IDENTIFICATION AND CHARACTERIZATION OF PUTATIVE ERYTHROCYTE BINDING PROTEIN GENES IN *Plasmodium falciparum*

by

PAUL BASSANIO DRUMMOND

(Under Direction the of DAVID S. PETERSON)

The erythrocyte binding proteins (EBP) of *Plasmodium falciparum* are ligands that play a functional role in erythrocyte invasion by merozoites. Six EBP genes have already been identified in the *P. falciparum* genome. However, their role in invasion and potential interaction is not completely understood. A series of experimental studies were conducted to identify, and to characterize these EBP genes.

A Polymerize Chain Reaction (PCR) approach was used to identify potential EBP genes in two *P. falciparum* isolates. Several clones sequenced were known EBP genes, which validated our approach to identified novel members of the EBP family. However, research to determine the identity of these sequences is on going.

A study was designed to investigate the level of sequence conservation within the Duffy binding-like domains of one member of the EBP family, the erythrocyte binding-like 1 (*EBL-1*) gene in ten *P. falciparum* isolates. The result indicates a degree of polymorphism in the Duffy binding-like domains comparable to that observed for erythrocyte binding antigen-175 (a member of the EBP family). Additionally, there was a clear bias towards nonsynonymous substitution in the *EBL-1* Duffy binding-like domains. Our result obtained from this study

suggests that *ebl-1* is not essential in some isolates. The results suggest that the diversity observed in the Duffy binding-like region of the *ebl-1* gene in *P.falciparum* may either be driven by host immune response, or may indicate receptor heterogeneity.

The final goal of this work was a comprehensive study of EBP gene expression during the intraerythrocytic developmental cycle in three *P. falciparum* isolates. Our results show that expression of EBPs is highly stage specific, with expression peaking at 36-hr in the 3D7 and Dd2 isolate, and at 44-hr in the Dd2-NM clone. We also found that *eba-175* had the highest level of expression, while *maebl* was the least expressed in all three isolates. We also found one gene that is not expressed in all isolates.

INDEX WORDS: *Plasmodium falciparum*, erythrocyte binding protein, Duffy binding-like, erythrocyte binding like-1, gene expression

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DEDICATION

This Dissertation is dedicated to the memory of my late parents, Lester and Rubena Drummond, whose exemplary life's, commitment, and source of inspiration had made indelible impression upon my life. To my other family members for being there for me; keeping me encouraged and for their understanding during the times I just could not be there for them. Thanks be to God.

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CHAPTER 1

INTRODUCTION

Malaria is caused by protozoan parasites of the genus *Plasmodium* that belong to the phylum Apicomplexa. Malaria remains a major health problem in many parts of the world, especially in the tropical and subtropical regions, particularly sub-Saharan Africa [1]. These obligate intracellular parasites depend on the successful invasion of an appropriate host cell for their survival. The parasite is transmitted to humans by the bite of an infected female (Anopheles sp.) mosquito. There are four species of *Plasmodium* (*P. falciparum, P. ovale, P.vivax* and *P. malariae*) that are responsible for the disease. The most dangerous of the *Plasmodium* species infecting humans is *P. falciparum*, which is responsible for an estimated 2 million deaths and 300-500 million infections per year worldwide [2]. Also, billions of individuals are at risk of acquiring malaria in over 100 countries [3, 4]. More than 90% of malaria cases and deaths occur in Africa south of the Sahara, mostly among children. It is estimated that malaria claims more than 100,000 lives per year elsewhere in the world and these deaths occur in all age groups [4].

Over the last eight years 1000–1500 (per year) reported cases of malaria with the onset of symptoms have been reported among persons in the United States or its territories [5-8]. In the United States most of the infections occur among persons who have traveled to an endemic area. However, other cases may occur through exposure to infected blood products, by local mosquito-borne transmission or by congenital transmission.

The disease pathology is caused by those parasite stages that multiply asexually in the red blood cells (RBC). The blood-stage development of malaria parasites is initiated by the invasion of merozoites into susceptible erythrocytes. The ability of *Plasmodium* merozoites to invade erythrocytes is contingent on a rapid cascade of specific interactions occurring between parasite molecules and host erythrocyte receptors. After random contact of the merozoite with the erythrocyte, a weak interaction between the surface components leads to reorientation to bring the apical end close to the erythrocyte. Proteins sequestered within the merozoite's apical organelles, including the rhoptries, micronemes and dense granules, then come into play. Microneme proteins that are involved in invasion include the erythrocyte binding antigen-175 (EBA-175) in *P. falciparum* and the Duffy antigen binding proteins (DABPs) in *P. vivax* and *P. knowlesi* [9, 10]. *P. falciparum* is known to use different receptors for invasion of erythrocytes and commonly invades via sialic acid residues present on glycophorin A or B [11]. Mutant RBCs with modifications or deficiencies in glycophorins are, in general, less susceptible to invasion by *P. falciparum* than normal erythrocytes [12]. However, *P. falciparum* appears not to be restricted to invasion via a single class of erythrocyte receptor. Indeed, there are at least five different pathways for ligand-receptor binding: glycophorin A, glycophorin B, glycophorin C, and at least two more pathways that have not yet been characterized, referred to as receptors E and X [11, 13-16]. The possibility of "switching" between, or up-regulating, novel phenotypes introduces a further element of complexity into the invasion process and was demonstrated through the ability of the Dd2 line of *P. falciparum* [17] to be selected for invasion via a pathway mediated by a different host cell receptor.

Recently, several novel members of the Duffy binding like (DBL) domain superfamily have been identified in open reading frames in the *P. falciparum* genome and described,

including *ebl-1* [18], *ebp-2/baebl* [19, 20], *eba-181*/*jesebl* [16, 21], *eba-165/pebl* [22], and *maebl* [21]. The identification and characterization of novel erythrocyte binding proteins (EBP) is an important step toward understanding the interactions between this class of parasite ligands and their host receptors. Since the members of this gene family have been proposed as vaccine candidates, against the erythrocytic stage of *P. falciparum*.

Development of an effective and deployable malaria vaccine seems possible in the malaria research community. For approximately 20 years, progress occurred are mainly in the form of experimental models instead of human vaccine trials [23]. The rate of clinical test for malaria vaccines has been increasing, in the last 5 years [24, 25]. Many potential vaccine candidates are at the point of preclinical assessment.

The intermediate goals of vaccine research can be divided into three areas: induction of strong, strain-transcending, durable immune responses [26]; identification of protective antigens for stage specific immunity; and successful combination of candidate immunogens. At present the best-characterized pre-erythrocyte antigen is the circumsporozoite protein (CSP) and vaccines made of CSP were shown to be safe and immunogenic in humans, but limited efficacy. However, a more effective approach been used today is a multi-gene, multi-stage DNA vaccine programmed. One of these DNA vaccine formulations, known as MuStDO9 consist of pre-erythrocyte antigens such as *CSP*, *LSA1* and *3*, *SSP2*, *EXP1* and erythrocytic antigens *EBA-175*, two alleles of *MSP-1*, and *AMA*-1 have been fairly effective against malaria [27]. Transmission-blocking vaccines (TBV) against malaria are intended to induce immunity against the stages (sexual) of the parasite that infect Anopheles mosquito, which would allow individuals who are immunized with TBVs will not be able to transmit malaria. *Pfs25* and *Pfs28* antigens, are the most widely know sexual antigens and are the

furthest along in developmental and testing.[28, 29]. Despite the level of difficulty that has been encountered in developing a malaria vaccine, recent progress such as the Malaria Genome Project gives us great hope of developing an effective vaccine.

The overall objective of this project was to identify novel erythrocyte binding protein (EBP) genes, to determine the level of sequence variation of known genes in different strains of *P. falciparum*, and to characterize the expression of known ebp genes. Specific aims of this study were:

(1) To determine if there are additional EBP's that may mediate red blood cell invasion

(2) To determine the level of sequence conservation within the ligand domains of the erythrocyte binding-like 1 gene

(3) To determine expression of EBP's in Dd2 and Dd2-NM clones

References

1. WHO, Malaria. Report No. 94 Fact Sheet. Geneva. 1996(Report No. 94 Fact sheet. Geneva).
2. WHO, WHO Technical Report Series, No. 892. 2000.
3. Trigg PI and Kondrachine AV, Commentary: malaria control in the 1990s. Bull World Health Organ, 1998. **76**(1): p. 11-6.
4. Kondrachine AV and Trigg PI, Global overview of malaria. Indian J Med Res, 1997. **106**: p. 39-52.
5. Williams HA, Roberts J, Kachur SP, et al., Malaria surveillance--United States, 1995. Mor Mortal Wkly Rep CDC Surveill Summ, 1999. **48**(1): p. 1-23.

6. Holtz TH, Kachur SP, MacArthur JR, et al., Malaria surveillance--United States, 1998. MMWR CDC Surveill Summ, 2001. **50**(5): p. 1-20.

7. Newman RD, Barber AM, Roberts J, et al., Malaria surveillance--United States, 1999. MMWR Surveill Summ, 2002. **51**(1): p. 15-28.

8. Filler S, Causer LM, Newman RD, et al., Malaria surveillance--United States, 2001. MMWR Surveill Summ, 2003. **52**(5): p. 1-14.

9. Adams JH, Hudson DE, Torii M, et al., The Duffy receptor family of Plasmodium knowlesi is located within the micronemes of invasive malaria merozoites. Cell., 1990. **63**(1): p. 141-153.

10. Sim BK, Toyoshima T, Haynes JD, et al., Localization of the 175-kilodalton erythrocyte binding antigen in micronemes of Plasmodium falciparum merozoites. Mol. Biochem. Parasitol., 1992. **51**(1): p. 157-159.

11. Dolan SA, Proctor JL, Alling DW, et al., Glycophorin B as an EBA-175 independent Plasmodium falciparum receptor of human erythrocytes. Mol. Biochem. Parasitol., 1994. **64**: p. 55-63.

12. Hadley TJ, Klotz FW, Pasvol G, et al., Falciparum malaria parasites invade erythrocytes that lack glycophorin A and B (MkMk). Strain differences indicate receptor heterogeneity and two pathways for invasion. J. Clin. Invest., 1987. **80**(4): p. 1190-1193.

13. Singh S, Pandey K, Chattopadhayay R, et al., Biochemical, biophysical, and functional characterization of bacterially expressed and refolded receptor binding domain of Plasmodium vivax duffy-binding protein. J Biol Chem, 2001. **276**(20): p. 17111-6.

14. Lobo CA, Rodriguez M, Reid M, et al., Glycophorin C is the receptor for the Plasmodium falciparum erythrocyte binding ligand PfEBP-2 (baebl). Blood, 2003. **101**(11): p. 4628-31.

15. Maier AG, Duraisingh MT, Reeder JC, et al., Plasmodium falciparum erythrocyte invasion through glycophorin C and selection for Gerbich negativity in human populations. Nat Med, 2003. **9**(1): p. 87-92.

16. Gilberger TW, Thompson JK, Triglia T, et al., A novel erythrocyte binding antigen-175 paralogue from Plasmodium falciparum defines a new trypsin-resistant receptor on human erythrocytes. J Biol Chem, 2003. **278**(16): p. 14480-6.

17. Dolan SA, Miller LH, and Wellems TE, Evidence for a switching mechanism in the invasion of erythrocytes by Plasmodium falciparum. J Clin Invest, 1990. **86**(2): p. 618-24.

18. Peterson DS and Wellems TE, EBL-1, a putative erythrocyte binding protein of Plasmodium falciparum, maps within a favored linkage group in two genetic crosses. Mol Biochem Parasitol, 2000. **105**(1): p. 105-13.

19. Mayer DC, Kaneko O, Hudson-Taylor DE, et al., Characterization of a Plasmodium falciparum erythrocyte-binding protein paralogous to EBA-175. Proc Natl Acad Sci U S A, 2001. **98**(9): p. 5222-7.

20. Thompson JK, Triglia T, Reed MB, et al., A novel ligand from Plasmodium falciparum that binds to a sialic acid-containing receptor on the surface of human erythrocytes. Mol Microbiol, 2001. **41**(1): p. 47-58.

21. Adams JH, Blair PL, Kaneko O, et al., An expanding ebl family of Plasmodium falciparum. Trends Parasitol, 2001. **17**(6): p. 297-9.

22. Triglia T, Thompson JK, and Cowman AF, An EBA175 homologue which is transcribed but not translated in erythrocytic stages of Plasmodium falciparum. Mol Biochem Parasitol, 2001. **116**(1): p. 55-63.

23. Kwiatkowski D and Marsh K, Development of a malaria vaccine. Lancet, 1997. **350**(9092): p. 1696-701.

24. Moorthy VS, Good MF, and Hill AV, Malaria vaccine developments. Lancet, 2004. **363**(9403): p. 150-6.

25. Moorthy V and Hill AV, Malaria vaccines. Br Med Bull, 2002. **62**(1): p. 59-72.

26. Beverley PC, Immunology of vaccination. Br Med Bull, 2002. **62**: p. 15-28.

27. Webster D and Hill AV, Progress with new malaria vaccines. Bull World Health Organ, 2003. **81**(12): p. 902-9.

28. Stowers A and Carter R, Current developments in malaria transmission-blocking vaccines. Expert Opin Biol Ther, 2001. **1**(4): p. 619-28.

29. Carter R, Transmission blocking malaria vaccines. Vaccine, 2001. **19**(17-19): p. 2309-14.

CHAPTER 2

LITERATURE REVIEW

History

Malaria is a disease caused by a protozoan parasite of the genus *Plasmodium* that belongs to the phylum Apicomplexa. Four parasite species of the genus *Plasmodium* infect humans (*P. falciparum, P. ovale, P. vivax* and *P. malariae*), however, *P. falciparum* and *P. vivax* are responsible for the majority of infections. This disease has been around since the dawn of history. It is suggested that malaria originated in Africa. Charles Louis Alphonse Laveran, identified the cause of malaria in 1880, and 18 years later, Ronald Ross discovered that mosquitoes (*Anopheles*) were responsible for transmission of the parasite. Giovanni Batista Grassi, who traced the course of the parasite through the mosquito, and proved that human malarias were transmitted by species of Anopheles [1, 2] confirmed this.

During a worldwide eradication effort organized by the World Health Organization (WHO) half a century ago malaria was eliminated or effectively suppressed in many areas around the world, although it persisted in most tropical and subtropical regions [3]. During the 1960's, the incidence of malaria was again on the rise because the parasite was becoming resistant to chloroquine, the most widely used antimalarial drug [4-8]. Also, more strains of *Anopheles* mosquitoes were developing resistance to insecticides such as dichloro-diphenyl-trichloroethane (DDT). As a consequence, the eradication of malaria through vector control had been abandoned in the late nineteen sixties, after some initial success [9]. It is estimated that the infection rate has increased by 40% between 1970 and 1997 in sub-Saharan Africa [10].

At present, malaria is responsible for the death of an estimated 2 million children, and 300-500 million infections per year worldwide, as depicted in Figure 2.1 [11]. Also, billions of individuals are at risk of acquiring malaria in over 100 countries [12]. More than 90% of malaria cases and deaths occur in Africa south of the Sahara, mostly among children. It is estimated that malaria claims more than 100,000 lives per year elsewhere in the world and these deaths occur in all age groups [13].

Life Cycle

The *Plasmodium* life cycle (Figure 2.2), is split between a vertebrate host and an insect vector [14]. Only *Anopheline* mosquitoes can act as vectors in the case human, and while there are about 380 species of *Anopheline* mosquito, only 60 can transmit the parasite. Only the female mosquitoes are involved, as the males do not feed on blood. During a blood meal, sporozoites (usually 8-15, but as many as 100) from the mosquito salivary gland are injected into the human host [2]. Once in the bloodstream, the sporozoites enter the liver and penetrate hepatocytes, where they remain and undergo a single round of merogony. Thousands of merozoites are released from the liver and can ultimately cause a parasite burden of greater than 10^{12} parasites in adults [2, 12], but not after red blood cell under go merogony. In the erythrocytic cycle parasites invade the red blood cells (RBC), and starts a 48-hour cycle of replication. Replication is followed by schizont rupture and invasion of new erythrocytes. The infection is brought under control by the immune system or death of the human host. Some of the blood stage merozoites differentiate into either micro- or macrogametocytes. Later, when another *Anopheline* mosquito takes a blood meal these gametocytes are subsequently ingested into its gut, where exflagellation of microgametocytes occurs, followed by fertilization of macrogametocytes.

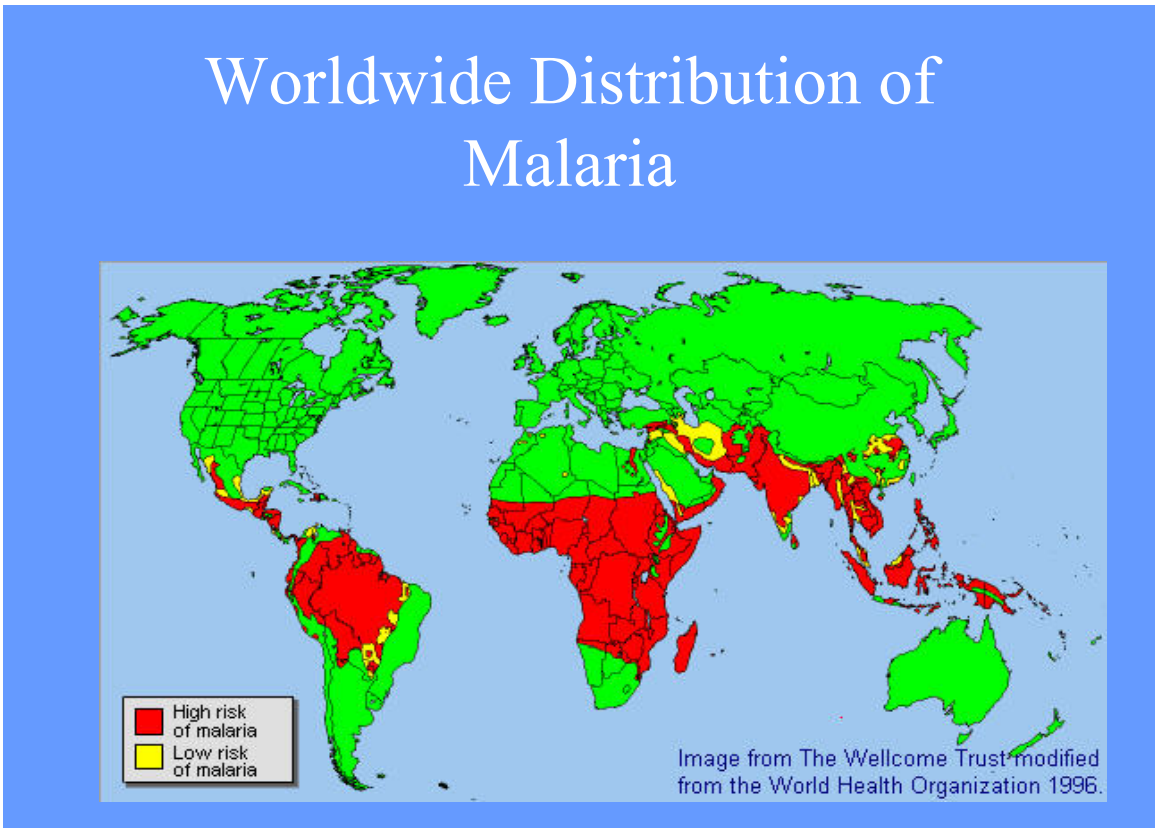


Figure 2.1 The map indicates the current distribution of indigenous malaria according to [11].

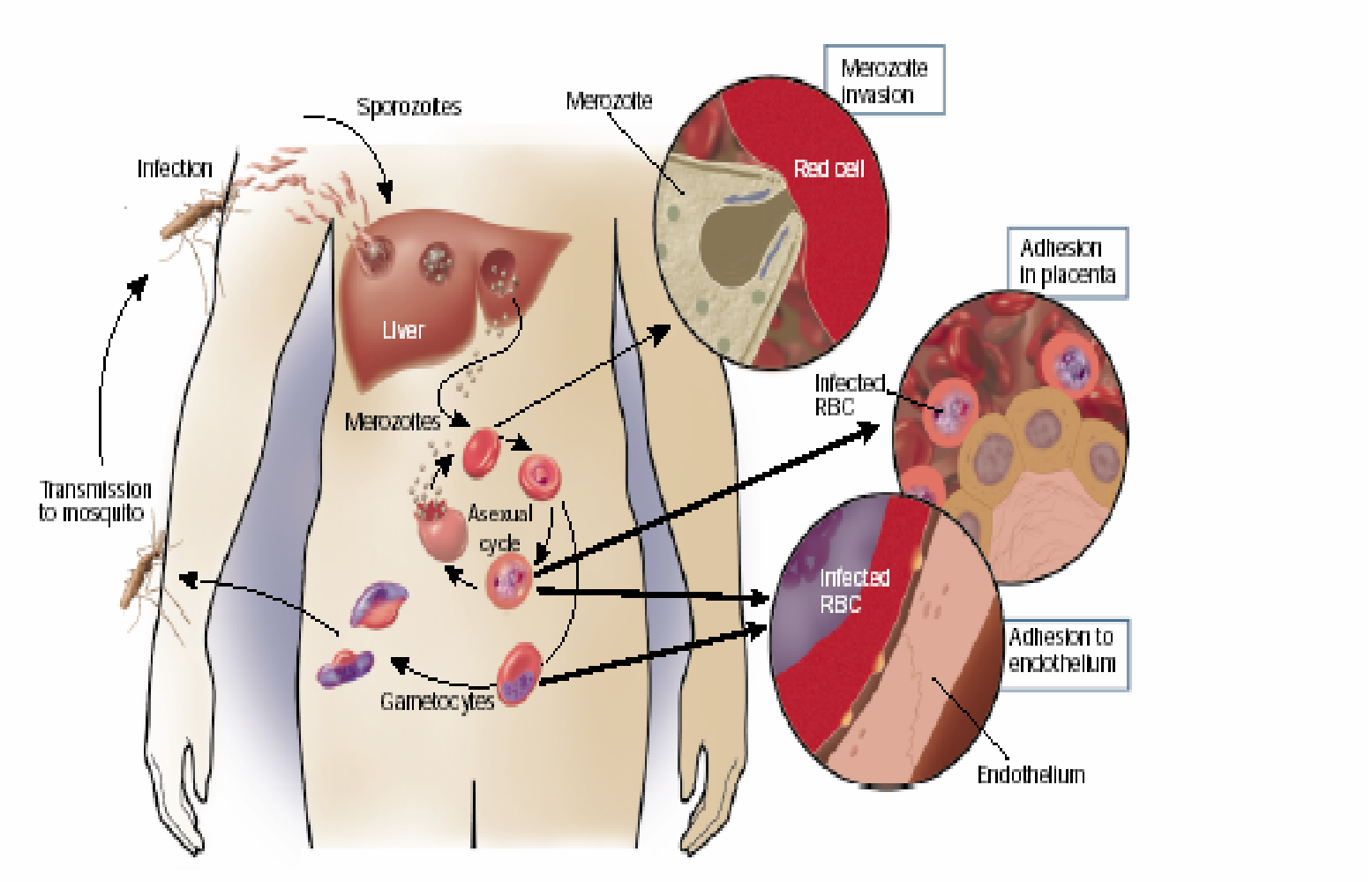


Figure 2.2 The life cycle of *Plasmodium falciparum* in human and the mosquito [14].

The resulting ookinete penetrates the midgut wall of the mosquito to develop into an oocyst. Sporogeny within the oocyst produces many sporozoites and, after rupture of the oocyst, the sporozoites migrate to the salivary gland, ready to be injected into the next host.

Invasion of the Erythrocyte

The invasion of host erythrocytes is a complex process illustrated in Figure 2.3. This figure shows the steps in erythrocyte invasion by a malaria parasite [15]. The ability of *Plasmodium* merozoites to invade erythrocytes is contingent on a rapid cascade of specific interactions occurring between parasite molecules and host erythrocyte receptors. After a random collision of the merozoite with the erythrocyte, a weak interaction between the surface components leads to re-orientation of the merozoites to allow the apical end to interact with the membrane of the host cell [16]. The contents of the apical organelles, the micronemes and rhoptries, are expelled, resulting in invagination of the RBC membrane. This is followed by the formation of a tight junction at the interface between the merozoite surface and the RBC membrane [16-19]. Once the tight junction is formed the merozoite is now irreversibly committed to an active invasion process. This invagination eventually envelops the invading merozoite in a membrane-lined cavity, the parasitophorous vacuole (PV) [19, 20]. After the merozoite is enclosed in the PV, and during the final stage of invasion, another set of apical organelles, the dense granules move to the surface of the parasite and release their contents into the PV, causing an expansion of the area of its membrane [19-21], and presumably causing the change in shape of the parasite to that of the ring stage.

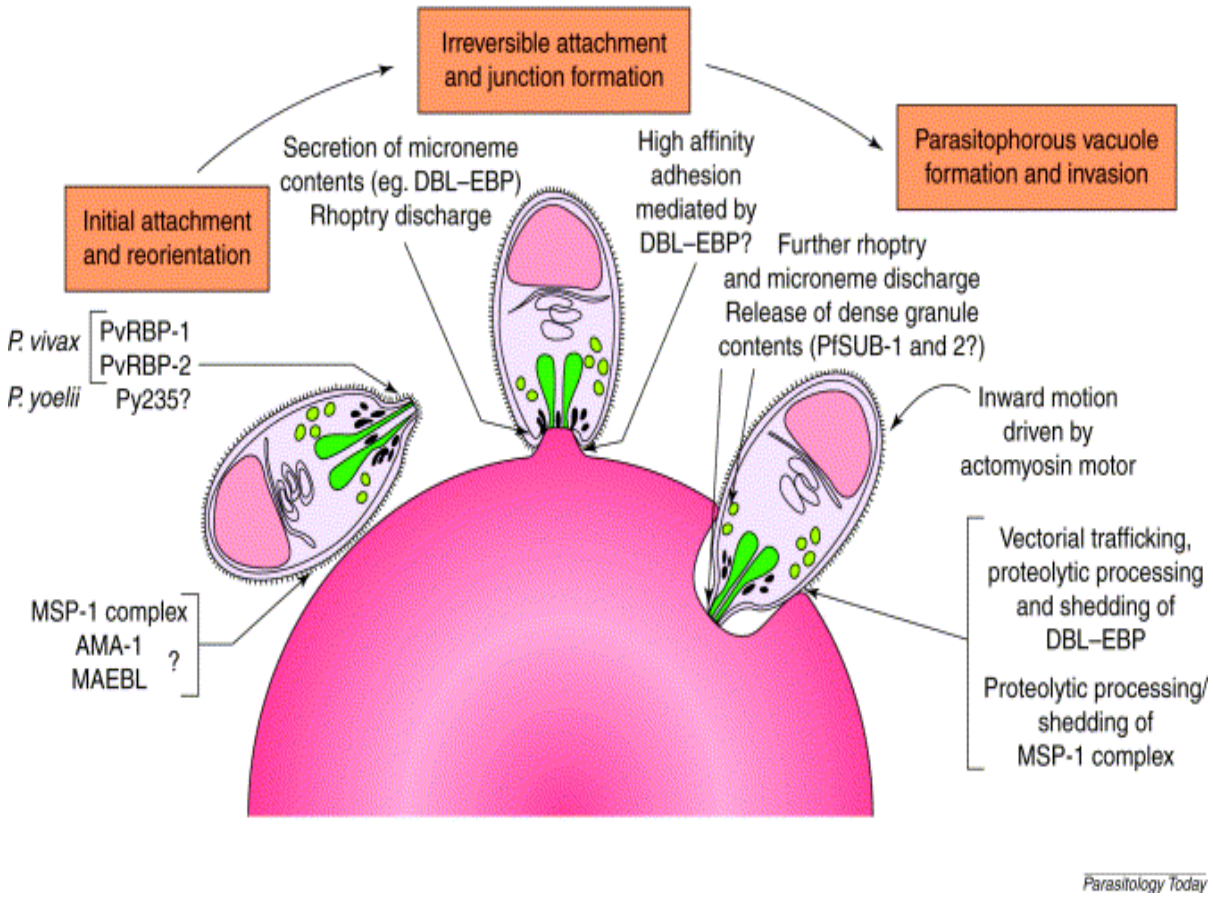


Figure 2.3 Schematic depicting the early stages of red blood cell (RBC) invasion by the malaria merozoite, and the putative roles of the various protein types discussed in the text [15].

Proteins involved in Invasion

Numerous proteins have been functionally implicated in this complex process. One of these proteins, merozoite surface protein 1 (*MSP1*), has been hypothesized to be involved in the initial interaction of the merozoite with the RBC surface [22, 23]. As its name implies, this GPI-anchored protein is found on the surface of the merozoite. This localization is shared with a number of other GPI-anchored proteins including *MSP2* [24], *MSP4* [25] *MSP6* [26] *MSP7*, [27], *MSP8* [28] and *MSP-9* [29]. However, the role of these *MSPs* during erythrocyte invasion by the merozoites has remained unknown.

The microneme proteins that are involved in invasion are members of the erythrocyte binding protein (EBP) family that includes erythrocyte binding antigen-175 (*EBA-175*) of *Plasmodium falciparum,* and the Duffy antigen binding proteins (*DABPs*) of the *P. vivax* and *P. knowlesi* [30-33]. *P. falciparum* is known to use different receptors for invasion of erythrocytes [34] and commonly invades via sialic acid residues present on glycophorin A or B [35]. Mutant RBCs with modifications or deficiencies in glycophorins are, in general, less susceptible to invasion by *P. falciparum* than normal erythrocytes [36]. Moreover, *P. falciparum* appears not to be restricted to invasion via a single class of erythrocyte receptor. Indeed there is mounting evidence, which suggests that the sialic acid-dependent invasion pathway is dispensable in *P. falciparum* malaria. The possibility of "switching" between, or up-regulating, novel phenotypes introduces a further element of complexity into the invasion process and was demonstrated through the ability of the Dd2 line of the *P. falciparum* to be selected for invasion via a pathway mediated by a different host cell receptor [37].

Several rhoptry proteins are thought to play a major role in host cell invasion including *MAEBL* [38], which is also a member of the EBP family and apical membrane antigen 1 [39].

Apical membrane antigen 1 (*AMA-1*) was first isolated from *P. knowlesi*. The precise biological role of *AMA-1* is unknown; however, studies using gene knockouts suggest that *AMA-1* fulfills a critical role during the invasion process across divergent *Plasmodium* species [40]. Also found in the rhoptries are the rhoptry associated proteins (*RAP-1*, *RAP-2*) [41, 42] and (*RAP-3*) [43]. The *RAP1-3* proteins exist in complexes and these complexes may even be maintained after RBC invasion and early parasite maturation. However, recent report by Baldi et al. show that by disrupting *RAP-1* gene did not associated with *RAP2* and *RAP 3* [41]. Homologues rhoptry proteins have been identified in a range of *Plasmodium* species [44-46]. It has been speculated that they are important in merozoite release from the mature schizont or that they may operate in merozoite attachment to, and invasion of, the RBC [43].

The dense granule (DG) proteins are released after the parasite has completed its entry, and these proteins are located in different areas within the newly invaded merozoites. Therefore, they are usually implicated in the modification of the host cell, or the PV membrane. For example, ring-infected erythrocyte surface antigen (RESA) is localized to dense granules in merozoites and is transported to the host erythrocyte membrane shortly after merozoite invasion [47]. However, subtilisin-like proteases (*Pf-SUB1-2*), which are implicated in the secondary proteolytic processing of *MSP-1*, have also been localized to *Plasmodium* dense granules [48, 49]. If *MSP-1* processing is catalyzed by these proteases, then at least some dense granules must be discharged at the time of invasion. Another protein associated with DGs is the ring membrane antigen (*RIMA*). The protein is detectable in the late stage of schizonts and free merozoites and is localized on the membrane of recently invaded ring stage parasites just after invasion [50].

DBL domain super-family

Infection of host cells by the malaria parasite requires host-parasite recognition events, which are mediated by adhesion molecules. This requires the specific recognition of molecules from the parasite by host cell surface receptor molecules. In *P. falciparum* it has previously been discovered that two groups of parasite adhesion proteins with distinct roles share a common feature and can therefore be grouped into a superfamily. The superfamily is defined by the presence of a cysteine rich region, homologous to that first described in the Duffy Antigen Binding Protein, and termed the Duffy Binding Like (DBL) domain. The dbl-domain superfamily is composed of two distinct sub-families; (1) the EBP family that participates in invasion and, (2) the *var* gene family encoding the variant antigen *Plasmodium falciparum* erythrocyte membrane protein 1 (*PfEMP-1*) which mediates binding of the infected RBC to the vascular endothelium resulting in sequestration. It is likely that the *PfEMP-1* DBL domain evolved from EBP proteins employed in erythrocyte invasion.

PfEMP-1

There are numerous observations that support an essential role for the *PfEMP-1* family as adhesion ligands in sequestration, along with other intercellular binding interactions displayed by the parasitised red blood cell. *PfEMP-1* is encoded by the highly diverse *var* gene family [51-54], which has at least four distinct functions. They are antigenic variation [51, 52], cytoadherence [55, 56], immunoregulatory activities on host immune cells [57], and rosetting of uninfected RBC [58].

The *var* genes contain a 5' exon, containing one to seven DBL domains, which is highly variable, and a more conserved 3' exon separated by an intron [53]. These DBL domains have been shown to be responsible for binding various ligands. These domains can be recognized by

conservation of a number of residues, particularly cysteines that occur in recognizable patterns. The *PfEMP-1* DBL-like domains vary greatly in both sequences and length in contrast to that observed in the DBL of the individual EBP genes, which are involved in erythrocyte invasion. It is important to note that although the EBP-DBL domains display natural variation due to point mutations, there is almost no length polymorphism.

The DBL domains in the erythrocyte binding proteins contain 12 invariant cysteine residues [55], however, the homology between PfEMP-1 and EBP DBL domains ends shortly after the 10th cysteine residue [59]. Some PfEMP-1 DBL types may no longer have a subset of these cysteine residues due to pairwise losses of cysteine. However, some types of *PfEMP-1* DBL appear to have gained cysteines. This gain of cysteines was not observed in DBL domains of the EBP family members, with the exception of *EBL-1*. It has not yet been determined where the disulfide bonding occurs between cysteines, which could help determine the actual binding domain.

***Erythrocyte binding-like* family**

In Plasmodium, a large number of proteins, many with similar structure and 'signature' domains, mediate host cell adhesion and invasion. Among the best-known merozoite ligands involved in invasion are products of the EBP family of adhesion molecules, which includes the *P.* falciparum erythrocyte-binding antigen-175 (*EBA-175*) and the *P.vivax* Duffy binding protein (DBP). Six EBP genes have been identified in the now complete *P. falciparum* genome [38]. In contrast *P.vivax* uses two erythrocyte surface proteins, DBP and reticulocyte binding protein (*PvRBP1*) [60] during erythrocyte invasion and is dependent upon a single erythrocyte receptor, the Duffy antigen/receptor for chemokines (DARC). Although the actual function of *PvRBP* in invasion is not known, it is believe to play a role in initial attachment of the parasite to the

reticulocyte. The EBP family of *P. falciparum* comprises proteins proposed to function as ligands to the various receptors on the erythrocyte surface during invasion. In spite of their differences in receptor specificity, an analysis of both *P. vivax* and *P. falciparum* adhesion molecules identified them as part of a homologous family of malarial erythrocyte-binding proteins which are now referred to as erythrocyte binding-like proteins [51]. The members of this *ebl* family have a similar exon-intron structure with conserved splicing boundaries, indicating a common evolutionary origin [38]. The DBP has only a single copy of the dbl-domain, where as the ebl members of *P. falciparum* have two dbl-domains (F1 and F2), although these tandem DBL domains appear to function as a single ligand domain.

In *P. falciparum* the *ebl* family members are present mostly on separate chromosomes, have the consensus gene structure and cysteine-rich dbl-domains, but otherwise lack any significant nucleotide identity, with each other. *ebl-1*, present on chromosome 13, was the first paralogue of *eba-175* that was identified [38, 61]. However, *ebl-1* is not found in the HB3 line of *P.falciparum* that is known to exhibit a slow proliferation phenotype in vitro, associated with the loss of a subtelomeric region of chromosome 13 [61]. Three other novel *P. falciparum* EBPs were identified through the Malaria Genome Project: *ebp-2/baebl*, *eba-165/pebl* and *eba-181/*jes*ebl* [38, 62-65]. The final gene that has been identified in this family is *maebl*. It is a unique paralogue that was initially identified in the rodent malaria parasites, *P. yoelii* and *P. berghei* [66]. The *P. yoelii* and *P. berghei maebl* has some of the characteristics of the family, except for its distinct ligand domains. It is a type 1 transmembrane protein with a carboxyl cysteine-rich region that is homologous to region VI of the EBL-EBPs. The *MAEBL* amino cysteine rich domain, which occurs as a tandem duplication (M1 and M2), has no similarity to the consensus DBL domain of the *ebl* family. Instead, the *MAEBL* ligand domain has a partial

similarity with the cysteine domains 1 and 2 of the *Plasmo*dium and *Toxoplasma* apical membrane antigen-1. However, in erythrocyte cytoadherence assays the M1 and M2 domains of *P. yoelii MAEBL* bound mouse erythrocytes and were shown to be functionally equivalent to the DBL ligand domains. Also *MAEBL* is localized first in the neck of the merozoite rhoptries, and then later on the surface of the merozoite [66], while the other members of the protein family are localized in the micronemes.

Recently, Blair et al. used quantitative real time RT-PCR to analyzed erythrocyte stage-specific expression [67]. They analyzed transcripts of the *P. falciparum eba-175* and other erythrocyte binding-like family genes in temperature-synchronized parasites and found these genes to have tightly controlled, stage-specific transcription. E*ba-175* transcripts were abundant only at the end of schizont development in a pattern common among these genes, including *baebl, pebl,* and *jesebl*. The *maebl* transcript pattern was unique, peaking at mid-late trophozoite stage but absent in late-stage schizont. *Ebl*-1 demonstrated another pattern of expression, which peaked during the mid-schizont stage and then significantly diminished in late-stage schizonts.

DBL domains are found in parasite ligands that mediate two key events, erythrocyte invasion and cytoadherence. To understand the structural basis of these receptor-ligand interactions, it is important to determine the three-dimensional structures of DBL and by mapping the regions within DBL that contain receptor-binding residues. The receptor binding domains of each EBP lie in a conserved, amino-terminal, cysteine-rich region; referred to as region II that contains around 330 amino acids with 12 to 14 conserved cysteines [68]. The binding domain of the *EBA-175* maps to the F2 dbl-domain, which specifically binds sialic acid residues on glycophorin A [68-70].

P. falciparum isolates differ in their erythrocyte requirements [34]; most if not all use sialic acid for optimal invasion, but the requirements for erythrocyte glycoproteins that carry sialic acid may vary. *P.falciparum* is known to use different receptors for invasion of erythrocytes and can invade by a minimum of five distinct pathways; using glycophorin A, glycophorin B, glycophorin C, and at least two more pathways that have not yet been characterized, referred to as receptor E and X. The existence of alternative invasion pathways will result in different *P. falciparum*, isolates to express different erythrocyte binding ligands.

Some strains (Camp, Dd2, and FCR-3) are restricted to the sialic acid-dependent pathway, because these parasites are unable to invade erythrocytes treated with neuraminidase, which removes sialic acid [37]. Other strains such as HB3, 7G8, and 3D7 are more flexible, having the potential to invade neuraminidase-treated erythrocytes (sialic acid-independent) [37]. In addition, it has been demonstrated that the Dd2 isolate can switch from a sialic acid-dependent to sialic acid-independent invasion phenotype at a switch rate of approximately 1 in 250 parasites [71]. The mechanism of this switch is currently unknown.

The complete genome sequenced of *P. falciparum*, will make it possible to characterize the entire family of erythrocyte binding ligands along with their respective erythrocyte receptors. This will further allow us to study the different ligands used by different field isolates in the invasion of erythrocyte.

The Genes and Protein of the EBL Family

EBA-175

A number of studies point to importance of *EBA-175* (molecule) in the invasion process, such as the binding of merozoites to red blood cell (RBC). *EBA-175* is a soluble antigen of *P. falciparum* micronemes, isolated from culture supernatants following rupture of schizont

infected RBC [72]. The *EBA-175* gene is highly conserved amongst different strains and encodes a protein of 1475 amino acids. *EBA-175* appears as a single-copy gene on chromosome 7, and does not cross-hybridize any other locus in the *P. falciparum* genome. *EBA-175* is believed to act as a bridge between the merozoite and the erythrocyte in the invasion process and binds to glycophorin A on the erythrocyte surface. *EBA-175* interacts directly with the erythrocyte surface via two mechanisms: an initial binding, which is dependent on sialic acid, and a secondary binding, which is not dependent on sialic acid. However, this binding involves recognition of both the sialic acid and the peptide backbone of glycophorin A [73].

A conserved region of 42 amino acid [74], has been implicated in the binding to the erythrocyte, although it is not essential for the initial sialic acid-dependent binding [68]. The erythrocyte-binding region of *EBA-175* is a 616 amino-acid region, designated region II, which lies in the amino terminal third of the molecule. The ability for native *EBA-175* to bind to susceptible erythrocytes generally correlates closely with the ability of *P. falciparum* to invaded erythrocytes, although some isolates can also invade neuraminidase-treated erythrocytes with a lower efficiency. Many studies [70, 75, 76] have shown that antibodies against region II may not only block invasion pathways involving sialic acid, but also block invasion pathways that may not include sialic acid. In addition to region II, some adjacent part of EBA-*175* most likely including the conserved 42 amino acid regions, may play a crucial role in merozoite invasion via a pathway that does not involve sialic acid.

EBA-175 antigen has been shown to be also express in pre-erythrocytic stages of P. *falciparum* [77]. This was demonstrated through parallel analyses of merozoite parasites and sporozoites at the transcriptional and the translation levels. Sporozoites were shown to contain *eba-175* mRNA, as determined by RT-PCR. Correctly spliced *eba-175* was present in

sporozoites, and splice sites were identical in merozoite parasites. The observation that the eba-175 gene is specifically transcribed in sporozoites suggests that the protein is synthesized at this stage of the parasite's life cycle [77]. Thus, one might speculate that *EBA-175* serves as an adhesion ligand both for RBC and for hepatocytes. This is consistent with the apparent absence in gametocytes, a noninvasive stage of the parasite life cycle. A functional role of *EBA-175* in the mammalian host and its presence in the hepatic stage present additional targets for immune responses induced by any vaccine based on *EBA-175*, thus improving the potential of such a vaccine to control the infection.

EBL-1

A paralog of *eba-175* was identified in the Dd2 isolate. *Ebl-1* is generated from a transcript of about 8-9.5 kb with a deduced protein sequence of 2647 amino acids [61]. *Ebl-1* is a single copy gene and is present on chromosome 13. Interestingly, ebl-1 is not found in the HB3 line of *P.falciparum* due to the loss of the terminal region of this chromosome. *Ebl-1* is expressed late in the trophozoites stage of the life cycle as determined by real-time PCR of synchronized parasites [67]. However, recent reports using microarray analysis indicate that it is expressed in the schizonts stage [78, 79]. Interestingly, information from the *Plasmodium* genome project indicated that there was a frame shift due to an insertion of 5 nucleotides just upstream of region II in isolate 3D7 [80], thus inactivating the gene in this isolate. Due to the similarity in sequence and gene structure, it has been suggested that *ebl-1* plays a role in invasion similar to that of *EBA-175*. However, a definitive role in erythrocyte invasion has not yet been demonstrated for this protein. The *ebl-1* gene also encodes a Carboxyl cysteine rich region homologous to that found in the *DABP* and *EBA-175*. The gene is located in the subtelomeric region of chromosome 13, in an area that is made up of extensive repeats, including the highly

diverse var and rifin gene family. Proteomic analysis suggests that it may also be expressed in sporozoite [81].

MAEBL

MAEBL has a gene structure similar to that of the EBP family, with two cysteine-rich regions, one each at the amino and the carboxyl terminus. The amino-terminal cysteine-rich region however, has no homology to that of other EBPs but resembles subdomain I and II (M1 and M2) of the apical membrane antigen-1 (*AMA-1*) protein. The carboxyl end of *MAEBL* contains a cysteine-rich domain homologous to region VI of EBPs. *MAEBL* can be detected in the rhoptries in the early schizont stage. It is also seen evenly distributed on the surface of mature merozoites in the late-stage of schizonts [82]. This pattern of localization and the fact that regions M1 and M2 of *MAEBL* bind erythrocytes suggest that *MAEBL* plays a role in invasion, possibly in the initial interaction with erythrocytes prior to apical reorientation and junction formation. *Maebl* is also a single copy gene and is present on chromosome 11. The predicted protein encoded by *maebl* comprises 2056 amino acids. In addition to blood-stage malaria parasites, *MAEBL* is expressed in sporozoites [77]. This may suggest why *MAEBL* plays an important conserved functional role in this multiple step process of invasion.

BAEBL (EBP2/EBA-140)

Another member of the gene family for erythrocyte invasion was mapped to the subtelomeric region of chromosome 13 from the *Plasmodium falciparum* genome sequence of the Sanger Center (Cambridge, U.K.). The *eba-140* gene is located in an area that is made up of extensive repeats that includes the highly diverse var and rifin gene family [63]. *EBA-140* protein is located in the micronemes and shares structural features and homology to *EBA-175* and *EBA-181*. The predicted protein encoded by *baebl* comprises 1210 amino acids. Thompson

et al. have also identified a parasite line (D10) that lacks the *baebl* gene[63]. There is no difference in invasion properties of D10 and E12 (a line containing the gene) in normal or neuraminidase treated erythrocytes, suggesting that this protein is not essential for in vitro invasion. *EBA-140* binds in a sialic acid-dependent manner to a receptor on the surface of human erythrocytes in a manner that is sensitive to neuraminidase and resistance to trypsin, proteinase K and pronase [83, 84]. The protease resistant properties of the erythrocyte receptor suggest that it is not glycophorin A or B. A recent report [84] has shown that *EBA-140* bind to glycophorin C (GPC) and is unable to bind to erythrocytes lacking GPC.

PEBL (EBA-165)

EBA-165 is a gene that is transcribed but not translated in the erythrocytic stage of *P. falciparum* [64]. It is possible that *EBA-165* is a transcribed pseudogene that has become redundant in the invasion of human red blood cells. The putative pseudogene *eba-165* consists of four exons, and has a structure analogous to that described for *eba-175*. The predicted protein would have a structure similar to *EBA175*, but the coding region of *EBA-165* contains frameshifts at two positions in some isolates, while other isolates have a single frameshift. It should be noted that the 3' frameshift is reported to be polymorphic [64, 85]. Antibodies that recognize *EBA-165* fusion proteins were unable to detect the protein in the *P. falciparum* line tested. *Eba-165* is map on chromosome 4 and the predicted protein encoded by *eba-165* comprises 1431 amino acids.

JESEBL /EBA-181

EBA-181 (also known as jesebl) is another member of the erythrocyte binding-like family and is a paralogue of *EBA-175*. *Eba-181* was identified from the *P.falciparum* genome sequence project [65, 86]; and consists of 4 exons and 3 introns. *Eba-181* maps to the telomeric region of

chromosome 1 and the predicted protein encoded by *eba-181* comprises 1567 amino acids. *EBA-181* is localized in the microneme organelles of the asexual stage parasites. A recent report suggests that the *EBA-181* receptor is neither glycophorin A, B or C nor the previously postulated receptor X [86]. It has been demonstrated that *EBA-165* binds to erythrocytes through a sialoglycoprotein that is chemotrypsin-sensitive and trypsin resistant. This novel receptor is called E [65].

Polymorphism in *Plasmodium falciparum*

The processes that maintain genetic polymorphism is of great importance and still debatable in population genetics. In the case of *Plasmodium falciparum*, the agent of most dangerous form of malaria is a matter of great clinical and epidemiological importance. Extensive polymorphism in *Plasmodium*'s proteins, particular those utilized during the invasion process (eg. *EBA-175*), remain a major impediment in the development of antimalarial vaccines and the use of antimalaria drugs. Since the erythrocytic stage is responsible for the clinical disease. The vaccine must either be based on conserved regions or incorporate multiple allelic forms of the antigen.

The receptor-binding domain is located in the functionally conserved, (5') N-terminal cysteine rich area called region II [76, 87]. The identification of the receptor-binding domain has led to the efforts at developing receptor-blocking therapy and a vaccine based on this ligand domain.

Highly polymorphic regions have been observed in the genes encoding surface and apical antigenic proteins such as *Msp-1, Msp-2, AMA-1, Pfs 48/45* and *Csp* [88-92]. These genes encode antigenic proteins that are normally recognized by the host immune system, and polymorphism observed in these genes is likely driven by immune pressure. This interpretation

is supported by a universal observation that synonymous nucleotide substitutions are less common than non-synonymous nucleotide substitutions in proteins under selective pressure [93].

Previous studies have examined the level of polymorphism in several members of the EBP family, EBA-175, EBA-140 and EBA-165 in region II and the Duffy binding protein in *P. falciparum* and *P. vivax* respectively [94-96].

Cysteine residues and the aromatic amino acids are conserved within the critical binding motifs in both DBP and EBP region II, whereas the point mutation observed in the intervening amino acid are few and scattered [68, 76, 87]. Liang et al. reported that they were seventeen polymorphic sites in region II of *eba-175* gene (detected in 16 different strains of *P.falciparum*), of which 1 or (6%) is synonymous and 16 (94%) nonsynonymous [68]. The point mutations leading to radical amino acid changes found among isolates were few and scattered. In a similar study looking at the level of sequence conservation in *ebl-1*, reported here in chapter 4, similar results were observed. A separate study was conducted to determine level of allelic dimorphisms in four *P.falciparum* merozoite proteins (*EBA-175, MSP-1, MSP-2 and MSP-3*). The results indicate that major dimorphic allelic sequences differences between merozoite proteins appear not to be related to differences in erythrocyte invasion phenotypes [97].

Quantification of EBP Gene Expression in *Plasmodium falciparum*

Invasion of erythrocytes by merozoites of *Plasmodium falciparum* is a complex process requiring interaction between many parasite and host proteins. Identification of genes expressed during the development of the asexual stages could give a better understanding on host-parasite interactions. Furthermore, an understanding of the expression of these genes at the different stage of the erythrocytic cycle can lead to the development of new strategies to interrupt the life cycle with drugs. A study by Blair and colleagues [67] suggested that the expression of the

maebl transcript peaked during mid-late trophozoite stage, *ebl-1* peaked at mid-schizont stage and *eba-175*, *eba-140*, *eba-165* and *eba-181* peaked at the end of schizont development. However, only a single isolate (3D7) was analyzed in this study. It is known that not all of the EBPs are expressed in all isolates [38, 61, 98] and it is possible that others are expressed at a higher level compensating for the lack of one or more genes in some isolates. Support for this come from recent studies by [98, 99] looking at the level of the *P.falciparum* rhoptry protein homolog (*PfRH2a*) and reticulocyte binding protein homolog (*PfRH2b*). These authors found that not all of these genes were transcribed in three *P. falciparum* isolates examined. While both genes are expressed in isolates 3D7 and T996, *PfRH2a* and *PfRH2b* were not expressed in FCB1.

During the erythrocytic stage of growth the parasite passes through stages described as rings, trophozoites and schizonts. As these stages have different morphological traits, their metabolic and expression profiles are expected to be different. There is strong stage-specific expression of genes such as dihydrofolate reductase-thymidylate synthase [67, 100] and all the EBPs [67]. It has been reported that var gene expression is also tightly regulated in the trophozoites stage [101]. A single trophozoite in an infected erythrocyte may express only one of fifty possible *var* genes and switch frequently between alleles [102]. However, the switching of expression amongst members of other surface protein families is less well understood.

Quantitative expression analysis of mRNA has been widely used in many experimental studies and has also found clinical applications. A number of methods are available for this purpose, such as northern blotting (NB), the RNase protection assay (RPA), quantitative competitive reverse transcription-PCR [103] and real-time reverse transcription PCR (RT-PCR) [104], and some of these procedures involved are rather time consuming. In addition both NB and RPA are limited by hybridization kinetics and require relatively large amounts of RNA. The

measurement of RNA transcription by real-time (kinetic) RT-PCR circumvents several obstacles known to limit the quantification potential of other PCR-based methods [105, 106].

Recent advances in the use of fluorogenic probes in conjunction with PCR have enabled the measurement of an accumulating PCR product in real time [107-109]. This allows the rapid generation of quantitative data showing changes in transcript in the samples, while the risk of a false positive result due to contamination with previously amplified products is dramatically reduced. The use of real-time quantitative PCR by the 5' fluorogenic nuclease assay (Taqman) in malaria has been validated in several recent studies [67, 110-112].

In the literature, a selection of housekeeping genes has been used to control for difference in RNA quantity and quality between samples. Three of the most widely used housekeeping genes are ribosomal 18S RNA, glycerol-dehyde-3-phosphate (GAPDH) and β-actin. Several of these studies determined that ribosomal 18S RNA [67, 113, 114] are more suitable for use as a normalizing gene in quantitative studies.

References

1. Joy DA, Feng X, Mu J, et al., Early origin and recent expansion of Plasmodium falciparum. Science, 2003. **300**(5617): p. 318-21.
2. White NJ and Ho M, The pathophysiology of malaria. Adv. Parasitol., 1992. **31**: p. 83-173.
3. Guerin PJ, Olliaro P, Nosten F, et al., Malaria: current status of control, diagnosis, treatment, and a proposed agenda for research and development. Lancet Infect Dis, 2002. **2**(9): p. 564-73.
4. Trape JF, The public health impact of chloroquine resistance in Africa. Am J Trop Med Hyg, 2001. **64**(1-2 Suppl): p. 12-7.

5. Paul RE, Brockman A, Price RN, et al., Genetic analysis of Plasmodium falciparum infections on the north-western border of Thailand. Trans R Soc Trop Med Hyg, 1999. **93**(6): p. 587-93.

6. White NJ, Delaying antimalarial drug resistance with combination chemotherapy. Parassitologia, 1999. **41**(1-3): p. 301-8.

7. Trape JF, Pison G, Preziosi MP, et al., Impact of chloroquine resistance on malaria mortality. C R Acad Sci III, 1998. **321**(8): p. 689-97.

8. Trape JF, Pison G, Spiegel A, et al., Combating malaria in Africa. Trends Parasitol, 2002. **18**(5): p. 224-30.

9. WHO, A global strategy to control Malaria. Report No. 839, Geneva. 1993.

10. USDHHS, *Malaria. National Institutes of Health.* 2002. p. 1-7.

11. WHO, Malaria. Report No. 94 Fact Sheet. Geneva. 1996(Report No. 94 Fact sheet. Geneva).

12. White NJ and Krishna S, Treatment of malaria: some considerations and limitations of the current methods of assessment. Trans R Soc Trop Med Hyg, 1989. **83**(6): p. 767-77.

13. WHO, WHO Technical Report Series, No. 892. 2000.

14. Miller LH, Baruch DI, Marsh K, et al., The pathogenic basis of malaria. Nature, 2002. **415**(6872): p. 673-9.

15. Chitnis CE and Blackman MJ, Host cell invasion by malaria parasites. Parasitol Today, 2000. **16**(10): p. 411-5.

16. Dvorak JA, Miller, L.H., Whitehouse, W.C., Invasion of erythrocytes by malaria merozoites. Science, 1975. **187**: p. 748-749.

17. Ward G. E. CCE, Miller, L.H., The invasion of erythrocytes by malarial merozoites, in *Bailliere'sclinical Infectious Diseases*, Tindall B, Editor. 1994. p. 155.

18. Barnwell JW and Galinski MR, The adhesion of malaria merozoite proteins to erythrocytes: a reflection of function? Res. Immunol., 1991. **142**(8): p. 666-672.

19. Atkinson CT and Aikawa M, Ultrastructure of malaria-infected erythrocytes. Blood Cells, 1990. **16**(2-3): p. 351-68.

20. Culvenor JG, Day KP, and Anders RF, Plasmodium falciparum ring-infected erythrocyte surface antigen is released from merozoite dense granules after erythrocyte invasion. Infect. Immun., 1991. **59**(3): p. 1183-1187.

21. Bannister LH and Mitchell GH, The fine structure of secretion by Plasmodium knowlesi merozoites during red cell invasion. J Protozool, 1989. **36**(4): p. 362-7.

22. Holder AA, Blackman, M.J., What is the function of MSP-1 on the Malaria Merozoite? Parasotology Today, 1994. **10**(5): p. 182-184.

23. Holder AA, Proteins on the surface of the malaria parasite and cell invasion. Parasitology, 1994. **108 Suppl**: p. S5-18.

24. Gerold P, Schofield L, Blackman MJ, et al., Structural analysis of the glycosyl-phosphatidylinositol membrane anchor of the merozoite surface proteins-1 and -2 of Plasmodium falciparum. Mol Biochem Parasitol, 1996. **75**(2): p. 131-43.

25. Marshall VM, Silva A, Foley M, et al., A second merozoite surface protein (MSP-4) of Plasmodium falciparum that contains an epidermal growth factor-like domain. Infect Immun, 1997. **65**(11): p. 4460-7.

26. Trucco C, Fernandez-Reyes D, Howell S, et al., The merozoite surface protein 6 gene codes for a 36 kDa protein associated with the Plasmodium falciparum merozoite surface protein-1 complex. Mol Biochem Parasitol, 2001. **112**(1): p. 91-101.

27. Pachebat JA, Ling IT, Grainger M, et al., The 22 kDa component of the protein complex on the surface of Plasmodium falciparum merozoites is derived from a larger precursor, merozoite surface protein 7. Mol Biochem Parasitol, 2001. **117**(1): p. 83-9.

28. Puentes A, Garcia J, Ocampo M, et al., P. falciparum: merozoite surface protein-8 peptides bind specifically to human erythrocytes. Peptides, 2003. **24**(7): p. 1015-23.

29. Li X, Chen H, Oo TH, et al., A co-ligand complex anchors plasmodium falciparum merozoites to the erythrocyte invasion receptor band 3. J Biol Chem, 2003.

30. Chitnis CE and Miller LH, Identification of the erythrocyte binding domains of Plasmodium vivax and Plasmodium knowlesi proteins involved in erythrocyte infection. J. Exp. Med., 1994. **180**: p. 497-506.

31. Adams JH, Hudson DE, Torii M, et al., The Duffy receptor family of Plasmodium knowlesi is located within the micronemes of invasive malaria merozoites. Cell., 1990. **63**(1): p. 141-153.

32. Camus D and Hadley TJ, A Plasmodium falciparum antigen that binds to host erythrocytes and merozoites. Science, 1985. **230**(4725): p. 553-6.

33. Orlandi PA, Klotz FW, and Haynes JD, A malaria invasion receptor, the 175-kilodalton erythrocyte binding antigen of Plasmodium falciparum recognizes the terminal Neu5Ac(alpha 2-3)Gal- sequences of glycophorin A. J. Cell. Biol., 1992. **116**(4): p. 901-909.

34. Pasvol G, How many pathways for invasion of the red blood cell by the malaria parasite? Trends Parasitol, 2003. **19**(10): p. 430-2.

35. Hadley TJ, Klotz FW, Pasvol G, et al., Falciparum malaria parasites invade erythrocytes that lack glycophorin A and B (MkMk). Strain differences indicate receptor heterogeneity and two pathways for invasion. J. Clin. Invest., 1987. **80**(4): p. 1190-1193.

36. Pasvol G, Wainscoat JS, and Weatherall DJ, Erythrocytes deficiency in glycophorin resist invasion by the malarial parasite Plasmodium falciparum. Nature, 1982. **297**(5861): p. 64-6.

37. Dolan SA, Miller LH, and Wellems TE, Evidence for a switching mechanism in the invasion of erythrocytes by Plasmodium falciparum. J Clin Invest, 1990. **86**(2): p. 618-24.

38. Adams JH, Blair PL, Kaneko O, et al., An expanding ebl family of Plasmodium falciparum. Trends Parasitol, 2001. **17**(6): p. 297-9.

39. Peterson MG, Marshall VM, Smythe JA, et al., Integral membrane protein located in the apical complex of Plasmodium falciparum. Mol Cell Biol, 1989. **9**(7): p. 3151-4.

40. Triglia T, Healer J, Caruana SR, et al., Apical membrane antigen 1 plays a central role in erythrocyte invasion by Plasmodium species. Mol Microbiol, 2000. **38**(4): p. 706-18.

41. Baldi DL, Andrews KT, Waller RF, et al., RAP1 controls rhoptry targeting of RAP2 in the malaria parasite Plasmodium falciparum. Embo J, 2000. **19**(11): p. 2435-43.

42. Howard RF, Jacobson KC, Rickel E, et al., Analysis of inhibitory epitopes in the Plasmodium falciparum rhoptry protein RAP-1 including identification of a second inhibitory epitope. Infect Immun, 1998. **66**(1): p. 380-6.

43. Howard RF, Narum DL, Blackman M, et al., Analysis of the processing of Plasmodium falciparum rhoptry-associated protein 1 and localization of Pr86 to schizont rhoptries and p67 to free merozoites. Mol Biochem Parasitol, 1998. **92**(1): p. 111-22.

44. Peterson MG, Nguyen-Dinh P, Marshall VM, et al., Apical membrane antigen of Plasmodium fragile. Mol Biochem Parasitol, 1990. **39**(2): p. 279-83.

45. Marshall VM, Peterson MG, Lew AM, et al., Structure of the apical membrane antigen I (AMA-1) of Plasmodium chabaudi. Mol Biochem Parasitol, 1989. **37**(2): p. 281-3.

46. Cheng Q and Saul A, Sequence analysis of the apical membrane antigen I (AMA-1) of Plasmodium vivax. Mol Biochem Parasitol, 1994. **65**(1): p. 183-7.

47. Culvenor JG, Day KP, and Anders RF, Plasmodium falciparum ring-infected erythrocyte surface antigen is released from merozoite dense granules after erythrocyte invasion. Infect Immun, 1991. **59**(3): p. 1183-7.

48. Blackman MJ, Fujioka H, Stafford WH, et al., A subtilisin-like protein in secretory organelles of Plasmodium falciparum merozoites. J Biol Chem, 1998. **273**(36): p. 23398-409.

49. Barale JC, Blisnick T, Fujioka H, et al., Plasmodium falciparum subtilisin-like protease 2, a merozoite candidate for the merozoite surface protein 1-42 maturase. Proc Natl Acad Sci U S A, 1999. **96**(11): p. 6445-50.

50. Trager W, Rozario C, Shio H, et al., Transfer of a dense granule protein of Plasmodium falciparum to the membrane of ring stages and isolation of dense granules. Infect Immun, 1992. **60**(11): p. 4656-61.

51. Peterson DS, Miller LH, and Wellems TE, Isolation of multiple sequences from the Plasmodium falciparum genome that encode conserved domains homologous to those in erythrocyte-binding proteins. Proc Natl Acad Sci U S A, 1995. **92**(15): p. 7100-4.

52. Smith JD, Chitnis CE, Craig AG, et al., Switches in expression of Plasmodium falciparum var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes [see comments]. Cell, 1995. **82**(1): p. 101-10.

53. Su XZ, Heatwole VM, Wertheimer SP, et al., The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes [see comments]. Cell, 1995. **82**(1): p. 89-100.

54. Baruch DI, Pasloske BL, Singh HB, et al., Cloning the P. falciparum gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes [see comments]. Cell, 1995. **82**(1): p. 77-87.

55. Smith JD, Subramanian G, Gamain B, et al., Classification of adhesive domains in the Plasmodium falciparum erythrocyte membrane protein 1 family. Mol Biochem Parasitol, 2000. **110**(2): p. 293-310.

56. Smith JD, Kyes S, Craig AG, et al., Analysis of adhesive domains from the A4VAR Plasmodium falciparum erythrocyte membrane protein-1 identifies a CD36 binding domain. Mol Biochem Parasitol, 1998. **97**(1-2): p. 133-48.

57. Urban BC, Ferguson DJ, Pain A, et al., Plasmodium falciparum-infected erythrocytes modulate the maturation of dendritic cells [see comments]. Nature, 1999. **400**(6739): p. 73-7.

58. Chen Q, Barragan A, Fernandez V, et al., Identification of Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) as the rosetting ligand of the malaria parasite P. falciparum. J Exp Med, 1998. **187**(1): p. 15-23.

59. Michon P, Stevens JR, Kaneko O, et al., Evolutionary relationships of conserved cysteine-rich motifs in adhesive molecules of malaria parasites. Mol Biol Evol, 2002. **19**(7): p. 1128-42.

60. Rayner JC, Vargas-Serrato E, Huber CS, et al., A Plasmodium falciparum homologue of Plasmodium vivax reticulocyte binding protein (PvRBP1) defines a trypsin-resistant erythrocyte invasion pathway. J Exp Med, 2001. **194**(11): p. 1571-81.

61. Peterson DS and Wellems TE, EBL-1, a putative erythrocyte binding protein of Plasmodium falciparum, maps within a favored linkage group in two genetic crosses. Mol Biochem Parasitol, 2000. **105**(1): p. 105-13.

62. Mayer DC, Kaneko O, Hudson-Taylor DE, et al., Characterization of a Plasmodium falciparum erythrocyte-binding protein paralogous to EBA-175. Proc Natl Acad Sci U S A, 2001. **98**(9): p. 5222-7.

63. Thompson JK, Triglia T, Reed MB, et al., A novel ligand from Plasmodium falciparum that binds to a sialic acid-containing receptor on the surface of human erythrocytes. Mol Microbiol, 2001. **41**(1): p. 47-58.

64. Triglia T, Thompson JK, and Cowman AF, An EBA175 homologue which is transcribed but not translated in erythrocytic stages of Plasmodium falciparum. Mol Biochem Parasitol, 2001. **116**(1): p. 55-63.

65. Gilberger TW, Thompson JK, Triglia T, et al., A novel erythrocyte binding antigen-175 paralogue from Plasmodium falciparum defines a new trypsin-resistant receptor on human erythrocytes. J Biol Chem, 2003. **278**(16): p. 14480-6.

66. Kappe SH, Curley GP, Noe AR, et al., Erythrocyte binding protein homologues of rodent malaria parasites. Mol Biochem Parasitol, 1997. **89**(1): p. 137-48.

67. Blair PL, Witney A, Haynes JD, et al., Transcripts of developmentally regulated Plasmodium falciparum genes quantified by real-time RT-PCR. Nucleic Acids Res, 2002. **30**(10): p. 2224-31.

68. Liang H and Sim BK, Conservation of structure and function of the erythrocyte-binding domain of Plasmodium falciparum EBA-175. Mol Biochem Parasitol, 1997. **84**(2): p. 241-5.

69. Ockenhouse CF, Barbosa A, Blackall DP, et al., Sialic acid-dependent binding of baculovirus-expressed recombinant antigens from Plasmodium falciparum EBA-175 to Glycophorin A. Mol Biochem Parasitol, 2001. **113**(1): p. 9-21.

70. Pandey K, Singh S, Pattnaik P, et al., Bacterially expressed and refolded receptor binding domain of Plasmodium falciparum EBA-175 elicits invasion inhibitory antibodies. Mol Biochem Parasitol, 2002. **123**(1): p. 23.

71. Soubes SC, Wellems TE, and Miller LH, Plasmodium falciparum: a high proportion of parasites from a population of the Dd2 strain are able to invade erythrocytes by an alternative pathway. Exp Parasitol, 1997. **86**(1): p. 79-83.

72. Orlandi PA, Sim BK, Chulay JD, et al., Characterization of the 175-kilodalton erythrocyte binding antigen of Plasmodium falciparum. Mol. Biochem. Parasitol., 1990. **40**(2): p. 285-294.

73. Kain KC, Orlandi PA, Haynes JD, et al., Evidence for two-stage binding by the 175-kD erythrocyte binding antigen of Plasmodium falciparum. J. Exp. Med., 1993. **178**(5): p. 1497-1505.

74. Sim BK, Carter JM, Deal CD, et al., Plasmodium falciparum: further characterization of a functionally active region of the merozoite invasion ligand EBA-175. Exp. Parasitol., 1994. **78**(3): p. 259-268.

75. Narum DL, Haynes JD, Fuhrmann S, et al., Antibodies against the Plasmodium falciparum receptor binding domain of EBA-175 block invasion pathways that do not involve sialic acids. Infect Immun, 2000. **68**(4): p. 1964-6.

76. Liang H, Narum DL, Fuhrmann SR, et al., A recombinant baculovirus-expressed Plasmodium falciparum receptor-binding domain of erythrocyte binding protein EBA-175 biologically mimics native protein. Infect Immun, 2000. **68**(6): p. 3564-8.

77. Kappe SH, Gardner MJ, Brown SM, et al., Exploring the transcriptome of the malaria sporozoite stage. Proc Natl Acad Sci U S A, 2001. **98**(17): p. 9895-900.

78. Bozdech Z, Llinas M, Pulliam BL, et al., The Transcriptome of the Intraerythrocytic Developmental Cycle of Plasmodium falciparum. PLoS Biol, 2003. **1**(1): p. E5.

79. Bozdech Z, Zhu J, Joachimiak MP, et al., Expression profiling of the schizont and trophozoite stages of Plasmodium falciparum with a long-oligonucleotide microarray. Genome Biol, 2003. **4**(2): p. R9.

80. Gardner MJ, Hall N, Fung E, et al., Genome sequence of the human malaria parasite Plasmodium falciparum. Nature, 2002. **419**(6906): p. 498-511.

81. Lasonder E, Ishihama Y, Andersen JS, et al., Analysis of the Plasmodium falciparum proteome by high-accuracy mass spectrometry. Nature, 2002. **419**(6906): p. 537-42.

82. Ghai M, Dutta S, Hall T, et al., Identification, expression, and functional characterization of MAEBL, a sporozoite and asexual blood stage chimeric erythrocyte-binding protein of Plasmodium falciparum. Mol Biochem Parasitol, 2002. **123**(1): p. 35-45.

83. Maier AG, Duraisingh MT, Reeder JC, et al., Plasmodium falciparum erythrocyte invasion through glycophorin C and selection for Gerbich negativity in human populations. Nat Med, 2003. **9**(1): p. 87-92.

84. Lobo CA, Rodriguez M, Reid M, et al., Glycophorin C is the receptor for the Plasmodium falciparum erythrocyte binding ligand PfEBP-2 (baebl). Blood, 2003. **101**(11): p. 4628-31.

85. Baum J, Thomas AW, and Conway DJ, Evidence for Diversifying Selection on Erythrocyte-Binding Antigens of Plasmodium falciparum and P. vivax. Genetics, 2003. **163**(4): p. 1327-36.

86. Gilberger TW, Thompson JK, Reed MB, et al., The cytoplasmic domain of the Plasmodium falciparum ligand EBA-175 is essential for invasion but not protein trafficking. J Cell Biol, 2003. **162**(2): p. 317-27.

87. Ranjan A and Chitnis CE, Mapping regions containing binding residues within functional domains of Plasmodium vivax and Plasmodium knowlesi erythrocyte-binding proteins. Proc Natl Acad Sci U S A, 1999. **96**(24): p. 14067-72.

88. Miller LH, Roberts T, Shahabuddin M, et al., Analysis of sequence diversity in the Plasmodium falciparum merozoite surface protein-1 (MSP-1). Mol Biochem Parasitol, 1993. **59**(1): p. 1-14.

89. Snewin VA, Herrera M, Sanchez G, et al., Polymorphism of the alleles of the merozoite surface antigens MSA1 and MSA2 in Plasmodium falciparum wild isolates from Colombia. Mol Biochem Parasitol, 1991. **49**(2): p. 265-75.

90. Escalante AA, Grebert HM, Isea R, et al., A study of genetic diversity in the gene encoding the circumsporozoite protein (CSP) of Plasmodium falciparum from different transmission areas--XVI. Asembo Bay Cohort Project. Mol Biochem Parasitol, 2002. **125**(1-2): p. 83-90.

91. Escalante AA, Grebert HM, Chaiyaroj SC, et al., Polymorphism in the gene encoding the Pfs48/45 antigen of Plasmodium falciparum. XI. Asembo Bay Cohort Project. Mol Biochem Parasitol, 2002. **119**(1): p. 17-22.

92. Escalante AA, Grebert HM, Chaiyaroj SC, et al., Polymorphism in the gene encoding the apical membrane antigen-1 (AMA-1) of Plasmodium falciparum. X. Asembo Bay Cohort Project. Mol Biochem Parasitol, 2001. **113**(2): p. 279-87.

93. Shi YP, Alpers MP, Povoa MM, et al., Diversity in the immunodominant determinants of the circumsporozoite protein of Plasmodium falciparum parasites from malaria-endemic regions of Papua New Guinea and Brazil. Am. J. Trop. Med. Hyg., 1992. **47**(6): p. 844-851.

94. Ampudia E, Patarroyo MA, Patarroyo ME, et al., Genetic polymorphism of the Duffy receptor binding domain of Plasmodium vivax in Colombian wild isolates. Mol Biochem Parasitol, 1996. **78**(1-2): p. 269-72.

95. Xainli J, Adams JH, and King CL, The erythrocyte binding motif of plasmodium vivax duffy binding protein is highly polymorphic and functionally conserved in isolates from Papua New Guinea. Mol Biochem Parasitol, 2000. **111**(2): p. 253-60.

96. Mayer DC, Mu JB, Feng X, et al., Polymorphism in a Plasmodium falciparum erythrocyte-binding ligand changes its receptor specificity. J Exp Med, 2002. **196**(11): p. 1523-8.

97. Binks RH and Conway DJ, The major allelic dimorphisms in four Plasmodium falciparum merozoite proteins are not associated with alternative pathways of erythrocyte invasion. Mol Biochem Parasitol, 1999. **103**(1): p. 123-7.

98. Taylor HM, Triglia T, Thompson J, et al., Plasmodium falciparum homologue of the genes for Plasmodium vivax and Plasmodium yoelii adhesive proteins, which is transcribed but not translated. Infect Immun, 2001. **69**(6): p. 3635-45.

99. Taylor HM, Grainger M, and Holder AA, Variation in the expression of a Plasmodium falciparum protein family implicated in erythrocyte invasion. Infect Immun, 2002. **70**(10): p. 5779-89.

100. Kumar VP and Datta S, Use of variability in the stage-specific transcription levels of Plasmodium falciparum in the selection of target genes. Parasitol Int, 2001. **50**(3): p. 165-73.

101. Scherf A, Hernandez-Rivas R, Buffet P, et al., Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in Plasmodium falciparum. Embo J, 1998. **17**(18): p. 5418-26.

102. Roberts DJ, Craig AG, Berendt AR, et al., Rapid switching to multiple antigenic and adhesive phenotypes in malaria [see comments]. Nature., 1992. **357**(6380): p. 689-692.

103. Wang AM, Doyle MV, and Mark DF, Quantitation of mRNA by the polymerase chain reaction. Proc Natl Acad Sci U S A, 1989. **86**(24): p. 9717-21.

104. Ginzinger DG, Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp Hematol, 2002. **30**(6): p. 503-12.

105. Ferre F, Quantitative or semi-quantitative PCR: reality versus myth. PCR Methods Appl, 1992. **2**(1): p. 1-9.

106. Freeman WM, Walker SJ, and Vrana KE, Quantitative RT-PCR: pitfalls and potential. Biotechniques, 1999. **26**(1): p. 112-22, 124-5.

107. Lie YS and Petropoulos CJ, Advances in quantitative PCR technology: 5' nuclease assays. Curr Opin Biotechnol, 1998. **9**(1): p. 43-8.

108. Heid CA, Stevens J, Livak KJ, et al., Real time quantitative PCR. Genome Res, 1996. **6**(10): p. 986-94.

109. Gibson UE, Heid CA, and Williams PM, A novel method for real time quantitative RT-PCR. Genome Res, 1996. **6**(10): p. 995-1001.

110. Polanco JC, Rodriguez JA, Corredor V, et al., Plasmodium vivax: parasitemia determination by real-time quantitative PCR in Aotus monkeys. Exp Parasitol, 2002. **100**(2): p. 131-4.

111. Lee MA, Tan CH, Aw LT, et al., Real-time fluorescence-based PCR for detection of malaria parasites. J Clin Microbiol, 2002. **40**(11): p. 4343-5.

112. Witney AA, Doolan DL, Anthony RM, et al., Determining liver stage parasite burden by real time quantitative PCR as a method for evaluating pre-erythrocytic malaria vaccine efficacy. Mol Biochem Parasitol, 2001. **118**(2): p. 233-45.

113. Goidin D, Mamessier A, Staquet MJ, et al., Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes as internal standard for

quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. Anal Biochem, 2001. **295**(1): p. 17-21.

114. Schmittgen TD and Zakrajsek BA, Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods, 2000. **46**(1-2): p. 69-81.

CHAPTER 3

IDENTIFICATION OF PUTATIVE ERYTHROCYTE BINDING PROTEIN IN *Plasmodium falciparum* BY HOMOLOGY POLYMERASE CHAIN REACTION (PCR) [1]

[1] Drummond, P. B. and D.S. Peterson. To be submitted to the Journal: Molecular and Biochemical Parasitology.

ABSTRACT

Invasion of erythrocytes by malaria parasites is mediated by specific molecular interactions between erythrocyte receptors and parasite ligands. *Plasmodium falciparum* is known to use several different receptors for invasion of erythrocytes and it appears not to be restricted to invasion of a single class of erythrocytes. The parasite ligands that bind these receptors belong to the erythrocyte-binding protein (EBP) family. This multigene family includes the erythrocyte-binding antigen-175 (*EBA-175*) and five others, four of which have significant homology to *EBA-175*. In this study we have used degenerate oligonucleotide primers derived from limited protein sequence information data to delineate conserved regions where members of the multigene family are related. Four forward and four reverse primers were synthesized, and 12 separate pairs of primer combinations were used to amplify potential ebp family members from the Dd2 and HB3 *P.falciparum* isolates. These amplicons were cloned and then subjected to sequencing. We have sequenced a total of 65 clones, of which 20 were from Dd2 and 45 from HB3. Several of these clones were known ebp genes, which validated our approach to isolating novel members of the EBP family. However, ongoing research to determine the identity of these amplified sequences is been done.

Keywords: Erythrocyte binding protein, *Plasmodium falciparum*, Degenerate primer

INTRODUCTION

The blood-stage development of malaria parasites is initiated by the invasion of merozoites into susceptible erythrocytes. Inhibition of erythrocyte invasion would prevent infection and consequently disease. Therefore, considerable effort has gone into the identification and characterization of the molecules involved in this process. The ability of *Plasmodium* merozoites to invade erythrocytes is contingent on a rapid cascade of specific interactions occurring between parasite molecules and host erythrocyte receptors [1]. After random contact of the merozoite with the erythrocyte, a weak interaction between the surface components leads to reorientation to bring the apical end close to the erythrocyte [2]. Proteins sequestered within the merozoite's apical organelles then come into play. Microneme proteins known to be involved in invasion include the erythrocyte binding antigen-175 (*EBA-175*) of *P.falciparum,* and the Duffy antigen binding proteins (*DABPs*) of *P. vivax* and *P. knowlesi* [3], [4], and [5]. *P.falciparum* is known to use different receptors for invasion of erythrocytes and commonly invades via sialic acid residues present on glycophorin A or B [6]. Mutant RBCs with modifications or deficiencies in glycophorins are, in general, less susceptible to invasion by *P.falciparum* than normal erythrocytes [7]. Moreover, *P.falciparum* appears not to be restricted to invasion via a single class of erythrocyte receptor. Indeed, there is mounting evidence of receptor heterogeneity in both laboratory [8] and field isolates [9, 10].

The possibility of "switching" between, or up-regulating, novel phenotypes introduces a further element of complexity into the invasion process and was demonstrated through the ability of the Dd2 line of the *P.falciparum* to be selected for invasion via a pathway mediated by a different host cell receptor [11]. Given that there are numerous pathways for invasion, mediated by at least 5 erythrocyte receptors, it is likely that many *P.falciparum* encoded erythrocyte

binding proteins are involved. The question therefore is how to identify these molecules and the genes encoding them.

Genome comparisons of distantly related organisms can reveal global biological patterns such as the existence and extent of conserved gene families [12];[13]. In addition, comparisons of more closely related species or different strains from a single species may contribute in identifying novel genes. Thus, gene families are best defined by related functions of individual gene products. However, in the absence of functional data, gene family members can be identified by amino acid sequence homology. The most used method to identify new family members within an organism, without the aid of a complete genome sequence, are amplification by PCR with degenerate primers [14]; [15-17].

In this study we sought to identify novel erythrocyte binding protein (EBP) genes in two isolates of *P.falciparum* by PCR with primers to motifs conserved in known EBP-family members. The identification and characterization of novel EBP's is an important step toward understanding the interactions between this class of parasite ligands and their host receptors. The recent completion of the genome of *P.falciparum* has provided a complete list of the EBP-family members present in one isolate of this parasite. The hypothesis to be tested here is that other isolates of *P.falciparum* may encode additional members of the EBP-family.

MATERIALS AND METHODS

P. falciparum clones and culture

The origins of *P.falciparum* clones Dd2 (IndoChina) and HB3 (Honduras) have been previously tabulated [18]. The parasites were culture in RPMI 1640 medium containing gentamicin (50mg/liter) 10% human plasma or 5% Albumax (Gibco-BRL, Gaithersburg, MD) at a hematocrit of 5% essentially as described previously [19].

DNA preparation and PCR amplification

Genomic DNA from *P.falciparum* clones Dd2 and HB3 was extracted from whole blood (20 ml) using the Roche Mammalian Blood DNA isolation Kit (Roche Indianapolis, IN) according to the manufacturer's protocol. Degenerate primers were designed from a multiple alignment of known EBP-Family sequences. The primers used in this study were, forward (ebpdbl-1) 5'-TAT (AT) (GC) (AGCT) TT (CT) GA (CT) GA (CT) TAT-3', and reverse (ebp3'rev-1) 5'-TTC (AG) TT TTC (AG) CA (CT) TT (AG) TT-3'; forward (ebpdbl-2) 5'-CCT CAA TTT TTG AGA TGG-3', and reverse (ebp3'rev-2) 5'-ATC (AGT) (GC) (AT) (AT) AT (AGT) (GC) (AT) ACA ACA TAA-3'; forward (ebpdblfor-1) 5'-CC (AT) CG (AT) (AC) G (AT) CA (AG) CA (AG) (CT) T (AT) TG-3', and reverse (ebpcterm-1) 5'-AAA A (AT) A TTT (CT) A (AT) ACA (AG) TA AT-3'; forward (ebpdblfor2) 5'- GAA TTG GG (AT) GAT GAT TTT-3', and reverse (ebpcterm-2) 5'- AAA A (AT) A TTT (CT) A (AT) ACA (AG) TA ATC-3'. These primers were used in different combination to detect candidate genes. This procedure is based on a PCR reaction, as described by [20] with minor modifications. The reaction mix consist of 20 mM Tris–HCl, 50 mM KCl, 1.5 mM MgCl2, 200 nM of each deoxynucleotide triphosphate, 2.5 U of Taq polymerase, 100 pg of each primer, and 50ng of DNA template in a final volume of 100 ul. The amplification cycle consisted of initial denaturation at 94 ^{o}C for 3 min followed by 30 cycles, each consisting of 1 min denaturation at 94 ^{o}C, 30s annealing at 48 ^{o}C and 5 min extension at 65 ^{o}C; the final cycle products are extended for 8min at 65 ^{o}C. PCR products were electrophoresed on 1% agarose gels, and DNA visualized on a Dark Reader transilluminator after SYBR Green staining.

The PCR products were purified from the low melting agarose gel, using a DNA purification kit (Bio-Rad Laboratories) according to manufacturer's protocol and cloned directly

into pCR2.1-TOPO cloning vector using a TOPO-TA cloning kit (In Vitrogen, La Jolla, CA). Inserts were sequenced using M13 forward and reverse primers. In those cases where amplicons were directly sequenced without cloning, excess dNTPs and unincorporated primers were eliminated using ethanol precipitation. Sequencing was performed on a Beckman CEQ-2000XL automated DNA sequencer using the dye terminator cycle sequencing chemistry.

Homology searches and sequence analysis

Open reading frame (ORF) predictions for the sequences of both *Plasmodium* isolates were performed using Vector Nti package v 6.0. To detect protein homology BLAST and FASTA searches were performed against the complete *P.falciparum* genome using the PlasmoDB database and against sequences in the GenBank database. Additional sequence analysis was done on the EMBL and NCBI servers.

RESULTS

Overview of approach to identify genes encoding novel EBPs

The approach used to identify novel EBP-Family members is depicted in Figure 3.1, and begins with the design of a set of degenerate primer pairs specific for the target gene family. Primers are called "degenerate" if they contain, at some positions, more than one possible nucleotide within the synthesized population. The degeneracy of the primers used here ranged from 2 to 256. The degeneracy of a primer is the product of the number of possible nucleotides at each position. The design is performed manually, (with the aid of a *Plasmodium* codon usage table) and, aims at striking a balance between degeneracy and specificity of the primers. PCR reactions were then carried out with these primers on genomic DNA from two different clonal isolates of *P.falciparum* HB3 and Dd2.

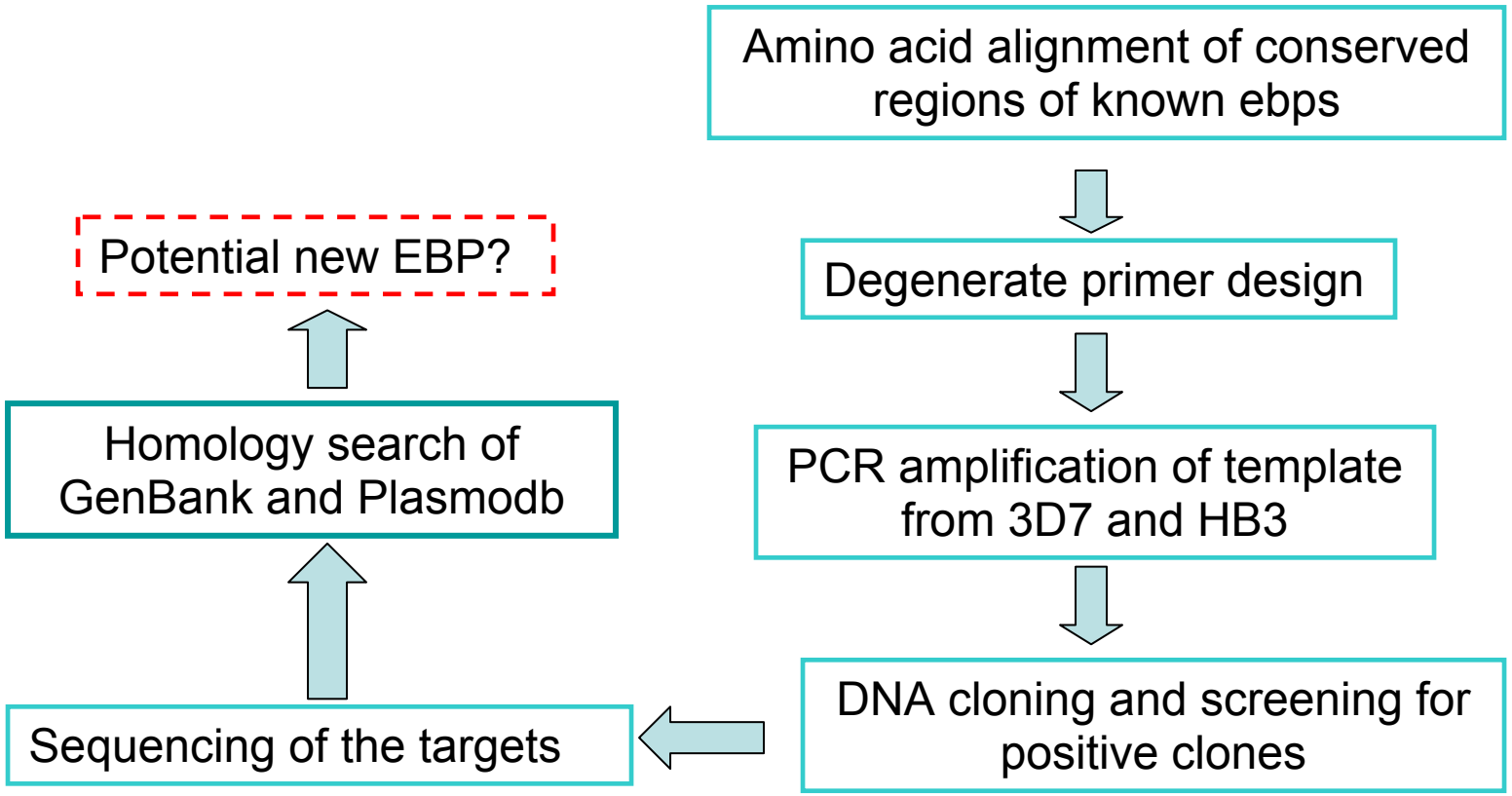


Figure 3.1. Overview of approach used to identify genes encoding novel EBPs. Starting from a know set of genes from the family of EBP, degenerate primers are designed manually from conserved amino acid sequence in order to amplify as many of these genes as possible, given a bound of the allowed degeneracy. Primer pairs are used to amplify genomic fragment from Dd2 and HB3 in PCR reactions. The products are cloned, sequenced and analyzed by homology searches against both GenBank and the Malaria Genome Project Database (PlasmoDB).

These two clones differ in their invasion phenotype and therefore may express different members of the EBP-family. After amplification,

the products are cloned, into the vector pCR2.1 and the resulting colonies screened for the presence of the insert. Positive clones were then sequenced and analyzed by homology searches against both GenBank and PlasmoDB.

Design of Degenerate Primers

Figure 3.2 shows that *EBA-175* and other members of the erythrocyte binding protein family have conserved N- and C-terminal cysteine-rich regions. The two cysteine-rich regions (often referred to as the 5' and 3' cys-rich regions of the EBP gene) bear numerous conserved cysteine residues, with the 5' cys-rich possessing the erythrocyte binding function. This region also shares homology with another adhesion protein**,** *PfEMP-1* encoded by the var gene family. Unfortunately the var gene family has upwards of 50 members, all of which contain one to seven cysteine-rich (cys-rich) regions with varying levels of homology to the 5' cys-rich region of the EBP genes. This represents an obstacle to the homology PCR approach as the 5' cysteine-rich region provides some of the best motifs among the EBP genes, but is shared with a much larger gene family. Still we believe that careful primer design can minimize amplification of the *PfEMP1* encoding var genes. Given a set of genes from a gene family of interest, one can design degenerate primers that embody an efficient balance between degeneracy and gene specificity. To accomplish this, conserved regions present in the EBP-family members *eba-175*, *ebl-1*, *eba-181*, and *eba-140* were identified by alignment of amino acid sequences. The degenerate primers were designed to match several highly conserved regions, as shown in Figure 3.3(a) and 3.3(b).

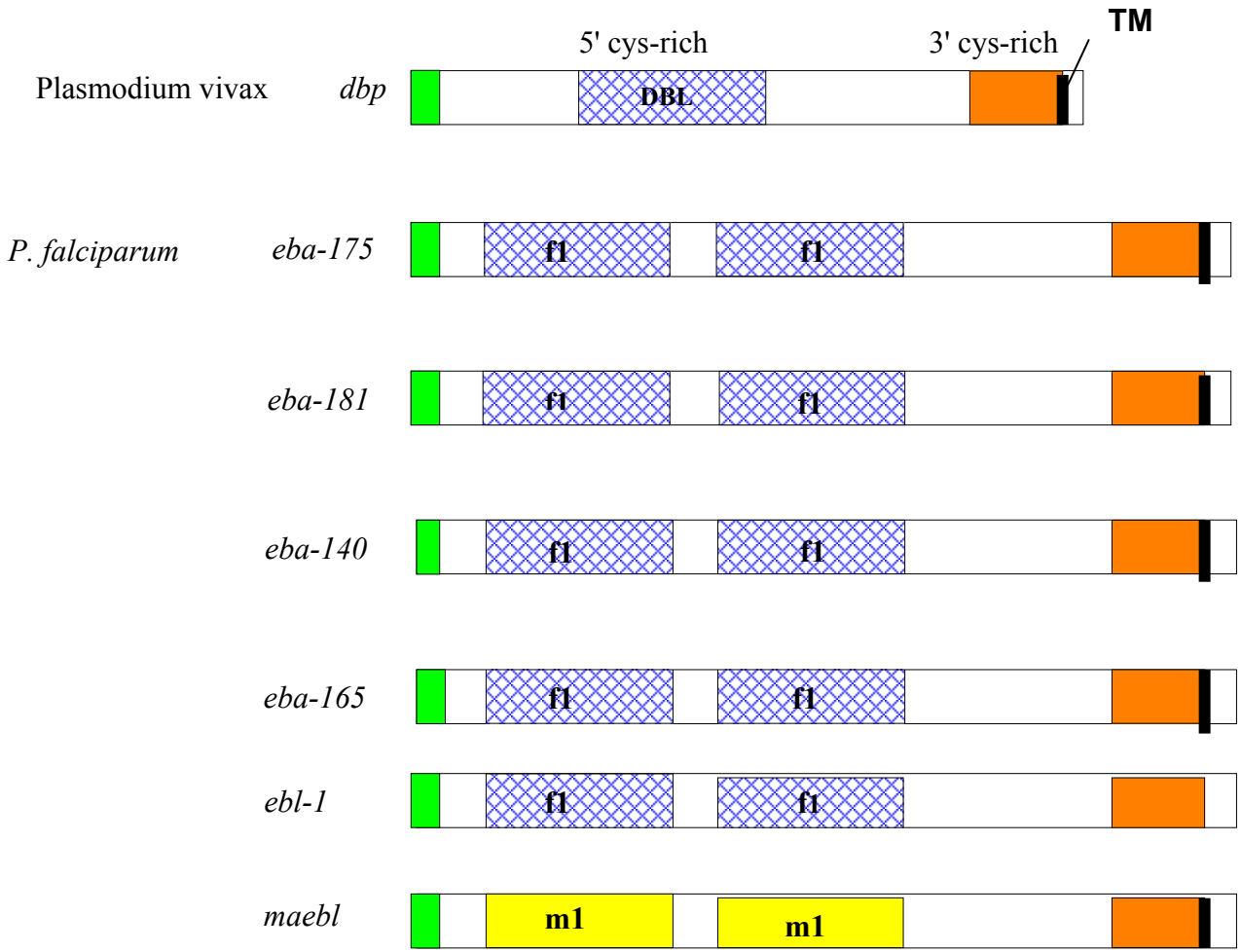


Figure 3.2. Schematic representations of the *Plasmodium vivax* and *P. falciparum EBP* multi-exon structure. There is a tandem duplication of the DBL domain in 5 of *P. falciparum ebl* family, with the exception of *maebl*. *MAEBL* ligand domain has duplication, which is similar to apical membrane antigen-1. Predicted signal sequences are indicated by black shading, TM = transmembrane Region. The DBL domain labeled as F1 and F2, while the *MAEBL* ligand domain is labeled as m1 and m2.

Unfortunately, the sequence between the 5' and 3' of cysteine-rich regions is highly diverse, with no detectable sequence similarity seen in the 5 family members. Four forward and four reverse degenerate primers were designed. Four map in the 5' cysteine-rich region, and the other 4 maps in the 3' cysteine-rich region.

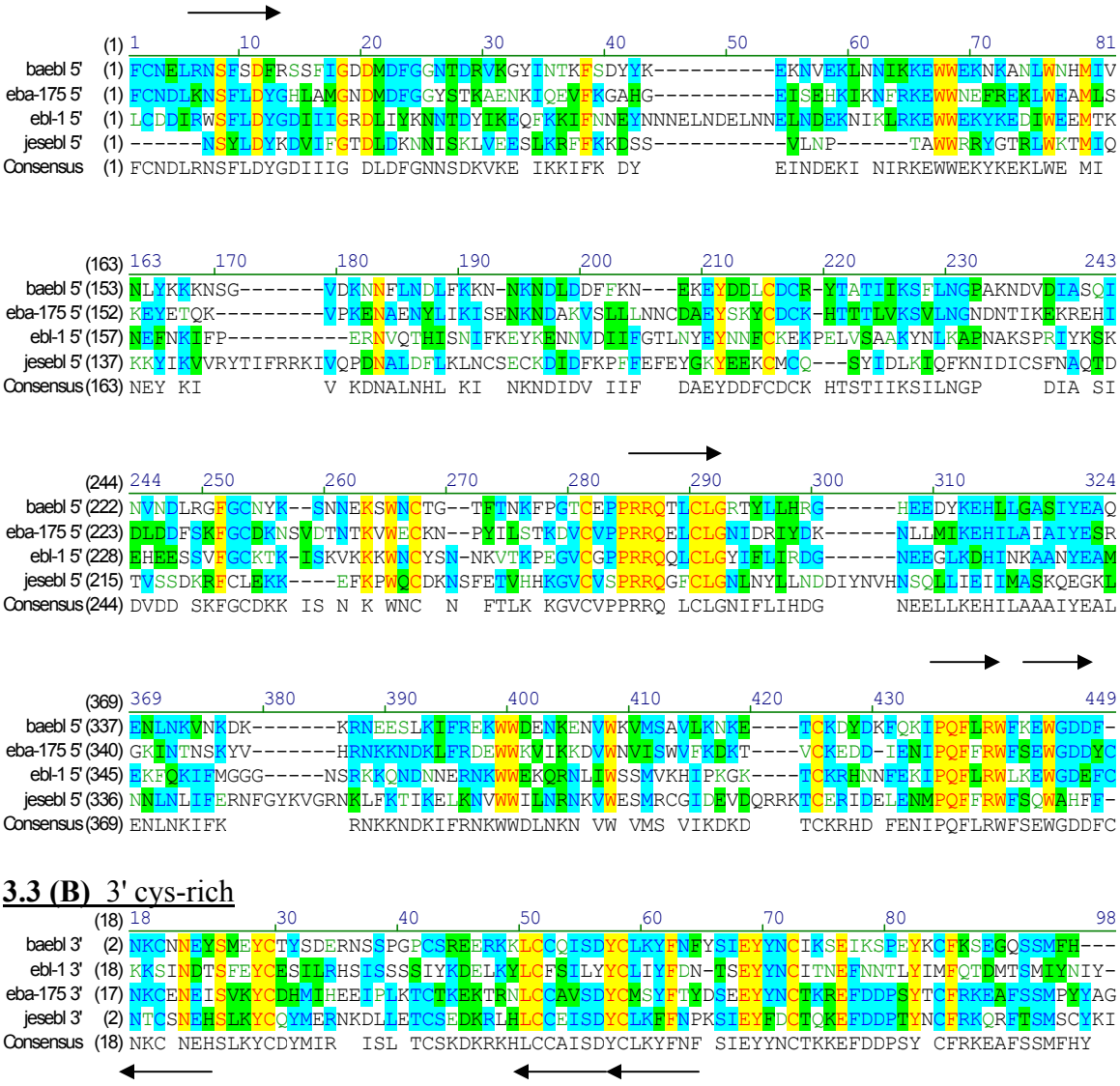
Twelve different primer combinations were generated based on the location of various conserved domains shown in Figure 3.4 (a). Therefore, the primer pairs used amplify a fragment that contains at its core a large region of non-conserved sequence as indicated in Figure 3.4 (b). Small variably sized regions originating from the 5' and 3' cysteine-rich regions flank this region. Only the terminal sequences therefore are of predictive value in determining if an amplified fragment is potentially a novel EBP gene. In addition to novel EBP genes, we expected to also amplify fragments from the five known EBP family genes with some of the primer pair combinations.

PCR Amplification of potential novel members of the EBP gene family

PCR amplification was performed with genomic DNA from clones Dd2 and HB3, using twelve different degenerate primer combinations as shown in Figure 3.5. The number of fragments amplified with each pair ranged from 3 to 7. Fragments to be cloned and sequenced were selected based upon the size expected from the relative location of primer pairs used. These fragments were then gel purified and cloned into the pCRII-TOPO vector. The Plasmid containing the inserted DNA was sequenced on the Beckman CEQ-2000XL.

All clones were sequenced from the orientation of the 5'cys-rich region, as this area was expected to provide sequence most useful in determining if the clone originated from a novel EBP gene. This is because only a small variably sized regions originating from the 5' and 3' cysteine-rich regions flank this region.

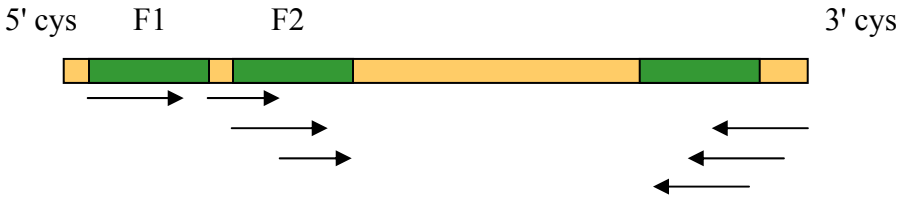
3.3 (A) 5' cys-rich



3.3 (B) 3' cys-rich

Figure 3.3 (A)-(B). Multiple amino acid sequence alignments of four *Plasmodium falciparum* ebl genes, baebl or *eba-140* (accession no. AF332918), *eba-175* (accession no M93397), *ebl-1* (accession no AF131999) and *eba-181* (accession no AB080796). Consensus sequences are shown below the alignment. Arrows indicates the annealing sites of the degenerate primers (ebpdbl-1, ebpdblfor-1, ebpdbl-2, ebpdblfor2, ebp3'rev-1, ebp3'rev-2, ebpcterm-1 and ebpcterm-2) respectively. However, a single line represents ebpcterm-1 and ebpcterm-2 because these primers are only different in a single nucleotide.

(A) Location of degenerate primers on EBP



(B) Region amplified

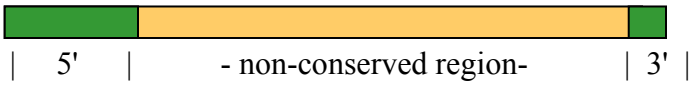


Figure 3.4. Diagram showing the relative location of the degenerate primers and the region that was amplified. (A) The arrows illustrate the regions where primer-to-template annealing during PCR. Twelve different primer combinations were generated based on the location of various conserved domains. (B) The amplified region consists of conserved 5' and 3' regions, separated by a large non-conserved central region.

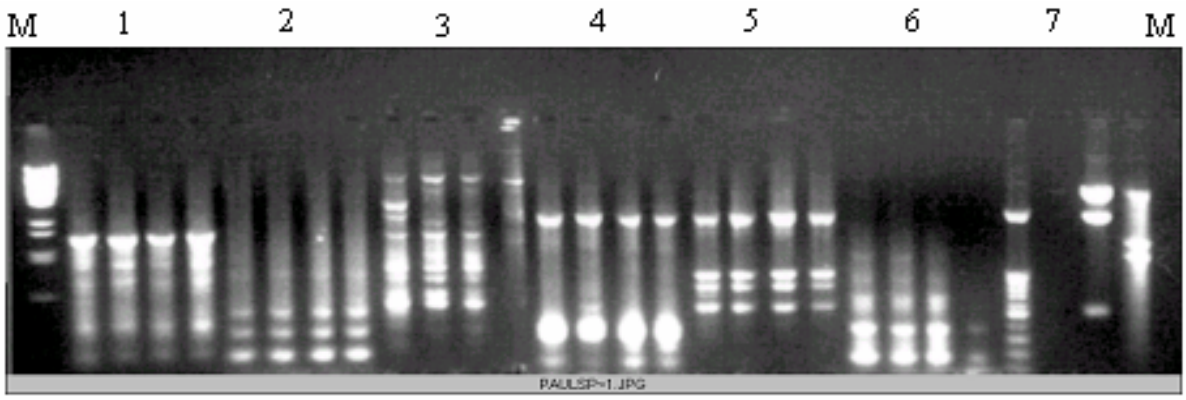


Figure 3.5. PCR amplifications for cloning novel EBP from genomic DNA from *P.falciparum* isolate HB3. PCR amplifications were performed using degenerate primers ebpdblf 1 and f 2 (5') and ebp 3' rev 1, 2 and ebpC-Term 1 and 2 (3') primer pairs. Agarose gel (0.8%) showing the PCR amplification products obtained from the different primer pairs. Section 1 (lane 1-4) representative reaction for primer pair ebpdblf 1/ ebpC-Term 1, section 2 (lane 5-8) ebpdblf 2/ ebpC-Term 1, section 3 (lane 9-12) ebpdblf 1/ ebp 3' rev 1, section 4 (lane 13-16) ebpdblf 2/ ebp 3' rev 1, section 5 (lane 17-20) ebpdblf 2/ ebp 3' rev 2, section 6 (lane 21-24) ebpdblf 2/ ebpC-Term 2 and section 7 (lane 25-28) ebpdblf 1/ ebp 3' rev 2. M- Molecular markers.

Once sequence information was obtained for an insert, the data was compared with sequences in Genbank and the Malaria Genome Database using BLAST. Table 3.1 presents a summary of the number of significant hits against the Plasmodium and GenBank databases. Five or 7.7% (forward) sequences showed significant homology to both EBPs and var genes and potentially represented novel members of the EBP or var gene family. Known EBPs accounted for 30.8% (20) of the total hits.

Although, not shown in the table, there was sequence from the 3' cys-rich end of the amplicons, which revealed an additional 3 with homology to known EBP s. There were also 23 significant hits obtained with known var genes, representing approximately 35.4% of the total hits. This was expected as both EBP and var genes are members of the larger Duffy Binding-Like superfamily, and some of the 5' primers can also anneal to some var genes. Finally, the remaining sequences represent genes with no clear homology to either EBP or var genes. The next step is to determine whether the 5 high scoring sequences do, in fact, encode EBP genes. This will be accomplished by obtaining additional sequence from flanking regions.

DISCUSSIONS

The goal of this study was to identify novel EBP gene family members in *P.falciparum*. *EBA-175*, the first EBP characterized in *P.falciparum*, was identified in culture supernatants following rupture of the schizont infected red blood cell [21]. This approach has not however yielded any further erythrocyte binding proteins, and the remaining members of the EBP family were identified by homology searches of the *P.falciparum* genome database [22].

Table. 3.1. Summary of blast search from both GenBank and Plasmodium database

CLONE	Total Hits	Non-dbl*	Probable var	Known EBP	Potential novel EBP/var
Dd2	20	5	5	7	3
HB3	45	12	18	13	2
Total	65	17	23	20	5

* Represents hit with significant homology to non-var related gene.

The PCR approach adopted in the present study has lead to the isolation of 5 potential ebp-derived PCR products from *P.falciparum* genomic DNA. However it remains unclear if the identified sequences are novel EBP-Family members, or result from amplification of related sequences from the more numerous var gene family.

With each PCR regime some genes amplify more efficiently than others; with hindsight, a PCR approach may have been more effective in amplifying potential ebp-related product if a new technique was used. This approach allows one to synthesize a specific oligonucleotide for each known family member so that it hybridizes to one strand of template, adjacent to the 3' end of the primer. This will allow the degenerate primer to bind yet prevents extension by DNA polymerase [23, 24]. Another method would be to use the degenerate primers to amplified members of the gene family, and then use oligofingerprinting of the cloned PCR products with clustering of the clones based on their fingerprints. This has been successfully applied to analyze members of multigene families as well as to isolate gene homology from different genomes [25] and should greatly decrease the number of clones to be sequenced by reducing the yielded of nonspecific products [26].

However, while this may have helped to inhibit amplification from the known members of the EBP-Family, it is unfortunately not applicable to the multi-copy var gene family. The var gene family contains approximately 50 members, and while the sequence of each gene is known in the *P.falciparum* isolate (3D7) used for the Malaria Genome Project, the family is highly diverse. This means that it is unlikely that any two *P.falciparum* isolates contain the same var gene sequences. Because both the EBP and var gene family originated from the Duffy binding like-domain super-family, {which share similar 5' cys-rich domains} it was difficult to design degenerate primer {represent in Figure 3.3 (a) and (b)} to amplify only EBPs.

Based on the above-mentioned limitation, it is important that further analysis would be required to determine if a novel ebp gene has been found. Although, the *Plasmodium falciparum* genome has been sequenced (22.8 mega-base), it is estimated that > 60% of the 5,409 predicted open reading frames (ORF) are yet to be annotated [27].These five ORF's however, show some homology with the erythrocyte binding proteins.

One of these ORF that was generated by ebpdblf 1(forward primer) and ebp 3' rev 1 (reverse primer) revealed a 20 % homology with chimpanzee malaria parasite *Plasmodium reichenowi* erythrocyte binding antigen-175 [28], a major gene that is required for erythrocyte invasion in *P. falciparum*. Therefore, the next step is to verify that the 5 high scoring sequences, do in fact, encode EBP genes.

CONCLUSION

The PCR approach with consensus-degenerate primer has led to the identification of potential EBP or var gene family members. This has validated our approach to isolate novel members of the EBP family. This is reasonable (five potential EBPs or var gene) when one take into consideration that only sixty-five clone was sequenced.

Further work will focus on the characterization of the five potential EBPs or var gene to determine their identity. We will use the vectorette PCR method to further characterized these potential EBPs or var gene. The best method would to used a *P. falciparum* cDNA library, however one is not available at this time.

Other possible enhancements might increase the effective of our method such as using computerized base program to design the degenerate primers and also include a hybridized probe as mention early in the discussion. These and other refinements should lead to even more efficient isolation of unknown EBPs or even var genes from other isolates.

REFERENCES

1. Sim BK, Toyoshima T, Haynes JD, et al., Localization of the 175-kilodalton erythrocyte binding antigen in micronemes of Plasmodium falciparum merozoites. Mol. Biochem. Parasitol., 1992. **51**(1): p. 157-159.
2. Dvorak JA, Miller, L.H., Whitehouse, W.C., Invasion of erythrocytes by malaria merozoites. Science, 1975. **187**: p. 748-749.
3. Dolan SA, Proctor JL, Alling DW, et al., Glycophorin B as an EBA-175 independent Plasmodium falciparum receptor of human erythrocytes. Mol. Biochem. Parasitol., 1994. **64**: p. 55-63.
4. Chitnis CE and Miller LH, Identification of the erythrocyte binding domains of Plasmodium vivax and Plasmodium knowlesi proteins involved in erythrocyte infection. J. Exp. Med., 1994. **180**: p. 497-506.
5. Adams JH, Hudson DE, Torii M, et al., The Duffy receptor family of Plasmodium knowlesi is located within the micronemes of invasive malaria merozoites. Cell., 1990. **63**(1): p. 141-153.
6. Hadley TJ, Klotz FW, Pasvol G, et al., Falciparum malaria parasites invade erythrocytes that lack glycophorin A and B (MkMk). Strain differences indicate receptor heterogeneity and two pathways for invasion. J. Clin. Invest., 1987. **80**(4): p. 1190-1193.
7. Pasvol G, Wainscoat JS, and Weatherall DJ, Erythrocytes deficiency in glycophorin resist invasion by the malarial parasite Plasmodium falciparum. Nature, 1982. **297**(5861): p. 64-6.

8. Sim BK, Carter JM, Deal CD, et al., Plasmodium falciparum: further characterization of a functionally active region of the merozoite invasion ligand EBA-175. Exp. Parasitol., 1994. **78**(3): p. 259-268.

9. Okoyeh JN, Pillai CR, and Chitnis CE, Plasmodium falciparum field isolates commonly use erythrocyte invasion pathways that are independent of sialic acid residues of glycophorin A. Infect Immun, 1999. **67**(11): p. 5784-91.

10. Baum J, Pinder M, and Conway DJ, Erythrocyte invasion phenotypes of Plasmodium falciparum in The Gambia. Infect Immun, 2003. **71**(4): p. 1856-63.

11. Dolan SA, Miller LH, and Wellems TE, Evidence for a switching mechanism in the invasion of erythrocytes by Plasmodium falciparum. J Clin Invest, 1990. **86**(2): p. 618-24.

12. Rubin GM, Yandell MD, Wortman JR, et al., Comparative genomics of the eukaryotes. Science, 2000. **287**(5461): p. 2204-15.

13. Chervitz SA, Aravind L, Sherlock G, et al., Comparison of the complete protein sets of worm and yeast: orthology and divergence. Science, 1998. **282**(5396): p. 2022-8.

14. Mack DH and Sninsky JJ, A sensitive method for the identification of uncharacterized viruses related to known virus groups: hepadnavirus model system. Proc Natl Acad Sci U S A, 1988. **85**(18): p. 6977-81.

15. Pytela R, Suzuki S, Breuss J, et al., Polymerase chain reaction cloning with degenerate primers: homology-based identification of adhesion molecules. Methods Enzymol, 1994. **245**: p. 420-51.

16. Preston GM, Cloning gene family members using PCR with degenerate oligonucleotide primers. Methods Mol Biol, 1997. **67**: p. 433-49.

17. Zhao ZY and Joho RH, Isolation of distantly related members in a multigene family using the polymerase chain reaction technique. Biochem Biophys Res Commun, 1990. **167**(1): p. 174-82.

18. Binks RH and Conway DJ, The major allelic dimorphisms in four Plasmodium falciparum merozoite proteins are not associated with alternative pathways of erythrocyte invasion. Mol Biochem Parasitol, 1999. **103**(1): p. 123-7.

19. Trager W and Jensen JB, Human malaria parasites in continuous culture. Science., 1976. **193**(4254): p. 673-675.

20. Sambrook J, Fritsch EF, and Maniatis T, Molecular Cloning. 1989, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

21. Orlandi PA, Sim BK, Chulay JD, et al., Characterization of the 175-kilodalton erythrocyte binding antigen of Plasmodium falciparum. Mol. Biochem. Parasitol., 1990. **40**(2): p. 285-294.

22. Adams JH, Blair PL, Kaneko O, et al., An expanding ebl family of Plasmodium falciparum. Trends Parasitol, 2001. **17**(6): p. 297-9.

23. Kunihiro S, Kawanishi Y, Sano M, et al., A polymerase chain reaction-based method for cloning novel members of a gene family using a combination of degenerate and inhibitory primers. Gene, 2002. **289**(1-2): p. 177-84.

24. Yuen PS, Brooks KM, and Li Y, RNA: a method to specifically inhibit PCR amplification of known members of a multigene family by degenerate primers. Nucleic Acids Res, 2001. **29**(6): p. E31.

25. Rose TM, Schultz ER, Henikoff JG, et al., Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. Nucleic Acids Res, 1998. **26**(7): p. 1628-35.

26. Fuchs T, Malecova B, Linhart C, et al., DEFOG: a practical scheme for deciphering families of genes. Genomics, 2002. **80**(3): p. 295-302.

27. Gardner MJ, Hall N, Fung E, et al., Genome sequence of the human malaria parasite Plasmodium falciparum. Nature, 2002. **419**(6906): p. 498-511.

28. Ozwara H, Kocken CH, Conway DJ, et al., Comparative analysis of Plasmodium reichenowi and P. falciparum erythrocyte-binding proteins reveals selection to maintain polymorphism in the erythrocyte-binding region of EBA-175. Mol Biochem Parasitol, 2001. **116**(1): p. 81-4.

CHAPTER 4

AN ANALYSIS OF SEQUENCE CONSERVATION WITHIN THE LIGAND DOMAINS OF THE ERYTHROCYTE BINDING-LIKE 1(EBL-1) GENE[2]

[2] Drummond, P. B. and D. S. Peterson. To be submitted to the Journal: Molecular and Biochemical Parasitology.

ABSTRACT

The erythrocyte binding proteins (EBP) of *Plasmodium falciparum* are ligand that plays a functional role in erythrocyte invasion by merozoites. These antigens are considered candidates for antimalarial vaccines; therefore it is important to understand the degree of sequence polymorphism within this family. The present study was designed to investigate the level of sequence conservation within the Duffy binding-like (*DBL*) domains of the erythrocyte binding-like 1 (*EBL-1*) gene of *Plasmodium falciparum*. This gene contains two putative ligand domains (the *DBL* domains) that may function in erythrocyte invasion. Ten isolates of *Plasmodium falciparum* originating from different parts of the world were used to study the conservation of the *ebl-1* gene. Relative to the published sequence for the *ebl-1* gene from isolate Dd2, we identified 27 point mutations in 9 isolates. Of the total polymorphisms, 7 were synonymous, and 20 caused changes in the amino acid encoded. The majority of the non-synonymous mutations were found in the second or F2 *DBL* domain, which contained 13 non-synonymous but only two synonymous mutations. A comparison of the level of polymorphism in the entire *ebl-1* coding region of isolate Dd2 relative to isolate 3D7 revealed 21 polymorphic sites, with 5 synonymous and 16 non-synonymous changes. In addition to the non-synonymous changes, a 5 nucleotide insertion is found upstream of the F1 *DBL* region of the *ebl-1* gene in isolate 3D7. Six of the 10 isolates examined here lack the insertion found in 3D7, and so contained an uninterrupted open reading frame. The preponderance of non-synonymous polymorphisms, notably in the F2 *DBL* domain suggests that the *ebl*-1 gene is under selective pressure, either by the host immune response or possible host cell receptor heterogeneity.

Keywords: Duffy binding-like, synonymous, non-synonymous, polymorphism, Erythrocyte binding like-1

INTRODUCTION

Malaria is a worldwide infectious disease caused by a protozoan parasite of the genus *Plasmodium*, and is transmitted to humans by Anopheline mosquitoes. The most dangerous of the species infecting humans is *P. falciparum*, which is responsible for the death of an estimated 2-3 million children, and 300-500 million infections per year worldwide [1]. *Plasmodium falciparum,* like other species of human *Plasmodium,* initiates erythrocyte invasion through expression of proteins on the surface and in apical organelles of the merozoite that bind to erythrocyte surface proteins. These parasite ligands are thought to be important mediators of invasion [2-5]. In *Plasmodium,* a large number of proteins, many with similar structures and 'signature' domains, mediate host cell adhesion and invasion. Of particular relevance to red cell invasion is the erythrocyte-binding protein (*EBP*) family, which includes the Duffy binding proteins (*DBP*) of *P. vivax* and six related proteins of *P. falciparum* [4, 6]. *P. vivax* utilizes the Duffy antigen as a receptor during invasion, however *P. falciparum* is less restricted in its invasion requirements, utilizing at least five different pathways for ligand-receptor binding mediated by glycophorin A, glycophorin B, glycophorin C, and at least two more receptors that have not yet been characterized, referred to as receptors E and X [3, 7-10]. In both *P. vivax* and *P. falciparum*, adhesion proteins of the *EBP* family show striking structural homology, possessing two cysteine-rich (cys- rich) domains with numerous conserved cysteine, a N-terminal domain that is duplicated in the *P. falciparum* proteins, and a C-terminal domain adjacent to a transmembrane domain [11]. The receptor-binding domain is located in the conserved, N-terminal cysteine rich area called region II [4, 12]. In *P. falciparum* the *DBL* ligand domains are present in a wide variety of proteins including the glycophorin A binding protein *EBA-175*. Recently, several novel members of the DBL domain super-family have been

identified in open reading frames in the *P. falciparum* genome and described, including *ebl-1* [11], *eba-140/baebl* [2, 13, 14], *eba-181/jesebl* [15] and *eba-165/pebl* [16].

Highly polymorphic regions have been observed in the genes encoding surface and apical proteins such as *Msp-1, Msp-2, AMA-1, Pfs 48/45* and *Csp* [17-21]. These genes encode antigenic proteins that are normally recognized by the host's immune system, and polymorphism observed in these genes is likely driven by immune pressure. Previous studies have examined the level of polymorphism in several members of the *EBP* family, including *EBA-175*, *EBA-140* and *EBA-165* in region II, and the Duffy binding protein in *P. falciparum* and *P. vivax* respectively [22-24].

Determining the level of polymorphism in the *DBL* ligand domain may provide some clues to the level of selective pressure on *P. falciparum* invasion proteins. Since the members of this gene family have been proposed as vaccine candidates, information on sequence polymorphism will be very important to the development of a potential vaccine against the erythrocytic stage of *Plasmodium falciparum.* In this study we have determined the level of sequence polymorphism in 10 *P. falciparum* clones within region II of the *ebl-1* gene.

MATERIALS AND METHODS

P. falciparum clones and cultivation

The origins of *P. falciparum* clones 3D7, Dd2, FCR, M camp, SL, ItB59, ItG2, ItD12, VI and 7G8 have been previously tabulated [25]. All parasites were maintained *in- vitro* in the presence of 10% human plasma or 5% Albumax (Gibco-BRL, Gaithersburg, MD) by standard methods [26].

Sequencing of DBL domain (region II) sampled from different isolates of *Plasmodium falciparum*.

DNA extraction was performed as described [27]. The DBL domain from each isolate was amplified with *ebl-1* specific primers (ebl-1fwd 5'-ATGTGGGAAGAATAAAGG-3' and ebl-1rev 5'-CCTCTATAGGAAACACATCC-3') complementary to regions flanking the ligand domains and including the entire region II. PCR and agarose gel electrophoresis were performed by standard methods [28]. For accurate amplification and sequence determination, the proofreading polymerase Ex Taq™ (Takara Mirus Bio, Madison, WI) was used. PCR products were purified using Wizard® PCR Preps a DNA purification kit (Promega) and ligated into pCRII-TOPO® vector using a TOPO-TA cloning kit (Invitrogen, La Jolla, CA) and transformed by heat shock into TOPO 10 One Shot® Chemically Competent Cells, following the supplier's protocol. Isolated plasmids were prepared using a Plasmid Miniprep (spin) column (QIAGEN). Two independent clones were isolated and sequenced (in both directions) from each isolate. Each template was sequenced using internal sequencing primers (primer walking) with the dye terminator cycle sequencing (DTCS) Quick Start Kit (Beckman Coulter, Fullerton, CA). Sequencing was performed on a Beckman Coulter CEQ™-2000XL Sequencer (Beckman Coulter). Polymorphic sites were identified in sequence alignments and were confirmed by reamplification from genomic DNA and direct sequencing to exclude any errors introduced by PCR or cloning.

Sequence Analysis

Individual sequencing runs were assembled using the Contig Express program (Informax Inc.). Once assembled all completed sequences were aligned with AlignX (Informax Inc.) for preliminary detection of polymorphisms. Non-synonymous and synonymous polymorphisms

were identified, and nucleotide diversity for this region was determined using DNAsp 3.99 software at http://www.bio.ub.es/~julio/Dnasp.html [29]. The rates of substitutions were estimated using the method of [30].

RESULTS

Analysis of Duffy binding-like domains of P. falciparum ebl-1

The ligand domain of the *ebl-1* gene was amplified from nine isolates originating from different parts of the world. The sequence of the *ebl-1* gene from isolate Dd2 (GenBank acc. # AF131999) was also included in the analysis. The size of the PCR product was 1865 bp, corresponding to nucleotides 663 – 2527 (amino acid 222-842), which includes region II (figure 4.1). No differences in sizes or in the fragment intensity were observed as shown in figure 4.1 suggesting that there are no gross changes in the *ebl-1* gene between the isolates studied.

Relative to the published sequence for the *ebl-1* gene of Dd2, we identified 27 point mutations within the other 9 isolates. Of the total mutations, 7 were synonymous (S) and 20 cause changes (non-synonymous or NS) in the amino acid encoded. There were three common mutations found in many of the isolates (Table 4.1). Seven isolates had a C > T mutation at position 1421 resulting in a change in amino acid of Thr > Ile, and eight isolates had a silent C > T mutation at position 1483. Six isolates contained both of these common polymorphisms.

Finally, 8 isolates had a G > A mutation at position 2303 resulting in an Arg to Lys coding change (Table 4.1). In addition there were two nonsynonymous and one synonymous mutation that were shared by two or more isolates. For the non-synonymous polymorphisms, 16 (88.8%) out of 18 result in a change in the family of the encoded amino acid. These changes included 7 resulting in a switch in the polarity of the amino acid, with 3 non-polar residues changing to polar and 4 polar residues changing to non-polar.

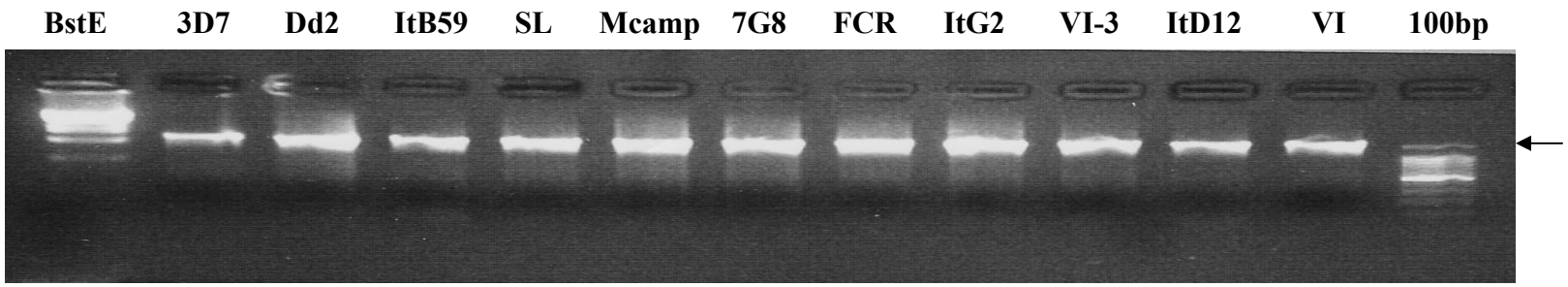


Figure 4.1. The PCR products of genomic DNA from the ligand domain encoding region region II of *ebl-1* of different *P. falciparum* isolates. All Amplified products were separated on a 1.2% agarose gel and stained with ethidium bromide showed a single band of 1865 bp (indicated by arrow) of region II of *ebl-1*. Lane 1 and 13 are DNA markers. Lane 2-12 is the different isolates.

Table 4.1. Polymorphic sites within the Duffy binding-like region of *P.falciparum* gene *ebl-1*. Only polymorphic sites are shown with position numbered vertically. Nonsynonymous (uppercase letters) and synonymous (lowercase letters) are shown relative to the reference sequence from isolate Dd2. F1and F2 subdomain is shown in solid boxes.

	854	969	1023	1187	1421	1425	1437	1474	1483	1561	1582	1617	1658	1682	1918	1945	1947	1952	2002	2067	2090	2171	2220	2273	2303	2312	2509
Dd2 ref:	C	A	A	A	C	C	A	T	C	A	T	T	A	C	A	G	A	T	A	C	A	G	G	G	G	G	G
Isolate:																											
ItB59	A	.	c	C	.	.	.	.	.	.	C	c	.	.	.	A	C	.	T	t	.	.	.	.	.	.	.
7G8	.	g	.	.	T	.	.	.	t	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
3D7	.	.	.	.	T	T	.	.	t	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A
ItD12	.	.	.	.	T	.	.	.	t	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
FCR3	.	.	.	.	T	.	.	.	t	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	A	.	.
V1	.	.	.	.	T	.	.	.	t	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
ItG2	.	.	.	.	T	.	.	.	t	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
SLD6	.	.	.	.	.	.	t	.	t	.	.	.	.	A	G	.	.	.	.	.	.	A	a	C	A	A	.
MCamp	.	.	.	.	T	.	.	C	t	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	.

F1 F2

Finally, seven of these amino acid changes lead to change in charge, and 2 changes affect aromatic amino acids. We found that all 21 cysteine residues (F1= 8 and F2 =13) were conserved in region II of the isolates studied

(Table 4.1). The presence of conserved aromatic amino acids is also observed in region region II of *EBP* family members. Within region II of the *ebl-1* gene of isolate Dd2 are 27 tyrosine, 17 tryptophan and 29 phenylalanine residues. All tryptophan residues are strictly conserved among all isolates; however, not all tyrosine and phenylalanine residues are conserved in all isolates. A phenylalanine is changed to leucine (aa 264) in the Mcamp clone and to tyrosine (aa 423) in the FCR clone; however, these are not among the aromatic residues conserved in all EBPs.

From a total of 1865 nucleotides that were studied, we observed a range of 3-9 changes for a particular isolate relative to Dd2. Two of these isolates, ItB59 and SL showed the greatest number of residue changes (9 each), while ItD12 and VI had the fewest with only 3 changes. These two isolates were identical on the basis of mutations in the nucleotides and their corresponding amino acids. Interestingly, all of the point mutations observed in ItB59 showed novel nucleotide changes not shared with other isolates. Six of the nucleotide changes observed in isolate SL were unique, with other isolates having either 1 or 2 novel mutations (Table 4.1).

Figure 4.2 shows the distribution of synonymous and nonsynonymous mutations in the *P falciparum* isolates. Isolates ItB59 and SL have the greatest number of synonymous (3) and nonsynonymous (6) mutations among all isolates. Both FCR and Mcamp have 1 synonymous and (4) non-synonymous mutation (though not the same ones). Only the 7G8 isolate contains an equal number of synonymous (2) and nonsynonymous (2) changes, while the 3D7 isolate contains 2 synonymous and 3 non-synonymous mutations. The smallest number of mutations,

nonsynonymous, 2 and 1 synonymous was observed in ItD12 and VI. Finally, only two (ItD12 and VI) isolates have the same allele (10 isolates define 9 allelic types).

The nucleotide diversity across the Duffy binding-like domains of *ebl-1* is depicted in Figure 4.3. The nucleotide diversity (π) in this region was calculated to be 0.00321. The distribution of polymorphisms was not even in the region sequenced, with the F1, inter domain region (IDR) and F2 having 9, 3 and 15 polymorphisms respectively. Within these 3 regions, the ratio of non-synonymous to synonymous was not constant, with the F2 region having significantly more non-synonymous polymorphisms. The number of synonymous and non-synonymous polymorphism observed in each domain is: F1, 4 S and 5 NS; IDR, 1 S and 2 NS; and F2, 2S and 13 NS. There were no insertions, deletions or gene rearrangements detected in the Duffy binding-like region of the *ebl-1* gene.

No clear correlation was observed with respect to geographic origin, since VI (Vietnam) and ItD12 (Brazil) are identical. We have found that overall South America and African isolates were the most diverse. However, it should be noted that only 10 isolates were compared in this study.

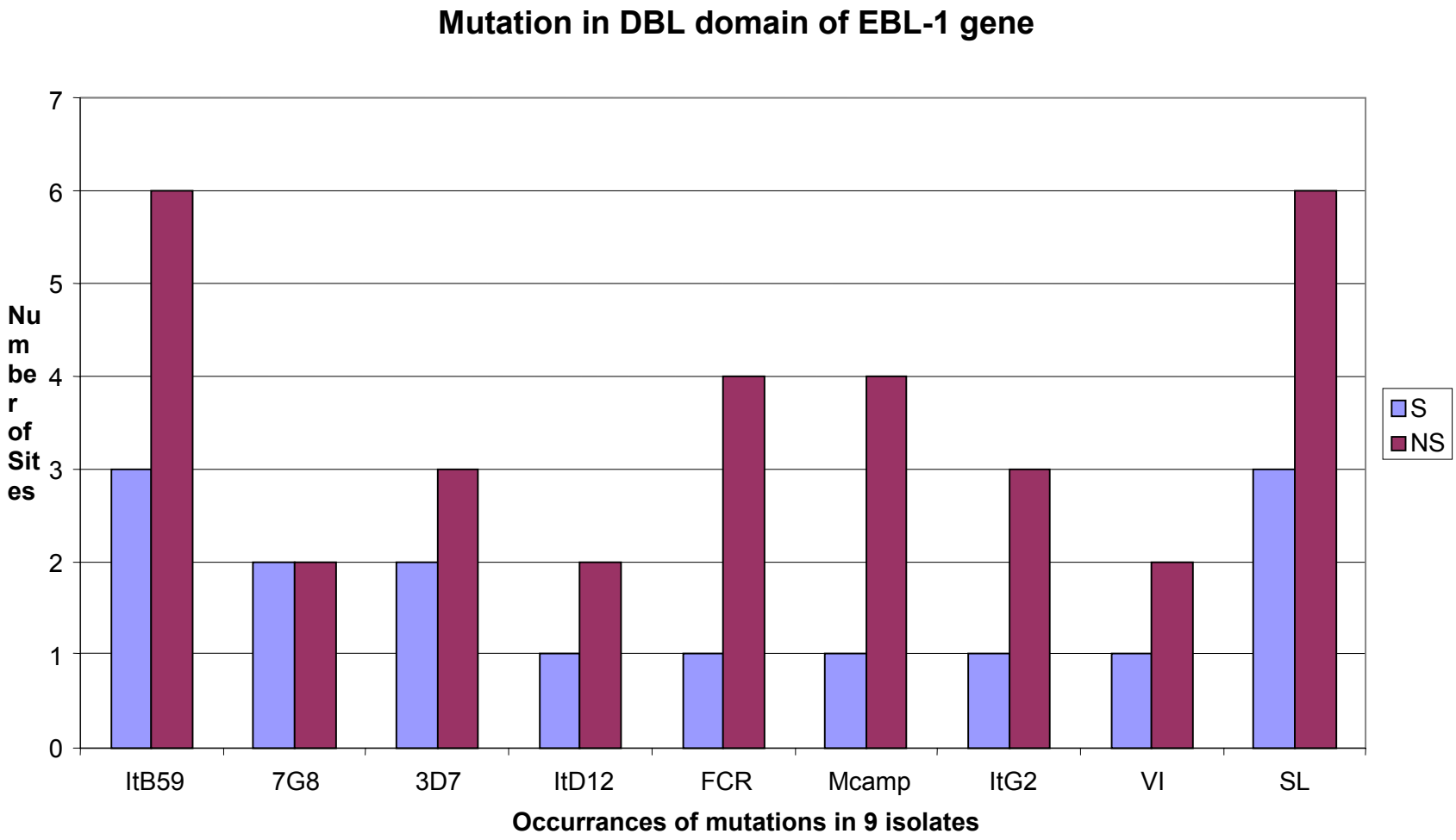


Figure 4.2 Occurrences of synonymous and nonsynonymous mutations in *Plasmodium falciparum* isolates.

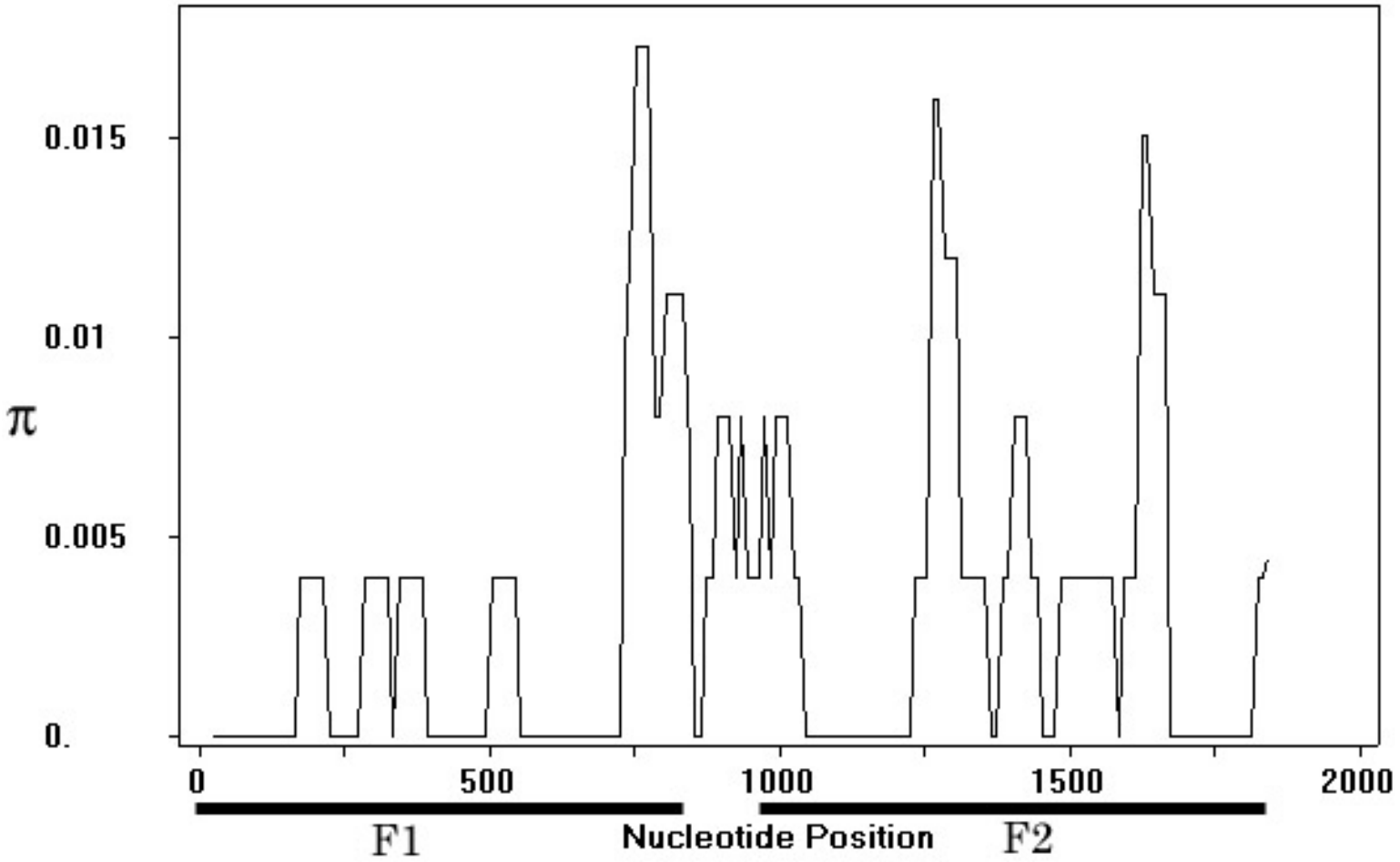


Figure 4.3. Nucleotides diversity across the Duffy binding-like domains of *ebl-1*. Nucleotide diversity (π) calculated according to Nei et al. [29] and graphed with a window size of 50 nt. and a step size of 10.

Insertion inactivates the *ebl*-1 gene in some *P.falciparum* isolates

Recent sequence information from the *Plasmodium* genome project indicates that there is a frame shift due to the insertion of 5 nucleotides upstream of the F1 DBL region of the *ebl-1* gene in isolate 3D7. We therefore decided to investigate whether there were similar insertions in the isolates examined in this study (as well as an 11th isolate, KD7). Figure 4.4 shows the position of the 5 T insertion within the reading frame of the *ebl-1* gene in some isolates. Six of the 10 isolates (Dd2, ItB59, FCR, VI, ItG2 and KD7), did not have the insertion while isolates ItD12, SL, Mcamp and 7G8 like 3D7 carried the insertion, Figure 4.4.

Polymorphism in the *ebl-1* coding region of 3d7 and Dd2 isolates

We also determined the number of polymorphisms in the entire ebl-1 gene in isolates 3D7 and Dd2. Figure 4.5 shows the location of polymorphisms with respect to the coding region of the *ebl-1* gene in these isolates. We identify 20 polymorphisms when comparing the entire *ebl-1* gene. Of the total polymorphisms detected in the coding region of *ebl-1* gene, five were synonymous, and 15 non synonymous. Thus, the nucleotide diversity (π) observed in the coding region of the *ebl*-1 gene between the two isolate was 0.00264, given that there are 7941 nucleotides in the entire coding region.

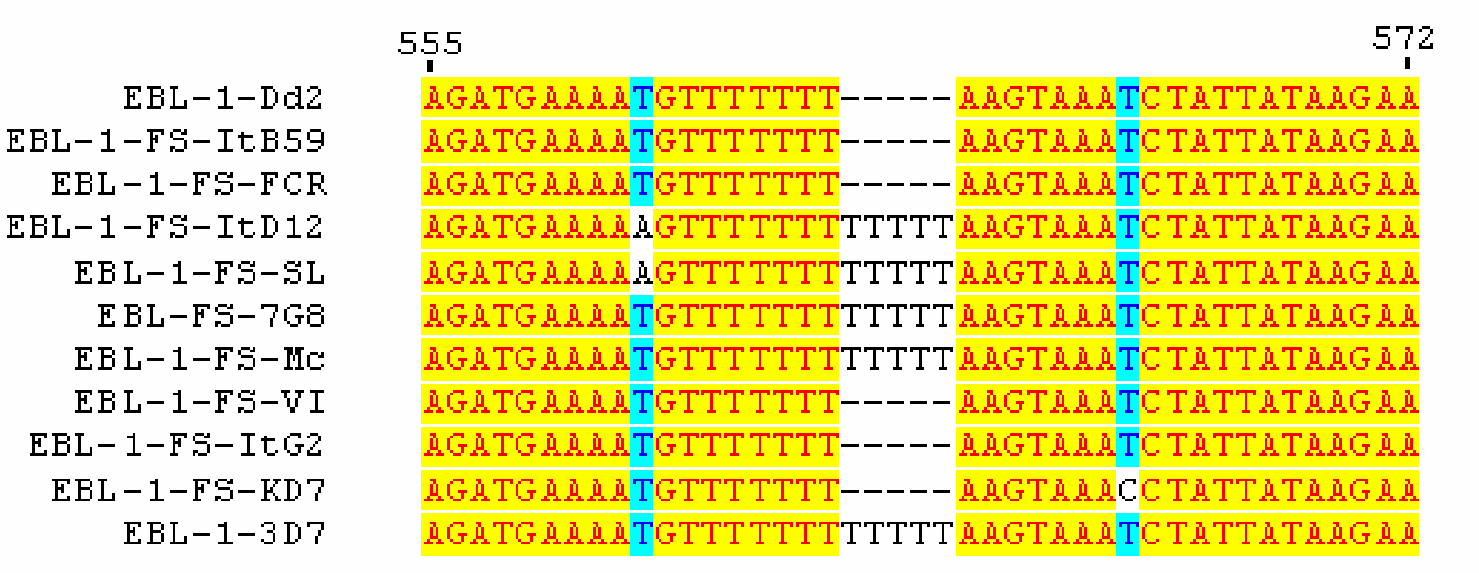


Figure 4.4. Insertion inactivates the *ebl-1* gene in some *P.falciparum* isolates. Sequence alignment of *ebl-1* gene from 10 *P.falciparum* isolates. Insertion of 5 Ts changes the reading frame resulting in premature translational termination

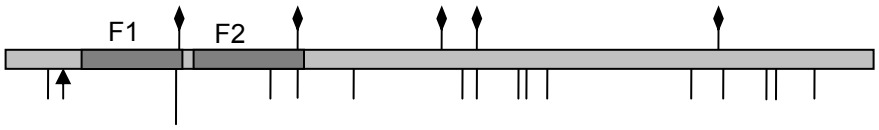


Figure 4.5. Locations of polymorphisms in the ebl-1 coding region of isolate Dd2 relative to isolate 3D7. The diamond tipped lines indicates synonymous changes, while the straight lines note non-synonymous changes. The arrow indicates the position of the 5-base insert present in 3D7. The darker gray areas denote the tandem dbl-domains. Longer line indicates two closely adjacent polymorphisms.

DISCUSSIONS

This study represents a survey of the level of polymorphism within the Duffy binding-like region of the *ebl-1* gene in 10 *P. falciparum* isolates. Our data show that DBL region of the *ebl-1* gene has a significant level of polymorphism in these isolates.

At the nucleotide level in region II of the *ebl-1* gene, there are 27 polymorphic sites present within 10 *P. falciparum* isolates, of which 20 (74%) are non-synonymous and 7 (26%) are synonymous. The number of polymorphic sites was somewhat higher than that observed in region II of *eba-175*, where Liang and Sim found 17 polymorphic sites in 15 isolates [25]. Both this study and that of Liang and Sim found a preponderance of non-synonymous polymorphisms. Overall region II was well conserved across all ten different *P. falciparum* isolate with an amino acid identity of 97.1%, similar to the sequence conservation in *eba-175* reported by [25] who observed a 98.2% (amino acid) sequence identity in region II among 16 different *P. falciparum* strains.

A study by [31]), investigating region II in the *P. vivax* in DBP Korean isolates revealed 5 positions changed from a total of 255 amino acids, corresponding to a polymorphism of 2.2%, which is comparable to our study . Previous reports have shown a 5% (Colombia) and 12.3 % (Papua New Guinea) level of polymorphism in the *P.* vivax Duffy binding domain [22, 32], a somewhat higher number of changes than observed here for *ebl-1*.

At the amino acid level in region II of the *EBL-1* gene, we identified 18 (2.9%) changes, 16 of which resulted in change in family of amino acid. Fourteen of the changes observed are in charge or polarity and two affect aromatic amino acids. None of the changes observed affected aromatic amino acids that are conserved in position between EBL-1, the DBP and EBA-175. The remaining changes observed in our study were between amino acids with similar

physicochemical properties. Such conservative amino acid substitutions might have relatively minor effects on protein structure and can likely be tolerated without compromising function.

The EBP family is defined in part by the presence of numerous conserved amino acids residues, particularly cysteine and aromatic amino acids within region II. The number and position of the 21 cysteine residues within region II was conserved. This result is similar to [25] where all 27 cysteine residues were conserved in region II of EBA-175. The conservation of cysteine residues observed in the *DBP, EBA-175* and as reported here in *EBL-1*, suggests they play a critical role in this ligand domain.

The number and distribution of polymorphic sites observed in the F1 and F2 domain of *EBL-1* was somewhat different than that seen in *EBA-175*. The *EBA-175* F1 and F2 domains contain almost the same number of polymorphic sites, 9 and 8 respectively [33], while, in *EBL-1* we find a greater number in the F2 domain (Table. 4.1). In the F2 domain of *EBL-1* we identified 2 synonymous and 13 non-synonymous mutations sites, which is similar to *EBA-175* where all mutations in the F2 domain were NS. However, the distribution of synonymous and non-synonymous mutations in the F1 domain was different from *EBA-175*. Both synonymous and non-synonymous mutations were approximately equal in *ebl-1* (Table. 4.1), while only non-synonymous mutation was detected in the F1 domain of EBA-175. It is assumed that polymorphism observed in genes encoding proteins exposed to the vertebrate host immune system is a strategic mechanism for evading the host's defense, suggesting that the *EBL-1* F2 domain has greater exposure to the host immune system.

CONCLUSION

The data presented show that the level of polymorphism in region II of *ebl-1* is comparable to that observed in *eba-175*, but differ to that observed in other members (*eba-140*

and *eba-165*) of the EBP family. None of the isolates examined are identical in sequence to the published sequence from clone Dd2, with the ten isolates comprising 9 different alleles. Also as previously observed for *EBA-175*, there is a clear bias towards non-synonymous substitutions in the *EBL-1* region II, particularly in the F2 domain. The diversity observed may be driven by host immune response, or potential receptor heterogeneity. However, the similarities in number and type of polymorphisms between *EBA-175* and *EBL-1* suggest both are exposed to similar selective pressures.

REFERENCES

1. WHO, Malaria. Report No. 94 Fact Sheet. Geneva. 1996(Report No. 94 Fact sheet. Geneva).
2. Narum DL, Fuhrmann SR, Luu T, et al., A novel Plasmodium falciparum erythrocyte binding protein-2 (EBP2/BAEBL) involved in erythrocyte receptor binding. Mol Biochem Parasitol, 2002. **119**(2): p. 159-68.
3. Gilberger TW, Thompson JK, Reed MB, et al., The cytoplasmic domain of the Plasmodium falciparum ligand EBA-175 is essential for invasion but not protein trafficking. J Cell Biol, 2003. **162**(2): p. 317-27.
4. Adams JH, Blair PL, Kaneko O, et al., An expanding ebl family of Plasmodium falciparum. Trends Parasitol, 2001. **17**(6): p. 297-9.
5. Duraisingh MT, Maier AG, Triglia T, et al., Erythrocyte-binding antigen 175 mediates invasion in Plasmodium falciparum utilizing sialic acid-dependent and -independent pathways. Proc Natl Acad Sci U S A, 2003. **100**(8): p. 4796-801.

6. Adams JH, Fang X, Kaslow DC, et al., Identification of a cryptic intron in the Plasmodium vivax Duffy binding protein gene. Mol. Biochem. Parasitol., 1992. **56**(1): p. 181-183.

7. Singh S, Pandey K, Chattopadhayay R, et al., Biochemical, biophysical, and functional characterization of bacterially expressed and refolded receptor binding domain of Plasmodium vivax duffy-binding protein. J Biol Chem, 2001. **276**(20): p. 17111-6.

8. Dolan SA, Proctor JL, Alling DW, et al., Glycophorin B as an EBA-175 independent Plasmodium falciparum receptor of human erythrocytes. Mol. Biochem. Parasitol., 1994. **64**: p. 55-63.

9. Lobo CA, Rodriguez M, Reid M, et al., Glycophorin C is the receptor for the Plasmodium falciparum erythrocyte binding ligand PfEBP-2 (baebl). Blood, 2003. **101**(11): p. 4628-31.

10. Maier AG, Duraisingh MT, Reeder JC, et al., Plasmodium falciparum erythrocyte invasion through glycophorin C and selection for Gerbich negativity in human populations. Nat Med, 2003. **9**(1): p. 87-92.

11. Peterson DS and Wellems TE, EBL-1, a putative erythrocyte binding protein of Plasmodium falciparum, maps within a favored linkage group in two genetic crosses. Mol Biochem Parasitol, 2000. **105**(1): p. 105-13.

12. Adams JH, Sim BK, Dolan SA, et al., A family of erythrocyte binding proteins of malaria parasites. Proc. Natl. Acad. Sci. U. S. A., 1992. **89**(15): p. 7085-7089.

13. Thompson JK, Triglia T, Reed MB, et al., A novel ligand from Plasmodium falciparum that binds to a sialic acid-containing receptor on the surface of human erythrocytes. Mol Microbiol, 2001. **41**(1): p. 47-58.

14. Mayer DC, Kaneko O, Hudson-Taylor DE, et al., Characterization of a Plasmodium falciparum erythrocyte-binding protein paralogous to EBA-175. Proc Natl Acad Sci U S A, 2001. **98**(9): p. 5222-7.

15. Gilberger TW, Thompson JK, Triglia T, et al., A novel erythrocyte binding antigen-175 paralogue from Plasmodium falciparum defines a new trypsin-resistant receptor on human erythrocytes. J Biol Chem, 2003. **278**(16): p. 14480-6.

16. Triglia T, Thompson JK, and Cowman AF, An EBA175 homologue which is transcribed but not translated in erythrocytic stages of Plasmodium falciparum. Mol Biochem Parasitol, 2001. **116**(1): p. 55-63.

17. Miller LH, Roberts T, Shahabuddin M, et al., Analysis of sequence diversity in the Plasmodium falciparum merozoite surface protein-1 (MSP-1). Mol Biochem Parasitol, 1993. **59**(1): p. 1-14.

18. Snewin VA, Herrera M, Sanchez G, et al., Polymorphism of the alleles of the merozoite surface antigens MSA1 and MSA2 in Plasmodium falciparum wild isolates from Colombia. Mol Biochem Parasitol, 1991. **49**(2): p. 265-75.

19. Escalante AA, Grebert HM, Isea R, et al., A study of genetic diversity in the gene encoding the circumsporozoite protein (CSP) of Plasmodium falciparum from different transmission areas--XVI. Asembo Bay Cohort Project. Mol Biochem Parasitol, 2002. **125**(1-2): p. 83-90.

20. Escalante AA, Grebert HM, Chaiyaroj SC, et al., Polymorphism in the gene encoding the Pfs48/45 antigen of Plasmodium falciparum. XI. Asembo Bay Cohort Project. Mol Biochem Parasitol, 2002. **119**(1): p. 17-22.

21. Escalante AA, Grebert HM, Chaiyaroj SC, et al., Polymorphism in the gene encoding the apical membrane antigen-1 (AMA-1) of Plasmodium falciparum. X. Asembo Bay Cohort Project. Mol Biochem Parasitol, 2001. **113**(2): p. 279-87.

22. Ampudia E, Patarroyo MA, Patarroyo ME, et al., Genetic polymorphism of the Duffy receptor binding domain of Plasmodium vivax in Colombian wild isolates. Mol Biochem Parasitol, 1996. **78**(1-2): p. 269-72.

23. Xainli J, Adams JH, and King CL, The erythrocyte binding motif of plasmodium vivax duffy binding protein is highly polymorphic and functionally conserved in isolates from Papua New Guinea. Mol Biochem Parasitol, 2000. **111**(2): p. 253-60.

24. Mayer DC, Mu JB, Feng X, et al., Polymorphism in a Plasmodium falciparum erythrocyte-binding ligand changes its receptor specificity. J Exp Med, 2002. **196**(11): p. 1523-8.

25. Liang H and Sim BK, Conservation of structure and function of the erythrocyte-binding domain of Plasmodium falciparum EBA-175. Mol Biochem Parasitol, 1997. **84**(2): p. 241-5.

26. Trager W and Jensen JB, Human malaria parasites in continuous culture. Science., 1976. **193**(4254): p. 673-675.

27. Peterson DS, Walliker D, and Wellems TE, Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. Proc. Natl. Acad. Sci. U. S. A., 1988. **85**(23): p. 9114-9118.

28. Sambrook J, Fritsch EF, and Maniatis T, Molecular Cloning. 1989, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

29. Rozas J, Sanchez-DelBarrio JC, Messeguer X, et al., DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics, 2003. **19**(18): p. 2496-7.

30. Nei M and Gojobori T, Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol, 1986. **3**(5): p. 418-26.

31. Kho WG, Chung JY, Sim EJ, et al., Analysis of polymorphic regions of Plasmodium vivax Duffy binding protein of Korean isolates. Korean J Parasitol, 2001. **39**(2): p. 143-50.

32. Tsuboi T, Kappe SH, al-Yaman F, et al., Natural variation within the principal adhesion domain of the Plasmodium vivax duffy binding protein. Infect Immun, 1994. **62**(12): p. 5581-6.

33. Baum J, Thomas AW, and Conway DJ, Evidence for Diversifying Selection on Erythrocyte-Binding Antigens of Plasmodium falciparum and P. vivax. Genetics, 2003. **163**(4): p. 1327-36.

CHAPTER 5

USE OF REAL-TIME PCR TO ANALYZE ERYTHROCYTE BINDING PROTEIN GENE EXPRESSION IN *Plasmodium falciparum* [3]

[3]Drummond, P. B. and D. S. Peterson. To be submitted to the Journal: Molecular and Biochemical Parasitology.

ABSTRACT

Invasion of erythrocytes by merozoites of *Plasmodium falciparum* is a complex process requiring interaction between many parasite and host proteins. Identification of genes expressed during the development of the asexual stages could give a better understanding of host-parasite interactions. Furthermore, an understanding of the expression of these genes at the different stages of the erythrocytic cycle could lead to the development of new strategies to interrupt the lifecycle. In this study, real-time quantitative RT-PCR was used to accurately quantify the relative expression of genes encoding six erythrocyte-binding proteins (EBP), during the intraerythrocytic development cycle. Three *Plasmodium falciparum* isolates, 3D7, Dd2 and Dd2-NM with unique invasion phenotypes were used in this study. Total RNA was isolated from synchronized parasites at four time-points (20-hr/ring, 28-hr/early-trophozoite, 36-hr/late-trophozoite and 44-hr/schizont). Our results show that expression of EBPs is highly stage specific, with expression peaking at 36-hr in the 3D7 and Dd2 isolates. In the Dd2-NM clone, expression of EBP genes peaked at 44-hr, with one exception, (*MAEBL*) which peaked at 36-hr. We also found that *eba-175* had the highest level of expression, while *maebl* was the least expressed in all three isolates. Our results indicate that *EBA-165* was not expressed in Dd2, but is expressed in Dd2-NM. These findings would suggest that during the switch from a sialic acid dependent phenotype to a sialic acid independent phenotype, expression of *EBA-165* was up regulated.

Keywords: Lifecycle, real-time PCR, stage specific, *Plasmodium falciparum*

INTRODUCTION

Invasion of erythrocytes by merozoites of *Plasmodium falciparum* is a complex process requiring interaction between many parasite and host proteins. Identification of genes expressed during the development of the asexual stages could give a better understanding of host-parasite interactions. Furthermore, an understanding of the expression of these genes at the different stages of the erythrocytic cycle could lead to the development of new strategies to interrupt the parasite's lifecycle. A study on the temporal expression of EBP genes by Blair and colleagues [1] suggests that the expression of the maebl transcript peaked during mid to late trophozoite stage; *ebl-1*, at mid-schizont stage; and *eba-175*, *eba-140*, *eba-165* and *eba-181*, nearly at the end of schizont development. However, only a single isolate (3D7) was analyzed in that study. It is known that not all of the EBPs are expressed in all isolates [2-4] and it is possible that others are expressed at a higher level compensating for the lack of one or more genes in some isolates. In support of this, recent studies [4, 5] looking at the level of the *P.falciparum* rhoptry protein homolog (*PfRH2a*) and reticulocyte binding protein homolog (*PfRH2b*) found that not all of these genes were transcribed in three *P.falciparum* isolates. While both genes are expressed in isolates 3D7 and T996, *PfRH2a* and *PfRH2b* were not expressed in FCB1.

During the erythrocytic stage of growth the parasite passes through stages described as ring, trophozoite and schizont. As these stages have different morphological traits, their metabolic and expression profiles are expected to be different. There is strong stage-specific expression of all the EBP genes [1]. It has been reported that var gene expression is also tightly regulated in the trophozoites stage [6].A single trophozoite in an infected erythrocyte may express only one of fifty possible *var* genes and switch frequently between alleles [7].

Quantitative expression analysis of mRNA has been widely used in many experimental studies and has also found clinical applications. A number of methods are available for this purpose, such as northern blotting (NB), the RNase protection assay (RPA), quantitative competitive reverse transcription-PCR [8] and real-time reverse transcription PCR (RT-PCR) [9], however some of these procedures are rather time consuming. In addition procedures such as NB and RPA are limited by hybridization kinetics and require large amounts of RNA. The measurement of RNA transcription by real-time (kinetic) RT-PCR circumvents several obstacles known to limit the quantification potential of other PCR-based methods [10, 11].

Recent advances in the use of fluorogenic probes in conjunction with PCR have enabled the measurement of an accumulating PCR product in real time [12-14]. The use of real-time quantitative PCR by the 5' fluorogenic nuclease assay (Taqman) in malaria has been reported in several recent studies [1, 15-17]. Real-time quantitative PCR allows for the rapid generation of quantitative data showing changes in transcript level in the samples, while the risk of a false positive result due to contamination with previously amplified products is dramatically reduced.

In the literature, a number of different housekeeping genes have been used to control for differences in RNA quantity and quality between samples. However, three of the most widely used housekeeping genes are ribosomal 18S RNA, glycealdehyde-3-phosphate (GAPDH) and β-actin. Several studies have determined that ribosomal 18S RNA [1, 18, 19] is more suitable for normalizing transcript levels between multiple samples.

This study had three separate goals. The first goal was to determine the extent to which expression of EBP genes varies among isolates. The second was to determine the extent to which lack of one EBP gene changes the expression of the other EBPs. The third was to quantitate the expression of EBPs in parasites that have been selected for a change in invasion

phenotype as [20]. To address this issue we have a used real-time PCR-base approach that allows effective, accurate, and reproducible quantitative measurement.

MATERIALS AND METHODS

Parasite and Cell Culture

Plasmodium falciparum isolates 3D7, Dd2 and Dd2/NM5 were used in this study. Parasite cultures in 5% hematocrit were maintained essentially as described [21] in RPMI-1640 supplemented with 25Mm HEPES, 20mM Na_2CO_3, 10% human serum and 0.1mg/ml gentamicin. The cultures were gassed with 5% O_2/5%CO_2/90%N_2 and the flasks sealed tightly, then incubated at 37^{o}C. Sorbitol synchronization of *P.falciparum* cultures were achieved as described [22]. Four different time points was selected for parasite collection, 20, 28, 36, 44 hours post invasion.

RNA Extraction and Quantification

Total RNA was isolated from different stages of the life cycle of 3D7, Dd2 and Dd2/NM5 isolates using the TriReagent protocol according to the manufacturer's instructions (Molecular Research Center, Inc., Cincinnati, OH). The isolated RNA was dissolved in a volume of 50 µ*l*. of RNA storage solution (Ambion, Inc., Austin TX). The concentration of RNA was determined spectrophotometrically and was found to be in the order of 0.5 to 1.5 µ*g* total RNA from each stage. RNA integrity was confirmed using agarose gel electrophoresis. One microgram of total RNA was treated with DNase I (Ambion, Inc) to remove contaminating DNA from RNA samples.

Reverse Transcription

One microgram of total RNA from each time point was used for each reaction with or without reverse transcriptase (RT). RT reactions were carried out as follows: 1 µ*g* of total RNA

(in 5-9 μ*l* water) was mixed with 1 μ*l* random hexamers primer and 1 μ*l* of dNTP mix and was incubated at 65^{o}C for 5 min. After cooling on ice the solution was mixed with 2 μ*l* 10X RT buffer, 4 μ*l* of $MgCI_2$, 1 μ*l* RNaseOUT and 2 μ*l* DTT and was incubated for 2 min at 25^{o}C. 1 μ*l* of SuperScript II RT was added to each tube and incubated at 25^{o}C for 10 min. Reactions were then incubated for 55 min at 42^{o}C and terminated by heating to 70 ^{o}C for 15 min. After cooling on ice the solution was mixed with 1 μ*l* RNase H and incubated at 37 ^{o}C for 20 min. After a preliminary RT-PCR assay amplifying the 18S rRNA and eba-175 genes to assess integrity, cDNA was stored at –85 ^{o}C until required for real-time PCR.

Primers and Fluorogenic Probes

TaqMan probe and primer sequences (Table 5.1) were designed using Primer Express (Version 1.5, PE Applied Biosystems, Foster City, CA) as close as possible to the 3'-coding region of target gene sequences obtained from GenBank. Where possible, probes and primers were designed based on manufacturer's guidelines, and as described [14]. However, due to the A/T richness of the *P. falciparum* genome the primer TM had to be decreased to 58^{o}C. Forward and reverse primers were positioned as close as possible to each other without overlapping the probe, and each had less than three Gs or Cs in the five most 3' nucleotides. Amplicons were between 70 and 133 base pairs (bp). Primers and probes were purchased from MWG-Biotech (High Point, NC) and each probe was synthesized with the fluorescent reporter dye FAM (6-carboxy-fluorescein) attached to the 5'-end and a quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine) to the 3'-end. All gene-specific primer pairs were checked for efficient amplification using *P. falciparum* genomic DNA. The resulting PCR products were run on a 2% agarose gel to verify amplification of a correct sized product.

Table 5.1 TaqMan Primers and Probes Used in this Study. Sequences are shown for forward (f) and reverse (r) primers and TaqMan probe (p).

Gene	GenBank Accession no.	p) 5'-(FAM)-probe-(TAMRA)-3' f) 5'-forward primer-3' r) 5'-reverse primer-3'	Amplicon size (bp)
EBA-175	M93397	p) 5'-CATGTGTGGTTCCCAAATCACCAACAGTT-3' f) 5'-AGAGTGATCAACAGAAAAACGATATGA-3' r) 5'-CTCCTGTAACAGGAACACTAATTTCG-3'	90
EBL-1	AF131999	p) 5'-ATTTCTGTTTCGCCTCCTAATGTATCTGTTACTTAT-3' f) 5'-GATGGAGATGTTTCAGAGAAGGA-3' r) 5'-ATACCTTGTCTTTTATCCCCTTCA-3'	89
MAEBL	AF400002	p) 5'-TGTCCACCAAGGTTCCCATTAAATCATACTAT-3' f) 5'-GGACCTATGCATCATCCTTCA-3' r) 5'-AATTATAGTACTGGAAAGTGTGAGACGT-3'	117
EBA-181	AB080796	p) 5'-AACAGGACGTTCAAAGTACACCACCCGA-3' f) 5'-AAAAAGAACTTCAATCTACTGTATCAA-3' r) 5'-GCATGCGGAGAAGTTAACA-3'	133
EBA-140	AF332918	p) 5'-CCCCCAGAAGACAAACTTTATGTCTTGGACG-3' f) 5'-GAACAAATTTCCTGGTACATGTGAA-3' r) 5'-TTCCTCATGACCACGATGTAAAA-3'	104
EBA-165	AY032735	p) 5'-CCGCTTGGTTTACTCTCACTCTCACGTGG-3' f) 5'-ACCGAAAGGTGGCACACAA-3' r) 5'-CAGTGCTTGGATCTTCTCTACCAT-3'	100
18S rRNA	M19172	p) 5'-TTGTACACACCGCCCGTCGCTC -3' f) 5'-GCTGACTACGTCCCTGCCC-3' r) 5'-ACAATTCATCATATCTTTCAATCGGTA-3'	70

Each probe was then checked with its appropriate primers in TaqMan reactions, using genomic DNA to determine optimum probe concentration.

TaqMan PCR

TaqMan PCR assays for each *P.falciparum* isolate, time point and gene target (Table 5.1) as well 18S rRNA were performed in triplicate on cDNA samples or genomic DNA standards (10 fold dilutions from 10 ng/reaction to 10 pg/reaction) in 384-well optical plates on an ABI Prism 7900 H Sequence Detection system (PE Applied Biosystems) according to the manufacturer's protocol. For each 20 µ*l* TaqMan reaction, 1 µ*l* cDNA was mixed with 7 µ*l* PCR-grade water, 10 µ*l* 2x TaqMan Universal PCR Master Mix (PE Applied Biosystems), 0.5 µ*l* foward primer (300 nM) 0.5 µ*l* reverse primer (300 nM) and 1 µ*l* TaqMan probe (100 nM). The PCR cycle condition was as follows: an initial step of 2 min. at 50 ^{o}C was used for AmpErase incubation followed by 10 min. at 95 ^{o}C to inactivate the AmpErase and to activate the *Taq* polymerase. This was followed by a denaturation step of 95 ^{o}C for 15 seconds and finally annealing/extension at 58 ^{o}C for 1 min for 40 cycles.

Data Analysis

All TaqMan PCR data were collected using Sequence Detector Software (SDS version 2.0; PE Applied Biosystems. For every sample, an amplification plot was generated showing the increase in the reporter dye fluorescence (ΔRn) with each cycle of PCR. A C_T value, defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value [13, 14] based on the variability of base line data in the first 15 cycles, was calculated for each reaction. C_T values were exported into Microsoft Excel worksheets for further analysis.

The correlation between the C_T value and the fold difference in the concentration was determined individually for each probe and primer set. When PCR efficiency is 100% the C_T values of two separate genes can be compared (ΔC_T) and the relative fold difference can be

determined. This was carried out using the Comparative C_T Method (Separate Tubes) as defined in the ABI analysis manual. The amount of target, normalized to an endogenous reference and relative to a calibrator is given by:

$$2^{-\Delta\Delta C_T}$$

For the $\Delta\Delta C_T$ calculation to be valid, the efficiency of the target and the efficiency of the reference amplification must be approximately equal. The absolute value of the slope of log input amount vs. ΔC_T should be <0.1. The ΔC_T value is determined by subtracting the average 18S RNA $C_T$value from the average EBPs C_T value. The calculation of $\Delta\Delta C_T$ involves subtraction by the ΔC_T calibrator value. The standard deviation of the difference is calculated from the standard deviation of the EBPs and 18S RNA values using the following formula:

$$s = \sqrt{s_1^2 + s_2^2}$$

The result represents mean and standard deviation of triplicate samples as reported earlier. Each experiment was repeated at three times.

RESULTS

EBP genes and design of TaqMan primers and probes

To determine EBP gene expression in the intraerythrocytic developmental cycle in *P.falciparum* isolates, we designed TaqMan primers and probes to amplify a portion of the coding region of the EBP genes. At present there are six EBP genes (*eba-175, ebl-1, eba-140, eba-165, eba-181 and maebl*) that have been identify in the *P. falciparum* genome [2, 3, 23-26]. Sequences for all six genes were obtained from GenBank (Table 5.1) and were imported into Primer Express Software to generate optimum TaqMan primer and probe positions. However, due to the A/T richness of the *P. falciparum* genome the primer TM had to be decreased to 58^{o}C. TaqMan primer and probe seta for each EBP gene was optimized and used to detect relative

expression of each gene. TaqMan primers and probes were also designed and optimized for the small subunit ribosomal RNA (*18S r RNA*) for use in normalizing expression values.

Relative Efficiency of Target and Reference Gene

Validation experiments were carried out to demonstrate that both the target and reference gene are amplified with approximately equal efficiencies. The 18S small subunit rRNA gene was chosen as the reference gene, allowing us to normalize the amount of total RNA used in each RT reaction. It is required that the absolute value of the slope of log input amount of template vs. ΔC_T should be < 0.1, as recommended by the manufacturer protocol (PE Applied Biosystems). To determine the PCR efficiency of both EBP and 18S RNA amplifications tenfold serial dilutions of 3D7 isolate DNA were performed and defined as standards. The slopes for these six EBP genes were as follows *EBA-175* (-0.068), *EBL-1* (-0.089), *EBA-140* (-0.071), *EBA-181* (-0.065), *MAEBL* (-0.041) and *EBA-165* (-0.098) indicating that all of them are amplified with equal efficiency. Figure 5.1 represents the relative efficiency plot of *EBA-181* and *18S rRNA*.

Correlation coefficients were obtained from the standard curve for all 6 genes studied in the three strains of *Plasmodium falciparum*. The average correlation coefficient for all experiments in this study was 0.989 (SD = 0.05), and ranged from 0.985- 0.998. Furthermore, the mean slope value for all genes tested was –3.51 (SD = 0.133), ranging from -3.71 to –3.31). At 100% amplification efficiency, the PCR slope should be –3.33, because it should require 3.3 cycles to generate a 10-fold increase in product.

***Plasmodium falciparum* Isolates**

Real-time RT-PCR was used to examine the temporal expression level of the six EBP genes found in *Plasmodium falciparum*. Two isolates differing in their ability to invade via a

sialic acid independent pathway were used in this study, 3D7 with a sialic acid independent phenotype, Dd2, with a sialic acid dependent phenotype. We also included isolate Dd2-NM, which was selected from Dd2, having switched to a sialic acid independent invasion phenotype. Samples were collected from four-time points during the 48-hr erythrocytic cycle. The time points were as follows: 20hrs (ring stage), 28hrs (early-trophozoites), 36-hrs (late-trophozoites) and 44-hrs (schizont). All parasite cultures used in this study were synchronized with sorbitol. Samples were collected at every time point for RNA isolation, and Giemsa stained thin smears were examined to determine the level of synchronization. All cultures were shown to be synchronous, with greater than 80% of parasites at each time point being the appropriate stage (Figure 5.2).

Relative Expression of EBPs in 3D7 Isolate

Figure 5.3 shows the relative expression of *EBA-175*, *EBL-1*, *EBA-140*, *EBA-181*, *MAEBL* and *EBA-165* as quantified in the 3D7 isolate at different stages of the erythrocytic cycle [20-hr (ring), 28-hr (early trophozite), 36-hr (late trophozite) and 44-hr (schizont). The expression at 28-hr, 36-hr and 44-hr is reported relative to the expression at 20-hr. The rationale for choosing the 20-h or ring-stage as a reference is that ring stage parasites are thought to be less transcriptionally active than the later stages. For all of the genes examined, the highest C_T values (hence the lowest expression) were found at that stage.

The relative increase in expression of the EBP genes in isolate 3D7 (Figure 5.3) varied from 17.62-fold (*MAEBL*) to 271.43-fold (*EBA-175*) when expression at 20-hr was compared to that at 36-hr. The maximal changes for each gene when compared to *MAEBL* were 15.4-fold for *EBA-175*, 7.4-fold for *EBL-1*, 5.8-fold for *EBA-140*, 11.7-fold for *EBA-181* and 1.5-fold for *EBA-165*.

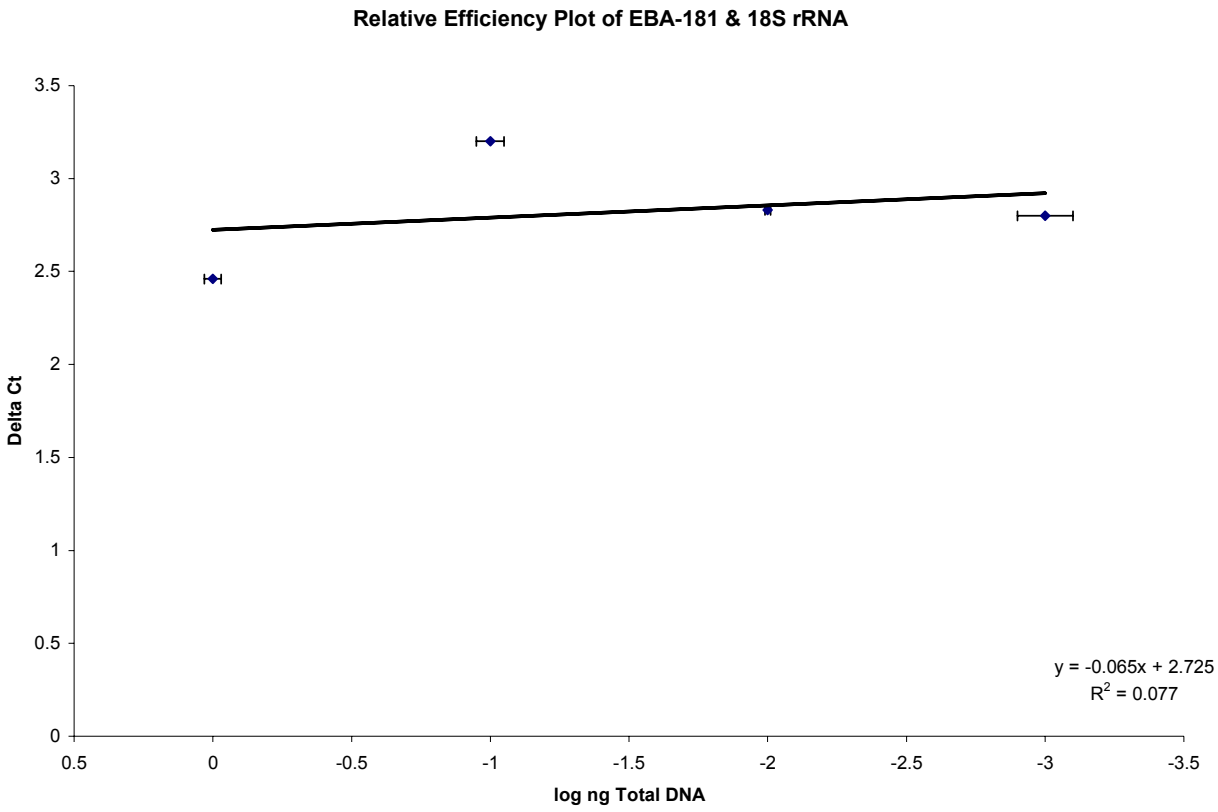
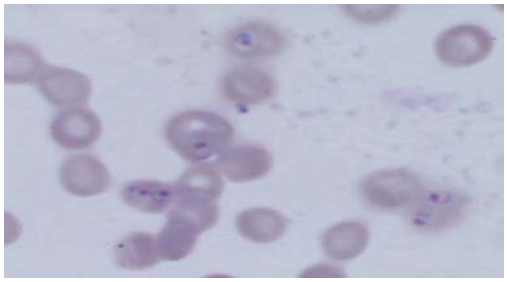
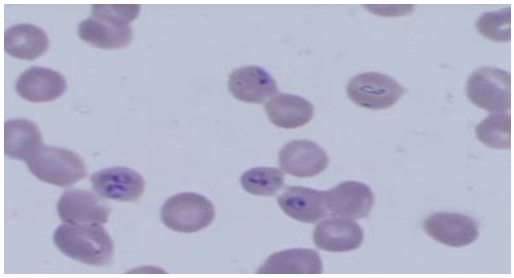


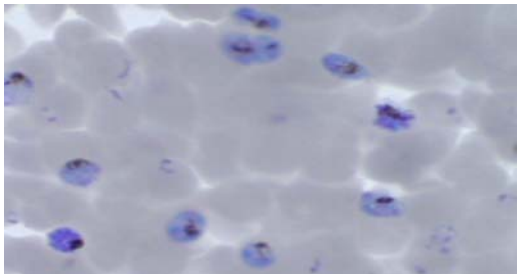
Figure 5.1. Plot of log input amount of template versus ΔC_T. Relative Efficiency Plot *EBA-181 & 18S rRNA*



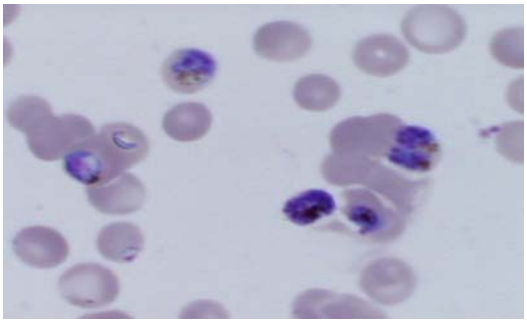
20hr



28hr



36hr



44hr

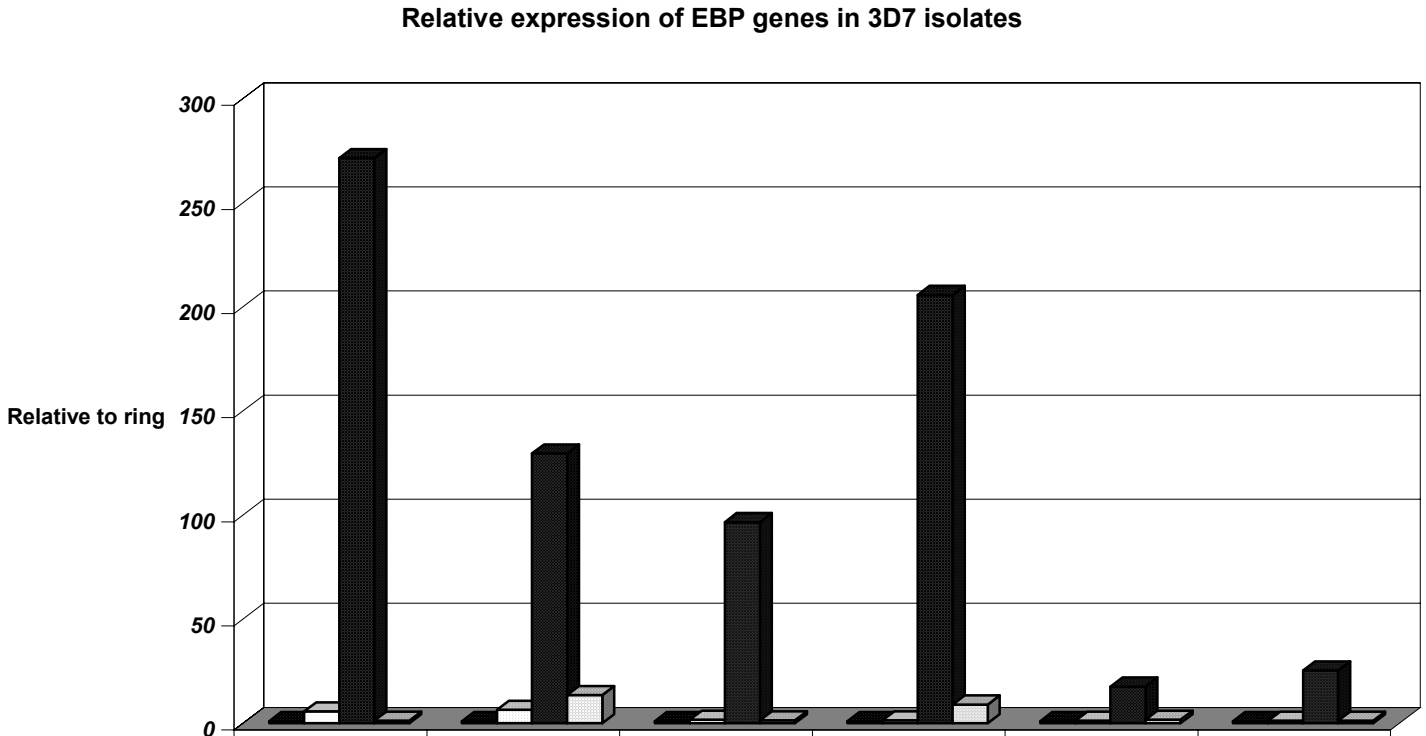
Figure. 5.2. Periodic (four) time points taken from synchronized cultures Dd2 during the erythrocytic cycle and was comparable to Dd2-NM and 3D7 isolate. All parasite cultures used in this study were synchronized with sorbitol. Samples were collected at every time point for RNA isolation, and Giemsa stained thin smears were examined to determine the level of synchronization. All cultures were shown to be synchronous, with greater than 80% of parasites at each time point being the appropriate stage.

Transcription of these genes was expected to be stage specific and the evidence presented here strongly supports this idea, as shown in Figure 5.3. The standard deviation (SD) for the triplicate samples ranged from 0.005 to 0.13. This level of SD was not detectable on the graph in Figure 5.3.

Relative Expression of EBPs in Dd2 Isolate

The Dd2 isolate of *P. falciparum* is unable to invade by the sialic acid independent pathway, suggesting that it may show a different EBP expression profile than 3D7. As seen for 3D7, the maximal expression for each gene was observed at the 36 hr time point.

The relative expression of EBP genes in the Dd2 isolate is depicted in Figure 5.4. *EBA-175* (36-hr) exhibited the greatest change in expression level, with a 353.63-fold increase when compared to the expression level at 20-hr. *EBL-1* (36-hr) showed the smallest increase (41.8-fold) and was used to generate fold differences in expression change between the genes. The maximal changes for each gene relative to *EBL-1* were 8.5-fold for *EBA-175*, 1.2-fold for *MAEBL*, 1.5-fold for *EBA-140* and 3.1-fold for *EBA-181*. The standard deviation (SD) ranged form 0.04 to 0.1. This level of SD was not detectable on the graph in Figure 5.4. Surprisingly, we did not detect any measurable level of expression from the *EBA-165* gene. PCR amplification of DNA confirmed that the gene was present in the Dd2 isolate at the DNA level. This suggests that *EBA-165* is not transcribed in this isolate.



	EBA-175	EBL-1	EBA-140	EBA-181	MAEBL	EBA-165
RING 20Hr	1	1	1	1	1	1
E TROPH 28Hr	5.68	6.46	1.71	1.44	1.21	1.27
L TROPH 36Hr	271.43	129.61	96.58	205.71	17.62	25.38
SCHIZONT 44Hr	1.28	13.56	1.41	9	1.68	1.27

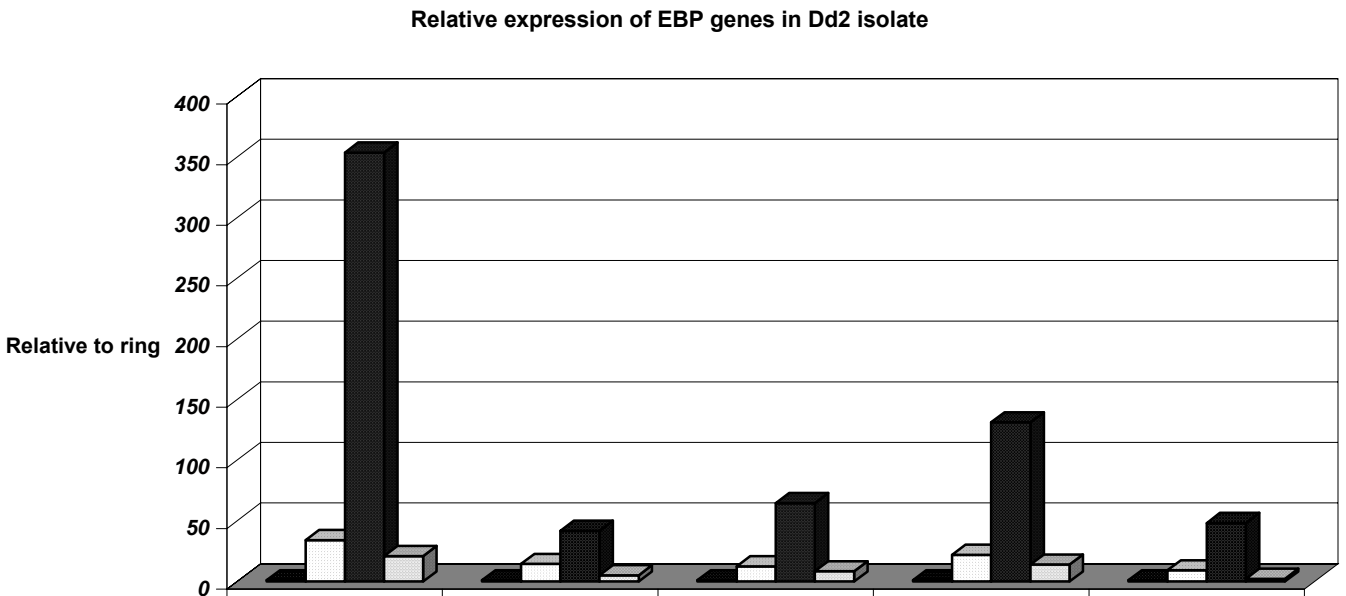
Figure 5.3. Real-time quantitative PCR of EBP genes, using cDNA from four-time point (20-hr, 28-hr, 36-hr and 48-hr) during the intraerythrocytic cycle in the 3D7 isolate. Ordinate values of the transcript levels are given in normalized to 18S rRNA gene. The results are comparable to the relative ring levels determined using the standard curve method. Relative expression values are given in the data table beneath the graph.

Relative Expression of EBPs in Dd2-NM Isolate

Dd2-NM was derived from isolate Dd2 by selecting for a switch to a sialic acid independent invasion phenotype. This switch in invasion phenotype could well be mediated by a change in EBP gene expression. Thus we decided to investigate the level of expression of EBP genes in this isolate. Figure 5.5 shows the temporal profile of EBP gene from the Dd2-NM isolate. Surprisingly *EBA-165* generates the highest relative increase in expression (277.9-fold difference at 44 hrs) when compared to the expression level at 20-hr. *EBL-1* (44-hr) showed the smallest increase (40.4-fold) and was used to generate fold differences. The maximal changes for each gene relative to *EBL-1* were 4.1-fold for *EBA-175*, 1.1-fold for *MAEBL*, 1.6-fold for *EBA-140* and 4.8-fold for *EBA-181* and 6.9-fold for *EBA-165*. The expression of all EBP genes in the Dd2-NM isolate peak at the 44-hr time point, except for *MAEBL*, which peaks at 36-hr. The standard deviation (SD) ranges from 0.03 to 0.25. This level of SD was not detectable on the graph in Figure 5.5.

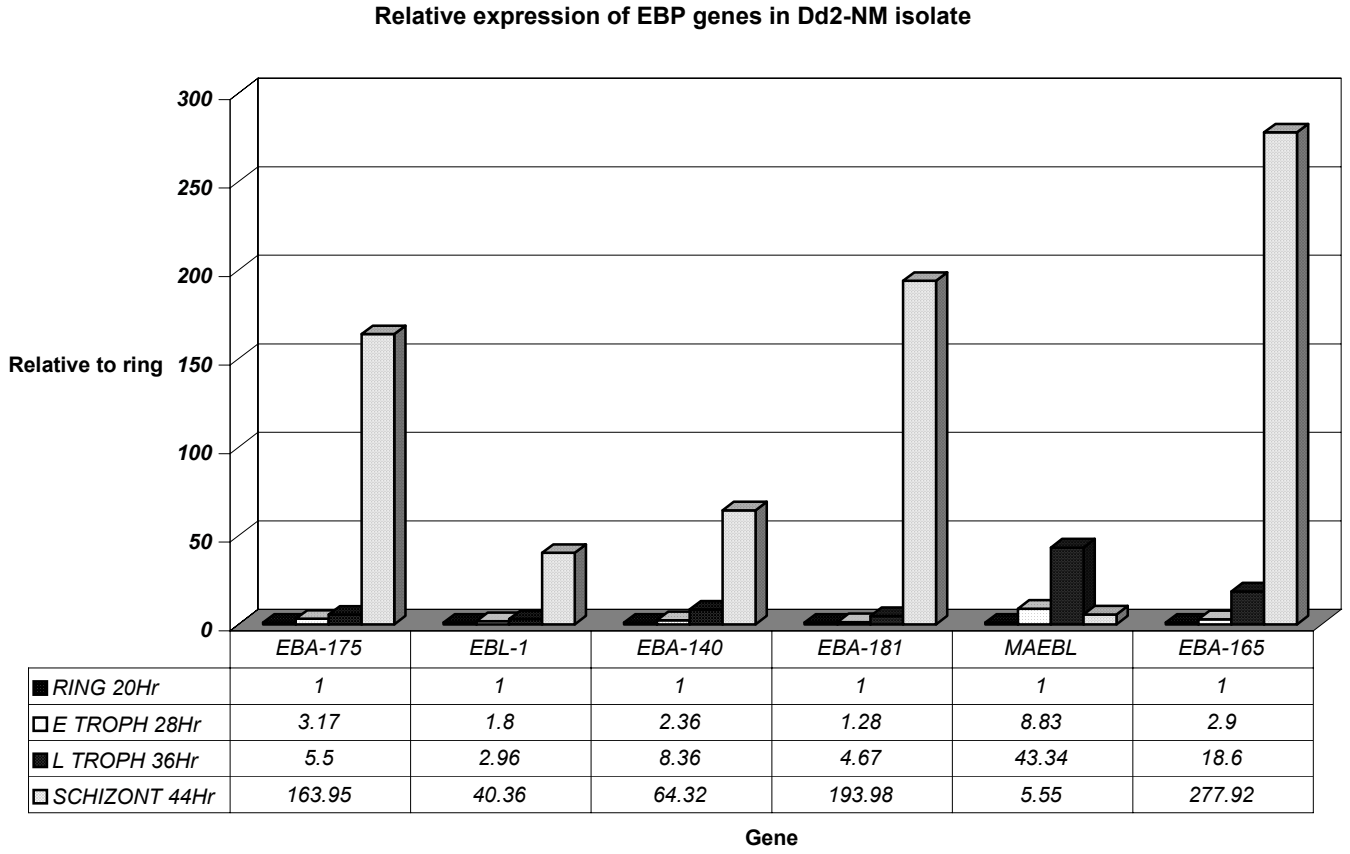
Low Variability Among *Plasmodium falciparum* Isolates in the Relative Expression of EBPs

A major goal of this study was to compare the relative changes in EBP gene expression between the 3 parasite isolates. All three isolates showed stage specific increase in the expression of the EBP genes, with the exception of the *EBA-165* gene in Dd2. Expression of this gene was not detectable in this isolate. Table 5.2 shows the relative difference in expression of each EBP gene between the three isolates. Taking the data shown in Figure 3-5, the fold difference for each gene was calculated by using the isolate with the lowest expression level for that gene. The greatest variation in gene expression among isolates was observed in Dd2-NM with a 11-fold difference relative to isolate 3D7 (Table 5.2).



	EBA-175	EBL-1	EBA-140	EBA-181	MAEBL
RING 20Hr	1	1	1	1	1
E TROPH 28Hr	34.11	14.5	12.44	22.05	9.21
L TROPH 36Hr	353.63	41.82	64.58	131.22	48.21
SCHIZONT 44Hr	21.02	5.2	8.41	13.99	2.2

Figure 5.4. Real-time quantitative PCR of EBP genes, using cDNA from four-time point (20-hr, 28-hr, 36-hr and 48-hr) during the intraerythrocytic cycle in the Dd2 isolate. Ordinate values of the transcript levels are given in normalized to 18S rRNA gene. The results are comparable to the relative ring levels determined using the standard curve method. Relative expression values are given in the data table beneath the graph.



	EBA-175	EBL-1	EBA-140	EBA-181	MAEBL	EBA-165
RING 20Hr	1	1	1	1	1	1
E TROPH 28Hr	3.17	1.8	2.36	1.28	8.83	2.9
L TROPH 36Hr	5.5	2.96	8.36	4.67	43.34	18.6
SCHIZONT 44Hr	163.95	40.36	64.32	193.98	5.55	277.92

Figure 5.5. Real-time quantitative PCR of EBP genes, using cDNA from four-time point (20-hr, 28-hr, 36-hr and 48-hr) during the intraerythrocytic cycle in the Dd2-NM isolate. Ordinate values of the transcript levels are given in normalized to 18S rRNA gene. The results are comparable to the relative ring levels determined using the standard curve method. Relative expression values are given in the data table beneath the graph.

However, minor differences in EBP gene expression were observed among the three isolates in this study, ranging from a 1.5 to 3.2-fold difference.

Expression of EBPs in parasites that have been selected for a change in invasion phenotype

There was no marked difference observed in the level of EBP gene expression between Dd2 and Dd2-NM, with the exception of the *EBA-165* gene. *EBA-165* is expressed at a very high level in Dd2-NM, but is undetectable in Dd2 (Table 5.2). The only other notable difference between Dd2 and Dd2-NM was the expression of *eba-175*, which showed 2.2-fold greater expression. A difference in the temporal staging of EBP gene expression was also noted, with the expression of all EBP genes in the Dd2-NM isolate peaking at the 44-hr time point, except for *MAEBL*, which peaks at 36-hr. In the Dd2 and 3D7 isolates all the EBP genes show maximal expression at 36-hrs.

DISCUSSION

In this study, we investigated the level of EBP gene expression in three *Plasmodium falciparum* isolates. EBPs are required for the parasite to invade red blood cells, and function as ligands for receptors on the erythrocyte surface. At present only four reports have been published with respect to quantifying expression of EBP genes. Three utilized a microarray analysis to compare gene expression between 3D7 and HB3 [27-29] isolates, and the other used a quantitative real time PCR to examine EBP gene expression in the 3D7 isolate [1]. We used the TaqMan real-time PCR assay to quantify the level of transcription of the EBP genes during the erythrocytic cycle. We found that the expression of the six-erythrocyte binding-like genes in both the 3D7 and Dd2 isolates peaked at the same time point (36-hr). This is in contrast to a report [1], suggesting that the maebl transcript peaks at mid-late 18-hr (trophozoite stage) in the 3D7isolate. However, a report [27] agrees with our data, showing that all EBP gene transcripts

peak at 36-hr of the erythrocytic cycle. Our results for the relative expression of the EBP genes in the 3D7 isolate were comparable to previously report [27]. A major drawback when studying developmental stage specific expression of any particular gene is the importance that a particular cell stage is not contaminated by other developmental stages, which can affect the result. Examination of Giemsa stained smears from each time point shows that greater than 80% of parasites are of the desired stage. Also, we observed during RNA isolation that the lowest amount of total RNA was always obtain from rings, making this stage most susceptible to contamination from other stages. There was one consistent difference in the timing of EBP gene expression in the Dd2-NM line relative to isolates 3D7 and Dd2. In Dd2-NM the *MAEBL* gene transcript peaked at 36-hr, while all other EBP genes peaked at 44-hr.

However, in the 3D7 and Dd2 isolate all six EBP genes peaked at the 36-hr mark. It is possible that the Dd2-NM is slightly less mature at each time point, while the other two isolates (3D7, Dd2) mature much faster. We report here two interesting and related findings; one was that *EBA-165* was not expressed in the Dd2 isolate of *P. falciparum*. However, the gene was detected at the DNA level, and so is present in the Dd2 isolate. The second finding was the high level (277-fold) of expression of *EBA-165* gene in the Dd2-NM isolate. It would be interesting to determine why ***EBA-165*** is highly expressed in Dd2-NM and not in Dd2. One explanation lies in the switch in invasion phenotype, as Dd2-NM which was derived from Dd2 through selection for new invasion phenotype [20]. What makes this more interesting is that *EBA-165* have been classified as a pseudogene in seven isolates [26].

Table 5.2 Relative difference in expression of each EBP gene between three isolates

Isolate	*eba-175*	*ebl-1*	*eba-140*	*eba-181*	*maebl*	*eba-165*
3D7	1.7*	3.2	1.5	1.6	1.0	1
Dd2	2.2	1.0	1.0	1	2.7	-
Dd2-NM	1.0	1.0	1.5	1.5	2.5	11

* Represents fold difference.

Each isolate examined had either one or two insertions, both of which disrupt the reading frame. Dd2 and Dd2-NM were not included in that study; however we have verified that they both contain these frameshifts. One possibility is that there might be a mechanism during the translation process, which may compensate for these frameshifts. Programmed translational frameshifting (in both the +1 and –1 direction) have been observed [30, 31] in bacteria and occurs due to a mutation in the tRNA or under amino acid starvation. Another possibility is that *EBA-165* is a part of a group of coordinately controlled genes and that another upregulated gene is responsible for the observed change in phenotype.

Comparative expression of EBP genes among different isolates indicates relative minor differences, with the exception of *EBA-165* in the Dd2-NM parasite line, (Table 5.2) and was comparable to previously reports [27]. Our results also show that some EBP genes were expressed at a higher level than others in all three isolates. Also, no significant difference was detectable based on change of invasion phenotype.

In this study, we have shown that not all EBP gene transcripts peak at the same stage in erythrocytic cycle in different isolate. Some ebl transcript was more abundant than others in different isolates, but it is unclear if the 2-3 fold differences observed are large enough to account for the observed differences in invasion phenotype.

REFERENCES

1. Blair PL, Witney A, Haynes JD, et al., Transcripts of developmentally regulated Plasmodium falciparum genes quantified by real-time RT-PCR. Nucleic Acids Res, 2002. **30**(10): p. 2224-31.

2. Peterson DS and Wellems TE, EBL-1, a putative erythrocyte binding protein of Plasmodium falciparum, maps within a favored linkage group in two genetic crosses. Mol Biochem Parasitol, 2000. **105**(1): p. 105-13.

3. Adams JH, Blair PL, Kaneko O, et al., An expanding ebl family of Plasmodium falciparum. Trends Parasitol, 2001. **17**(6): p. 297-9.

4. Taylor HM, Triglia T, Thompson J, et al., Plasmodium falciparum homologue of the genes for Plasmodium vivax and Plasmodium yoelii adhesive proteins, which is transcribed but not translated. Infect Immun, 2001. **69**(6): p. 3635-45.

5. Taylor HM, Grainger M, and Holder AA, Variation in the expression of a Plasmodium falciparum protein family implicated in erythrocyte invasion. Infect Immun, 2002. **70**(10): p. 5779-89.

6. Scherf A, Hernandez-Rivas R, Buffet P, et al., Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in Plasmodium falciparum. Embo J, 1998. **17**(18): p. 5418-26.

7. Roberts DJ, Craig AG, Berendt AR, et al., Rapid switching to multiple antigenic and adhesive phenotypes in malaria [see comments]. Nature., 1992. **357**(6380): p. 689-692.

8. Wang AM, Doyle MV, and Mark DF, Quantitation of mRNA by the polymerase chain reaction. Proc Natl Acad Sci U S A, 1989. **86**(24): p. 9717-21.

9. Ginzinger DG, Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp Hematol, 2002. **30**(6): p. 503-12.

10. Ferre F, Quantitative or semi-quantitative PCR: reality versus myth. PCR Methods Appl, 1992. **2**(1): p. 1-9.

11. Freeman WM, Walker SJ, and Vrana KE, Quantitative RT-PCR: pitfalls and potential. Biotechniques, 1999. **26**(1): p. 112-22, 124-5.

12. Lie YS and Petropoulos CJ, Advances in quantitative PCR technology: 5' nuclease assays. Curr Opin Biotechnol, 1998. **9**(1): p. 43-8.

13. Heid CA, Stevens J, Livak KJ, et al., Real time quantitative PCR. Genome Res, 1996. **6**(10): p. 986-94.

14. Gibson UE, Heid CA, and Williams PM, A novel method for real time quantitative RT-PCR. Genome Res, 1996. **6**(10): p. 995-1001.

15. Polanco JC, Rodriguez JA, Corredor V, et al., Plasmodium vivax: parasitemia determination by real-time quantitative PCR in Aotus monkeys. Exp Parasitol, 2002. **100**(2): p. 131-4.

16. Lee MA, Tan CH, Aw LT, et al., Real-time fluorescence-based PCR for detection of malaria parasites. J Clin Microbiol, 2002. **40**(11): p. 4343-5.

17. Witney AA, Doolan DL, Anthony RM, et al., Determining liver stage parasite burden by real time quantitative PCR as a method for evaluating pre-erythrocytic malaria vaccine efficacy. Mol Biochem Parasitol, 2001. **118**(2): p. 233-45.

18. Goidin D, Mamessier A, Staquet MJ, et al., Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. Anal Biochem, 2001. **295**(1): p. 17-21.

19. Schmittgen TD and Zakrajsek BA, Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods, 2000. **46**(1-2): p. 69-81.

20. Dolan SA, Miller LH, and Wellems TE, Evidence for a switching mechanism in the invasion of erythrocytes by Plasmodium falciparum. J Clin Invest, 1990. **86**(2): p. 618-24.

21. Trager W and Jensen JB, Human malaria parasites in continuous culture. Science., 1976. **193**(4254): p. 673-675.

22. Lambros C and Vanderberg JP, Synchronization of Plasmodium falciparum erythrocytic stages in culture. J Parasitol, 1979. **65**(3): p. 418-20.

23. Mayer DC, Kaneko O, Hudson-Taylor DE, et al., Characterization of a Plasmodium falciparum erythrocyte-binding protein paralogous to EBA-175. Proc Natl Acad Sci U S A, 2001. **98**(9): p. 5222-7.

24. Sim BK, Orlandi PA, Haynes JD, et al., Primary structure of the 175K Plasmodium falciparum erythrocyte binding antigen and identification of a peptide which elicits antibodies that inhibit malaria merozoite invasion. J. Cell. Biol., 1990. **111**(5): p. 1877-1884.

25. Thompson JK, Triglia T, Reed MB, et al., A novel ligand from Plasmodium falciparum that binds to a sialic acid-containing receptor on the surface of human erythrocytes. Mol Microbiol, 2001. **41**(1): p. 47-58.

26. Triglia T, Thompson JK, and Cowman AF, An EBA175 homologue which is transcribed but not translated in erythrocytic stages of Plasmodium falciparum. Mol Biochem Parasitol, 2001. **116**(1): p. 55-63.

27. Bozdech Z, Llinas M, Pulliam BL, et al., The Transcriptome of the Intraerythrocytic Developmental Cycle of Plasmodium falciparum. PLoS Biol, 2003. **1**(1): p. E5.

28. Bozdech Z, Zhu J, Joachimiak MP, et al., Expression profiling of the schizont and trophozoite stages of Plasmodium falciparum with a long-oligonucleotide microarray. Genome Biol, 2003. **4**(2): p. R9.

29. Le Roch KG, Zhou Y, Blair PL, et al., Discovery of gene function by expression profiling of the malaria parasite life cycle. Science, 2003. **301**(5639): p. 1503-8.

30. Urbonavicius J, Stahl G, Durand JM, et al., Transfer RNA modifications that alter +1 frameshifting in general fail to affect -1 frameshifting. Rna, 2003. **9**(6): p. 760-8.

31. Farabaugh PJ and Bjork GR, How translational accuracy influences reading frame maintenance. Embo J, 1999. **18**(6): p. 1427-34.

SUMMARY AND CONCLUSIONS

In an effort to determine if there are additional erythrocyte binding proteins that may mediate red blood cell, invasion a Polymerize Chain Reaction (PCR) approach was used to identify 5 potential EBP genes in two *P. falciparum* isolates. Several clones sequenced were known EBP genes, which validated our approach for identifying novel members of the EBP family. The 5 candidate genes are currently being characterized.

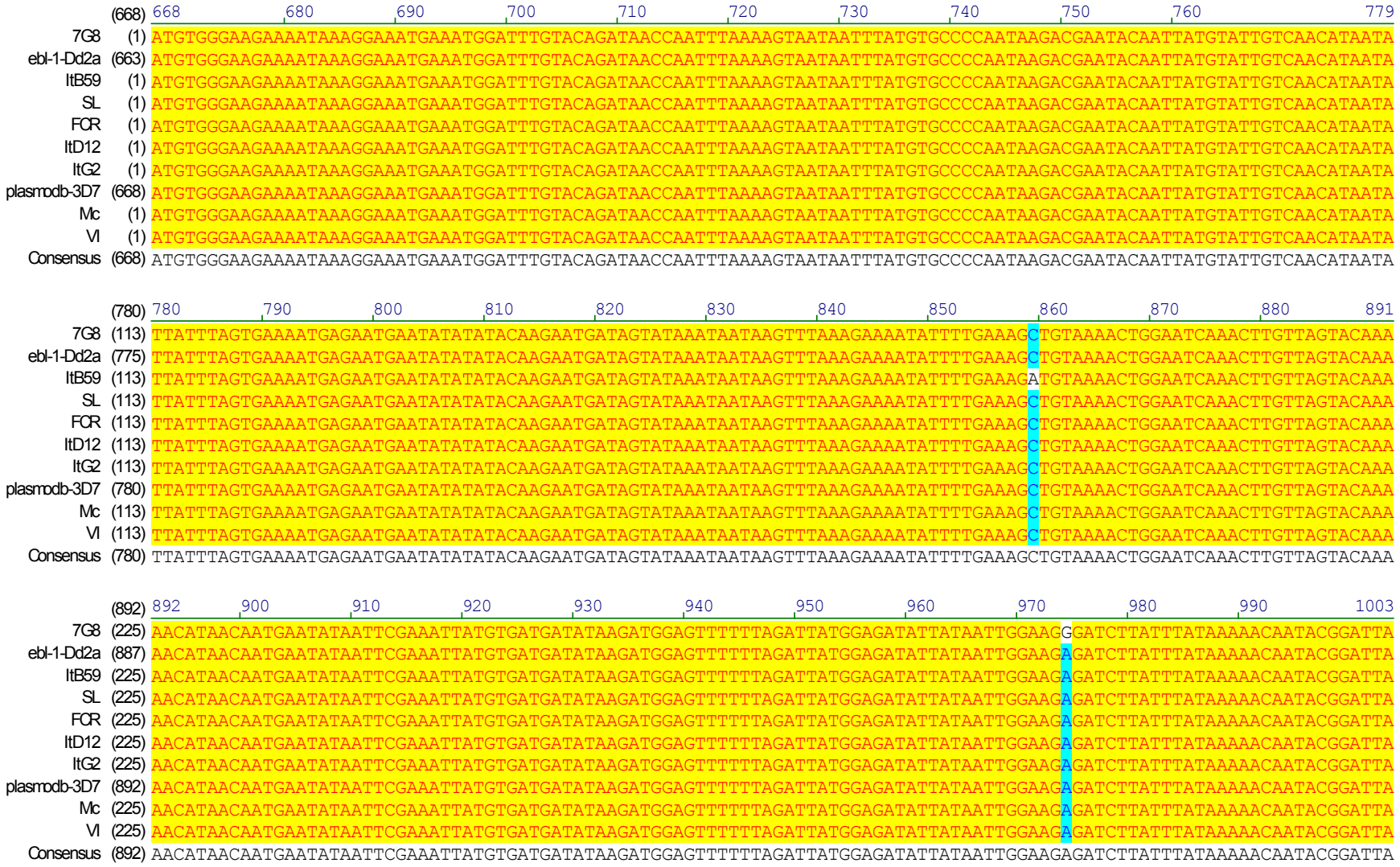
Evaluation of the level of sequence conservation within the ligand domains of the erythrocyte binding-like 1 gene has revealed a strong bias towards non-synonymous substitutions as compared to synonymous substitutions. The similarities in number and type of polymorphisms between *EBA-175* and *EBL-1* suggest both are exposed to similar selective pressures. However our results also suggest that *ebl-1* is not essential in some parasite isolates.

In order to determine whether expression of EBP genes varies among isolates or is altered in a parasite that has been selected for a change in invasion phenotype, we monitored EBP gene expression by real-time PCR. Significant differences in invasion phenotype between 3D7 and Dd2 were isolates not reflected in gross changes in EBP gene expression. The erythrocyte binding antigen-165 gene is not expressed in the Dd2 isolate. Surprisingly, increased expression of *eba-165*, (a pseudogene) was observed in the Dd2-NM isolate.

The studies reported herein will contribute significantly towards the understanding of EBP genes and the proteins they encode. When combine with existing information, the findings in these studies provide additional information to guide development of asexual-stage vaccines.

APPENDIX A

SEQUENCE ALIGNMENT OF THE DBL DOMAIN OF EBL-1 GENE IN 10 *Plasmodium falciprium* ISOLATES


(668) 668 680 690 700 710 720 730 740 750 760 779
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ebl-1-Dd2a (663) ATGTGGGAAGAAAATAAAGGAAATGAAATGGATTTGTACAGATAACCAATTTAAAAGTAATAATTTATGTGCCCCAATAAGACGAATACAATTATGTATTGTCAACATAATA
ItB59 (1) ATGTGGGAAGAAAATAAAGGAAATGAAATGGATTTGTACAGATAACCAATTTAAAAGTAATAATTTATGTGCCCCAATAAGACGAATACAATTATGTATTGTCAACATAATA
SL (1) ATGTGGGAAGAAAATAAAGGAAATGAAATGGATTTGTACAGATAACCAATTTAAAAGTAATAATTTATGTGCCCCAATAAGACGAATACAATTATGTATTGTCAACATAATA
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plasmodb-3D7 (668) ATGTGGGAAGAAAATAAAGGAAATGAAATGGATTTGTACAGATAACCAATTTAAAAGTAATAATTTATGTGCCCCAATAAGACGAATACAATTATGTATTGTCAACATAATA
Mc (1) ATGTGGGAAGAAAATAAAGGAAATGAAATGGATTTGTACAGATAACCAATTTAAAAGTAATAATTTATGTGCCCCAATAAGACGAATACAATTATGTATTGTCAACATAATA
Vl (1) ATGTGGGAAGAAAATAAAGGAAATGAAATGGATTTGTACAGATAACCAATTTAAAAGTAATAATTTATGTGCCCCAATAAGACGAATACAATTATGTATTGTCAACATAATA
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ItG2 (113) TTATTTAGTGAAAATGAGAATGAATATATATACAAGAATGATAGTATAAATAATAAGTTTAAAGAAAATATTTTGAAAGCTGTAAAACTGGAATCAAACTTGTTAGTACAAA
plasmodb-3D7 (780) TTATTTAGTGAAAATGAGAATGAATATATATACAAGAATGATAGTATAAATAATAAGTTTAAAGAAAATATTTTGAAAGCTGTAAAACTGGAATCAAACTTGTTAGTACAAA
Mc (113) TTATTTAGTGAAAATGAGAATGAATATATATACAAGAATGATAGTATAAATAATAAGTTTAAAGAAAATATTTTGAAAGCTGTAAAACTGGAATCAAACTTGTTAGTACAAA
Vl (113) TTATTTAGTGAAAATGAGAATGAATATATATACAAGAATGATAGTATAAATAATAAGTTTAAAGAAAATATTTTGAAAGCTGTAAAACTGGAATCAAACTTGTTAGTACAAA
Consensus (780) TTATTTAGTGAAAATGAGAATGAATATATATACAAGAATGATAGTATAAATAATAAGTTTAAAGAAAATATTTTGAAAGCTGTAAAACTGGAATCAAACTTGTTAGTACAAA
(892) 892 900 910 920 930 940 950 960 970 980 990 1003
7G8 (225) AACATAACAATGAATATAATTCGAAATTATGTGATGATATAAGATGGAGTTTTTTAGATTATGGAGATATTATAATTGGAAGGGATCTTATTTATAAAAACAATACGGATTA
ebl-1-Dd2a (887) AACATAACAATGAATATAATTCGAAATTATGTGATGATATAAGATGGAGTTTTTTAGATTATGGAGATATTATAATTGGAAGAGATCTTATTTATAAAAACAATACGGATTA
ItB59 (225) AACATAACAATGAATATAATTCGAAATTATGTGATGATATAAGATGGAGTTTTTTAGATTATGGAGATATTATAATTGGAAGAGATCTTATTTATAAAAACAATACGGATTA
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FCR (225) AACATAACAATGAATATAATTCGAAATTATGTGATGATATAAGATGGAGTTTTTTAGATTATGGAGATATTATAATTGGAAGAGATCTTATTTATAAAAACAATACGGATTA
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plasmodb-3D7 (892) AACATAACAATGAATATAATTCGAAATTATGTGATGATATAAGATGGAGTTTTTTAGATTATGGAGATATTATAATTGGAAGAGATCTTATTTATAAAAACAATACGGATTA
Mc (225) AACATAACAATGAATATAATTCGAAATTATGTGATGATATAAGATGGAGTTTTTTAGATTATGGAGATATTATAATTGGAAGAGATCTTATTTATAAAAACAATACGGATTA
Vl (225) AACATAACAATGAATATAATTCGAAATTATGTGATGATATAAGATGGAGTTTTTTAGATTATGGAGATATTATAATTGGAAGAGATCTTATTTATAAAAACAATACGGATTA
Consensus (892) AACATAACAATGAATATAATTCGAAATTATGTGATGATATAAGATGGAGTTTTTTAGATTATGGAGATATTATAATTGGAAGAGATCTTATTTATAAAAACAATACGGATTA


(1004) 1004 1010 1020 1030 1040 1050 1060 1070 1080 1090 1100 1115
7G8 (337) TATAAAAGAACAATTTAAGAAAATATTTAATAATGAATATAATAATAATGAATTAAATGACGAATTAAATAACGAATTAAATGATGAAAAGAATATAAAGTTACGAAAAGAA
ebl-1-Dd2a (999) TATAAAAGAACAATTTAAGAAAATATTTAATAATGAATATAATAATAATGAATTAAATGACGAATTAAATAACGAATTAAATGATGAAAAGAATATAAAGTTACGAAAAGAA
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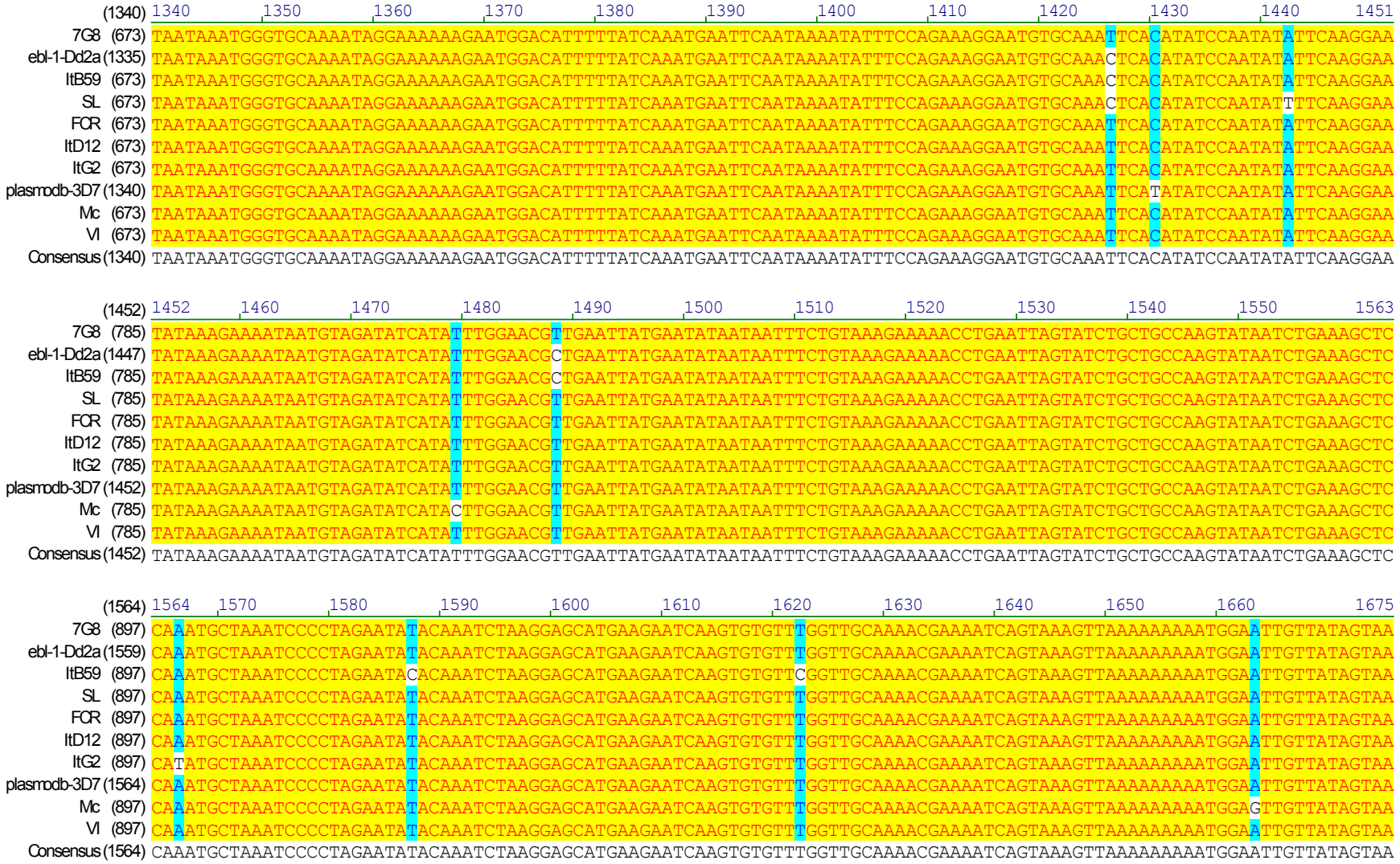
FCR (337) TATAAAAGAACAATTTAAGAAAATATTTAATAATGAATATAATAATAATGAATTAAATGACGAATTAAATAACGAATTAAATGATGAAAAGAATATAAAGTTACGAAAAGAA
ItD12 (337) TATAAAAGAACAATTTAAGAAAATATTTAATAATGAATATAATAATAATGAATTAAATGACGAATTAAATAACGAATTAAATGATGAAAAGAATATAAAGTTACGAAAAGAA
ItG2 (337) TATAAAAGAACAATTTAAGAAAATATTTAATAATGAATATAATAATAATGAATTAAATGACGAATTAAATAACGAATTAAATGATGAAAAGAATATAAAGTTACGAAAAGAA
plasmodb-3D7 (1004) TATAAAAGAACAATTTAAGAAAATATTTAATAATGAATATAATAATAATGAATTAAATGACGAATTAAATAACGAATTAAATGATGAAAAGAATATAAAGTTACGAAAAGAA
Mc (337) TATAAAAGAACAATTTAAGAAAATATTTAATAATGAATATAATAATAATGAATTAAATGACGAATTAAATAACGAATTAAATGATGAAAAGAATATAAAGTTACGAAAAGAA
VI (337) TATAAAAGAACAATTTAAGAAAATATTTAATAATGAATATAATAATAATGAATTAAATGACGAATTAAATAACGAATTAAATGATGAAAAGAATATAAAGTTACGAAAAGAA
Consensus (1004) TATAAAAGAACAATTTAAGAAAATATTTAATAATGAATATAATAATAATGAATTAAATGACGAATTAAATAACGAATTAAATGATGAAAAGAATATAAAGTTACGAAAAGAA
(1116) 1116 1130 1140 1150 1160 1170 1180 1190 1200 1210 1227
7G8 (449) TGGTGGGAAAAATACAAAGAAGATATTTGGGAAGAAATGACAAAGGAACATAATGATAAATTTATTGAGAAATGTAAATATTTTGCTAAGGATGAACCACAAATTGTTCGAT
ebl-1-Dd2a (1111) TGGTGGGAAAAATACAAAGAAGATATTTGGGAAGAAATGACAAAGGAACATAATGATAAATTTATTGAGAAATGTAAATATTTTGCTAAGGATGAACCACAAATTGTTCGAT
ItB59 (449) TGGTGGGAAAAATACAAAGAAGATATTTGGGAAGAAATGACAAAGGAACATAATGATAAATTTATTGAGAAATGTACATATTTTGCTAAGGATGAACCACAAATTGTTCGAT
SL (449) TGGTGGGAAAAATACAAAGAAGATATTTGGGAAGAAATGACAAAGGAACATAATGATAAATTTATTGAGAAATGTAAATATTTTGCTAAGGATGAACCACAAATTGTTCGAT
FCR (449) TGGTGGGAAAAATACAAAGAAGATATTTGGGAAGAAATGACAAAGGAACATAATGATAAATTTATTGAGAAATGTAAATATTTTGCTAAGGATGAACCACAAATTGTTCGAT
ItD12 (449) TGGTGGGAAAAATACAAAGAAGATATTTGGGAAGAAATGACAAAGGAACATAATGATAAATTTATTGAGAAATGTAAATATTTTGCTAAGGATGAACCACAAATTGTTCGAT
ItG2 (449) TGGTGGGAAAAATACAAAGAAGATATTTGGGAAGAAATGACAAAGGAACATAATGATAAATTTATTGAGAAATGTAAATATTTTGCTAAGGATGAACCACAAATTGTTCGAT
plasmodb-3D7 (1116) TGGTGGGAAAAATACAAAGAAGATATTTGGGAAGAAATGACAAAGGAACATAATGATAAATTTATTGAGAAATGTAAATATTTTGCTAAGGATGAACCACAAATTGTTCGAT
Mc (449) TGGTGGGAAAAATACAAAGAAGATATTTGGGAAGAAATGACAAAGGAACATAATGATAAATTTATTGAGAAATGTAAATATTTTGCTAAGGATGAACCACAAATTGTTCGAT
VI (449) TGGTGGGAAAAATACAAAGAAGATATTTGGGAAGAAATGACAAAGGAACATAATGATAAATTTATTGAGAAATGTAAATATTTTGCTAAGGATGAACCACAAATTGTTCGAT
Consensus (1116) TGGTGGGAAAAATACAAAGAAGATATTTGGGAAGAAATGACAAAGGAACATAATGATAAATTTATTGAGAAATGTAAATATTTTGCTAAGGATGAACCACAAATTGTTCGAT
(1228) 1228 1240 1250 1260 1270 1280 1290 1300 1310 1320 1339
7G8 (561) GGATAGAGGAGTGGAGTAAACAATTTTTGGATGAGAAAAATTATATGTTGTTTACTTTGAGAAACACATATAATGAAATGAATATAATTCATGAGAATAATTGTAAACAATA
ebl-1-Dd2a (1223) GGATAGAGGAGTGGAGTAAACAATTTTTGGATGAGAAAAATTATATGTTGTTTACTTTGAGAAACACATATAATGAAATGAATATAATTCATGAGAATAATTGTAAACAATA
ItB59 (561) GGATAGAGGAGTGGAGTAAACAATTTTTGGATGAGAAAAATTATATGTTGTTTACTTTGAGAAACACATATAATGAAATGAATATAATTCATGAGAATAATTGTAAACAATA
SL (561) GGATAGAGGAGTGGAGTAAACAATTTTTGGATGAGAAAAATTATATGTTGTTTACTTTGAGAAACACATATAATGAAATGAATATAATTCATGAGAATAATTGTAAACAATA
FCR (561) GGATAGAGGAGTGGAGTAAACAATTTTTGGATGAGAAAAATTATATGTTGTTTACTTTGAGAAACACATATAATGAAATGAATATAATTCATGAGAATAATTGTAAACAATA
ItD12 (561) GGATAGAGGAGTGGAGTAAACAATTTTTGGATGAGAAAAATTATATGTTGTTTACTTTGAGAAACACATATAATGAAATGAATATAATTCATGAGAATAATTGTAAACAATA
ItG2 (561) GGATAGAGGAGTGGAGTAAACAATTTTTGGATGAGAAAAATTATATGTTGTTTACTTTGAGAAACACATATAATGAAATGAATATAATTCATGAGAATAATTGTAAACAATA
plasmodb-3D7 (1228) GGATAGAGGAGTGGAGTAAACAATTTTTGGATGAGAAAAATTATATGTTGTTTACTTTGAGAAACACATATAATGAAATGAATATAATTCATGAGAATAATTGTAAACAATA
Mc (561) GGATAGAGGAGTGGAGTAAACAATTTTTGGATGAGAAAAATTATATGTTGTTTACTTTGAGAAACACATATAATGAAATGAATATAATTCATGAGAATAATTGTAAACAATA
VI (561) GGATAGAGGAGTGGAGTAAACAATTTTTGGATGAGAAAAATTATATGTTGTTTACTTTGAGAAACACATATAATGAAATGAATATAATTCATGAGAATAATTGTAAACAATA
Consensus (1228) GGATAGAGGAGTGGAGTAAACAATTTTTGGATGAGAAAAATTATATGTTGTTTACTTTGAGAAACACATATAATGAAATGAATATAATTCATGAGAATAATTGTAAACAATA


(1340) 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440 1451
7G8 (673) TAATAAATGGGTGCAAAATAGGAAAAAAGAATGGACATTTTTATCAAATGAATTCAATAAAATATTTCCAGAAAGGAATGTGCAAATTCACATATCCAATATATTCAAGGAA
ebl-1-Dd2a (1335) TAATAAATGGGTGCAAAATAGGAAAAAAGAATGGACATTTTTATCAAATGAATTCAATAAAATATTTCCAGAAAGGAATGTGCAAACTCACATATCCAATATATTCAAGGAA
ItB59 (673) TAATAAATGGGTGCAAAATAGGAAAAAAGAATGGACATTTTTATCAAATGAATTCAATAAAATATTTCCAGAAAGGAATGTGCAAACTCACATATCCAATATATTCAAGGAA
SL (673) TAATAAATGGGTGCAAAATAGGAAAAAAGAATGGACATTTTTATCAAATGAATTCAATAAAATATTTCCAGAAAGGAATGTGCAAACTCACATATCCAATATTTTCAAGGAA
FCR (673) TAATAAATGGGTGCAAAATAGGAAAAAAGAATGGACATTTTTATCAAATGAATTCAATAAAATATTTCCAGAAAGGAATGTGCAAATTCACATATCCAATATATTCAAGGAA
ItD12 (673) TAATAAATGGGTGCAAAATAGGAAAAAAGAATGGACATTTTTATCAAATGAATTCAATAAAATATTTCCAGAAAGGAATGTGCAAATTCACATATCCAATATATTCAAGGAA
ItG2 (673) TAATAAATGGGTGCAAAATAGGAAAAAAGAATGGACATTTTTATCAAATGAATTCAATAAAATATTTCCAGAAAGGAATGTGCAAATTCACATATCCAATATATTCAAGGAA
plasmodb-3D7 (1340) TAATAAATGGGTGCAAAATAGGAAAAAAGAATGGACATTTTTATCAAATGAATTCAATAAAATATTTCCAGAAAGGAATGTGCAAATTCATATATCCAATATATTCAAGGAA
Mc (673) TAATAAATGGGTGCAAAATAGGAAAAAAGAATGGACATTTTTATCAAATGAATTCAATAAAATATTTCCAGAAAGGAATGTGCAAATTCACATATCCAATATATTCAAGGAA
Vl (673) TAATAAATGGGTGCAAAATAGGAAAAAAGAATGGACATTTTTATCAAATGAATTCAATAAAATATTTCCAGAAAGGAATGTGCAAATTCACATATCCAATATATTCAAGGAA
Consensus (1340) TAATAAATGGGTGCAAAATAGGAAAAAAGAATGGACATTTTTATCAAATGAATTCAATAAAATATTTCCAGAAAGGAATGTGCAAATTCACATATCCAATATATTCAAGGAA
(1452) 1452 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1563
7G8 (785) TATAAAGAAAATAATGTAGATATCATATTTGGAACGTTGAATTATGAATATAATAATTTCTGTAAAGAAAAACCTGAATTAGTATCTGCTGCCAAGTATAATCTGAAAGCTC
ebl-1-Dd2a (1447) TATAAAGAAAATAATGTAGATATCATATTTGGAACGCTGAATTATGAATATAATAATTTCTGTAAAGAAAAACCTGAATTAGTATCTGCTGCCAAGTATAATCTGAAAGCTC
ItB59 (785) TATAAAGAAAATAATGTAGATATCATATTTGGAACGCTGAATTATGAATATAATAATTTCTGTAAAGAAAAACCTGAATTAGTATCTGCTGCCAAGTATAATCTGAAAGCTC
SL (785) TATAAAGAAAATAATGTAGATATCATATTTGGAACGTTGAATTATGAATATAATAATTTCTGTAAAGAAAAACCTGAATTAGTATCTGCTGCCAAGTATAATCTGAAAGCTC
FCR (785) TATAAAGAAAATAATGTAGATATCATATTTGGAACGTTGAATTATGAATATAATAATTTCTGTAAAGAAAAACCTGAATTAGTATCTGCTGCCAAGTATAATCTGAAAGCTC
ItD12 (785) TATAAAGAAAATAATGTAGATATCATATTTGGAACGTTGAATTATGAATATAATAATTTCTGTAAAGAAAAACCTGAATTAGTATCTGCTGCCAAGTATAATCTGAAAGCTC
ItG2 (785) TATAAAGAAAATAATGTAGATATCATATTTGGAACGTTGAATTATGAATATAATAATTTCTGTAAAGAAAAACCTGAATTAGTATCTGCTGCCAAGTATAATCTGAAAGCTC
plasmodb-3D7 (1452) TATAAAGAAAATAATGTAGATATCATATTTGGAACGTTGAATTATGAATATAATAATTTCTGTAAAGAAAAACCTGAATTAGTATCTGCTGCCAAGTATAATCTGAAAGCTC
Mc (785) TATAAAGAAAATAATGTAGATATCATACTTGGAACGTTGAATTATGAATATAATAATTTCTGTAAAGAAAAACCTGAATTAGTATCTGCTGCCAAGTATAATCTGAAAGCTC
Vl (785) TATAAAGAAAATAATGTAGATATCATATTTGGAACGTTGAATTATGAATATAATAATTTCTGTAAAGAAAAACCTGAATTAGTATCTGCTGCCAAGTATAATCTGAAAGCTC
Consensus (1452) TATAAAGAAAATAATGTAGATATCATATTTGGAACGTTGAATTATGAATATAATAATTTCTGTAAAGAAAAACCTGAATTAGTATCTGCTGCCAAGTATAATCTGAAAGCTC
(1564) 1564 1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1675
7G8 (897) CAAATGCTAAATCCCCTAGAATATACAAATCTAAGGAGCATGAAGAATCAAGTGTGTTTGGTTGCAAAACGAAAATCAGTAAAGTTAAAAAAAAATGGAATTGTTATAGTAA
ebl-1-Dd2a (1559) CAAATGCTAAATCCCCTAGAATATACAAATCTAAGGAGCATGAAGAATCAAGTGTGTTTGGTTGCAAAACGAAAATCAGTAAAGTTAAAAAAAAATGGAATTGTTATAGTAA
ItB59 (897) CAAATGCTAAATCCCCTAGAATACACAAATCTAAGGAGCATGAAGAATCAAGTGTGTTCGGTTGCAAAACGAAAATCAGTAAAGTTAAAAAAAAATGGAATTGTTATAGTAA
SL (897) CAAATGCTAAATCCCCTAGAATATACAAATCTAAGGAGCATGAAGAATCAAGTGTGTTTGGTTGCAAAACGAAAATCAGTAAAGTTAAAAAAAAATGGAATTGTTATAGTAA
FCR (897) CAAATGCTAAATCCCCTAGAATATACAAATCTAAGGAGCATGAAGAATCAAGTGTGTTTGGTTGCAAAACGAAAATCAGTAAAGTTAAAAAAAAATGGAATTGTTATAGTAA
ItD12 (897) CAAATGCTAAATCCCCTAGAATATACAAATCTAAGGAGCATGAAGAATCAAGTGTGTTTGGTTGCAAAACGAAAATCAGTAAAGTTAAAAAAAAATGGAATTGTTATAGTAA
ItG2 (897) CATATGCTAAATCCCCTAGAATATACAAATCTAAGGAGCATGAAGAATCAAGTGTGTTTGGTTGCAAAACGAAAATCAGTAAAGTTAAAAAAAAATGGAATTGTTATAGTAA
plasmodb-3D7 (1564) CAAATGCTAAATCCCCTAGAATATACAAATCTAAGGAGCATGAAGAATCAAGTGTGTTTGGTTGCAAAACGAAAATCAGTAAAGTTAAAAAAAAATGGAATTGTTATAGTAA
Mc (897) CAAATGCTAAATCCCCTAGAATATACAAATCTAAGGAGCATGAAGAATCAAGTGTGTTTGGTTGCAAAACGAAAATCAGTAAAGTTAAAAAAAAATGGAGTTGTTATAGTAA
Vl (897) CAAATGCTAAATCCCCTAGAATATACAAATCTAAGGAGCATGAAGAATCAAGTGTGTTTGGTTGCAAAACGAAAATCAGTAAAGTTAAAAAAAAATGGAATTGTTATAGTAA
Consensus (1564) CAAATGCTAAATCCCCTAGAATATACAAATCTAAGGAGCATGAAGAATCAAGTGTGTTTGGTTGCAAAACGAAAATCAGTAAAGTTAAAAAAAAATGGAATTGTTATAGTAA

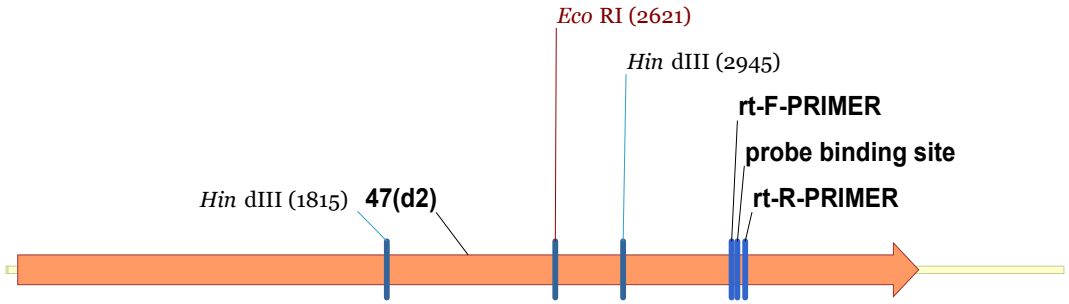

(1676) 1676 1690 1700 1710 1720 1730 1740 1750 1760 1770 1787
7G8 (1009) TAATAAAGTAACTAAACCTGAAGGTGTATGTGGACCACCAAGAAGGCAACAATTATGTCTTGGATATATATTTTTGATTCGCGACGGTAACGAGGAAGGATTAAAAGATCAT
ebl-1-Dd2a (1671) TAATAAAGTAACTAAACCTGAAGGTGTATGTGGACCACCAAGAAGGCAACAATTATGTCTTGGATATATATTTTTGATTCGCGACGGTAACGAGGAAGGATTAAAAGATCAT
ItB59 (1009) TAATAAAGTAACTAAACCTGAAGGTGTATGTGGACCACCAAGAAGGCAACAATTATGTCTTGGATATATATTTTTGATTCGCGACGGTAACGAGGAAGGATTAAAAGATCAT
SL (1009) TAATAAAGTAAATAAACCTGAAGGTGTATGTGGACCACCAAGAAGGCAACAATTATGTCTTGGATATATATTTTTGATTCGCGACGGTAACGAGGAAGGATTAAAAGATCAT
FCR (1009) TAATAAAGTAACTAAACCTGAAGGTGTATGTGGACCACCAAGAAGGCAACAATTATGTCTTGGATATATATTTTTGATTCGCGACGGTAACGAGGAAGGATTAAAAGATCAT
ItD12 (1009) TAATAAAGTAACTAAACCTGAAGGTGTATGTGGACCACCAAGAAGGCAACAATTATGTCTTGGATATATATTTTTGATTCGCGACGGTAACGAGGAAGGATTAAAAGATCAT
ItG2 (1009) TAATAAAGTAACTAAACCTGAAGGTGTATGTGGACCACCAAGAAGGCAACAATTATGTCTTGGATATATATTTTTGATTCGCGACGGTAACGAGGAAGGATTAAAAGATCAT
plasmodb-3D7 (1676) TAATAAAGTAACTAAACCTGAAGGTGTATGTGGACCACCAAGAAGGCAACAATTATGTCTTGGATATATATTTTTGATTCGCGACGGTAACGAGGAAGGATTAAAAGATCAT
Mc (1009) TAATAAAGTAACTAAACCTGAAGGTGTATGTGGACCACCAAGAAGGCAACAATTATGTCTTGGATATATATTTTTGATTCGCGACGGTAACGAGGAAGGATTAAAAGATCAT
Vl (1009) TAATAAAGTAACTAAACCTGAAGGTGTATGTGGACCACCAAGAAGGCAACAATTATGTCTTGGATATATATTTTTGATTCGCGACGGTAACGAGGAAGGATTAAAAGATCAT
Consensus (1676) TAATAAAGTAACTAAACCTGAAGGTGTATGTGGACCACCAAGAAGGCAACAATTATGTCTTGGATATATATTTTTGATTCGCGACGGTAACGAGGAAGGATTAAAAGATCAT
(1788) 1788 1800 1810 1820 1830 1840 1850 1860 1870 1880 1899
7G8 (1121) ATTAATAAGGCAGCTAATTATGAGGCAATGCATTTAAAAGAGAAATATGAGAATGCTGGTGGTGATAAAATTTGCAATGCTATATTGGGAAGTTATGCAGATATTGGAGATA
ebl-1-Dd2a (1783) ATTAATAAGGCAGCTAATTATGAGGCAATGCATTTAAAAGAGAAATATGAGAATGCTGGTGGTGATAAAATTTGCAATGCTATATTGGGAAGTTATGCAGATATTGGAGATA
ItB59 (1121) ATTAATAAGGCAGCTAATTATGAGGCAATGCATTTAAAAGAGAAATATGAGAATGCTGGTGGTGATAAAATTTGCAATGCTATATTGGGAAGTTATGCAGATATTGGAGATA
SL (1121) ATTAATAAGGCAGCTAATTATGAGGCAATGCATTTAAAAGAGAAATATGAGAATGCTGGTGGTGATAAAATTTGCAATGCTATATTGGGAAGTTATGCAGATATTGGAGATA
FCR (1121) ATTAATAAGGCAGCTAATTATGAGGCAATGCATTTAAAAGAGAAATATGAGAATGCTGGTGGTGATAAAATTTGCAATGCTATATTGGGAAGTTATGCAGATATTGGAGATA
ItD12 (1121) ATTAATAAGGCAGCTAATTATGAGGCAATGCATTTAAAAGAGAAATATGAGAATGCTGGTGGTGATAAAATTTGCAATGCTATATTGGGAAGTTATGCAGATATTGGAGATA
ItG2 (1121) ATTAATAAGGCAGCTAATTATGAGGCAATGCATTTAAAAGAGAAATATGAGAATGCTGGTGGTGATAAAATTTGCAATGCTATATTGGGAAGTTATGCAGATATTGGAGATA
plasmodb-3D7 (1788) ATTAATAAGGCAGCTAATTATGAGGCAATGCATTTAAAAGAGAAATATGAGAATGCTGGTGGTGATAAAATTTGCAATGCTATATTGGGAAGTTATGCAGATATTGGAGATA
Mc (1121) ATTAATAAGGCAGCTAATTATGAGGCAATGCATTTAAAAGAGAAATATGAGAATGCTGGTGGTGATAAAATTTGCAATGCTATATTGGGAAGTTATGCAGATATTGGAGATA
Vl (1121) ATTAATAAGGCAGCTAATTATGAGGCAATGCATTTAAAAGAGAAATATGAGAATGCTGGTGGTGATAAAATTTGCAATGCTATATTGGGAAGTTATGCAGATATTGGAGATA
Consensus (1788) ATTAATAAGGCAGCTAATTATGAGGCAATGCATTTAAAAGAGAAATATGAGAATGCTGGTGGTGATAAAATTTGCAATGCTATATTGGGAAGTTATGCAGATATTGGAGATA
(1900) 1900 1910 1920 1930 1940 1950 1960 1970 1980 1990 2000 2011
7G8 (1233) TTGTAAGAGGTTTGGATGTTTGGAGGGATATAAATACTAATAAATTATCAGAAAAATTCCAAAAAATTTTTATGGGTGGTGGTAATTCTAGGAAAAAACAAAACGATAATAA
ebl-1-Dd2a (1895) TTGTAAGAGGTTTGGATGTTTGGAGGGATATAAATACTAATAAATTATCAGAAAAATTCCAAAAAATTTTTATGGGTGGTGGTAATTCTAGGAAAAAACAAAACGATAATAA
ItB59 (1233) TTGTAAGAGGTTTGGATGTTTGGAGGGATATAAATACTAATAAATTATCAACAAATTCCAAAAAATTTTTATGGGTGGTGGTAATTCTAGGAAAAAACAAAACGATTATAA
SL (1233) TTGTAAGAGGTTTGGATGTTTGGGGGGATATAAATACTAATAAATTATCAGAAAAATTCCAAAAAATTTTTATGGGTGGTGGTAATTCTAGGAAAAAACAAAACGATAATAA
FCR (1233) TTGTAAGAGGTTTGGATGTTTGGAGGGATATAAATACTAATAAATTATCAGAAAAATACCAAAAAATTTTTATGGGTGGTGGTAATTCTAGGAAAAAACAAAACGATAATAA
ItD12 (1233) TTGTAAGAGGTTTGGATGTTTGGAGGGATATAAATACTAATAAATTATCAGAAAAATTCCAAAAAATTTTTATGGGTGGTGGTAATTCTAGGAAAAAACAAAACGATAATAA
ItG2 (1233) TTGTAAGAGGTTTGGATGTTTGGAGGGATATAAATACTAATAAATTATCAGAAAAATTCCAAAAAATTTTTATGGGTGGTGGTAATTCTAGGAAAAAACAAAACGATAATAA
plasmodb-3D7 (1900) TTGTAAGAGGTTTGGATGTTTGGAGGGATATAAATACTAATAAATTATCAGAAAAATTCCAAAAAATTTTTATGGGTGGTGGTAATTCTAGGAAAAAACAAAACGATAATAA
Mc (1233) TTGTAAGAGGTTTGGATGTTTGGAGGGATATAAATACTAATAAATTATCAGAAAAATTCCAAAAAATTTTTATGGGTGGTGGTAATTCTAGGAAAAAACAAAACGATAATAA
Vl (1233) TTGTAAGAGGTTTGGATGTTTGGAGGGATATAAATACTAATAAATTATCAGAAAAATTCCAAAAAATTTTTATGGGTGGTGGTAATTCTAGGAAAAAACAAAACGATAATAA
Consensus (1900) TTGTAAGAGGTTTGGATGTTTGGAGGGATATAAATACTAATAAATTATCAGAAAAATTCCAAAAAATTTTTATGGGTGGTGGTAATTCTAGGAAAAAACAAAACGATAATAA

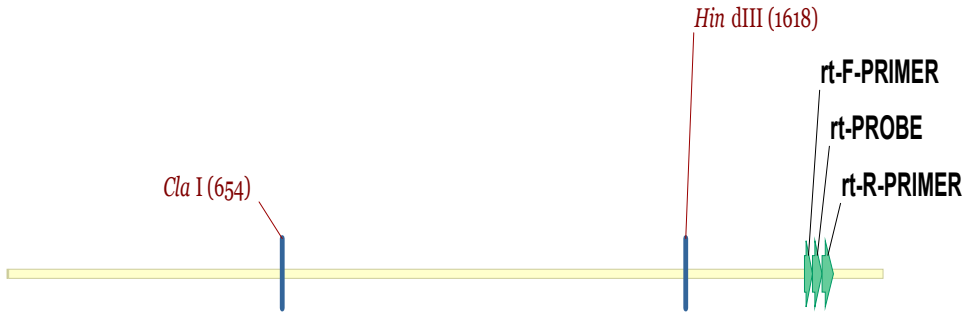

(2012) 2012 2020 2030 2040 2050 2060 2070 2080 2090 2100 2110 2123
7G8 (1345) TGAACGTAATAAATGGTGGGAAAAACAAAGGAATTTAATATGGTCTAGTATGGTAAAACACATTCCAAAAGGAAAAACATGTAAACGTCATAATAATTTTGAGAAAATTCCT
ebl-1-Dd2a (2007) TGAACGTAATAAATGGTGGGAAAAACAAAGGAATTTAATATGGTCTAGTATGGTAAAACACATTCCAAAAGGAAAAACATGTAAACGTCATAATAATTTTGAGAAAATTCCT
ItB59 (1345) TGAACGTAATAAATGGTGGGAAAAACAAAGGAATTTAATATGGTCTAGTATGGTAAAACATATTCCAAAAGGAAAAACATGTAAACGTCATAATAATTTTGAGAAAATTCCT
SL (1345) TGAACGTAATAAATGGTGGGAAAAACAAAGGAATTTAATATGGTCTAGTATGGTAAAACACATTCCAAAAGGAAAAACATGTAAACGTCATAATAATTTTGAGAAAATTCCT
FCR (1345) TGAACGTAATAAATGGTGGGAAAAACAAAGGAATTTAATATGGTCTAGTATGGTAAAACACATTCCAAAAGGAAAAACATGTAGACGTCATAATAATTTTGAGAAAATTCCT
ItD12 (1345) TGAACGTAATAAATGGTGGGAAAAACAAAGGAATTTAATATGGTCTAGTATGGTAAAACACATTCCAAAAGGAAAAACATGTAAACGTCATAATAATTTTGAGAAAATTCCT
ItG2 (1345) TGAACGTAATAAATGGTGGGAAAAACAAAGGAATTTAATATGGTCTAGTATGGTAAAACACATTCCAAAAGGAAAAACATGTAAACGTCATAATAATTTTGAGAAAATTCCT
plasmodb-3D7 (2012) TGAACGTAATAAATGGTGGGAAAAACAAAGGAATTTAATATGGTCTAGTATGGTAAAACACATTCCAAAAGGAAAAACATGTAAACGTCATAATAATTTTGAGAAAATTCCT
Mc (1345) TGAACGTAATAAATGGTGGGAAAAACAAAGGAATTTAATATGGTCTAGTATGGTAAAACACATTCCAAAAGGAAAAACATGTAAACGTCATAATAATTTTGAGAAAATTCCT
Vl (1345) TGAACGTAATAAATGGTGGGAAAAACAAAGGAATTTAATATGGTCTAGTATGGTAAAACACATTCCAAAAGGAAAAACATGTAAACGTCATAATAATTTTGAGAAAATTCCT
Consensus (2012) TGAACGTAATAAATGGTGGGAAAAACAAAGGAATTTAATATGGTCTAGTATGGTAAAACACATTCCAAAAGGAAAAACATGTAAACGTCATAATAATTTTGAGAAAATTCCT
(2124) 2124 2130 2140 2150 2160 2170 2180 2190 2200 2210 2220 2235
7G8 (1457) CAATTTTTGAGATGGTTAAAAGAATGGGGTGATGAATTTTGTGAGGAAATGGGTACGGAAGTCAAGCAATTAGAGAAAATATGTGAAAATAAAAATTGTTCGGAAAAAAAAT
ebl-1-Dd2a (2119) CAATTTTTGAGATGGTTAAAAGAATGGGGTGATGAATTTTGTGAGGAAATGGGTACGGAAGTCAAGCAATTAGAGAAAATATGTGAAAATAAAAATTGTTCGGAAAAAAAAT
ItB59 (1457) CAATTTTTGAGATGGTTAAAAGAATGGGGTGATGAATTTTGTGAGGAAATGGGTACGGAAGTCAAGCAATTAGAGAAAATATGTGAAAATAAAAATTGTTCGGAAAAAAAAT
SL (1457) CAATTTTTGAGATGGTTAAAAGAATGGGGTGATGAATTTTGTGAGGAAATGGATACGGAAGTCAAGCAATTAGAGAAAATATGTGAAAATAAAAATTGTTCAGAAAAAAAAT
FCR (1457) CAATTTTTGAGATGGTTAAAAGAATGGGGTGATGAATTTTGTGAGGAAATGGGTACGGAAGTCAAGCAATTAGAGAAAATATGTGAAAATAAAAATTGTTCGGAAAAAAAAT
ItD12 (1457) CAATTTTTGAGATGGTTAAAAGAATGGGGTGATGAATTTTGTGAGGAAATGGGTACGGAAGTCAAGCAATTAGAGAAAATATGTGAAAATAAAAATTGTTCGGAAAAAAAAT
ItG2 (1457) CAATTTTTGAGATGGTTAAAAGAATGGGGTGATGAATTTTGTGAGGAAATGGGTACGGAAGTCAAGCAATTAGAGAAAATATGTGAAAATAAAAATTGTTCGGAAAAAAAAT
plasmodb-3D7 (2124) CAATTTTTGAGATGGTTAAAAGAATGGGGTGATGAATTTTGTGAGGAAATGGGTACGGAAGTCAAGCAATTAGAGAAAATATGTGAAAATAAAAATTGTTCGGAAAAAAAAT
Mc (1457) CAATTTTTGAGATGGTTAAAAGAATGGGGTGATGAATTTTGTGAGGAAATGGGTACGGAAGTCAAGCAATTAGAGAAAATATGTGAAAATAAAAATTGTTCGGAAAAAAAAT
Vl (1457) CAATTTTTGAGATGGTTAAAAGAATGGGGTGATGAATTTTGTGAGGAAATGGGTACGGAAGTCAAGCAATTAGAGAAAATATGTGAAAATAAAAATTGTTCGGAAAAAAAAT
Consensus (2124) CAATTTTTGAGATGGTTAAAAGAATGGGGTGATGAATTTTGTGAGGAAATGGGTACGGAAGTCAAGCAATTAGAGAAAATATGTGAAAATAAAAATTGTTCGGAAAAAAAAT
(2236) 2236 2250 2260 2270 2280 2290 2300 2310 2320 2330 2347
7G8 (1569) GTAAAAATGCATGTAGTTCCTATGAAAAATGGATAAAGGAACGAAAAAATGAATATAATTTGCAATCAAAGAAATTTGATAGTGATAAAAAATTAAATAAAAAAAACAATCT
ebl-1-Dd2a (2231) GTAAAAATGCATGTAGTTCCTATGAAAAATGGATAAAGGAACGAAAAAATGAATATAATTTGCAATCAAAGAGATTTGATAGTGATAAAAAATTAAATAAAAAAAACAATCT
ItB59 (1569) GTAAAAATGCATGTAGTTCCTATGAAAAATGGATAAAGGAACGAAAAAATGAATATAATTTGCAATCAAAGAGATTTGATAGTGATAAAAAATTAAATAAAAAAAACAATCT
SL (1569) GTAAAAATGCATGTAGTTCCTATGAAAAATGGATAAAGGAACCAAAAAATGAATATAATTTGCAATCAAAGAAATTTGATAATGATAAAAAATTAAATAAAAAAAACAATCT
FCR (1569) GTAAAAATGCATGTAGTTCCTATGAAAAATGGATAAAGGAACGAAAAAATGAATATAATTTGCAATCAAAGAAATTTGATAGTGATAAAAAATTAAATAAAAAAAACAATCT
ItD12 (1569) GTAAAAATGCATGTAGTTCCTATGAAAAATGGATAAAGGAACGAAAAAATGAATATAATTTGCAATCAAAGAAATTTGATAGTGATAAAAAATTAAATAAAAAAAACAATCT
ItG2 (1569) GTAAAAATGCATGTAGTTCCTATGAAAAATGGATAAAGGAACGAAAAAATGAATATAATTTGCAATCAAAGAAATTTGATAGTGATAAAAAATTAAATAAAAAAAACAATCT
plasmodb-3D7 (2236) GTAAAAATGCATGTAGTTCCTATGAAAAATGGATAAAGGAACGAAAAAATGAATATAATTTGCAATCAAAGAAATTTGATAGTGATAAAAAATTAAATAAAAAAAACAATCT
Mc (1569) GTAAAAATGCATGTAGTTCCTATGAAAAATGGATAAAGGAACGAAAAAATGAATATAATTTGCAATCAAAGAAATTTGATAGTGATAAAAAATTAAATAAAAAAAACAATCT
Vl (1569) GTAAAAATGCATGTAGTTCCTATGAAAAATGGATAAAGGAACGAAAAAATGAATATAATTTGCAATCAAAGAAATTTGATAGTGATAAAAAATTAAATAAAAAAAACAATCT
Consensus (2236) GTAAAAATGCATGTAGTTCCTATGAAAAATGGATAAAGGAACGAAAAAATGAATATAATTTGCAATCAAAGAAATTTGATAGTGATAAAAAATTAAATAAAAAAAACAATCT


(2348) 2348 2360 2370 2380 2390 2400 2410 2420 2430 2440 2459
7G8 (1681) TTATAATAAATTTGAGGATTCTAAAGCTTATTTAAGGAGTGAATCAAAACAGTGCTCAAATATAGAATTTAATGATGAAACATTTACATTTCCTAATAAATATAAAGAGGCT
ebl-1-Dd2a (2343) TTATAATAAATTTGAGGATTCTAAAGCTTATTTAAGGAGTGAATCAAAACAGTGCTCAAATATAGAATTTAATGATGAAACATTTACATTTCCTAATAAATATAAAGAGGCT
ItB59 (1681) TTATAATAAATTTGAGGATTCTAAAGCTTATTTAAGGAGTGAATCAAAACAGTGCTCAAATATAGAATTTAATGATGAAACATTTACATTTCCTAATAAATATAAAGAGGCT
SL (1681) TTATAATAAATTTGAGGATTCTAAAGCTTATTTAAGGAGTGAATCAAAACAGTGCTCAAATATAGAATTTAATGATGAAACATTTACATTTCCTAATAAATATAAAGAGGCT
FCR (1681) TTATAATAAATTTGAGGATTCTAAAGCTTATTTAAGGAGTGAATCAAAACAGTGCTCAAATATAGAATTTAATGATGAAACATTTACATTTCCTAATAAATATAAAGAGGCT
ItD12 (1681) TTATAATAAATTTGAGGATTCTAAAGCTTATTTAAGGAGTGAATCAAAACAGTGCTCAAATATAGAATTTAATGATGAAACATTTACATTTCCTAATAAATATAAAGAGGCT
ItG2 (1681) TTATAATAAATTTGAGGATTCTAAAGCTTATTTAAGGAGTGAATCAAAACAGTGCTCAAATATAGAATTTAATGATGAAACATTTACATTTCCTAATAAATATAAAGAGGCT
plasmodb-3D7 (2348) TTATAATAAATTTGAGGATTCTAAAGCTTATTTAAGGAGTGAATCAAAACAGTGCTCAAATATAGAATTTAATGATGAAACATTTACATTTCCTAATAAATATAAAGAGGCT
Mc (1681) TTATAATAAATTTGAGGATTCTAAAGCTTATTTAAGGAGTGAATCAAAACAGTGCTCAAATATAGAATTTAATGATGAAACATTTACATTTCCTAATAAATATAAAGAGGCT
VI (1681) TTATAATAAATTTGAGGATTCTAAAGCTTATTTAAGGAGTGAATCAAAACAGTGCTCAAATATAGAATTTAATGATGAAACATTTACATTTCCTAATAAATATAAAGAGGCT
Consensus (2348) TTATAATAAATTTGAGGATTCTAAAGCTTATTTAAGGAGTGAATCAAAACAGTGCTCAAATATAGAATTTAATGATGAAACATTTACATTTCCTAATAAATATAAAGAGGCT
(2460) 2460 2470 2480 2490 2500 2510 2520 2530 2540 2550 2560 2571
7G8 (1793) TGTATGGTATGTGAAAATCCTTCATCTTCGAAAGCTCTTAAACCTATAAAAACGGATGTGTTTCCTATAGAGG----------
ebl-1-Dd2a (2455) TGTATGGTATGTGAAAATCCTTCATCTTCGAAAGCTCTTAAACCTATAAAAACGGATGTGTTTCCTATAGAGGAAACAAAAAAATCTGAGTTATCAAGTTTAACAGATAAAT
ItB59 (1793) TGTATGGTATGTGAAAATCCTTCATCTTCGAAAGCTCTTAAACCTATAAAAACGGATGTGTTTCCTATAGAGG----------
SL (1793) TGTATGGTATGTGAAAATCCTTCATCTTCGAAAGCTCTTAAACCTATAAAAACGGATGTGTTTCCTATAGAGG----------
FCR (1793) TGTATGGTATGTGAAAATCCTTCATCTTCGAAAGCTCTTAAACCTATAAAAACGGATGTGTTTCCTATAGAGG----------
ItD12 (1793) TGTATGGTATGTGAAAATCCTTCATCTTCGAAAGCTCTTAAACCTATAAAAACGGATGTGTTTCCTATAGAGG----------
ItG2 (1793) TGTATGGTATGTGAAAATCCTTCATCTTCGAAAGCTCTTAAACCTATAAAAACGGATGTGTTTCCTATAGAGG----------
plasmodb-3D7 (2460) TGTATGGTATGTGAAAATCCTTCATCTTCGAAAGCTCTTAAACCTATAAAAACGAATGTGTTTCCTATAGAGGAATCAAAAAAATCTGAGTTATCAAGTTTAACAGATAAAT
Mc (1793) TGTATGGTATGTGAAAATCCTTCATCTTCGAAAGCTCTTAAACCTATAAAAACGGATGTGTTTCCTATAGAGG----------
VI (1793) TGTATGGTATGTGAAAATCCTTCATCTTCGAAAGCTCTTAAACCTATAAAAACGGATGTGTTTCCTATAGAGG----------
Consensus (2460) TGTATGGTATGTGAAAATCCTTCATCTTCGAAAGCTCTTAAACCTATAAAAACGGATGTGTTTCCTATAGAGG

APPENDIX B

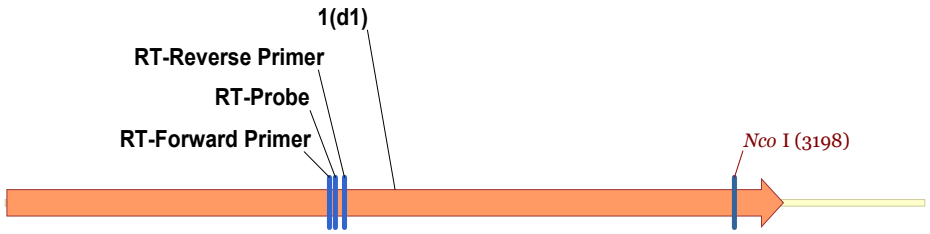
DIAGRAM OF EBP GENES AND ssu rRNA DEPICTING THE LOCATIONS OF THE PRIMERS AND PROBE BINDING SITES


Eco RI (2621)
Hin dIII (2945)
rt-F-PRIMER
probe binding site
rt-R-PRIMER
Hin dIII (1815)
47(d2)
eba-175a
5057 bp



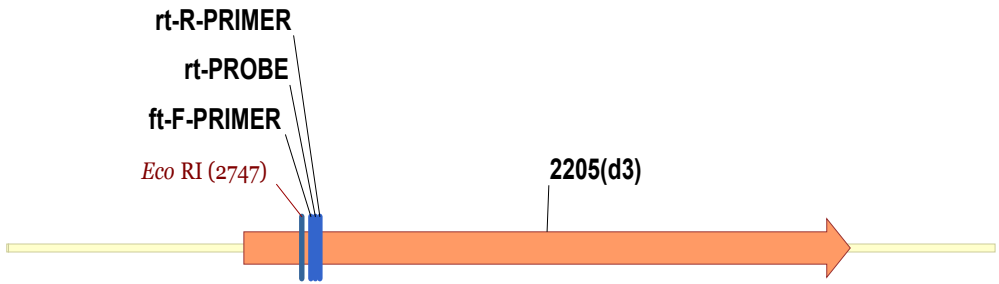
18S rRNA

2090 bp



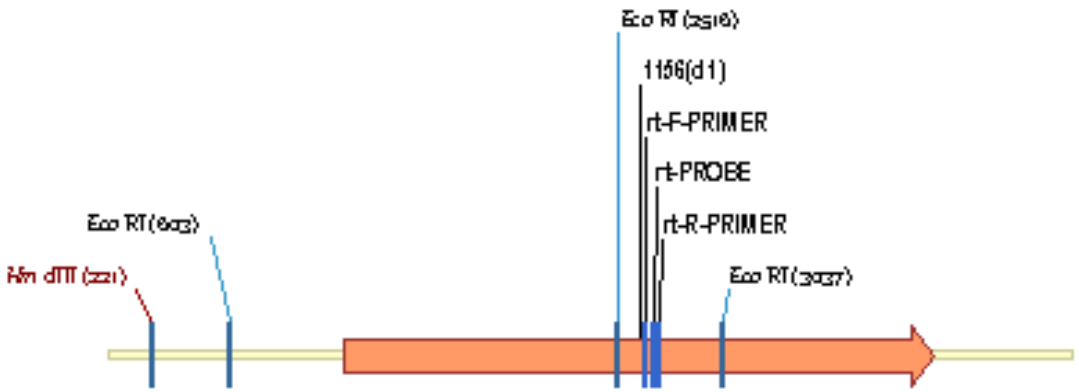
EBA-140-1

4034 bp

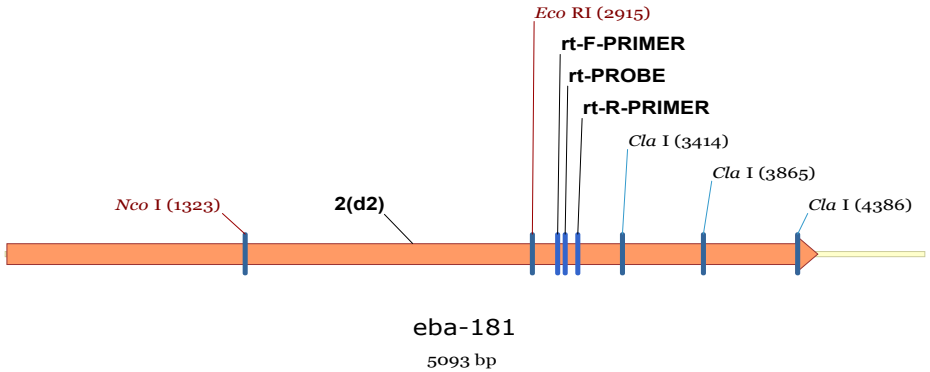


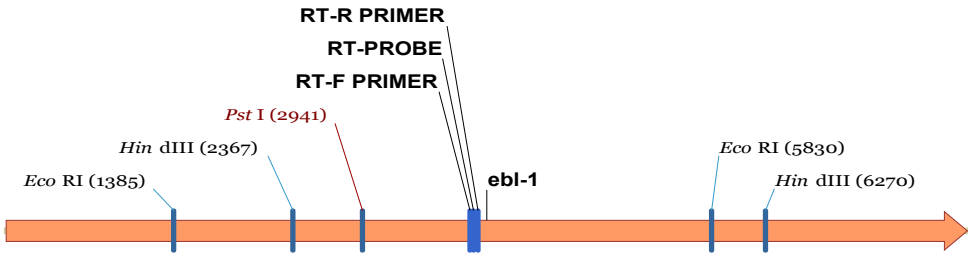
maebl

9240 bp


Eco RI (2516)
1156(d1)
rt-F-PRIMER
rt-PROBE
rt-R-PRIMER
Eco RI (603)
Eco RI (3037)

eba-165


Eco RI (2915)
rt-F-PRIMER
rt-PROBE
rt-R-PRIMER
Cla I (3414)
Cla I (3865)
Cla I (4386)
Nco I (1323)
2(d2)
eba-181
5093 bp


RT-R PRIMER
RT-PROBE
RT-F PRIMER
Pst I (2941)
Hin dIII (2367)
Eco RI (1385)
ebl-1
Eco RI (5830)
Hin dIII (6270)

ebl-1

7941 bp