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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Timothy Griffin Clark

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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

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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

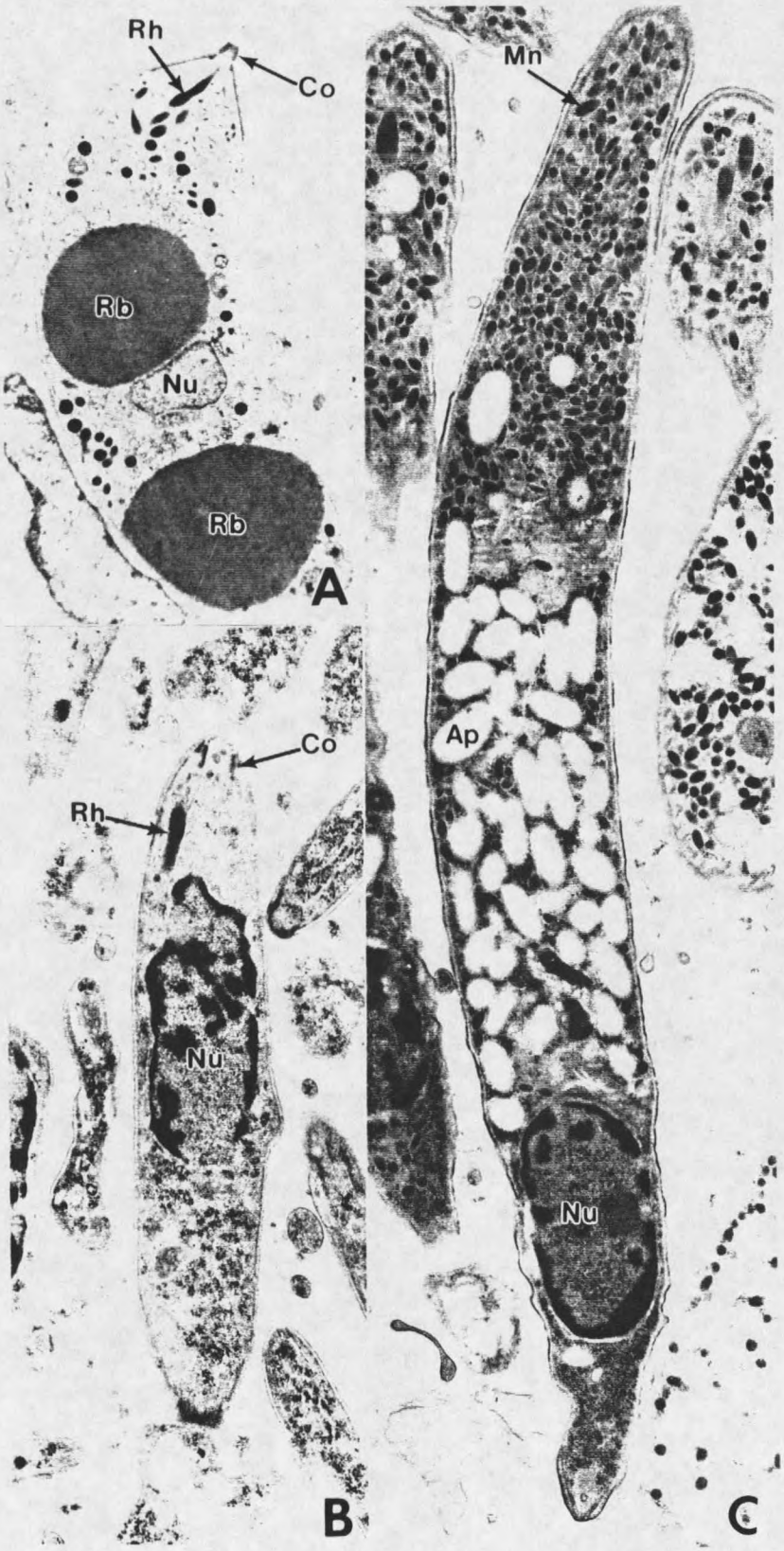
the central lacteals in the terminal ileum. The sporozoite penetrates the endothelial cell and is contained in a parasitophorous vacuole surrounded by the parasitophorous vacuolar membrane (PVM). In the endothelial cells, the sporozoites replicate by merogony, where a single sporozoite develops into 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
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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

centrifuged at 12,500 g for 45 min and washed in 70% ethanol. The pellet was then resuspended in injection grade H_2O. Merozoite RNA was prepared by lysing the merozoites directly in the 4 M GIT solution and mixing by vortex for 1 min. The sample was then processed as described for the oocyst lysates.

cDNA Library Construction and Screening

To isolate developmentally regulated cDNAs of *E. bovis*, libraries in λgt11 were constructed from mRNA isolated from oocysts sporulated for 36 hours (partially sporulated) and first-generation merozoites that had been produced in cell culture [54,55]. Total RNA prepared from each stage as described above [61] was enriched for poly-A+ RNA by oligo dT cellulose chromatography (Stratagene, La Jolla, CA) and double stranded cDNA was synthesized by the method of Gubler and Hoffman [56] using oligo dT for priming first-strand synthesis. Differential reciprocal screens [57] were performed using ^{32}P-labeled first-strand cDNA prepared from partially-sporulated oocyst poly-A+ RNA and first-generation merozoite poly-A+ RNA. Sixty thousand phage, (15,000 phage/150 mm plate) were plated and duplicate nitrocellulose lifts were made. The lifts were then hybridized overnight at 50 C in hybridization solution consisting of 6 x standard saline citrate (SSC) [58], 100μg/ml yeast tRNA, 50% formamide, 2 x Denhardt's solution and the ^{32}P-labeled cDNA probes at 6 x 10^5 cpm/ml. The nitrocellulose lifts were washed 3 times in 2X SSC, 0.1% (w/v) sodium dodecyl sulfate (SDS) for 20 minutes at room temperature followed by two washes in

0.1 X SSC, 0.1% SDS for 30 minutes at 55 C. The filters were then air dried and exposed to X-ray film at -70 C for 4-16 hours with an intensifying screen. Plaques that hybridized to the target probe and not the control probe were selected and the phage eluted overnight in SM buffer [58]. The selected phage were then rescreened until the phage were plaque pure. The selected inserts were then subcloned into the plasmid pBS-SK+.

Northern Blot Analysis

Northern blot analysis was performed using total RNA isolated from unsporulated, partially-sporulated, fully-sporulated oocysts, and first-generation merozoites produced *in vitro*. Briefly, total RNA was isolated from these stages as described above and 5μg of each was electrophoresed on 1.2% (w/v) agarose gels containing 6.7% (v/v) formaldehyde, 1 mM EDTA, 20 mM sodium phosphate, pH 7.0, and 5 mM sodium acetate. The RNA was transferred to nitrocellulose by capillary blotting using 20 x SSC. The blots were hybridized (1×10^6 cpm/ml) with gel-purified cDNA inserts that had been ^{32}P-labeled by nick translation to specific activities ranging from $2 - 4 \times 10^8$ cpm/μg DNA. The blots were then hybridized and washed as described for screening of the cDNA libraries. The filters were then exposed to X-ray film using intensifying screens.

DNA Sequencing and Analysis

cDNA sequencing was performed on double stranded templates by the dideoxy method of Sanger [59] using the Sequenase 2 kit (USB, Gaithersburg, MD). The deduced sequences were used to search the GenBank® database by the FASTA algorithm of Pearson and Lipman [60].

Results

Development of a Method for Isolating RNA from Coccidian Oocysts.

A major hindrance to the study of coccidian parasites has been the inability to produce the quantity of biomolecules necessary for biochemical studies. Of major import to our studies was the isolation of total RNA from the various life cycle stages. Although RNA could be readily isolated from first-generation merozoites, isolation of intact total RNA from oocysts was problematic. Methods had been reported for isolating RNA from excysted sporozoites by established protocols. These, however, suffered from either low yields or from poor quality. Additionally, they provided for only the isolation of RNA from the sporozoite stage and not from the developing sporozoite.

To address this problem, we tested methods for isolating intact total RNA from fully-sporulated oocysts by various methods. We chose fully-sporulated oocysts because in our previous work we had found them to be the most resistant to disruption

(Abrahamsen, M.S., T.G. Clark and M.W. White, unpublished observations). In this study [61], we compared the quantity and quality of RNA isolated from fully-sporulated oocysts disrupted by grinding in a Diamonite mortar in the presence of 4M GIT to oocysts disrupted by passage through a French pressure cell at 20,000 psi in GIT. The French pressure cell yielded much higher quality total RNA, as assayed by denaturing electrophoresis and Northern analysis, than did grinding in the mortar (data not shown) [61]. Additionally, the yield of RNA from the French press method was 22-fold higher than that from grinding (Table 1). These studies provided methods by which comprehensive biochemical and genetic studies could be undertaken with the parasite *E. bovis*.

Table 1.
Comparison of Oocyst Breakage Methods

Oocyst breakage method	Oocysts broken	RNA yield (μg)	RNA (pg/oocyst)
French Press	$>7.6 \times 10^7$	134	1.68
Liquid N_2 Grinding	6.4×10^6	6	0.080

From Abrahamsen et al., 1993 [61].

Differential Screening of *E. bovis* cDNA libraries.

To isolate developmentally regulated *E. bovis* genes, cDNA libraries were constructed from oocysts sporulated for 36 hr (partially sporulated) and first-generation merozoites produced in cell culture. Reciprocal differential screens [57]

were performed using ^{32}P-labeled first-strand cDNA prepared from partially-sporulated oocyst poly A+ RNA and first-generation merozoite poly A+ RNA. From each library a total of 60,000 phage were screened. Sixty-seven plaques from the merozoite cDNA library preferentially hybridized with the merozoite cDNA probe, and over 250 plaques from the partially-sporulated oocyst library preferentially hybridized with the partially-sporulated oocyst cDNA probe. On average, only 2% of the plaques screened hybridized to either probe, with the majority (>80%) of these hybridizing to both probes. Due to the limiting concentration of individual cDNA molecules available for hybridization, practical limits for detection by differential screening have been estimated to be those mRNAs that are at concentrations >0.1% of total mRNA species [57]. The remaining non-hybridizing phage can be expected to represent either low abundance mRNAs or DNAs that are artifacts of reverse transcription.

Seven phage that preferentially hybridized with the merozoite cDNA probe and 3 phage that preferentially hybridized with the partially-sporulated oocyst cDNA probe were plaque-purified and the cDNA fragments subcloned into plasmid pBS-SK+. The size of each cDNA clone was estimated on agarose gels by comparison to DNA standards of known length (Table 2). The cDNA inserts varied in size from 100 to 2800 nucleotides. The oocyst-specific clone SZ17 contained an internal EcoRI restriction site that was identified during characterization. Both fragments were subcloned and designated SZ 17L and SZ 17S. A series of Southern blots were generated to analyze the extent, if any of cross-hybridization of the 10 cDNA clones to

each other. No cross-hybridization was found, indicating that each of the clones was unique (data not shown).

Table 2.

Estimate of the insert size of cDNAs isolated from the differential screening of merozoite and oocyst cDNA libraries

Merozoite cDNA[a]	Fragment Size (bp)	Oocyst cDNA[b]	Fragment Size (bp)
MZ 1.1	1700	SZ 17L	2800
MZ 1.4	100	SZ 17S	450
MZ 13.1	700	SZ 22	500
MZ 19.1	1250	SZ 27	900
MZ 20.1	200		
MZ 25.1	950		

[a] Merozoite cDNA library was prepared from first-generation merozoites produced in cell culture.
[b] Oocyst cDNA library was prepared from 36-h partially-sporulated oocysts.
From Abrahamsen et al., 1993 [54].

Characterization of mRNA expression.

To assess the success of the differential screens and to examine the mRNA expression pattern of the corresponding cDNA clones, total RNA isolated from unsporulated oocysts, partially-sporulated oocysts, fully-sporulated oocysts, and first-generation merozoites produced *in vitro* was subjected to Northern blot analysis. Total RNAs were separated on formaldehyde/agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled cDNA inserts. Hybridization of the partially-sporulated oocyst cDNA clones revealed a common pattern of mRNA expression during sporulation (Fig. 2A). The expression of the mRNAs was low in unsporulated oocysts, strongly induced in partially-sporulated oocysts and down regulated in fully-sporulated oocysts to levels

comparable to that found in unsporulated oocysts. No hybridization to merozoite mRNA was detected with any of the partially-sporulated oocyst cDNA clones, which is consistent with the lack of hybridization in the differential screen of these cDNA clones with ^{32}P-labeled first-strand merozoite cDNA. Ethidium bromide staining demonstrated equal loading and equivalent quality of each of the oocyst and merozoite RNA samples (Fig 2B). As expected, if the phage clone SZ 17 contained an internal EcoRI site, both SZ 17S and SZ 17L hybridized to mRNAs of identical size and expression kinetics (Fig. 2A). Sequence analysis later confirmed that SZ 17L and SZ 17S are the result of an internal EcoRI site (data not shown).

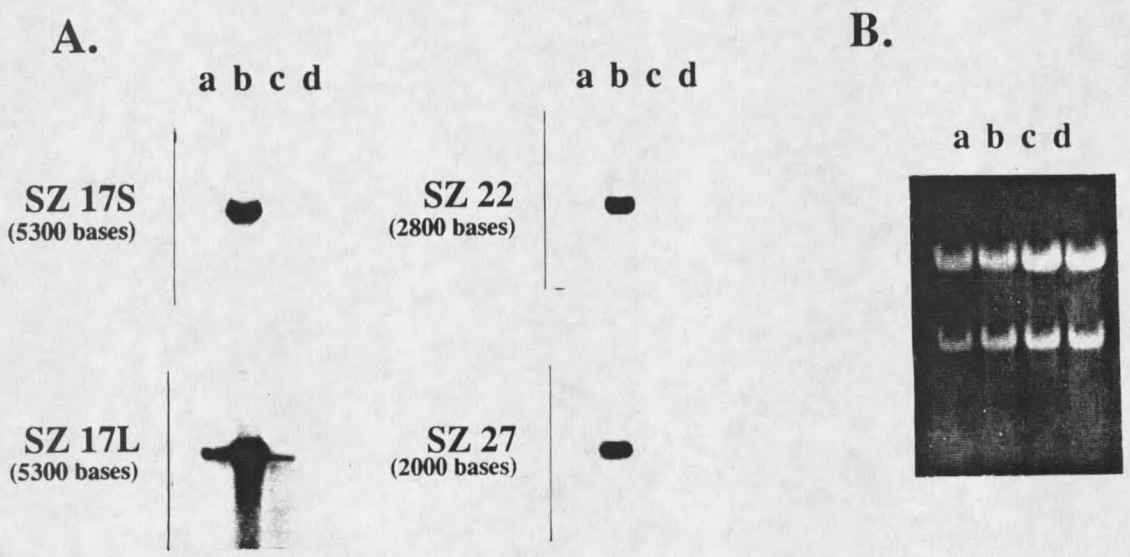


Figure 2. **See Previous Page.** Developmental expression of the partially-sporulated oocysts cDNA clones. **A.** Northern-blot analysis was performed on *E. bovis* total RNA isolated from unsporulated (lane a), partially-sporulated (lane b), fully-sporulated oocysts (lane c) and merozoites (lane d). 5 μg of each RNA in the presence of 2 μg of ethidium bromide were separated on 1.2% formaldehyde agarose gels, transferred to nitrocellulose and hybridized with ^{32}P-labeled cDNA fragments. mRNA sizes indicated in parentheses were estimated from the position of the *E. bovis* major ribosomal RNAs and are only for comparison purposes. **B.** Photograph of a representative gel visualized under ultraviolet light demonstrates equivalent loading and quality of the RNA samples. From Abrahamsen et al., 1993 [54].

Consistent with the results of the differential screen, the merozoite cDNA clones, which were isolated based on their preferential hybridization to the merozoite first-strand cDNA probe as compared to the partially-sporulated oocyst cDNA probe, displayed little or no hybridization to partially-sporulated oocyst RNA and exhibited strong hybridization to merozoite RNA (Fig. 3). Unlike the partially-sporulated oocyst clones, which all displayed a common pattern of mRNA expression, the merozoite cDNAs recognized 3 different classes of mRNAs based on the patterns of expression (Fig. 3). The first class of merozoite cDNAs, represented by MZ 1.4 and MZ 20.1, hybridized to mRNAs expressed to some extent in all of the RNA samples, with the highest level of expression occurring in merozoites. Clones MZ 1.1, MZ 13.1 and MZ 25.1, which represent the second class of merozoite genes, hybridized to mRNAs which were low or absent in unsporulated oocysts, slightly elevated in partially-sporulated oocysts and most abundantly expressed in fully-sporulated oocysts and merozoites. In addition, this class of genes hybridized to multiple mRNA species, the levels of which are regulated relative to each other during development as well. It has

not been determined if these multiple mRNA species are the result of alternate post-transcriptional processing or represent members of a multiple gene family. The third class of genes, which included MZ 19.1 and MZ 12.2, were expressed at low but significant levels in unsporulated oocysts, completely absent in partially-sporulated oocysts and expressed at high levels in both fully-sporulated oocysts and merozoites.

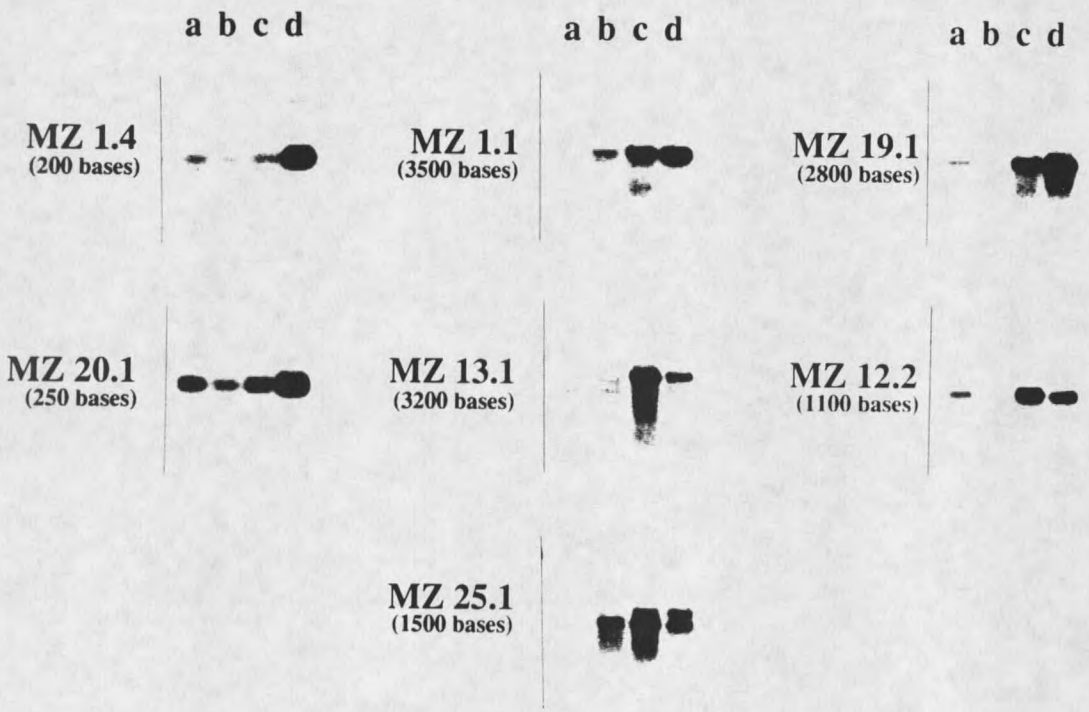


Figure 3. Expression of developmentally regulated mRNAs recognized by the merozoite cDNA clones. Northern-blot analysis was performed on *E. bovis* total RNA isolated from unsporulated (lane a), partially-sporulated (lane b), and fully-sporulated oocysts (lane c) and merozoites (lane d). mRNA sizes indicated in parentheses were estimated from the position of the *E. bovis* major ribosomal RNAs and are only for comparison purposes. From Abrahamsen et al., 1993 [54].

DNA Sequencing and Analysis

The complete nucleotide sequence has been determined for each of the oocyst and merozoite clones, with the exception of MZ 1.1. Sequences with significant identity to the *E. bovis* cDNA clones, as determined by FASTA searches [60] of the GenBank database, are shown in Table 3.

Table 3.

Sequences with significant homology to *E. bovis* cDNA clones

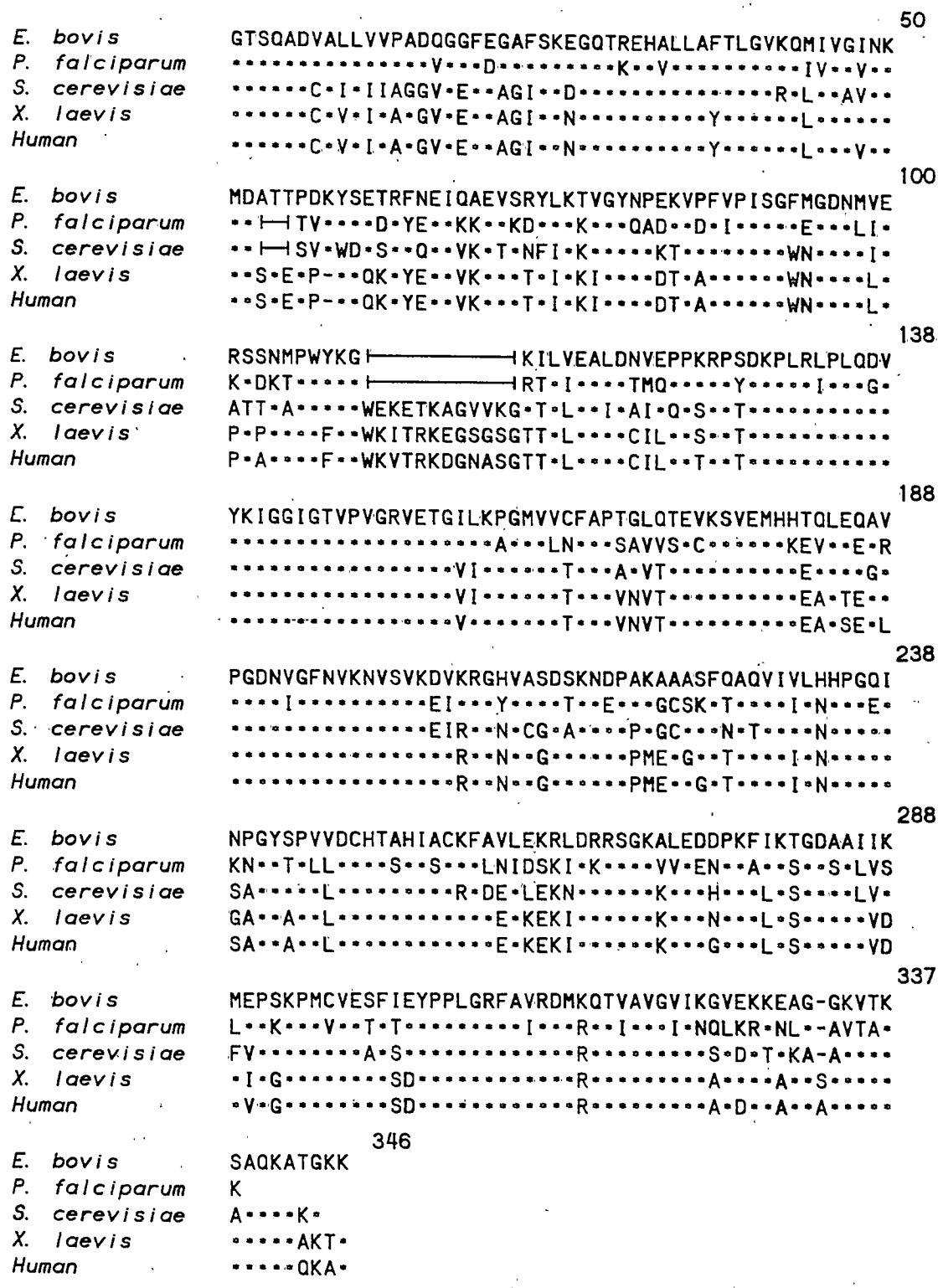
Clone	Percent homology[a]	Database sequence
SZ17S	56.0% in 273 nt	[b]Drosophila insulin-degrading enzyme
SZ 17L	55.8% in 328 nt	[b]Drosophila insulin-degrading enzyme
MZ 1.4	81.4% in 102 nt	[f]Toxoplasma mitochondrial-like DNA
MZ 12.2	72.6% in 496 nt	[c]Drosophila ubiquitin fusion protein
MZ 19.1	65.7% in 825 nt	[d]Saccharomyces elongation factor 1α
MZ 20.1	85.8% in 162 nt	[e]Plasmodium cytochrome c oxidase

[a]nt=nucleotide
[b]Genbank Accession No. M58465; [c] Accession No. X53059; [d] Accession No. M15666; [e] Accession No. M33978; [f] Accession No X60241.
From Abrahamsen et al., 1993 [54].

Clones SZ 22, SZ 27, MZ 25.1, MZ 13.1 and MZ 1.1 displayed only limited identity over short regions to sequences present in the GenBank database and appear to represent genes that either have not been previously identified in other organisms or that are unique to *E. bovis*. Conversely, the other cDNAs displayed varying degrees of identity to other known genes, and two in particular, MZ 19.1 and MZ12.2, appear to be *E. bovis* homologs of previously identified eukaryotic genes. The sequence of MZ 19.1 indicates that it encodes the *E. bovis* homolog of elongation factor 1α (EF-1α), as 70 of the 75 genes with the greatest identity to MZ 19.1 were identified as EF-

1α from several different species (Fig. 4). Sequence analysis of MZ 12.2 revealed that it encodes the *E. bovis* ubiquitin-52 amino acid fusion protein. MZ 12.2 displayed over 70% nucleotide identity with the ubiquitin fusion protein from human, *Drosophila, Trypanosoma,* yeast and other species (Fig. 5). The 52 amino acid extension of ubiquitin has been previously shown to be a ribosomal protein which is incorporated into the large ribosome subunit [62, 63]. The cDNA clone of MZ 12.2 is 673 nucleotides long and contains the entire coding sequence for the 129 amino acids of the ubiquitin-52 amino acid fusion protein. The putative amino acid sequence encoded by MZ 12.2 is aligned for comparison with the ubiquitin fusion proteins from *Trypanosoma brucei* [64], *Clamydomonas reinhardtii* [65], *Drosophila melanogaster* [66] and human [67] (Fig. 5). Sequences 5' of the arrow encode the ubiquitin portion of the protein and sequences 3' of the arrow encode the fused 52 amino acid ribosomal protein. As expected, the ubiquitin monomer encoded by MZ 12.2 displays high conservation, with only a single amino acid change between *E. bovis* and human ubiquitin. In addition, the ribosomal protein extension is also highly conserved displaying 6 conservative out of 10 total substitutions between *E. bovis* and human.

Figure 4. **See Next Page.** Amino acid sequence alignment and comparison of EF-1α from *E. bovis* (MZ 19.1, this report), *P. falciparum* [68], *S. cerevesiae* [69,70], *X. laevis* [71], and human [72]. Amino acid residues identical to those of *E. bovis* MZ 19.1 are indicated by *. Bracketed lines indicate gaps in the amino acid sequences. From Abrahamsen et al., 1993 [54].


50
E. bovis GTSQADVALLVVPADQGGFEGAFSKEGQTREHALLAFTLGVKQMIVGINK
P. falciparum ••••••••••••••V•••D••••••••K••V••••••••••IV••V••
S. cerevisiae ••••••C•I•IIAGGV•E••AGI••D••••••••••••••R•L••AV••
X. laevis ••••••C•V•I•A•GV•E••AGI••N••••••••••Y••••••L••••••
Human ••••••C•V•I•A•GV•E••AGI••N••••••••••Y••••••L•••V••
100
E. bovis MDATTPDKYSETRFNEIQAEVSRYLKTVGYNPEKVPFVPISGFMGDNMVE
P. falciparum ••|—|TV••••D•YE••KK••KD•••K•••QAD••D•I•••••E•••LI•
S. cerevisiae ••|—|SV•WD•S••Q••VK•T•NFI•K•••••KT••••••••WN•••••I•
X. laevis ••S•E•P•••QK•YE••VK•••T•I•KI••••DT•A••••••WN•••••L•
Human ••S•E•P•••QK•YE••VK•••T•I•KI••••DT•A••••••WN•••••L•
138
E. bovis RSSNMPWYKG|————————|KILVEALDNVEPPKRPSDKPLRLPLQDV
P. falciparum K•DKT•••••|————————|RT•I••••TMQ•••••Y•••••I•••G•
S. cerevisiae ATT•A•••••WEKETKAGVVKG•T•L••I•AI•Q•S••T••••••••••
X. laevis P•P••••F••WKITRKEGSGSGTT•L••••CIL••S••T••••••••••
Human P•A••••F••WKVTRKDGNASGTT•L••••CIL••T••T••••••••••
188
E. bovis YKIGGIGTVPVGRVETGILKPGMVVCFAPTGLQTEVKSVEMHHTQLEQAV
P. falciparum ••••••••••••••••••••A•••LN•••SAVVS•C•••••••KEV••E•R
S. cerevisiae ••••••••••••••••VI•••••T•••A•VT••••••••••E••••G•
X. laevis ••••••••••••••••VI•••••T•••VNVT••••••••••EA•TE••
Human ••••••••••••••••••V•••••••T•••VNVT••••••••••EA•SE•L
238
E. bovis PGDNVGFNVKNVSVKDVKRGHVASDSKNDPAKAAASFQAQVIVLHHPGQI
P. falciparum ••••I••••••••••EI•••Y••••T••E•••GCSK•T••••I•N•••E•
S. cerevisiae ••••••••••••••••EIR••N•CG•A••••P•GC•••N•T••••N•••••
X. laevis ••••••••••••••••••R••N••G•••••••PME•G••T••••I•N•••••
Human ••••••••••••••••••R••N••G•••••••PME••G•T••••I•N•••••
288
E. bovis NPGYSPVVDCHTAHIACKFAVLEKRLDRRSGKALEDDPKFIKTGDAAIIK
P. falciparum KN••T•LL••••S••S•••LNIDSKI•K••••VV•EN••A••S••S•LVS
S. cerevisiae SA•••••L•••••••••R•DE•LEKN•••••K•••H•••L•S••••LV•
X. laevis GA••A••L••••••••••••E•KEKI•••••K•••N•••L•S•••••VD
Human SA••A••L••••••••••••E•KEKI•••••K•••G•••L•S•••••VD
337
E. bovis MEPSKPMCVESFIEYPPLGRFAVRDMKQTVAVGVIKGVEKKEAG-GKVTK
P. falciparum L••K•••V••T•T••••••••I•••R••I•••I•NQLKR•NL••AVTA•
S. cerevisiae FV••••••••A•S••••••••••••R•••••••••S•D•T•KA-A••••
X. laevis •I•G••••••••SD••••••••••••R•••••••••A••••A••S•••••
Human •V•G••••••••SD••••••••••••R•••••••••A•D••A••A•••••
346
E. bovis SAQKATGKK
P. falciparum K
S. cerevisiae A••••K•
X. laevis •••••AKT•
Human •••••QKA•

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E. bovis          MQIFVKTLTGKTITLDVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQL 50
T. brucei         ------------A-E--A------------------------------
C. reinhardtii    -------------E--S------------------------------
D. melanogaster   -------------E------------------------------
Human             -------------E------------------------------

                                                  ↓
E. bovis          EDGRTLSDYNIQKESTLHLVLRLRGGIIEPSLALLASKYNCEKKICRKCY 100
T. brucei         -E----A-----------------VM--T-EA--K---W---V--R--
C. reinhardtii    ------A-------------------QA--R---Q--M------
D. melanogaster   ----------------------------RI--Q----D-M------
Human             ----------------------------RQ--Q----D-M------

E. bovis          ARLPPRATNCRKKKCGHTSQLRPKKKPKN 129
T. brucei         ----V--------G---C-N--M---
C. reinhardtii    ---H---K-----S----N-------L-
D. melanogaster   ---H-------------NN------L-
Human             ---H---V----------NN------V-
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Figure 5. Amino acid sequence alignment and comparison of the ubiquitin-52 amino acid fusion protein from *E. bovis* (MZ 12.2, this report), *T. brucei* [74], *C. reinhardtii* [75], *D. melanogaster* [73], and human [67]. Amino acid residues identical to those of *E. bovis* MZ 12.2 are indicated by *. Amino acid sequences 5' of arrow encode the ubiquitin moiety and amino acid sequences 3' of arrow encode the fused ribosomal protein. From Abrahamsen et al., 1993 [54].

Discussion

Identification of genes that are regulated during the coccidian life cycle is critical to the understanding of the unique developmental biology of these parasites. In this report, we successfully employed methods utilized in other eukaryotic models to isolate developmentally regulated genes of *E. bovis*. Differential screening of cDNA libraries generated from merozoites and partially-sporulated oocysts resulted in the identification of numerous cDNAs which displayed preferential hybridization to either the merozoite or partially-sporulated oocyst cDNA probe. Each of the characterized partially-sporulated oocyst cDNA clones displayed similar kinetics of expression during

sporulation (Fig. 2) and were specific to the oocyst stage as no hybridization was detected in merozoite mRNA even after long exposure times (data not shown). The identification of a single expression class of oocyst-specific genes is primarily due to the choice of probes (partially-sporulated oocyst vs. merozoite) and does not necessarily mean that other oocyst genes with different patterns of expression do not exsist. Generation and differential screening of cDNA libraries prepared from RNA isolated before, during and after sporulation will be necessary to identify different classes of genes that are involved at various times during sporulation.

In contrast to the oocyst cDNA clones, the 7 merozoite cDNA clones fell into 3 different classes depending on their pattern of mRNA expression during development (Fig. 3). One characteristic that was common to all of the merozoite clones was that no mRNA species was identified in which the expression was restricted exclusively to the merozoite stage. Each of the clones was expressed to some extent in some or all of the oocyst RNA samples, but in each case expression was higher in merozoites than in partially-sporulated oocysts which is consistent with the restriction of expression defined by the differential screen.

All of the *E. bovis* cDNA clones examined were expressed to some extent in fully-sporulated oocysts, with clones MZ 13.1 and MZ 25.1 displaying maximal expression after completion of sporulation. These steady-state message levels must be the result of either continuing gene transcription in fully-sporulated oocysts or the mRNA molecules having extremely long half-lifes. The high level of expression of MZ

19.1 and MZ 12.2 in fully-sporulated oocysts and the lack of any detectable message in partially-sporulated samples suggest that fully-sporulated oocysts are metabolically competent for transcription of at least some genes. These are likely to be genes in which the products of which are needed for maintaining the viability of the environmentally resistant oocyst and those genes which are required by the sporozoite to initiate the processes of excystation and host cell penetration. These results are consistent with observations of Ellis and Thurlby [47] who demonstrated a transient increase in the expression of a variety of mRNAs during the initial phase of sporulation in *E. maxima*, which was followed by an increase in transcription of several specific genes during the later stages of sporulation. Our results extend these observations by identifying multiple classes of specific genes which were induced and suppressed at different times during sporulation.

Sequence analysis of the *E. bovis* clones has identified several cDNAs of which the high degree of identity to mammalian genes provides clues for understanding the biology of these parasites. The cDNAs MZ 12.2 and MZ 19.1 are most likely the *E. bovis* homologs of well-characterized genes involved in protein metabolism. Not surprisingly, the differential expression of these genes suggests that protein metabolism, both synthesis and degradation, is highly regulated during *E. bovis* development and is likely to be important for the major morphological changes associated with the process of sporozoite and merozoite formation.

MZ 19.1 appears to be the *E. bovis* homolog of EF-1α. EF-1α functions in the elongation step of protein synthesis, where it binds GTP and aminoacyl-tRNA and mediates the codon-dependent placement of this aminoacyl-tRNA at the A site on the ribosome [73]. Multiple variants of EF-1α have been cloned from *X. laevis* which are developmentally regulated during oocyte maturation [74]. This is consistent with the regulated expression of EF-1α (MZ 19.1) during *E. bovis* sporulation. Studies have revealed a number of regulated activities of EF-1α beyond delivering aminoacyl -tRNA to ribosomes including sequestration of tRNA or tRNA synthetases in ribonucleotide-protein particles [75,76], which may be compartmentalized in cellular organelles through interactions with membrane or cytoskeletal structures [76,77,78]. These observations are not well understood but may indicate a role for EF-1α in compartmental regulation of protein synthesis [79,80]. This hypothesis is particularly relevant to coccidian sporulation which must proceed through a series of highly defined cytokinetic steps in which the sporont cleaves twice to form 4 sporoblasts, and within each sporoblast 2 sporozoites are formed.

MZ 12.2 is predicted to encode the ubiquitin-52 amino acid fusion protein which exhibits extreme evolutionary conservation over a broad range of eukaryotic species [81]. The ubiquitin fusion gene is translated into a single ubiquitin moiety fused to ribosomal proteins. After proteolytic processing in yeast cells, ubiquitin functions as a post-translational modifying group which signals the degradation of acceptor proteins

[82], and the fused 52 amino acid ribosomal protein is incorporated into the large ribosome subunit [62,63]. The presence of ubiquitin at the N-terminus of these ribosomal proteins has been shown to greatly facilitate their incorporation into nascent ribosomes and is required for efficient ribosome biogenesis [62]. The identification of the ubiquitin fusion protein in *Eimeria* is consistent with a 'chaperone' function for ubiquitin being established early during eukaryotic evolution and its differential expression provides additional evidence that protein metabolism is highly regulated during *E. bovis* development.

A third cDNA, the putative function of which is based on the similarity of its sequence to another enzyme involved in protein metabolism, raises some interesting questions concerning coccidian biology. SZ17 (containing SZ17L and SZ17S sequences) displays a 54.4% identity in a 680 nucleotide overlap and a 51.1% identity in a 656 nucleotide overlap to *D. melanogaster* [83] and human [84] insulin-degrading enzymes (IDE) respectively. Overall, this nucleotide identity is reflected in a 30% amino acid identity with human IDE. However, regions at both the N-terminal and C-terminal domains contain at least 6 stretches of 11-28 amino acids that have 55-72% identity among SZ17, human and *Drosophila* IDE (data not shown). In mammalian cells, IDE is involved in the initial cleavage of internalized insulin before removal to lysosomes [85,86]. Recently, both mammalian and *Drosophila* IDE have been shown to be able to degrade transforming growth factor-α *in vitro* [87] and *in vivo* [88]. In addition, IDE may be a critical factor in mediating the onset of differentiation of

smooth muscle cells in culture [89], suggesting that IDE may function in both growth factor removal and cellular differentiation processes. As in the case for SZ17, the expression of which is specific to the oocyst, IDE has been demonstrated to be developmentally regulated in *Drosophila* [90]. These observations suggest that the protein encoded by SZ17 may be involved in the degradation of a proteins(s) which is an important component of a signal transduction pathway regulating oocyst sporulation. Whether the protein encoded by SZ 17 has any activity on insulin remains to be determined.

The success in isolation of developmentally regulated *E. bovis* genes demonstrates the power of using established developmental approaches in studying intracellular protozoan parasites. The amount of information that has been generated by quickly examining a relatively small number of genes suggests that similar approaches to isolate additional gene subsets should provide valuable information concerning specific developmental stages including the intracellular schizont. One of the drawbacks to the differential screens used in these studies is that only cDNAs corresponding to abundant mRNAs are identified [57]. To isolate low abundance, developmentally regulated mRNAs, it will be necessary to employ technically more demanding cDNA library construction techniques.

CHAPTER 3

DEVELOPMENTAL CHARACTERIZATION OF THE *EIMERIA BOVIS* HSP90 HOMOLOG

Introduction

When oocysts of the protozoan parasite *Eimeria bovis* are ingested by a bovine host, the sporozoites excyst from the sporocyst and ultimately invade endothelial cells of the central lacteals in the terminal ileum. The invasion initiates a complex developmental process that procedes through several asexual stages and a final sexual stage that results in the formation of the oocyst and the completion of the life cycle [8]. *E. bovis* development is highly programmed with little or no deviation observed between individual infections. This programming is likely dictated by changes in gene expression. Several groups have studied changes in mRNA and protein expression associated with the different developmental stages in *Eimeria* spp. [47,49,91,92] and have found that there are large changes in gene expression that occur between the various developmental stages. We have used techniques of differential screening to isolate differentially regulated cDNAs from different stages of *E. bovis* [61] to study the developmental biology of this important organism. From these experiments, we have identified the *E. bovis* homolog of heat shock protein 90 (hsp90).

The 90-kDa stress protein, heat shock protein 90 (hsp90), like other stress proteins such as hsp70, is conserved among all organisms from bacteria to humans [93,94]. Protein hsp90 is constituitively expressed in eukaryotic cells but, similar to the hsp70 family, can be induced by mitogenic stimulation or heat stress [95]. This stress response has been observed in every organism examined, including prokaryotes and eukaryotes [94]. The hsp90 proteins have been found in association with many intracellular 'substrate proteins' including calmodulin, actin, tubulin, several kinases and steroid receptors [96] and their activity is in agreement with a broad range chaperone function. Consistent with this role, hsp90 is the most abundant cytosolic protein in eukaryotic cells [96] and it is proposed that hsp90 recognizes immature, non-native proteins and facilitates their transport and folding to their native and functional form. This function requires ATP hydrolysis and is thought to require the accessory proteins p50, hsp70, and hsp56 [97,98,99,100]. The hsp90 proteins are found as heterodimers in mammalian cells and are likely highly autophosphorylated [101]. Previous attempts to identify heat shock proteins in *E. bovis* identified hsp-like proteins in the range of 43, 72 and 75 kDa, but not in the 90 kDa range[102]. Interestingly, our isolation of *E. bovis* hsp90 was based upon its lack of mRNA expression in sporulating oocysts, which is unexpected since hsp90 appears to be constituitively expressed in other eukaryotic cells [95].

Materials and Methods

Parasite Production

One month old Holstein-Friesian calves were inoculated with 250,000 *E. bovis* sporozoites [49] through injection into the small intestine 1 meter proximal to the ileo-cecal junction. Purification of the oocysts followed the procedure of Speer [103]. Briefly, 18 days following infection, feces was collected from the animals, passed through a 60 mesh screen, and sedimented at 1500 g by continuous centrifugation. The fecal pellet was then resuspended in sucrose to a final concentration of 1M, centrifuged at 2000 rpm for 20 min and the supernatant recovered. The supernatant was diluted 1:1 with with H_2O and re-centrifuged for 20 min at 2000 rpm, pelleting the oocysts. The purified oocysts were either harvested as unsporulated, or potassium dichromate was added to a final concentration of 2.5% (w/v) and the oocysts were incubated in roller bottles for 36 h (partially-sporulated) or 72 hours (fully-sporulated) at room temperature. The potassium dichromate was removed from the oocysts by repeated washings in phosphate-buffered saline (PBS).

Merozoites were produced by infection of embryonic bovine tracheal cells (EBTr, ATCC, CCL# 144) with excysted sporozoites [49] at an infection rate of 2 sporozoites/EBTr cell. Cultures were either harvested for RNA isolation at various time points or were allowed to complete their developmental cycle and produce merozoites. Merozoites were harvested from the culture supernatant following 14 days

of infection and pelleted at 1400 g. The merozoites were then washed in PBS and re-pelleted before RNA isolation.

RNA Isolation

RNA was isolated from un-, partially-, and fully-sporulated oocysts and first-generation merozoites produced in cell culture as described in Chapter 2 and by Abrahamsen et al. [61]. *E. bovis* sporozoite-infected EBTr cells were harvested by lysis in 4 M guanidine isothiocyanate solution (GIT; see Chapter 2) at day 2, 4, 6, 10 and 12 following sporozoite inoculation. The RNA was processed as described in Chapter 2.

Library Construction and Differential Screening

Poly A+ RNA (2 μg), isolated from merozoite total RNA using oligo-dT cellulose columns (Stratagene, La Jolla, CA), was converted to double stranded cDNA by the method of Gubler and Hoffman [56] using a Pharmacia cDNA synthesis kit. The cDNA was ligated to λgt11 phage arms (Pharmacia) and packaged using phage packaging extracts (Stratagene, La Jolla, CA). Differential screens were performed on the merozoite library using first strand cDNA probes generated from both merozoites and partially-sporulated oocysts. Poly A+ RNA from both merozoites and partially-sporulated oocysts was reverse transcribed using Superscript MMLV reverse transcriptase (BRL, Gaithersburg, MD) with oligo-dT as a primer. The merozoite cDNA library was plated at a density of 15,000 phage/150 mm plate and duplicate

nitrocellulose lifts were made. The lifts were then hybridized overnight at 50 C in hybridization solution [6X standard saline citrate (SSC), yeast tRNA (100 μg ml^{-1}), 50% formamide, 2X Denhardt's solution and 50 mM sodium phosphate, pH 7.0 (SSC is 150 mM NaCl, 15 mM sodium citrate; 50X Denhardt's solution is 1% (w/v) Ficoll 400, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) bovine serum albumin)] with either the merozoite or partially-sporulated oocyst first strand ^{32}P-labeled cDNA. The filters were washed 3 times in 2X SSC, 0.1% (w/v) sodium dodecyl sulfate (SDS) for 20 min at room temperature and then 2X in 0.1X SSC, 0.1% SDS for 30 min at 55 C. The filters were then exposed to X-ray film at -70 C with an intensifying screen for 4-16 hr, and the plaques that hybridized only to the merozoite probe selected and further purified through two more rounds of screening. The cDNA fragment of the clone of interest (MZ2.5) was subcloned into the NotI site of pBluescript SK+ (Stratagene, La Jolla, CA).

Northern Analysis

Five μg of total RNA isolated from un-, partially- and fully-sporulated oocysts and merozoites or 10 μg of infected-cell mRNA was electrophoresed on formaldehyde-agarose gels and transferred to nitrocellulose by blotting in 20X SSC [54]. The blots were hybridized (1×10^6 cpm ml^{-1}) with gel-purified MZ2.5 or MZ1.1 [54] cDNA inserts that had been ^{32}P-labeled by nick translation to a specific activity of $2\text{-}4 \times 10^8$ cpm μg^{-1} DNA using the hybridization and wash conditions described for screening.

MZ1.1 was used for comparison because it had shown a similar pattern of expression during sporulation. The probed blots were exposed to X-ray film overnight at -70 C with an intensifying screen. To quantitate the levels of MZ2.5 and MZ1.1 mRNA with respect to *E. bovis* ribosomal RNA in sporozoite-infected cells, the Northern blots were washed in 90 C water for 15 min and rehybridized with a 5'-end ^{32}P-labeled oligonucleotide (5'-CTCAAACTTCCTCGCGTTAGACACGCAAA-3') which was based upon sequence comparison of coccidian small subunit rRNAs [104]. Hybridization was then performed as described above. Quantitation of autoradiograms was performed by densometric scanning (LKB Ultroscan XL Laser scanner).

Nucleotide Sequence Analysis

Dideoxy DNA sequencing was performed, using double stranded MZ2.5 cDNA in pBS-SK+ as a template, with the Sequenase version 2 kit (USB, Cleveland, OH). T3 and T7 primers were used to initally sequence the cDNA. The complete sequence of the cDNA insert was determined by using primers complementary to the deduced sequence. The primers used were TC-5 (5'-CCACCGCCATATACATA-3'), TC-6 (5'-GTCGAGGGTCAATTAGA-3'), TC-14 (5'-GTAAGGAGGGCGGTATC-3'), TC-15 (5'-CATTCAAGGAATACTGG-3'), TC-16 (5'-CAAGTCCTTCTTTCG-3'), TC-17 (5'-TAAAGAAGTGTTGCACG-3'), TC-18a (5'-GATACCGCCCTCCTTACCTCTGG-3'), TC-19a (5'-GCTACAGTTTGACGAGATTCCCC-3') and TC-30 (5'-

CTTGACGAAGTTCAGCCATTCAGG-3'). Sequences were analyzed using Lasergene™ (DNASTAR, Madison, WI) computer software and the alignments performed with the BLAST program [105] and the GenBank database.

Southern Analysis

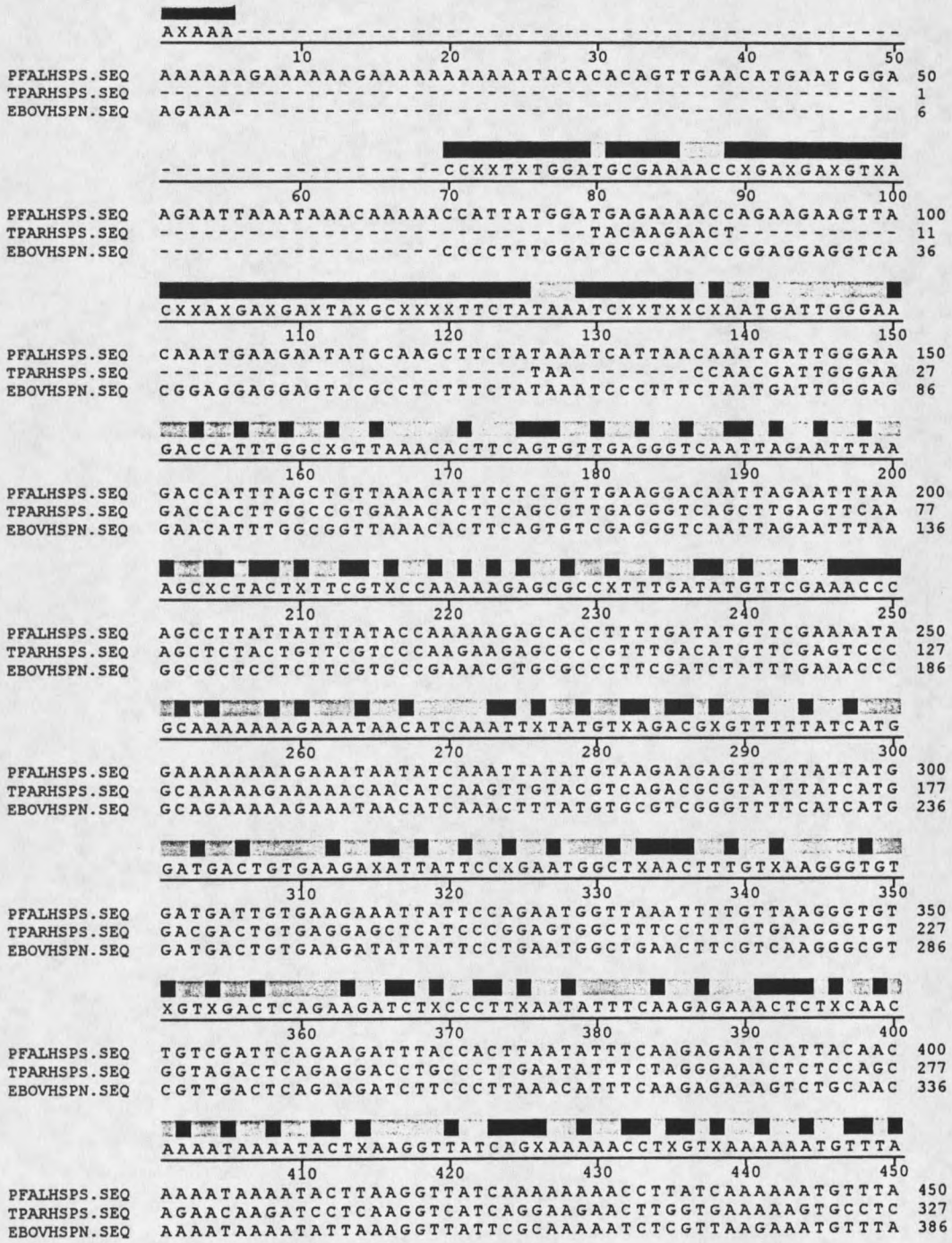
Genomic DNA was isolated from *E. bovis, Eimeria acervulina, Toxoplasma gondii, Cryptosporidium parvum* and EBTr cells by alkaline lysis [106]. The DNA was incubated with various restriction enzymes in the appropriate buffer and electrophoresed in 0.8% agarose gels, after which the gel was treated with 0.25 M HCl for 10 minutes, 0.5 M NaOH, 1.5 M Tris pH 7.0, for 10 minutes, 0.5 M NaCl, 1.5 M Tris pH 7.0 for 10 minutes, and 20X SSC for 10 minutes. The DNA was transferred to Duralose nylon membrane (Stratagene, La Jolla, CA) and hybridized with nick-translated ^{32}P-labeled MZ2.5 cDNA insert for 12-16 hours at 37-42 C. Blots were then washed three times with 2X SSC, 0.5% SDS for 20 min, followed by 2 washes in 0.1X SSC, 0.5% SDS at the hybridization temperature (normal stringency), or washed three times with 2X SSC, 0.5% SDS for 20 min at room temperature, followed by 2 washes in 0.5X SSC, 0.5% SDS at the hybridization temperature (low stringency). The blots were then exposed to X-ray film with an intensifying screen at -70 C.

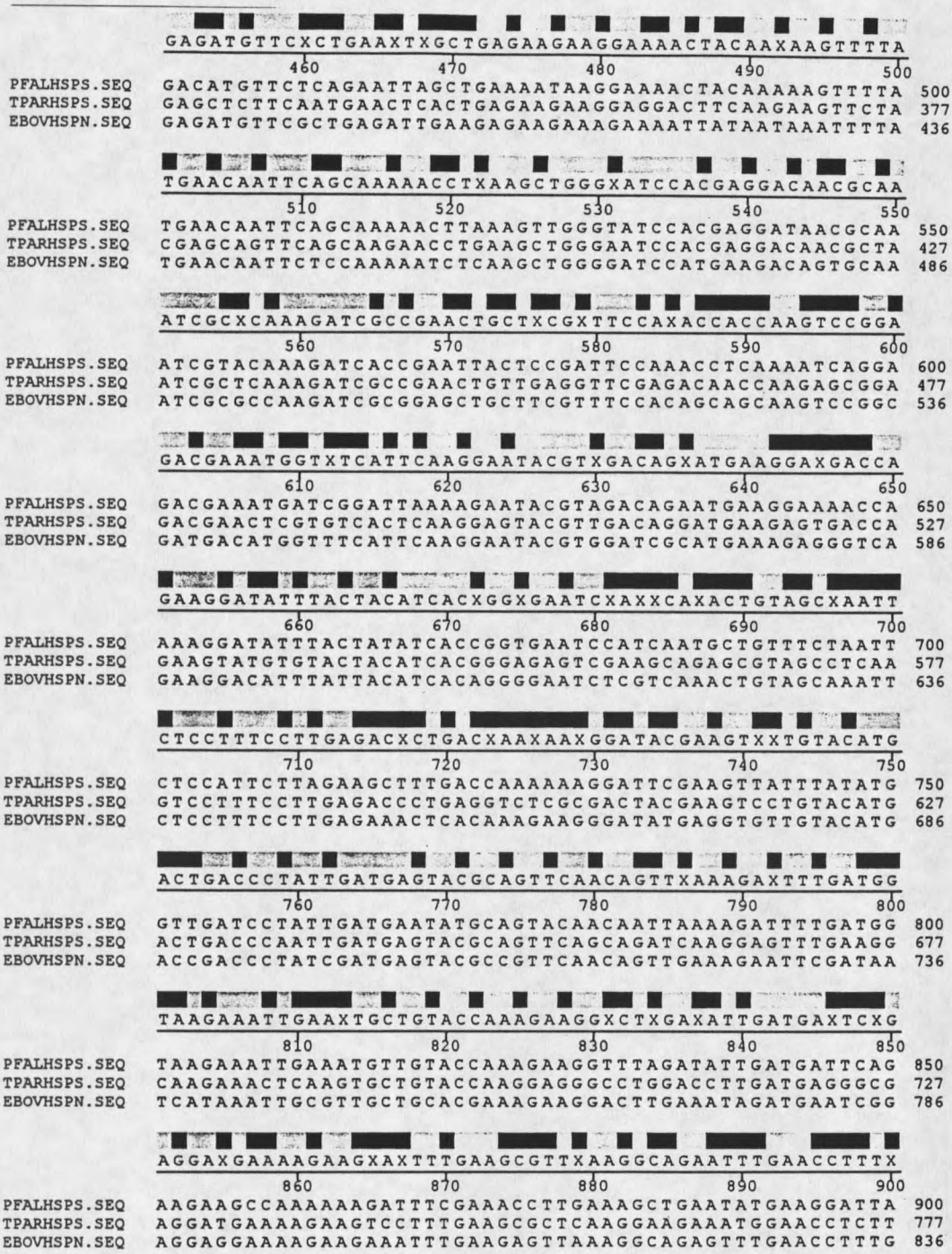
Results

Isolation of the Differentially Regulated cDNA MZ2.5.

A cDNA library constructed from total RNA extracted from first-generation merozoites produced in tissue culture was differentially screened using ^{32}P-labeled first-strand cDNA generated from first-generation merozoites and partially-sporulated (36 h) oocysts. From a total of 60,000 plaques screened, 67 merozoite specific plaques were isolated [61]. The clone MZ2.5 was isolated based on its hybridization to the first-strand cDNA probe from merozoites and lack of hybridization to the first-strand cDNA probe from partially-sporulated oocysts. The cDNA MZ2.5 was subcloned into pBS-SK+, and sequence analysis revealed a 1628 base pair AT rich (60%) fragment whose sequence, when used to search the Genbank ® database, showed 69% identity to *Plasmodium falciparum* hsp90 [107] and 67% identity to *Theileria parva* hsp90 [108] (Fig. 6). The *E. bovis* hsp90 sequence is incomplete and corresponds to the 3' end of the known hsp90 coding regions. The *E. bovis* hsp90 deduced amino acid sequence revealed a 427 amino acid reading frame with 79% identity to the amino acid sequence of *P. falciparum* hsp90. This similarity increases to 89% when conservative substitutions are included (Fig. 7). In addition, we have identified a putative ATP binding site at residue 69 (Fig. 7) and a putative calmodulin binding site at residue 204 that are highly similar to the ATP and calmodulin binding concensus sequences [107] (Fig. 7).

Figure 6. **Pages 46-49.** Nucleotide sequence alignment and comparison of hsp90 from *E. bovis*, *P. falciparum* [107] and *T. parva* [108]. Regions of greater identity are indicated by the light gray bars above the consensus sequence. The black bars indicate regions of lower conservation.

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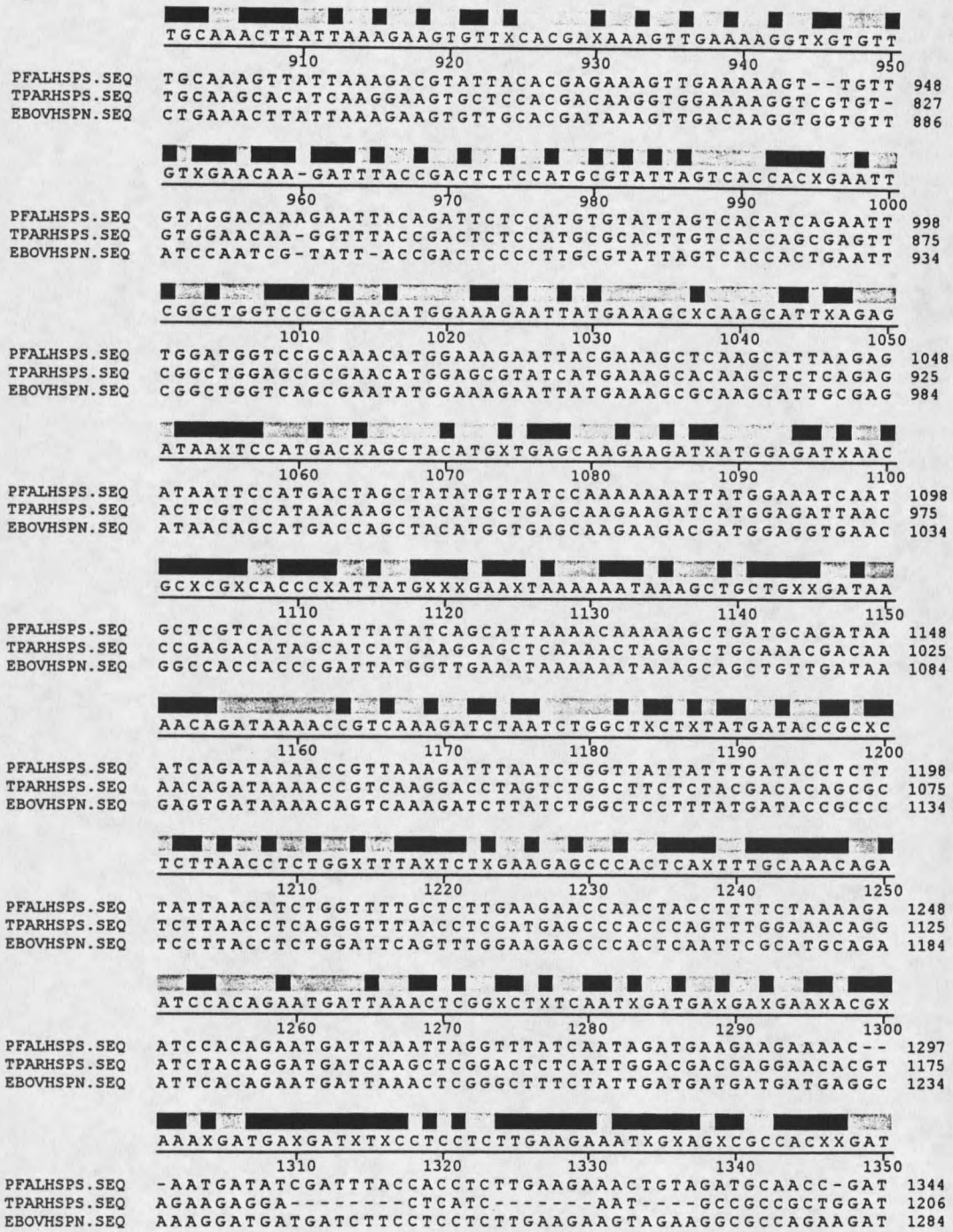
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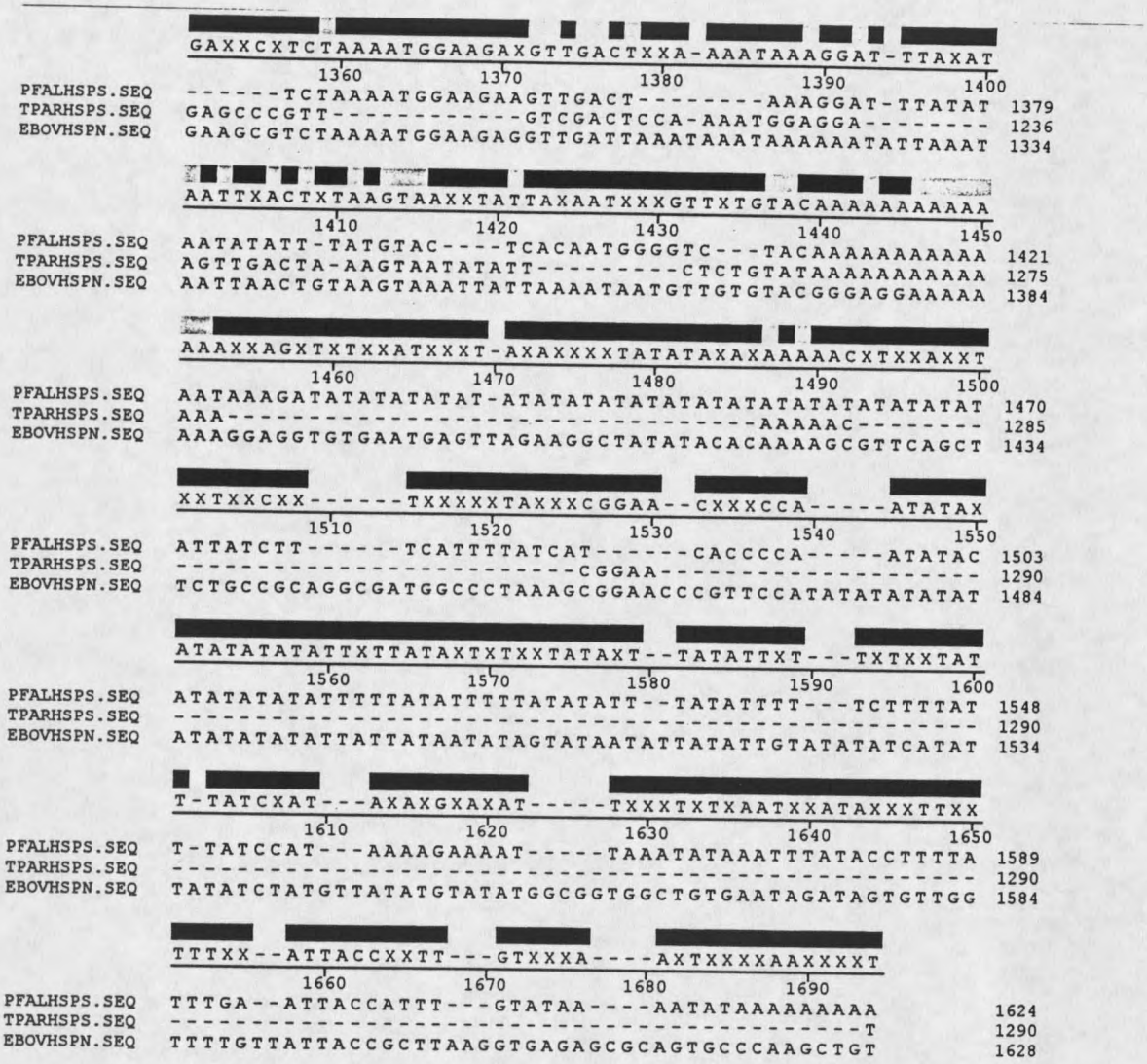
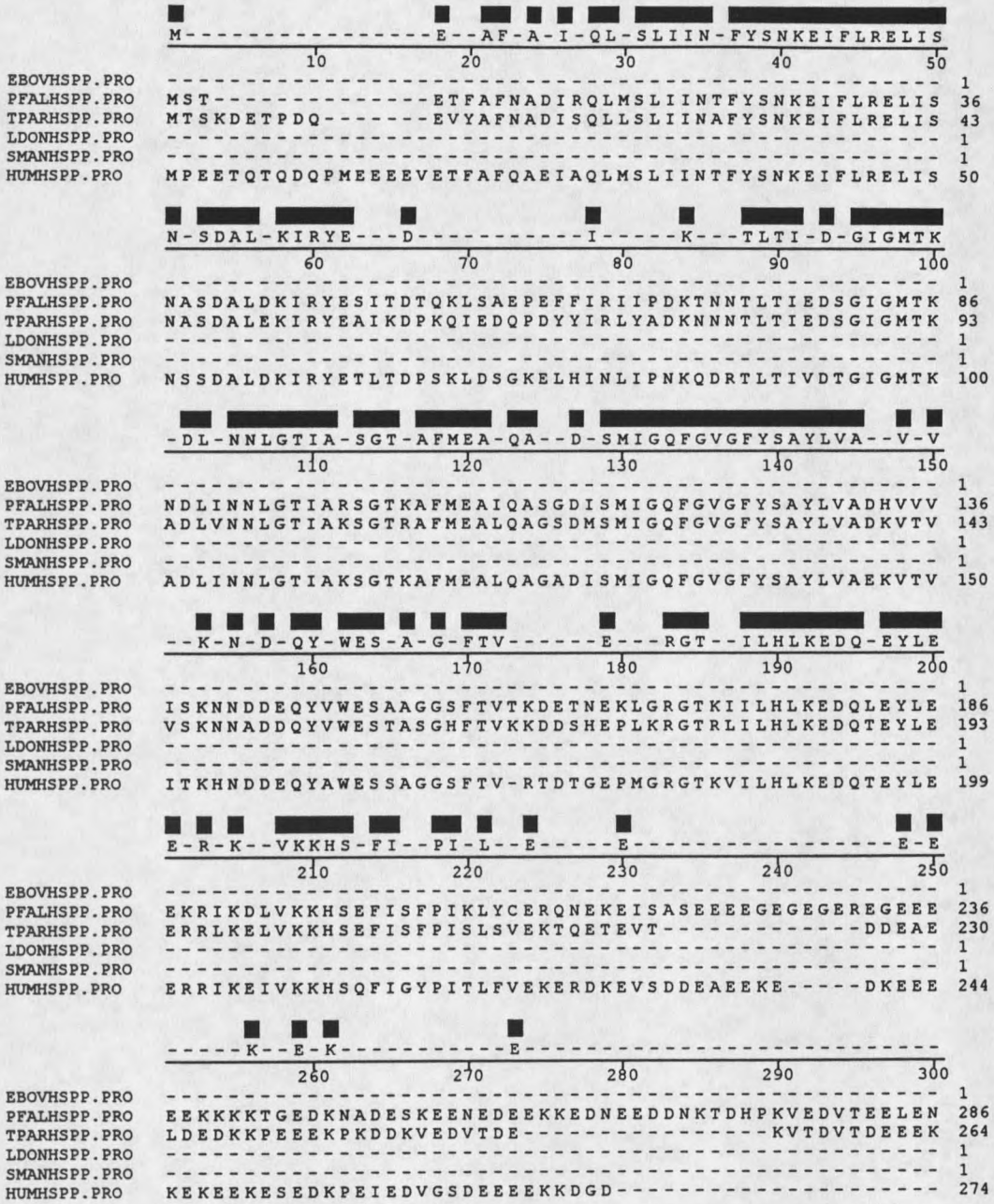


Figure 7. **Pages 50-52.** Amino acid sequence alignment and comparison of hsp90 from *E. bovis* , *P. falciparum* [107], *T. parva* [108], *S. mansonii* [109], *L. donovani* [110] and human [111]. The bar above the consensus sequence indicates regions of identity ranging from low identity (black bars) to high identity (light gray bars).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LDONHSPP.PRO ---------- 1
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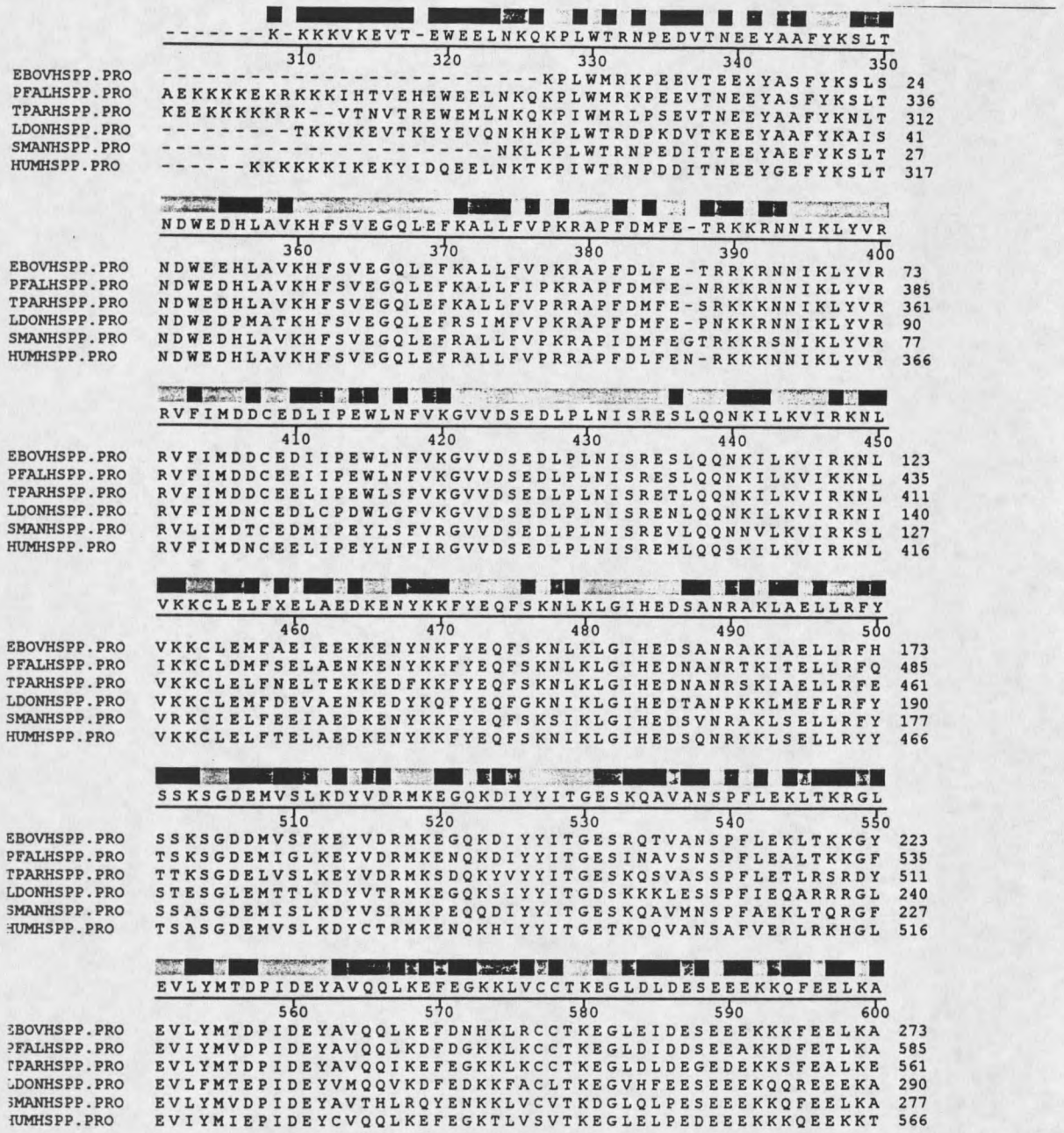
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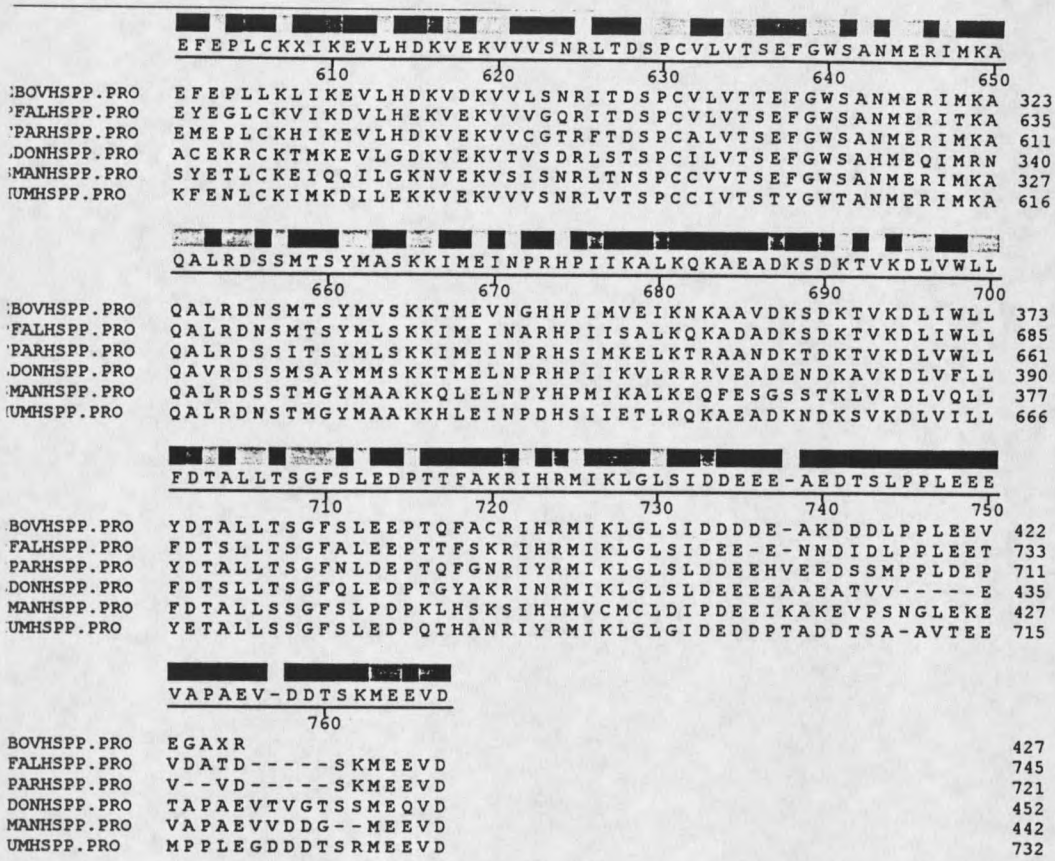
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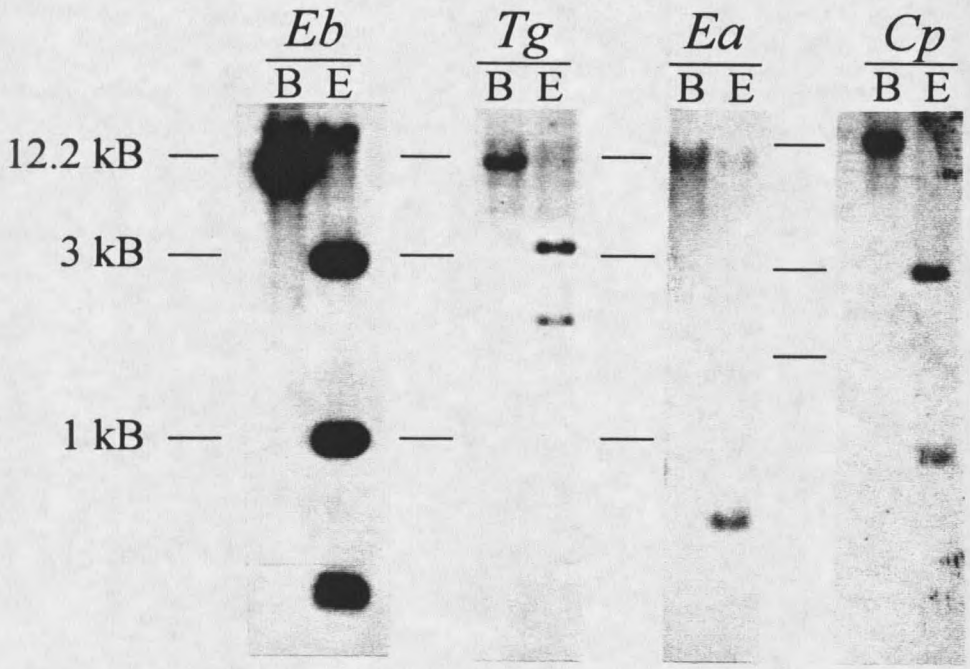
MANHSPP.PRO VAPAEVVDDG--MEEVD 442
UMHSPP.PRO MPPLEGDDDTSRMEEVD 732

***Eimeria bovis* hsp90 Cross-reacts with Genomic DNA from Other Coccidians.**

To determine if *E. bovis* hsp90 was present as a single gene, Southern blot analysis was performed on *E. bovis* genomic DNA. The genomic DNA was digested with the restriction enzymes *Eco*R I and *Bam*H I. Hybridization of the Southern blot with *E. bovis* hsp90 cDNA insert indicated the presence of a single gene with two internal *EcoRI* sites that were predicted by the cDNA sequence (Fig. 8). To determine the degree of cross-hybridization to other coccidians, the *E. bovis* hsp90 cDNA was used to probe genomic DNA isolated from *E. acervulina*, *T. gondii* and *C. parvum.* These genomic DNAs showed hybridization to the *E. bovis* hsp90 probe (Fig. 8). Further, one of the *Eco*R I restriction sites was conserved in all coccidian DNAs tested. *E. bovis* hsp90 does not, however, cross-react with bovine genomic DNA, at low stringency (37 C hybridization; 0.5X SSC, 37 C final wash) using a 150-fold excess amount of DNA (data not shown).

Figure 8. **See Next Page**. Southern blot analysis of *E. bovis (Eb)*, *T. gondii (Tg)*, *E. acervulina (Ea)*, and *C. parvum (Cp)*, genomic DNA. A Southern blot was prepared using genomic DNA isolated from the various species and digested with either BamHI (B) or EcoRI (E). The blot was hybridized with ^{32}P-labeled MZ 2.5 cDNA insert.



Stage Specific Expression of *E. bovis* hsp90 mRNA.

To confirm the developmental regulation of *E. bovis* hsp90, Northern blot analysis was performed using RNA isolated from un-, partially-, and fully-sporulated oocysts, and first-generation merozoites. The blots were probed with *E. bovis* hsp90 cDNA insert that had been ^{32}P-labeled by nick-translation. Figure 9 shows that *E. bovis* hsp90 mRNA is strongly expressed in fully-sporulated oocysts and merozoites but is not expressed in un-, or partially-sporulated oocysts. For comparison, MZ1.1, a cDNA isolated by differential screening [54], shows no mRNA expression in unsporulated oocysts, but is induced slightly in partially-sporulated oocysts and

expressed at high levels in fully-sporulated oocysts and merozoites. The observation that *E. bovis* hsp90 is not expressed in un-, and partially-sporulated oocysts is consistent with the results obtained by the differential screen, in which cDNAs were selected based on their hybridization to merozoite first-strand cDNA probe and lack of hybridization to the partially-sporulated oocyst first-strand cDNA probe.

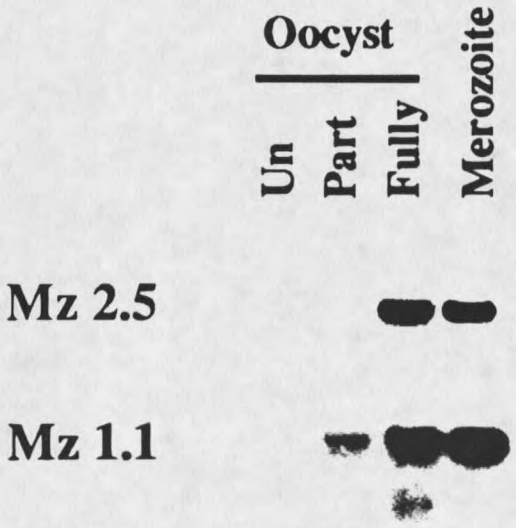


Figure 9. Developmental regulation of MZ 2.5 mRNA during sporulation. Total RNA from un-, partially-, and fully-sporulated oocysts and merozoites was separated on a formaldehyde agarose gel and blotted to nitrocellulose. The resulting blots were probed with ^{32}P-labeled MZ 2.5 insert. For comparison, an identical blot was probed with MZ 1.1 [54].

RNA expression during *E. bovis* merogony was examined by Northern analysis using RNA isolated from EBTr cells inoculated with *E. bovis* sporozoites and harvested at 2, 4, 6, 10, and 12 days post-inoculation. The blots were probed with ^{32}P-labeled *E. bovis* hsp90 and MZ1.1 cDNA inserts. To account for the differences in sporozoite infection rates between timepoints, the blots were reprobed for the *E. bovis*

major small subunit rRNA. The resultant autoradiograms were normalized to the levels of *E. bovis* 18s ribosomal RNA by densometric scanning [50]. The relative levels of hsp90 and MZ1.1 mRNAs with respect to 18s rRNA are plotted in Figure 10. The level of the rRNA remains fairly constant during the first 10 days of infection and then greatly increases at day 12. Both *E. bovis* hsp90 and MZ1.1 mRNA show peak expression with respect to rRNA at day 6. However, hsp90 mRNA is expressed at moderately high levels at day 2 relative to day 6, which results in a bimodal pattern of expression (Fig. 10). By contrast, MZ1.1 is expressed at low levels early in merogony and increases to a peak at day 6.

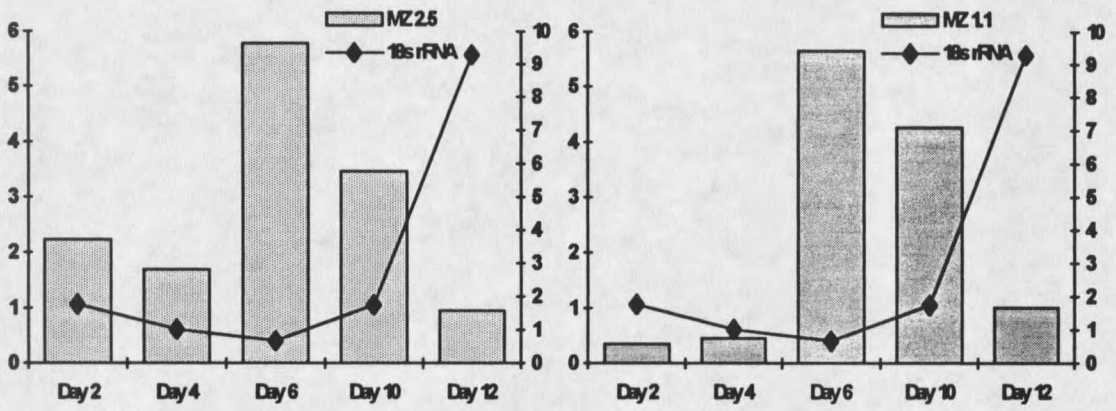


Figure 10. Northern analysis of total RNA isolated from *E. bovis* sporozoite-infected EBTr cells. Total RNA (5μg) from day 2, 4, 6, 10 and 12 *E. bovis* sporozoite-infected cells was separated by formaldehyde agarose gels and blotted to nitrocellulose. The blots were hybridized with ^{32}P-labeled MZ 2.5, MZ 1.1, and 18s ribosomal RNA probe. Autoradiograms of Northern blots probed with MZ 2.5, MZ 1.1 and 18s rRNA were quantitated by densometric scanning and and the levels of MZ 2.5 and MZ 1.1 normalized to the level of 18s ribosomal RNA.

Discussion

Robertson et al., 1988 identified hsp-like proteins from *E. bovis* in the 43, 72 and 75 kDa range using antibodies directed against *Plasmodium falciparum* hsp 75, but were unable to detect hsp-like proteins in the 90 kDa range. We were also unsuccessful in detecting *E. bovis* hsp90 protein using antibodies directed against *Theileria parva* hsp90 [108] and *Achlya ambisexualis* hsp90 (StressGen, Victoria, British Columbia) (data not shown). However, by differential screening of a merozoite cDNA library we isolated the *Eimeria bovis* homolog of hsp90. The clone was approximately 69% identical to *P. falciparum* hsp90 and 67% identical to *T. parva* hsp90 at the nucleotide level. The putative amino acid sequence of *E. bovis* hsp90 was 79% identical to that of *P. falciparum.* The *E. bovis* hsp90 homolog appears to be encoded by a single gene that is highly conserved among coccidians, cross-reacting at high stringency with genomic DNA from *T. gondii, C. parvum* and *E. acervulina,* but not with bovine genomic DNA. An internal EcoRI site also appears to be conserved in all the coccidian genomic hsp90 DNA examined. Putative ATP and calmodulin binding sites have also been identified that correspond to sites predicted by the *P. falciparum* hsp90 sequence [107].

E. bovis hsp90 mRNA was highly expressed in fully-sporulated oocysts and continued to be expressed throughout merogony. When normalized to parasite 18s rRNA, peak expression for *E. bovis* hsp90 mRNA occurred 6 days following sporozoite infection. Similarly, MZ 1.1 mRNA expression peaked at day 6, relative to 18s rRNA, but was expressed at much lower levels during early merogony. The decrease observed in the relative levels of hsp90 and MZ 1.1 mRNAs at day 12 was the result of the corresponding large increase in rRNA. Interestingly, although meront size increases greatly during merogony, there was no change in the level of rRNA until just before merozoites were formed. Thus, the synthesis of new ribosomes destined for merozoites occurs quite late in merogony and the accumulation of merozoite mRNAs proceeds this event. This raises questions concerning the translational capacity of the meront.

The presence of hsp90 mRNA during merogony does not necessarily indicate the presence of protein, as Gerhards et al. [108] have shown with hsp90 of *T. parva* in the piroplasm stage. However, protein and mRNA can be coordinately regulated, as we have shown with the refractile body proteins of *E. bovis* [See Chapter 4]. In contrast to what we observe in sporozoites and during merogony, we could not detect any hsp90 mRNA in un- or partially-sporulated oocysts indicating that hsp90 protein synthesis cannot occur during these stages.

Hsp90 proteins have been hypothesized to be molecular chaperones that escort proteins to their site of function and help proteins fold to their native conformation [96]. Because hsp90 proteins are constituatively expressed, their presence in the cell is likely to be necessary for normal cell function. The hsp90 protein in *Plasmodium falciparum* has been shown to be concentrated within the parasitophorous vacuole in the ring stages of erythrocytic infection [112] and to be associated with the proteins SERP and ABRA which localize to the parasitophorous vacuole [113] indicating that the role of hsp90 may involve the chaperoning and localization of parasite proteins to the parasitophorous vacuole. This observation is in agreement with our finding that there is no hsp90 expression in sporulating *E. bovis* oocysts, in contrast to the fact that most hsp90 proteins are constituatively expressed [95]. Together these data indicate that the function of hsp90 in *E. bovis* is integrally associated with the intracellular stages of development and may play a role in adaptation of the parasite to the host environment. Further studies of the sexual stages of *E. bovis* and confirmation of the expression of protein within the intracellular stages will provide more definitive proof to the function of hsp90 in eimerian parasites.

In addition to the significance of hsp90 protein function in the parasite, recent findings have indicated that parasite hsp90 may also be an important target of the host immune response. Studies with *P. falciparum* hsp90 in squirrel monkeys (*Saimiri sciureus*) have shown that approximately 60% of monkeys immunized with a 90-110

kDa protein fraction, containing *P. falciparum* hsp90, show resistance to parasitemia and that the primary immune response is directed against *P. falciparum* hsp90 [113]. Additionally, parasite hsp90 has been reported to be immunogenic in patients infected with *Trypanosoma cruzi* and *Schistosoma mansonii* [114,115]. These data indicate that *E. bovis* hsp90 might be a good candidate for use as a vaccine against coccidiosis.

In summary, we have identified, by differential screening of a merozoite cDNA library, the *E. bovis* homolog of hsp90. *E. bovis* hsp90 mRNA, in contrast to other eukaryotic systems, is developmentally regulated with no mRNA expressed in the unsporulated or sporulating oocyst. These observations indicate that the role of hsp90 might be unique in coccidian parasites.

CHAPTER 4

CHARACTERIZATION OF THREE DISTINCT REFRACTILE BODY ASSOCIATED PROTEINS IN *EIMERIA BOVIS*

Introduction

The protozoan parasite, *Eimeria bovis,* is one of the causitive agents of bovine coccidiosis. *E. bovis* has a homoxenous life cycle that proceeds through a series of developmental stages common to most coccidian parasites. A striking feature of the *E. bovis* life cycle is the development of the sporozoite into more than 100,000 first-generation merozoites [9]. This biotic potential allows for the occurrence of severe disease by infection with very few oocysts. Certain features, such as the apical complex, are shared by both sporozoites and merozoites of *E. bovis,* but there are also many morphological and biochemical differences (see Chapter 1) that are likely regulated by changes in gene expression. Consistent with this hypothesis, Reduker and Speer [49] have shown by Western analysis that there are large differences between the protein profiles of sporozoites and merozoites. Further, Abrahamsen et al. [50] have estimated that there are up to 5% differences in mRNA species between sporozoites and first-generation merozoites.

Ultrastructurally, the most prominent difference between *E. bovis* sporozoites and merozoites are the refractile bodies, which are found only in the sporozoite stage.

Refractile bodies are moderately electron dense inclusions that are generally found both anterior and posterior to the nucleus of the sporozoites (Fig 1A, Chapter 1). Collectively, refractile bodies can comprise up to 40% of the cellular volume of the sporozoite. Within the *Eimeria* species, three distinct proteins have been identified that localize to the refractile body. The cDNA clones encoding these refractile body proteins (RB-proteins) have been isolated. Clones 6S2 and Ea1A were isolated from an *Eimeria acervulina* cDNA library and encode RB-proteins that show low homology to a cysteine protease and a transhydrogenase, respectively [118,119]. The cDNA clone, Eb25/50, was initially identified as a developmentally regulated protein found in sporozoites and not merozoites which is consistent with the lack of refractile bodies in *E. bovis* first-generation merozoites [120]. This report discusses our investigation of the temporal expression of Eb 25/50 *in vivo,* and compares the expression of Eb25/50, 6S2, and Ea1A at the mRNA and protein level during *E. bovis* development. The results of these studies raise interesting questions concerning the regulation and function of RB-proteins in *E. bovis.*

Materials and Methods

Parasite Production

Eimeria bovis oocysts were produced and isolated as described in Abrahamsen et al., 1993 [61] and in Chapter 2. to study *in vitro* merogony, first-generation merozoites and *E. bovis* sporozoite-infected cells were produced by sporozoite inoculation of EBTr cells at a ratio of 2 sporozoites/cell, as described [120].

cDNA screening.

Total RNA was isolated from a mixture of partially-, and fully-sporulated oocysts of *E. bovis* according to methods described in chapter 2 [61]. A cDNA library was constructed in λgt11 by Stratagene (La Jolla, CA). The library was immunoscreened with the anti-RB protein monoclonal antibody 2.4 [120] using the CLIK II immunoscreening kit (Clonetech, Palo Alto, CA). cDNA inserts from the positive lambda phage were subcloned into the EcoRI site of the plasmid pBS-SK+ (Stratagene, La Jolla, CA). The cDNA library was then screened by hybridization with the largest cDNA insert isolated by immunoscreening. Hybridization was performed at standard conditions as described below. Two additional inserts were isolated and subcloned. The double stranded cDNAs were sequenced by the dideoxy chain termination method as described in Chapter 1. Analysis of the sequencing results was performed by Genepro™ (Riverside Scientific, Seattle, WA) and Lasergene™

(DNASTAR, Madison, WI) computer software. Sequence comparisons against the GenBank database were performed as described in Chapter 1.

Northern analysis.

Northern blots were performed on total RNA isolated from un-, partially-, and fully-sporulated oocysts and first-generation merozoites produced in cell culture as described in Chapter 3.

Recombinant protein expression and antibody preparation.

The largest open reading frame of all of the cDNA clones isolated was subcloned into the expression vector pQE-40 in frame with mouse dihydrofolate reductase (DHFR) and a 6 x Histidine (His) tag used for purification [121]. The construct was made by amplifying the coding region using the polymerase chain reaction (PCR) with primers MA62 and MA60. MA62 (5' ATTTAGGATCCGCAGAAGCATCCACTTTC 3') contains a BamHI site needed for subcloning and binds to nucleotides 4-21 of Eb-20. MA60 (5' ATTAGGATCCGCAACACTGAAGCCGTTC 3') also contains a BamHI site and binds to nucleotides 673-690 of Eb-20. Following PCR, the resulting fragment was purified, digested with *BamHI* and subcloned into the *BglII* site of the expression vector pQE-40 (Qiagen, Chatsworth, CA). The protein was then expressed as a fusion to DHFR with a 6 x His affinity tag that allowed for purification to virtual homogeneity by one step nickel chelate chromatography as described by the manufacturer.

Purified protein 20 was used to generate rat polyclonal antibodies that recognized the *E. bovis* portion of the fusion protein. Immune serum was raised against the recombinant protein by immunizing 7-week old Wistar Furth rats (Harlan Sprague Dawley, Indianapolis, IN) 3 times intraperitoneally at 2-week intervals with ≈50μg of the purified fusion protein (protein 20) emulsified in TiterMax adjuvant (Sigma, St. Louis, MO). Reactivity of immune serum against the DHFR portion of protein 20 was removed using acetone powders prepared from *E. coli* strain M15 containing pQE-40 and expressing DHFR [122].

Western Blot Analysis.

Western blots were performed on protein extracts as described by Abrahamsen et al. [120]. Un-, partially-, and fully-sporulated *E. bovis* oocysts were disrupted in a French press (Aminco, Urbana, IL) in PBS in the presence of 1 mM phenylmethylsulfonyl fluoride, 1mM benzamidine HCl, 10 mM 1,10-phenanthroline, and 10 μg/ml each of pepstatin A, chymostatin, leupeptin, and antipain. Merozoites, excysted sporozoites [49], and sporozoite-infected EBTr cells were disrupted in lysis buffer (2% Nonidet-P40, 150 mM NaCl, 5 mM EDTA, 0.02% NaN_3, 50 mM Tris, pH 8.0) with the same protease inhibitors described above. Recombinant proteins were prepared as described above. Proteins were separated by SDS-PAGE, using reducing or non-reducing conditions, on 12% acrylamide gels. The proteins were electroblotted to nitrocellulose and blocked with horse serum (Quad-5, Ryegate, MT). The blots were

then incubated with primary antibodies followed by incubation with a secondary antibody in a staining apparatus (Miniblotter 25; Biotec, Madison, WI). The mAb 2.4 [120], anti-Ea1A [119], and Dreg 55 [123] were recognized by alkaline phosphatase conjugated anti-mouse (Sigma, St. Louis). Poly 20 and preimmune rat serum were recognized by alkaline phosphatase conjugated anti-rat IgG (Sigma, St. Louis). The polyclonal serum anti-6S2 [118] was recognized by alkaline phosphatase conjugated anti-rabbit (Sigma, St. Louis). Alkaline phosphatase was detected with nitro-blue tetrazolium and BCIP as described previously [123,124,].

In situ protein localization.

Three week old Holstein calves were infected with excysted *E. bovis* sporozoites [49] by injection directly into the ileum. Briefly, laparotomies were performed and the ileo-cecal junction was exposed. One hundred thousand to two million sporozoites were injected into the ileum 45 cm prior to the ileo-cecal junction. The calves were sacrificed and tissues were taken 6, 10, 12, 14, 16, and 18 days following injection of the sporozoites. Calves to be sacrificed at days 6, 10, 12 and 14 were injected with 2 x 10^6 sporozoites to facilitate the localization of the parasites. Calves sacrificed at days 16 and 18 were inoculated with 1 x 10^5 sporozoites to prevent severe disease from occuring. The tissues were cut into ≈3 mm sections and frozen in O.C.T. (Miles Inc., Elkhart, IN). Five μM thin sections were cut on a cryostat microtome (Reichert-Jung Cryocut 1800, Deerfield, IL). The sections were allowed to

air dry after which they were fixed in acetone for 10 min. The sections were again allowed to air dry and then either frozen at -70 C or used immediately. Tissue sections were blocked with 50% fetal calf serum in PBS for 30 min followed by reaction with the primary antibody for 30 min in a humidity chamber at room temperature. The slides were then washed 3 times in 1X PBS followed by 1 wash in PBS containing ≈0.4% rabbit serum. The sections were reacted with secondary antibody conjugated to biotin (1:250) (Tago, Caramillo, CA) in the presence of 2.5% goat serum, 2.5% fetal calf serum for 30 min at room temperature and washed as before. The sections were incubated with horseradish peroxidase conjugated with streptavidin (1:500) (Tago) for 30 min at room temperature followed by washing and reacted with a solution containing 0.0297% hydrogen peroxide, 264μg/ml amino-ethylcarbazol (Sigma, St. Louis, MO), 6.6% N,N dimethylformamide (Sigma) and 0.09M sodium acetate, pH 5.2 for 10 min. The sections were washed in H_2O and then stained for 30 sec in hematoxylin (Richard Allan, Richland, MI) followed by mounting with Gelmount (Biomeda, Foster City, CA).

Results

Cloning and recombinant protein expression of Eb25/50 cDNA

The λgt11 cDNA library prepared from poly-A+ RNA isolated from partially- and fully-sporulated oocysts of *E. bovis* was immunoscreened using the mAb 2.4 [120].

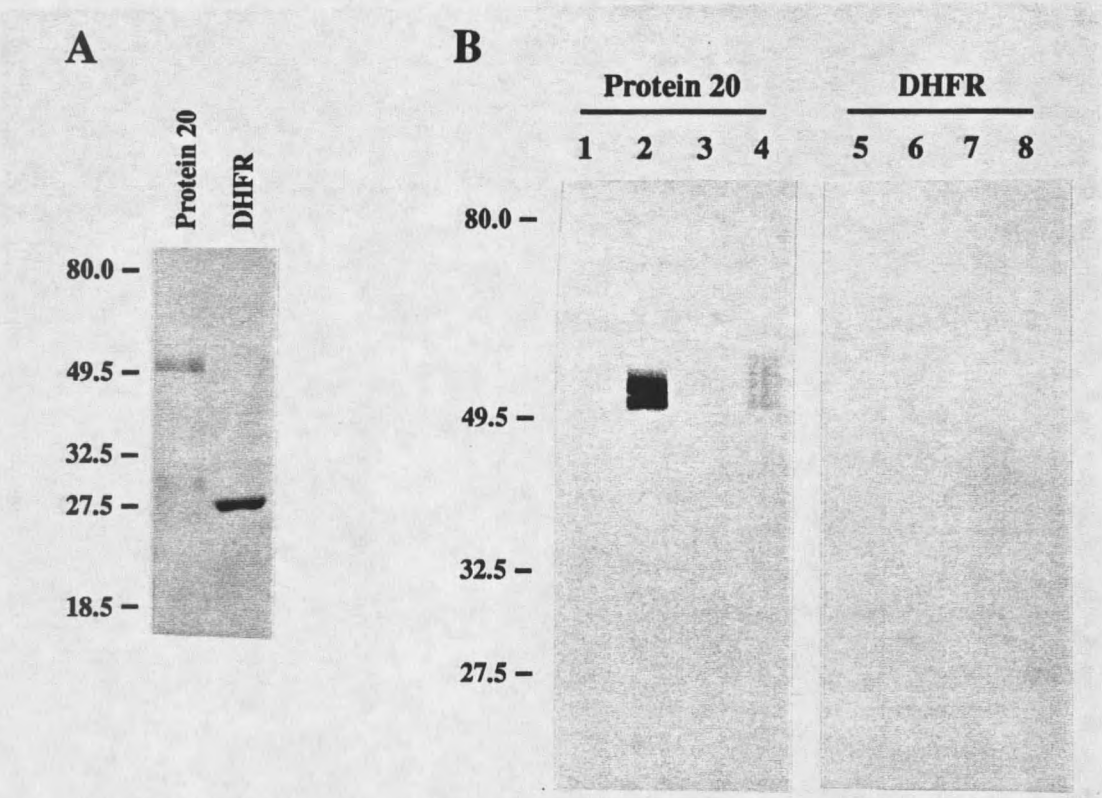
Forty-eight immunoreactive phage were identified and six of these phage were plaque purified and subcloned into pBS-SK+. The clones were then sequenced, revealing inserts of various sizes, but all less than 400 nucleotides. The largest clone, Eb-9.1 was then used to screen the same cDNA library by hybridization to isolate larger cDNA fragments. The two largest clones, Eb-20 and Eb-29 were purified and the inserts subcloned into pBS-SK+ and sequenced. The sequencing revealed fragments of 1275 bp (Eb-20) and 1223 bp (Eb-29) that contained overlapping sequences resulting in a total of 1504 bp of unique sequence designated Eb25/50 (GenBank accession number UO9588). A 627 bp continuous open reading frame beginning with an ATG start codon and ending with a stop codon was identified. An in-frame stop codon was identified 36 bp upstream of the ATG codon, suggesting that the complete coding sequence had been identified. The predicted protein has 208 amino acids and a mass of 22 kDa.

To confirm that the cDNA identified by mAb 2.4 encoded a native *E. bovis* RB protein, the predicted coding sequence was subcloned into the expression vector pQE-40 in frame with mouse dihydrofolate reductase (DHFR) containing a 6 x histidine tag at the amino terminus used for purification by nickel chelate chromatography. Expression of the fusion protein construct produced a protein (protein 20) that migrated at approximately 50 kDa as determined by SDS-PAGE (Fig 11A). The parent construct, pQE-40, produced a protein of 27 kDa, approximately 23 kDa smaller than the fusion protein. The coding sequence of Eb-20 would be expected to add

approximately 22 kDa to the DHFR protein, which is nearly identical to what is observed. The smaller fragments seen with protein 20 could be degradation products or, because the 6 x histidine tag is located at the amino-terminus of the DHFR-fusion protein, premature translational termination products.

Purified protein 20 was used to generate rat polyclonal antibodies that recognized the *E. bovis* portion of the fusion protein. Immune serum prepared from a rat immunized with purified protein 20 (poly 20) reacted with the 50 kDa fusion protein (Fig. 11B, lane 4) but failed to detect purified DHFR (Fig. 11B, lane 8). Preimmune rat serum showed no reactivity with the purified bacterial proteins (Fig. 11B, lanes 3 and 7). As expected, mAb 2.4 [120] displayed the same specificity as poly 20, reacting strongly with protein 20 (Fig. 11B, lane 2), but not the DHFR fusion partner (Fig. 11B, lane 6).

Figure 11. **See Next Page**. Reactivity of polyclonal antiserum to recombinant Protein 20 (Poly 20). **A**. Coomassie brilliant blue staining of affininty-purified recombinant Protein 20 and DHFR (2.5 μg of each protein) separated on SDS-PAGE gels. **B**. Western blot analysis of recombinant Protein 20 and DHFR. Affinity-purified proteins (12 μg) were separated on SDS-PAGE gels and electroblotted to nitrocellulose. The nitrocellulose blots were reacted with mouse secondary antibody alone (lanes 1 and 5), mAb 2.4 (lanes 2 and 6), preimmune rat antiserum (lanes 3 and 7), or Poly 20 (lanes 4 and 8). Bound antibodies were detected by alkaline phosphatase conjugated goat anti-mouse (lanes 2 and 6) or goat anti-rat (lanes 3,4,6 and 8) secondary antibodies. Molecular mass standards are indicated in kDa to the left of each figure. From Abrahamsen et al., 1994 [112].



Poly 20 reacted on Western blots of *E. bovis* sporozoite proteins with multiple antigens in the ranges of 23-28 kDa and 50-60 kDa prepared under both non-reducing and reducing conditions (Fig. 12, lanes 5 and 11). This pattern of reactivity was identical to that of mAb 2.4 (Fig. 12, lanes 1 and 7). Immune serum prepared from a rat immunized with Chinese hamster ovary cells (L3) showed no reactivity with the sporozoite proteins (Fig. 12, lanes 4 and 10). Dreg 55 [123] served as an irrelevant mouse IgG control in these experiments.

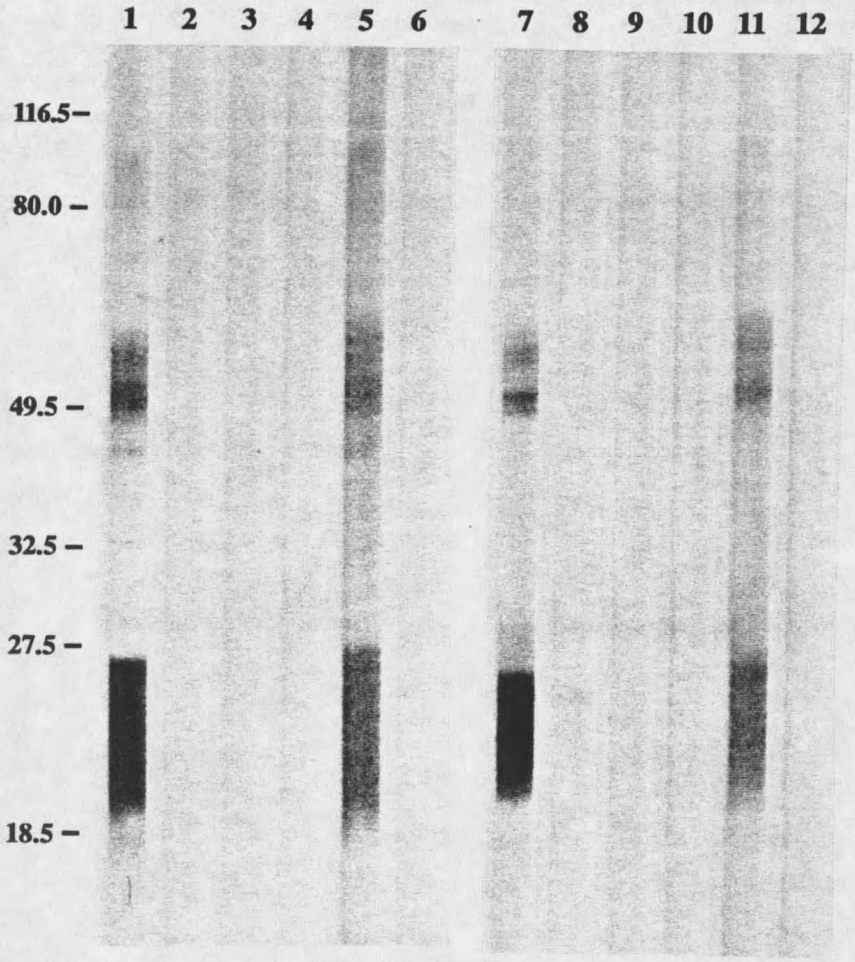


Figure 12. Western blot analysis of *E. bovis* sporozoite proteins reacted with poly 20 antiserum or mAb 2.4. Protein extracts from purified *E. bovis* sporozoites were electrophoresed under non-reducing (lanes 1-6) and reducing (lanes 7-12) conditions on SDS-PAGE gels. Following transfer to nitrocellulose, proteins recognized by mAb 2.4 or Poly 20 were detected as described in Figure 11. Lanes 1 and 7, mAb 2.4; lanes 2 and 8, irrelevant control mAb Dreg 55 [123]; lanes 3 and 9, mouse secondary antibody alone; lanes 4 and 10, irrelevant rat immune serum, L-3; lanes 5 and 11, poly 20; lanes 6 and 12, rat secondary antibody alone. Molecular mass standards are indicated in kDa.

Eb25/50 is homologous to an *E. tenella* sporozoite RB-protein.

Comparison of the Eb25/50 cDNA sequence to those contained in the GenBank database, as determined by FASTA searches [60], revealed a significant identity to a

previously cloned *E. tenella* sporozoite antigen [125,126]. The predicted amino acid sequence of Eb25/50 is aligned for comparison with the *E. tenella* sporozoite protein (Fig. 13). These proteins displayed a 62% identity over their entire amino acid sequences that increased to over 84% when allowing for conservative substitutions.

To determine if the mAb 1209 [127], an antibody that recognizes the *E. tenella* sporozoite antigen described above, would also recognize Eb25/50 protein, we performed Western blot analysis, under reducing conditions, using protein extracts from partially- and fully-sporulated oocysts. In addition, a second Western blot was performed, under the same conditions, using purified protein 20 and recombinant DHFR. Figure 14A shows that mAb 2.4 and mAb 1209 recognize identical patterns of protein in partially- and fully-sporulated oocysts and both antibodies are capable of recognizing protein 20 but do not recognize recombinant DHFR (Fig. 14B).

To further investigate the conservation of Eb25/50 in other eimerian species, an immunofluorescence assay was performed on acetone-fixed *E. bovis* and *E. acervulina* sporozites (Fig. 15). The mAb 2.4 specifically reacted with the *E. acervulina* sporozoites when compared to negative controls, with the staining predominantly localized to the large refractile body (Fig. 15C). This antibody also stains the posterior and anterior refractile bodies in *E. bovis* sporozoites (Fig. 15A).

```
E. bovis      MAEASTFFSGLMGGVVGAVAAVPVP--G------SPGTDWSACYSKLQESGRELEGFV  50
E. tenella    ••D---L••••V•••••••••ADL•AE•ERAPRPA•••A•TC•C•••••GA•••••••

E. bovis      QQLVFIAGKMASCLCAGGDILSRCLAEGRVP---SGSSCCP---IDKSDVDQGLDAAK 102
E. tenella    •••S•V•••L•C••RV•AEQ•A••A••••L•SSS•S••••ALLQLE•Q•LE•S•E•G•

E. bovis      QGANYLMRGGRLVLEALMEGAKVAATRTLVAVEGGKEVVLRNLPYTQDKLSQAYSSFL 160
E. tenella    •••EC•L•SSK•A••••L•••R•••••G•LL••SS•DT•••SI•H••E••A•••••••

E. bovis      RGYQ--SGGRSLGYQGYQAPSYHHQERPSGYGAPQHQQQQPQQPSGGFFW 208
E. tenella    ••••GAAA•••••••AP•AA•GQ•QQ••S•••••••PASS•••••••••
```

Figure 13. Amino acid sequence alignment of the predicted Eb25/50 protein product with an *E. tenella* sporozoite protein [125,126]. Asterisks (*) indicate residues in the *E. tenella* sporozoite protein that are identical with Eb25/50. Gaps in the amino acid sequences are indicated by hyphens. GenBank™ accession numbers; *E. tenella*, X15898; *E. bovis* , UO9588. From Abrahamsen et al., 1994 [112].

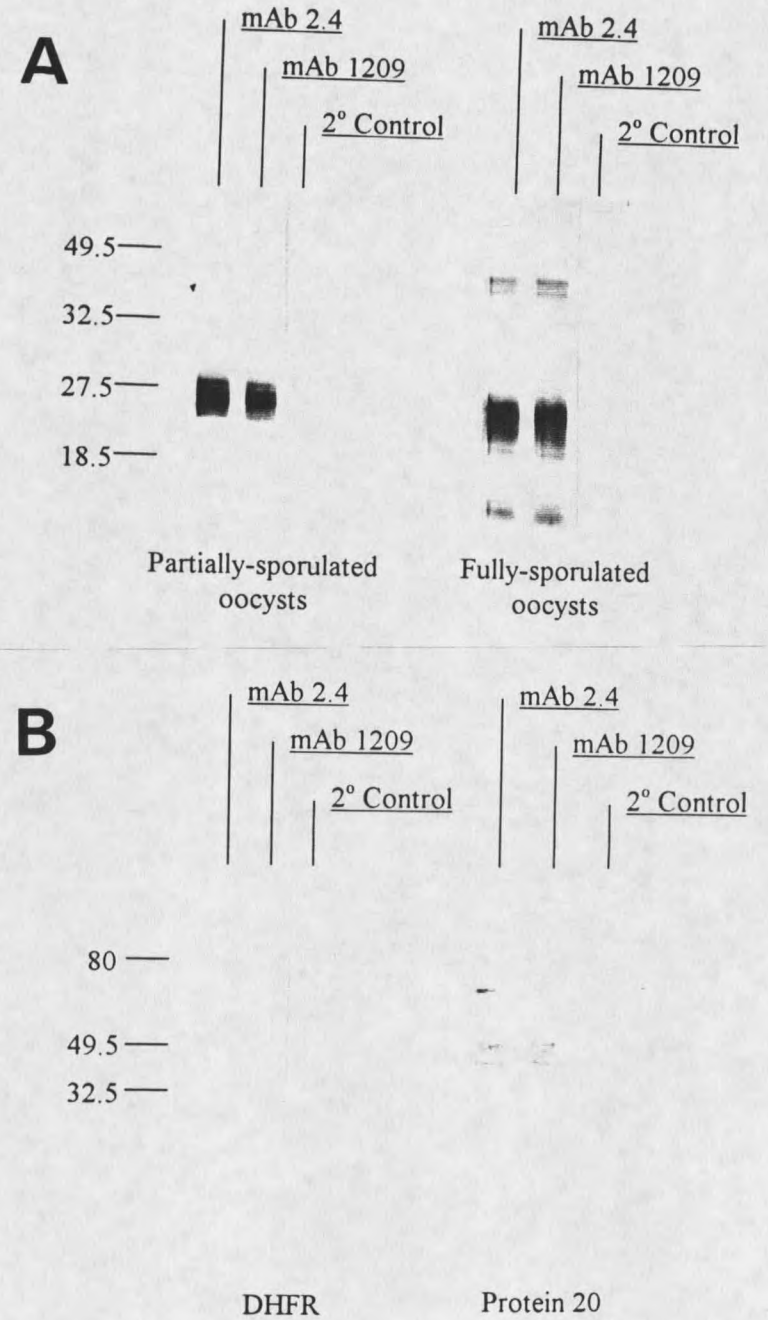


Figure 14. Comparison of mAb 2.4 and mAb 1209 by Western analysis. **A**. Protein extracts from *E. bovis* partially- and fully-sporulated oocysts were electrophoresed on an SDS-PAGE gel, blotted to nitrocellulose, and reacted with primary antibodies mAb 2.4 and mAb 1209 which were detected with an alkaline phosphatase conjugated anti-mouse secondary reagent. **B.** Recombinant Protein 20 and recombinant DHFR were separated by SDS-PAGE , blotted to nitrocellulose, and reacted with mAb 2.4 and mAb 1209 as above.

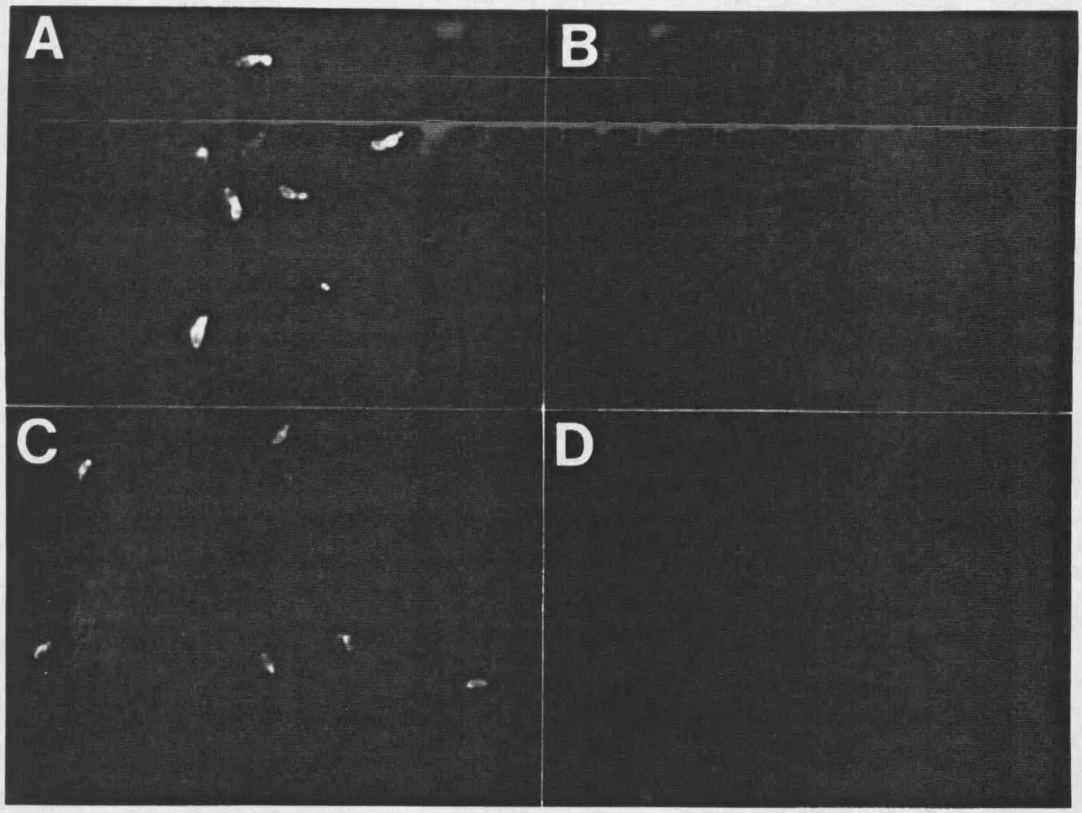


Figure 15. Immunofluorescence photomicrographs (x120) of acetone-fixed *E. bovis* (A and B) and *E. acervulina* (C and D) sporozoites. Sporozoites were purified from oocysts as described in Materials and Methods, applied to microscope slides, and fixed with acetone. The fixed-sporozoites were incubated with mAb 2.4 (A and C) or an irrelevant mAb DREG 55 [123] (B and D). Positive staining was detected with affinity-purified goat anti-mouse Ig conjugated to fluorescein isothiocyanate. From Abrahamsen et al., 1994 [112].

Expression of Refractile Body Protein Eb25/50 is Similar *in vivo* and *in vitro*.

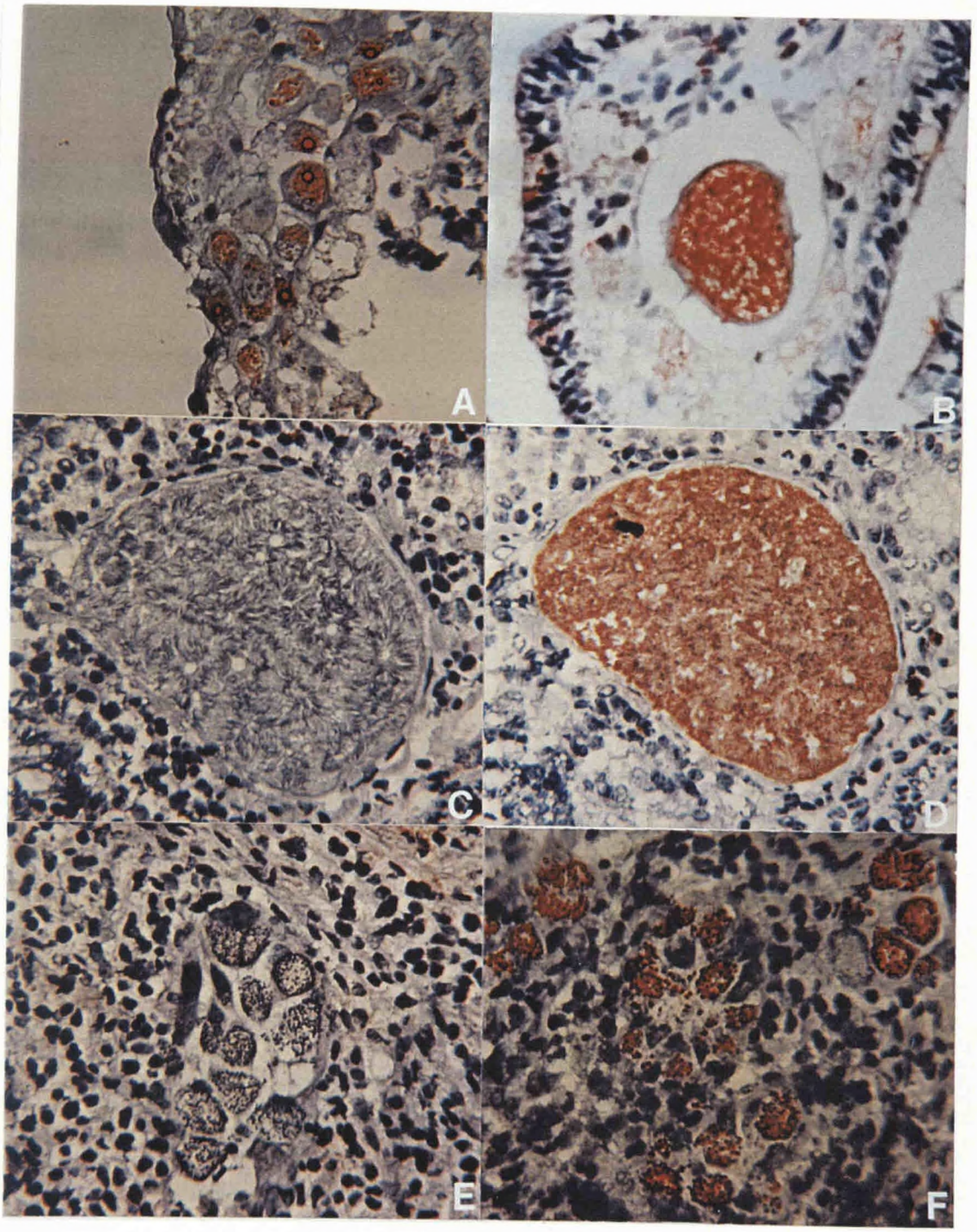
Abrahamsen et al [120] has shown that Eb25/50 protein is expressed in *E. bovis* throughout merogony and the protein is found localized to the host cell early in merogony, restricted to the meront during late merogony, and is turned off as merozoites are released. To determine if the *in vitro* model reflects *in vivo* expression,

an experiment was performed to examine the expression of Eb25/50 in the calf. Sections of various tissues from the G.I. tract of infected Holstein calves, inoculated by surgical implantation of *E. bovis* sporozoites, were stained with mAb 2.4 [120] followed by detection with horseradish peroxidase conjugated secondary reagents. Tissue sections were isolated from infected calves 6, 10, 14, and 18 days following sporozoite inoculation. Sections were taken from tissues representing the G.I. tract of the calves starting at the pyloric valve and continuing to the rectum. *E. bovis* meronts were identified in tissue sections from the terminal ileum of the small intestine 6, 10, 12 and 14 days following sporozoite inoculation. Meronts from these sections were found localized to the endothelial cells of the central lacteals as described [128]. Neither meronts or any other developmental stge of *E. bovis* were found in other tissues examined from these timepoints. *E. bovis* sexual stages were identified in calves 18 days after sporozoite inoculation and were found in the epithelial cells of the crypts in the cecum and large intestine. No other *E. bovis* developmental stages were identified in the cecum, large intestine or other gut tissues examined in the 18 day infected animals. Because of the small size of the sporozoite and early meront [129], we were unable to detect the parasites earlier than 6 days following inoculation.

Meronts from the terminal ileum of an *E. bovis* infected calf 6 days post-inoculation showed positive staining with mAb 2.4 throughout the parasitophorous vacuole, with some diffuse host cell staining similar to what was observed *in vitro* [120] (Figure 15 A). Meronts from a 10 day *E. bovis*-infected calf show strong

staining restricted to the meront. Mature meronts containing fully formed first-generation merozoites from 14 day *E. bovis* infected calves, however, reveal no expression of Eb25/50 protein (Figure 16 C). To determine if the lack of staining of day 14 meronts was due to lack of Eb25/50 protein or an artifact associated with the inability to stain the tissues, we stained serial sections with the monoclonal antibody MZ25 which stains mature meronts and first-generation merozoites [Clark, T.G., R.R. Johnson, M.A. Jutila and M.W. White, unpublished observations]. The monoclonal MZ25 stained the day 14 meronts very strongly (Fig. 16 D). Similar to the staining observed with mAb 2.4 on day 14 meronts, mAb 2.4 does not react with the sexual stages from day 18 *E. bovis* infected proximal colon (Fig. 16 E). However, EA7, a monoclonal antibody generated against sexual stages from *E. tenella* [130], shows strong staining of the wall forming bodies in the day 18 proximal colon sections (Fig. 16 F).

Figure 16. **See Next Page.** In situ localization of the Eb25/50 antigens during *in vivo* development of *E. bovis* . Calves that had been surgically inoculated with *E. bovis* sporozoites were sacrificed 6, 10, 14, and 18 days after infection. Tissues sections (5 μm) from the ileum and colon were prepared and incubated with mAb 2.4, mAb MZ25 or mAb EA7. Antibody reactivity was visualized using a three-stage avidin-biotin immunoperoxidase staining kit (Tago Inc., Camarillo, CA) and the sections were counterstained with hematoxylin. **A.** Day 6 section from ileum stained with mAb 2.4. **B.** Day 10 section from ileum stained with mAb 2.4. **C.** Day 14 section from ileum stained with mAb 2.4. **D.** Day 14 section from ileum stained with mAb MZ25. **E.** Day 18 section from colon stained with mAb 2.4. **F.** Day 18 section from colon stained with mAb EA7.


A
B
C
D
E
F

Expression of Eb25/50 mRNA and Protein During Sporulation

Northern blots of total RNA isolated from unsporulated, partially-sporulated and fully-sporulated oocysts and first-generation merozoites were probed with ^{32}P-labeled cDNA insert from Eb-9.1. A single mRNA species of ≈1.4 kilobase pairs (kbp) in size was detected that was expressed at low levels in unsporulated oocysts, abundantly expressed in partially-sporulated and fully-sporulated oocysts and absent in first-generation merozoites (Fig. 16C). Several minor mRNA species were also detected but at substantially lower levels.

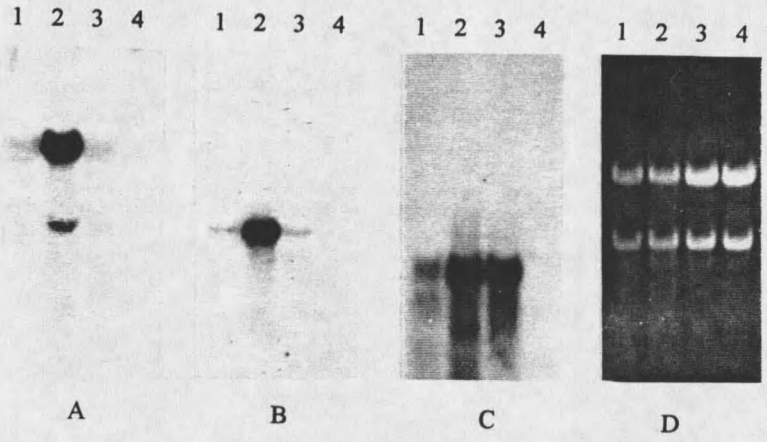


Figure 17. Developmental expression of RB protein mRNAs during sporulation. Northern blots were prepared using total RNA (5 μg) isolated from unsporulated (lane 1), partially-sporulated (lane 2) and fully-sporulated (lane 3) *E. bovis* oocysts and first-generation merozoites (lane 4). The nitrocellulose blots were then hybridized to ^{32}P-labeled Eb-9.1 cDNA insert, 6S2 cDNA insert, or Ea1A cDNA insert. **A.** Northern blot of Ea1A. **B.** Northern blot of 6S2. **C.** Northern blot of Eb25/50. **D**. Ethidium bromide stain prior to nitrocellulose transfer to demonstrate equal loading and quality of the RNA samples and show size of mRNAs relative to ribosomal RNAs.

To determine if expression of Eb25/50 protein expression correlates with Eb25/50 mRNA expression during sporulation, Western blots were performed using protein extacts from un-, partially-, and fully-sporulated oocysts and *E. bovis* infected EBTr cells harvested 1 and 3 days following sporozoite infection. The gels were loaded with approximately 177,000 oocysts/lane and 660,000 sporozoites/lane in the infected cells. The mAb 2.4 antigens were expressed at low levels as a single band in unsporulated oocysts, but were highly expressed in partially-sporulated and fully-sporulated oocysts and were also expressed in day 1 and day 3 *E. bovis* infected EBTr cells during merogony (Fig. 18). This pattern of protein expression correlates well with the kinetics of Eb25/50 mRNA expression during sporulation.

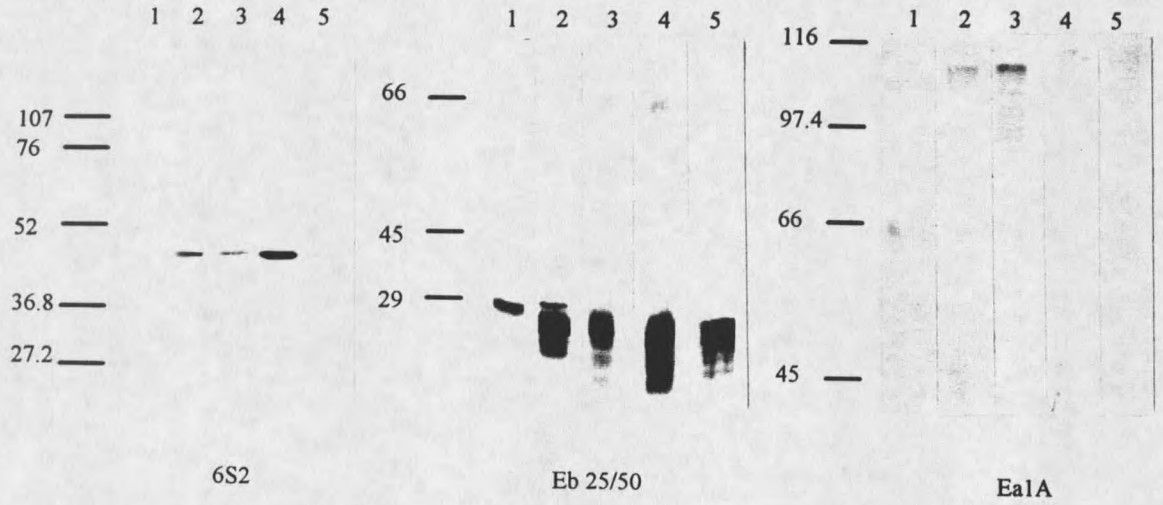


Figure 18. **See Previous Page.**Western blot analysis of RB Proteins in *E. bovis* . Extracts of un-, partially-, and fully sporulated oocysts and day 1 and day 3 infected EBTr's were separated by SDS-PAGE under reducing conditions. The equivalent of 5 x 10^5 sporozoites were run for the oocyst extracts and the equivalent of 2 x 10^6 sporozoites were run for the infected cell extracts. The proteins were transferred to nitrocellulose by electroblotting and incubated with either mAb 2.4, polyclonal anti-6S2 [118] or mAb anti-Ea1A [119]. The antibodies were detected with either anti-mouse conjugated alkaline phosphatase or anti-rabbit conjugated alkaline phosphatase. Lane 1, unsporulated oocysts; lane 2, partially sporulated oocysts; lane 3, fully sporulated oocysts; lane 4, day 1 sporozoite infected EBTr; lane 5, day 3 sporozoite infected EBTr.

Expression of Ea1A and 6S2 during sporulation and merogony.

Proteins encoded by 6S2 and Ea1A cDNAs have previously been shown to be associated with the refractile body of eimerian sporozoites [118,119]. To determine if these RB-proteins possessed similar temporal expression patterns to that of Eb25/50 we initiated studies on both mRNA and protein expression *in vitro*.

We examined by Northern blot the kinetics of 6S2 and Ea1A mRNAs during sporulation. Northern analysis of *E. bovis* total RNA using ^{32}P-labeled Ea1A and 6S2 cDNA inserts showed that Ea1A and 6S2 mRNAs are expressed at high levels in partially-sporulated oocysts (Fig. 17, panel A and B, lane 2) with un- (Fig. 17, panel A and B, lane 1), and fully-sporulated (Fig. 17, panel A and B, lane 3) oocysts showing only low expression, and no expression in merozoites (Fig. 17, panel A and B, lane 4). We could detect no Ea1A or 6S2 mRNA during merogony (data not shown).

The 6S2 cDNA insert identified only one species of mRNA that migrated at approximately 2800 nt. The Ea1A cDNA insert, however, identified two species of mRNA that possessed the same kinetics. The larger species migrated with a predicted

size of 6200 nt and the smaller species migrated at approximately 3100 nt. The two mRNA species identified by the Ea1A probe may be the result of alternative mRNA processing, although further work is necessary to confirm this.

Expression of 6S2 and Ea1A RB-proteins during sporulation and early merogony follows closely the pattern of expression observed for their encoded mRNAs. RB-protein 6S2 was not detected in unsporulated oocysts (Fig. 18, lane1), but revealed substantial levels in partially-sporulated oocysts (Fig. 18, lane 2) (consistent with the large increase in mRNA) and fully-sporulated oocysts (Fig. 18, lane 3). The level of 6S2 protein remained high in day 1 infected cells (Fig. 18, lane 4) but was substantially reduced by day 3 of merogony (Fig. 18, lane 5). RB-protein Ea1A showed similar kinetics, although the protein was nearly undetectable in day 1 and day 3 infected cells. Unlike the complex staining pattern of Eb25/50 (Fig. 18) anti-Ea1A and anti-6S2 antibodies identified, bands at 100 kDa and 51 kDa, respectively, which are in agreement with their predicted molecular weights [118,119].

Discussion

Critical to the understanding of *E. bovis*, and ultimately to the treatment of bovine coccidiosis is knowledge of the unique biochemical pathways that distinguish *E. bovis* from its host. We have focused our studies on identifying characteristics that are both unique to the parasite, to distinguish it from its host, and unique to a particular developmental stage so that developmentally regulated genes that contribute to these

characteristics can be identified. In this manuscript we report our studies on the expression of RB-proteins, Eb25/50, 6S2 and Ea1A. Our findings show that all three RB-proteins and their corresponding mRNAs begin to be expressed during oocyst sporulation but are not expressed in merozoites. These observations are in agreement with the ultrastructural evidence that *E. bovis* sporozoites possess refractile bodies whereas merozoites do not [8].

Although all three refractile body proteins begin their expression during sporozoite formation, there is a significant difference in their expression during merogony. Ea1A and 6S2 mRNAs are not expressed during merogony and their protein products are only detectable during early merogony, whereas Eb25/50 is expressed throughout merogony until just before merozoite release [120]. Our data also demonstrate that regulation of Eb25/50 *in vivo* is similar to what is observed *in vitro*. These data confirm that the absence of Eb25/50 in merozoites is not an artifact of the *in vitro* system.

Eb25/50 protein is highly processed and the processing appears to be developmentally regulated. Eb25/50 protein is present as a single 21 kDa protein species in unsporulated oocysts and a complex smear in sporozoites and meronts. This pattern is not the result multiple genes because Eb25/50 is present as a single copy gene [112] encoding a single mRNA species. Further, there are no introns present in the coding region that could lead to differential splicing (data not shown). There are many possibilites to explain the complex protein profile observed for Eb25/50, including lipid

conjugation, phosphorylation and glycosylation. If glycosylation is responsible for the complex pattern observed with Eb25/50, it is likely O-linked because there are no consensus N-linked glycosylation sites within the amino acid sequence.

Although the 6S2 and Ea1A proteins do not appear to be post-translationally modified, as Eb25/50, all three proteins localize to the refractile body, indicating that at least a portion of their trafficking pathway is similar. Supporting this observation, a motif was identified at the amino terminus of each of the RB-proteins that was highly similar (Table 4). These sequences are also similar to the consensus signal sequences that have been deduced for exported proteins [131]. The refractile body motif begins with a serine followed by a strong hydrophobic region, containing double valines. A glycine is present 8 to 9 residues from the serine and is conserved across all sequences. Finally, a proline is found 15 to 16 residues from the serine and denotes the end of the motif. Interestingly, no motifs have been identified in proteins that traffic to dense granules (GRA1, 2, 4 and 5, Table 4), another inclusion body of coccidian parasites, or in proteins destined for the surface of coccidian parasites (SAG1 and 3, Table 4).

Dense Granule Associated Proteins	
GRA 1	MVRVSAIVGAAASVFVCLSAGAYAAEGGDN
GRA 2	MFAVKHLCLLVVAVGALVNVSVRAAEFSGV
GRA 4	MQGTWFSLFVVVMVSHLACGGECSFGSHLA
GRA 5	MASVKRVAVMIVNVLALIFGVAGSTRDVGS
SAG Proteins	
SAG 1	MSVSLHHFIISSGFLTSMFPKAVRRAVTAG
SAG 3	MQLWRRRAAGPASLGRQSLPLGCFFAAFGL
Refractile Body Proteins	
Eb 25/50	MAEASTFF**S**GLMGGVV**G**AVAAV**P**VPGSPGT
Et1a1	MSEVNPEMS**S**YDVVLVV**G**ANDTVN**P**AALEP
6S2	MR**S**LLVVAGLA**G**CSSFA**P**TDARHRFLSETL

Table 4. Comparison of Amino Terminal Sequences of Coccidian Proteins. The amino terminal sequences of the RB-proteins are shown with the putative signal sequences underlined. A serine (in bold) begins the motif with a glycine (in bold) midway through the motif and a proline (in bold) at the end of the sequence. Valine pairs are double underlined. The amino terminal sequences of SAG and GRA proteins are shown for comparison.

Refractile bodies are the most prominent inclusions in eimerian sporozoites but their function is still undetermined. Doran [132] has shown that refractile bodies appear

to diminish in size and disappear during first-generation merogony leading several authors to speculate that the refractile body is an energy reserve for the parasite that is metabolized during early merogony. RB-protein, Ea1A, which has been described as a putative transhydrogenase and glucose transporter [119], supports the proposal that the refractile bodies may be involved in energy metabolism. Vermeulen et al. [119] have suggested that RB-protein Ea1A might be involved in mannitol metabolism by regulating the levels of the pyridine nucleotides NAD(P) and NAD(P)H+, required for the conversion of fructose-6-phosphate to mannitol-1-phosphate.

Results from our and others studies, however, are not consistent with the consumption of refractile bodies to meet the energy needs of *E. bovis*. The RB-protein Ea1A is barely detectable 3 days following sporozoite infection, whereas previous reports [133,134] have indicated that the refractile bodies of eimerian sporozoites are not consumed but are instead found dispersing throughout the cytoplasm of the meront as small globules at least 6 days after sporozoite infection. Further, Danforth and Augustine [135] have proposed that some refractile body material may need to be continually synthesized throughout merogony, which is consistent with their observations that the protein recognized by mAb 1209 is found throughout the meront in *E. tenella* during merogony, and our observations that the RB-protein Eb25/50 is continually synthesized throughout merogony.

Although the function of the refractile bodies is still unknown, it appears they are essential for merozoite development. When *E. tenella* sporozoite infected cells are

incubated with mAb 1209, preventing the redistribution of the mAb 1209 protein, merozoite production is diminished [135]. Further, attenuated strains of *Eimeria* species, which produce fewer merozoites than corresponding wild-type strains, possess fewer refractile bodies than the wild-type strains, and in some strains the refractile bodies are completely absent [136]. These data suggest that refractile-body formation is directly associated with merozoite production.

The RB-proteins show strong conservation across many coccidians. RB-proteins 6S2 and Ea1A were isolated from different *Eimeria* species of avian coccidia and have also been found in *E. bovis*. RB-protein Eb25/50 has been shown to be conserved across many different species of eimerian parasites [112]. Eb25/50 is recognized by mAb 1209 which also recognizes the *E. tenella* sporozoite antigen isolated by Liberator et al. [125,137]. Furthermore, the mAb 1209 has been used in immunoelectron microscopic studies to identify a protein present in *Lankesterella minima* sporozoites, a coccidian that infects frog erythrocytes [138]. Collectively, the high degree of conservation of the RB-proteins implies that their functions are likely important in the development of coccidian sporozoites.

CHAPTER 5

CONCLUSIONS

The findings in this thesis are the result of a new approach to the study of eimerian biochemistry. Through these techniques we have been able to identify many stage specific cDNA of both known and unknown function, that possess unique patterns of regulation. From these studies we have made the following conclusions:

1. Although *E. bovis* sporozoites and merozoites are morphologically similar, there are many differences in gene expression. These differences may be up to 5% of total mRNA expression. The differences between sporozoite and merozoite mRNA expression is much higher than the differences in mRNA expression observed between resting and activated mammalian cells, which is approximately .01% [50].

2. Our studies (Chapter 1, published Molecular and Biochemical Parasitology [54]) have shown that many of the genes described as constitutively expressed in other systems are differentially expressed in *Eimeria bovis*. Genes such as EF-1α, ubiquitin and hsp90 mRNAs are highly developmentally regulated in *E. bovis*. For example, *E. bovis* hsp90 mRNA is not expressed during sporulation, which is in contradiction to observations that hsp90 is constitutively expressed in other eukaryotes. The pattern of

expression of *E. bovis* hsp90, being found only in stages that are or will be intracellular, is consistent with the proposed role of hsp90 in *Plasmodium falciparum* where it may chaperone proteins destined for the parasitophorous vacuole. If hsp90 in *E. bovis* is responsible for or assists in the adaptation of the parasitophorous vacuole to the parasite then it becomes a strong candidate for drug development provided it is sufficiently dissimilar from host hsp90. Drugs designed to specifically inhibit parasite hsp90 function might prevent intracellular adaptation enabling the host cell to kill the parasite. Further, use of hsp90 as a potential vaccine candidate has already been proposed. *Plasmodium falciparum* hsp90 is one of the most immunodominant antigens in parasite preparations, indicating that parasite hsp90 may be a potential target for vaccine development.

3. Consistent with the observation that there are major differences between sporozoites and merozoites is the observation that only sporozoites possess refractile bodies. Not surprisingly, proteins that localize to the refractile body are developmentally regulated. Refractile-body proteins in eimerian sporozoites begin to be expressed during mid-sporulation and are found in fully-formed sporozoites. However, the RB-proteins are not expressed in the same fashion during merogony. The RB-proteins 6S2 and Ea1A are expressed only in the oocyst and in early merogony, but not during late merogony, whereas the RB-protein Eb25/50 is continually expressed throughout merogony. Eb25/50 is turned off just prior to merozoite release and is not expressed during the

sexual stages, which is consistent with their lack of refractile bodies (Eb25/50 data published in Molecular and Biochemical Parasitology [112]).

4. The expression of Eb25/50 has been found to be identical *in vitro* and *in vivo*. Studies of tissues from calves infected with *E. bovis* sporozoites have shown that Eb25/50 is expressed throughout merogony *in vivo* in a manner that is similar in both spatial and temporal expression to *in vitro* expression. This represents the first *E. bovis* gene that has been characterized by its pattern of protein expression both *in vivo* and *in vitro*, and further validates the *in vitro* system for the study of gene expression.

5. A sequence has been identified at the amino terminal ends of the three RB-proteins that is similar to signal sequences of the exocytic pathway of higher eukaryotes. This motif may be the signal responsible for trafficking and localization of the RB-proteins to the refractile bodies. In addition to the signal motif identified in the RB-proteins, Eb25/50 is highly modified post-translationally and there is some indication that these modifications are the result of glycosylation, consistent with its localization via the exocytic pathway. There are, however, no N-containing glycosylation sites within the coding region of Eb25/50, indicating that if the post-translational modifications to Eb25/50 are glycosylations, then they must be O-linked. Finally, it also appears that the post-translational modification of Eb25/50 is developmentally regulated, implying that

the function of Eb25/50 may be dependent on the post-translational modifications and that the processing of modified proteins may also be developmentally regulated.

6. The expression of the RB-proteins is not consistent with a proposed role for refractile bodies as energy stores. Although the function of the refractile body remains unknown, it is clear that refractile bodies are necessary for merozoite development. Refractile body material can be observed at least 6 days following sporozoite invasion of host cells and Eb25/50 is continually synthesized throughout merogony. Further, if infected cells are incubated with an antibody that recognizes Eb25/50 protein, merozoite production is inhibited. The differences in the characteristics of the three RB-proteins indicates that the refractile body may simply be a store to hold various components that will be necessary for merogony. The specific roles of the RB-proteins may, therefore, be unrelated except in the sense that they all assist in adaptatation of the host by the parasite. Both 6S2 and Ea1A have been shown to be homologous to previously identified families of proteins, the aspartyl proteases and transhydrogenases, respectively. Eb25/50, however, has no significant homology to any protein with known function, although it appears that Eb25/50 protein function is required for a longer duration during merogony than 6S2 or Ea1A. Eb25/50 also appears to directly interact with the host cell as shown by Abrahamsen et al. [120] where Eb25/50 protein can be found throughout the host cell cytoplasm. This indicates that the role of Eb25/50 may be to modify the host cell through direct interaction.

7. The abrupt down-regulation of Eb25/50 protein immediately before merozoite release indicates that Eb25/50 protein expression may be deleterious to merozoite function. Supporting this suggestion is the observation that merozoites produced in culture, that cannot develop further, possess some Eb25/50 protein immediately after release [120]. Our studies have shown that, in vivo, Eb25/50 protein is completely absent in mature meronts prior to merozoite release. This difference may be a clue to the arrested development of *E. bovis* merozoites in culture. Eb25/50 will provide an excellent marker to study this phenomenon.

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