Developmental gene expression in Eimeria bovis : characterization of stage specific genes of sporozoites and merozoites
by Timothy Griffin Clark

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

Abstract:
Eimeria bovis, a causative agent in bovine coccidiosis, is responsible for several hundred million dollars in losses in beef and dairy production annually. The greatest loss is due to morbidity during acute infection. Although anticoccidials have been used for over 20 years, their efficacy has been limited and resistant strains of coccidia are emerging. To better understand the biochemistry of eimerian parasites, which will hopefully lead to new insights for prophylaxis and treatment, we have undertaken the study of developmental gene expression in Eimeria bovis. We have constructed cDNA libraries from both developing sporozoites and merozoites of E. bovis and have isolated cDNAs by differential screening or expression screening. From these screens we isolated cDNAs that were either homologous to previously identified genes or showed no homology to any known genes. Further the cDNAs fell into 5 classes based upon their patterns of mRNA expression during sporulation. Two of the cDNAs, MZ 2.5 and Eb25/50, were selected for further characterization. MZ 2.5, the E. bovis homolog of heat shock protein 90, is highly developmentally regulated with its mRNA being expressed in sporozoites, throughout merogony, and in merozoites. MZ 2.5 is, however, not expressed during sporulation, in contrast to other known hsp90s that are constituatively expressed. Additionally we have identified a refractile body associated protein that is highly developmentally regulated. In contrast to other refractile body proteins identified, which are expressed in the sporozoite and then quickly turned off during early merogony, Eb25/50 is expressed throughout merogony and down-regulated just prior to the release of merozoites indicating that Eb25/50 protein is likely necessary throughout merogony.

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APPROVAL

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This thesis has been read by each member of the thesis 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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Date 7/31/95

I dedicate this thesis to my parents, Jack and Peggy Clark, and Melanee Olson in thanks for their unending support.

ACKNOWLEDGMENTS

Materials contained within several chapters of this document were taken either completely or in part from three previously published manuscripts of which I was an author. Although I am not first author on these three papers, I did perform a significant role in the research contained therein and participated in writing and/or editing of each of the manuscripts. In Chapter 2 taken in part from the publication Developmental gene expression in *Eimeria bovis* by Abrahamsen, Clark, Mascolo, Speer and White, my specific contributions were as follows 1) the isolation of the RNA for and the construction of both partially-sporulated oocyst and merozoite cDNA libraries; 2) differential screening of the merozoite cDNA library; 3) assisting with the differentially screening of the partially-sporulated oocyst cDNA library; 4) subcloned all merozoite specific cDNAs; 5) assisted in the Northern blot analysis of the merozoite cDNAs; 6) participated in sequencing of all merozoite cDNAs; and 7) conducted the computer analysis of all partially-sporulated oocyst and merozoite cDNAs.

Also contained in Chapter 2 is data taken from the manuscript entitled An improved method for isolating RNA from coccidian oocysts by Abrahamsen, Clark and White published in the Journal of Parasitology. In this project I participated in all aspects of the research but focused primarily on the oocyst disruption techniques. Finally, in Chapter 4 data was included from the manuscript Developmental regulation of an *Eimeria bovis* mRNA encoding refractile body-associated proteins by Abrahamsen, Johnson, Clark and White published in the journal Molecular and Biochemical Parasitology. For this project I 1) developed the recombinant protein constructs; 2) expressed the recombinant proteins; 3) raised and isolated the polyclonal antisera, and 4) participated in the screening of the antisera by Western blot analysis.

The findings presented in this document would not have been possible without the cooperation of many individuals who either worked directly with or advised me in the pursuit of this project. First I would like to thank my advisor Michael White, not only for

his valuable advice, but also for his recreational diversions that kept me sane during my time here. Similarly I would like to thank Mitch Abrahamsen for his helpful insight and also for the opportunity for recreational diversions. I would like to thank the members of my committee Mark Jutila, C.A. Speer, Bruce Granger, and Larry Jackson for their guidance during this project. I would also like to acknowledge the people that have provided technical assistance during this project; Rhonda Johnson and Cheryl Lancto helped with many experiments and performed most of the parasite purifications, Maria Jerome was involved in parasite production and recently provided technical assistance; James Thompson and Kerry Rask cared for the animals and assisted with parasite production; Patrice Mascolo was involved in the initial isolation of developmentally regulated cDNAs; John Smart provided technical assistance with antibody production; and many others too numerous to name who helped in various aspects of the project. To all who helped in the project I am indebted and I thank you.

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ABSTRACT

Eimeria bovis, a causative agent in bovine coccidiosis, is responsible for several hundred million dollars in losses in beef and dairy production annually. The greatest loss is due to morbidity during acute infection. Although anticoccidials have been used for over 20 years, their efficacy has been limited and resistant strains of coccidia are emerging. To better understand the biochemistry of eimerian parasites, which will hopefully lead to new insights for prophylaxis and treatment, we have undertaken the study of developmental gene expression in *Eimeria bovis*. We have constructed cDNA libraries from both developing sporozoites and merozoites of *E. bovis* and have isolated cDNAs by differential screening or expression screening. From these screens we isolated cDNAs that were either homologous to previously identified genes or showed no homology to any known genes. Further the cDNAs fell into 5 classes based upon their patterns of mRNA expression during sporulation. Two of the cDNAs, MZ 2.5 and Eb25/50, were selected for further characterization. MZ 2.5, the *E. bovis* homolog of heat shock protein 90, is highly developmentally regulated with its mRNA being expressed in sporozoites, throughout merogony, and in merozoites. MZ 2.5 is, however, not expressed during sporulation, in contrast to other known hsp90s that are constituatively expressed. Additionally we have identified a refractile body associated protein that is highly developmentally regulated. In contrast to other refractile body proteins identified, which are expressed in the sporozoite and then quickly turned off during early merogony, Eb25/50 is expressed throughout merogony and down-regulated just prior to the release of merozoites indicating that Eb25/50 protein is likely necessary throughout merogony.

CHAPTER 1

INTRODUCTION

Impact of Protozoan Diseases

Protozoan parasites account for some of the most devastating diseases both in terms of mortality and morbidity in humans, with over 600 million people affected each year [1]. Additionally, protozoan parasites cause enormous economic losses in agriculture. A phylum of protozoa that contains genera responsible for some of the most widespread and costly diseases is the Apicomplexa (also known as Sporozoa). Apicomplexans comprise a diverse group of parasites infecting a variety of definitive and intermediate hosts; however, all members of this phylum possess a characteristic apical complex in certain developmental stages. The Apicomplexa contain four classes, Gregarinea, Piroplasmea, Haemosporidiea and Coccidea [2]. The class of Gregarinea is composed of three orders of parasites, which infect mainly invertebrates. Piroplasmea contains two orders of parasites and includes the genus *Theileria* which is responsible for severe lymphoproliferative disease in both man and animals [3]. Haemosporidiea, the most studied class of Apicomplexans, includes the *Plasmodium* species which are responsible for malaria in both man and animals. It is estimated that 500,000 children die each year as a result of infections with *Plasmodium* species [4]. Finally, the class of Coccidea contains four orders that include the medically important genera *Toxoplasma* and *Cryptosporidium*, responsible for serious secondary infections in immunocompromised patients, and the economically important genus, *Eimeria*.

Eimeria bovis, in addition to *Eimeria zuernii* and *Eimeria auburnensis,* are the principal species responsible for bovine coccidiosis, an important disease affecting beef production in the United States. It is estimated that over 70,000,000 beef and dairy animals are exposed each year to *Eimeria* species, resulting in an annual economic loss of several hundred million dollars due to weight loss and death [5]. Coccidiosis is generally observed in calves following weaning, with infected animals exhibiting bloody diarrhea, dehydration, and morbidity [6]. The disease is self-limiting, with animals becoming immune to further infections after recovery from the disease.

Life Cycle of *Eimeria bovis*

Eimeria bovis is an obligate intracellular parasite that develops through a series of morphologically distinct extracellular stages characteristic of coccidians: unsporulated oocyst, sporozoite, merozoite, and gametocytes [7]. The life cycle begins with the ingestion of a fully-sporulated oocyst by the bovine host. Fully sporulated oocysts of *E. bovis* possess four sporocysts, each of which contains two sporozoites. The oocyst of *E. bovis* is ovoid in appearance and is typically 27-29 μm by 20-21 μm [8], distinguishing it from the oocysts of *E. zuernii* (15-18 x 15-18 μm) and *E. auburnensis* (36-41 x 22-26 μm). Upon ingestion of an oocyst, the sporocysts are released; through the action of trypsin (which acts on the Steida body at the apical end of the sporocyst) and bile salts, the sporozoites then exit from the sporocysts. Once the motile sporozoites are free from the sporocysts, they migrate to the endothelial cells of

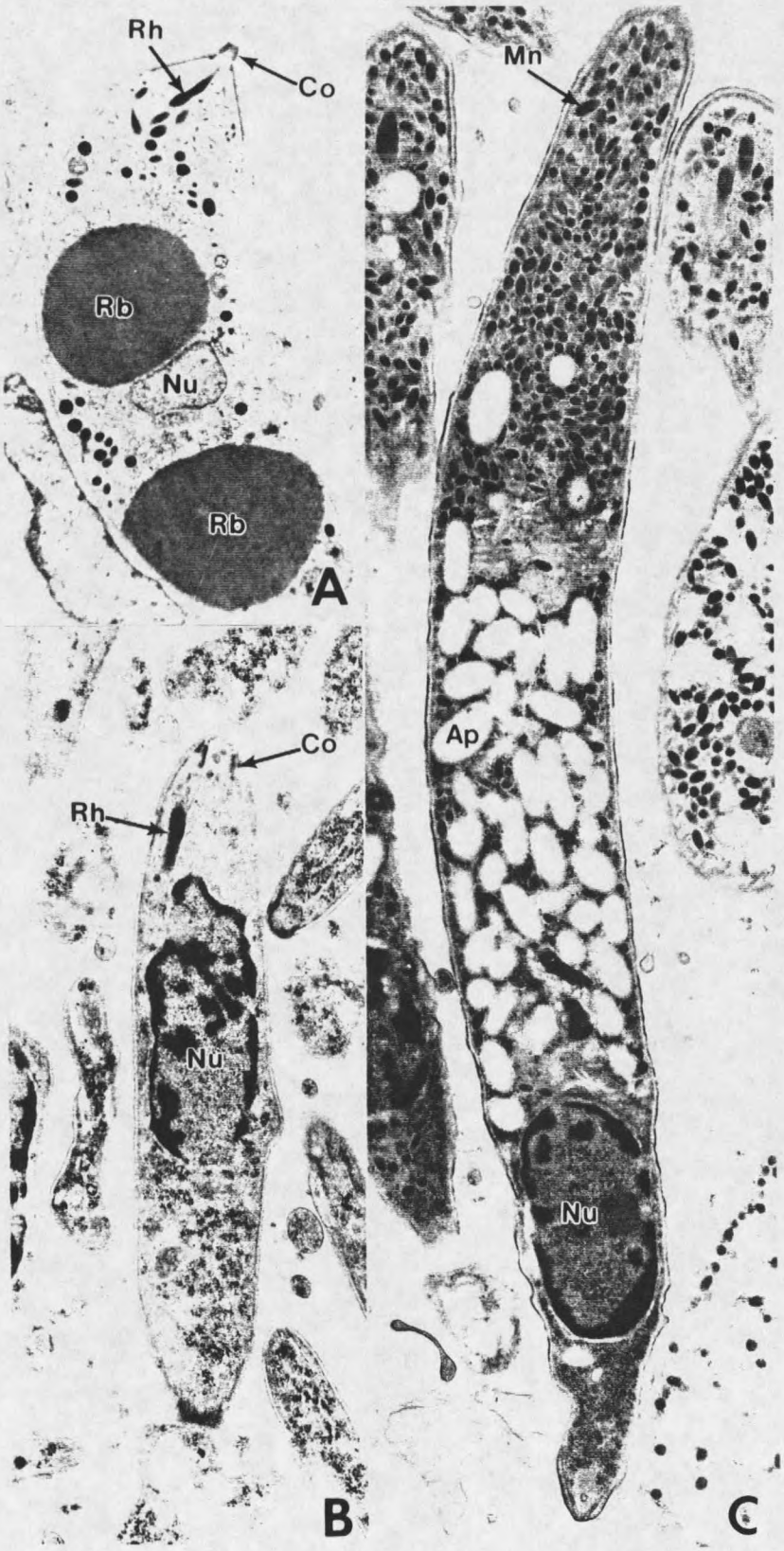
the central lacteals in the terminal ileum. The sporozoite penetrates the endothelial cell and is contained in a parasitophorous vacuole surrounded by the parasitophorous vacuolar membrane (PVM). In the endothelial cells, the sporozoites replicate by merogony, where a single sporozoite develops into over 100,000 first-generation merozoites; a process that requires ≈14 days [9]. During merogony the sporozoite undergoes DNA replication until over 100,00 nuclei are formed. The nuclei are found initially arranged peripherally around the meront after which they migrate inward forming compartments that give rise to blastophores. Each blastophore has a single layer of nuclei around the periphery. Merozoites are formed as radial outgrowths around individual nuclei and bud from the blastophore within the meront. Two types of *E. bovis* first-generation merozoites have been observed to be released from *in vivo* meronts [10]. Type I merozoites are large, crescent-shaped and highly motile with a posteriorly located nucleus. Type II merozoites are small, spindle shaped and relatively immobile with a centrally-localized nucleus. The type I merozoites contain many more micronemes and amylopectin granules than type II merozoites and are capable of penetrating cultured cells whereas type II merozoites cannot. Although the role of these two types of merozoites is unknown, it is thought that they may play very different roles in further infection.

The release of these two types of merozoites ruptures the host cell and the merozoites then migrate, through an unknown mechanism, to the epithelial cells within the crypts of the large intestine and cecum. Once in the epithelial cells, the merozoites

undergo another round of merogony, after which the second generation merozoites enter the sexual phase. During the sexual phase the merozoites either develop into micro- or macrogametes. After the microgametes are formed they are released and penetrate the cells containing the macrogametes where they fuse with the macrogametes to form the diploid zygote. This feature of the coccidian life cycle indicates that a single haploid sporozoite is capable of forming both gametes. The oocyst containing the zygote then ruptures the host cell and is released and shed with the feces. Once outside the host the oocyst begins the process of sporulation developing into the sporocysts that contain the sporozoites. The interval from oocyst ingestion to oocyst shedding is approximately 18-23 days.

Although the tissue sites and cell types in which the various stages of *E. bovis* develop are well defined, the biochemical mechanisms responsible for this specificity are unknown. It has been proposed that *E. tenella* sporozoites may enter lymphocytes and remain dormant over extended periods of time [11]. Additionally, sporozoites and merozoites may use the lymphocytes to rapidly transit from one site of development to another, although these models remain largely unproven.

Figure 1. **See Next Page.** Transmission electron micrographs of *Eimeria bovis*. **A.** Sporozoite in cultured bovine monocyte; Co, conoid; Nu, nucleus; Rb, refractile body; Rh, rhoptry X5,500. Provided by C.A. Speer. **B.** Type II first-generation merozoite; Co, conoid; Nu, nucleus; Rh, rhoptry. X18,000. **C.** Type I first-generation merozoite; Ap, amylopectin granule; Mn, microneme; Nu, nucleus. X18,000. Figures B and C from Speer, 1988 [10].


Rh
Co
Rb
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Rb
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C

Host-immune response to eimerian infections

Several key observations have greatly increased our knowledge of the host immune response to eimerian infections. Both the humoral and cellular arms of the immune system participate in the response against eimerian parasites, but the cellular response appears to be the most critical. Initial studies with avian *Eimeria* species have shown that some protection can be passively transfered by serum. Studies in B-cell-depleted animals (bursectomized and bursa-diseased chickens, Biozzi, low-responder, and CBA/N mice) have shown that, although these animals are less resistant to primary infection and slightly more susceptible to challenge than control animals, they retain the ability to resist a challenge infection [12]. These data indicate a contributory, but not fundamental, function for the humoral immune response.

T-cell mediated immunity appears to be much more crucial to the immune response against eimerian infections, although the function of the individual components is uncertain. Athymic mice and rats, upon initial infection, show more severe disease and no evidence of any resistance to challenge infections [13,14]. Depletion of CD8+ T-cells *in vivo* caused an increase in the oocyst production in *E. vermiformis* infected mice. However, depletion of CD4+ T-cells, *in vivo*, increased the severity of primary infection of *Eimeria vermiformis* in mice to a greater extent than that observed with CD8+ T-cell depletion [15]. Further *in vitro* depletion of CD4+ but not CD8+ T-cells collected from *E. vermiformis* immune mice prevented the transfer

of immunity to naive mice [16], indicating that protective immunity may be mediated by CD4+ T-cells. Thus it appears, as observed with B-cells, the role of CD8+ T-cells is contributory, but not fundamental. The role of CD4+ T-cells appears critical since the CD4+ cell is responsible for mediating aspects of both the cell mediated and humoral immunity. The important regulatory role played by CD4+ cells makes a definitive search for the effector cells directly responsible for immunity to eimerian infections extremely difficult.

CD8+ T-cells have, however, been shown to be present as a significant portion of the population of T-cells during the recovery phase of eimerian infection [17]. Recent studies have shown that the related coccidian, *Toxoplasma gondii,* can stimulate CD8+ T-cells in the context of major histocompatibility complex (MHC) class II independent of antigen processing, indicating a superantigen activity [18]. The stimulation results in proliferation and production of interferon γ by CD8+ T-cells. This observation is in contrast to other superantigens that stimulate CD8+ T-cells in conjuction with MHC class I. The response observed with CD8+ T-cells in *Eimeria tenella* infected chickens is consistent with the superantigen response observed with *T. gondii,* indicating the CD8+ response to eimerian infections might be superantigen-mediated. This is intriguing because most superantigen responses have deleterious effects upon the host.

One of the results of stimulation of T-cells is the release of cytokines directly or through T-cell stimulation of macrophages. Although there are likely many cytokines

produced in response to eimerian infections, interferon (IFN) γ appears to play a pivotal role. BALB/c mice that have been depleted of IFNγ by treatment with an anti-IFNγ antibody lose their ability to regulate the course of primary infections, which increases the oocyst output [19,20]. Further, IFNγ has been shown to inhibit the development of eimerian species in cell cultures [21]. This treatment does not, however, have an effect upon development of immunity. The immune response to eimerian infections, therefore, appears to utilize CD8+ T-cells, humoral immunity and the production of IFNγ, but relies strongly on the CD4+ T-cell response.

Vaccines Against Eimerian Infections

Vaccine development to prevent eimerian infections has met with limited success. Such development is hindered by a lack of knowledge of both the immune response against eimerian infections and biochemistry of eimerian development. The greatest concern with current vaccine use is that with respect to cost, no vaccine available is competitive with chemotherapy. Previous efforts have focused on the development of vaccines for poultry coccidiosis with two types of vaccine being pursued: attenuated live vaccines and subunit vaccines. To date, the only vaccines that are commercially available for immunization against coccidiosis are attenuated live vaccines directed against chicken *Eimeria* spp. [22]. These vaccines suffer from a lack of efficacy against the same species as evidenced by reports showing poor protection from a virulent coccidian strain by a vaccine developed from an attenuated line of a

different isolate of the same species [22,23,24]. Thus, the issue of strain variation makes the development of a live vaccine composed of multiple species and strains an enormous task. Although research on subunit vaccines is at a very early stage, there have been a number of reports on potential vaccine candidates. Protection has been reported against chicken *Eimeria* using refractile body proteins of *E. tenella* and *Eimeria acervulina* [25,26] and gametocyte antigens of *Eimeria maxima* [27] as immunogens. The most promising candidate, a refractile body protein isolated from *E. tenella* and encoded by the cDNA S07, has been shown to cross-protect against four species of *Eimeria*: *E. tenella*, *E. acervulina*, *E. maxima*, and *E. necatrix*. Interestingly, the immune response against the S07 protein appears to be cell-mediated, which is in agreement with the observation that cell-mediated response is necessary for immune protection from eimerian infections [28,29].

Biochemistry of Eimerian Development

Initial studies on the biochemistry of eimerian parasites occurred over 30 years ago, and although the difficulty in studying eimerian parasites has hindered our progress, some interesting and crucial observations have been made. Some of the research aimed at understanding the biochemistry of eimerian development has been initiated based on scientific curiosity, however, a great deal of emphasis has focused on the identification of biochemical pathways that might be exploited for drug targeting. Previous speculation had suggested that coccidian parasites are vastly different from

their hosts and lack many critical biosynthetic pathways making them dependent upon the host for important macromolecular precursors. However, Pfefferkorn [30], through the study of *T. gondii,* has recently proposed that coccidians lack only a very few biosynthetic pathways and their dependence upon the host cell is limited. This is supported by the observation that *T. gondii* can grow in enucleated cells, ruling out the possibility that *T. gondii* requires host cell DNA or RNA synthesis for its development.

Toxoplasma gondii, like other coccidian parasites, is fully capable of *de novo* pyrimidine synthesis [40] and furthermore does not appear to have access to the pyrimidine nucleotide pools of the host. *T. gondii* mutants deficient in pyrimidine salvage cannot utilize either UTP or TTP from the host [31]. Parasite *de novo* pyrimidine synthesis was the first metabolic target exploited to develop chemotherapies for treatment of coccidian caused diseases. Because folate is required for pyrimidine synthesis, and the parasites have folate metabolism enzymes similar to prokaryotic folate metabolism enzymes, inhibitors of prokaryotic folate metabolism, such as the sulfonamides, are used extensively for the treatment of coccidian infections [32].

It is also clear that coccidian parasites do not have access to ATP from the host cell. Metabolic labeling studies utilizing ATP that is double labeled with ^{3}H in the purine ring and ^{32}P in the alpha phosphate revealed that only the purine ring is incorporated into *T. gondii* tachyzoite RNA [33]. Additionally, host cells that lack functional mitochondria support the growth of these parasites. These data are not surprising since coccidian parasites have fully functional mitochondria and are capable

of both glycolysis and oxidative respiration [34]. This raises the question as to what energy source coccidian parasites scavenge from the host cell. All of the stages of *E. tenella* contain amylopectin (poly-glucose) which was thought to be the parasite's only carbohydrate reserve [35]. However, a unique pathway has been identified in coccidians in which mannitol is synthesized from glucose [36,37,38], a biochemical pathway that had previously only been identified in plants and bacteria. All four of the enzyme activities unique to the mannitol cycle have been found in cell-free extracts of oocysts, sporozoites and merozoites of *E. tenella*. It has been proposed that by synthesizing mannitol from glucose, the parasite removes glucose, decreasing the concentration and allowing more glucose to enter the parasite. Mannitol then serves as an energy reserve. Enzyme activities involved in the mannitol cycle have been shown to be developmentally regulated. Enzymes responsible for the synthesis of mannitol, mannitol-1-phosphate dehydrogenase and mannitol-1-phosphatase, are present in unsporulated oocysts, whereas the enzyme involved in mannitol utilization, mannitol dehydrogenase, is present at low levels, resulting in the accumulation of mannitol. The levels of these enzymes reverse during late sporulation, when mannitol levels decrease dramatically. The developmental regulation of the enzymes involved in mannitol metabolism, therefore, determines when mannitol is synthesized or catabolized. It is not known if the mannitol cycle is a common feature of all coccidians, or whether the enzymes are active at all stages of *E. tenella* development. However, because of the

uniqueness of this pathway, the mannitol cycle is being pursued as a potential drug target.

It is unclear if coccidian parasites require host-cell protein synthesis for their development. Studies indicate that *T. gondii* can grow in host cells that are temporarily deficient in protein synthesis. Drug studies in which host cells are treated and then washed free of muconomycin A, a nearly irreversible inhibitor of protein synthesis, reveal that the parasite grows normally and incorporates ^{3}H-leucine in these cells. Other experiments have demonstrated that host cells containing a thermolabile leucyl-tRNA synthetase support normal growth of *T. gondii* when grown at elevated temperatures. Thus, it appears that *T. gondii* can grow, at least temporarily, in a host deficient in protein synthesis.

Coccidian parasites are probably reliant upon their host cells for some lipids, although these pathways are not well explored. It is known that *E. tenella* and the related apicomplexan *Plasmodium berghei* are incapable of cholesterol synthesis, thereby relying on the host cell for this membrane component. One of the central questions of Apicomplexa biochemistry is the source of lipids in the parasitophorous vacuolar membrane (PVM). Pouvelle et al. [39] have recently demonstrated, using non-exchageable lipophilic dyes, that the PVM is derived from the host cell membrane in *Plasmodium falciparum*-infected erythrocytes. Further, dye localized to the PVM can become integrated into the parasite plasma membrane; however, this dye exchange is unidirectional, and once incorporated into the parasite plasmalemma does not transfer

back to the PVM. Therefore, it appears that the parasite must rely on host lipids to initially form the PVM and can then integrate the host lipids into its plasma membrane.

To date the only host biochemical pathway coccidian parasites are absolutely dependent on is purine biosynthesis. All parasitic protozoa that have been examined are unable to synthesize the purines *de novo* [40] and therefore must utilize classical purine salvage pathways to obtain purines from the host cell. Studies have shown that when host cells deficient in the enzyme responsible for transferring formate groups to developing purine rings are infected with *T. gondii*, ^{14}C-labeled formate is not incorporated into *T. gondii* purines indicating *de novo* purine synthesis is not occurring. The labeled formate is incorporated as the methyl group in parasite thymine indicating that formate is accessable to the parasite [30]. Further, studies with *E. tenella* unsporulated oocyst extracts have shown that amidophosphoribosyl transferase, which is the first enzyme in the *de novo* purine synthetic pathway, is absent [41]. To compensate for their lack of *de novo* purine synthesis, *E. tenella* parasites possess approximately 10-fold higher hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT, responsible for transferring the phosphorylated ribose groups to the purine rings) activity as compared to the host cell. Interestingly, HXGPRT, the enzmye of coccidian parasites differs from the enzyme homolog found in vertebrate host cells in that coccidian HXGPRT can utilize xanthine. The utilization of xanthine by coccidian parasites has recently been exploited to develop selection strategies for the introduction of DNA into *T. gondii*. Mutant *T. gondii* strains that are deficient in

HXGPRT can be transfected and selected for the presence of HXGPRT by the addition of mycophenolic acid, which blocks conversion of inosine monophosphate to xanthine monophosphate forcing the parasite to use xanthine. Thus, only HXGPRT+ parasites can grow under these conditions. Conversely, *T. gondii's* ability to utilize xanthine results in its susceptibility to 6-thioxanthine which can be used to select against HXGPRT+ cells.

The observation that coccidians cannot synthesize purine *de novo* raises the issue of how these parasites salvage purines from the host cell. The most likely candidate for salvage is ATP because of its high concentration in the host cell. However, recent studies have shown that *T. gondii,* because of the impermeability of the plasma membrane to nucleotides, salvages adenosine and adenine [42]. This conclusion is supported by the demonstration that NTPase is present in the parasitophorous vacuole and in contact with the PVM [43]. The NTPase, in association with a 5'-nucleosidase and an adenine transporter that has a higher K_m than mammalian adenine transporters, implies that adenine is the main source of purine for the parasite.

Considering the various attempts at rational drug design, it is interesting that the most successful group of anti-coccidials are the polyether ionophores. The ionophores have outperformed the drugs of all other classes [44] in their efficacy against coccidians. However, it has been speculated that the mode of action of ionophores is to act on the host-cell directly and the parasite indirectly. Wang [40] has postulated that the effect of oral administration of ionophores to eimerian infected

animals results in the breakdown of the ion balance of the host plasma membrane which results in the decreased import of carbohydrates needed by the parasite thereby killing the parasite. However, parasites resistant to monensin have been shown to be deficient in monensin transport, indicating that the effect is directly upon the parasite [45]. Tartakoff [46] has shown that monensin has the greatest effect upon the Golgi, where it inhibits protein trafficking and glycosylation. The effect of Golgi inhibition on parasite protein trafficking might result in the parasite being unable to synthesize the glycoproteins necessary for the apical complex.

Developmental Gene Expression in *Eimeria.*

Eimerian parasites undergo developmental processes that are precisely coordinated with very little variation observed in the duration of their life cycles. In order for these parasites to precisely regulate their development, one would expect tight control in the gene expression specific for each developmental stage. Several observations have been made that suggest that large changes in gene expression occur between the various developmental stages in eimerians. Ellis and Thurlby [47] have shown that the levels of translatable RNAs, as measured by *in vitro* translation, change significantly between un-, partially-, and fully-sporulated oocysts of *E. maxima.* Additionally, Hebert et al. [48] have isolated cDNAs that are expressed only in unsporulated oocysts. In the transition from sporozoite to merozoite during merogony, there are large differences in gene expression. Reduker and Speer [49] have shown that there are numerous differences in the species of proteins expressed in sporozoites and

merozoites of *E. bovis* indicating that although the two stages are similar morphologically (Fig. 1), there are major changes in gene expression during merogony. The differences in gene expression between sporozoites and merozoites may be as high as 5% [50]. Finally, Mencher et al. [51] have identified antigens by *in vitro* translation of gametocyte RNA from *E. maxima* that show different electrophoretic patterns than proteins produced by translation of oocyst RNA. These data indicate that major changes in gene expression occur between the various developmental stages of eimerian species.

To further investigate the mechanisms responsible for the developmental regulation of eimerian parasites, with the ultimate goal of identifying biochemical pathways that might be exploited for drug development, we have undertaken the study of developmental gene expression in these organisms. The focus of our studies is on the transition of eimerian parasites from the sporozoite to the merozoite. It is during this stage in *E. bovis* that the greatest biotic potential occurs; where a single sporozoite can develop into over 100,000 merozoites. We have used various techniques to isolate developmentally regulated genes of *E. bovis* sporozoites and merozoites and have characterized these genes based upon their patterns of spatial and temporal expression both at the mRNA and protein levels.

CHAPTER 2

ISOLATION OF DEVELOPMENTALLY REGULATED cDNAS FROM *EIMERIA BOVIS* SPOROZOITES AND MEROZOITES

Introduction

The coccidia belong to the phylum Apicomplexa and comprise a diverse group of intracellular protozoan parasites of vertebrates and invertebrates [8]. Coccidian life cycles are complex with certain species being homoxenous, while others are facultatively or obligatorily heteroxenous. Regardless of whether a species is homoxenous or heteroxenous, the coccidia are known to progress sequentially through a series of common developmental stages [8]. Although detailed ultrastructural descriptions of the developmental stages of some coccidians have been available for the past 20 years, little is known regarding the biochemical mechanisms that are critical for, or regulate the development of, coccidian parasites. Recently, a limited number of reports have identified genes and antigens that are developmentally regulated in coccidian parasites as described in chapter 1.

To identify specific changes in gene expression characteristic of the coccidian life cycle, we explored methods to rapidly isolate a large pool of developmentally regulated genes. We have used differential screening of stage-specific *E. bovis* cDNA

libraries to isolate genes that are regulated during development of sporozoites and merozoites. Northern-blot analysis of a limited number of cDNA clones identified a single class of oocyst-specific genes and 3 classes of genes expressed in merozoites, based on their patterns of mRNA expression during development. Several of these developmentally regulated cDNA clones display a high degree of identity to mammalian genes involved in protein synthesis and degradation.

Materials and Methods

Parasite Isolation

Oocysts were isolated from the feces of experimentally infected Holstein-Friesian calves as previously described [52]. Partially- and fully-sporulated oocysts were obtained by incubating unsporulated oocysts in 2.5% potassium dichromate at room temperature for 36 h and 72 h, respectively. Embryonic bovine tracheal cells (EBTr; ATCC #CCL44) were used for *in vitro* production of first-generation merozoites [52]. Un-, partially-, or fully-sporulated oocysts of *Eimeria bovis* in 2.5% potassium dichromate were washed in sterile deionized, distilled H_2O (ddH_20) and collected by centrifugation at 830 g. This process was repeated three times. The oocysts were sterilized with 50% Clorox (5.25% sodium hypochlorite) for 1 hr at room temperature. An equal volume of ddH_2O was added and the oocysts collected by centrifugation. The oocysts were then washed two additional times as above.

RNA Isolation

The oocysts were then resuspended in 3 ml of 4M guanidine isothiocyanate (GIT) containing 0.024 M sodium citrate, 0.005% (w/v) Sarcosyl, and 8% (v/v) 2-mercaptoethanol. The oocysts were either quick frozen in liquid N_2 in a Diamonite mortar (Fisher Scientific, Pittsburgh, PA) that had been precooled with liquid N_2 and ground for 30 min in the presence of liquid N_2 , or they were added to a French pressure cell (Aminco cat. no. FA-003, Urbana, IL), that had been precooled to -20 C, and disrupted at 20,000 psi. The lysates were then centrifuged to remove cell debris and processed by a modified version of Chomzynski and Sacchi [53]. Injection grade H_2O (Baxter Healthcare Corp., cat no. 2B0304, Deerfield, IL) was added to the samples to reduce the GIT concentration to 2.5 M. Sodium acetate (3 M), pH 4.0 was added to a final concentration of 0.18 M, followed by the addition of an equal volume of H_2O-saturated phenol and 0.2 volumes $CHCl_3$. The solution was mixed with a vortex for 1 min and the lysate incubated on ice for 15 min. The lysates were then centrifuged at 12,100 g for 20 min and the upper aqueous layer transferred to a fresh tube. An equal volume of isopropanol was added and the lysates precipitated for 1-2 hr at -20 C. The RNA was collected by centrifugation at 12,100 g for 20 min. The pellets were then redissolved in 2.5 M GIT containing 8% (v/v) 2-mercaptoethanol and precipitated with 2 volumes 100% ethanol at -20 C for 1 hr. The samples were