CELLULAR FUNCTIONS OF DNA GYRASE AND CONDITIONAL GENE REGULATION IN THE MALARIA PARASITE Plasmodium falciparum

By

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2008
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<tr>
<td>ATc</td>
<td>anhydrotetracyline</td>
</tr>
<tr>
<td>BDS</td>
<td>blasticidin deaminase</td>
</tr>
<tr>
<td>C3</td>
<td>pTOCAM-Δpm4 transformant clone C3</td>
</tr>
<tr>
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<td>pTOCAM-Δpm4 transformant clone C3 with piggyBac transposase-mediated insertion of the tati2 expression cassette using pGYBSD-TA</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindol</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
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<td>FISH</td>
<td>fluorescent in situ hybridization</td>
</tr>
<tr>
<td>FISH/IFA</td>
<td>fluorescent in situ hybridization / immunofluorescence assay</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>hDHFR</td>
<td>human di-hydrofolate reductase</td>
</tr>
<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>K+-SDS</td>
<td>potassium chloride / sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Min. cam.</td>
<td>minimal calmodulin promoter</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>TA-01</td>
<td>culture transformed with pGYBSD-TA using the piggyBac transformation system</td>
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<tr>
<td>TA-02</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>TaTi2</td>
<td>transactivator</td>
</tr>
<tr>
<td>TetO</td>
<td>tetracycline-inducible operator</td>
</tr>
<tr>
<td>VM-26</td>
<td>podophyllotoxin derivative tenoposide</td>
</tr>
<tr>
<td>VP-16</td>
<td>podophyllotoxin derivative etoposide</td>
</tr>
<tr>
<td>WR99210</td>
<td>Walter Reed compound 99210</td>
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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

CELLULAR FUNCTIONS OF DNA GYRASE AND CONDITIONAL GENE REGULATION IN THE MALARIA PARASITE Plasmodium falciparum

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Major: Veterinary Medical Sciences

Malaria is among the greatest disease threats to global public health. As part of the effort to combat the disease, the development of new anti-malarials remains imperative. The causative agent of malaria is a parasitic pathogen of the genus Plasmodium which is transmitted to humans through the bite of an anopheline mosquito. The parasite contains a relic, non-photosynthetic plastid (called the apicoplast) of red algal origin that was acquired by an ancestral form through a secondary endosymbiotic event. The apicoplast supports critical biosynthetic processes in the parasite that are divergent from the equivalent host pathway, providing substantial opportunity for the identification of possible drug targets. The Plasmodium DNA gyrase is a eubacterial type II topoisomerase that is expected to function within the apicoplast, with a principal role in regulating the topological transitions of the 35 kb circular apicoplast genome. In this study, the anti-bacterial ciprofloxacin was used to probe the cellular functions of DNA gyrase. For further characterization of enzyme functions, transgenic parasites were generated to develop a system for conditional gene regulation. Results from this study indicated that targeting DNA gyrase with ciprofloxacin inhibited the replication of apicoplast DNA and the accumulation of apicoplast RNA transcripts, and blocked the production of apicoplast ribosomal RNA. The apicoplast nucleoid appeared reduced in size and did not segregate normally, and the apicoplast displayed
abnormal morphology. In contrast, mitochondrial DNA replication, transcription, and segregation were unaffected. The DNA gyrase appeared to associate with the apicoplast DNA at non-specific loci, implicating numerous functional roles for the enzyme. Results from the conditional gene regulation studies indicated that the Tet-transactivator system has potential applicability for controlling endogenous gene expression in *P. falciparum*. Artificial promoters were successfully targeted to the *pfpm4* genomic locus, and *piggyBac* insertion of transactivator (TaTi2) expression cassettes resulted in stable TaTi2 expression. In washout studies, the temporal pattern of *pfpm4* RNA expression closely followed the expression of *tati2*. In the induced state of target transgene expression the steady-state TaTi2 levels decreased, a phenomenon that will be further investigated.
Malaria Overview

Malaria is among the greatest disease threats to global public health. Every year an estimated 300-500 million episodes of malaria-related illness result in 700,000 – 2,700,000 deaths (National Center for Infectious Diseases 2004). Malaria is caused by infection with the protozoan pathogen *Plasmodium* and is transmitted to humans by anopheline mosquitoes (Tuteja 2007). There are over 100 identified species of *Plasmodium* that infect diverse vertebrate life forms, including five species (*P. malariae, P. ovale, P. vivax, P. falciparum and P. knowlesi*) that infect humans (CDC, Tuteja 2007, White 2008). Ongoing endeavors to combat disease transmission include promoting the use of insecticide-treated bed nets, increasing the application of potent insecticides, improving water quality, and advancing social welfare and healthcare infrastructure (Chareonviriyaphap et al. 2000, Tseng et al. 2008). Also, substantial effort has been directed toward developing a vaccine for malaria (Vekemans and Ballou 2008). Some vaccine candidates show promise in terms of reducing the severity of disease, however protection rates are low and vaccination has not induced long-term immunity (Maher 2008).

therapies (ACTs) are now widely used as a first-line anti-malarials and show great promise for reducing malaria-related morbidity and mortality over the years to come (Greenwood et al. 2008). However, the clinical failure of ACTs due to development of resistance to the partner drug has been documented, and there are rising concerns about artemisinin toxicity (Greenwood et al. 2008).

The global eradication of malaria is the ultimate goal of all the endeavors aimed at preventing transmission, protecting against infection, and curing illness. As a part of this effort, the development of new anti-malarials remains a high priority.

**Plasmodium Lifecycle**

Successful completion of the *Plasmodium* lifecycle requires passage through both a vertebrate and invertebrate host where the parasite undergoes dramatic metamorphoses into various distinctive, specialized forms. The deadliest of the human malaria parasites, *Plasmodium falciparum*, initiates infection of its human host through the bite of an infected anopheline mosquito. Parasites in the form of sporozoites are deposited into the human dermis where they enter the circulatory system through blood vessels in the skin (Ponnudurai et al. 1982). Within 30 minutes the sporozoites amass in the liver for the exoerythrocytic stage of development, transiting through Kupffer cells ultimately residing in hepatocytes (Druilhe et al. 1982, Kappe et al. 2003). Each sporozoite replicates intracellularly within the hepatocyte by schizogony, forming thousands of merozoites (Mazier et al. 1983). Between 6-16 days post inoculation, hepatocytes rupture and release merosomes containing aggregates of merozoites that are released into the blood where they invade mature erythrocytes to initiate the asexual blood-stage of infection (Mazier et al. 1990). The parasite enters a vegetative trophozoite stage where it begins to degrade hemoglobin, eventually consuming ~ 70 % of the hemoglobin within in infected
erythrocyte (Lew et al. 2003). The parasite then enters a replicative phase where nuclear chromosomes undergo 3-5 mitotic replications by schizogony, producing multiple discrete nuclei that emerge approximately 30-40 hours post erythrocytic invasion. Schizogony is culminated around 44 hours post invasion by the formation of 8-32 daughter merozoites. Newly formed merozoite progeny rupture from the schizont and rapidly invade fresh host erythrocytes to repeat the cycle of asexual multiplication (Pinder et al. 2000). Concurrent with the asexual cycle, a small subset of *P. falciparum* parasites suspends mitotic division and enters an alternative pathway of differentiation to form sexual stage gametocytes (Smalley et al. 1981). There are five recognized stages of gametogenesis that develop within 8-17 days and result in the formation of male (micro) and female (macro) gamete precursors (Day et al. 1998). During a blood meal, gametocytes are extracted from the human host by an anopheline mosquito (Lensen 1996). In the mosquito gut the gametocytes differentiate into male and female gametes, and in the case of male gamete formation, additional mitotic replications occur rapidly in the mosquito gut to produce up to 8 flagellated cells (Mendis et al. 1994). The male gamete fertilizes the female gamete to produce a diploid zygote which elongates to form an ookinete (Carter and Kaushal 1984). The ookinete burrows through the mosquito mid-gut wall and attaches to the outside of the basil epithelium where it undergoes multiple rounds of mitotic division during development of the oocyst (Vinetz 2005). The oocyst becomes filled with thousands of haploid sporozoites (Carter et al. 2007), and finally the sporozoites rupture from the oocyst and migrate through hemolymph to the mosquito salivary glands, where they are poised to infect another human host (Ponnudurai et al. 1989, Vaughan et al. 1994, Al-Olayan et al. 2002).
The Apicoplast

Origin of the Apicoplast

*Plasmodium falciparum* contains a relic, non-photosynthetic plastid (McFadden et al. 1996) (called the apicoplast) of red algal origin that was acquired by an ancestral form through a secondary endosymbiotic event (Fast et al. 2001). Plastids are derived from a primary endosymbiotic event where a phagotrophic eukaryote engulfed a photosynthetic cyanobacterium (Gray 1993, Delwiche et al. 1995). In the case of apicomplexans, a secondary endosymbiosis occurred where a eukaryotic phagotroph engulfed a eukaryotic cell of the red algal lineage (Delwiche 1999, McFadden 1999, McFadden and van Dooren 2004). The apicomplexan lineages (Coccidia, Haemosporida, Gregarinia, and Piroplasma) are believed to have a single origin, all evolving from the same secondary endosymbiotic event (McFadden and Waller 1997). Furthermore, phylogenetic analysis suggests that all alveolates (apicomplexans, dinoflagellates, and ciliates) together with the chromists (heterokonts), haptophytes, and cryptophytes evolved from the same heterotrophic ancestor giving rise to all members of the supergroup chromalveolata (Cavalier-Smith 1999, Fast et al. 2001). In apicomplexans, the 4 membranes bounding the apicoplast attest to these evolutionary events (Kohler et al. 1997, McFadden and Roos 1999, Diniz et al. 2000), with each having a distinct evolutionary origin (Cavalier-Smith 2000). The innermost 2 membranes bounding the apicoplast stroma are derived from the cyanobacterial envelope, the third bounding membrane is derived from the endosymbiont plasma membrane, and the fourth bounding membrane is derived from the host phagosome membrane (Cavalier-Smith 2000).
The Apicoplast Genome


The content and arrangement of genes encoded by the *Plasmodium* and *Toxoplasma* 35 kb apicoplast genomes are nearly identical (Williamson et al. 2001). However, the genome topology and the primary modes for apicoplast DNA replication differ. In *Plasmodium* the apicoplast DNA is present as covalently closed circles and is replicated by a twin displacement-loop (D-loop) mechanism (Williamson et al. 2002). In *Toxoplasma* the apicoplast DNA is arranged in linear tandem arrays that occur in multiples of one to twelve, which are the result of a rolling circle mode of DNA replication (Williamson et al. 2001). The ends of the linear *Toxoplasma* 35 kb DNA molecules begin and terminate within the center region of the inverted repeat (Williamson et al. 2001).

Through evolution, the plastid genome was drastically reduced as the initial cyanobacterial endosymbiont, followed by the secondary algal endosymbiont, lost autonomy and became an organelle. Plastid genes were either transferred to the host cell nucleus, replaced by a host cell homologue, or lost if the gene no longer conferred a selective advantage (Martin and Schnarrenberger 1997, Delwiche 1999). Today, the 35 kb apicoplast genome primarily encodes genes required for protein translation and RNA
transcription (Wilson et al. 1996). There are 2 copies each of SSU and LSU rRNAs, 25
tRNAs, 17 ribosomal proteins, the elongation factor \textit{tufA}, and subunits B, C1, and C2 of a
eubacterial RNA polymerase. The different tRNA species encoded in the 35 kb DNA are
expected to provide a complete set of tRNAs required for translation in the apicoplast
(Preiser et al. 1995). The apicoplast genome also encodes \textit{clpC}, a stromal chaperone and
member of the Tic complex for protein import; \textit{sufB}, a protein involved in iron
homeostasis and iron-sulfur cluster formation, and 7 additional putative open reading
frames with unassigned function. Figure 2-1 shows a map of the \textit{P. falciparum} apicoplast
DNA.

Using in situ hybridization in combination with fluorescent or electron
microscopy, studies in \textit{Toxoplasma} confirm that the 35 kb DNA localizes to the
apicoplast. In \textit{Toxoplasma} the apicoplast DNA is visualized as discrete foci, indicating
that the DNA is organized into nucleoid compartments similar to what has been observed
for chloroplast DNA (Striepen et al. 2000, Matsuzaki et al. 2001). Chloroplast nucleoids
are organized by a bacterial-derived histone-like protein (HU) (Kobayashi et al. 2002)
and both \textit{Plasmodium} and \textit{Toxoplasma} contain an apicoplast targeted HU homologue
(Vaishnava and Striepen 2006, Arenas et al. 2008). The histone-like HU protein is likely
to play an important role in apicoplast genome organization, expression, and proper
segregation. During apicoplast segregation in \textit{Toxoplasma}, the apicoplast DNA localizes
to the ends of the organelle near the centrosomes, perhaps indicating that nucleoid
positioning may be linked to an association with the centrosome (Vaishnava and Striepen
2006). Proteins such as histone-like HU could be important players in this association
(Vaishnava and Striepen 2006).
**Replication of the Apicoplast Genome**

Pulsed-field gel electrophoresis and ionizing radiation have revealed that the *P. falciparum* 35 kb apicoplast DNA has a circular topology (less than 3 % are linear), and is found in relaxed and twisted monomeric forms (Williamson et al. 2002). Furthermore, two-dimensional gel electrophoresis and electron microscopy have indicated that the apicoplast DNA is replicated by 2 mechanisms: by the formation of twin D-loops within the inverted repeat region of the circle, and by a rolling circle mechanism that initiates outside of the inverted repeat region (Williamson et al. 2002). The apicoplast DNA contains multiple origins of replication that are differentially activated and initiate from within the inverted repeat region (Singh et al. 2003, Singh et al. 2005). An unusual multi-domain polypeptide (PfPREX) containing DNA primase, DNA helicase, DNA polymerase, and 3’ to 5’ exonuclease activity is targeted to the apicoplast and is proposed to play a central role in apicoplast DNA replication (Seow et al. 2005).

**Protein Targeting to the Apicoplast**

Most of the apicoplast proteome, > 500 predicted genes, are encoded in the nuclear genome (Foth et al. 2003) and post-translationally targeted to the apicoplast by means of a bipartite N-terminal extension (Waller et al. 1998, Ralph et al. 2004). The N-terminal presequence is comprised of a signal and transit peptide, and both domains are required for proper subcellular localization to the apicoplast. The signal peptide mediates entry into the endomembrane system, and is cleaved during translation and import into the endoplasmic reticulum (Waller et al. 1998, Waller et al. 2000, Foth et al. 2003). The transit peptide allows transport through the apicoplast envelope (innermost 2 membranes) and entry into the organelle stroma where the transit peptide is cleaved by a stromal processing peptidase (van Dooren et al. 2002). Currently, the mechanism for transport out
of the endoplasmic reticulum and through the periplastid membrane is unknown (Foth and McFadden 2003).

Studies have demonstrated that apicoplast trafficking is not affected by the Golgi-disrupting agent Brefeldin A (DeRocher et al. 2005, Tonkin et al. 2006). Furthermore, the addition of an ER retention signal (XDEL motif) to apicoplast targeted proteins does not result in cis-Golgi mediated retrieval by ERD2 (Tonkin et al. 2006). These findings strongly suggest that apicoplast targeting is independent of passage through the Golgi, and thus proteins are transported directly from the ER to the apicoplast. There are three primary models proposed for ER to apicoplast transport (Vaishnava and Striepen 2006). Trafficking may occur by general vesicular transport from the ER to the apicoplast, there maybe an extension of the ER that cradles the apicoplast where proteins are transported in vesicles or through specialized tubules that span the ER and apicoplast membranes, and/or the outermost membrane of the apicoplast is simply continuous with the ER and proteins are directly imported. The latter scenario could imply that all secretory proteins are subject to an initial apicoplast sorting step before processing by the Golgi, which could be a fitting assumption since the apicoplast is the final destination for half of the secreted proteins in *Plasmodium* (Tonkin et al. 2008).

**Apicoplast Division**

Most of what is known about plastid division comes from studies in *Arabidopsis*. There are highly conserved proteins of cyanobacterial origin that govern the process of plastid division, in addition to various eukaryotic players. In *Arabidopsis*, cyanobacterial Ftz homologues (which are tubulin-like GTPases) form a ring-like structure (Z-ring) at the plastid division site. Members of the cyanobacterial minicell gene family (min D and min E) secure the positioning of the Z-ring for a symmetrical plastid division. Additional
cyanobacterial proteins such as Ftn2/ARC6 and the eukaryotic dynamin-related protein ARC5 are also required for the division process. While FtsZ and ARC5 mediated division is conserved in most plastid containing organisms, *Plasmodium* and *Toxoplasma* do not contain genes with significant homology to any of the above plastid division proteins (Vaishnava and Striepen 2006).

In the absence of conserved division proteins, it is proposed that apicoplast fission is accomplished during the final period of pellicle formation in the daughter cell (Striepen et al. 2000). The elongating apicoplast shows a clear association with the centrosomes in *Toxoplasma* and *Sarcosystis*, raising the possibility that the apicoplast is pulled into daughter buds via a connection to the mitotic spindle apparatus (Striepen et al. 2000, Vaishnava et al. 2005). The simultaneous downward movement of the daughter cell pellicle might further constrict the apicoplast, resulting in fission (Vaishnava et al. 2005). Moreover, regions of apicoplast constriction in *Toxoplasma* co-localize with ring structures containing membrane occupation recognition nexus protein (MORN1) (Gubbels et al. 2006). Time lapse imaging studies have revealed that motile MORN1 rings move over the daughter cell bud and constrict the posterior end of the bud where final cytokinesis occurs (Gubbels et al. 2006, Vaishnava and Striepen 2006).

The process of cell division in apicomplexans varies across species. While *Plasmodium, Eimeria, Babesia*, and *Theileria* divide by schizogony, *Toxoplasma* utilizes the process of endodyogeny for cell division, and *Sarcocystis* undergoes endopolygeny. The shared characteristic between these different modes of cell division is the ultimate formation of the apicomplexan daughter cell, termed the zoite, which contains the full complement of cellular organelles, membranes, cytoskeletal elements, cytoplasmic
contents and genetic material. A growing body of evidence suggests that regardless of how many times the cell is replicated prior to division, ultimately the organization and formation of the zoite follows a similar pattern, and this process is tightly coupled to nuclear division and association with the centrosomes (Striepen et al. 2007). In apicomplexa, a specialized invaginating compartment of the nucleus called the centricone or spindle pole plaque houses the mitotic spindle apparatus (Bannister and Mitchell 1995, Morrissette and Sibley 2002). The nuclear centricone directly interacts with the cytoplasmic centrosomes and the centricone can be found throughout the entire cell cycle in *Toxoplasma* and *Sarcocystis*. The continuous presence of the centricone may be reflective of a mitotic spindle that also remains present throughout the cell cycle. It has been hypothesized that attachment of spindle microtubules to the kinetochore of nuclear chromosomes may also occur throughout the cell cycle, and acts as a means of properly distributing replicated DNA during polyploid stages. Furthermore, the apicoplast of *Toxoplasma* and *Sarcocystis* also maintains a clear association with the centrosomes during the cell cycle, and interestingly, the apicoplast nucleoid is also found at the site of interaction. These observations lead to the intriguing hypothesis that a physical link between apicoplast DNA and the centrosomes ensures proper organelle segregation into daughter cells.

Well defined centrioles have not been identified in *Plasmodium*, but the parasite does contain replicating spindle pole plaques that serve as a microtubule organizing center (MTOC) during mitosis (Bannister and Mitchell 1995, Morrissette and Sibley 2002). If the *Plasmodium* apicoplast associates with the spindle pole plaques, it would be
anticipated that an interaction is maintained between the organelle and all nuclei, however this is not always observed (van Dooren et al. 2005).

Throughout asexual development, the apicoplast and mitochondrion retain various points of contact (Hopkins et al. 1999, van Dooren et al. 2005). It has been hypothesized that contact between the organelles is to allow the exchange of metabolites from shared biochemical pathways (Hopkins et al. 1999). However, live cell studies in transgenic parasites containing fluorescent apicoplast and mitochondrion targeted proteins demonstrated that during the most metabolically active stages of asexual parasite development, the number of contact points between the apicoplast and mitochondrion did not increase (van Dooren et al. 2005). An additional idea was put forth postulating that the association between the apicoplast and mitochondrion is for the purpose of organization in cell division (van Dooren et al. 2005). Critical to understanding organelle division in plasmodia will be the examination of the process in the context of what is known in other apicomplexans, most notably determining whether or not the apicoplast associates with the spindle pole plaques or other MTOCs.

**Functions of the Apicoplast**

The apicoplast supports numerous biosynthetic processes that are vital for the apicomplexan cell (Surolia and Padmanaban 1992, Jomaa et al. 1999, Surolia and Surolia 2001, Sato and Wilson 2002, Ralph et al. 2004). Since the apicoplast is cyanobacterial in origin, equivalent pathways in the host are divergent and this provides substantial opportunity for the identification of possible drug targets. Functions of the apicoplast include a synthesis of type II fatty acids, production of isopentyl diphosphate precursors (IPP) using a non-mevalonate deoxyxylulose phosphate (DOXP) isoprenoid synthesis pathway, synthesis of heme, and generation of reducing power in the form of ferredoxin,

**Apicoplast as a Drug Target**

The *Plasmodium* apicoplast, being prokaryotic in origin, serves as an excellent target for therapeutic intervention (Wiesner and Seeber 2005). Several anti-bacterial compounds exert a parasiticidal effect against *P. falciparum in vitro*, and studies have validated that the apicoplast is the primary target of these drugs (Gardner et al. 1991b, McConkey et al. 1997, Weissig et al. 1997, Jomaa et al. 1999, Williamson et al. 2002, Dahl et al. 2006). In fact, some anti-bacterials such as doxycycline and clindamycin are currently used for malaria prophylaxis and in combination with other drugs for malaria treatment (Wiesner et al. 2003, Borrmann et al. 2005). Even anti-bacterials with limited *in vivo* or *in vitro* efficacy are extremely valuable as lead compounds for the development of more specific inhibitors. With rigorous validation of anti-bacterial drug targets at the cellular and molecular level in *Plasmodium*, a framework can be established for future pursuits in developing novel inhibitors against defined targets.

The anti-bacterials tested against *Plasmodium* can be divided into two broad categories: those that target housekeeping functions of the apicoplast (e.g. DNA replication, RNA transcription, and protein translation), and those that target metabolic pathways of the apicoplast (e.g. type II fatty acid synthesis, non-mevalonate isopentenyl diphosphate synthesis, and components of the heme biosynthesis pathway). The efficacy of anti-bacterials against *P. falciparum* is quite variable in terms of the period of time it takes to kill the parasite. Many of the anti-bacterials that target housekeeping functions of the apicoplast show a dramatic increase in potency when drug inhibition is evaluated at 96 h instead of 48 h, following a 48 h drug treatment. Most notably, when parasite counts
are taken at 96 h (after 2 asexual cycles), the IC$_{50}$ concentrations of the 50S ribosomal subunit inhibitors azithromycin and clindamycin decrease by ~100-fold and >1,000-fold respectively, compared to the IC$_{50}$ values calculated after 1 asexual cycle at the 48 h time point (Dahl and Rosenthal 2007). This phenomenon is referred to as a ‘delayed-death’ phenotype, and was first described in *Toxoplasma gondii* (Fichera and Roos 1997, He et al. 2001). In *Toxoplasma gondii*, treatment with ciprofloxacin, clindamycin, and chloramphenicol causes delayed-death and can be correlated with a defect in apicoplast segregation and distribution into daughter cells (He et al. 2001). The apicoplast does not segregate into daughter cells that develop in the first asexual cycle of drug treatment, but these plastid-less parasites are able to invade a new host cell and establish a parasitophorous vacuole, where they subsequently die in the second asexual cycle. In *P. falciparum*, delayed-death does not correlate with an apicoplast segregation defect, given that all second generation parasites contain the organelle (Dahl and Rosenthal 2007, Goodman et al. 2007).

In comparison to clindamycin and azithromycin, the 30S ribosomal subunit inhibitor doxycycline shows more modest delayed-death effects in *P. falciparum*, where the IC$_{50}$ decreases by 10-fold after 2 asexual cycles. Thiostrepton on the other hand, which targets the 50S ribosomal subunit, immediately causes parasite death in the first asexual cycle (McConkey et al. 1997). Also resulting in immediate death is treatment with rifampacin, an inhibitor of the apicoplast eubacterial RNA polymerase. Interestingly, the effects of the DNA gyrase inhibitor ciprofloxacin appear to be strain-dependent in *P. falciparum* (Dahl and Rosenthal 2008). In one study, the difference in IC$_{50}$ values obtained at 48 vs. 96 h was negligible for the chloroquine-sensitive strain
3D7, while in the chloroquine resistant strain W2 the drug was 10-fold more potent at 96 h (Dahl and Rosenthal 2007). Chloroquine targets the parasite digestive vacuole and interferes with heme polymerization (Slater and Cerami 1992). In another report testing ciprofloxacin against the chloroquine-resistant strain D10, the difference in IC$_{50}$ values obtained at 48 vs. 96 h could also be considered negligible (Goodman et al. 2007). The latter report defined a specific set of criteria to define delayed-death (treatment with 10 times the 96 h IC$_{50}$ concentration should not inhibit growth after 48 h, and the growth inhibition should not be affected by the presence or absence of drug in the second asexual cycle). With these criteria it was determined that treatment with ciprofloxacin as well as thiostrepton and rifampacin resulted in immediate death, whereas treatment with clindamycin and tetracycline resulted in a delayed death response (Goodman et al. 2007).

In contrast with the parasite response to anti-bacterials that target housekeeping functions of the apicoplast where delayed-death is sometimes an outcome, anti-bacterials that target metabolic functions of the apicoplast result in immediate death within the first asexual cycle (Waller et al. 2003, Goodman and McFadden 2007, Ramya et al. 2007, Wiesner and Jomaa 2007). For example, the anti-bacterial triclosan targets the enoyl-acyl carrier protein (ACP) reductase of the fatty acid biosynthesis pathway and treatment of *Plasmodium* and *Toxoplasma* with triclosan results in inhibition during the first asexual cycle of treatment (McLeod et al. 2001, Ramya et al. 2007). The use of fosmidomycin to target DOXP reductoisomerase of the isoprenoid synthesis pathway also results in parasite inhibition during the first asexual cycle of treatment (Jomaa et al. 1999, Wiesner et al. 2002)
DNA Gyrase

DNA gyrase is a eubacterial type II topoisomerase that functions in regulating the topological transitions of circular DNA molecules. The regulation of DNA topology by DNA gyrase is required for the processes of DNA replication, DNA segregation, and RNA transcription (Wang 2002). *Plasmodium falciparum* contains nuclear gene sequences encoding both subunits of this bacterial enzyme, *gyrA* and *gyrB*, each of which share good homology with its *E. coli* orthologue (42% and 51% amino acid similarity, respectively). The DNA gyrase sequences contain an apicoplast targeting element located at their amino terminus, whereas other putative topoisomerase genes in *P. falciparum* lack this feature (Khor et al. 2005) (PlasmoDB). Since this putative gyrase is the only type II topoisomerase that is predicted to function within the apicoplast, this enzyme is likely to play a central role in maintaining a functional structure of the apicoplast DNA that may be required for DNA replication, DNA segregation, and RNA transcription.

**Functions of the Bacterial Type II Topoisomerase**

Two eubacterial type II topoisomerases have been identified, DNA gyrase and topoisomerase IV, and each of these enzymes functions as a heterotetramer (Champoux 2001). DNA gyrase is comprised of two A subunits and two B subunits (*gyrA2gyrB2*), and topoisomerase IV is comprised of two very similar subunits (*parC2parE2*). Both enzymes create transient double stranded breaks in DNA to allow for the passage of another segment of duplex DNA through the break (Wang 1996, Champoux 2001). The DNA break is stabilized by the formation of a protein bridge created by the covalent attachment of the active site tyrosine to both 5’ phosphoryl ends of the broken DNA (Liu and Wang 1979, Been and Champoux 1980, Liu et al. 1980). The process of strand
passage is required for the resolution of topological problems that occur as a result of DNA replication, recombination, and RNA transcription.

DNA gyrase is the only type II topoisomerase that is capable of generating negative supercoils in DNA, and this is an ATP-dependent process that is essential for maintenance of circular genomes (Bates and Maxwell 1989, Maxwell 1997, Champoux 2001). During DNA replication, topoisomerase IV plays an important role in unlinking precatenanes that form behind the replication fork, whereas gyrase relieves positive supercoiling that accumulates in front of the replication fork (Liu and Wang 1987, Wu et al. 1988). Topoisomerase IV is also responsible for resolving the catenanes that result from failure of gyrase to completely remove positive supercoils that accumulate between two converging replication forks, and also helps to relax excessive negative supercoiling induced by DNA gyrase (Wang 2002, Wang and Shapiro 2004).

The type II Topoisomerase Reaction

All type II topoisomerases are predicted to work using a two-gated mechanism (Roca and Wang 1994, Kampranis et al. 1999, Champoux 2001). Below is a description of the process as described in (Roca and Wang 1994, Kampranis et al. 1999, Champoux 2001). The enzyme has two gated cavities, one on either side of the G-segment (gated segment) of the DNA substrate. The N-gate is comprised of the B subunits and ATPase domains. The exit gate is called the C-gate and is comprised of the N-terminal portions of gyrA. The DNA capture domains lie in a cavity of the N-gated region, where positively charged amino acid residues in this area facilitate the binding of DNA. It is thought that binding of ATP to one of the ATPase domains induces a local conformation change which results in closing of the N-gate and also allows for the binding of a second ATP molecule. ATP hydrolysis is required to open the gate, and free forms of topoisomerase II
are thought to oscillate between N-gate open and closed forms. The first step in the topoisomerase reaction process is capture of G-segment DNA through the DNA capture domains that line the base of the N-gated cavity and extend over the CAP-like regions of the protein which contain the active site. Once the G-segment DNA is bound, conformation changes induce an increased rate of N-gate opening and closing using the ATP dependent mechanism described above. Next, another segment of DNA (T-segment) is trapped within the N-gated cavity (perhaps by topology recognition and interactions with the DNA binding domains), and through binding to ATP the N-gate is closed. The G-segment is then cleaved by nucleophilic attack of the active site tyrosine on the DNA phosphate backbone. Cleavage of the G-DNA induces a conformation change and the CAP regions are pulled apart to allow passage of the T-segment DNA into the lower cavity. Another conformation change thought to result from the hydrolysis of ATP enhances the transport of the T-segment to open the G-segment DNA gate. Following strand passage the G-DNA gate is closed and the G-DNA is re-ligated, which causes the C-gate to open and release the T-segment. A second ATP is hydrolyzed to facilitate the closing of the N-gate. In the case of DNA gyrase, the negative supercoiling reaction is dependent upon the C-terminal wrapping function of the A subunit which wraps DNA in a right-handed orientation to ensure that the nodal arrangement of G and T segments is positive, thus giving rise to a reaction that produces predominantly negative supercoils.

**Inhibitors of Type II Topoisomerase**

The two major classes of inhibitors that target the bacterial topoisomerases DNA gyrase and topoisomerase IV are 1) the fluoroquinolone compounds which are derivatives of the quinolone nalidixic acid, and 2) the coumerin compounds which are derived from *Streptomyces* species (Maxwell 1999, Drlica and Malik 2003). Examples of
the fluoroquinolones include: norfloxacin, biprofloxacin, levofloxacin, gemifloxacin, and moxifloxacin. Examples of the coumerins include: novobiocin, chlorobiocin, and coumermycin A1. A commonly used and potent fluoroquinolone against the bacterial type II topoisomerases is ciprofloxacin which is made of a nalidixic acid quinolone core molecule containing fluorine at C-6 position, a piperazinyl ring at the C7 position, a substituted carbon for the position 8 nitrogen, and an N-1 cyclopropyl. The most commonly discussed coumerin derivative is novobiocin, which consists of a 3-amino-4,7-dihydroxy-coumerin core attached to the sugar novoise. The fluoroquinolones, like the mammalian topoisomerase II inhibitors, are considered poisons. These inhibitors promote the rate at which covalent enzyme-DNA reaction intermediates are formed through causing distortions in the DNA where topoisomerases are bound (Wilstermann and Osheroff 2003). Replication forks and transcription complexes are unable to bypass these trapped ternary complexes. Ultimately, it is thought that the cytotoxic action of these inhibitors is the accumulation of double stranded lesions in the DNA that cannot be overcome by the DNA repair machinery (Wilstermann and Osheroff 2001). In mammalian cells these events trigger apoptosis. Coumerins directly inhibit topoisomerase II activity by competing for binding sites in the ATPase regions. With the exception of DNA gyrase relaxing activity, without the energy from ATP the conformation changes in the enzyme required to drive DNA relaxation, catenation/ decatenation, induction of negative supercoils (by DNA gyrase) do not occur. Bacterial and mammalian type II topoisomerases are largely inhibited by the same mechanism which is by inducing cytotoxic lesions in the DNA by stabilizing protein-DNA covalent bonds (Chen et al. 1984, Ross et al. 1984, Rowe et al. 1984, Rowe et al. 1986, Byl et al. 2001). Mammalian
enzymes however are sensitive to different classes of topoisomerase poisons including VP-16/VM-26 etoposides, amsacrine, and ellipticines.

**Characterization of the *Plasmodium* DNA Gyrase**

*Plasmodium falciparum* contains two pharmacologically distinguishable type II topoisomerase activities, one of which is sensitive to ciprofloxacin and is specific to the apicoplast DNA (Weissig et al. 1997). Furthermore, proliferation of *P. falciparum in vitro* is inhibited by the fluoroquinolone compound ciprofloxacin and coumerin drug novobiocin (Dahl and Rosenthal 2007, Goodman et al. 2007, Raghu Ram et al. 2007). Parasites treated with ciprofloxacin also show characteristic molecular effects of DNA cleavage and protein linked 5' DNA ends that are specific to the apicoplast DNA (Weissig et al. 1997). Recently a report was published where GyrA and GyrB subunits were detected by western blot from *P. falciparum* lysates using antisera generated against recombinant portions of the two polypeptides (Raghu Ram et al. 2007). In addition, the sub-cellular location of PfGyrA and PfGryB appear to be within the apicoplast (Dar et al. 2007, Raghu Ram et al. 2007). Analysis of recombinant *P. falciparum* and *P. vivax* GyrB has revealed that the ATPase activity of GyrB increases linearly with the concentration of enzyme (Khor et al. 2005, Raghu Ram et al. 2007). The ATPase activity of recombinant PfGyrB is further stimulated in the presence of a segment of plasmid DNA containing a strong DNA gyrase binding site (Raghu Ram et al. 2007). Treatment of *P. falciparum* with novobiocin results in a selective loss of apicoplast DNA (compared to nuclear and mitochondrial DNA) in the second asexual cycle after treatment (Raghu Ram et al. 2007). However, the recombinant enzyme has a reduced sensitivity to novobiocin (compared to *E. coli*) and it has been suggested that the *Plasmodium* GyrB may be able to dimerize in the absence of ATP, which could explain this reduced sensitivity since novobiocin binds
monomeric GyrB forms (Khor et al. 2005, Dar et al. 2007, Raghu Ram et al. 2007). Even with these differences, PfGyrB is able to complement *E. coli* temperature sensitive strains (Dar et al. 2007). Furthermore, recombinant PfGyrB in combination with *E. coli* GyrA or an N-terminal segment of PfGyrA shows cleavage activity that increases in the presence of ciprofloxacin in a dose-dependent manner (Dar et al. 2007). The complex of PfGyrB with *E. coli* GyrA can also transform relaxed DNA into a supercoiled state, however this activity is not very robust (Dar et al. 2007).

**Conditional Gene Regulation**

The complete sequencing of the *Plasmodium falciparum* genome provides a starting platform for efforts to develop vaccines and drugs by providing detailed molecular information about the genetic resources and metabolic pathways of the parasite. Although the complete *P. falciparum* genome sequence is available (Gardner et al. 2002), few methods for genetic manipulation have proven successful. Interruption or deletion of gene coding sequences through the use of single and double cross-over vector systems is currently the most common approach used to screen for essential *P. falciparum* gene products. The inability to successfully establish parasite culture following targeted gene knockout experiments provides weak, negative evidence that a gene is essential, and this is unsatisfactory as the foundation for a major project to undertake rational drug design. Unfortunately, the key enzymes enabling the RNA interference (RNAi) pathway are not present in the malarial genome, rendering this approach to gene knockdown unfeasible. Although there are a few isolated reports of some success with this approach (McRobert and McConkey 2002), the RNAi method is generally considered unreliable in identifying essential genes in the malaria parasite. New and robust methods employing alternative reverse genetic technologies are urgently
needed to identify proteins required by the parasite to perform critical cellular functions. Recent reports of functional tetracycline (Tet)-regulated gene expression and high-efficiency transposon-based transformation in *P. falciparum* demonstrate that alternative approaches for genetic manipulation of the parasite may be feasible (Balu et al. 2005, Meissner et al. 2005).

Episomal transgene expression can be conditionally regulated in *Plasmodium falciparum* through the use of a Tet-transactivator system (Meissner et al. 2005). The transactivator was created by random genomic insertion of the tetracycline repressor (TetR) gene in the apicomplexan *T. gondii* which was used to trap a transactivator sequence motif at the C-terminus of the TetR gene (Meissner et al. 2002). In combination with a TetR-responsive operator sequence (TetO) and minimal promoter, the resulting functional tetracycline-responsive transactivator (TaTi2) allowed conditional regulation of a green fluorescent protein (GFP) reporter gene in *P. falciparum*. Expression of TaTi2 was under the control of the 5’ region of *msp2*, the gene encoding merozoite surface protein 2, and follows the temporal pattern of *msp2* expression. In the absence of the tetracycline analogue anhydrotetracycline (ATc), TaTi2 binds the TetO and stimulates transcription of a downstream transgene. When ATc is present, it binds TaTi2 causing a conformational change in TaTi2 that does not allow it to bind TetO, thus transcription is minimal. In plasmid pTGPI-GFP, seven tandem repeats of the TetO sequence are positioned 5’ to the minimal CAM promoter sequence forming a tet-responsive expression cassette, which lies 5’ of the transgene insertion site containing GFP (Meissner et al. 2005). A concentration of 0.5µg/ml ATc was non toxic to *P. falciparum* and allowed 50-fold regulation of GFP protein expression. (Meissner et al. 2005).
2005). A potential application of this technology is the conditional regulation of native
*Plasmodium* gene transcription.

**Study Rationale and Specific Objectives**

Prevention and treatment of human malaria is heavily dependent upon the availability of effective antimalarial drugs, since there are no effective vaccines currently available. Unfortunately, the repertoire of antimalarial drugs effective against *Plasmodium falciparum*, the principal causative agent of human malaria, is dwindling due to widespread drug resistance. Gene products that perform an essential cellular function in the asexual blood stage have the greatest potential as targets for novel drugs. Target verification, the initial required step in rational drug development, is greatly hampered without the proper genetic tools. Numerous presumptive targets for rational drug design may be proposed among the ~5,300 predicted gene sequences from the *P. falciparum* genome, but the currently available methods for identifying essential gene products in *P. falciparum* are inefficient and for the present purpose yield inconclusive negative results.

In *Plasmodium* spp., apicoplast function is essential for parasite survival as it supports numerous biochemical pathways such as fatty acid, heme and isoprenoid biosynthesis (Ralph et al. 2001, Foth and McFadden 2003). Accordingly, preservation of the apicoplast genome by enzymes such as DNA gyrase are expected to be essential for survival, since they may be required for maintenance of the organelle (Fichera and Roos 1997, Weissig et al. 1997, Dar et al. 2007). It is especially urgent to investigate the importance of enzymes in *Plasmodium* that are already established drug targets of bacterial pathogens, such as DNA gyrase. DNA gyrase has no mammalian counterpart, and is the target of the clinically important quinolone and coumerin antibiotics.
The goals of this study were to further characterize the cellular and molecular functions of the *P. falciparum* DNA gyrase using ciprofloxacin as a pharmacological probe, and to define parameters of Tet-regulated gene expression in *P. falciparum* with the intent of developing an inducible gene-knockdown system that will allow the conditional regulation of endogenous genes. Conditional gene regulation will serve as an independent and important means for specifically probing gene functions, and enable the positive identification of genes that are essential for the growth of the asexual, intraerythrocytic parasite.

**Hypothesis**

I hypothesize that DNA gyrase inhibition by ciprofloxacin in *Plasmodium falciparum* selectively targets the normal remodeling of apicoplast DNA topology, which elicits cytotoxic effects due to apicoplast dysfunction that result in an immediate rather than a delayed arrest in the cell cycle.

**Specific Aims**

Aim 1: Utilize the DNA gyrase inhibitor ciprofloxacin to characterize the role of the *P. falciparum* DNA gyrase in the malaria parasite. a) Determine whether the DNA gyrase acts on the apicoplast DNA and/or whether it acts on the mitochondrial DNA. b) Examine the effects of drug treatment on organelar DNA replication, organelar RNA transcription and organelar DNA segregation. c) Determine whether the DNA gyrase interacts with the plastid DNA at specific locations.

Aim 2: Demonstrate the feasibility of converting the *Plasmodium falciparum* Tet-transactivator system into a tool that will allow the conditional regulation of gene expression within a gene’s native locus. a) Replace the native 5' regulatory sequences of the non-essential gene *pfpm4* with a tetracycline-analogue-regulated promoter by single-
crossover homologous recombination. b) Use the piggyBac transformation system to stably integrate single-copy transactivator (tati2) expression cassettes into the P. falciparum genome. c) Demonstrate stable transgene expression of TaTi2 in the piggyBac transformants. d) Use the piggyBac transformation system to integrate tati2 expression cassettes into parasites where pfpm4 is under the regulation of the TetO / min. cam promoter. e) Conditionally regulate the expression of pfpm4. f) Measure the range of control that is afforded by utilizing a genomic integration strategy to demonstrate Atc-regulation of gene expression in P. falciparum
CHAPTER 2
MATERIALS AND METHODS

Parasite Culture

Asexual stages of *Plasmodium falciparum* (strain 3D7) were cultured in vitro within human red blood cells (RBCs) at 4-5 % hematocrit in complete medium [RPMI 1640, 0.005 % hypoxanthine, 0.225 % sodium bicarbonate, 25 mM Hepes, 0.5 % Albumax (Invitrogen), 0.01 mg/ml gentamycin, sterile deionized water; pH 6.8 and 0.22 µM filtered] (Trager and Jensen 1976). Cultures were maintained at 37 ºC in a controlled atmosphere of 1% O₂, 5% CO₂, and 94% N₂. ºC

Synchronization of Asexual Parasite Stages

Sorbitol Method

Culture medium was aspirated and replaced with the same volume of sorbitol solution (5 % sorbitol in sterile deionized water; 0.22 µM filtered) (Lambros and Vanderberg 1979). Parasites were resuspended in the sorbitol solution, transferred to 50 ml conical tubes, and incubated at room temperature for 15 minutes. Cells were collected by centrifugation at 800 x g for 5 minutes and the supernatant was poured off. Cells were washed twice with 30 – 50 ml of complete medium and collected by centrifugation. Fresh RBCs were added to adjust the parasitemia and cultures were re-plated with complete medium and incubated as previously described. The procedure was repeated at 2 days and 4 days after the first treatment to obtain synchronous parasites.

Percoll / Sorbitol Density Gradient Centrifugation

Percoll was diluted to the desired concentration with deionized water with a 1X final concentration of incomplete media (RPMI 1640, 0.005 % hypoxanthine, 0.6 % Hepes, sterile
deionized water; pH 6.8 and 0.22 µM filtered) and with a final concentration of 6 % (w/v) sorbitol (Fernandez et al. 1998). Five ml of a 70 % solution of percoll was first pipetted into a 15 ml conical tube and an equal volume of the 40 % solution was carefully overlaid, taking care not to introduce any bubbles. Parasite suspension was carefully layered over the 40 % solution. Tubes were centrifuged at 1000 x g for 10 minutes and the parasite layer at the interface of the 40 % and 70 % solutions was recovered to isolate late stage parasites, and/or the pellet was collected to recover ring stage parasites. For preparation of highly synchronized cultures, the late stage parasites were allowed to invade fresh RBCs for a 3 h period, after which cultures were treated with one round of sorbitol lysis as described above to leave only the newly invaded rings.

**Magnetic Isolation**

Twenty milliliters of culture (3-5 % parasitemia, 4 % hematocrit) containing predominately schizont-stage parasites (obtained by 2-3 successive sorbitol treatments) were concentrated to 4 ml in complete medium. The magnetic column (type LS, Miltenyi Biotec) was pre-conditioned with 4 ml of complete medium. Next, the column was attached to a MidiMACSTM magnet and the parasite concentrate was applied to the column reservoir and allowed to pass by gravity flow (Balu et al. 2005). The column was washed 2 times with 5 ml of incomplete medium. The column was detached from the magnet and the late-stage parasites were eluted with 2 ml of complete medium. The parasites in the column eluent were collected by centrifugation, and transferred back into culture with fresh RBCs for 3 hours to allow for re-invasion. Cultures were treated with sorbitol once as described above to eliminate any remaining schizonts, leaving only newly-invaded ring forms.
Parasite Transfection

Direct Electroporation Method

Predominately ring-stage cultures at 5-6 % parasitemia were obtained by successive sorbitol treatments. Cells and plasmid DNA were resuspended in cytomix solution [120 mM KCl; 0.15 mM CaCl₂; 10 mM K₂HPO₄/KH₂PO₄, pH 7.6; 25 mM Hepes, pH 7.6; 2 mM EGTA, pH 7.6; 5 mM MgCl₂; pH adjusted with KOH] (Wu et al. 1995). A volume of 400 µl containing cells and plasmid DNA (100 µg) was loaded into pre-chilled (-20 ºC) 2 mm cuvettes and electroporated under low voltage, high capacitance (0.31 kV, 960 µF) conditions using a Gene Pulser II (BioRad) (Fidock and Wellems 1997). Cells were immediately transferred into culture and media was changed after 1 hour, and daily thereafter. Drug selection was started at 2 days post-transfection. Parasites were stably transformed with pSSPF2/PfACP-GFP (plasmid kindly provided by Shigeharu Sato, National Institute for Medical Research, London, UK) to generate culture ACP-GFP. Parasites were stably transformed with pTGPI-GFP (plasmid kindly provided by Brendon Crabb, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) to generate culture pTGPI-GFP. In cultures ACP-GFP and pTGPI-GFP, plasmid DNA was maintained extrachromosomally and selection was conducted with 2.5 nM WR99210. For pTGPI-GFP, anhydrotetracycline (1 µg/ml) was added to the culture medium. Parasites were stably transformed with pTOCAM-Δpm4 which was targeted for integration by single-crossover homologous recombination. Selection was conducted with 5 nM WR99210. Plasmid maps are shown in Figures 2-1, 2-2, & 2-3. Clonal parasite lines were obtained by limiting dilution (Rosario 1981).

RBC-loading Method

The RBC-loading method (Deitsch et al. 2001) was used for piggyBac transformation. Plasmid pGYBSD-TA or pGYBSD-TATK (300 µg) containing the ITR recognition sequences,
and 150 µg of helper plasmid were combined and precipitated in 0.3M sodium acetate pH 5.2, 67% ethanol. The pellet was washed with 70 % ethanol, air dried, and resuspended in 50 uL TE buffer. For each transformation 1.5 ml of 50 % (packed cell volume) RBCs was washed with 1.5 ml of cytomix solution, the volume was brought back to 3 ml with cytomix, and plasmid DNA was added. RBCs were electroporated as described above and washed with complete medium. Plasmid-loaded RBCs were brought to a 5 % hematocrit with complete medium. Approximately 1 x 10^6 schizonts isolated from the magnetic column were added to the plasmid-loaded RBCs and the cells were placed in culture (Balu et al. 2005). After two days in culture, the media was replaced with selective media containing 2.5 µg / ml blasticidin-S and maintained with daily media changes for 5 days. Selective media was then removed and cultures were maintained with daily media changes without drug selection until parasites were detected in thin smears prepared from the cultures. Selective media containing blasticidin-S was used for an additional 5 days. This procedure of alternately culturing in the presence and absence of drug was continued until episomal plasmid was lost and only parasites with chromosomally-integrated plasmid remained. Cultures pGYBSD-TA, pGYBSD-TATK, and C3TA were generated using this method. Clonal parasite lines were obtained by limiting dilution (Rosario 1981).

**Drug Assays**

**Parasitemia Counts**

Ciprofloxacin (5 µg/ml, 10 µg/ml, and 25 µg/ml) was added to highly synchronous cultures at the newly-invaded ring stage. Parasitemia was determined approximately every 12 hours by counting stained blood smears. At least 10 fields of view were counted, with a minimum of 1000 cells counted.
Parasite Growth Inhibition Measured by [$^3$H]-hypoxanthine Incorporation

Drug dilutions were added to *P. falciparum* 3D7 asynchronous culture (0.5-1.0% parasitemia, 1.5-2.0% hematocrit) in triplicate, in 96-well plates at a final volume of 200 µl / well. The parasites were cultured in low-hypoxanthine (2.5 mg/L) containing medium for 48 h. After 48 h, 100 µl of medium was removed and replaced with 100 µl of ‘low-hypoxanthine’ medium containing [$^3$H]-hypoxanthine at a concentration of 0.5 µCi/mL (Chulay et al. 1983, Fidock et al. 1998). After an additional 24 h, cells were harvested onto glass fiber filters and washed thoroughly with distilled water. Dried filters were placed in sample bags and immersed in scintillation fluid (PerkinElmer), and radioactive emissions were counted in a 1450 MicroBeta reader (PerkinElmer). Percentage reduction in hypoxanthine uptake (a marker of growth inhibition) was calculated as follows: reduction = 100 × [(geometric mean cpm of no-drug samples)-(geometric mean cpm of test samples)] / (geometric mean cpm of no-drug samples). The percentage reductions were plotted as a function of drug concentration using the variable-slope sigmoidal dose-response nonlinear regression equation (Systat Software Inc.).

Parasite Proliferation Rate by Flow Cytometry

The proliferation rate for parasite clones 3D7, C3 and B3 were determined in duplicate cultures and were sampled every 24 hours for 12 days. For collection, 100 µL of well-mixed parasite culture was gently pelleted by centrifugation for 3 minutes at 800 x g. The parasite pellet was washed with PBS and resuspended in 500 µL of 2% paraformaldehyde in PBS. Samples were stored at 4 °C. Twenty-four hours prior to flow cytometry analysis, the fixative solution was replaced with 500 nM Cyto24 (Molecular Probes) in PBS and samples were stored at 4 °C. Fifty thousand cells from each sample were sorted based on fluorescence, and the geometrical mean of duplicate series 1 and 2 were plotted with the standard deviation.
**Derivation of Plasmid Constructs**

Plasmid pTGPI-GFP (described in Meissner et al 2005) was the kind gift of Brendan Crabb of The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia (Figure 2-1), plasmid pXLBacII derivative #352 was the kind gift of Alfred Handler (USDA, ARS, CMAVE, Gainesville, FL). Plasmids pBSD-CAM and pHHT-TK were the kind gift of David Fidock (Columbia University, New York, NY). All PCR products and digested fragments were gel isolated and extracted using the QiaQuick gel extraction kit (Qiagen). All PCR products were sub-cloned into the pCR2.1 vector from the TOPO-TA cloning kit (Invitrogen), transformed into chemically-competent *E. coli* (TOP10 cells), and sequenced. Large plasmids were transformed into chemically-competent XL10-Gold cells (Stratagene). Restriction enzymes were purchased from NEB, and DNA ligase was purchased from Invitrogen.

To create plasmid pTOCAM-Δpm4 (Figure 2-2), the first 727 bp of *pfpm4* were amplified from 3D7 gDNA by PCR and the fragment was cloned into pCR2.1. The fragment was re-amplified with primers containing restriction enzyme linkers for *Pst* I and *Bgl* II and re-cloned into pCR2.1. Both the *pfpm4*-pCR2.1 plasmid and pTGPI-GFP were digested with *Pst* I and *Bgl* II and fragments were gel isolated and purified. The *pfpm4* fragment was ligated into pTGPI-GFP, replacing GFP and the transactivator expression cassette

To create plasmids pGYBSD-TATK and pGYBSD-TA (Figure 2-3), first the 5’ UTR from *msp2* was PCR amplified from plasmid pTGPI-GFP, using primers containing *Sac* II and *Not* I restriction sites, and cloned into the *Sac* II / *Not* I cloning site in plasmid pBSD-CAM. The *tati2/Pbdhfr* 3’ UTR was PCR amplified with primers containing *Age* I and *Not* I restriction sites, and cloned into the *Age* I / *Not* I cloning site of pBSD-CAM-5’ *msp2* to make plasmid pBSD-TA. Next, the 5’ ITR from pXLBacII was PCR amplified to contain *Bgl* II and *Sac* II restriction linkers and the 5’ ITR was cloned into the *Bgl* II / *Sac* II restriction sites in pXLBacII to create...
pXL BacII-5’2X. Plasmids pXL BacII-5’2X and pHHT-TK were digested with Spe I and Not I and the 4.1 kb and 2.9 kb fragments were isolated respectively and cloned together to make pGYTK. Plasmid pBSD-TA was digested with Kpn I and Cla I and the 4.1 kb fragment was isolated and cloned into the Kpn I / Cla I site in pGYTK to create plasmid pGYBSD-TATK. To create pGYBSD-TA, pGYBSD-TATK was digested with EcoR I and the large fragment was re-ligated and cloned.

Quantitative PCR standards were generated by cloning one target amplicon into the TA cloning site of pCR2.1, and the other target amplicon into the Pst I site of the pCR2.1 plasmid.

**Southern Blot Analysis**

Parasites were treated with 0.05% saponin and DNA was released by SDS lysis. Lysates were incubated in the presence of 100 µg/ml proteinase K for 2 – 15 h at 65 ºC. DNA was purified by phenol-chloroform extraction, precipitated with isopropanol, and resuspended with nuclease-free water. DNA was digested with restriction enzymes overnight, and 5µg of the digested DNA was electrophoresed in a 0.75 % agarose gel for 6 hours at 60 V. The gel was exposed to UV light for 5 minutes to nick DNA, and was denatured with 1.5 M NaCl, 0.5 N NaOH (2 x 20 min). The gel was neutralized with 1 M Tris-HCl (pH 7.5), 1.5 M NaCl (2 x 20 min), and soaked in 10 X SSC (2 x 15 min). DNA was transferred to positively-charged nylon membranes and UV cross-linked for 2 minutes (Brown 1999). Membranes were blocked with 2 % casein, 0.2 % SDS, 0.1 % Sarkosyl, 5X SSC for 2 hrs at 50 ºC – 60 ºC depending on which probe was used (Roche 2000). Membranes were hybridized overnight (at 50 ºC – 60 ºC) with DIG-labeled DNA probes. The membrane was washed 2 × 5 min with 2 × SSC / 0.1 % SDS at room temperature and 2 × 20 min with 0.1 × SSC / 0.1 % SDS at 55 ºC – 60 ºC. Membranes were blocked with 4 % casein in 100 mM maleic acid and 150 mM NaCl for 2 hours and alkaline phosphatase-conjugated anti-DIG was added at a 1:10,000 dilution in the same blocking solution.
for 1 hour (Roche 2000). Membranes were washed with $2 \times 30$ minutes with 0.3 % Tween-20 in 100 mM maleic acid and 150 mM NaCl and equilibrated with detection buffer (100 mM Tris-HCl, 100 mM NaCl, and 50 mM MgCl$_2$, pH 9.5). The chemiluminescent substrate CDP-Star (Roche) was applied and the membrane was exposed to film.

**Northern Blot Analysis**

RNA was extracted from saponin-lysed cultures using Trizol (Invitrogen) according to the manufacturer’s protocol. Purified RNA was added to 3 volumes of loading buffer (Sigma), heated at 60 ºC for 10 minutes then placed on ice. Approximately 5 µg of RNA was electrophoresed at 50 V through a 1 % agarose gel containing formaldehyde (Brown et al. 2004). The gel was soaked in 20 X SSC for 2 x 15 minutes then the RNA was blotted to positively-charged nylon membrane overnight by capillary transfer (Brown et al. 2004). RNA was cross-linked to the membrane by exposure to UV-light for 2 minutes. The membranes were pre-hybridized for 1 hour in DIG Easy Hyb (Roche) at 50 ºC. The membranes were hybridized with DIG-labeled probe overnight at 50 ºC. DIG-labeled DNA probes that were generated using gene-specific primers, DIG-labeled dUTP (Roche), and exo(-) Klenow DNA polymerase (Stratagene). Membranes were washed with 2X SSC and 0.1 % SDS for 2 x 5 minutes at room temperature then with 0.2 X SSC and 0.1 % SDS for 2 x 20 minutes at 50 ºC. For detection, the membranes were further processed with the DIG wash and block buffer set (Roche) according to manufacturer’s instructions. The chemiluminescent substrate CDP-Star (Roche) was applied and the membrane was exposed to film.

**Western Blot Analysis**

Asynchronous parasites were saponin treated (final 0.05%, w/v) for 5 min and washed 3 times with PBS to remove RBC ghosts. All centrifugations were performed at 10,000 X g for 10 minutes. Extracellular parasites were resuspended in SDS sample buffer [100 mM Tris-Cl (pH
Parasite lysates were electrophoresed through either 10 % or 12 % SDS-PAGE gels and transferred by electroblotting onto a PVDF (0.22 μM) membrane (Coligan 1996). Membranes were blocked for 1-8 hours with 100 mM Tris, 150 mM NaCl, pH 7.5, 0.1% Tween-20, 5 % skim milk and probed with dilutions of antisera (1:10,000 of α-PfPM4, 1:10,000 of α-BIP, 1:800 of α-TetR, 1:1000 of α-GFP, 1:6,000 of α-FP3, or 1:6,000 of α-PfPM1) in 100 mM Tris, 150 mM NaCl, pH 7.5, 2 % skim milk overnight at 4 ºC. After incubation with primary antibody, membranes were washed 10 times with 25 ml of 100 mM Tris, 150 mM NaCl and 0.1% Tween-20 over 2 hrs. Secondary antibody (1:150,000 HRP-conjugated goat anti-rabbit or HRP-conjugated goat anti-rat was added in 100 mM Tris, 150 mM NaCl, pH 7.5, 2 % skim milk. After 1.5-hours of incubation with secondary antibody, membranes were washed 10 times with 25 ml of 100 mM Tris, 150 mM NaCl and 0.1% Tween-20 over 1-2 hours. Membranes were soaked in SuperSignal West Dura chemiluminescent substrate (Pierce/Thermo Scientific) for 5 minutes and exposed to film.

**Quantitative PCR and Quantitative Reverse- Transcriptase PCR**

Parasites were synchronized to obtain a population of parasites proceeding through the asexual development cycle within a 3 h window of development. Initially cultures were treated with 5 % sorbitol every 40 – 48 hours, for three consecutive treatments. Late schizont-stage parasites were then isolated by percoll / sorbitol density gradient centrifugation, introduced to red blood cells and cultured for 3 hours to allow merozoites to re-invade. Cultures were next treated with 5 % sorbitol to leave only the newly invaded ring-stages. Parasites were cultured both in the presence and absence of 5 μg/ml ciprofloxacin and samples were collected at specific developmental time points based upon the development of the untreated controls (t₀ = newly-invaded rings). DNA was released from saponin-lysed parasites using 1 % Triton X-100, 1 mM
EDTA, and 10 mM Tris-HCl (pH 7.5). The lysates were incubated at 65 °C for 2 hours in the presence of 60 µg/ml proteinase K, boiled for 10 minutes and immediately placed in an ice bath. The samples were diluted 1:10 in deionized water for quantitative PCR analysis. RNA was isolated from saponin-lysed parasites using Trizol (Invitrogen) following the manufacturer’s protocol. Purified RNA was treated with DNase I (Promega), and reverse-transcribed using random hexamers and SuperScript III reverse transcriptase (Invitrogen). The cDNA synthesis reactions were seeded with an RNA transcript generated in vitro off the T7 promoter from a pCR2.1 cloned segment of the Cryptosporidium parvum COWP gene. The RNA transcript served as an inhibition control. Cryptosporidium DNA was the kind gift of Michael Riggs (University of Arizona, Tucson).

Quantitative PCR analysis was performed using a DNA Engine Opticon 2 continuous fluorescence detection thermocycler. Quantities of target gene were calculated based on a titration of recombinant plasmid DNA containing both a segment of a single copy nuclear gene (pm4) and a segment of either a plastid gene (clpC or LSU) or mitochondrial gene (cytB or cox1). Samples of DNase treated RNA were run for each cDNA sample to verify DNA removal. Data plots are representative of 5 separate experiments. In the case of the qPCR analysis for the GFP FACS experiment, the plasmid standard contained all 4 gene targets (tati2, pfpm4, gfp, β-lactamase) to allow for normalization of primer amplification efficiencies. For analysis of pfpm4 5’ and 3’ cDNA, data was normalized to apicoplast LSU cDNA levels.

**Fluorescent in situ Hybridization (FISH)**

Parasites were saponin-treated, washed with PBS and deposited as monolayers on microscope slides (Bond-Rite, Richard-Allan Scientific). A hydrophobic barrier was drawn around the cell monolayer using a PAP-PEN and slides were air-dried at room temperature overnight. Cells were fixed for 15 min with 4 % paraformaldehyde and permeabilized with 0.1 %
NP-40. Ice-cold acetone was used to clear lipids and cells were treated with 20 µg/ml RNase in 2X SSC at 37 ºC for 30 minutes. Slides were washed 3 times for 3 minutes in PBS after each of the above steps. The cell monolayers were denatured then hybridized overnight at 37 ºC in the presence of 2X SSC, 10 % dextran sulphate, 50 % deionized formamide, 250 ng/ml herring sperm DNA, and 1 ng of DIG-labeled probe (Mancio-Silva et al. 2004). The hybridization solution was sealed on the monolayer by a coverslip placed over the hydrophobic barrier. Slides were washed in 2X SSC at 50 ºC for 10 minutes, 2X SSC at 55 ºC for 10 minutes, and 4X SSC at 22C for 10 minutes (Mancio-Silva et al. 2004). Slides were blocked with 1% Roche blocking reagent and 4% bovine albumin then incubated at for 2 hours at 22 ºC with 1 µg/ml sheep anti-DIG. Secondary antibody (Alexa 594 goat anti-sheep) was applied at 2 µg/ml for 45 minutes. Slides were washed 3 times in 100 mM Tris, 150 mM NaCl and 0.3% tween-20 (Mancio-Silva et al. 2004) and counterstained with 1 µg/ml Hoechst 33342.

**Combined FISH / IFA**

Ten milliliters of 1-3 % parasitemia culture was washed 3 times with PBS and the cells were resuspended in 3-4 ml of PBS. Saponin (5 %, w/v stock in PBS) was added to a final concentration of 0.05 % and tubes were immediately centrifuged at >10,000 x g for 10 min. The parasite pellet was washed 3 times with PBS, taking care to remove the residual RBC ghosts. The pellet was finally resuspended in 20 – 60 ul of PBS. Approximately 2 ul of the suspension was spread onto a Bond-Rite slide (Richard-Allan Scientific) to distribute cells as a monolayer. The slide was air dried for 30 min to overnight. A hydrophobic barrier was drawn around the monolayer using a PAP pen. Slides were fixed in with 4 % ultra pure formaldehyde (16 % stock from Polysciences Inc., No:18814-20) in PBS for 15 min, and washed 3 x 5 min with PBS. Next, cells were permeabilized with 0.05 % Triton-X in PBS for 15 min, and washed 3 x 5 min with PBS (no agitation in Coplin jar). The slides were gently blotted dry with Kimwipes. The
monolayers were blocked for 1 hour with blocking buffer (1 % w/v Roche blocking reagent in 100 mM maleic acid, 150 mM NaCl, pH 7.5) and incubated with anti-GFP rabbit polyclonal antibodies (1:100 dilution) in blocking buffer overnight at 4 °C in a moist chamber. Primary antibody solution (15-20 µl) was added to the monolayer, and a coverslip was used to seal liquid within the PAP pen markings. Slides were washed 3 x 20 min in washing buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.5 % v/v Tween-20) in Coplin jars. Next, the monolayers were blocked for 1 hour with blocking buffer and incubated with goat anti-rabbit Alexa Fluor 488 or 568 (1:400 dilution) for 1-2 hours at room temperature. Slides were washed 3 x 20 min in washing buffer with no agitation in Coplin jars and gently blotted dry. Slides were fixed again with 4 % ultra pure formaldehyde in PBS for 15 min, washed 3 x 5 min with PBS, and gently blotted dry. Next, slides were incubated with 70 % deionized formamide in 2X SSC at 70 °C for 10 min (~ 20 µl of solution was added to the monolayer and sealed with coverslip) (Freitas-Junior et al. 2005). The coverslip was quickly and gently removed and the slide was immediately dehydrated in an ice-cold ethanol series (50 %, 70 %, 90 %, 100 % EtOH for 1 minute each) and the slides were air-dried (Freitas-Junior et al. 2005). FISH probes were denatured for 5 min at 95 °C in hybridization solution (50 % deionized formamide v/v, 10 % dextran sulphate w/v, 2X SSC, 250 µg/ml herring sperm DNA). While the probe was denaturing, slides were briefly heated to 70 °C. Hybridization solution (10 µl) was added to the monolayer and the area was sealed with cover slip. Slides were incubated at 70 °C for 7 min, then overnight at 37 °C in dark (Freitas-Junior et al. 2005). The coverslips were gently removed and the monolayers were incubated with 50 % v/v deionized formamide in 2 X SSC for 30 min at 37 °C. Slides were washed with 2× SSC for 10 min at 55 °C, 2× SSC for 15 min at 60 °C, and 4× SSC for 10 min at room temperature. For DIG-labeled FISH probes, the monolayers were blocked for 15 min with
blocking buffer, and incubated with sheep anti-DIG (1:200) in blocking buffer for 1.5 hours at room temperature in the dark. Slides were washed 3 x 20 min with washing buffer in a 50 ml conical centrifuge tube with periodic inversion. The monolayers were incubated with donkey anti-sheep Alexa Fluor 594 (1:400) in blocking buffer for 1 hour at room temperature in dark. The slides were washed 3 x 20 min with washing buffer in a 50 ml conical centrifuge tube with periodic inversion. The slides were gently blotted dry and mounted with Vectashield with DAPI.

**FISH Probe Synthesis**

Sixteen gene segments from different regions of the apicoplast DNA were PCR amplified and gel extracted. The gene regions encompassed 7 kb of sequence and included both LSU rRNA genes of the inverted repeat, both SSU rRNA genes of the inverted repeat, *sufB*, ORF 51, *rpoB*, *rpoC*, *rpoC2*, ORF 79, *clpC*, *tufA*, *rps7*, *rps12*, *rps3*, *rps19* (Figure 2-1). The amplicons were either pooled or individually labeled with DIG-dUTP using exo – Klenow and specific primers. The labeled amplicons were sonicated to an average fragment size of 300 bp.

Plasmid clone #410 was the kind gift of Akhil Vaidya (Drexel University, Philadelphia, PA) and contained 5.8 kb of the *P. falciparum* mitochondrial DNA. The plasmid was digested with *Eco*RI and *Bam*HI, the mitochondrial DNA sequence was isolated and was DIG-labeled with random hexamers. The labeled DNA was sonicated to produce fragments with an average size of 300 bp.

**Production of DNA Gyrase Antibody**

Anti-GyrA rabbit antiserum was generated against the peptide VEYIKNFDGNEREPK by Sigma-Aldrich. Serum was collected from the immunized rabbit (#887) every 3 – 4 weeks, and booster immunizations were administered between each collection. Anti-GyrA antibody was affinity purified against the immunizing peptide using the SulfoLink Immobilization Kit for Peptides (Pierce/Thermo-Scientific).
Immunoprecipitation of Protein-DNA complexes

Parasites were cultured in the presence of drug for 3 hours (ciprofloxacin: 25 µg/ml, VP16: 50 µM, VM26 25 µg/ml), and control cultures received the equivalent volume of dH2O or DMSO. Cells were collected and treated with saponin (0.05% w/v) for 10 min at room temperature and centrifuged at 10,000 x g for 10 min. The parasite pellet was washed 2 times with complete medium and resuspended in drug (ciprofloxacin: 1 µg/ml, VP16: 10 mM, VM26 10 µg/ml), dH2O, or DMSO for 15 min at 37 ºC. One milliliter of preheated (65 ºC) lysis solution (1 % SDS, 0.4 mg/ml herring sperm DNA, 5 mM EDTA, pH 8) was added to the parasite pellet and lysates were incubated for 10 min at 65 ºC (Rowe et al. 2001). Next, KCl was added to a final concentration of 65 mM and the lysates were vigorously vortexed for 10 s to fragment DNA (Rowe et al. 2001). Samples were placed on ice for 10 min to separate the DNA covalently bound to protein (precipitate), from the protein-free DNA (supernatant) (Rowe et al. 2001). Samples were centrifuged at 10,000 x g for 10 min at 4 ºC. The supernatant was transferred to a clean microcentrifuge tube and placed on ice for later. The pellet was resuspended in wash solution (10 mM Tris-HCl, 0.1 mg/ml herring sperm DNA, 5 mM EDTA, pH 8.0) and incubated at 65 ºC for 10 min (Rowe et al. 2001). Samples were placed back on ice for 10 min to allow precipitation to occur, and centrifuged at 10,000 x g for 10 min at 4 ºC. The wash step was repeated. A portion of the supernatant and pellet fraction (1/6th total volume) was transferred to a clean microcentrifuge tube for direct extraction and analysis. For direct extraction, samples were adjusted to contain a final concentration of 1 % SDS, 100 mM NaCl, and 60 mM EDTA with 500 µg/ml proteinase-K in 500 ul, and were incubated at 65 ºC overnight. DNA was extracted by phenol/chloroform/isoamyl 25:24:1 followed by chloroform/isoamyl 24:1. DNA was precipitated with a final concentration of 0.3 M sodium acetate and 2 volumes of 100 % ethanol at -20 ºC for
1 hour. The DNA pellet was washed with 70 % ethanol and resuspended in 40 µl of H2O. The DNA (2.5 µl of a 1:10 dilution) was analyzed in duplicate by quantitative PCR.

For immunoprecipitation, the SDS-KCl pellet was resuspended in 1 ml of non-denaturing lysis buffer [1 % Triton X-100, 50 mM Tris-HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, protease inhibitor cocktail (complete, Mini; Roche)] (Bonifacino and Dell'Angelica 1998). Recombinant protein G agarose beads (Invitrogen) were washed 5 times with PBS and equilibrated in non-denaturing lysis buffer for 30 min at room temperature on a rotating wheel. To reduce non-specific binding to protein G agarose, 50 µl of a 50 % agarose bead slurry in non-denaturing buffer was added to the 1 ml sample lysate (Bonifacino and Dell'Angelica 1998). The mixture was incubated at room temperature for 3 hours with constant mixing using a rotating wheel. After the pre-clearing step, samples were centrifuged at 10,000 x g for 10 minutes to pellet the agarose beads and other cellular debris. The supernatant was taken and divided equally into 3 new microcentrifuge tubes. Samples received one of the following: 10 µg of affinity purified gyrase subunit A antibody (anti-GyrA #887), 10 µg of pre-immune sera (Pre-I #887), or no antibody. Tubes were affixed to a rotating wheel to supply constant mixing for 1 hour at room temperature, and were kept at 12 ºC overnight. Fifty microliters of a 50 % suspension of protein G agarose with herring sperm DNA was added to the samples and tubes were incubated at room temperature for 1 hour on a rotating wheel (Bonifacino and Dell'Angelica 1998). The protein-G beads were collected by centrifugation at 8,000 x g for 2 minutes. The beads were washed 2 times with ChIP lysis buffer [50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % SDS, protease inhibitor (complete, Mini; Roche)] (Yan et al. 2004), 2 times with TE buffer with protease inhibitors, and 2 times with ice-cold TE (Bonifacino and Dell'Angelica 1998). The agarose beads were resuspended in 30 µl of PCR lysis buffer (1 %
Triton X-100, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.5), and incubated for 2 hours with 200 µg/ml proteinase-K. Samples were boiled for 10 minutes and placed on ice. The samples were directly analyzed by quantitative PCR.
Figure 2-1. Map of the 34,682 bp (‘35’ kb) *Plasmodium falciparum* apicoplast DNA. Gene sequences were obtained from PlasmoDB (plastid IRA and IRB) and the circle was constructed in Vector NTI (Invitrogen) based on information published in Wilson et al 1996. *Xba* I and *Hind* III restriction sites are shown.
Figure 2-2. Plasmid map of pTGPI-GFP (described in Meissner et. al. 2005). Plasmid pTGPI-GFP was the kind gift of Dr. Brendan Crabb of the Walter and Eliza Hall Institute of Medical Research. This plasmid allows stable transgene expression of an anhydrotetracycline (ATc)–responsive transactivator (TaTi2) in *P. falciparum*. In the absence of ATc, TaTi2 interacts with the Tet-operator (TetO), and in combination with a minimal promoter (min cam) stimulates the expression of GFP. In the presence of ATc, GFP expression is silenced.
Figure 2-3. Plasmid map of pTOCAM-Δpm4. Plasmid pTOCAM-Δpm4 was designed to target the pfpm4 locus for single cross-over homologous recombination. The recombination event places the TetO/min cam promoter directly upstream of the full length pfpm4 gene. The hDHFR gene was used for a drug-selectable marker.
Figure 2-4. Plasmid maps of pGYBSD-TATK and pGYBSD-TA. Plasmids pGYBSD-TATK (A) and pGYBSD-TA (B) were engineered with the *piggyBac* transposase inverted terminal repeat sequences (5’ and 3’ ITR), the BSD gene for a drug-selectable marker, and the *tati2* expression cassette. When transfected with a helper plasmid encoding the piggyBac transposase, the sequence elements flanked by the ITRs in pGYBSD-TATK and pGYBSD-TA are randomly integrated into TTAA genomic sites. pGYBSD-TATK was further engineered with a second 5’ ITR to allow re-mobilization of the thymidine kinase expression cassette, leaving the remaining integrated sequence unable to re-mobilize.
1) 1 kb+ ladder  
2) 50 bp ladder  
3) clpC amplicon  
4) LSU amplicon  
5) cytB amplicon  
6) cox1 amplicon  
7) ASL amplicon  
8) pm4 amplicon

Figure 2-5. Agarose gel electrophoresis of the PCR amplicons generated from the quantitative PCR (qPCR) primer and probe sets that were used to measure apicoplast (clpC, LSU) and mitochondrial (cytB, cox1) DNA and cDNA copies (A). For apicoplast and mitochondrial genome copy number determinations, the apicoplast and mitochondrial qPCR targets were normalized to the quantity of a single-copy nuclear gene segment (pm4). Plasmid standards were generated for the qPCR assay that contained both a segment of pm4 and the target organellar gene, which allowed for the normalization of different primer set efficiencies, and provided precision in calculating the relative quantities of 2 different PCR targets (B). To test whether nuclear DNA quantity represented by pm4 (chr 14) was representative of other nuclear genes, preliminary experiments were conducted comparing levels of pm4 to ASL (chr 2). Results indicated that the quantities of two gene segments from different chromosomes were similar with respect to the timing and temporal pattern of replication through the P. falciparum asexual cell cycle. Prior to cDNA synthesis, an in vitro transcript was generated from the Cryptosporidium parvum COWP gene and was seeded into each sample as an inhibition control (B).
Figure 2-6. Schematic representations of genomic loci containing Tet-transactivator elements showing the anticipated transgene affect of TaTi2 in the absence and presence of anhydrotetracycline (ATc). (A) Single crossover homologous recombination event using plasmid pTOCAM-Δpm4. (B) In the absence of ATc, TaTi2 binds the operator (TetO) turning expression of pfpm4 ON. (C) In the presence of ATc, TaTi2 does not bind the TetO maintaining transcription of pfpm4 OFF.
CHAPTER 3
RESULTS

Drug Studies

Effect of DNA Gyrase Inhibitors on Parasite Proliferation

Proliferation of *P. falciparum in vitro* (measured by \(^3\)H-hypoxanthine uptake) was inhibited by the fluoroquinolone compound ciprofloxacin in a dose-dependent fashion with an IC\(_{50}\) value of 4.9 µg/ml ± 1.3 (Figure 3-1A). Parasite proliferation was similarly inhibited in a dose-dependent fashion by coumermycin with an IC\(_{50}\) value of 5.2 µg/ml (Figure 3-1B); and novobiocin with an IC\(_{50}\) value of 15.8 µM (Figure 3-1C).

Effects of Ciprofloxacin on Parasite Proliferation, Growth, and Viability

Results of the \(^3\)H-incorporation assay were confirmed by following the parasitemia of synchronous parasite cultures treated with ciprofloxacin at three different concentrations (5 µg/ml, 10 µg/ml and 25 µg/ml). At 5 µg/ml ciprofloxacin, approximately half the numbers of newly invaded rings (compared to control) were present in the second asexual cycle (Figures 3-2 and 3-3). At a concentration of 25 µg/ml ciprofloxacin, parasites were immediately inhibited within the first asexual cycle and did not produce new progeny over the next 60 hours that were followed (Figure 3-2). Even in cultures treated with 5 µg/ml ciprofloxacin (which approximates the IC\(_{50}\)) an immediate effect of the drug was observed as asexual development was delayed (Figure 3-3). Differential counts of all asexual parasite stages revealed that ciprofloxacin induced a developmental delay during the trophozoite growth stage (Figure 3-4A). Subsequent entry into the replicative stage (schizont) was delayed (5 µg/ml and 10 µg/ml) or arrested (25 µg/ml) (Figure 3-4A). The arrested parasites in the 25 µg/ml treatment accumulated as trophozoites were not viable as assessed by the microscopic identification of energized mitochondria.
following 1 h incubation with 5 nM DIOC₆. Gametocytes were only observed with the 5 µg/ml ciprofloxacin treatment.

After 34 hours in culture, a portion of culture from the control and 5 µg/ml ciprofloxacin treated parasites was taken and mature (schizonts) and less mature (trophozoites) forms were separated using percoll/sorbitol density gradient centrifugation. The parasites were placed back into culture with drug and after 86 hours were assessed for viability using DIOC₆ staining. A significantly greater proportion of the developmentally-delayed parasites were viable (45 %) compared to the parasites that continued developing along the prescribed developmental path (10 %) as if drug were not present (i.e. developing similar to the control culture at the time of collection) (Figure 3-4B).

**Organelle Genome Copy Number and DNA Loss with Drug Treatment**

**Apicoplast and Mitochondrial Genome Copy Number**

The determination of apicoplast DNA copy number was initially calculated from data obtained with different apicoplast genes which were selected based on their proximity to the 35 kb DNA origin of replication: clpC (distant from the origin of replication) and LSU (near the origin of replication) (Figure 2-1). The ratios of apicoplast to nuclear DNA were approximately 4 fold greater based on the LSU gene as compared to ratios based on the clpC gene (Figure 3-5). There are 2 copies of the LSU gene and 1 copy of clpC per apicoplast genome, which in part explains the higher copy number determined by LSU. The LSU genes are also in close proximity to the origins of replication, and the gene copies may be present at higher numbers due to the formation of replication intermediates within that region.

Based on ratios of the clpC gene to a single copy nuclear encoded gene (pfpm4), *Plasmodium falciparum* contains on average 2 copies of the 35 kb apicoplast DNA per every nuclear genome copy in non-replicating ring forms (Figures 3-5 & 3-6A). This is an average of
the total population and greater variability may be present in individual cells. The copy number of the mitochondrial DNA was more variable, ranging from 10 – 30 copies in the ring stage (Figure 3-6B). Each parasite contains a single apicoplast and a single mitochondrion in trophozoite stages, and a single apicoplast or mitochondrial network in schizont stages (Figures 3-14 & 3-15).

**Effects of Ciprofloxacin Treatment on Organellar DNA Replication**

Apicoplast DNA was selectively lost in *P. falciparum* cultures treated with a 50% inhibitory concentration of ciprofloxacin. Using the ratio of *clpC*/*pm4* the copy number of the apicoplast DNA was determined to be 2 copies per parasite in non-replicating ring forms. After 96 h in culture with 5 µg/ml ciprofloxacin the copy number of the apicoplast DNA was reduced to 0.5 copies per parasite (Figure 3-6A). The ratio of *cytB*/*pm4* was used to calculate the mitochondrial DNA copy number, which did not decrease after 96 h in culture with 5 µg/ml ciprofloxacin (Figure 3-6B).

The relative quantities of apicoplast, mitochondrial and nuclear gene targets were also determined and these data are presented in Figure 3-7. The apicoplast-encoded gene segment (*clpC*) showed a >100 fold decrease in the ciprofloxacin-treated parasites compared to the control after 120 h in culture (Figure 3-7B). In contrast, the quantity of the single copy nuclear-encoded gene segment (*pm4*) decreased by approximately 10-fold (Figure 3-7A). Like the nuclear gene, a similar 10-fold loss was observed in the quantity of a mitochondria-encoded gene segment (*cytB*) (Figure 3-7C).

Southern blot analysis was also used to follow the time course of the DNA loss with ciprofloxacin treatment. Both copies of the LSU gene within the inverted repeat region of the apicoplast DNA were detected with the same probe. Two other genes that are distal from the origin of replication were also probed (*clpC* and *tufA*). By 72 hours, parasites treated with
ciprofloxacin showed a decrease in the hybridization intensity of the apicoplast targets. By 108 hours, the apicoplast signal from each of the 4 genes was almost completely lost (Figure 3-8). The possibility that the *P. falciparum* DNA gyrase interacts with the mitochondrial DNA was also considered. The mitochondrial DNA was probed by southern blot and in contrast to the apicoplast DNA, there was no loss of mitochondrial DNA over 144 hours (Figure 3-8). Further, in the 5 µg/ml drug treatment there was no loss observed with a nuclear encoded gene (*pm4*) and no loss of plastid, mitochondrial or nuclear gene targets when parasites accumulated as pycnotic forms in hyperparasitemic culture (Figure 3-8). The latter result demonstrated that parasite death by an alternative manner did not result in the selective loss of apicoplast DNA.

**Effects of Ciprofloxacin on Organellar DNA Transcription Accumulation**

To examine whether DNA gyrase activity is involved in RNA transcription processes, levels of LSU rRNA and *clpC* were determined in synchronous parasites every 12 h over 2 asexual cycles of ciprofloxacin (5 µg/ml) treatment. The RNA levels were normalized to nuclear DNA copies and the data was reported as rRNA or transcripts / nuclear genome. Both LSU and *clpC* showed a temporal pattern of RNA expression where levels were highest in late trophozoites and lowest in rings (Figure 3-9). With drug treatment, both LSU and *clpC* RNA showed a significant decrease (~ 2 log) in the second asexual cycle. In the first asexual cycle, *clpC* transcript levels were not significantly different from the control (Figure 3-9B); however there was a significant decrease in LSU rRNA observed after 12 h and LSU rRNA steadily decreased over the 96 h experiment (Figure 3-9A). Two mitochondrial transcripts were also analyzed. The *cytB* and *cox1* transcripts also displayed stage-specific expression where levels were highest during the late trophozoite stage and lowest during the ring stage (Figure 3-10). Unlike the apicoplast RNAs, in the second asexual cycle of drug treatment, both *cytB* and *cox1* RNA levels increased (Figure 3-10). There was an approximate 24 h delay in the accumulation...
of mitochondrial RNA which most likely reflected the developmental delay in the drug treated parasites (Figures 3-10 and 3-3).

**Drug-induced Cleavage**

Extensive Southern blot analysis was conducted to determine whether DNA gyrase was associated with specific regions of the apicoplast 35 kb DNA. *Ex vivo* parasites were treated with ciprofloxacin, VP16/VM26, or for controls dH20 and DMSO. SDS denaturant was used to irreversibly trap DNA cleavage at sites of type II topoisomerase-DNA interactions (Liu et al. 1983, Chen et al. 1984). DNA was treated with 200 µg/ml proteinase-K overnight and isolated by phenol-chloroform extraction and ethanol precipitation. The purified DNA was digested with either EcoR I, Nsi I, Hpa I, Xba I, or Hind III and analyzed by southern blotting using a DIG-labeled mixed apicoplast target probe (same as FISH probe described in Materials and Methods). The locations of the restriction sites are shown in Figure 3-11. Restriction digests that produced linear apicoplast DNA (*EcoR* I, *Nsi* I) showed a distinctive smear in ciprofloxacin treated parasites that was not present in the control (Figure 3-12). The DNA smear was also present in the VP16 treated parasites in the *Nsi* I digest. In the *Nsi* I digest the smear extended to approximately 13 kb, and in the *Eco* RI digest the smear extended to somewhat lower molecular weight bands reaching 11 kb (Figure 3-12). There was no smearing observed in probed fragments that were 22.5 kb and smaller in size. The blot was also hybridized with a specific probe for the SSU rRNA gene which also detected the smear (Figure 3-12). In a different experiment, *Hpa* I digests were specifically probed with SSU and LSU. Results indicated that either one or both of the *Hpa* I sites within the inverted repeat region was blocked from digestion (Figure 3-13).
Immunoprecipitation of DNA

Affinity purified GyrA rabbit antibodies were used to immunoprecipitate DNA that was covalently bound to GyrA through drug-induced cleavable complex formation and subsequent addition of protein denaturant. The procedure first entailed precipitation of covalent protein-DNA complexes using the K$^+$-SDS precipitation method (Rowe et al. 2001). Results from three K$^+$-SDS precipitation experiments are listed in Table 1. Treatment with VM26 resulted in the precipitation of nuclear, apicoplast and mitochondrial DNA targets. Treatment with ciprofloxacin did not result in precipitation of nuclear DNA, but resulted in a small enrichment of apicoplast DNA and to a lesser extent mitochondrial DNA.

Immunoprecipitated DNA was analyzed by quantitative PCR for apicoplast (clpC and LSU), mitochondrial (cytB and cox1) and nuclear gene (pfpm4) targets and the data is presented as the fold increase in gene target relative to the control. Table 2 lists the results from VM26 treated cells and Table 3 lists the results from ciprofloxacin treated cells. The percentage of input DNA for each target after immunoprecipitation (IP) was calculated, and the data was normalized to no antibody controls and the IP data from non-drug treated samples, and finally a ratio of IP by specific GyrA antibody to IP by pre-immune antibodies was calculated. After VM26 treatment and immunoprecipitation, the fold enrichment of apicoplast DNA targets relative to untreated controls in one experiment was 7-fold for LSU and 20-fold for clpC, while the mitochondrial and nuclear DNA targets were enriched by less than 3-fold. In another experiment with VM26 treatment, IP resulted in a greater enrichment of apicoplast DNA targets compared to mitochondrial and nuclear DNAs, although the enrichment was less dramatic. IP of ciprofloxacin treated cells did not result in good enrichment of DNA targets.
Organellar DNA Segregation

Visualization of Apicoplast and Mitochondrial DNAs during Asexual Parasite Development

The morphology of the apicoplast and mitochondrion was observed during asexual development. Images of the organelles in different stage parasites are shown in Figures 3-14 and 3-15. Fluorescent in situ hybridization was used to visualize the apicoplast and mitochondrial DNAs throughout asexual parasite development. The apicoplast DNA was localized to a single punctuate spot in trophozoites. In mature trophozoites the intensity of the hybridization signal increased and during schizogony numerous hybridization foci were observed (Figure 3-16). The mitochondrial DNA was also observed as a single punctuate spot in early trophozoites and the number of hybridization foci steadily increased during schizogony (Figure 3-17). It was observed that the first apicoplast and mitochondrial DNA segregations commonly preceded the onset of nuclear DNA segregation. Interestingly, the apicoplast and mitochondrial DNAs did not show a clear association with the developing nuclei until the culmination of schizogony when organellar division was complete and for each only a single punctuate spot corresponding to the nidus of a minimal organelle was observed within each daughter merozoite.

Effects of Ciprofloxacin Treatment on Organellar DNA Division

The effects of DNA gyrase inhibition by ciprofloxacin were studied on an individual cellular level by using fluorescent in situ hybridization (FISH). Parasites that were subjected to a 24 hour treatment with 5 µg/ml ciprofloxacin showed a dramatic reduction in the intensity and number of plastid DNA hybridization foci. Typically 8 – 10 foci were seen in 36-hour schizonts, however just 1-2 foci were seen in the drug treated schizonts (Figure 3-18). Conversely, mitochondrial DNA segregation was unaffected (Figure 3-18). A concentration of 50 µg/ml ciprofloxacin was added to ring stage parasites and the in situ effects of drug treatment were examined 24 h later. The plastid DNA signal was not detected in most cells, whereas the
mitochondrial DNA was clearly visible and even appeared to have undergone 1 or more rounds of replication and division (Figure 3-18).

**Visualization of the Apicoplast and Apicoplast DNA during Asexual Parasite Development**

Combined immunofluorescence and fluorescent *in situ* hybridization were used to follow both organellar morphology and nucleoid distribution in asexual stages of *P. falciparum*. Figure 3-19 shows representative images from different parasite stages. As was observed in the FISH studies described above, the apicoplast DNA was predominately organized as single discrete nucleoid structures in non-replicating rings and trophozoites. In trophozoites the apicoplast DNA signal colocalized with a large proportion of the apicoplast as defined by the signal from antibody binding to apicoplast GFP (Figure 3-19A & B). In maturing trophozoite stages when the apicoplast began to elongate, the nucleoid was often visualized as semi-discrete foci at both ends of the elongating apicoplast (Figure 3-19C). As the apicoplast elaborated and began to branch, multiple nucleoids were observed within the apicoplast that varied in shape, size and hybridization intensity, and often appeared to encompass the entire stromal compartment (Figure 3-19D, E & F). In mature and segmenting schizonts the nucleoid signal was smaller and discrete PDHF were associated with nuclei (Figure 3-19G & H).

The increased luminal staining observed in combined FISH/IFA as compared to FISH alone could be a result of hybridization to rRNAs. By electron microscopy analysis, the texture of the apicoplast stroma usually appears to be homogeneous, showing a fine granular texture that is indicative of plastid 70S ribosomes (McFadden et al. 1996, McFadden and Waller 1997). By FISH alone in which the protocol included an RNase treatment step, the apicoplast DNA signals were more commonly visualized as discrete foci
Effects of Ciprofloxacin on the Apicoplast and Apicoplast DNA

The DNA gyrase inhibitor ciprofloxacin was used to probe the effects of apicoplast DNA loss on organellar morphology. Synchronous ring stage parasites were treated with ciprofloxacin or cultured without drug. Parasites were then collected at 24 h and 72 h for analysis of apicoplast DNA and the apicoplast by combined FISH / IFA. Figures 3-20 and 3-21 show representative images of the control parasites at 24 h and 72 h. Figures 3-22 and 3-23 show representative images of the drug-treated parasites at 24 h and 72 h. In ciprofloxacin treated parasites, the nucleoid signal was reduced in size, intensity and number, while the apicoplast signal became fragmented and was often localized to the perinuclear region. Also, in many instances the apicoplast DNA did not fully co-localize with the apicoplast signal. These observations were most prevalent at the 72 h analysis; however similar effects were also observed after 24 h.

Conditional Gene Regulation
Analysis of Parasites Transformed with pTGPI-GFP

Parasites were transfected with plasmid pTGPI-GFP and selected in the presence of anhydrotetracycline (ATc) to maintain the GFP expression cassette in a silenced state. Once stable episomal transformants were obtained, ATc was washed out and parasites were observed by fluorescence microscopy every 24 h. Fluorescent schizonts were observed, and the GFP signal showed a pattern that was consistent with ER and membrane localization (Meissner et al. 2005) (Figure 3-24). At 72 h post washout, approximately 20 % of schizont-stage parasites were visibly expressing GFP (Figure 3-25). The percentage of parasites expressing GFP was maximal after 72 h in culture without ATc, and slowly decreased thereafter. The level of GFP expression observed from cell to cell was not uniform. Some cells were intensively fluorescent whereas other cells gave off a weak fluorescence signal (Figure 3-25). Fluorescent parasites were not observed when culture media was continuously supplemented with ATc (1.0 µg/ml).
FACS analysis was used to assess how rapidly expression of GFP was silenced upon addition of ATc. Asynchronous cultures were propagated in the absence of ATc for 72 hours and the number of GFP-expressing parasites was counted (Day 0) from 1 million sorted cells. ATc (1 µg/ml) was applied to cultures after the initial count on Day 0 and the number of GFP-expressing parasites were counted from 1 million sorted cells every 24 hours for 3 days. When ATc was added to the culture for as little as 24 hrs, there was a 10 fold decrease in the percentage of fluorescent parasites reaching background levels observed with parasites in continuous culture with ATc (Figure 3-26).

Fluorescently sorted parasites were also analyzed by quantitative PCR to determine the copy number of pTGPI-GFP in GFP-expressing vs. GFP non-expressing cells. At 90 days and 180 days post-transfection, ATc was washed out and the GFP-expressing parasites and the non GFP-expressing parasites were sorted from the mixed culture by FACS. For both time points, 8,000 GFP positive cells were collected. Quantitative PCR was used to determine the plasmid copy number in GFP +, GFP -, and unsorted parasites. The plasmid standard for the qPCR assay contained gene segments for each of the 3 plasmid targets (tati2, gfp, B-lactamase) and a gene segment of a single-copy nuclear gene used for normalization. At 90 d post-transfection, the GFP+ cells contained on average 1 copy plasmid per parasite (Figure 3-27A) while at 180 d post-transfection the GFP + cells contained roughly between 10 – 30 copies of plasmid (Figure 3-27B). The range of fluorescent intensity in the GFP + parasites at 90d and 180 d did not appear dramatically different when viewed by fluorescent microscopy. FACS data indicated that Further, the percentage of parasites that expressed GFP only slightly increased at 180 d.

Additional fluorescent sorts were used to isolate the GFP-expressing cells which were then placed back into culture. The progeny of these parasites were not uniformly fluorescent and
number of fluorescent parasites gradually decreased as observed when ATc was removed from
the unsorted culture. The GFP sorted parasites were also placed back into culture in the presence
of ATc for 14 d. Upon washout of ATc, the number of GFP expressing parasites was similar to
what was observed for the washout experiments using unsorted cells (i.e. ~ 20 % of schizonts).

**Targeted Genomic Insertion of the Tet-Operator / Min Cam Promoter**

The *pfpm4* gene was targeted by single-crossover homologous recombination using
plasmid pTOCAM-Δpm4. Clonal parasite lines were obtained by limiting dilution. Southern blot
analysis and PCR for integration verified that *pfpm4* was partially duplicated in the clones which
resulted in the insertion of a 7X repeat of the tet-operator sequence and a truncated segment of
the *P. falciparum* calmodulin promoter immediately 5’ to the full length copy of *pfpm4* and
natural *pfpm4* 3’-UTR (Figures 3-28 and 3-39). PCR products were cloned and sequenced to
verify that *pfpm4* remained in frame. Southern blot analysis also indicated that one or more extra
copies of the plasmid were integrated in the pTOCAM-Δpm4 clones (Figure 3-28). Clones C3
and B3 initially appeared to contain the stoichiometric equivalent of one extra plasmid copy and
thus C3 was selected as the parasite line in which to introduce transactivator expression
cassettes. The proliferation characteristics of clones C3 and B3 were very similar to that of the
parental 3D7 strain (Figure 3-30). By western blot, PfPM4 protein expression was not detected in
any of the pTOCAM-Δpm4 clones analyzed (Figure 3-31). Furthermore, 3D7 protein lysates
were diluted in C3 protein lysates and by western blot it was determined that PfPM4 levels in C3
were at least 16-fold less than in 3D7 (Figure 3-31).

**Creation and Characterization of the TA Parasite Lines**

In other parasites, the piggyBac transformation system was utilized to stably integrate
transactivator (*tati2*) expression cassettes into the *P. falciparum* genome to create cultures TA-
01, TA-02, TATK-03, and TATK-04. Southern blot analysis revealed that there were
approximately 20 unique insertion events in the uncloned \textit{piggyBac} transformants (Figure 3-32). The level of transactivator expression in the uncloned lines was analyzed by western blotting and compared to TaTi2 expression from episomally transformed pTGPI-GFP. Most of the \textit{piggyBac} lines with integrated \textit{tati}2 copies showed steady-state TaTi2 levels that appeared to be similar to those expressed by parasites carrying pTGPI-GFP as episomal copies (Figure 3-33). Cultures were cloned by limiting dilution and southern blot and quantitative PCR analysis indicated that most TA-01 parasites contained either 2 or 3 copies of \textit{tati}2, while TA-02, TATK-03, and TATK-04 parasites contained 1 copy of \textit{tati}2 (Figure 3-34). Clones containing more than 1 integration of \textit{tati}2 showed correspondingly greater steady-state expression of TaTi2 protein (Figures 3-34 and 3-35). Furthermore, the transactivator-expressing parasites were always cultured in the absence of anhydrotetracycline (ATc) and still maintained apparently stable expression of TaTi2, suggesting that TaTi2 alone was not toxic for the cell.

\textbf{Creation and Characterization of the C3-TA Parasite Line}

The \textit{piggyBac} transformation system was next used to introduce transactivator expression cassettes into pTOCAM-Δpm4 (clone C3) to produce culture C3TA. Post-transfection, cultures C3TA-B1 and C3TA-B2 were selected in the absence of ATc to allow for expression of \textit{pfpm4}. Stable transformants were selected and verified for \textit{tati}2 integration by southern blot (Figure 3-36). By western blot however, PfPM4 and TaTi2 protein expression were not initially detected. After C3TA-B1 was cultured for 96h and 196h in the presence of ATc, expression of TaTi2 protein was readily detected at each time point. TaTi2 levels did not appear to increase between 96 and 196 hours, indicating that parasites achieved maximum steady-state TaTi2 expression levels within 96 hours of culture with ATc (data not shown).

For subsequent experiments, C3TA-B1 parasites were cultured in the presence of ATc and a drug wash-out approach was used in an effort to regulate the expression of \textit{pfpm4}. Even upon
accumulation of TaTi2 and subsequent wash-out of ATc, induction of PfPM4 protein expression was only potentially detected upon over-exposure of the western blot (data not shown). The expression of two functionally related proteins (PfPM1 and FP3) was not affected during the washout experiments (Figure 3-41). Numerous antibodies were used for western blot detection of PfPM4: rabbit antiserum generated to both N-terminal and internal regions of PfPM4 both before and after affinity purification, and PfPM4 monoclonal antibodies that were the kind gift of Dan Goldberg (Washington University, St. Louis, MO). TaTi2 protein levels remained consistently less in C3TA-B1 cultured without ATc (Figure 3-37A, B, & C). To examine whether the nature of this effect was extended to other genes under the control of the TetO, parasites transformed with pTGPI-GFP were analyzed after culture without ATc for 40 days. The same TaTi2 silencing phenomenon was observed with these parasites (Figure 3-37D).

The regulation of pfpm4 expression in the wash-out studies was also investigated at the RNA level. By quantitative RT-PCR it was found that pfpm4 3′ transcripts were reduced by 100-fold in pTOCAM-Δpm4 clone C3, but the 5′ pfpm4 transcript levels were essentially the same as wild-type 3D7 (Figure 3-38). The 5′ pfpm4 transcripts in C3TA-B1 (in continuous culture with and without ATc) were also detected at similar levels to C3 and 3D7, however the 3′ transcripts were on average 50-fold less than 3D7 (Figure 3-38). In the first of three separate experiments, upon wash-out of ATc from culture C3TA-B1 there was a 12.7-fold increase of 3′ pfpm4 transcripts and a 6.8-fold increase in 5′ transcripts was detected by qPCR after 72 h (Figure 3-38A). By northern blot analysis, the increase in the 5′ transcripts was readily observed (Figure 3-43A). However, even upon induction, pfpm4 3′ transcripts remained below the detection limits of the northern blot. The following 2 washout experiments did not result in an induction of pfpm4 3′ RNA expression. Nevertheless an important observation was made. The
temporal pattern of pfpm4 3’ RNA expression closely followed $tati2$ expression, while 5’ $pfpm4$ expression varied independently (Figures 3-38 & 3-39).

The C3 and C3TA parasite lines were analyzed by southern blot using a restriction enzyme ($Bsr$ GI) flanking the $pfpm4$ locus to determine how many plasmid copies of pTOCAM-$\Delta pm4$ were integrated. Prior to the introduction of $tati2$ expression cassettes, pTOCAM-$\Delta pm4$ clone C3 was comprised mostly of parasites with either 1 or 2 extra plasmid copies integrated at the $pfpm4$ locus. After introduction of $tati2$ expression cassettes by piggyBac transformation, parasite line C3TA-B1 mostly contained parasites with 3 extra plasmid copies integrated (Figure 3-40). Parasite lines C3TA-B2 and –A1 mostly contained 4 or more extra plasmid copies while C3TA-A2 contained approximately equal numbers of parasites with either no extra copies, 1 extra copy, or 2 extra plasmid copies (Figure 3-40). pTOCAM-$\Delta pm4$ clone B3 started with parasites containing either no extra plasmid copies or 2 extra copies, and following additional rounds of selection after attempted piggyBac transformation one of the resulting parasite lines contained no extra copies, but the other parasite line mostly contained 1 extra integrated copy (Figure 3-40). These data demonstrate that DNA integrated by single-crossover recombination was prone to expansion and contraction, and the copy number did not remain stable over time in culture.

**Summary of Conditional Gene Regulation Findings**

In summary, efforts to configure the Tet-transactivator system into a tool that will provide conditional regulation of endogenous $P. falciparum$ genes have led to the following key findings: 1) A single copy of $tati2$ is sufficient to regulate transgene expression in parasites episomally transformed with pTGPI-GFP; 2) Higher steady-state transactivator expression is achieved in parasites that contain more than 1 integrated copy of the expression cassette; 3) Transactivator protein is stably expressed in the absence of the Tet-operator / minimal calmodulin promoter sequence; 4) In the absence of ATc, transactivator expression is silenced in parasites that contain
the Tet-operator / minimal calmodulin promoter sequence; 5) Transactivator silencing in the presence of Tet-operator / minimal calmodulin promoter is apparently independent of the chromosomal positioning of Tet-transactivator elements in cis; 6) Continual stimulation of pfpm4 expression using the Tet-transactivator system was not achieved and was confounded by the silencing of TaTi2; 7) Only a slight induction of pfpm4 occurred upon transient stimulation of expression.
Figure 3-1. Dosage-dependent response of *P. falciparum* proliferation in *in vitro* culture to DNA gyrase inhibitors using [3H]-hypoxanthine uptake assays. A variable-slope sigmoidal dose-response nonlinear regression equation (Systat Software Inc.) was used to generate the fifty-percent inhibitory concentrations (IC$_{50}$) for each drug. DNA gyrase is a heterotetramer (2 gyrA: 2 gyrB) and ciprofloxacin targets the active site of gyrA while coumermycin and novobiocin target the ATPase domain on gyrB. The IC$_{50}$ values for gyrase inhibitors were as follows: ciprofloxacin, 4.9 µg/ml (Panel A); coumermycin, 5.2 µg/ml (Panel B); novobiocin, 15.8 µM (Panel C). The percentage reductions were plotted as a function of drug concentration where the concentrations of ciprofloxacin used were 40 µg/ml, 20 µg/ml, 10 µg/ml, 5.0 µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.625 µg/ml; the concentrations of coumermycin used were 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml, 1.56 µg/ml, 0.781 µg/ml and the concentrations of novobiocin were 400 µM, 200 µM, 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM.
Figure 3-2. Proliferation of parasites cultured in complete media with different concentrations of ciprofloxacin. Percent parasitemia (% of number of infected red blood cells/total number of red blood cells) is shown in (A). Counts were determined from microscopic analysis of Hema 3 stained blood smears. Parasitemia did not increase over 110 h with the 25 μg/ml ciprofloxacin treatment. DNA quantity determined by qPCR of a single-copy gene is shown in (B).
Figure 3-3. Developmental delay in parasites treated with 5 µg/ml ciprofloxacin. Differential counts of asexual parasites were determined every 12 h. The percentage of each stage was plotted over time. ET, early trophozoites; LT, late trophozoites; S, schizonts, R, rings. Closed circles, control; open boxes, ciprofloxacin treated.
Figure 3-4. Parasite differentiation in untreated control cultures and cultures treated with 5 µg/ml ciprofloxacin (A). Highly synchronized parasites at the newly invaded ring stage were used to initiate cultures at t₀ with or without addition of ciprofloxacin. Differential counts of parasites in each of the morphologically recognized stages of development in the asexual cycle were counted at each of 9 time points over a 120 hr period and expressed as a percent of the total. Data is representative of 5 separate experiments. Arrows indicate time point during drug treatment (34 h) at which a portion of the culture was taken and mature and immature forms separated by density gradient centrifugation and used to establish separate cultures under continued drug treatment. Viability of the isolated mature and immature parasites was assessed by DIOC6 staining after an additional 86 h in culture and results of microscopic counts from 20 fields of view are shown in (B).
Figure 3-5. Apicoplast 35kb genome copy number determined by the ratio of apicoplast and nuclear DNA using quantitative PCR. Measurements of apicoplast 35kb DNA copy number in control and ciprofloxacin-treated (5 µg/ml) cultures were based upon the copy number of the clpC gene (A) and LSU rRNA gene (B). The apicoplast DNA quantities were divided by the nuclear DNA quantities based on the nuclear encoded pfpm4 gene. Data plots show the mean and standard deviation of 2 separate experiments. Quantities of target gene were calculated based on a titration of recombinant plasmid DNA containing both a segment of a single copy nuclear DNA encoded gene (pfpm4) and a segment of an apicoplast DNA encoded gene (clpC or LSU).
Figure 3-6. Copy number of apicoplast and mitochondrial DNA with and without ciprofloxacin treatment. Data is reported as the average of five separate experiments and error bars represent the standard deviation of the mean. Copy number was calculated based on the ratio of \textit{clpC} and \textit{pm4} for apicoplast DNA or \textit{cytB} and \textit{pm4} for mitochondrial DNA. Highly synchronous rings were used to initiate the experiment and samples were collected every 12 h over 2 asexual cycles. Drug was added to the ring stage parasites and maintained in culture throughout the experiment. Time points correspond to the following developmental stages in the control samples: 12 & 60 h, mid-trophozoites; 24 & 72 h, late-trophozoites; 36 & 84 h, schizonts; 48 & 96 h, rings.
Figure 3-7. Analysis of nuclear, apicoplast, and mitochondrial DNA replication and selective loss of apicoplast DNA in *P. falciparum* cultures treated with ciprofloxacin. Highly synchronous rings were used to initiate the experiment and 8 samples were taken over 120 h and analyzed by quantitative PCR. The copy number of a nuclear-encoded gene segment (*pm4*) decreased by ~10-fold in ciprofloxacin-treated parasites (A). A similar fold loss was observed in the copy number of a mitochondrial gene segment (*cytB*) (C). The copy number of an apicoplast gene segment (*clpC*) showed >100-fold decrease in ciprofloxacin-treated parasites (B). Data plots represent similar data obtained from 5 separate experiments.
Figure 3-8. Selective loss of plastid DNA in *P. falciparum* cultures treated with ciprofloxacin determined by southern blot hybridization. Cultures treated with a 50% inhibitory concentration of ciprofloxacin (5 µg/ml) showed a depletion of plastid DNA targets while the mitochondrial and nuclear signals were not lost. Parasites were stage synchronized by sorbitol and cultured with no drug (control), 5 µg/ml ciprofloxacin, or 10 µg/ml ciprofloxacin. Samples were collected every 36 hours. DNA was released from saponin-treated parasites by SDS lysis and lysates were incubated in the presence of 100 µg/ml proteinase K for 2 hours at 65 °C. DNA was purified by phenol-chloroform extraction and digested overnight with *Hind* III. DNA (5 µg) was electrophoresed for 6 hours at 60 V and transferred to a nylon membrane. Membranes were hybridized with DIG-labeled DNA probes for apicoplast DNA targets (*clpC*, *tufA* LSU IRA, LSU IRB), mitochondrial DNA targets (*cytB*, *cox1*, *coxIII*) and a nuclear DNA target (*pm4*).
Figure 3-9. Effects of ciprofloxacin treatment on apicoplast RNA. Highly synchronous parasites were cultured with or without ciprofloxacin were sampled every 12 hours. The levels of LSU rRNA (A) and clpC transcripts (B) were determined by qRT-PCR and normalized the quantity of the genomic DNA target pfpm4. Data is plotted as the mean of 2 separate experiments, and error bars reflect the standard deviation of the mean.
Figure 3-10. Effect of ciprofloxacin treatment on mitochondrial RNA. Highly synchronous parasites were cultured with or without ciprofloxacin were sampled every 12 hours. The levels of cytB transcripts (A) and cox1 transcripts (B) were determined by qRT-PCR and normalized the quantity of the genomic DNA target pfpm4. Data is plotted as the mean of 2 separate experiments, and error bars reflect the standard deviation of the mean.
Figure 3-11. Map of restriction enzyme sites used for cleavage analysis on the apicoplast DNA. Black bars inside the circle denote the locations of the amplicons used to generate the FISH probe and mixed apicoplast DNA probe used for southern blotting.
Figure 3-12. DNA cleavage analysis of the apicoplast 35 kb DNA. DNA from drug-treated and control parasites was digested and hybridized to a mixed apicoplast DNA probe detecting 7 kb of the 35 kb circle (see Figure 3-11 for location of the probe fragments) (A). The EcoRI and NsiI digests linearized the 35 kb DNA and a region of smearing was observed below the linear 35 kb in the ciprofloxacin treatment. VP16 treatment produced a similar smear in the NsiI digest. The same blot was hybridized with a probe for SSU (B).
Figure 3-13. Analysis of the apicoplast 35 kb DNA inverted repeat topology. DNA from drug-treated and control parasites was digested with HpaI and hybridized to a probe for the LSU (A) or SSU (B) rRNA genes. Treatments were conducted on an asynchronous culture (mixed culture), late trophozoites/early schizonts (Troph/Schiz), and schizonts (Schiz). The higher molecular weight bands observed in the SSU blot correspond to a block in digestion of one or more HpaI sites located in the inverted repeat. In the LSU blot an additional distinct band was observed in the VP16 treated Troph/Schiz sample corresponding to a block in the IRB HpaI site of the inverted repeat.
Table 3-1. Results of SDS/KCl precipitation from 3 different experiments listing the fold enrichment of gene targets in drug treated parasites relative to controls. Data for both the pellet and supernatant fractions are shown.

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Table 3-2. Fold enrichment of apicoplast and mitochondrial DNA gene targets relative to control DNA after VM26 treatment and immunoprecipitation with GyrA antibody.

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Table 3-3. Fold enrichment of apicoplast and mitochondrial DNA gene targets relative to control DNA after ciprofloxacin treatment and immunoprecipitation with GyrA antibody.

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Figure 3-14. Morphology of the apicoplast in asexual stages of *P. falciparum*. Parasites (strain 3D7) were transformed with plasmid ACP-GFP (plasmid DNA kindly provided by S. Sato) to target GFP to the plastid. Fluorescent images show the plastid in green (left panel) and the right panel shows the fluorescence merged with a differential interference contrast image of the parasite-infected erythrocyte. The apicoplast is visible as a small oval shaped structure in rings (Panel A) and takes on a more spherical shape in young trophozoites (Panel B). In maturing trophozoites the plastid elongates and forms looped structures (Panels C, D, and E). As the parasite enters schizogony the plastid branches (Panels F and G) and segments of the organelle are distributed to daughter merozoites in mature, segmented schizonts (Panel H). At the time of schizont rupture, plastids are seen as small ovals within merozoites (Panel I).
Figure 3-15. Morphology of the mitochondrion in asexual stages of *P. falciparum*. Parasites (strain 3D7) were cultured for 1 hour in the presence of 2 nM DIOC6 and 1 µg/ml Hoechst dye 33342. Differential interference contrast (left panel) and fluorescent (right panel) micrographs show parasites at various developmental stages in the asexual cycle. Green, mitochondrion; blue, nuclear DNA. At low concentrations, DIOC6 selectively accumulates in energized mitochondria. The mitochondrion is visible as a small rod shaped structures in young trophozoites (Panels A and B). In maturing trophozoites the mitochondrion elongates and form a branched structure (Panels C and D). As the parasite continues to mature in the schizont stages the mitochondrion becomes more elongated and intertwined amongst the dividing nuclei (Panels E, F, and G). Panels H and I show rupturing schizonts where each newly formed merozoite has received a segment of mitochondria.
Figure 3-16. Analysis of apicoplast DNA segregation by fluorescence *in situ* hybridization (FISH). Red is the hybridization signal from the apicoplast DNA probe and nuclear DNA in blue was counterstained with 1 µg/ml Hoechst 33342. A) The apicoplast DNA is visible as a single punctate spot in trophozoites. B) and C) The apicoplast DNA segregates independently of nuclear DNA during schizogony. D) In segmented schizonts the apicoplast DNA is distributed amongst daughter merozoites.
Figure 3-17. Analysis of mitochondrial DNA by fluorescence in situ hybridization. A) Mitochondrial DNA (red signal) was visible as single spots in merozoites with the nuclear DNA in blue labeled with Hoechst Dye 33258. B) Mitochondrial DNA segregating prior to nuclear DNA segregation in a trophozoite, C) Early in schizogony the mitochondrial DNA was actively segregated. D) and E) Mitochondrial DNA was distributed to daughter merozoites in mature and segmented schizonts.
Figure 3-18. Effects of ciprofloxacin on apicoplast and mitochondrial DNAs detected by FISH. Parasite cultures were treated for 24 hours in culture with a 50 % inhibitory concentration of ciprofloxacin (5 µg/ml) or a completely inhibitory concentration of ciprofloxacin (50 µg/ml) and analyzed by FISH. In parasites treated with 5 µg/ml ciprofloxacin there was a significant decrease in the apicoplast DNA signal (panels A & B) which was most pronounced in later stage parasites, while the signal for mitochondrial DNA was not reduced (panels C & D). Mitochondrial DNA was still detected as multiple spots in parasites treated with high dosages of ciprofloxacin for 24 hours (panels G and H), whereas apicoplast DNA was only seen as a single spot in ≤10% of parasites (panels E & F).
Table 3-19. Visualization of the apicoplast DNA and the apicoplast in asexual stages of *P. falciparum* by combined FISH / IFA. The apicoplast signal is delineated by IFA for GFP (green), the apicoplast DNA was detected by FISH (red). Parasite nuclei were stained with DAPI (blue). Trophozoites (A & B), Early Schizont (C), Schizont (D, E, F), Mature Schizont (G), Segmenting Schizont (H).

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Figure 3-19. Visualization of the apicoplast DNA and the apicoplast in asexual stages of *P. falciparum* by combined FISH / IFA. The apicoplast signal is delineated by IFA for GFP (green), the apicoplast DNA was detected by FISH (red). Parasite nuclei were stained with DAPI (blue). Trophozoites (A & B), Early Schizont (C), Schizont (D, E, F), Mature Schizont (G), Segmenting Schizont (H).
Control 24h

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Figure 3-20. Combined FISH / IFA on control 24 h parasites. Images are representative of the parasite population at the time of sampling. Apicoplast, green; apicoplast DNA, red; nuclei, blue.
Control 72 h

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Figure 3-21. Combined FISH / IFA on control 72 h parasites. Images are representative of the parasite population at the time of sampling. Apicoplast, green; apicoplast DNA, red; nuclei, blue.
Figure 3-22. Combined FISH / IFA on ciprofloxacin treated parasites at 24 h. Images are representative of the parasite population at the time of sampling. Apicoplast, green; apicoplast DNA, red; nuclei, blue.
Figure 3-23. Combined FISH / IFA on ciprofloxacin treated parasites at 72 h. Images are representative of the parasite population at the time of sampling. Apicoplast, green; apicoplast DNA, red; nuclei, blue.
Figure 3-24. GFP expression in *P. falciparum* cells stably transformed with pTGPI-GFP. The 3D7 clone of *P. falciparum* was transfected with pTGPI-GFP and stably transformed parasites were selected with 2.5 nM WR99210. Culture medium was supplemented with ATc (0.5 μg/ml) for the duration of the selection process. When ATc was removed, GFP expressing parasites were observed by fluorescence microscopy within 48 hours. Only schizont stages were fluorescent which is consistent with transactivator (TaTi2) expression being regulated by the stage-specific promoter of *msp2*. Images of GFP fluorescence, phase contrast, and GFP merged with phase contrast are shown of A) an early schizont, B) a developing schizont, C) a mature and segmented schizont.
Figure 3-25. Induction of GFP expression in a *P. falciparum* culture stably transformed with pTGPI-GFP. Parasites were cultured in the absence of ATc for 72 hours to show GFP expression (A), or continuously in presence of ATc (B). Cells were counterstained with Hoechst, saponin-treated, and viewed at 400X magnification. Approximately 20% of schizont-stage parasites became fluorescent when ATc was removed (A), while fluorescence was not observed in parasites that were in continuous culture with ATc (B).
Figure 3-26. Counts of GFP expressing cells after the addition of ATc determined by flow cytometry. Asynchronous cultures were propagated in the absence of ATc for 72 hours and the number of GFP-expressing parasites was counted (Day 0) from 1 million sorted cells. ATc (1 µg/ml) was applied to cultures after the initial count on Day 0 and the number of GFP-expressing parasites was counted from 1 million sorted cells every 24 hours for 3 days. Flow cytometry data is shown in (A) where R1 indicates the counted cell population. Parasitemia was also determined by flow cytometry using Paraformaldehyde-fixed cells stained with 250 ng/ml Syto 24. The cell sorting results were normalized to parasitemia and compared to control non-transfected parasites (B). The number of fluorescent parasites is reduced by approximately 10 fold.
Figure 3-27. Analysis of plasmid copy number in parasites transformed with pTGPI-GFP after time in culture. *Plasmodium falciparum* (strain 3D7) was transformed with pTGPI-GFP and selected with 2.5 nM WR99210 and cultured with ATc. At 90 days and 180 days post-transfection, ATc was washed out. The GFP-expressing parasites and the non GFP-expressing parasites were sorted from the mixed culture by FACS. For both time points, 8,000 GFP positive cells were collected. Quantitative PCR was used to determine the plasmid copy number in GFP +, GFP -, and unsorted parasites. At 90 d post-tranfection, the GFP + cells contained on average 1 copy plasmid (A) per parasite while at 180 d post-transfection the GFP + cells contained roughly between 10 – 30 copies of plasmid (B).
Figure 3-28. Southern blot analysis of transfectant pTOCAM-Δpm4. Analysis showed that the expected 5' and 3' integration occurred at the pfpm4 locus. Probing with sequences from the pro-region of pfpm4, the EcoRI/NotI digest produced a 4.4 kb fragment indicating 5' integration and produced a 1.8 kb fragment indicating 3' integration. Hybridization of the EcoRI/NotI digest with hDHFR similarly yielded a 4.4 kb fragment which was expected to co-localize with the 4.4 kb fragment probed with pro-pfpm4. Wild-type pfpm4 produced a band at 2.6 kb and plasmid produced a 3.6 kb band. TF, transfected parasites; WT, wild-type non-transfected parasites; PL, plasmid.
Figure 3-29. PCR verification of the integration of the TetO/min cam promoter upstream of full length *pfpm4* in uncloned cultures. For PCR, two primer sets (P1 and P2) were designed to detect 3’ integration in transfection cultures. The locations of the primers are shown in the schematic locus in figure 3-29 for pTOCAM-Δpm4 which produce amplicons of 893 bp (P1) and 1379 bp (P2). The PCR products were visualized by agarose gel electrophoresis, cloned into the pCR-4 Topo plasmid (Invitrogen), and sequenced for confirmation.
Figure 3-30. Flow cytometry results for a 264 hr growth study (12 days) for wild type 3D7 and pTOCAM-Δpm4 clones B3 and C3. Growth study was conducted with duplicate cultures using 24 well plates. Cultures were split 1/10 at 24 hrs, 96 hrs, 168 hrs, and 216 hrs. At each collection time period, 100 μL of sample was added to a 1.5 mL microcentrifuge tube and centrifuged for 5 minutes at 2,000 rpm. Slides were made (2 μL) and the remaining sample was resuspended in 500 μL of 2% paraformaldehyde. Samples were stored at 4°C. At 24 hrs before Flow cytometry analysis, Cyto24 dye (500 nM) was added to each sample. The flow cytometer was set to sort 50,000 cells. Samples were done in duplicate, series 1 (B) and series 2 (C). The geometrical mean from series 1 and 2 were used for graph (A), with standard deviation bars shown.
Figure 3-31. Western blot analysis of PfPM4 protein expression in pTOCAM-Δpm4 clones. PfPM4 protein expression was not detected in any clones analyzed (A). 3D7 was run as a positive control and a pfpm4 knockout parasite line (TX4) was run for a negative control. By the detection limits of western blotting, PFPM4 protein levels in clone C3 were at least 16-fold less than PfPM4 levels in wild-type 3D7 (B).
Figure 3-32. Genomic integration of transactivator expression cassettes using *piggyBac*-mediated transformation. Two plasmids (pGYBSD-TA and pGYBSD-TATK) were engineered to integrate *tati2* using the *piggyBac* transformation system. Mature asexual-stage parasites were isolated on a magnetic column, introduced to plasmid-loaded red blood cells and selected with 2.5 µg/ml blasticidin-S. Parasite DNA from transformed cultures was digested with *EcoR* I and hybridized with DIG-labeled probes for the blasticidin deaminase gene (BSD) and for *tati2*. Cultures TA-01 and TA-02 contain multiple integrations while a single integration site predominates in cultures TATK-03 and TATK-04 (B). Episomal plasmid would be detected as an 8.1 kb fragment.
Figure 3-33. Expression of transactivator (TaTi2) protein in *piggyBac* transformants by western blot. Parasite lysates prepared from asynchronous cultures were probed with α-TetR to detect TaTi2 expression, and α-BIP for a measure of protein loading. TaTi2 was detected in both parasites transformed with episomal copies of *tati2* (pTGPI-GFP), and in parasites cultures transformed with integrated *tati2* by *piggyBac* (TA-01, TA-02, TATK-03, TATK-04). Non-transfected parasites were also probed as a control. Both the episomal and integrated *tati2* are under control of the *msp2* promoter. TaTi2 expression levels appear to differ among the various *piggyBac* transformants and the levels of TaTi2 in the *piggyBac* transformants was similar to what was observed in the episomally transformed parasites.
Figure 3-34. Analysis of piggyBac transformants TA-01 and TA-02 for transactivator expression cassette integration. Putative clones and subcultures of TA-01 and TA-02 were analyzed by southern blotting and quantitative PCR which revealed that the majority of the TA-01 parasites contained 2 or even 3 copies of tati2 inserted into each genome, whereas the TA-02 parasites contained 1 copy of tati2 inserted in the genome.
Figure 3-35. Western blot analysis of TaTi2 expression in TA-01 and TA-02 parasites with BiP detection for a loading control. Clones containing differential copy numbers of integrated *tati2* showed that those parasites with more than one insertion of the transactivator expression cassettes contained correspondingly higher levels of transactivator protein. Also, all transformations were selected in the absence of ATc and parasites were still able to maintain stable TaTi2 expression suggesting that TaTi2 alone is not toxic for the cell.
Figure 3-36. Southern blot analysis of *piggyBac* insertion sites in culture C3TA. The transactivator expression cassette was introduced into pTOCAM-Δpm4 clone C3 using the *piggyBac* transformation system. After the establishment of WR99210 and BSD drug-resistant parasites, genomic DNA from sub-cultures C3TA-B1 and C3TA-B2 was digested with *Eco*RI and probed with *tati2*. Plasmid DNA from the pGYBSD-TA construct and genomic DNA from wild-type 3D7 were run for controls. A single *piggyBac* insertion site predominates in C3TA-B1, while plasmid DNA was still detected in C3TA-B2.
Figure 3-37. Analysis of protein regulation in C3TA-B1 cultured in the absence and presence of ATc. C3TA-B1 was continuously cultured in the absence of ATc (-ATc), and in the presence of ATc (+ATc). ATc was washed out of the +ATc culture and parasites were sampled after 24h, 48h and 72h in the absence of ATc. PfPM4 protein expression was not detected in 3 separate experiments (A, B, & C). TaTi2 expression was silenced in C3TA-B1 (-ATc) (A, B, C), and TaTi2 expression was similarly silenced in pTGPI-GFP (–ATc) (D).
Figure 3-38. Analysis of steady-state *pfpm4* RNA transcripts by qRT-PCR. Both the 5’ (black bars) and 3’ (grey bars) *pfpm4* levels were determined. Error bars represent the standard deviation of samples run in duplicate. Three separate washout experiments are shown.
Figure 3-39. Analysis of steady-state *pfpm4* 3’ RNA and *tati2* RNA by quantitative RT-PCR. Steady-state levels of *pfpm4* 3’ RNA (light grey bars) fluctuate similarly to *tati2* RNA (dark grey bars). Error bars represent the standard deviation of duplicate samples. Three separate washout experiments are shown.
Figure 3-40. Southern blot analysis of the number of plasmid integrations in clones of pTOCAM-Δpm4 and C3TA. A) Illustration of the pfpm4 locus after a single plasmid integration showing BsrGI restriction sites, and the gene segment probed for during southern blotting in blue. B) DNA fragment sizes from the BsrGI digest resulting from the integration of extra plasmid copies. C) & D) Southern blots revealing that the majority of parasites in these clones contain extra plasmid copies integrated at the pfpm4 locus. The non-transfected parental line 3D7 is also shown.
Figure 3-41. Western blot analysis of PfPM1 and FP3 expression in C3TA washout experiments. The expression of two functionally related proteins of PFPM4 (PfPM1 and FP3) was examined by western blot in the washout experiments. Levels of BiP were used as a loading control.
Figure 3-42. Analysis of steady-state pfpm4 5' and 3’RNA transcripts in 3D7 and pTOCAM-Δpm4 clone C3 by northern blot. Nuclear rRNA is shown as a loading control. Total cellular RNA was blotted and hybridized to a probe for the 5’ end of pfpm4 and the 3’ end of pfpm4. In 3D7 a dominate transcript at ~ 3.3 kb and two smaller bands were detected by both probes. Three transcripts were detected in C3 with the 5’ probe, and two faint transcripts were detected by the 3’ probe in C3.
Figure 3-43. Analysis of pfpm4 and tati2 RNA transcripts in 3D7, C3, and C3TA-B1 by northern blot. In washout experiments tati2, pfpm4 5’ and pfpm4 3’ RNAs were followed by northern blot. The schematic locus in (A) shows the locations of the pfpm4 probes: pfpm4 5’ probe, blue; pfpm4 100 bp probe, orange; pfpm4 3’ probe, green. Two different washout experiments are shown in B & C.
CHAPTER 4
DISCUSSION

DNA Gyrase and Apicoplast Studies

The cellular and molecular response of *P. falciparum* to ciprofloxacin confirms that a prokaryotic type II topoisomerase activity in the apicoplast is a target of this drug. While cyanobacteria utilize both DNA gyrase and topoisomerase IV enzymes, available sequence data indicates that apicomplexans retained only one of these enzymes (NCBI, NLM). Similarly, the green algae *Clamydomonas* and *Ostreococcus* retained only 1 enzyme; and the Cryptomonad *Guillardia theta* has just one nucleomorph-encoded eubacterial type II topoisomerase (NCBI, NLM). Based on phylogenetic analysis, it is uncertain whether the apicomplexan DNA gyrase/Topo IV enzyme evolved from a DNA gyrase or Topoisomerase IV precursor (Appendix A). Most of the apicomplexan orthologues are annotated as DNA gyrase, although this enzyme may have more diverse functional roles and/or perform additional specialized functions. Functional analysis will be required to define the origin and provide classification of the apicomplexan DNA gyrase/Topo IV enzyme.

The two major classes of DNA gyrase inhibitors caused a dosage-dependent inhibition of *P. falciparum* proliferation in *in vitro* culture. Fluoroquinolones (e.g. ciprofloxacin) are derivatives of the quinolone nalidixic acid, and coumerins (e.g. coumermycin and novobiocin) are natural products of *Streptomyces spp.* The fluoroquinolones promote the rate at which covalent enzyme-DNA reaction intermediates are formed by causing distortions in the DNA where DNA gyrase is bound. The trapped enzyme complex interferes with the passage of replication forks and transcription complexes (Drlica and Malik 2003). Coumerins directly inhibit DNA gyrase activity by competing for binding sites in the ATPase regions (Maxwell 1999). The energy from ATP induces conformation changes in type II topoisomerases that are
required to drive DNA relaxation (with the exception of DNA gyrase which has an ATP-independent relaxation activity), catenation/ decatenation, and in the case of DNA gyrase, induction of negative supercoils (Liu et al. 1980). Ultimately, it is thought that the cytotoxic action of these inhibitors is the accumulation of double stranded lesions in the DNA that cannot be overcome by the DNA repair machinery. Interestingly, parasites which were developmentally-delayed after 34 h of treatment with the IC_{50} of ciprofloxacin showed greater viability after 120 h compared to parasites that showed developmental progression similar to the control population after 34 h of drug treatment. This developmental delay may provide an opportunity for a small subset of parasites to enter a quiescent state and undergo apicoplast DNA repair and remodeling in response to the drug.

In contrast to the delayed-death phenotype that occurs with ciprofloxacin treatment in the closely related apicomplexan parasite *Toxoplasma gondii* (Fichera and Roos 1997), ciprofloxacin caused immediate death in *Plasmodium falciparum*. While the *T. gondii* and *P. falciparum* 35 kb plastid genomes are extremely similar in nucleotide sequence and gene organization, the topology and mode in which the plastid genomes are replicated are very different. The *T. gondii* plastid is present as mostly linear tandem arrays and is replicated through a rolling circle mechanism (Williamson et al. 2001). The *P. falciparum* apicoplast DNA is present primarily as covalently closed circular monomers in either relaxed or twisted forms, with a small proportion of highly twisted forms (Williamson et al. 2002). The apicoplast DNA is replicated by 2 mechanisms: the formation of twin displacement-loops (D-loops) within the inverted repeat region of the circle, and by a rolling circle mechanism that initiates outside of the inverted repeat region (Williamson et al. 2002). Of these 2 modes of apicoplast DNA replication, the twin D-loop mechanism was found to be considerably more sensitive to ciprofloxacin (Williamson et al. 2002).
It was found in this study that mitochondrial DNA replication was not affected by ciprofloxacin. Like the *Toxoplasma* apicoplast DNA, the *Plasmodium* mitochondrial DNA is present as linear tandem arrays and utilizes a rolling circle method of DNA replication. However, *Toxoplasma* has an apicoplast targeted DNA gyrase, and treatment with ciprofloxacin results in a selective loss of apicoplast DNA (Fichera and Roos 1997). The *P. falciparum* DNA gyrase is specifically localized to the apicoplast and not the mitochondrion (Raghu Ram et al. 2007). A type I topoisomerase (topoisomerase III, PlasmoDB ID PF13_0251) encoded in the nucleus contains putative N-terminal transit peptide, suggesting it could be targeted to the mitochondrion (Appendix B). Presence of a type I topo in the mitochondrion further explains the lack of mitochondrial sensitivity to both bacterial (ciprofloxacin) and eukaryotic (VP-16/VM-26) topo poisons. While in the land plants *Arabadopsis thaliana* and *Nicotiana benthamiana* there is a DNA gyrase which is targeted to both the plastid and mitochondrion (Cho et al. 2004, Wall et al. 2004), this may not be the case in more ancient plastid-harboring organisms. For example, treatment of the red alga *Cyanidioschyzon merolae* with the gyrase subunit A inhibitor naladixic acid results in unequal distribution of the plastid nucleoid in divided chloroplasts, whereas the mitochondrial DNA remained unaffected by the drug treatment (Itoh et al. 1997). In *P. falciparum*, a recent study that followed the morphology of the apicoplast and mitochondrion after treatment with a lethal dosage of ciprofloxacin showed that the apicoplast never elongated, while normal elongation and branching of the mitochondrion was observed in some cells (Goodman et al. 2007). Results from this study support the findings of Goodman et al. (2007).

Loss of DNA with fluoroquinolone treatment is a characteristic molecular phenotype of DNA Gyrase-DNA interactions and in this study ciprofloxacin treatment resulted in a selective loss of apicoplast DNA. The loss of apicoplast DNA did not preferentially occur in the inverted
repeat region as may have been expected based on previous work demonstrating that DNA replication of the inverted repeat region is sensitive to ciprofloxacin (Williamson et al. 2002). A similar trend in the loss of apicoplast DNA was observed for both LSU genes of the inverted repeat and two genes that are distant from the inverted repeat (clpC and tufA). This result can be explained by an increased reliance on the rolling circle method of DNA replication. Eventually however, once the circular template from which the linear multimers are generated during rolling circle replication is depleted as a result of drug-induced cleavage, replication would cease. In fact, DNA gyrase activity may be required for regenerating the circular templates from the linear DNA (Williamson et al. 2002), and this could further inhibit DNA replication.

In this study the effects of ciprofloxacin on the steady-state RNA levels of a protein coding gene (clpC) and the rRNA gene (LSU) were examined over a 96 hour time course in synchronous parasites. For these experiments the IC\textsubscript{50} concentration of ciprofloxacin (5 \(\mu\text{g/ml}\)) was used. Both clpC transcripts and LSU rRNA showed a significant decrease in the second asexual cycle (~ 100-fold) compared to the untreated controls. However, the effects of drug treatment on clpC transcripts were less pronounced in the first asexual cycle, and the temporal pattern of clpC expression appeared normal, with the highest abundance of transcripts accumulating in the late trophozoite stage. Conversely, drug treatment resulted in an immediate effect on LSU rRNA levels, which were detected at levels significantly lower than the controls after just 12 h of drug treatment. The abundance of LSU rRNA steadily decreased over the next 84 h.

Protein coding genes of the apicoplast DNA are actively transcribed in polycistronic units and it proposed that at least 4 primary transcripts originate from the inverted repeat region (Gardner et al. 1991b, Feagin and Drew 1995, Preiser et al. 1995, Wilson et al. 1996). By
probing with \textit{rpoB} and \textit{rpo C}, Feagin and Drew detected four low abundance transcripts at approximately 15 kb, 12.5 kb, 11 kb, and 7.8 kb which were reproducibly found within a smear of transcripts (1995). It has also been found that probing with \textit{tuf A} gives a smear, and \textit{rpl2} and \textit{rpl23}, and \textit{rps3} and \textit{rps19} transcripts are linked (Wilson et al. 1996). In contrast, the ribosomal RNAs are detected as discrete bands. Reports indicate that the LSU rRNA is detected as a single band at 2.9 kb, and the SSU rRNA is detected as 3 bands at 1.7 kb, 1.35 kb, and 1.2 kb (Gardner et al. 1991a, Feagin and Drew 1995). Based on this published information, it can be deduced that RNA expression of the apicoplast ribosomal genes occurs independently of the protein coding genes. Further, results from this study indicate that rRNA expression and/or accumulation was sensitive to ciprofloxacin, whereas protein coding transcripts were less sensitive. The decrease in \textit{clpC} transcripts could be mostly attributed to a cognate loss of DNA template resulting from the inhibition of DNA replication.

Among the most striking sub-cellular morphological features of the \textit{P. falciparum} cell are the elaborate configurations taken on by the developing apicoplast and mitochondrion. During the asexual cycle, a segment of each organelle is segregated into all newly formed daughter merozoites just before rupture of the schizont. On average 16 merozoites are produced during schizogony, and in addition to orchestrating organelle segregation, a mechanism for organellar genome segregation must be in place. This process is expected to be directed in part by components of the eukaryotic division apparatus, and may also be linked to the structural characteristics and positioning of the nucleoid, to ensure proper segregation of the apicoplast genome. DNA gyrase is expected to play a critical role in maintaining the fidelity of apicoplast DNA segregation into new apicoplasts. There are two global systems that govern binary fission in bacteria: 1) Min system-regulated positioning of the Z-ring at the midcell, and 2) inhibition of
septation by either the SOS-mediated SulA division inhibitor, or more specific septation inhibition by nucleoid exclusion (Rothfield et al. 2005). In bacteria, when DNA replication and/or segregation are blocked, the system of nucleoid exclusion (NO) prevents septation from occurring over the nucleoid, but allows division to take place in a different area of the cell (Woldringh et al. 1991, Bernhardt and de Boer 2005). If the Plasmodium DNA gyrase is required for DNA replication and nucleoid segregation, it could be anticipated that ciprofloxacin treatment would give rise to DNA-deficient apicoplasts (that appear otherwise normal) in daughter cells. However, results from the FISH/IFA analysis strongly suggest that apicoplasts did not develop normally in drug treated cells. Results from qRT-PCR experiments showed that the accumulation of ribosomal RNA was immediately inhibited with ciprofloxacin treatment. Whereas LSU rRNA levels climbed to 505 ± 142 molecules per nuclear genome during the first asexual cycle in untreated parasites, in ciprofloxacin treated cultures the number of LSU rRNA molecules per nuclear genome levels reached a maximum of 64 ± 24. The reduction in rRNA would immediately affect translation of apicoplast encoded genes. The additive consequences of impaired protein translation of apicoplast encoded genes (further reduction of translation from lack of ribosomal proteins, reduction in transcription, and decreased protein import) would result in dysfunctional and morphologically abnormal apicoplasts.

Two 50S ribosomal inhibitors (clindamycin and azithromycin) and a 30S ribosomal inhibitor (doxycycline) cause delayed-death in P. falciparum, whereas another 50S ribosomal inhibitor (thiostrepton) causes immediate death. The variation in the potencies of these different inhibitors may be a result of where they bind the rRNA, how well inhibitors gain access to the apicoplast stroma, different half-lives, etc. In the case of the delayed-death inhibitors, washout studies have demonstrated that the ultimate lethality was due to consequences of drug treatment
within the first asexual cycle. The precise reason for the delayed death remains unknown. It is proposed that thiostrepton (which causes immediate death) interferes with the association of elongation factors with the ribosome (Jonker et al. 2007). Thiostrepton is a thiopeptide antibacterial that binds both the 23S rRNA and the L11 ribosomal protein, and inhibits ribosome-mediated GTP hydrolysis and peptidyltransferase activity (Harms et al. 2008). Conversely, another thiopeptide inhibitor micrococcin stimulates GTPase activity of the ribosome, yet it is extremely potent against *P. falciparum* with a low nM IC₅₀ (Rogers et al. 1998). Interestingly, thiostrepton causes an immediate reduction in the levels of apicoplast *rpoB/rpoC* transcripts and the reduction is similar to what is observed for the eubacterial RNA polymerase inhibitor rifampicin, which causes immediate death (McConkey et al. 1997). Therefore, inhibiting apicoplast RNA transcription, whether directly or indirectly, may be a common thread in antibacterials that cause immediate death target in *P. falciparum*. While ciprofloxacin did not cause an immediate reduction in the transcript levels of the protein coding gene *clpC*, the levels of rRNA were significantly reduced, and the drug caused immediate parasite death. The cytotoxic action of ciprofloxacin may be a result of inhibiting rRNA production and thus protein translation. FISH studies demonstrated that the apicoplast nucleoid signal was reduced both in size and intensity in parasites cultured with ciprofloxacin. After 72 h in culture with ciprofloxacin (5 µg/ml) there was a significant reduction in the quantity of apicoplast DNA and RNA, which would result in disruption of protein synthesis of apicoplast encoded genes. In the absence of apicoplast encoded proteins, in particular the molecular chaperone and member of the Tic protein import complex CLPC, protein transport into the apicoplast would be severely impeded. Indeed, studies have shown that inhibition of apicoplast translation with anti-bacterial
drugs leads to the accumulation of unprocessed apicoplast-targeted protein (Goodman et al. 2007).

However, compared to thiostrepton the inhibitory effect of ciprofloxacin on protein translation was not immediate. The finding that \textit{clpC} transcription (an indirect measure of apicoplast protein translation based on affecting the translation of the apicoplast DNA encoded RNA polymerase subunit genes \textit{rpoB}, \textit{rpoC}, and \textit{rpoC2}) was not significantly reduced within the first cycle of ciprofloxacin treatment could indicate that apicoplast ribosome function was not completely inhibited. It is also possible that the remaining pool of RNA polymerase was able to continue transcription.

If inhibiting protein translation in the apicoplast is a major cytotoxic consequence of ciprofloxacin, it may be difficult to uncover other DNA gyrase functions using ciprofloxacin as a probe. Also, during schizogony DNA replication and segregation can not be delineated into specific time frames where one process was occurring exclusively of the other, leaving it difficult to assess the effect of DNA gyrase inhibition by ciprofloxacin solely on one process in the absence of the other.

The \textit{in situ} analysis of ciprofloxacin treated parasites by combined FISH / IFA provided important insight into the mechanism of DNA gyrase inhibition by the drug. In many cells the apicoplast signal from GFP became fragmented and was localized to the perinuclear region. Furthermore, in many instances the apicoplast DNA did not fully co-localize with the GFP signal. If a consequence of ciprofloxacin treatment is inhibition of protein transport into the apicoplast, then GFP detected in the drug treated cells may have been trapped within the apicoplast membranes. In addition, the observation that the GFP signal was perinuclear raised the possibility that apicoplast targeted proteins were trapped in the membranes of the ER. It is
also possible that the fragmented appearance of the GFP delineated structures indicates that the apicoplast was disintegrating and this could result in the release of the apicoplast DNA. If protein import was also impeded in the apicoplast disintegration scenario, this would result in a lack of proper structural organization of the nucleoid by DNA associated proteins such as the apicoplast targeted HU-like proteins.

Typically in a dividing eukaryotic cell, the nuclear membrane dissolves into vesicles following karyokinesis (Anderson and Hetzer 2008). In apicomplexa a unique situation exists where the nuclear membrane persists throughout the cell cycle (Striepen et al. 2007). The nuclear lamina coating the inside of the nuclear membrane is essential in orchestrating chromatin organization, DNA replication, and cell cycle regulation (Gruenbaum et al. 2005). Like the nuclear membrane, the inner plastidic membrane and associated components could serve as an attachment point for the apicoplast nucleoid. In spinach chloroplasts the nucleoid is bound to the chloroplast envelope early in development and to the thylakoid membrane later in development, with the attachment sites residing near the inverted repeat region containing 16S and 23S rRNA genes (Liu and Rose 1992). In this study a novel transmembrane and DNA binding protein was identified with predicted apicoplast targeting sequences (PFL1055c, Appendix C). This protein has structural features that indicate it is a PEND-like protein found in chloroplasts (N-terminal bzip-like DNA binding domain, C-terminal transmembrane domains, internal repeat sequences). In chloroplasts this protein is predicted to bind the chloroplast DNA, and to attach to the inner plastidic membrane through its transmembrane domains (Sato et al. 1999, Sato and Ohta 2001). The PEND protein binds specifically to the canonical sequence TAAGAAGT (Sato and Ohta 2001), and the P. falciparum apicoplast DNA contains 2 copies of this sequence in each side of the inverted repeat (total of 4 copies). Furthermore, the P. falciparum PEND-like protein
contains a predicted bipartite apicoplast targeting sequence (Appendix C). In the absence of a protein such as the PEND-like homologue and other membrane bound proteins to provide the scaffolding for the PEND-like homologue, the apicoplast DNA could become disassociated from the apicoplast envelope.

Like nuclear chromatin, the bacterial nucleoid has functional structure (Razin 1999, Dorman and Deighan 2003, Thanbichler et al. 2005, Cremer et al. 2006). The bacterial nucleoid is comprised of short-range structures that are governed by histone-like proteins and long range structures in the form of looped domains radiating from the inner bacterial membrane (Kavenoff and Ryder 1976, Brunetti et al. 2001, van Noort et al. 2004). DNA segments that are compartmentalized in long-range loop domains exhibit independent supercoiling topology, and in eukaryotic cells nuclear topoisomerase II is found at the chromosomal loop anchorage sites associated with the nuclear matrix/scaffold (Adachi et al. 1989, Laemmli et al. 1992). Drug-induced DNA cleavage by topoisomerase II results in excision of DNA loops and is used as an important tool for understanding the higher order structure of chromatin. Similarly, in bacteria drug-induced cleavage by DNA gyrase and topoisomerase IV results in excision of loop-sized DNA fragments (Hsu et al. 2006).

In the present study, DNA cleavage analysis was performed to further characterize the potential sites that DNA gyrase associates with the apicoplast DNA. The topoisomerase poisons ciprofloxacin and VM26/VP-16 were used to stabilize potential ‘cleavable complexes’. SDS, which induces double stranded breaks in the DNA of the cleavable complex (Liu et al. 1983, Rowe et al. 1984) was added after drug treatment. The DNA remains attached at the 5’ phosphoryl end to the active site tyrosine of GyrA, and this property was exploited for
immunoprecipitation experiments using GyrA antibody. For cleavage analysis DNA was treated overnight with proteinase-K.

Results from the cleavage analysis indicated that the DNA gyrase did not associate with highly specific regions of the apicoplast DNA, as discrete bands were not observed in the vast majority of experiments conducted. Instead, a smearing of apicoplast DNA was observed in the ciprofloxacin treated samples that were digested with single cutting enzymes (EcoRI and NsiI). In the Nsi I digest, the smearing was similarly produced in the VM26 treated sample. A typical feature of inverted repeats is the formation of cruciform structures, and cruciform structures have been identified in P. falciparum apicoplast DNA by electron microscopy (Williamson et al. 2002). Cruciform structures often coincide with origins of replication and typically exhibit independent supercoiling topology (Pearson et al. 1996). Furthermore, type II topoisomerases can interact with specific recognition sites based on secondary structure (Froelich-Ammon et al. 1994), raising the possibility that the cruciform structure of the apicoplast DNA could act as a preferential substrate for DNA gyrase. However, in the present study preferential sites of interaction were not identified. Of note were the results of the Hpa I digest, where either one or both of the Hpa I sites located within the inverted repeat appeared to be blocked in a subset of DNA. The blockage was most pronounced in drug-treated late trophozoite / early schizont stages. A potential explanation for the blocked DNA digestion is the formation of transient single-stranded extensions within the inverted repeat region, which are characteristic of D-loop replication. Drug treatment may have resulted in an accumulation of replication intermediates by obstructing movement of the replication complex. A similar obstruction may have led to lack of LSU rRNA accumulation.
Conditional Gene Regulation

Prior to attempting the conditional regulation of DNA gyrase expression, efforts were focused on demonstrating that conditional regulation of a characterized non-essential gene within its native locus would be feasible. The plasmepsin 4 gene was selected for this investigation after considering previous work demonstrating that a) the plasmepsin locus is permissive to homologous recombination, and b) disruption/deletion of pfpm4 is not lethal for blood-stage P. falciparum cultured in vitro (Omara-Opyene et al. 2004, Bonilla et al. 2007a, Bonilla et al. 2007b).

FACS analysis of parasites transformed with pTGPI-GFP indicated that a single copy of the transgene under the TetO / min. cam and a single copy of transactivator were sufficient to control GFP expression. Also encouraging was the finding that GFP expression was silenced within 24 h of adding ATc. However, the finding that the progeny of GFP expressing parasites did not uniformly express GFP raised some concern. Initially it was thought that some instability could arise due to the fact that the Tet-transactivator elements were being expressed from an episome. Plasmid episomes in P. falciparum can form large concatamers that may be subject to spurious recombination events, and episomes are not equally segregated into daughter cells (O'Donnell et al. 2001, O'Donnell et al. 2002). The toxic effects of the GPI-anchor fused to the C-terminus of GFP (to promote turnover) were also considered as a potential explanation for the phenotypic instability (Meissner et al. 2005). In the present study, the regulatory elements of the Tet-transactivator system were integrated into the P. falciparum genome to confer greater genetic stability. First, the TetO/min.cam promoter was successfully targeted for integration immediately upstream of pfpm4 to create the pTOCAM-Dpm4 parasite lines. Insertion of the artificial promoter reduced the level of full-length pfpm4 RNA expression by > 100-fold compared to the wild-type pfpm4 levels, and PfPM4 protein was not detected by western blot.
Next, *piggyBac* transformation was used to insert the *tati2* expression cassette into the genome and parasites were selected in the absence of ATc to create parasite line C3TA. If restoration of PfPM4 expression was advantageous for parasite development, perhaps this would promote the selection of parasites where the Tet-transactivator system was properly functioning. However, the proliferation rates of two pTOCAM-Δpm4 clones tested did not differ from the wild-type 3D7 strain, leaving little opportunity to select C3TA parasites based on increased proliferation.

In fact, after the successful introduction of the *tati2* expression cassette, PfPM4 expression was not restored in culture C3TA. It was also found that although *tati2* RNA was in high abundance, TaTi2 protein was not correspondingly high. Interestingly however, when C3TA was cultured in the presence of ATc for as little as 96 h, TaTi2 protein expression was restored to near maximum steady-state levels.

In subsequent experiments, C3TA-B1 parasites were cultured in the presence of ATc and a drug wash-out approach was used in an effort to regulate the expression of *pfpm4*. Western blot analysis of the wash-out experiments revealed that TaTi2 protein levels remained consistently less in C3TA-B1 cultured without ATc, and the same TaTi2 silencing phenomenon was observed with pTGPI-GFP transformed parasites in which *gfp* was the transgene under the regulation of the TetO/min. cam promoter. However, TaTi2 expression was stable in the absence of ATc in parasite clones that did not contain a TetO/min. cam promoter (TA-01, TA-02, and TATK-04 parasite lines). The reduction in TaTi2 protein in C3TA-B1 in the absence of ATc was unlikely attributable only to transcriptional silencing, since the RNA levels of *tati2* differed by less than 2-fold while the protein levels differed on average by 10-fold. Furthermore, the silencing effect on the transactivator appeared to be dependent upon a trans interaction of TaTi2 with the operator sequence, and was not dependent on the nuclear location of TaTi2 or the TetO.

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sequence, and was not dependent on the particular gene that is placed under control of the TetO. The rapid accumulation of TaTi2 in the presence of ATc (where TaTi2 cannot bind the TetO) additionally supports the reasoning that TaTi2 silencing results as a consequence of interaction with the TetO.

Binding of the transactivator to the TetO induces a conformational change in the protein (Orth et al. 2000), and perhaps in this conformation the protein is less stable when released from the DNA. Alternatively, perhaps the protein is more readily degraded if bound to the TetO. There is an intimate association between the potency of transactivators, ubiquitination, and proteasome activity, (Kodadek et al. 2006). Proteosome mediated turnover of transactivators can restrict promoter activity, or keep gene transcription in a potentiated state to continuously respond to environmental or cellular stimuli (Reid et al. 2003, Szutorisz et al. 2006). In future studies, to investigate whether the nature of the silencing affect was caused by increased TaTi2 turnover, experiments may be conducted with C3TA-B1 parasites using proteasome inhibitors in the absence and presence of ATc. Experiments may also be conducted to compare the degree of ubiquitination of TaTi2 in C3TA-B1 in the presence and absence of ATc and compare the result to the level of TaTi2 ubiquitination in the absence and presence of ATc in one of the TA parasite lines.

In the washout experiments, pfpm4 expression was expected to be stimulated upon washout of ATc. TaTi2 was first allowed to accumulate by culturing in the presence of ATc, and then the drug was washed out to permit binding of the protein to the operator sequence. It was encouraging that 3’ pfpm4 RNA levels closely followed the levels of tati2 RNA, as it would be expected that the temporal pattern of TaTi2 expression would directly influence the expression of the gene under regulation of the TetO. These results indicate that at least some TaTi2 was
binding the TetO to stimulate transcription, although the process was not robust enough to restore PfPM4 protein production to detectable levels. The lack of robust stimulation could be the result of numerous factors. First, three extra plasmid copies of pTOCAM-Δpm4 were integrated in clone C3TA-B1, and it may be the configuration of the locus that was interfering with the capacity to regulate *pfpm4* expression. While a single *pfpm4* RNA transcript predominated in wild-type 3D7, there were three dominant RNA transcripts detected in C3 and C3TA-B1. Northern blot and qRT-PCT data indicated that all three transcripts in C3 and C3TA-B1 originated from the natural *pfpm4* promoter and the smaller two transcripts were break-down products of the larger transcript. The absence of a 3’ UTR and associated stabilizing elements was likely responsible for increased rate of RNA breakdown of *pfpm4* transcripts originating from the natural promoter. Very little RNA was detected from the full length copy of *pfpm4* under the control of TetO/min. cam promoter. The additional *pfpm4* copies that were truncated at the 3’ end and under the regulation of TetO/min. cam promoter were likely to be similarly minimally active. Further supporting that all TetO/min. cam promoters were very minimally active were the findings that regardless of how many additional plasmid copies were integrated the same three transcripts were detected by northern blot, the transcripts were in the sense strand orientation, and levels of 5’ *pfpm4* RNA determined by qRT-PCR were not statistically significantly different in 3D7, C3 and C3TA-B1 (Figures 3-43 & 3-44).

The majority of the eukaryotic genome is transcribed (Cheng et al. 2005, Kapranov et al. 2005). Non-coding RNAs regulate chromatin structure and the epigenome in the eukaryotic cell, and can directly regulate transcription processes (Amaral et al. 2008). Also, alternatively spliced RNA transcripts from protein coding genes, and antisense transcripts are also much more common than previously thought (Mendes Soares and Valcarcel 2006). Although the functional
diversity of alternative mRNA isoforms remains to be discovered, studies are beginning to link
the presence of alternative mRNAs with specific biological functions (Relogio et al. 2005, Ule et
al. 2005, Mendes Soares and Valcarcel 2006). The natural 5’ promoter in the pTOCAM-Δpm4
and C3TA parasite lines created in this study remained pervasively active. The activity of the
pfpm4 natural promoter and the presence of abundant 5’ pfpm4 RNA and its breakdown products
originating from this promoter might negatively impact the potential of inducing expression of
the additional pfpm4 copies under the TetO/min. cam promoter. Other potential explanations
may also need to be examined. The minimal cam promoter could be lacking sequence that is
required for RNA polymerase to be in a poised state for transcription. In eukaryotic cells RNA
transcription requires recruitment of RNA polymerase and the pre-initiation complex,
transcription initiation, and pause at promoter proximal sites prior to escape from the pause sites
and elongation (Core and Lis 2008). Perhaps, for the Tet-transactivator system to be functional
additional sequence elements may need to be present in close proximity to the TetO.
Recombination of episomal DNA that commonly occurs in P. falciparum (K. Deitsch, pers.
comm.), may lead to the occasional association of required elements with the TetO min CAM
promoter for the operator/promoter to work as expected, and this may explain why only 20% of
the pTGPI-GFP transformed clones express GFP. Other potential explanations for the lack of
induction could be that TaTi2 does not contain a sufficiently strong transactivating domain and
that proximity to a strong promoter (such as 5’ pfpm4 or in pTGPI-GFP msp2) could be
detrimental for target transgene expression as the strong promoter could titrate out transcriptional
factors.

Consideration must also be given to the timing of tati2 expression and the subcellular
localization of TaTi2. The expression of tati2 is under the regulation of the msp2 promoter,
which peaks in activity during the late-trophozoite and early schizont stages (Wickham et al. 2003). The peak of pfpm4 expression however is during the early ring stages. This difference in the timing of expression may result in deficient co-localization of the pfpm4 locus with TaTi2, and TaTi2 may localize to the nucleus in a time frame where the pfpm4 locus is not poised for transcriptional stimulation. Furthermore, the timing of transactivator expression may result in trafficking of the protein to a specific subcellular location other than the nucleus. Studies have demonstrated that the timing of transgene expression in Plasmodium is critical for appropriate sub-cellular localization (Kocken et al. 1998, Triglia et al. 2000). In future studies, the N-terminal 90 bases of the P. falciparum histone H2B containing a predicated 17 amino acid nuclear targeting sequence may be appended to the N-terminus of the transactivator protein. It is possible that both proper timing of expression and the addition of the appropriate targeting sequence may be required to achieve superior nuclear localization. Once in the nucleus however, the transactivator must also reach the appropriate nuclear compartment also containing the gene under the regulation of the TetO promoter. To properly achieve so many concerted levels of cellular regulation will be difficult using a cogent approach, and thus future efforts will focus on setting up functional screens.

As illustrated by P. falciparum episomally transformed with pTGPI-GFP, the level of Tet-transactivator regulated transgene expression is highly variable among individual cells. Experiments targeting non-essential endogenous loci for conditional regulation may prove to be even more problematic. Because the protein product of a non-essential gene is dispensable for the parasite, pressure to maintain stable expression of this gene may be more costly (perhaps by affecting the timing and levels of expression of nearby genes) than not expressing the gene. However, it remains critical to evaluate how robustly the system works when applied to an
essential gene, since in this case the parasite is under pressure to consistently drive the cascade of
events required to maintain stable gene expression.

For future studies, a functional approach will be used to select for parasites where Tet-
regulated gene expression is most uniformly operational and persists over generations. In one set
of experiments, a hDHFR expression cassette under the regulation of the TetO/min. cam
promoter will be randomly integrated by piggyBac transformation into the genome of low,
medium, and high TaTi2-expressing lines. Cultures will be selected with WR99210 at a range of
concentrations to obtain parasites where the Tet-transactivator system is able to drive hDHFR
expression. Mapping of the integration site, combined FISH / IFA, and chromatin
immunoprecipitation will be used to examine the configuration of nuclear environments that are
permissive and non-permissive for Tet-regulated expression.

In another approach, parasites may first be randomly transformed by piggyBac with a
plasmid construct containing the hDHFR with TetO/min. cam promoter expression cassette and
BSD as a selectable marker. Next, piggyBac transformation will be used to introduce
transactivator expression cassettes lacking a selectable marker. Instead, WR99210 will be used to
select, thus only parasites with functional Tet-regulation driving hDHFR expression will emerge
from the population pool. Also, tati2 expression cassettes lacking a promoter sequence and/or
terminator sequence will be used. Once the transgenic parasite lines are produced, ATc will be
added to the culture medium to turn off hDHFR expression, and hDHFR protein and RNA levels
will be followed.

In a third approach to determine whether the reduction in TATi-2 levels can be overcome
by increased expression, a plasmid construct with a bidirectional promoter will be used to drive
both tati2 and bsd expression cassettes. By increasing the selective concentration of blasticidin-
S, the plasmid copy number will increase to drive bsd expression which will also increase tati2 transcription.

**Conclusion**

In conclusion, results from this study indicated that targeting DNA gyrase with ciprofloxacin inhibited the replication of apicoplast DNA and the accumulation of apicoplast RNA transcripts, and blocked the production of apicoplast ribosomal RNA. Also, with ciprofloxacin treatment the apicoplast nucleoid appeared reduced in size and did not segregate normally, and the apicoplast displayed abnormal morphology. In contrast, mitochondrial DNA replication, transcription, and segregation were unaffected. The DNA gyrase appeared to associate with the apicoplast DNA at non-specific loci, implicating numerous functional roles for the enzyme. For further characterization of the *Plasmodium* DNA gyrase cellular functions independent of probing with drug, efforts were focused on developing a system for conditional gene regulation. Results from the conditional gene regulation studies indicated that the Tet-transactivator system has potential applicability for controlling endogenous gene expression in *P. falciparum*, but must be further optimized. Artificial promoters were successfully targeted to the *pfpm4* genomic locus, and *piggyBac* insertion of transactivator (TaTi2) expression cassettes resulted in stable TaTi2 expression. In washout studies, the temporal pattern of *pfpm4* RNA expression closely followed the expression of tati2. In the induced state of target transgene expression the steady-state TaTi2 levels decreased, a phenomenon that will be further investigated. Attention will also be given to optimizing the configuration of the locus containing the ATc-responsive promoter, and providing optimal timing of transactivator expression and nuclear localization. The ultimate goal of these studies is to establish a system for conditional gene regulation in *P. falciparum* to characterize the cellular functions of parasite proteins such as DNA gyrase, and other potential drug targets. These studies will also help to illuminate the
mechanisms of gene regulation utilized by *Plasmodium falciparum*, one of the world’s deadliest pathogens.
Maximum parsimony tree showing the phylogenetic relatedness of DNA gyrase GyrA and Topo IV ParC. Tree was created in Mega 3.1 using a CLUSTAL-W alignment and numbers at the nodes were derived from an interior branch test (5,000 replicates).
Minimum evolution tree showing the phylogenetic relatedness of DNA gyrase GyrA and Topo IV ParC. Tree was created in Mega 3.1 using a CLUSTAL-W alignment and numbers at the nodes were derived from an interior branch test (5,000 replicates).
Minimum evolution tree of topoisomerase III created with Mega 3.1 using a CLUSTAL-W alignment. Numbers at the nodes generated by an interior branch test (5,000 replicates)
APPENDIX C

CLUSTAL-W ALIGNMENT of Plasmodium PEND-like Homologues

Clustal-W alignment of the PEND-like homologue in Plasmodium spp. showing putative structural features. The signal peptide (red box) and transit peptide (green box) were identified using the PATS server. A bZIP-like domain with a basic region (purple box) and leucine repeats (asterisks) comprise a potential DNA binding region. Transmembrane helices were identified with TMHMM and are highlighted in yellow. Tandem repeats were identified with XSTREAM (small black boxes).


Roche. 2000. DIG application manual for filter hybridization. Roche Diagnostics, Germany.


BIOGRAPHICAL SKETCH

Tonya Bonilla earned a Bachelor of Science in biology from the University of Minnesota. She moved to south Florida and earned a Master of Science degree in marine biology from Nova Southeastern University. Tonya next attended the University of Florida where she earned a Doctor of Philosophy in infectious diseases and pathology.