Growth and development in Toxoplasma gondii
by Michael Nicholas Guerini

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Molecular Biology
Montana State University

Abstract:
The significance of Toxoplasma gondii to human and animal health, along with its value as a model for other pathogenic protozoa, makes this microorganism an important model in the field of Apicomplexa research. It is clear from numerous studies of the diseases caused by this family of microorganisms that parasitaemia itself is key to pathogenesis, and thus, an understanding of the growth processes in these pathogens could provide better treatments. In this work, the relationship/regulation between the cell cycle and development was investigated. A G2 population arose during tachyzoite-to-bradyzoite differentiation thus, providing a mechanism of linking the cell cycle with development. To gain further insight into the regulation of the parasite cell cycle, both a reverse and forward genetic approach was used. One approach used bioinformatics and cDNA library screening to identify cell cycle related proteins. Two distinct proliferating cell nuclear antigen (PCNA) genes in T. gondii were identified and this work demonstrated that these two genes were differentially expressed during development and the tachyzoite cell cycle. Further work showed that each PCNA likely acts independently and provided evidence that TgPCNA1 potentially serves as the major replisomal PCNA. The function of PCNA2 in T. gondii remains unknown. Finally, a forward genetics approach focused on the complementation of the tachyzoite temperature sensitive (ts) mutant 11C9, which arrests within 1 to 2 divisions at the non-permissive temperature (40°C), and approximately half of the parasites arrest with a single undivided nucleus (2N) placing the defect at some point in mitosis. Complementation of tel 11C9 with a homolog of the eukaryotic XPMC2 suggests that the cyclinB/cdk or wee1/chk1 pathway in this mutant is likely affected. In T. gondii, a proposed linkage between growth and development seem consistent with a developmental timer system (“clock mechanism”), yet the presence of checkpoints suggests that the domino model plays at least a partial role in regulating the tachyzoite cell cycle.

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Michael Nicholas Guerini

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate studies.

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ABSTRACT

The significance of *Toxoplasma gondii* to human and animal health, along with its value as a model for other pathogenic protozoa, makes this microorganism an important model in the field of Apicomplexa research. It is clear from numerous studies of the diseases caused by this family of microorganisms that parasitaemia itself is key to pathogenesis, and thus, an understanding of the growth processes in these pathogens could provide better treatments. In this work, the relationship/regulation between the cell cycle and development was investigated. A G2 population arose during tachyzoite-to-bradyzoite differentiation thus, providing a mechanism of linking the cell cycle with development. To gain further insight into the regulation of the parasite cell cycle, both a reverse and forward genetic approach was used. One approach used bioinformatics and cDNA library screening to identify cell cycle related proteins. Two distinct proliferating cell nuclear antigen (PCNA) genes in *T. gondii* were identified and this work demonstrated that these two genes were differentially expressed during development and the tachyzoite cell cycle. Further work showed that each PCNA likely acts independently and provided evidence that TgPCNA1 potentially serves as the major replisomal PCNA. The function of PCNA2 in *T. gondii* remains unknown. Finally, a forward genetics approach focused on the complementation of the tachyzoite temperature sensitive (*ts*) mutant 11C9, which arrests within 1 to 2 divisions at the non-permissive temperature (40°C), and approximately half of the parasites arrest with a single undivided nucleus (2N) placing the defect at some point in mitosis. Complementation of *ts*11C9 with a homolog of the eukaryotic XPMC2 suggests that the cyclinB/cdk or wee1/chk1 pathway in this mutant is likely affected. In *T. gondii*, a proposed linkage between growth and development seem consistent with a developmental timer system ("clock mechanism"), yet the presence of checkpoints suggests that the domino model plays at least a partial role in regulating the tachyzoite cell cycle.

CHAPTER 1

TOXOPLASMA GONDII AND APICOMPLEXANS

Toxoplasma gondii

Life cycle

The phylum Apicomplexa is divided into four classes, the Gregarinea, Haemosporidea, Piroplasmea and Coccidea, which together comprise over 5,000 named species of protozoan parasites [1]. The coccidea are the largest class (~1,500 species) and include various pathogens of man and animals [*Eimeria*, *Sarcocystis*, *Neospora*, *Cryptosporidium*, *Cyclospora* and *Toxoplasma gondii*]. *T. gondii*, the subject of this thesis, is capable of infecting an unusually broad range of hosts and many different cell types. *Toxoplasma* is facultatively heteroxenous (two host life cycle) and causes one of the most common parasitic zoonoses in the world (Fig. 1-1) [2-3]. There are three infectious stages in the life cycle of *T. gondii;* sporozoites, tachyzoites and bradyzoites. *T. gondii* is unique among heteroxenous coccidians in that all three of these parasite forms are infectious to both intermediate and definitive hosts. Three routes are responsible for *Toxoplasma* infection: 1) horizontal transmission by oral ingestion of oocysts (sporozoites) from the environment, 2) horizontal transmission of tissue cysts (bradyzoites) by oral ingestion of raw or undercooked meat, 3) vertical transmission of

tachyzoites via the placenta of infected mothers [3-4]. It has also been reported that tachyzoites may be transmitted through milk from mother to offspring [3].

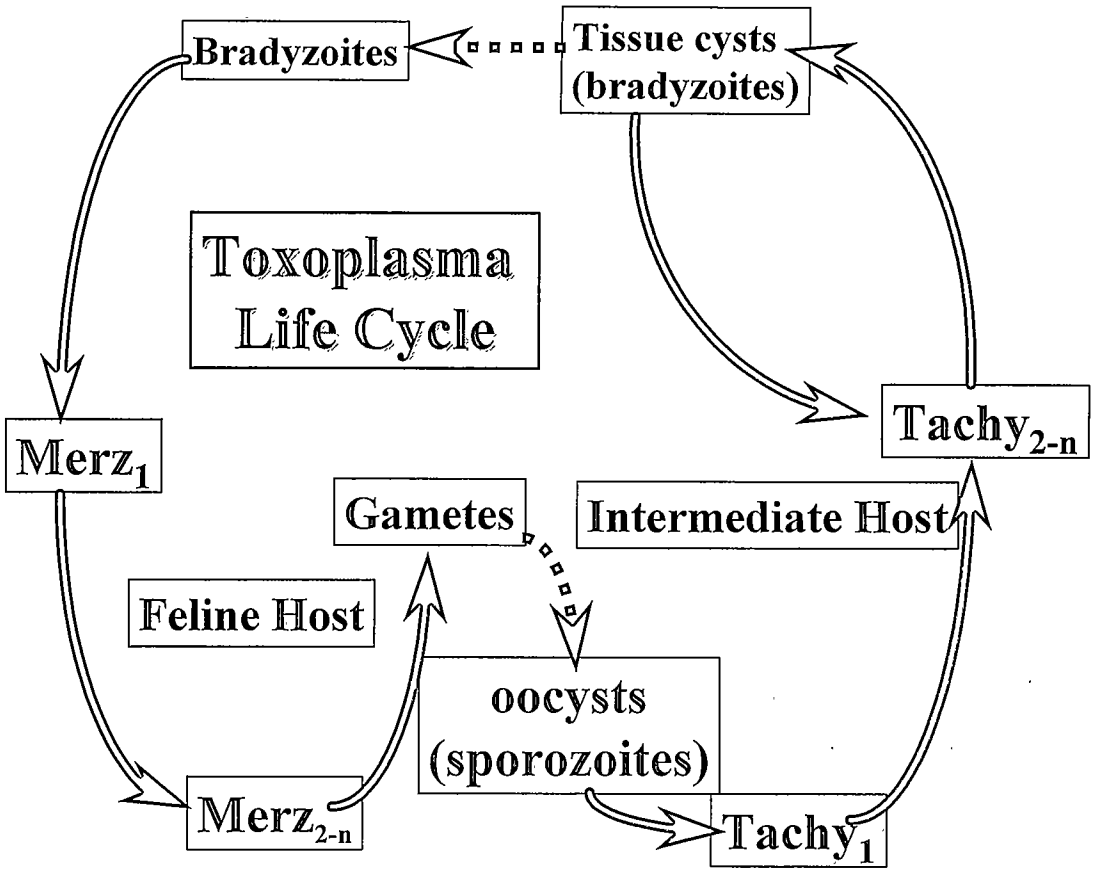

Figure 1-1: Life cycle of *Toxoplasma gondii*.

As with other coccidians, the *T. gondii* oocyst is the environmentally protective housing for the sporozoite [2]. When oocysts are ingested by an intermediate host, sporozoites excyst and actively penetrate cells of the intestinal tract where they differentiate into replicating tachyzoites. Tachyzoites multiply rapidly by repeated rounds of endodyogeny and will ultimately develop into bradyzoites contained within a protective tissue cyst. Tissue cysts are the end-stage of the intermediate life-cycle and

are capable of persistence for the life of the host. It is postulated that tissue cysts break down periodically and bradyzoites differentiate back into tachyzoites, which renews the pathogenic cycle (recrudescence) and is the basis of chronic disease in immunocompromised individuals [3]. Alternatively, bradyzoites are released from tissue cysts when ingested by a new animal host and differentiate into a tachyzoite to begin the intermediate life cycle. The location and number of tissue cysts in animals differ with respect to host and *T. gondii* strain [5]. In mice and rats, tissue cysts were more prevalent in the brain [5] whereas higher mammals present more cysts in muscular tissues [5].

Within the feline host, bradyzoite development follows the definitive life-cycle [2]. Bradyzoite invasion of feline enterocytes results in differentiation into merozoites that replicate for several cycles prior to forming gametocytes. The ensuing processes of gamogony and oocyst formation take place within the epithelium of the small intestine. After fertilization of a macrogametocyte by a microgamete, the zygote forms within an oocyst that is passed into the intestinal lumen and shed in the feces. The zygotes (the only diploid stage) in the oocyst undergo meiosis to form eight haploid sporozoites, which are subcompartmentalized into two groups of four, each group is contained in a sporocyst. Sporozoites and tachyzoites ingested by a feline must first develop into bradyzoites prior to initiation of the definitive life cycle. Thus, the incubation period prior to oocyst shedding is characteristic of which developmental stage is present in the inoculum. Oocysts are shed 3 to 10 days after ingesting tissue cysts, >13 days after ingesting tachyzoites and >18 days after ingesting oocysts [3].

Disease and clinical relevance of *Toxoplasma* infections

A relatively small number of Apicomplexa species are responsible for the diseases of medical and veterinary significance (Table 1-1) [6-7]. Examples include *Plasmodium spp.,* which cause malaria, *Eimeria spp.*, which cause coccidiosis in animals, and *Toxoplasma gondii* which causes toxoplasmosis [2]. Toxoplasmosis in healthy, immunocompetent humans is common yet clinical presentation is often inapparent or misdiagnosed. Toxoplasmosis can be categorized into three states; acute, sub-acute and chronic. In the acute state (caused by tachyzoite parasitaemia), the most common symptoms are swollen lymph glands, associated fever headaches, and anemia, all of which are symptoms typically confused with influenza [2-3,8]. The sub-acute stage is characterized by formation of cysts in tissues such as liver, heart, brain and eyes [2-3,8]. The recrudescence of the tissue cyst (bradyzoite) back into the proliferative tachyzoite leads to a chronic infection. A number of factors influence the severity of disease symptoms including pathogen strain, susceptibility and age of the host, and the state of acquired immunity within the host.

ORGANISM	DISEASE	AFFLICTS
Toxoplasma	Toxoplasmosis	All warm-blooded animals, birds (30+ spp.)
Plasmodium	Malaria	Humans and Animals
Sarcocystis	Sarcocystosis	Humans, Mammals, Birds and Reptiles
Eimeria	Coccidiosis	Mammals and Birds
Cyclospora	Cyclosporiasis	Humans
Isospora	Isosporosis	Humans and Animals
Cryptosporidium	Cryptosporidiosis	Humans and Animals
Babesia	Babesiosis (redwater fever)	Cattle, other animals, man (rare)
Theileria	Theileriosis (East Coast Fever)	Cattle

Table 1-1: Apicomplexan diseases of medical and veterinary significance [6,7].

T. gondii infection within healthy individuals in the United States is thought to occur most commonly through the consumption of contaminated meat products, although the relative contribution of contaminated food versus environmental sources remains an open question [3-4]. Tissue cysts are present in edible tissues of approximately 30% of sheep and swine in the United States [2,9-11]. Outside of Europe and North America, the prevalence of infected animals increases to >50% (ex. in pigs) thereby leading to high rates of infection in some countries [3]. A higher percentage of food source infections have been found in populations of individuals within France, Panama and the continent of Africa where the seropositive rate can exceed 65% [4]. These findings support the hypothesis that crowded living conditions and cultural/eating habits contribute to the wide range of seropositivity among various populations [3].

Documented epidemics of oocyst infection have occurred raising concerns that environmental contact with oocysts is a threat to urban populations [12-14]. Two cases in North America have provided the foundation for this concern. At a riding stable in Atlanta Georgia 43% of individuals exposed to an oocyst source became seropositive [15]. In a case in Vancouver, Canada, 110 individuals presented with *T. gondii* infections as a result of contaminated water from a municipal water source [12]. In this local epidemic, it was estimated that several thousand individuals were exposed to contaminated water. High seropositive rates are seen in farm workers, gardeners [4], and a vegetarian life-style does not seem to minimize the risk of exposure [4] suggesting environmental transmission is a significant cause of toxoplasmosis.

The unborn and the immunocompromised are at significant risk for acquiring a *T. gondii* infection. The symptoms presented by these two groups include encephalitis, sepsis syndrome/shock, myocarditis, hepatitis and retinochoroiditis [3]. Congenital toxoplasmosis usually occurs when the mother acquires a primary *T. gondii* infection during pregnancy and may result in an abortion, neonatal death or fetal abnormalities. Vertical transmission occurs in mothers previously exposed to *T. gondii* who enter into an immunocompromised state during pregnancy either through the use of steroids, systemic lupus erythematosus or an HIV infection [3]. It has been suggested that in these women, the reactivation of a latent infection (recrudescence of the bradyzoite) resulted in transplacental transmission [3].

Children who survive a prenatal infection may have a significantly reduced quality of life with defects in vision, hearing, and central nervous system (CNS) deficiencies [3]. The frequency of prenatal infection varies from 1 to 100 per 10,000

births [3]. The risk of an interuterine infection increases during pregnancy but the most severe manifestations of toxoplasmosis occur when transmission comes at an early stage of pregnancy. In some states of the U.S.A. and various European countries (Austria, Italy, Denmark, Poland and France) programs have been initiated that are aimed at early detection of prenatal infection in neonates [3]. The prophylactic use of drugs on women of childbearing age is an unreasonable proposal. Through the implementation of effective screening programs, mothers can be treated with pyrimethamine and sulfadiazine providing an *in utero* therapy for the fetus. These drugs have been shown to decrease the severity of symptoms in infected children. More encouraging news comes from a study in which pregnant women who showed evidence of a recently acquired infection exhibited an ~40% decrease in the number of prenatal infections when given spiromycin during pregnancy [3]. Additionally, spiromycin has been shown to decrease the percentage of fetuses with severe disease or intrauterine death from 11 to 3% [3].

In immunocompromised individuals, either a primary infection or a previously acquired latent infection can lead to severe complications [3,16]. Encephalitis is the leading affliction in immunocompromised individuals infected with *T. gondii* [3,16]. In AIDS patients, the unique ability of bradyzoites and tachyzoites to interconvert is considered to be the underlying cause of *Toxoplasma*-induced encephalitis [16]. Consequently, AIDS patients are treated prophylactically with a combination of trimethoprin/sulfamethoxyzole which is often not well tolerated. Prophylactic treatment for this pathogen along with recent advances in antiretroviral therapies and immune reconstitution has reduced the incidence of CNS toxoplasmosis in AIDS patients [3,16].

Apicomplexan Biology

Genetics

Apicomplexan parasites have a wide range of genome sizes (8 Mb to >200 Mb). The genome of *Cryptosporidium parvum* is 8 Mb, *Plasmodium falciparum* 30 Mb, *Eimeria* spp. ~60 Mb and *Sarcocystis cruzi* may have the largest genome at 200 Mb [17]. *T. gondii* posseses two organellar genomes (apicoplast, ~35 kb and mitochondria, ~7 kb) and a nuclear genome estimated to be ~80 Mb (haploid) partitioned into at least 11 chromosomes ranging in size from 1.9 Mb to greater than 10 Mb [18]. Based on genome size and a rudimentary knowledge of intron/exon abundance, it is estimated that Apicomplexan genomes may contain as many as 10-15,000 genes [J. Ajioka, personal communication]. This is likely an overestimate, particularly for the small *Cryptosporidium* genome, although there appear to be few introns in this unusual member of the Apicomplexa [M. Abrahamsen personal communication]. *T. gondii* nuclear DNA has a 55% GC content as compared to *Plasmodium* spp., which have a 20% GC content [16]. Unlike other coccidian parasites, *T. gondii* codon usage is relatively unbiased [17].

Phylogenetic analysis indicates that three genotypic lineages of *T. gondii* account for most of the strains found in the world [21]. The RH strain (Type I) is the most extensively used strain for experimental and genetic manipulations [22]. Type I strains have an essentially identical genotype leading to the hypothesis that they are derived from a single clonal lineage [23]. Type I strains have a reduced ability to undergo the definitive life cycle in cats and are highly virulent in mice as opposed to Type II and III

strains which are comparatively avirulent but are capable of efficiently initiating the definitive life cycle in the feline. The laboratory strain ME49-Plk represents the Type II variant most often identified within HIV patients that present with acute *Toxoplasma*-encephalitis [21]. The Type III strain is most commonly found in livestock and is the second most common strain found in HIV patients. Interestingly, karyotype comparisons between the three major clonal lineages show very little chromosome size variation as compared to other Apicomplexa. The karyotype of RH strain, for example, has remained virtually unchanged in more than 50 years of culture [18,23-24]. T. gondii isolates can be divided into two virulence classes based on experiments in mice. The 50% lethal dose (LD_{50}) for most strains is dose dependent (requires 10^2-10^5 parasites) [25]. The acutely virulent Type I strains cause mortality at a very low parasite inoculum (LD_{50} <10^2) [25].

Three Apicomplexan genomes have been sequenced; *P. falciparum*, *C. parvum* and *Theileria parvum* [26-28]. The combined efforts of an EST project and a recently funded genome sequencing project have begun to provide resources for *T. gondii* [29-31]. The current *Toxoplasma* EST database includes ~3200 ESTs derived from sporozoite cDNA libraries (1262 unique), 17,000 tachyzoite ESTs representing ~6600 unique sequences and ~3300 ESTs obtained from bradyzoite cDNA libraries which represent 1289 unique sequences.

T. gondii has proven to be an important Apicomplexan model due to the relative ease of *in vitro* genetic manipulations in the tachyzoite stage. More than 50% of parasites express transiently after electroporation and non-homologous integration of DNA can occur in 1/20 viable parasites (RH strain) transfected using one of several selectable markers; hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT),

chloramphenicol, pyrimethamine, or phleomycin [32-35]. *Toxoplasma* expression vectors with a variety of promoter strengths, fluorescent markers (CFP, YFP, GFP, RFP), epitope tags, and targeting sequences are now available [36]. Using fluorescent markers, proteins associated with the cytoskeleton [36], the nucleus [37], or targeted to specialized organelles including the plastid [36,38-39] have all been characterized. Antisense protocols have been used to study the functions of nucleoside triphosphate hydrolase [40] and HXGPRT [41]. Recently a rudimentary tetracycline-inducible system was described [42].

The high level of non-homologous integration in *Toxoplasma* allows for a large portion of the parasite genome to be marked [43]. Through genomic tagging by insertional mutagenesis, a gene can be identified whose inactivation is not lethal and for which a suitable selection screen is available [43-45]. Initial studies of gene function and expression rely on the use of cDNA sequences (typically <2kb) that typically incorporate into the genome by non-homologous recombination. Larger fragments of genomic DNA can switch the targeting from a non-homologous to homologous type of integration. Using genomic sequences >8kb, of which ~ 4 kb flank the selectable marker, has resulted in ~50% of integrations occurring by homologous recombination. Examples of homologous integrations generating gene replacements include: dihydrofolate-reductase (DHFR) [46], HXGPRT [47], bradyzoite antigen 1 [48], adenosine kinase [49], uracil phosporibosyl transferase [50], rhoptry protein 1 [33] and dense granule protein two (GRA2) [51]. Until recently, the major deficiency in the *Toxoplasma* genetic "tool-kit" was a robust forward genetic model for complementation. An episomal library containing *T. gondii* genomic fragments [52] was unsuccessful due to the high integration

rate. A new strategy has now been developed that combines phage recombination [53] with the high level of stable integration in the tachyzoite [See Chapter #5 and 54].

Cell Biology of Growth

Apicomplexan parasites undergo three types of nuclear/cellular division. Schizogony results when multiple nuclei are formed by alternating rounds of DNA synthesis (S) and mitosis (M) ((S-M-ND)n–C-G1) (representative organisms: *Eimeria callospermophilli* and *T. gondii* merozoites) [55, Speer unpublished observations]. Budding of daughter parasites (cytokinesis - C) is delayed until the last round of nuclear division. In endopolygeny ((S–M)n–ND/C–G1) (representative organisms: *Sarcocystis tenella* and *Plasmodium berghei*), multiple rounds of DNA replication occur in the absence of mitosis, thereby generating a single polyploid nucleus that subsequently undergoes multiple mitotic events in conjunction with concerted parasite budding [56-57]. Endodyogeny, the mode of division of the *T. gondii* tachyzoite, has long been considered a binary form of schizogony [58-59]. In endodyogeny (S-M/C-G1), DNA synthesis is followed by mitosis and parasite budding, which results in a process that is indistinguishable from the final cycle of schizogony.

Interestingly, the scale, if not the number, of schizogonous nuclear divisions appears to be predetermined in many of the Apicomplexa. For example, *Eimeria bovis* sporozoites invariantly produce very large schizonts (with >100,000 first-generation merozoites) [60], while sporozoites of *Cryptosporidium parvum* only form small schizonts that produce 6-8 merozoites [61]. In each case the relative size of the schizont

is predetermined and obligatory to further development. Thus, it is evident that growth control can directly affect the course of parasite development. The idea that growth control and development are linked was recently explored in *T. gondii*, and it was established that tachyzoites emerging from sporozoite-initiated infections undergo a rapid growth for 20 divisions, followed by a spontaneous shift to a slower growth rate that precedes differentiation into the bradyzoite [62]. This growth shift occurs at least 24 h prior to the expression of bradyzoite markers.

The structural features of parasite budding in *T. gondii* have been examined in detail [58] by electron microscopy. Budding is first recognized by division of the Golgi and by the appearance of conoid structures (apical complexes) in the tachyzoite cytoplasm. These structures appear prior to nuclear division and are almost always anterior to the nuclear envelope [63]. As budding continues, two inner membrane complexes emerge and extend posteriorly from each of the central apical complex structures in order to enclose their half of the mother cell organelles. Into each immature daughter parasite a divided nucleus migrates while the inner membrane complexes continue to expand. Ultimately, the daughter cells consume most of the mother cell, although they may remain joined posteriorly (rozetta) for several divisions.

Recently, the features of tachyzoite endodyogeny have been placed within the framework of the eukaryotic cell cycle. The tachyzoite cell cycle is unusual in that it is comprised primarily of a G1 and a bimodal S-phase [37,64]. Tachyzoite division can be partitioned into ~60% G1, ~30% S phase and ~10% G2+M phases [37,64]. A significant fraction of tachyzoites possess a 1.7-1.8 N DNA content, indicating that DNA synthesis may be unequal with ~20% of the genome replicating just prior to entering mitosis. This

cell cycle feature remains to be confirmed; however, it is noteworthy that gametocytes of *Toxoplasma* and *Plasmodium* spp. appear to pause or slow chromosome replication (1.7 N) prior to invasion of the next host cell where chromosome replication is completed [17, 65-66]. In this regard, the *Plasmodium* microgametocyte is remarkable, as chromosome replication is interrupted indefinitely until the gametocytes move into the insect host at the time of a blood meal. Whether this pause in DNA synthesis represents a novel cell cycle checkpoint remains to be determined.

The subsequent chapters describe work in *T. gondii* which has provided insight into the mechanisms involved in the linkage between growth and development. The hypothesis for chapters 2 and 4 is as follows: Proliferating cell nuclear antigen (PCNA) is an essential co-factor of DNA replication and repair in eukaryotes, and is present as a single gene. Apicomplexa have two PCNAs and we hypothesize that one PCNA is essential for DNA replication and the second PCNA functions in repair. The initial characterization of these two genes was reported in Guerini et al. [67] and appears as chapter 2 in this thesis. The work presented in chapter 3 begins with experiments that use TgPCNA1 and 2 as a marker for proliferation and this work is based on the hypothesis that a cell cycle switching mechanism is involved in differentiation. Further work in this chapter characterizes the recrudescent pathway. Chapter 5 describes work using a forward genetics approach [54] to complement a loss-of-function mutant in *Toxoplasma*. This work was performed to prove that a forward genetics approach to complementing temperature sensitive (*ts*) tachyzoite mutants was functional and that the Cyclin B pathway might play a role in the defect associated with *ts*11C9 [68].

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CHAPTER 2

TWO GENES ENCODING UNIQUE PROLIFERATING-CELL-NUCLEAR-ANTIGENS ARE EXPRESSED IN *TOXOPLASMA GONDII.*

Introduction

The haploid genome of *Toxoplasma gondii* consists of 80 mb partitioned between 11 chromosomes [1]. Little is understood about *T. gondii* chromosome structure or the mechanisms of chromosome replication, although a low-resolution genetic map [2-3] based, in part, on expressed-sequence-tags has been generated for this parasite [4]. What is known about *T. gondii* division derives primarily from decades old ultrastructural observations [5]. Members of the phylum Apicomplexa, to which *T. gondii* belongs, replicate by simple binary division (endodyogeny) or by multinuclear processes (schizogony) that either involve multiple rounds of S and M phase prior to cytokinesis or the synthesis of polyploid chromatin followed by a single, multifocal mitosis and cytokinesis [6]. Schizogonous mechanisms appear to govern *T. gondii* multiplication in its definitive feline host, whereas in the intermediate host, asexual replication of the tachyzoite is exclusively by endodyogeny [6]. Rapid tachyzoite division is closely associated with virulence, which parallels reports that virulent isolates have elevated DNA polymerase activity [7]. This observation is potentially important to understanding acute disease caused by Type-I virulent strains [3]; however, further work is needed in

order to identify specific components of the DNA replication machinery that may be linked to virulence.

Proliferating Cell Nuclear Antigen (PCNA) is a ubiquitous co-factor of eukaryotic DNA synthesis and repair [8-9]. Attempts to generate ▲PCNA diploids in yeast have been unsuccessful, demonstrating this protein is essential for cell survival and implying a critical role in DNA replication [10-11]. PCNA is thought to specifically function by increasing the processivity of DNA polymerases δ and ε and in this way possesses a similar function to the β-subunit of the *E. coli* DNA polymerase III holoenzyme and the product of gene-45 of bacteriophage-T4 [12]. Animal and yeast PCNAs are relatively small proteins (~30 kDa) that are thought to function as homotrimeric ring structures. Assembly of the PCNA ring on chromosomes appears to be initiated by the binding of replication factor-C, which then recruits PCNA and polymerase δ to form an active replication complex [13-15].

Dramatic increases in PCNA levels have been shown to occur as animal cells enter S phase following a period of growth arrest [16]. Although originally thought to be S-phase specific, it is now accepted that PCNA levels marginally increase (~2-fold) in cycling animal cells and is more accurately considered an indicator of cell cycle entry from a G_0 state [8,16]. In *Plasmodium falciparum,* PCNA expression is tightly regulated during intraerythrocytic development [17]. Early, uninucleate ring stages (G1 phase) contain little, if any, PCNA mRNA or protein. PCNA induction is first detected in the late ring stage and is maximally expressed in trophozoites. This progression correlates with the onset of parasite DNA replication in the intraerythrocytic cycle, and thus, PCNA expression is also observed in schizonts undergoing mitosis and cytokinesis [17-18].

These studies illustrate the value of PCNA as a marker to delineate the periods of the cell cycle during the developmental transitions of other apicomplexan parasites. In this chapter, we describe the cloning and expression of two unique PCNA genes in *T. gondii*.

Materials and Methods

Cell culture and parasite growth

Human foreskin fibroblasts (HFF) were grown in Dulbecco's Modified Eagle Medium (Gibco BRL, Grand Island NY) supplemented with either 10% (v/v) newborn calf serum or fetal bovine serum (Hyclone Laboratories Inc., Logan UT). *T. gondii* tachyzoites of three strains: RH (Type I), ME49-PLK (Type II) and VEG (Type III) were maintained by serial passage in HFF cells according to standard methods [19-21]. Tachyzoites were purified from host cell monolayers by filtration through 3.0 μM Nucleopore filters (Costar, Cambridge, MA).

PCNA cDNA Cloning

Initial clones were obtained for TgPCNA1 by using reverse-transcription/PCR and degenerate oligonucleotide primers to conserved N-terminal (-Y/FRCDR-) and C-terminal (Y/FLAPK) PCNA sequences. Preliminary TgPCNA2 sequence information was discovered by examination of the *T. gondii* EST database (TgESTzy03b07.r1) [4,22]. Complete sequences for each of the TgPCNAs were then isolated by screening a λ ZAPII