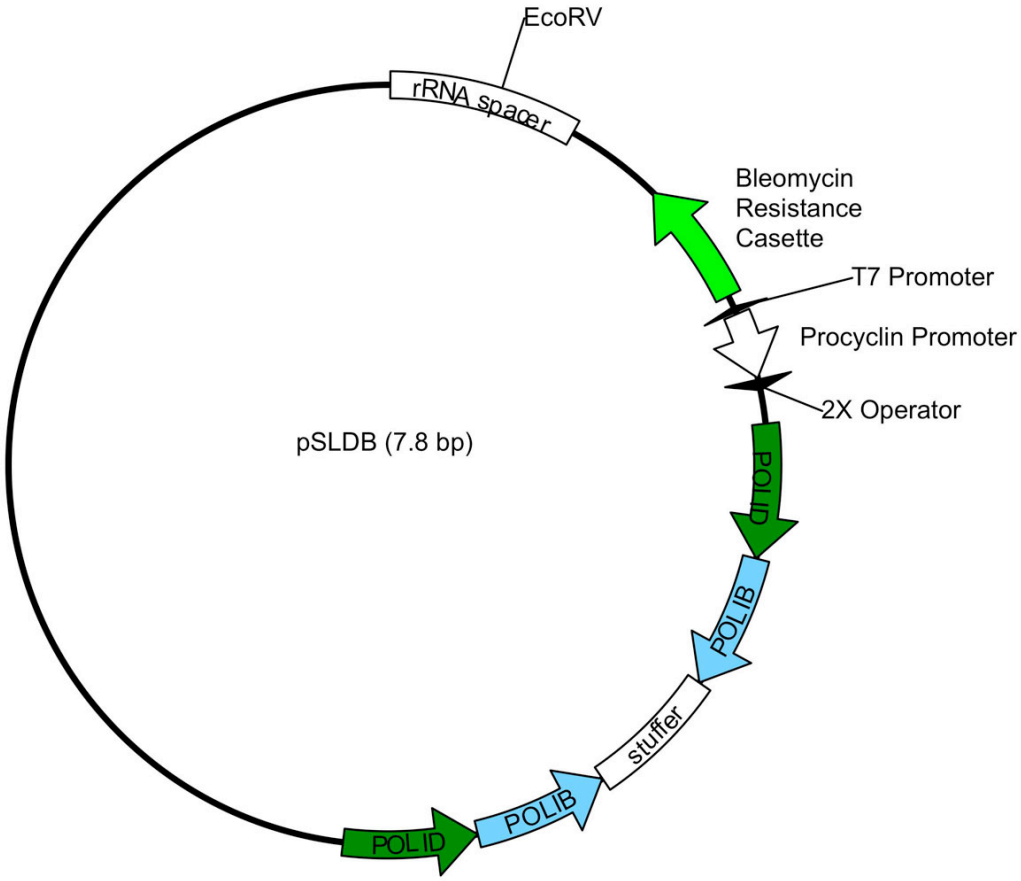
5.6.2 pSLIC


EcoRV
rRNA spacer
Bleomycin
Resistance
Casette
T7 Promoter
pSLIC (6.8 bp)
Procyclin Promoter
2X Operator
POLIC
stuffer
POLIC

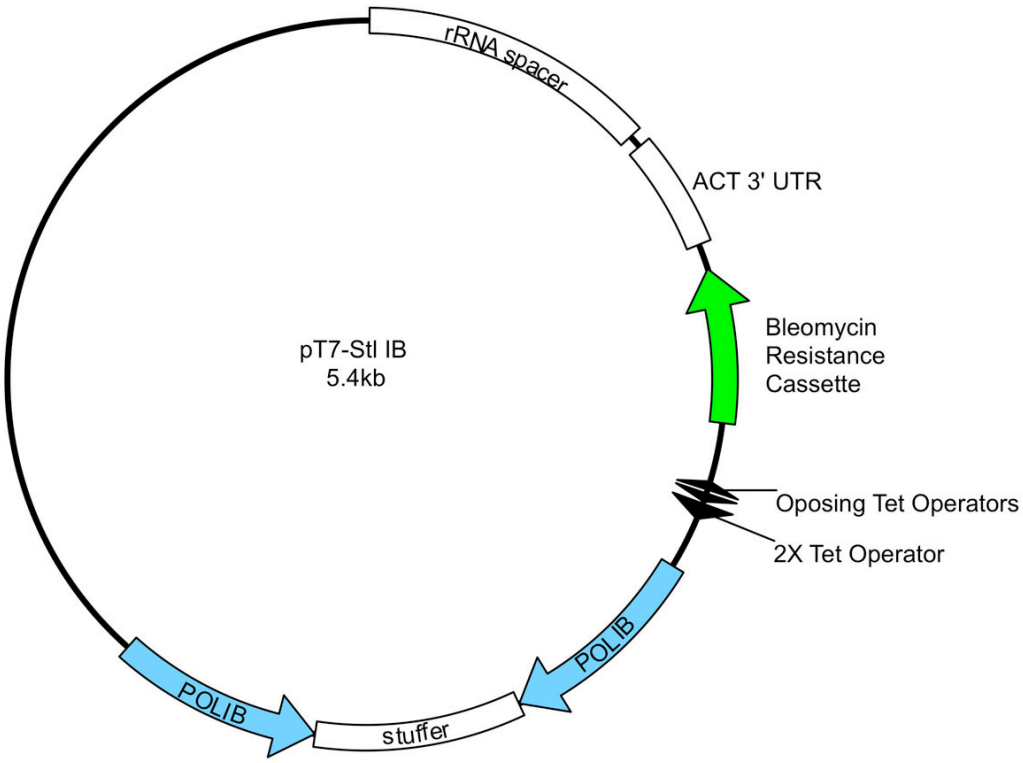
5.6.3 pSLID


EcoRV
rRNA spacer
Bleomycin
Resistance
Casette
T7 Promoter
pSLID (6.8 bp)
Procyclin Promoter
2X Operator
POLID
stuffer
POLID

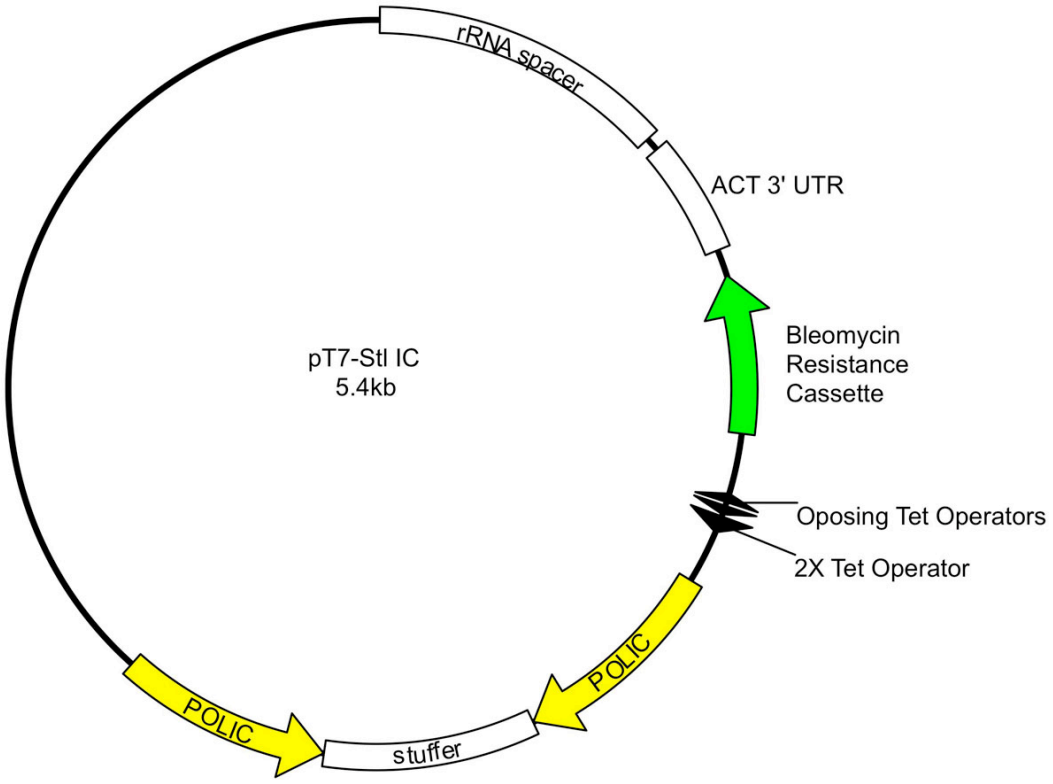
5.6.4 pSLDB


EcoRV
rRNA spacer
Bleomycin
Resistance
Casette
T7 Promoter
Procyclin Promoter
2X Operator
pSLDB (7.8 bp)
POLID
POLIB
stuffer
POLIB
POLID

5.6.5 pT7-Stl IB



5.6.6 pT7-Stl IC



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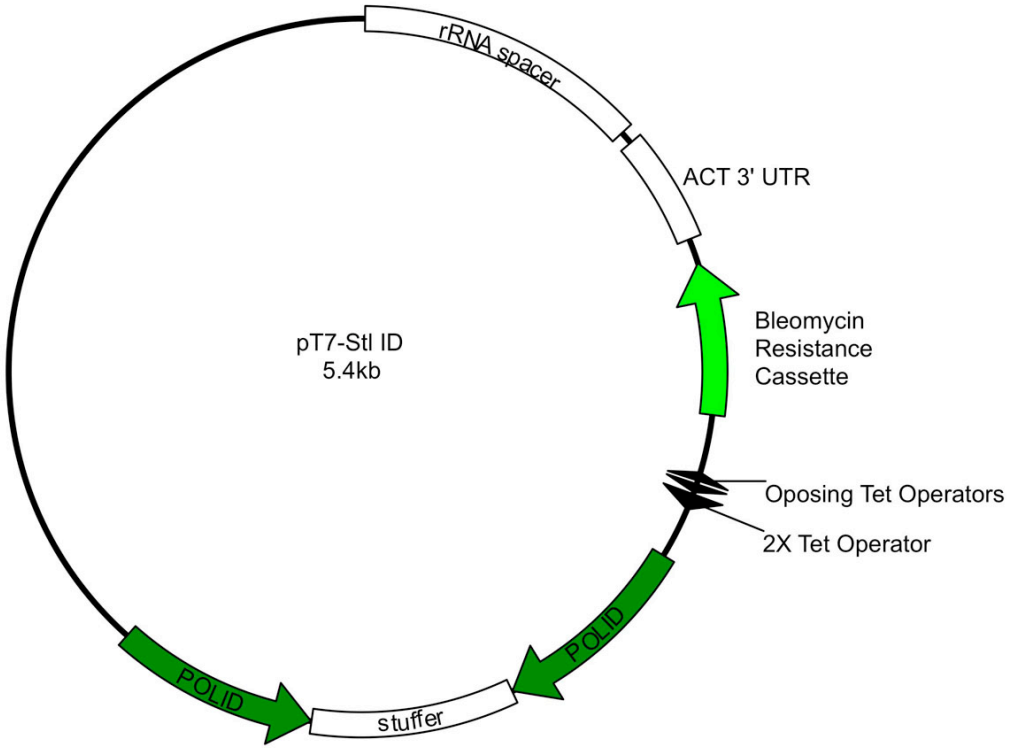

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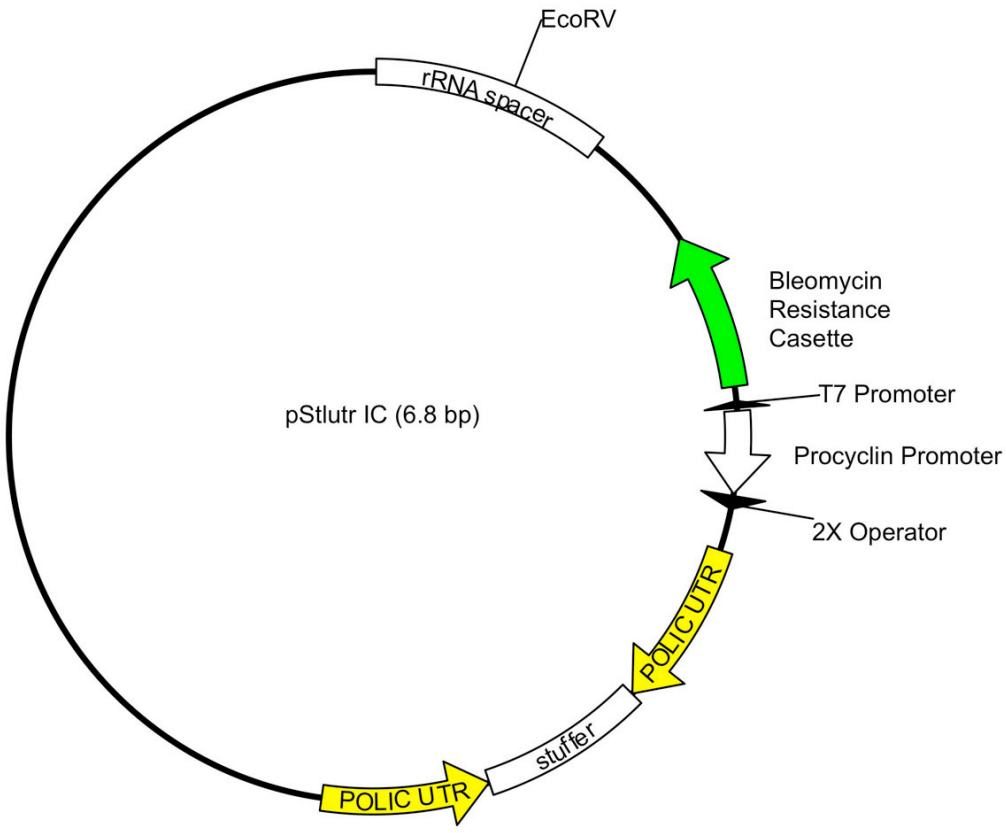
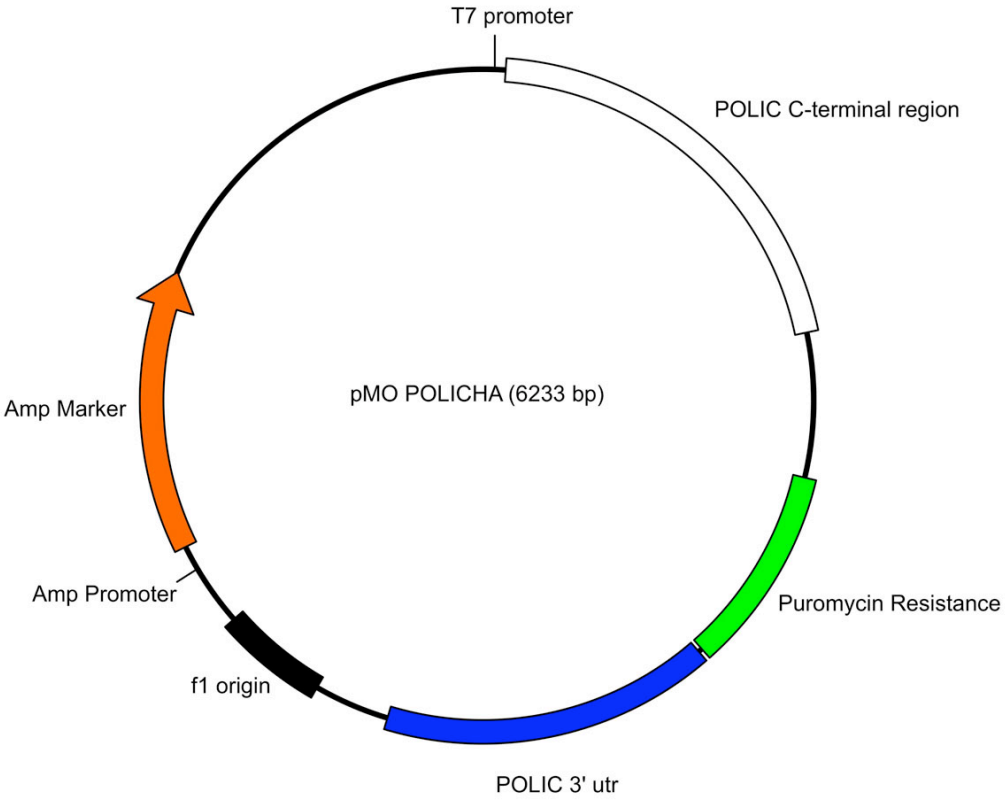
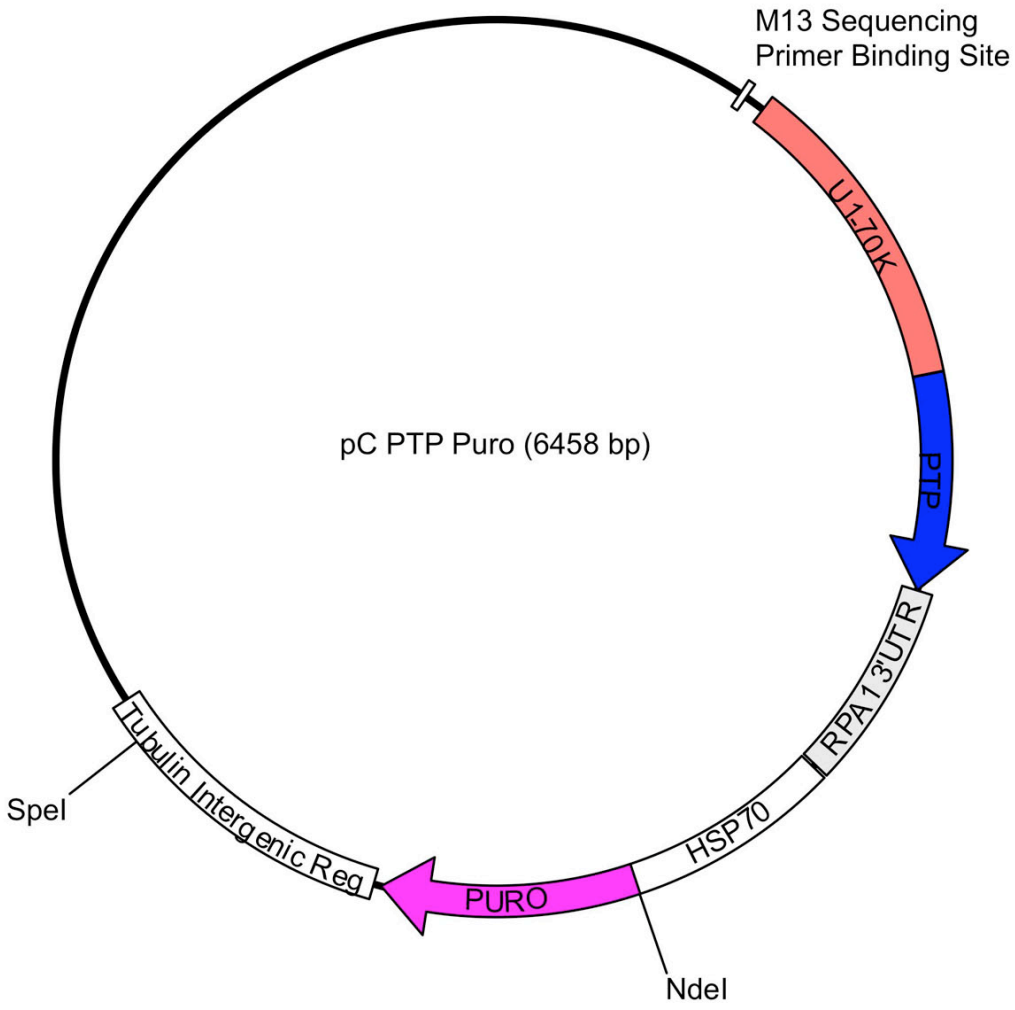


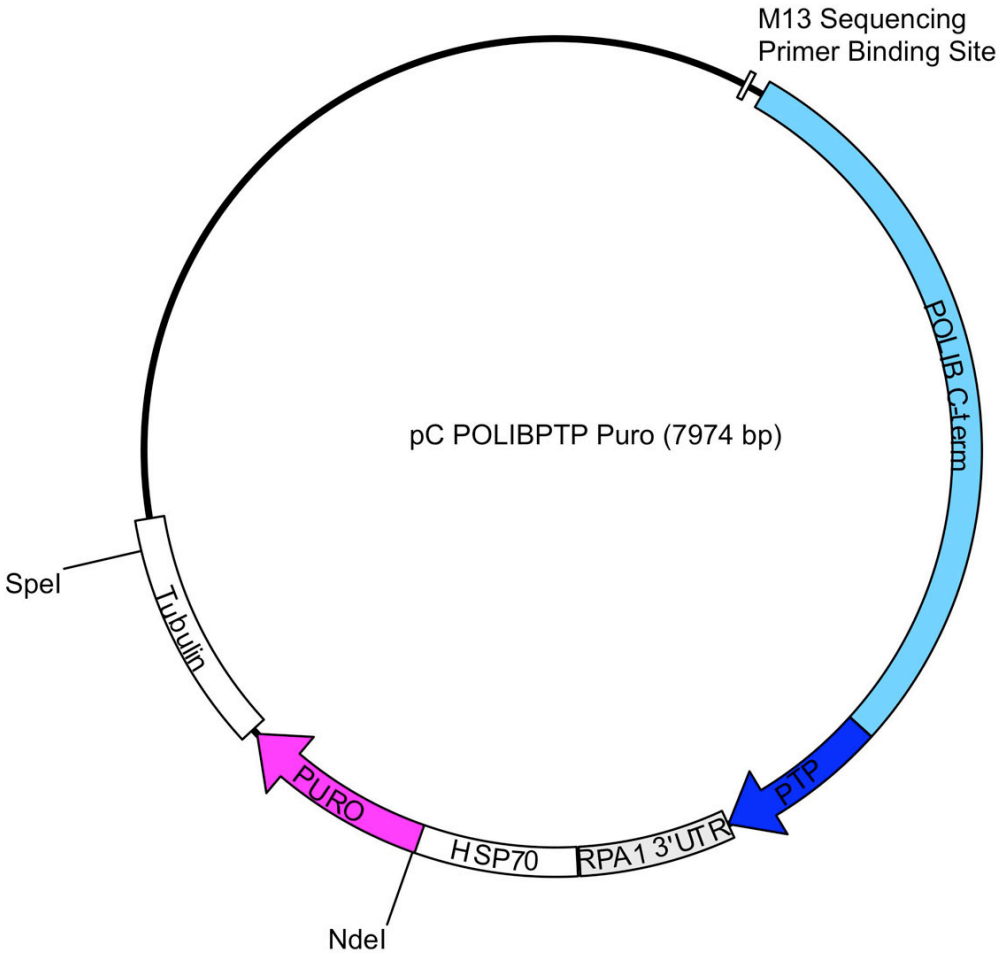
5.6.9 pMO POLICHA



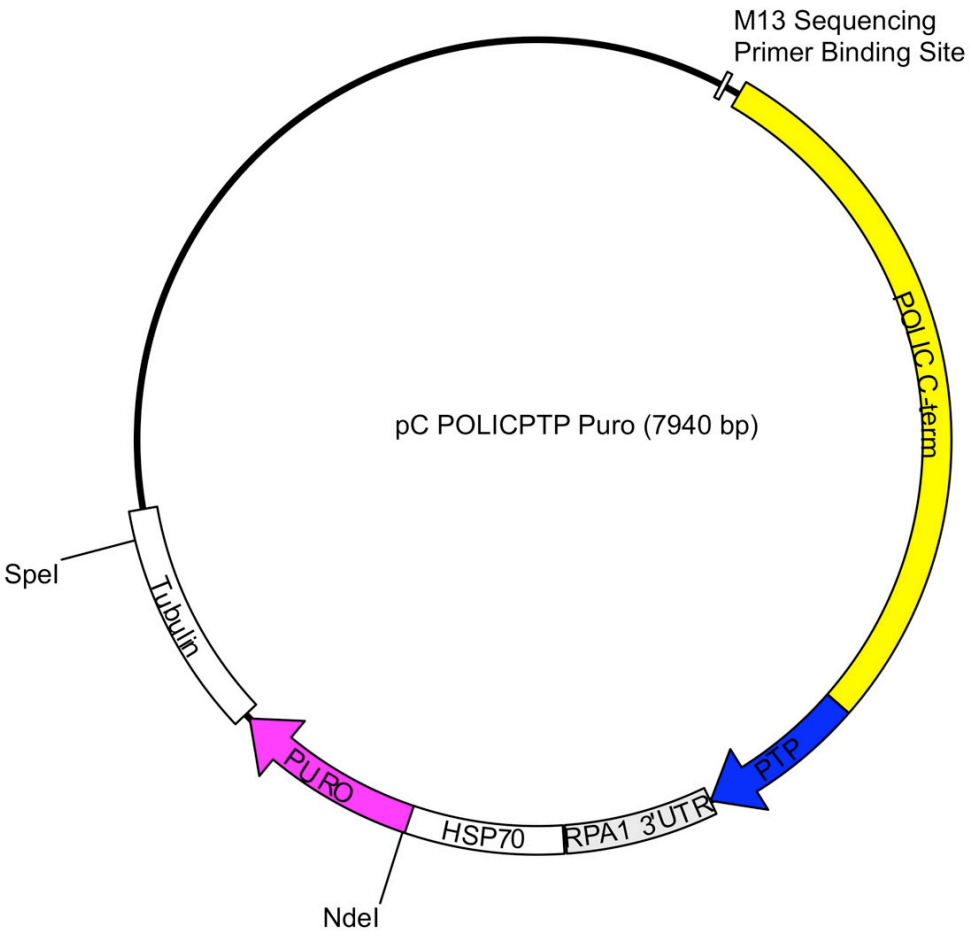
5.6.10 pC PTP Puro



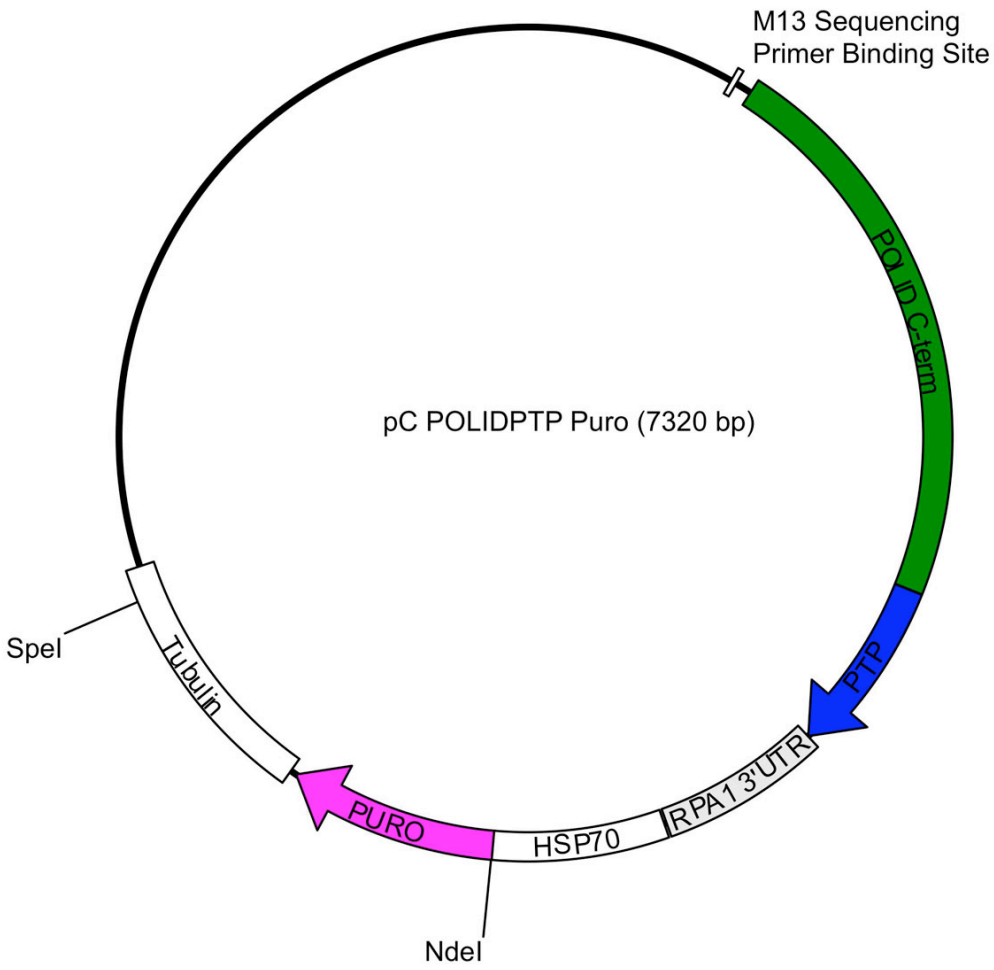
5.6.11 pC POLIBPTP Puro



5.6.12 pC POLICPTP Puro



5.6.13 pC POLIDPTP Puro



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